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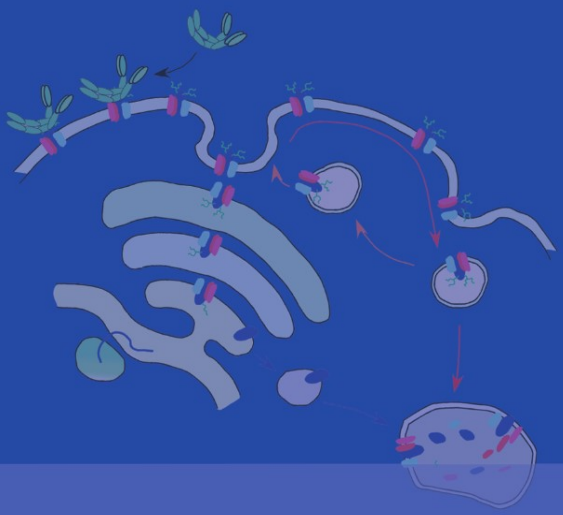

Allergy Frontiers:

Classification and Pathomechanisms

Volume 2

Springer

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Lanny J. Rosenwasser
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Foreword

When I entered the field of allergy in the early 1970s, the standard textbook was a few hundred pages, and the specialty was so compact that texts were often authored entirely by a single individual and were never larger than one volume. Compare this with *Allergy Frontiers: Epigenetics, Allergens, and Risk Factors*, the present six-volume text with well over 150 contributors from throughout the world. This book captures the explosive growth of our specialty since the single-author textbooks referred to above.

The unprecedented format of this work lies in its meticulous attention to detail yet comprehensive scope. For example, great detail is seen in manuscripts dealing with topics such as "Exosomes, naturally occurring minimal antigen presenting units" and "Neuropeptide S receptor 1 (NPSR1), an asthma susceptibility gene." The scope is exemplified by the unique approach to disease entities normally dealt with in a single chapter in most texts. For example, anaphylaxis, a topic usually confined to one chapter in most textbooks, is given five chapters in *Allergy Frontiers*. This approach allows the text to employ multiple contributors for a single topic, giving the reader the advantage of being introduced to more than one viewpoint regarding a single disease.

This broad scope is further illustrated in the way this text deals with the more frequently encountered disorder, asthma. There are no fewer than 26 chapters dealing with various aspects of this disease. Previously, to obtain such a comprehensive approach to a single condition, one would have had to purchase a text devoted solely to that disease state.

In addition, the volume includes titles which to my knowledge have never been presented in an allergy text before. These include topics such as "NKT ligand conjugated immunotherapy," "Hypersensitivity reactions to nano medicines: causative factors and optimization," and "An environmental systems biology approach to the study of asthma."

It is not hard to see that this textbook is unique, offering the reader a means of obtaining a detailed review of a single highly focused subject, such as the neuropeptide S receptor, while also providing the ability to access a panoramic and remarkably in-depth view of a broader subject, such as asthma. Clearly it is intended primarily for the serious student of allergy and immunology, but can also serve as a resource text for those with an interest in medicine in general.

I find it most reassuring that even though we have surpassed the stage of the one-volume, single-author texts, because of the wonderful complexity of our specialty and its broadening scope that has evolved over the years, the reader can still obtain an all-inclusive and comprehensive review of allergy in a single source. It should become part of the canon of our specialty.

Phil Lieberman, M.D.

Foreword

When I started immunology under Professor Kimishige Ishizaka in the early 1950s, allergy was a mere group of odd syndromes of almost unknown etiology. An immunological origin was only suspected but not proven. The term "atopy," originally from the Greek word *à-topòs*, represents the oddness of allergic diseases. I would call this era "stage 1," or the primitive era of allergology.

Even in the 1950s, there was some doubt as to whether the antibody that causes an allergic reaction was really an antibody, and was thus called a "reagin," and allergens were known as peculiar substances that caused allergy, differentiating them from other known antigens.

It was only in 1965 that reagin was proven to be an antibody having a light chain and a unique heavy chain, which was designated as IgE in 1967 with international consensus. The discovery of IgE opened up an entirely new era in the field of allergology, and the mechanisms of the immediate type of allergic reaction was soon evaluated and described. At that point in time we believed that the nature of allergic diseases was a mere IgE-mediated inflammation, and that these could soon be cured by studying the IgE and the various mediators that induced the inflammation. This era I would like to call "stage 2," or the classic era.

The classic belief that allergic diseases would be explained by a mere allergen-IgE antibody reaction did not last long. People were dismayed by the complexity and diversity of allergic diseases that could not be explained by mere IgE-mediated inflammation. Scientists soon realized that the mechanisms involved in allergic diseases were far more complex and that they extended beyond the conventional idea of a pure IgE-mediated inflammation. A variety of cells and their products (cytokines/chemokines and other inflammatory molecules) have been found to interact in a more complex manner; they create a network of reactions via their receptors to produce various forms of inflammatory changes that could never be categorized as a single entity of inflammation. This opened a new era, which I would like to call the modern age of allergology or "stage 3."

The modern era stage 3 coincided with the discovery that similar kinds of cytokines and cells are involved in the regulation of IgE production. When immunologists investigated the cell types and cytokines that regulate IgE production, they found that two types of helper T cells, distinguishable by the profile of cytokines they produce, play important regulatory roles in not only IgE production

but also in regulating allergic inflammation. The advancement of modern molecular technologies has enabled detailed analyses of molecules and genes involved in this extremely complex regulatory mechanism. Hence, there are a number of important discoveries in this area, which are still of major interest to allergologists, as can be seen in the six volumes of this book.

We realize that allergology has rapidly progressed during the last century, but mechanisms of allergic diseases are far more complex than we had expected. New discoveries have created new questions, and new facts have reminded us of old concepts. For example, the genetic disposition of allergic diseases was suspected even in the earlier, primitive era but is still only partially proven on a molecular basis. Even the molecular mechanisms of allergic inflammation continue to be a matter of debate and there is no single answer to explain the phenomenon. There is little doubt that the etiology of allergic diseases is far more varied and complex than we had expected. An immunological origin is not the only mechanism, and there are more unknown origins of similar reactions. Although therapeutic means have also progressed, we remain far from our goal to cure and prevent allergic diseases.

We have to admit that while we have more knowledge of the many intricate mechanisms that are involved in the various forms of allergic disease, we are still at the primitive stage of allergology in this respect. We are undoubtedly proceeding into a new stage, stage 4, that may be called the postmodern age of allergology and hope this era will bring us closer to finding a true solution for the enigma of allergy and allergic diseases.

We are happy that at this turning point the editors, Ruby Pawankar, Stephen Holgate, and Lanny Rosenwasser, are able to bring out such a comprehensive book which summarizes the most current knowledge on allergic diseases, from epidemiology to mechanisms, the impact of environmental and genetic factors on allergy and asthma, clinical aspects, recent therapeutic and preventive strategies, as well as future perspectives. This comprehensive knowledge is a valuable resource and will give young investigators and clinicians new insights into modern allergology which is an ever-growing field.

Tomio Tada, M.D., Ph.D., D.Med.Sci.

Foreword

Allergic diseases represent one of the major health problems in most modern societies. The increase in prevalence over the last decades is dramatic. The reasons for this increase are only partly known. While in former times allergy was regarded as a disease of the rich industrialized countries only, it has become clear that all over the world, even in marginal societies and in all geographic areas—north and south of the equator—allergy is a major global health problem.

The complexity and the interdisciplinary character of allergology, being the science of allergic diseases, needs a concert of clinical disciplines (internal medicine, dermatology, pediatrics, pulmonology, otolaryngology, occupational medicine, etc.), basic sciences (immunology, molecular biology, botany, zoology, ecology), epidemiology, economics and social sciences, and psychology and psychosomatics, just to name a few. It is obvious that an undertaking like this book series must involve a multitude of authors; indeed, the wide spectrum of disciplines relevant to allergy is reflected by the excellent group of experts serving as authors who come from all over the world and from various fields of medicine and other sciences in a pooling of geographic, scientific, theoretical, and practical clinical diversity.

The first volume concentrates on the basics of etiology, namely, the causes of the many allergic diseases with epigenetics, allergens and risk factors. Here, the reader will find up-to-date information on the nature, distribution, and chemical structure of allergenic molecules, the genetic and epigenetic phenomena underlying the susceptibility of certain individuals to develop allergic diseases, and the manifold risk factors from the environment playing the role of modulators, both in enhancing and preventing the development of allergic reactions.

In times when economics plays an increasing role in medicine, it is important to reflect on this aspect and gather the available data which—as I modestly assume—may be yet rather scarce. The big effort needed to undertake well-controlled studies to establish the socio-economic burden of the various allergic diseases is still mainly ahead of us. The Global Allergy and Asthma European Network (GA2LEN), a group of centers of excellence in the European Union, will start an initiative regarding this topic this year.

In volume 2, the pathomechanisms of various allergic diseases and their classification are given, including such important special aspects as allergy and the bone marrow, allergy and the nervous system, and allergy and mucosal immunology.

Volume 3 deals with manifold clinical manifestations, from allergic rhinitis to drug allergy and allergic bronchopulmonary aspergillosis, as well as including other allergic reactions such as lactose and fructose intolerances.

Volume 4 deals with the practical aspects of diagnosis and differential diagnosis of allergic diseases and also reflects educational programs on asthma.

Volume 5 deals with therapy and prevention of allergies, including pharmacotherapy, as well as allergen-specific immunotherapy with novel aspects and special considerations for different groups such as children, the elderly, and pregnant women.

Volume 6 concludes the series with future perspectives, presenting a whole spectrum of exciting new approaches in allergy research possibly leading to new strategies in diagnosis, therapy, and prevention of allergic diseases.

The editors have accomplished an enormous task to first select and then motivate the many prominent authors. They and the authors have to be congratulated. The editors are masters in the field and come from different disciplines. Ruby Pawankar, from Asia, is one of the leaders in allergy who has contributed to the understanding of the cellular and immune mechanisms of allergic airway disease, in particular upper airway disease. Stephen Holgate, from the United Kingdom, has contributed enormously to the understanding of the pathophysiology of allergic airway reactions beyond the mere immune deviation, and focuses on the function of the epithelial barrier. He and Lanny Rosenwasser, who is from the United States, have contributed immensely to the elucidation of genetic factors in the susceptibility to allergy. All three editors are members of the Collegium Internationale Allergologicum (CIA) and serve on the Board of Directors of the World Allergy Organization (WAO).

I have had the pleasure of knowing them for many years and have cooperated with them at various levels in the endeavor to promote and advance clinical care, research, and education in allergy. Together with Lanny Rosenwasser as co-editor-in-chief, we have just started the new *WAO Journal* (electronic only), where the global representation in allergy research and education will be reflected on a continuous basis.

Finally, Springer, the publisher, has to be congratulated on their courage and enthusiasm with which they have launched this endeavor. Springer has a lot of experience in allergy—I think back to the series *New Trends in Allergy*, started in 1985, as well as to my own book *Allergy in Practice,* to the *Handbook of Atopic Eczema* and many other excellent publications.

I wish this book and the whole series of *Allergy Frontiers* complete success! It should be on the shelves of every physician or researcher who is interested in allergy, clinical immunology, or related fields.

Johannes Ring, M.D., Ph.D.

Preface

Allergic diseases are increasing in prevalence worldwide, in industrialized as well as industrializing countries, affecting from 10%–50% of the global population with a marked impact on the quality of life of patients and with substantial costs. Thus, allergy can be rightfully considered an epidemic of the twenty-first century, a global public health problem, and a socioeconomic burden. With the projected increase in the world's population, especially in the rapidly growing economies, it is predicted to worsen as this century moves forward.

Allergies are also becoming more complex. Patients frequently have multiple allergic disorders that involve multiple allergens and a combination of organs through which allergic diseases manifest. Thus exposure to aeroallergens or ingested allergens frequently gives rise to a combination of upper and lower airways disease, whereas direct contact or ingestion leads to atopic dermatitis with or without food allergy. Food allergy, allergic drug responses and anaphylaxis are often severe and can be life-threatening. However, even the less severe allergic diseases can have a major adverse effect on the health of hundreds of millions of patients and diminish quality of life and work productivity. The need of the hour to combat these issues is to promote a better understanding of the science of allergy and clinical immunology through research, training and dissemination of information and evidence-based better practice parameters.

Allergy Frontiers is a comprehensive series comprising six volumes, with each volume dedicated to a specific aspect of allergic disease to reflect the multidisciplinary character of the field and to capture the explosive growth of this specialty. The series summarizes the latest information about allergic diseases, ranging from epidemiology to the mechanisms and environmental and genetic factors that influence the development of allergy; clinical aspects of allergic diseases; recent therapeutic and preventive strategies; and future perspectives. The chapters of individual volumes in the series highlight the roles of eosinophils, mast cells, lymphocytes, dendritic cells, epithelial cells, neutrophils and T cells, adhesion molecules, and cytokines/chemokines in the pathomechanisms of allergic diseases. Some specific new features are the impact of infection and innate immunity on allergy, and mucosal immunology of the various target organs and allergies, and the impact of the nervous system on allergies. The most recent, emerging therapeutic strategies are discussed, including allergen-specific immunotherapy and anti-IgE treatment,

while also covering future perspectives from immunostimulatory DNA-based therapies to probiotics and nanomedicine.

A unique feature of the series is that a single topic is addressed by multiple contributors from various fields and regions of the world, giving the reader the advantage of being introduced to more than one point of view and being provided with comprehensive knowledge about a single disease. The reader thus obtains a detailed review of a single, highly focused topic and at the same time has access to a panoramic, in-depth view of a broader subject such as asthma.

The chapters attest to the multidisciplinary character of component parts of the series: environmental, genetics, molecular, and cellular biology; allergy; otolaryngology; pulmonology; dermatology; and others. Representing a collection of state-of-the-art reviews by world-renowned scientists from the United Kingdom and other parts of Europe, North America, South America, Australia, Japan, and South Africa, the volumes in this comprehensive, up-to-date series contain more than 150 chapters covering virtually all aspects of basic and clinical allergy. The publication of this extensive collection of reviews is being brought out within a span of two years and with the greatest precision to keep it as updated as possible. This six-volume series will be followed up by yearly updates on the cutting-edge advances in any specific aspect of allergy.

The editors would like to sincerely thank all the authors for having agreed to contribute and who, despite their busy schedules, contributed to this monumental work. We also thank the editorial staff of Springer Japan for their assistance in the preparation of this series. We hope that the series will serve as a valuable information tool for scientists and as a practical guide for clinicians and residents working and/or interested in the field of allergy, asthma, and immunology.

Ruby Pawankar, Stephen Holgate, and Lanny Rosenwasser

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Part I
Classification and Pathomechanisms

Basic Aspects of Allergy and Hypersensitivity Reactions

Isabella Pali-Schöll and Erika Jensen-Jarolim

Introduction

The immune system of higher organisms has evolved to defend pathogens like viruses, bacteria, or parasites, and for tolerance against nutrients and self-antigens. For this, two different strategies have been developed, on the one hand soluble, easily distributable factors like antibodies, and on the other hand cells that are able to actively migrate and penetrate tissues in a target-oriented fashion. In addition, cytokines, chemokines, and several mediator substances contribute to the immunological homeostasis. This very potent system has to be kept under control and in a certain balance between attack and tolerance. If an imbalance between reactivity and tolerance occurs, the immune system may turn against self-antigens or harmless foreign antigens, which results in autoimmunity or hypersensitivity reactions. Hyperimmune reactions have been classified by Gell and Coombs more than 40 years ago into four classes (Fig. 1), according to the responsible mechanisms evoking the symptoms in the organism [1]. There have been novel findings showing that mixed pathophysiologies and additional mechanisms can also underlie harmful immune reactions and that the Gell-and-Coombs scheme has several limitations [2, 3]. Nevertheless, this classification is helpful and still widely used today, and it shall serve as the frame scheme for this chapter, with the focus set on the basics of type I hypersensitivity reactions.

Type I Hypersensitivity Reactions

After several decades of increasing numbers of atopic and/or allergic diseases, the prevalence of allergic disorders like hay fever and eczema seems to have reached a plateau lately [4]. Nevertheless a considerable number of people, namely 30–40%

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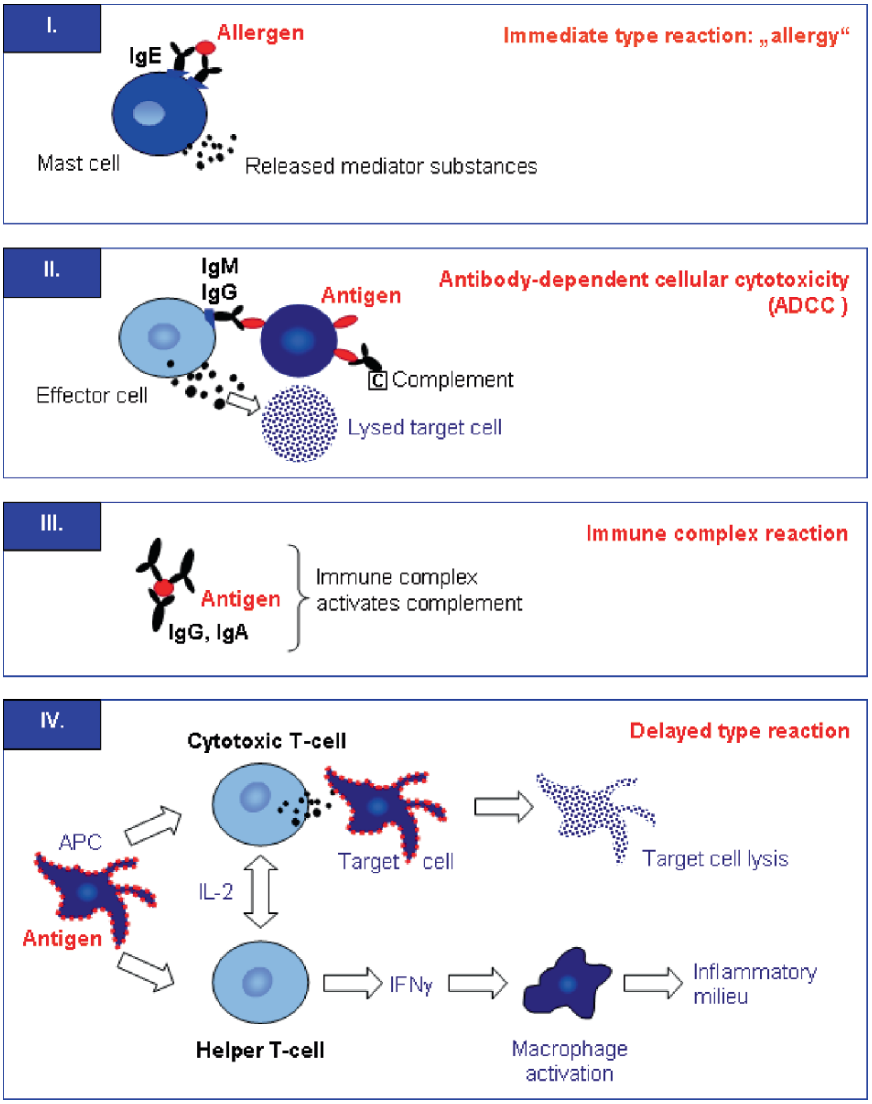

Fig. 1 Classification of hypersensitivity reactions by Gell and Coombs [1]. The type I–III reactions are mediated by antibodies, whereas the type IV hypersensitivity is mediated by cells. In type I hyperreactivity, the IgE-cross-linking on mast cells by the respective allergen leads to the release of mediator substances, thereby causing allergic symptoms. Type II reactions are evoked by IgG or IgM antibodies attached to antigens on the target cells, which in consequence is lysed via cytotoxicity. The immune complexes formed during type III reactions in excess of antigen are deposited in different tissues and induce local damage via complement activation and cell recruitment. Alternatively, an excess of preformed antibodies can precipitate antigen already on the entrance site and induce inflammation. In type IV hypersensitivity, antigens are presented to T cells by antigen-presenting cells and activate these cells to effector cells. During a consecutive antigen contact, these effector cells target and lyse the antigen-bearing cell

of adults, are still affected by symptoms of hay fever [4], and around 3% of adults suffer from adverse reactions to food [5] in industrialized countries.

Mechanism of Type I Allergy

Antibodies are versatile tools to target antigens at almost any place of the body. Five antibody classes have evolved adapted for special compartments of the body and interact with various effector cells, thereby potentiating the effects. IgE antibodies have natural functions in the defense of parasites [6] and are also very effective against tumors [7], but when turned against harmless antigens they cause allergies with symptoms ranging from harmless itch to anaphylactic shock. Despite the high occurrence of these disorders, only a few factors have been identified that are held responsible for the induction and maintenance of allergy.

The underlying mechanism of type I (i.e., immediate type) allergies is the switch from a physiological IgM/IgG antibody response produced by B cells against an antigen/allergen to an IgE-dominated response in the so-called sensitization phase. Thus type I hypersensitivity reactions are characterized as IgE-mediated allergic reactions. These IgE-antibodies bind to effector cells like mast cells and basophils. Through a consecutive contact with the respective allergen the effector phase of allergy can be initiated. Bridging or cross-linking of at least two IgE-antibodies on these effector cells induces the release of preformed mediator substances like histamine, neutral proteases (tryptase, chymase, kininogenase), and proteoglycans, e.g., heparin. These released factors lead to the development of the classical symptoms involving the skin, respiratory tract, circulation, and gastrointestinal tract. Typically, this immediate phase reaction occurs within a short time, mostly already several minutes after allergen contact. Mast cells which lose membrane area due to degranulation get activated, and, while trying to recover, start a de novo synthesis of prostaglandins and leukotrienes from membrane arachidonic acid [8, 9]. The newly synthesized chemokines and cytokines initiate recruitment and activation of additional inflammatory cells, including basophils, eosinophils, and Th2 lymphocytes [10], and lead to the inflammatory late phase reaction with tissue edema and enduration several hours to days after allergen contact.

The mechanisms occurring during the effector phase of type I allergy are well-defined and it is accepted today that the conformation of allergenic molecules plays a key role for their ability to induce immediate type hypersensitivity in the organism [11]. The question about common features of allergens is long standing and it was hypothesized that there is a limited number of amino acid sequence motifs that are found in allergenic molecules which can be exploited to predict allergenicity [12, 13]. However, no common feature has been identified yet that applies to *all* molecules which are known as allergens today [14], apart from their ability to cross-link IgE antibodies.

Multimerization of Allergens Facilitates IgE Cross-linking

The most important properties of allergens are (i) to bind to IgE-antibodies, (ii) to cross-link IgE being bound to effector cells like mast cells, inducing allergic symptoms [15], and (iii) to cross-link B cell receptors (BCR), leading to internalization, processing, and presentation of the allergen, again resulting in IgE antibody production and triggering [16].

For cross-linking processes in general, which are key mechanisms for biological activation, it seems to be clear that several epitopes have to be displayed on the surface of allergenic molecules. This can be performed in several ways. (i) Different epitopes can be displayed on one allergen molecule in a multivalent fashion. (ii) The same epitope can also be presented several times in a repetitive manner, for instance in the allergen Bla g 1 from cockroach [17], Hev b 5 from latex [18], or tropomyosin from shrimp [19]. (iii) When the same type of allergen molecules approximates in a multimeric way either via covalent binding, disulfide bridges, or just by simple aggregation, they again present the same epitope in a continual fashion (Fig. 2). The list showing that allergens have a tendency to multimerize is growing longer every year (Table 1). Importantly it includes not only airborne allergens, but also injective and food allergens.

In our own studies, we analyzed whether this phenomenon also applies to the major birch pollen allergen Bet v 1, a rather small globular protein. By different in vitro tests it could be demonstrated that a solution of Bet v 1 contained monomers, but even more dimers and oligomers of this allergen. Comparing solutions which contained either solely Bet v 1 monomers or oligomers it could be confirmed that dimerization is a crucial step for activating B memory cells as well as for eliciting histamine release in a murine in vivo model [20]. Thus in both, the sensitization and the effector phase of type I allergy, oligomerization of allergens is an important principle.

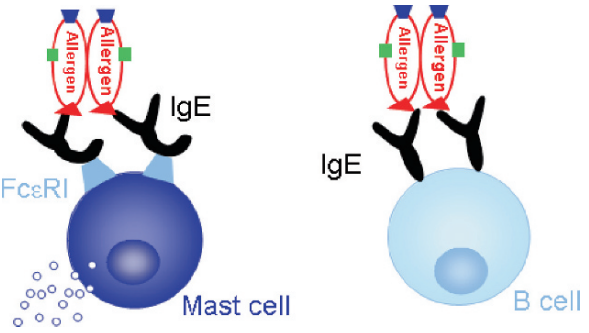

Fig. 2 Allergens have to display several epitopes for the cross-linking of IgE-antibodies on effector cells like mast cells (**a**) or B cells (**b**). The presentation of these epitopes can either occur in multivalent form of different epitopes, in a repetitive form of the same epitope, or after multimerization of the allergen again in a repetitive manner of the same epitope

Table 1 Examples of allergens, which are able to form dimers or multimers (in alphabetical order)

Allergen (source)	Citation	Year of publication
ABA 1 (*Ascaris*, nematodes)	McGibbon et al. Mol Biochem Parasitol 39:163	1990
ABA-1 (*Ascaris*, nematodes)	Xia et al. Parasitology 120:211	2000
Alt a 1 (*Alternaria*, fungi)	de Vouge, Int Arch Allergy Immunol 111:385	1996
Ara h 1 (peanut)	Shin et al. J Biol Chem 273:13753	1998
Ara h 1 (peanut)	Maleki et al. J Immunol 164:5844	2000
Ara h 2 (peanut)	Sen et al. J Immunol 169:882	2002
Bet v 1 (birch pollen)	Wellhausen et al. Z Ernährungswiss 35:348	1996
Bet v 1 (birch pollen)	Schöll et al. J Immunol Dec 15;175:6645	2005
Bovine dander allergens	Rautiainen et al. Allergy 51:378	1996
Can f 1 and 2 (dog dander)	Kamate Y et al. Int Arch Allergy Immunol 142:301	2007
Dac g 5 (grass pollen)	van OOrt et al. Int Arch Allergy Immunol 136:113	2005
Der p 1 (house dust mite)	de Halleux et al. J Allergy Clin Immunol 118:971	2006
Equ c 1 (horse dander)	Gregoire et al. Acta Cryst D Biol Cryst 55:880	1999
Equ c 1 (horse dander)	Lascombe et al. J Biol Chem 275:21572	2000
Fel d 1 (cat dander)	Grönlund et al. J Biol Chem 278:40144	2003
Parvalbumin (fish)	Das Dores et al. Allergy 57 (Suppl 72):79	2002
Phl p 1 (grass pollen)	Petersen et al. Clin Exp Allergy 28:315	1998
Phl p 1 (grass pollen)	Fedorov et al. RCSB Protein Data Bank;1 N10	2003
Phl p 5b (grass pollen)	Rajashankar et al. Acta Cryst D Biol Cryst 58:1175	2002
Phl p 7 (grass pollen)	Verdino et al. EMBO J 21:5007	2002
Profilin (panallergen)	Wopfner et al. Biol Chem 383:1779	2002
Sol i II (fire ant)	Hoffmann J, Allergy Clin Immunol 91:71	1993
Tropomyosin (e.g. fish)	Gimona et al. PNAS 92:9776	1995
Tropomyosin (seafood)	Reese et al. Int Arch Allergy Immunol 119:247 (review)	1999
Ves v 5 (wasp venom)	Suck et al. Int Arch Allergy Immunol 121:284	2000

In line with our results it has been shown that the allergenic potency is even reduced if dimerized proteins are broken down to their single-protein structure, for instance in grass pollen group 5 allergens [21]. Therefore, in contrast to earlier suggestions [22, 23], the polymerization of allergens does not necessarily lead to

hypoallergenicity, but rather to the contrary if epitope spacing is narrow enough to cross-link IgE. This was also shown with Ara h 1, a major peanut allergen, where IgE-binding was retained when higher polymers were created [24]. These data are further underlined by the recent observation that wild-type dimers and trimers of Dau c 1, the carrot allergen, showed an enhanced immunogenicity compared to monomers and simultaneously retained their IgE-cross-linking capacity [25]. The authors therefore assumed that oligomerization might not be a universal method to create hypoallergenic molecules [25, 26].

Oligomerization further provides a quite good explanation for the activation of B cells in triggering IgE-production, because the BCR possesses only one certain specificity. Moreover, different methods have revealed that allergens often comprise only one predominant IgE epitope [27–29], which can sometimes be simply due to their small size [30]. In this setting, the presentation of contiguous epitopes able to cross-link BCR or cytophilic antibodies could only occur via oligomerization. In summary, the presentation of several identical epitopes on the surface of proteins might substantially contribute to their allergenicity and immunogenicity.

Allergenic Potential of Proteins

Other facts described to influence the allergenic capacity of proteins are related to their concentration, their stability, their behavior after treatment like heating or digestion, and their modification arising from environmental influences like chemicals, ozone, diesel exhaust particles, etc. Importantly, some might again lead to the multimerization of the proteins, which is discussed above. In this respect, the influence of ozone on the concentration of allergenic protein produced in pollen is important. It was shown in grass pollen that the allergen concentration could reach up to 6 mg/ml. At these high concentrations unspecific aggregation of proteins occurs, likely to cause an enhancement of the allergic potential of these pollen proteins [31].

Not directly affecting the allergenic molecules but improving their tissue penetration or uptake by phagocytes are several accompanying substances with adjuvant properties. For pollen allergens, it was revealed that in an aqueous extract from pollen grains of grass or birch not only the allergenic proteins are solubilized, but additionally products from linolenic and linoleic acid are present [32]. Added to isolated polymorphonuclear granulocytes in vitro, these lipid mediators caused migration and activation of cells from allergic as well as from nonallergic individuals. Furthermore, tested on dendritic cells (DCs) in vitro, the aqueous extract of birch pollen led to the inhibition of IL-12 p70 production of activated DC, and changed the chemokine receptor and chemokine production profile in a way that resulted in DC with an enhanced Th2-attracting and a reduced Th1-cell polarizing capacity. The responsible factors were identified as phytoprostanes [33, 34]. Therefore, when pollen grains reach the mucosal surfaces of the organism and release their bioactive substances, these pollen-associated lipid mediators (PALMs) [35] may act as proinflammatory stimuli, independent of the allergen.

The immunologic potential of proteins is further influenced by air pollutants and nitration of proteins [36, 37]. The nitration process investigated in vitro as well as in vivo in a mouse model did not only enhance the allergenic potency of the airborne allergen Bet v 1, but did also influence the allergenicity of ovalbumin, a major egg allergen. This modification up-regulated the T cell stimulatory capacity as well as the antibody production in the mouse model. Importantly, also the IgE-binding and the IgE-cross-linking efficacy of both proteins was enhanced in vitro in a hexosaminidase-release assay using human sera of allergic patients. The underlying mechanism could be ascribed to a facilitated uptake and presentation by antigen-presenting cells (APCs) by improved recognition of epitopes on the antigens after the nitration process. It is tempting to speculate that these effects were also promoted by oligomerization, as it was shown that chemical treatment of the glutathione S-transferase with peroxynitrite, which is also associated with protein oxidation and/or tyrosine nitration [38], and the nitration of the presynaptic protein a-synuclein [39] led to increased formation of dimers, trimers, or higher oligomers.

Requirements for Food Allergens

The physiological demand of food proteins is to get properly digested to achieve nutritional value and at the same time be ignored by the immune system. However, for alimentary proteins a number of variables may affect the digestibility and have to be taken into consideration. Already, before being applied to the organism, they could have undergone a variety of treatments, like heating, high pressure, boiling, etc. Only proteins that present a certain stability of their conformation resist these food-processing methods and also the harsh environment in the stomach and intestine. In these organs, the pH changes dramatically and several proteolytic enzymes are present [40]. Thus, it was believed that only conformationally stable food proteins are able to reach the intestine in an intact form and induce or bind antibodies which are specific for their native epitopes. However, there are several allergens, which have been shown to be labile in in vitro gastric digestion experiments and nevertheless can per se induce allergy without any crossreactivity to pollen [41, 42]. A dysfunctional peptic digestion in the stomach would provide an explanation for the induction of adverse humoral immune responses to a normally digestion-labile food protein. Circumstances which affect gastric digestion are numerous, among them the widely practiced intake of anti-acid drugs. Their main function is the increase of the gastric pH. For the treatment of dyspeptic disorders like reflux, gastritis, and stomachache a level of $pH > 4$ is the treatment goal. Under these conditions the inactive protease pepsinogen cannot be converted into its active form and the breakdown of proteins is hindered (Fig. 3). When the hence persisting food proteins reach the immune cells in the intestine, they indeed provoke an IgE-antibody response resulting in sensitization and clinically relevant allergy against food [41, 43]. In human gastroenterologic patients, the relative risk is increased by 10.5 for sensitization against food after anti-acid treatment [41, 44]. Importantly,

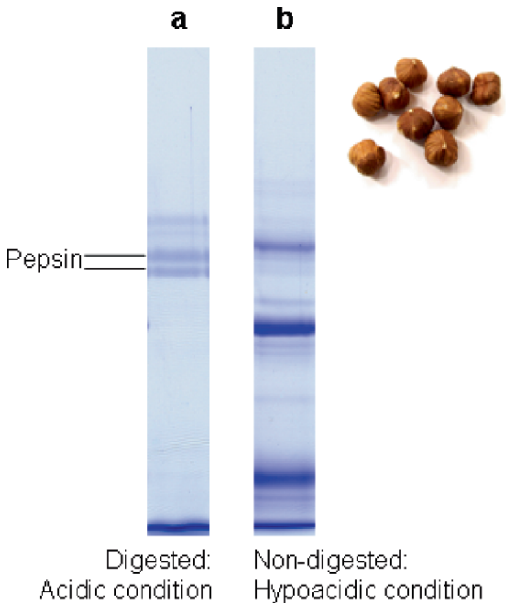

Fig. 3 (**a**, **b**) A number of digestion-labile food proteins, like milk, codfish or hazelnut, can induce food allergy when persisting gastrointestinal digestion. The example shows that proteins of hazelnut are readily degraded in simulated gastric fluid at pH 1.2 after 30 s. (**a**) However, they resist peptic digestion for more than 2 h when the pH is increased to 5.0 (**b**)

taken during pregnancy, anti-acid drugs did not only induce sensitization against food in the mother in murine studies, but could also shift the immune response towards a Th2-dominance in the offspring [45]. The prerequisite for the induction of an allergic response even against a digestion-labile food protein therefore seems to be a conformational stabilization and persistence in the gastrointestinal tract.

Type II Hypersensitivity Reactions

These reactions are mediated by IgM- or IgG-antibodies, evoking two mechanisms: on the one hand phagocytes (macrophages, neutrophils, eosinophils) and killer cells (K- or NK-cells) can attach to the antibody-opsonized cells via their Fcγ-receptors. This cross-linking induces antibody-dependent cellular cytotoxicity (ADCC), phagocytosis, and the release of inflammatory mediators, which can finally lead to cellular destruction and the amplification of the inflammatory response [46–48]. On the other hand, complement-mediated lysis and induction of the membrane attack complex may occur.

The antigens which provoke the antibody production can either be endogenous, i.e., part of the patient's own cells (autoimmune reaction); or they are of exogenous origin (e.g., drugs or other haptens) and are attached to cell membranes (hypersensitivity reaction). Examples are drug-induced hemolytic anemia, granulocytopenia, and thrombocytopenia with symptoms like petechiae or glomerulonephritis [49] (e.g., Goodpasture syndrome), where autoantibodies against the basal membrane of

the kidney glomeruli are formed. The onset of this immune reaction after antigen contact takes place within minutes to hours.

Type III Hypersensitivity Reactions

The underlying mechanism of these reactions is the formation of immune complexes, consisting of an antibody and the respective antigen. In situations with antigen excess, these complexes are deposited in the form of precipitates in different tissues, mainly endothelia of blood vessels in lung, kidneys, joints, and skin. They induce local inflammation and damage via complement activation and recruitment of macrophages, neutrophils, and platelets [50]. The resulting disease is serum sickness, a historical term derived from the early days of immunology, when antisera raised in animals, preferentially horses, were applied to patients as passive immunotherapy. Today, elicitors are infusions of foreign (i.e., heterologous) sera, for instance anti-thymocyte globulins, anti-snake venom [51], or anti-tetanus toxin serum [52]. Also antibodies used for therapy, which are chimeric and/or have been humanized, may still act as antigens and cause serum sickness due to minimal foreign antigenic determinants. Vasculitis and pruritus in legs or hands have also been observed during the maintenance phase of allergen-specific immunotherapy [53, 54]. Symptoms can take as long as 14 days to appear after exposure, and may include rashes, arthralgia, fever, lymphadenopathy, shock, hypotension, and splenomegaly. In the case that preformed antibodies precipitate antigen at the site of entrance, the so-called Arthus reaction may occur. Symptoms can especially be evoked by the repeated application of drugs [55]. Allergens which cause type III hypersensitivity according to the Arthus reaction are mold (farmer's lung) [56]; bird-related antigens from excreta, blood, or feather (bird breeder's lung) [57]; and a lot of other organic reagents [58]. The final consequence is loss of elasticity of the lungs through pulmonary fibrosis.

Type IV Hypersensitivity Reactions

Type IV hypersensitivity is also known as cell-mediated or delayed-type hypersensitivity. The mechanism involves T-lymphocytes and macrophages. The antigen is processed and presented to T-lymphocytes by APC [59]. After sensitization, subsequent antigen contact causes specific cytotoxic T-cells to damage the tissues, whereas helper T-cells secrete cytokines, thereby activating cytotoxic T-cells and recruiting and activating macrophages. All of these cells in concert cause the inflammation, where symptoms usually develop within 2–14 days after exposure to the antigen or allergen.

The classical example of this hypersensitivity is the tuberculin (Montoux) reaction, which peaks 48 h after the injection of the mycobacterial antigen in a sensitized individual. After intense exposure with antigen an "early delayed hypersensitivity" can

already occur after 6 h [60]. Type IV hypersensitivity is also involved in the pathophysiology of many autoimmune and infectious diseases (leprosy, blastomycosis, histoplasmosis, toxoplasmosis, leishmaniasis, etc.) and in granuloma formation due to infections and foreign antigens. Another form of delayed hypersensitivity is contact dermatitis (caused by poison ivy, chemicals, heavy metals like nickel, etc.) [61].

Combined Hypersensitivity Reactions

There are several examples, where different mechanisms from the hypersensitivity classes described above in parallel are responsible for the induction of symptoms. Here, only two prototypes of diseases shall be mentioned, namely atopic dermatitis (AD) and celiac disease.

Atopic dermatitis can occur due to genetic predisposition and different immunological imbalances [62–64]. Immunological triggers for exacerbation are airborne allergens, alimentary allergens, products from microbes, or contact allergens. Currently, the proposed model describes a predominant Th2-milieu in the initial stage of acute lesions. In this phase, symptoms can arise due to IgE-mediated reactions in allergic patients, for instance when IgE specific for airborne allergens or food allergens is present. The high affinity receptor for IgE FcεRI has been found to be overexpressed on Langerhans cells in atopic dermatitis. These APCs therefore act as allergen trappers and further can efficiently process and present antigen peptides to T cells, which then are responsible for the Th2-cytokine release [65]. Eosinophils and their toxic products in the dermis account for a major part of the inflammation [66], and mast cells are held responsible for induction of pruritus and proinflammation via cytokines. Peripheral monocytes in addition produce prostaglandin E2, which reduces IFN-γ-production by T cells and thereby favors the initial Th2-milieu. Furthermore, it directly enhances IgE-production with increased secretion of IL-4, IL-5, and IL13.

AD can also present as a combination of type I and type IV mechanisms, where IgE-antibodies are induced due to atopic predisposition and in addition T cells are responsible for the chronic manifestation of eczema. Here, a mixed Th1/Th2 environment is associated with chronicity. The involvement of the delayed-type component can be proved by patch testing with allergens, where especially contact and nutritional allergens play a critical role.

Celiac disease results from type II, III, and IV hypersensitivity (Fig. 4), affecting the small intestinal mucosa of genetically predisposed individuals and leading to symptoms like diarrhea, weight loss, retarded growth, and secondary anemia.

The exogenous trigger responsible for the type IV reaction is gliadin from gluten in food sources like wheat, rye, and barley [67]. It is presumed that some undigested products of the cereals are presented to the small bowl [68], and can activate specific T cell populations. Additionally, gluten/gliadin derivates, which can stem from deamidation by the tissue transglutaminase (tTG), bind to HLA-DQ2 or DQ8 restricted CD4 T cells and evoke the type IV reaction mediated by cytotoxic T cells or T helper cells.

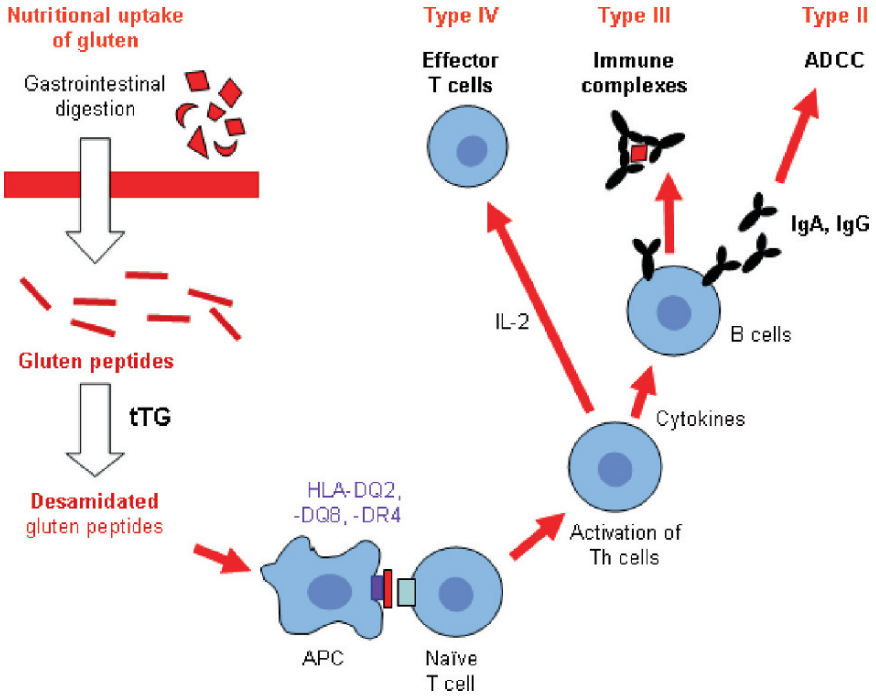

Fig. 4 Pathophysiology of celiac disease. Celiac disease is a combined hypersensitivity reaction of type II, III, and IV, where the exogenous trigger is gliadin from gluten, and the endogenous antigens are tissue transglutaminse, endomysium, and enterocytes

Furthermore, an antibody-dependent response is induced by cytokine release from the previously activated T cells. Predominantly, IgA and IgG against gliadin (exogenous) and against multiple auto-antigens including the tissue enzyme tTG [69] as well as calreticulin of enterocytes [70] and endomysium [71], are produced. These antibodies contribute to hypersensitivity reactions of type II (ADCC) and type III (immune complex formation).

Summary

For type I allergy reactions – the focus of the present chapter – the importance of the conformation of allergens, the presentation of multiple epitopes, e.g., via dimerization, and the stability thereof have been shown. Important novel findings show that modification of allergens by environmental factors, or persistence of their conformation during the gastrointestinal passage are key factors determining the allergenicity of a protein.

The knowledge about such basic mechanisms may help to design strategies to prevent a further increase in allergy prevalence in the future.

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Pathogen Recognition and New Insights into Innate Immunity

Joel N. Kline

Introduction

The vertebrate immune system is tasked with detection and elimination of "foreign agents" while causing minimal harm to the host. These responses may be clustered as "adaptive" or "innate." Adaptive immunity is specific and elegant, but slow; recognition of antigens requires random and diverse arrays of antigen receptors (T and B cell receptors) and clonal selection and expansion of effector and antibody-producing cells. Repeat offenders induce more rapid responses due to immunological memory, but newly encountered dangerous organisms could overwhelm the host while differentiation and expansion occurred. In contrast, innate immunity (the most ancient and conserved elements of the immune system) can respond to a range of potentially harmful organisms in a rapid, vigorous, and nonspecific manner; innate responses require no previous exposure but rely on pattern-recognition of "danger signals" for host defense.

Pattern recognition receptors (PRRs) have evolved as an important arm of the innate immune system; examples include mannose receptors (that bind to terminal mannose groups on microbial glycoproteins, facilitating their endocytosis), nucleotide-binding oligomerization domain proteins (NODs, that promote intracellular recognition of microbial peptidoglycans), and the Toll-like receptors (TLRs). TLRs are highly conserved receptors originally identified in drosophila that share both structural and functional characteristics. TLRs are found both on the cell surface and within the cell, where they facilitate recognition of and response to microbes and their components (Pathogen-Associated Molecular Pattern, PAMPs, such as endotoxin [TLR-4], bacterial flagellin [TLR-5], viral RNA, [TLR-3, -7, -8], and bacterial DNA [TLR-9]). Downstream of ligand/TLR engagement are cascades that induce the transcription of cytokines, maturation of inflammatory

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cells, and ultimately engagement of both additional innate and adaptive immune mechanisms.

Mechanisms of Innate Immunity: PAMPs and PRRs

The last decade has witnessed an explosion of information regarding PRRs and the PAMPs that they recognize. PAMPs are conserved products of microbial metabolism that are generally unique to microorganisms and absent from the host (examples include lipopolysaccharides [LPS], lipoproteins, peptidoglycan, and lipoteichoic acids): this allows distinction between "self" and (potentially dangerous) non-self. PAMP recognition emerged early in the evolution of host-defense systems; many PAMPs are detected by innate immune systems of invertebrates and plants. PAMPS are often essential for microbial survival, preventing the generation of escape mutants, and are invariant between microorganisms of a given class, allowing a limited number of germ-line-encoded PRRs to detect nearly any microbial infection. Although the target molecule may differ between microbial species, common molecular patterns can be found in all. For example, the lipid-A portion of LPS is invariant and serves as a PAMP; in contrast, the O-antigen varies between species and is not a strong activator of the innate immune system. Important PRRs that both serve in host defense against microorganisms and also appear to play a role in innate immune responses in atopy and asthma include the TLRs, CD14, and the NODs. These receptors are found both on the cell surface (where they may serve to transduce signals from randomly encountered as well as cell-presented PAMPs) and within the cytosol of cells (recognizing endocytosed PAMPs as well as those that have the ability to cross the cell membrane).

PAMPs and Asthma: The Hygiene Hypothesis

Early life exposure to and recognition of microbes and microbial products (PAMPs) is important for development and programming of immune responses. It has been proposed that a reduction in such exposures, due to modern public health and medical practices as well as social and environmental changes, has had the unanticipated consequence of immune dysregulation, resulting in an increased prevalence of inflammatory disorders. The "Hygiene Hypothesis" speculates that our immune system, evolved to optimize protection from/coexistence with environmental microorganisms, is now faced with a paucity of appropriate targets [39]. Reduced exposures to PAMPS, and thus more limited engagement of PRRs and innate immune responses, has been linked to increased inflammatory disorders of a variety of types.

The prevalence and severity of asthma and atopic disorders have increased substantially over the last 4 decades, especially in developed nations [2]. Currently,

over 20 million people in the United States currently have asthma and over 50 million suffer from rhinosinusitis or other atopic conditions; these disorders may begin early in childhood or later in life. Underdeveloped or third world nations have been relatively spared this epidemic, as are certain populations. Among others groups with reduced risk are included: individuals raised in rural, agricultural settings with early-life exposure to barns [5, 35]; late birth-order children from larger families [43] and those who enter day-care at an early age [39]; and survivors of certain non-respiratory infections [38]. Children at high-risk (e.g., with atopic parents) for developing asthma are somewhat protected if they grow up with a dog in the house [44], and children who are raised in parasite-endemic areas demonstrate increased rates of asthma if they are dewormed [47, 48]. These observations and others suggested a link between childhood infections (e.g., illnesses brought home from school by an older sibling) or microbes and their products (e.g., those found in high concentrations in agricultural settings) and resistance to atopic disorders. In addition to the modern epidemic of asthma and atopy, type I diabetes, multiple sclerosis, and inflammatory bowel disease have also risen dramatically in prevalence; these maladies are most common in industrialized nations and are also on the rise in traditional societies that are undergoing modernization or westernization.

The linking of reduced susceptibility to asthma with early-life exposures to pathogens or microbial products was initially puzzling, as infections and the resulting inflammation have long been associated with asthma exacerbations rather than prevention. Atopic disorders are characterized by a skewing of immune responses towards a Th2 pattern; Th2 cytokines promote eosinophilia, class switching of B cells to the production of IgE antibodies, goblet cell metaplasia and airway mucus hypersecretion, and airway hyperreactivity among other effects. Although the fetal immune system is typically Th2-oriented [32], newborns ordinarily exhibit a rapid decline in this tendency; one hypothesis is that early-life exposure to microbial products (a number of PAMPs have been proposed to account for the Hygiene Hypothesis – see Table 1) induces a Th1 milieu, and since Th1 and Th2 responses are counterregulatory, suppression of Th2 activity may result. It was initially proposed, therefore, that this suppression does not occur (perhaps resulting in a life-long tendency towards Th2 responses to otherwise innocuous antigens) in the absence of early-life infections or related exposures, providing an immunologic basis for the

Table 1 Potential mediators of hygiene hypothesis

PAMP	Source	PRR
Endotoxin	Gram-negative bacteria	TLR-4, CD-14
CpG DNA	Bacteria, viruses, protozoa	TLR-9
Muramic Acid/Peptidoglycans	Bacteria	TLR-2, NOD
ES-62 [phosphorylcholine-containing glycoprotein]	Helminths	???
Glucans	Fungi	
Lipoarabinomannan (LAM)	Mycobacteria	

Hygiene Hypothesis. Considerable doubt has arisen regarding the accuracy of this proposed schema; although increased Th2 responses would account for a higher prevalence of atopy, this would not account for increases in Th1-skewed conditions such as multiple sclerosis and inflammatory bowel disease, nor would they explain the ability of Th2-skewing organisms (e.g., helminths) to protect against atopic disorders. More compelling is an alternative proposal, that a lack of exposure to PAMPs impairs development of appropriate regulatory responses.

Toll-Like Receptors, NODs, and Surfactant Proteins

The family of Toll-like receptors (TLRs) is among the best characterized PRRs. The Toll protein was first identified in *Drosophila* through its control of embryonic dorsal-ventral patterning. It was subsequently found to play an important role in *Drosophila* immunity, as *Toll* mutants were inordinately susceptible to fungal infections. Other related fly proteins, also related to host defense, include 18-wheeler, Tollo, and Tehao [40]; these promote antimicrobial peptide production (defensins) among other activities. The first human homologue (hToll, later TLR-4) was identified in 1997 by Medzhitov [28]; other TLRs have been since recognized, with the current family including 13 mammalian TLRs (TLR1-TLR11) that recognize distinct microbial PAMPs (Table 2) [41].

Members of the TLR family are type I transmembrane PRRs, most of which include a conserved intracellular region, the Toll/IL-1 Receptor (TIR) domain,

Table 2 TLR, ligand [synthetic in brackets], natural source

TLR	Ligand	Source of natural ligand
TLR-1	Triacyl Lipopeptides	Bacteria
TLR-2 [with TLR-1, TLR-6]	Lipopeptides, lipoteichoic acid, peptidoglycan; zymosan, phospholipomannan; GPI Anchor; Envelope Protein	Bacteria, fungi, protozoa, virus
TLR-3	Double stranded RNA; [Poly (I:C)]	Virus
TLR-4	Lipopolysaccharides; mannan, glucuronoxylomannan; Glycoinositolphospholipids; RSV fusion protein	Gram-negative bacterial cell wall, fungi, protozoa, virus
TLR-5	Flagellin	Bacteria
TLR-6	Diacyl lipopeptides; Zymosan	Bacteria, fungi
TLR-7	Single-stranded viral RNA; [Imiquimod]	Virus
TLR-8	Single-stranded viral RNA, Imiquimod	Virus
TLR-9	DNA containing unmethylated CpG motif; Hemozoin	Bacteria, protozoa, virus; protozoa
TLR-10	Unknown	
TLR-11 (murine)	Profilin-like molecule	Protozoa
TLR-12 (murine)	Unknown	
TLR-13 (murine)	Unknown	

which mediates protein–protein interactions with downstream signal transduction elements and varying numbers of extracellular leucine-rich repeats (LRR), involved in ligand binding and TLR dimerization. Supporting its conserved role in innate immunity, TIR is also found in a variety of transmembrane and cytoplasmic proteins in animals and plants that play a role in host defense. In addition to the Toll family, other proteins with TIR domains include IL-1 receptors (IL-1R), Myeloid Differentiation Factor-88 (MyD88), and TIR-containing Adaptor Protein (TIRAP).

Engagement of TLRs by specific, but broadly expressed ligands (PAMPs such as lipoteichoic acid from gram-positive bacteria [TLR-2], lipopolysaccharides in gram-negative bacterial cell walls [TLR-4], and CpG motifs in bacterial DNA [TLR-9]) activate differential, but overlapping signaling pathways. TLRs signal through a group of adaptor proteins that activate downstream kinases and can lead to activation of nuclear factor κB (NF-κB), mitogen-activated protein kinases (MAPK) and other cascades, resulting in induction of immune response gene activation. TLR engagement leads to both MyD88-dependent and MyD88-independent responses. Specificity of responses to PAMPs is promoted by differential patterns of receptor expression (including cell surface and intranuclear) and cell types. Some TLRs are broadly expressed whereas others (e.g., TLR-9) demonstrate a more restricted expression pattern.

Well before identification of the TLR system, it was recognized that bacterial DNA is immunostimulatory. More than a decade ago, Krieg first reported that the cytosine–guanine dinucleotide (known, because of the phosphate bond, as CpG), in specific base sequences (CpG motifs) in bacterial DNA, were immunostimulatory and capable of strong B cell activation [24]; we now understand that these effects are due to ligation of TLR-9. Prokaryotic DNA contains the expected frequency of CpG dinucleotides (1:16 base pairs) which are suppressed in eukaryotic DNA (1:50–1:100 base pairs); moreover, when present, the cytosine in eukaryotic DNA is often methylated, which silences or reduces the immunostimulatory properties of the motif. Subsequent studies demonstrated that synthetic oligodeoxynucleotides (ODNs) centered on CpG motifs (CpG ODN) recapitulated the patterns of activation induced by bacterial DNA, setting the stage for the use of CpG ODN as immunotherapeutic agents.

CpG oligodeoxynucleotides are categorized based on their structure; three major classes of CpG-ODN have been described with distinct patterns of activity: A-Class CpG ODNs are based on a phosphodiester backbone; they have phosphorothioate poly-G motifs at the 5′ and/or 3′ ends that can form stable higher-ordered structures. These ODN are strong inducers of IFN-α secretion by plasmacytoid Dendritic Cells (pDCs), and poorly induce B cell proliferation. B-class CpG ODN (of which CpG 7,909, the ODN intended for use in these proposed studies, is a member) consist of a phosphorothioate backbone; these ODN strongly induce B cell proliferation and pDC maturation, strongly induce IL-10, but poorly induce IFN-α secretion from pDCs. C-class ODN have a hexameric CpG motif linked by a T spacer to GC-rich palindromic sequences; they are entirely phosphorothioate and have no poly-G sequences. C-class CpG ODN induce both B cell activation and IFN-α secretion.

When encountered by immune cells, CpG DNA undergoes endocytosis, binds to TLR-9, and is translocated to the nucleus where it induces a series of downstream immune responses. In plasmacytoid dendritic cells (pDCs), TLR-9 activation is dependent on IL-1 Receptor-associated kinase (IRAK)-4 and interferon regulatory factor (IRF)-7 and requires direct interactions between IRF7 and MyD88, TNF-α-receptor activated factor (TRAF)-6, and IRAK-1 [16, 18, 19, 42, 46, 49, 50]. TLR9 activation induces NF-κB and other intracellular pathways that initiate a rapid innate immune response characterized by cytokines and chemokines including IL-6, TNF-α, type I interferons, IFN-inducible protein-10 (IP-10), and IL-10. Downstream responders to these signals include NK-cells, T cells, and other cells, which amplify and modulate the immune response. Later effects include induction of costimulatory receptors, immunoglobulin isotype switching by B cells, and activation of a cascade of cellular responses p. B cells activated via TLR9 are more responsive to antigen stimulation and readily differentiate into plasma cells, thus modulating the adaptive immune response. The sum total of these inflammatory cascades was initially viewed as Th1-skewing; as a result of the induction of IFN-γ from NK-cells [9], IFN-γ, and IL-12 from lymphocytes [22], and IL-12 from antigen presenting cells (APCs) [7], TLR-9 activation can strongly promote Th1 responses [8]. It is increasingly understood, however, that the effect of TLR-9 on regulatory responses (characterized by increased IL-10 and suppression of both Th1 and Th2 effects) may be more important in the Hygiene Hypothesis as well as in developing novel therapies.

Based on the apparent role played by TLR ligand exposure in modulating disease susceptibility, a search for linkages between *TLR* genetic variants and asthma and atopy has been eagerly pursued. Swedish investigators examined a single common *TLR4* polymorphism in a cohort of Swedish school children and found an association with asthma and reduced LPS-induced IL-12 (p70) release [4]. In contrast, two family-based cohorts were evaluated for association between *TLR4* polymorphisms and asthma and found no evidence of association for any of the polymorphisms tested and asthma- /atopy-related phenotypes [33]. Further muddying the waters, a UK family study (including 336 families containing at least 2 asthmatic siblings) found no association between *TLR4* polymorphism and an asthma diagnosis or severity, but did find linkage of atopy severity scores (skin-prick tests and specific IgE), with higher values in subjects with Asp/Gly or Gly/Gly genotypes (1.8 ± 1.1, $n = 39$) compared to those with Asp/Asp genotype (1.2 ± 1.0, $n = 279$) ($P = 0.003$) [45]. Similarly, disparate findings have been reported for *TLR2* [11, 34], *TLR9* [3, 25, 30], and *TLR10* polymorphisms [26], among others.

Although critical in innate immunity, TLR engagement also plays a role in controlling adaptive immune responses. Engagement of TLRs on APCs upregulates costimulatory factors (CD80 and CD86) and MHCII molecules and induce cytokines (e.g., IL-12), chemokines, and their receptors, triggering maturation and activation of dendritic cells (DCs). Induction of CD80/86 on APCs by TLRs leads to the activation of T cells specific for pathogens that trigger TLR signaling. B cell activation and immunoglobulin class switching may be modulated both directly by TLR engagement and indirectly, secondary to elaboration of cytokines by other

immune cells. DCs appear to have a significant role in linking innate and adaptive immunity. Their maturation and activation develops in response to engagement of TLRs and other PRRs, such as NOD2, Diectin1 (a β-glucan receptor), and the mannose receptor. These exposures promote a phenotype that skew responses to a presented antigen towards a Th1 or Th2 pattern.

Other important PRRs in innate immunity include the family of NODs and surfactant proteins. NODs are cytosolic proteins that recognize muramic acid and bacterial peptidoglycans (a ubiquitous component of bacterial cell wall) [12]. NOD1 and NOD2 recognize different peptidoglycan motifs, and may confer some specificity to this system (NOD1 appears to respond more to peptidoglycan from gram-negative bacteria). Like TLRs, NOD activation can induce upregulation of nuclear factor – κB, and also serves to regulate inflammation through enhancement of casapase-1 and caspase-9 actions, which promote apoptosis. NODs interact with other components of innate immunity, such as the TLR system; peptidoglycans can activate TLR2 as well as NODs. Surfactant protein A and B are members of the collectin family of C-type lectins. Like TLRs, they link adaptive and innate immunity; like TLRs, specific polymorphisms have been linked to susceptibility to chronic lung disorders, as well as infections [31].

Novel "Anti-hygenic" Therapeutic Approaches to Asthma and Atopic Disorders

An improved understanding of the innate immune responses associated with an increased prevalence and severity of atopy and asthma (and, perhaps more importantly, those mechanisms which provide protection) has led to the development and investigation of several novel therapeutic approaches. Rook and colleagues have proposed that saprophytic mycobacteria, such as *Mycobacterium vaccae*, along with lactobacillae and certain helminths serve as immunologic "old friends," historically responsible for immunologic balance and lacking in modern society [36]. Murine studies have found that heat-killed *M. vaccae* can suppress asthmatic inflammation through induction of allergen-specific regulatory cells [51]. Based on these and other studies, a clinical trial was conducted that demonstrated a substantial (but not statistically significant) reduction in late allergic response and a trend towards reduced IgE [6]. Endotoxin exposure can induce Th1 responses and inhibit atopic inflammation in murine models [14, 23], and TLR4 analogs are in development that may prove to provide therapeutic benefit for human asthma [37].

CpG DNA, the TLR-9 agonist, is also being developed as a therapeutic agent for asthma and atopic disorders. We first proposed the potential therapeutic use of CpG-ODN for asthma and atopy based on murine studies in which we were able to prevent atopic sensitization. Using a schistosome egg model of atopic asthma, we found that coadministration of CpG-ODN along with the sensitizing antigen suppressed both pulmonary (airway eosinophilia, bronchial hyperreactivity) and systemic (elevated serum IgE levels, Th2 cytokine production) manifestations of atopic asthma [21].

Although demonstration that CpG ODN administered at the time of allergen sensitization prevents the manifestations of atopic asthma provides a plausible mechanism to explain a potential role for PAMPs in protecting against atopic disorders, this situation is quite different from that encountered in clinical settings. We therefore next explored the effects of CpG ODN on established atopic asthma. Using an ovalbumin (OVA) model of atopic asthma, we examined the effects of treating previously sensitized and challenged mice [20]. We found that mice treated with OVA and CpG-ODN demonstrated nearly complete reversal of eosinophilic inflammation, and marked suppression of bronchial hyperreactivity and IgE responses. Using a mucosal (trans-nasal) approach, mice that received CpG-ODN alone as well as those treated with CpG-ODN and allergen had profound therapeutic responses [17].

There is a growing experience with the effects of CpG ODN in humans; in addition to normal subjects, it has been studied as a treatment or adjunct in infectious disease, malignancy, atopic rhinitis, and asthma. In general, administration of CpG-ODN has been found to be safe. Phosphorothioate oligonucleotides are known to induce sequence-independent backbone-related effects. Chronic dosing of phosphorothioate ODN in rodents results in a dose-dependent mononuclear cell infiltration of the kidney and liver, but these effects have not been seen in monkeys or humans [27]; no adverse effects on renal function has been seen in clinical trials using phosphorothioate ODN. The major dose-limiting acute toxicity of phosphorothioate ODN in primates stems from activation of the alternative complement pathway; leukocyte activation can increase vascular permeability resulting in significant hypotension [15]. This toxicity requires a threshold blood concentration of 40–50 μg/ml, which is generally the result of rapid intravenous administration [29].

CpG-specific toxicity may also result from TLR9 activation. The safety profile of several TLR9 agonists in humans has been observed in clinical trials over a more than 1,000-fold dose range from 0.0025–0.81 mg/kg; a maximal tolerated dose in humans has not been reported to date. The most prominent adverse events are dose-dependent local injection reactions (such as erythema, pain, swelling, induration, pruritus, or warmth at the site of injection) and systemic reactions including headache, rigors, myalgia, pyrexia, nausea, and vomiting. Depending on the dose, systemic symptoms typically develop within 12–24 h of dosing and persist for 1–2 days. At the low doses used in vaccine trials there seems to be a slight increase in the frequency of injection-site reactions, which are generally mild, above the frequency observed with the vaccine alone. Early concerns regarding the risk of inducing autoimmune responses have not been demonstrated in human trials, with no reports of anti-human antibodies or other markers of autoimmune responses. The most common adverse events include injection-site reactions (local pain, erythema, and induration) and mild systemic symptoms (fever, chills, fatigue, myalgias, arthralgias, and headache) [1].

A recent study has evaluated the effect of a novel CpG/allergen immunotherapy (AIC: the ragweed allergen, *Amb a 1*, conjugated to CpG oligonucleotides) for ragweed-induced allergic rhinitis [10]. Subjects were treated with increasing doses of AIC prior to being followed for two sequential ragweed seasons. The study

demonstrated that subjects treated with AIC had significantly better rhinitis scores during the ragweed season that immediately followed the course of therapy as well as during the following year. This study, although limited by a lack of comparison with standard immunotherapy, provides encouragement for the investigation of immune mechanisms invoked by CpG-ODN that are relevant to the induction of tolerance in allergic hypersensitivity.

One published report on the use of CpG ODN in human asthma [13], examined the effect of inhaled CpG-ODN on the pulmonary immune response and airflow obstruction resulting from allergen inhalation challenge. In that study, the group treated with CpG-ODN demonstrated induction of interferon-inducible cytokines and chemokines but no changes in allergen-induced airflow obstruction (neither early nor late) or sputum eosinophilia. These results support the conclusion that CpG-ODN are immunoactive, inducing a pattern of (interferon-related) responses that would be expected to suppress atopic inflammation; the failure to demonstrate reduction in clinically relevant manifestations of the asthma phenotype, however, suggests that these cytokines alone may not be sufficient to provide a therapeutic benefit. Other trials examining CpG-ODN in clinical asthma are ongoing.

Summary

The modern emphasis on the value of hygiene (e.g., "cleanliness is next to godliness") has led to a remarkable reduction in the epidemic and endemic infectious diseases that were the most significant cause of mortality from the earliest expression of human civilization until the nineteenth century. Resulting measures, including waste management strategies, improved housing, control of the food chain, and both preventive and therapeutic medical practices, may now be leading to unanticipated consequences, most notably an alarming increase in inflammatory disorders, including diabetes, inflammatory bowel disease, multiple sclerosis, and asthma and atopy. The Hygiene Hypothesis of asthma and atopy proposes that early-life exposure to microbes and microbial products (PAMPs) is required for appropriate programming of the developing innate immune system; an absence of such exposures leads to dysregulated inflammation.

The mechanistic basis for the Hygiene Hypothesis is the innate immune PRRs that identify PAMPs by recognition of critical and characteristic motifs. These receptors are believed to have developed secondary to strong evolutionary pressure to survive (coexist with) otherwise dangerous microbes. Recognition of a PAMP by a PRR initiates a cascade of signals that have not only immediate consequences (i.e., engagement of mechanisms that lead to elimination of the threat) but also developmental sequelae (i.e., instruction and programming of the immature immune system), especially when this occurs in early life.

Although numerous PRRs have been identified, the family of TLRs remains the best studied. These receptors (13 of which have been identified in mammals) act individually and in concert to transduce recognition of microbial patterns

into characteristic inflammatory pathways. The same mechanisms that protect against microbes are now being exploited in the development of novel therapeutic approaches to asthma and atopic disorders. CpG-ODN, the TLR-9 ligand, has been shown, in preclinical models, to be effective in the prevention and therapy of atopic asthma. Human studies have supported safety of its use; recent trials suggest benefit in the management of clinical disease.

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New Nomenclature and Clinical Aspects of Allergic Diseases

S.G.O. Johansson

Introduction

The incidence of allergic diseases has been increasing in all industrialized countries ever since 1819 when Dr. John Bostock in London reported about his own hay fever. Today the prevalence is in the order of 30%. Allergic reactions can occur in all age groups and in many different organs. Increased knowledge of these diseases and their mechanisms has improved patient care. However, the understanding of allergy and the nomenclature used to describe allergy and allergy-like reactions over the years has been confusing. In order to facilitate the cooperation and communication between scientists, allergy specialists, primary care doctors, and patients it is essential that we all use one and the same terminology.

The European Academy of Allergy and Clinical Immunology (EAACI) realized the importance of an unambiguous use of terms and in the late 1990s established a committee to work with this issue. The group's task was to propose a revised nomenclature for allergic and other related reactions. It should be possible to use regardless of type of allergic disease or organ involved and lead to a better understanding and better communication. After a period of interesting discussions the group formulated its proposal, which was published as an official EAACI Position Statement in *Allergy* in September 2001 [1]. The EAACI proposal was widely recognized and so far has been published, in its entirety or in summary, in more than ten different languages. The Position Statement has received a high impact factor ranking. On the EAACI's website a glossary of the most important terms in 24 languages is available.

In order to facilitate the global application of the revised nomenclature the World Allergy Organization (WAO) in 2002 created a Nomenclature Review Committee, with the task to update the EAACI proposal and, if necessary, suggest revisions

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that would facilitate the global acceptance. The report of the WAO committee was published in May 2004 in *Journal of Allergy and Clinical Immunology* [2] and basically agreed with EAACI's proposal, with a few additions. Special attention was given to skin diseases, an area where the terminology has been especially confusing, and where the EAACI had proposed some provisional terms awaiting the conclusion of further discussions among the specialists in the field.

Historic Background

The field of allergy has developed rapidly during the last decades. Knowledge of immunological mechanisms and pharmacological effects has improved our understanding [3].

The classic nomenclature for allergic reactions with the well-known types I–IV was introduced by Gell and Coombs in 1968 [4], and has been very useful. But too much importance has been given to the presumably antagonistic roles of antibodies and immunocompetent cells. This dichotomy is not in line with our current view of a dynamic cooperation within the immune system, triggered by dendritic cells, B-cells, and T-cells, mediated by effector cells of various types and involving antibodies, complements, chemokines, and cytokines. IgE as well as the last two groups have mainly been identified after Gell and Coombs introduced their classification of allergic reactions. Thus a revised nomenclature based on disease-initiating mechanisms, rather then roman figures, can now be developed.

Many of the terms used are, however, older still and they have been given varying meaning at different points in time and by different scientists. Not least does this concern the central term *atopy*. In the early 1920s Coca and Cooke [5] introduced the term "atopy" to denote certain hypersensitivity phenomena in man. Among other things they believed "atopy" to be hereditary, limited to a small group of humans, and associated with skin reactions of the immediate type ("wheal and flare"). At the time they were obviously not familiar with the work published by Prausnitz and Küstner in 1921 on passive transfer of immediate hypersensitivity by serum [6].

Coca and Grove continued their atopy research [7] and elucidated some important aspects, but they also arrived at some strange conclusions [8]. They reported the presence of heat-sensitive "bodies" which they called "reagins" due to their similarity in this respect to the heat-sensitive reagins active in the Wassermann reaction, i.e., complement. They concluded that it was "advisable for the present to avoid the term 'antibodies'," because there was "no evidence that these bodies appeared as the result of immunological stimulation."

In their original definition of atopy, Coca and Cooke only included allergic rhinitis and asthma. For what was called Prurigo Besnier, "confusing types of localized and generalized lichenification, generalized neurodermatitis or manifestations of atopy" a similar designation "atopic dermatitis" was proposed [9]. But Rajka pointed out early the unfortunate choice of the term "atopic": "This … is

an unfortunate choice of term, even when considered in the light of the definition (of 'atopy') by Coca in 1953 [10]. The flaw lies in the conclusion from recent experience that the disease can no longer be considered a typical atopic disease" [11].

In 1968 WHO's International Reference Center for Immunoglobulins decided that enough critical data were available to announce the existence of a fifth isotype of immunoglobulin, IgE [12]. The classic "reagin activity" could be linked to IgE [13, 14]. In the mid-1970s classic, IgE-mediated allergic reactions to inhalant allergens were given the name "atopic allergy" by Pepys [15]. The term "atopic" has been used synonymously with "IgE-mediated" by many physicians and scientists working with allergy. But there are others, especially pediatricians, who also view "atopy" as a constitutional property. They consider "atopy" to be a clinically useful alternative to the genetic marker that we still do not have, as IgE-mediated allergy is often inherited in the family and is common among children and adolescents.

General Terms

As similar clinical symptoms can be triggered by different mechanisms, it is of great importance to scientists as well as to physicians that the initiating mechanism is correctly identified. Misunderstandings can lead to false conclusions, unsuitable advice, and ineffective treatment. The revised nomenclature proposed by the EAACI and the WAO is therefore based on the mechanism that initiates the reaction and causes the symptoms and signs of allergic disease.

Hypersensitivity

The term *hypersensitivity* should be used to describe *objectively reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal persons*.

Classic responses to infection, autoimmunity, and toxic reactions are not covered by this definition. A consequence of this strict definition is that some dubious concepts, e.g., "total drug sensitivity" and "multiple clinical sensitivity" [16], and multisymptomatic conditions, like those attributed to amalgam in dental fillings [17] and electromagnetic waves [18], do not fulfill the criteria, and thus should not be called hypersensitivity. If there is no objective link between exposure and symptoms, preventive measures cannot be successfully applied.

Allergy

Allergy is a hypersensitivity reaction initiated by specific immunological mechanisms. When such mechanisms cannot be demonstrated or other mechanisms are

mediating the reactions, as in hypersensitivity to salicylic acid [19], the term hypersensitivity can be used, or, if you want to be more specific, the term *nonallergic hypersensitivity* can be used. Allergy can be *antibody-mediated* or *cell-mediated*. In most patients with allergic symptoms in the respiratory organs or the gastrointestinal tract, the antibody involved belongs to the IgE isotype, and these patients can thus be said to have an *IgE-mediated allergy*. In this context the term IgE refers to an IgE antibody towards an allergen. Nonantibody-active IgE molecules are not known to have any allergy-related biological activity. Therefore, allergy cannot be defined based on an elevated level of IgE, often called "total IgE," or in the presence of IgE on a cell surface.

It seems possible that the inflammatory reaction causing the symptoms in the more chronic stage of an originally IgE-mediated allergic inflammation is dominated by allergen-specific lymphocytes. As a result of the allergic inflammation in the mucosal membranes, an increased sensitivity, hyperreactivity, or other allergic symptoms can be triggered or aggravated by nonimmunological factors like infection, irritants, physical exertion, etc.

IgG antibodies are regularly produced in response to normal antigen exposure, as is the case also for lymphocyte sensitization. Thus, IgG antibodies to antigens in our local environment often occur without causing symptoms, while high concentrations of IgG, as well as IgE, antibodies have been shown to be of importance in allergic bronchopulmonary aspergillosis [20].

Thus, moderate levels of IgG antibodies to an antigen or a positive lymphocyte stimulation test with moderate to high (>1 μg/mL) antigen concentrations is not necessarily a sign of allergic disease. The presence of IgE antibodies, on the other hand, always constitutes a potential risk for allergic inflammation.

In *non-IgE-mediated allergy* the inflammation can be mediated by allergen-specific lymphocytes, as in allergic contact dermatitis, or by antibodies of the IgG isotype, as in anaphylaxis caused by complement activated by immunocomplexes containing dextran [21], and the classic, but today unusual, serum sickness, which actually gave rise to the term allergy [22].

After chronic inhalation of high concentrations of certain materials containing proteins, e.g., from *Actinomycetes* and certain moulds in farmer's lung, and bird droppings in bird fancier's disease, airway symptoms referred to as alveolitis can occur [23]. The term *allergic alveolitis* should be used for such diseases.

Atopy

Atopy is a personal and/or familial tendency, usually in childhood or adolescence, to become sensitized and produce IgE antibodies in response to ordinary exposures to allergens, usually proteins. As a consequence, these persons can develop typical symptoms of asthma, rhinoconjunctivitis, or eczema.

The term *atopy* should be reserved to describe the genetic predisposition to become IgE-sensitized against allergens that commonly occur in the environment and

to which we are all exposed, but to which most individuals do not react by producing antibodies. Interestingly, the allergen dose is usually very low: the annual dose of grass pollen allergen has been calculated to be in the order of 1 μg [24]. Atopy is thus a clinical definition of an individual who has a great propensity to produce IgE antibodies. Unfortunately, today the atopics cannot be identified until an IgE sensitization has already occurred, and then the atopic state must be documented by the demonstration of IgE antibodies in serum or by a positive skin test. Even if one can get close with the aid of a skillfully interpreted case history or a good questionnaire, the only way to establish atopy is to demonstrate sensitization.

If the allergen exposure is not low or does not occur through a mucosal membrane, nonatopic persons can also be IgE-sensitized. Thus, neither a positive skin test nor the presence of IgE antibodies to a less common allergen occurring in large amounts in the environment, e.g., most drugs, is a diagnostic criterion for atopy. Another example of high-dose exposure is when the allergen is not introduced in the body through the mucosal membranes but directly by injection. Typical examples of the latter are bee and wasp stings. Such patients should instead be termed *skin-test-positive* or *IgE-sensitized*, depending on what tests have been performed. However, allergic symptoms in a typical atopic individual can be called *atopic*, as in atopic asthma.

Allergen

An *allergen* is an antigen causing allergy. Immunotherapy with allergens [25], which is an allergen-specific immunomodulation, is best referred to as *allergen-specific immunotherapy* (ASIT). The injection of monoclonal antibodies against IgE [26] can in the same way be called *anti-IgE-specific immunotherapy* (ESIT).

Allergic Diseases

Asthma

Asthma [27] mediated by immunological reactions should be called *allergic asthma* (Fig. 1). In most cases it is initiated by IgE antibodies, and if one wants to stress this, a suitable term is *IgE-mediated allergic asthma*. In order to clarify the significance of other immunological mechanisms initiating the inflammation associated with allergic asthma, further research is required. Eighty percent of childhood asthma [28] and more than 50% of asthma in adults [29] has been reported to be allergic. The mechanisms initiating *nonallergic asthma* are not well defined, but similar inflammatory changes are present in both forms of asthma. There are

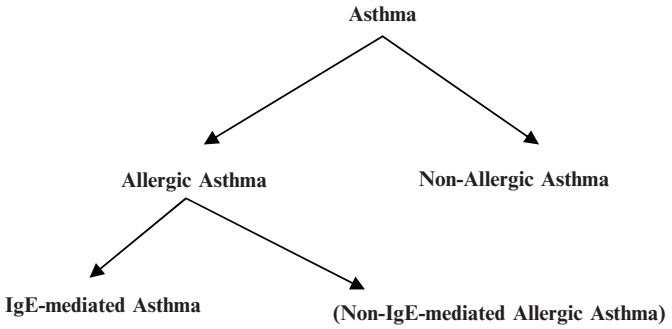

Fig. 1 Structure of asthma according to the revised nomenclature

indications that the prevalence of allergic asthma increases at the same rate as the general increase of allergic diseases [29].

Rhinitis

Hypersensitivity symptoms from the nose, e.g., itching, sneezing, increased secretion, and congestion, should, when they are immunologically mediated, be called *allergic rhinitis*. The overwhelming majority of cases are mediated by IgE antibodies, and for especially drawing attention to this, a suitable term can be *IgE-mediated allergic rhinitis*. If the symptoms are seasonal, e.g., allergic rhinitis caused by pollen, the term *seasonal allergic rhinitis* is formally correct, but here the classic term "hay fever" is really fitting. A new classification of allergic rhinitis based on duration and the degree of symptoms has been suggested by the WHO project "Allergic Rhinitis and Its Impact on Asthma" (ARIA) [30]. According to this classification the words *intermittent* and *persistent* denote the duration, and *mild* and *moderate–severe* define the effect of the symptoms on sleep, work, and other activities. The term *nonallergic rhinitis* covers several different forms of rhinitis which will not be discussed in this context.

Conjunctivitis

IgE-mediated allergic conjunctivitis often occurs together with allergic rhinitis, so it is suitable to call this disease *allergic rhinoconjunctivitis*. In addition to IgE-mediated conjunctivitis there is also contact allergic conjunctivitis, involving T_H1 mechanisms [31]. *Nonallergic conjunctivitis* also often occurs together with nonallergic rhinitis. The relationship between allergic and nonallergic conjunctivitis on the one hand and atopic keratoconjunctivitis and vernal keratoconjunctivitis [32] on the other requires further studies.

Dermatitis

The overall term for a local inflammation in the skin should be *dermatitis* (Fig. 2).

The term *eczema* is proposed to replace the provisional term atopic eczema/dermatitis syndrome (AEDS). The term covers a couple of skin disease entities based on a genetically determined defect in the skin barrier [33–35]. This genetically determined organ sensitivity [36] constitutes the basis for eczema, in line with the sensitive mucosae seen in asthma and rhinitis.

In infants and adolescents with an atopic constitution the underlying inflammation is dominated by an IgE antibody-related reaction, which enables the use of the term *atopic eczema*. As long as the immunological mechanism behind eczema remains unclear [37], the disease should simply be called eczema. We must remember that the classification of atopy, and consequently of atopic eczema, is based on IgE sensitization and consequently cannot be done without the determination of IgE antibodies or a skin test. In chronic cases the inflammation appears to be affected by IgE antibodies to a lesser extent and the dominating cells in biopsies are lymphocytes. The mechanisms initiating the skin disorder in *nonatopic eczema* require further studies.

Eczema without any signs of an atopic constitution is common in preschool children [38] and prevalences between 45% and 64% have been reported in studies [39, 40], but also among adults figures as high as 40% have been reported [41]. Nonatopic children with eczema have been reported to have a lower risk for developing asthma in youth than atopic children with eczema [42, 43]. But nonatopic eczema in children can develop into atopic eczema. The differentiation between atopic eczema and eczema in general seems to have significant prognostic implications [41].

In some countries the term “atopic dermatitis” is still used, which is most confusing, since only the subgroup of eczema affecting an atopic individual could deserve the term, according to the revised nomenclature. Thus, when the term “atopic dermatitis” is used it is not possible for the reader/listener to understand if reference is made to the umbrella term eczema or only the atopic subgroup.

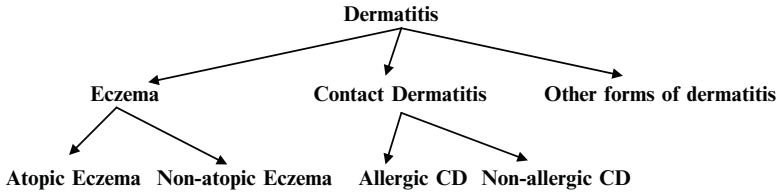

Fig. 2 The term eczema should be used to cover the disease both in atopic individuals, *atopic eczema*, and in non-atopic individuals, *non-atopic eczema*

Contact Dermatitis

Close contact with chemicals of low molecular weight or irritants can cause a local inflammation in the skin. When the reaction is mediated by immunological mechanisms, primarily T_H1 lymphocytes, it should be called *allergic contact dermatitis*. Typical allergens functioning as haptens include nickel, chromium, perfumes, and preservatives. Exposure can occur through oral intake, so-called *systemic allergic contact dermatitis*. If no immunological mechanism is involved, the correct term is *nonallergic contact dermatitis*, but terms such as *irritant/toxic contact dermatitis* can also be used to describe the disease.

A subgroup of contact dermatitis, protein contact dermatitis, is probably an IgE-mediated reaction caused by absorption of proteins through damaged skin [44]. It can be termed *allergic protein contact dermatitis*, or for stressing the role played by IgE, *IgE-mediated allergic protein contact dermatitis*. The relationship to eczema requires further studies. There are also a number of other types of nonallergic dermatitis but they will not be discussed here.

Urticaria

When the reaction is mediated by immunological mechanisms it should be termed *allergic urticaria*. This is commonly IgE-mediated, but it can also be immune complex-associated. These different mechanisms can be highlighted by the choice of terms, e.g., *immune complex-associated allergic urticaria*. Urticaria can also develop locally, after local contact with the allergen, e.g., on the hands of a latex-allergic person wearing latex gloves [45, 46], or a person allergic to dogs being licked by a dog. Such urticaria, which can be IgE-mediated, should be called *allergic contact urticaria*. In certain cases of chronic urticaria autoantibodies to the high-affinity IgE receptor, FcεRI, can be involved [47], in which case the pheno-menon should be regarded as an autoimmune variant of allergic urticaria.

Food Hypersensitivity

An adverse reaction to food should be called *food hypersensitivity*. The term *food allergy* should not be used until an immunological mechanism has been demonstrated. The presence of food-specific IgG antibodies in serum is not of clinical or diagnostic importance, but only indicates previous exposure to the food in question. When IgE is involved in the reaction, the term *IgE-mediated food allergy* is suitable. All other reactions should be termed *nonallergic food hypersensitivity* [48, 49].

Drug Hypersensitivity

As for other possible allergens, only when immunological mechanisms have been demonstrated, either antibody or cell-mediated, is it appropriate to refer to the reactions as *drug allergy*. By adding the adjectives *immediate* and *late* or *delayed* the time for the occurrence of the symptoms in relation to drug contact can be indicated, and also the probable responsible immunological mechanism, mediated by IgE antibodies or by lymphocytes [50]. *IgE-mediated drug allergy* constitutes only a minor part of the hypersensitivity reactions to drugs compared with *nonallergic drug hypersensitivity*. The immunological mechanism is often difficult to identify, as the allergen is a small molecule or may be a drug metabolite, both functioning as a hapten. The allergenic determinant might be a configurational structure depending on the hapten–carrier conjugation, which complicates selection or production of appropriate allergens for testing. A positive intradermal test, a weak skin-prick test (<3 mm), or a basophil-provocation test with a high concentration of the substance (e.g., >1 mg/mL) is not sufficient to identify a truly immunopathologic mechanism since many drugs have a direct triggering effect on mast cells and basophils. The demonstration of IgG antibodies or a positive lymphocyte stimulation test only indicates previous exposure, unless, in the latter case, the dose of the antigen used for testing is very low (<1 μg/ml).

Hypersensitivity to Insect Stings or Bites

Hypersensitivity to insect venoms mediated by an immunological mechanism should be termed *venom allergy*, e.g., *bee venom allergy*. To stress the major role of IgE antibodies the term *IgE-mediated bee venom allergy* can be used. The large amount of venom allergen transferred by a sting is comparable to inhaling pollen allergens for years. This high-dose sensitization probably explains why the prevalence of atopy among patients with IgE sensitization to insect venoms is only marginally higher than in the normal population [51]. The high-allergen dose and the exposure through the skin barrier mean that moderate serum concentrations of IgE antibodies should also be of concern.

Anaphylaxis

The term *anaphylaxis* is used differently by physicians in different parts of the world [52]. The proposal is to use anaphylaxis as an umbrella term for the acute reaction, since the immediate clinical decision on case handling is independent of mechanisms. Anaphylaxis is defined as follows: *Anaphylaxis is a severe, life-threatening, generalized or systemic hypersensitivity reaction*. Usually anaphylaxis

develops gradually, often starting locally with itching of the throat, the palms, or the soles, and urticaria, developing into a multiple-organ reaction often with a severe asthma culminating in hypotension, shock, heart failure, and death.

The term *allergic anaphylaxis* should be used when the reaction is mediated by an immunological mechanism, e.g., IgE, IgG, or immune complex-related. An anaphylactic reaction mediated by IgE antibodies, e.g., a food anaphylaxis caused by peanut, can be termed *IgE-mediated allergic anaphylaxis*. Anaphylaxis with nonimmunological causes should be called *nonallergic anaphylaxis*.

Conclusions

Allergy and other types of hypersensitivity affect almost every other citizen in industrialized countries. Today not only allergists, but above all primary care physicians and pediatricians, come in everyday contact with probable cases, quite a different situation compared to 50 years ago, when the prevalence was less than 10%. Allergy care is discussed by politicians, is a major subject for debate in the school environment, is highlighted in the media, and is an everyday problem for the allergic individuals and their families. It is obvious that in order to avoid misunderstandings all must speak the same language, i.e., use the same terms and give them the same contents. Some 70 allergy associations all over the world have realized the problem and agreed on an updated nomenclature for allergy. It will probably need updating again in 5–10 years, when our knowledge of allergy has developed even further. But until then the current updated nomenclature is of great importance. We must all join forces to use it and spread it.

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IgE and the High-Affinity Receptor, FcεRI: The IgE–CD23 Interaction

Donald MacGlashan, Jr.

Introduction

In the 1960s, the antibody responsible for conferring an immediate hypersensitivity reaction was identified as immunoglobulin E (IgE), but it required another 25 years to adequately describe the receptors that mediate the binding of IgE to cells. As with IgE itself, immediate hypersensitivity is defined by the presence of the high-affinity IgE receptor, FcεRI, because only by binding to this receptor on the plasma membrane of cells is the antigen specificity of IgE conferred to the cellular reaction. With a more general perspective, atopy may also be defined by the presence of the low-affinity IgE receptor, FcεRII, since this receptor mediates a variety of regulatory events that bias the immune response to or away from atopy. Both low- and high-affinity receptors have interesting life cycles that continue to provide new perspectives on how they regulate the immune response. However, FcεRI and FcεRII are quite different proteins and share almost nothing in common except for their role in binding IgE antibody. Each protein will be considered in turn.

FcεRI

The life cycle of FcεRI is depicted in Fig. 1. Not surprisingly, at the crude level of this cartoon, it is not very different than any other cell surface receptor. However, there are some details that provide a unique set of characteristics that have important implications for IgE-mediated diseases. In humans, the mature receptor is composed of two or three subunits depending on the cell type. The alpha subunit contains the IgE-binding domains, the beta subunit is optional in humans and the gamma subunit possesses immunotyrosine activation motifs (ITAMs) that allow the receptor to interact with intracellular enzymes. The beta subunit also possesses an atypical ITAM that

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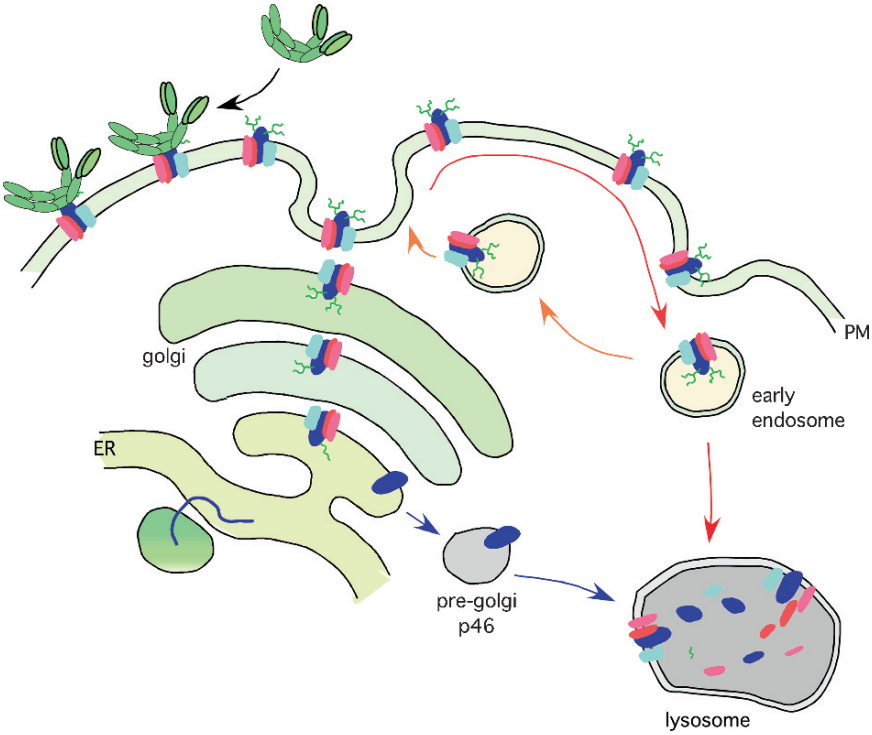

Fig. 1 Cartoon of FcεRI receptor generation, cycling, and degradation in human basophils. ER = endoplasmic reticulum, PM = plasma membrane

confers some unexpected properties to the receptor [1], although the full details of the role this ITAM plays in signaling is still evolving.

Translation and Early Processing

The transcribed alpha subunit contains an ER retention signal that determines its fate depending on the presence of either the beta or gamma subunits [2–4]. Either beta or gamma can mask the retention signal allowing the receptor to progress through the Golgi and on to the plasma membrane. In the absence of both subunits, alpha is shunted into a degradative pathway [5]. As noted earlier, in humans, the presence of FcεRIβ is optional for cell surface expression of FcεRI [6]. But it appears that the beta subunit provides a better masking signal to protect this shunting step because the presence of beta increases cell surface receptor expression 3–6-fold above that observed with FcεRIαγ2 in cell line models of receptor expression [7]. The effect may be even more extreme in wild-type cells. Although FcεRI is expressed on monocytes and dendritic cells (as well as several other cell

types), its expression level is 10–100-fold less than observed for peripheral blood basophils from the same subjects. Monocytes and dendritic cells do not express the beta subunit, so that, the cell differences notwithstanding, these observations might suggest the "beta-effect" to be more profound in normal cells. A transcriptional variant of the beta subunit has been identified [5]. The so-called β_T splice variant includes the fifth intron, which happens to contain a stop codon that leads to truncation of FcεRIb translation. The truncated beta protein does not include the masking sequence and this leads to partial shunting of FcεRIα into the degradation pathway and in cells expressing more β_T, there is less cell surface FcεRIα.

In eosinophils, where the beta subunit does not appear to be expressed, there is a large pool of intracellular FcεRIα that can be readily found in the extracellular medium during culture of these cells [8], although there remains some controversy about surface expression of FcεRIα on eosinophils [9]. The mass of intracellular FcεRIα remains constant during culture suggesting that there is a continual synthesis and secretion of the alpha subunit. The biological significance of this secreted alpha subunit is not clear. In human basophils, where the beta subunit is well expressed, there continues to be an intracellular pool of FcεRIα that is rapidly lost when the cell is cultured [10]. In contrast to the eosinophil, however, this pool does not appear to be secreted or maintained in cultured basophils. The biological significance of an intracellular pool in basophils is also not clear, but it is worth noting that the ratio of FcεRIβ to FcεRIα in basophils from different donors is not a constant as would be expected from a simple model of expression dynamics. These observations suggest that even in a cell that could be considered professional in its role as a cell of immediate hypersensitivity reactions, FcεRIβ expression could be used to control cell surface receptor levels. Indeed, there is good relationship between the ratio of FcεRIβ to FcεRIα and total cell surface receptor expression in basophils. There is at least one known cytokine, IL-3, that appears to regulate FcεRIβ protein and mRNA levels in human basophils [11] and there may be others for basophils and possibly a different profile that alter mast cell FcεRIβ expression levels.

These intracellular pools of FcεRIα have a lower molecular weight than receptor on the plasma membrane. Ranging from 45 to 50 kD, they contain the level of glycosylation associated with pre-Golgi apparatus processing; the core protein is approximately 28 kD, so these endoglycosidase H-sensitive carbohydrates account for approximately 20 kD of the mass. As FcεRIα is processed through the Golgi organelle, these sugars are removed and replaced with a larger glycosylation core that is also quite heterogeneous. Surface FcεRIα has a broad molecular weight ranging from 58 to 70 kD with a peak around 60 kD. This Golgi-matured form of the receptor is difficult to observe in most non-basophil/mast cell types that express this receptor.

Time on the Surface

One characteristic of this receptor that determines much of its biology in the organism is its loss from the cell surface in the absence of its ligand. This behavior, while

not unique, is the opposite of a typical receptor that is internalized when ligand binds. Instead, unoccupied FcεRIα is lost from the cell surface. Recent studies suggest that unoccupied receptor is internalized and enters an early endosomal pool that partially recycles receptor back to the cell surface [12]. These studies also suggest that at least one half of the receptor experiences this recycling, but that the other half is degraded by a Bafilomycin A-sensitive process that is therefore, likely to be lysosomal degradation (but the amount of recycling may be dependent on the cytokine environment). It is the binding of IgE antibody that stabilizes the receptor on the cell surface, but from one perspective, this is a passive effect. IgE binding appears to change receptor confirmation in such a way that it is no longer recognized by a constitutively active process of receptor clearance. For example, there is no other signal that is generated to induce more receptor synthesis; the presence of IgE does not alter the steady state of mRNA expression or change posttranslational processing. Instead, at least with respect to the presence or absence of IgE antibody, FcεRIα synthesis is constitutive. This is not to say that there are no other factors that alter receptor synthesis (see above and below).

Parenthetically, there now exists a complex story about the ability of monomeric IgE to induce signaling in mast cells [13, 14]. The full details of this story are still being developed, but the current understanding is that in murine mast cells, there are certain IgE antibodies that induce signals similar to those found to follow aggregation of FcεRI. Early signaling elements become active and the cell secretes some cytokines. There are several hypotheses about the nature of the IgE that allows this behavior to be manifest but notably, this story has not been well recapitulated using human cells [15] with the exception of some isolated reports [16]. There is reasonable evidence that some monomeric IgE antibodies may cross-link FcεRI to another cell surface structure. It has also been shown that this process is sensitive to the presence or absence of early signaling tyrosine kinases while receptor stabilization on the cell surface by IgE is not dependent on these kinases [12, 17].

Because receptor is stabilized on the cell surface after IgE binds, the steady state level of receptor expression is dependent on the affinity of IgE for the receptor. Studies have shown that because the dissociation of IgE from receptor is very slow, stabilization is primarily sensitive to forward rate constant of IgE binding to receptor. An interesting characteristic of IgE binding to receptor is the notably slow on-rate, about 10–50-fold slower than would be expected for comparably sized proteins [18–20]. This characteristic shifts the EC50 for IgE-mediated up-regulation of FcεRIα to approximately 250 ng/ml, or 100-fold greater than the affinity for the receptor would suggest. But this unusual property of IgE binding introduces some potential for biological variation in receptor up-regulation. Unpublished studies from this laboratory have found that not all IgE antibodies are equipotent for inducing up-regulation. While several show an EC50 of 250 ng/ml, an EC50 for one IgE of 50–100 ng/ml has been observed. The possibility that different IgE antibodies differentially alter up-regulation due to differences in forward binding constants needs further exploration, but suggests that there may be a new source of biological variation controlling receptor expression.

Consequences of FcεRI Properties

There are several biological consequences to these receptor characteristics. First, in the presence of IgE, receptor can accumulate to extremely high levels. It is not unusual to observe 500,000 FcεRI per basophil and one million receptors is occasionally observed. It is possible that this high level has a functional consequence since modeling shows that there will be many spontaneous aggregates of receptor at these densities. Some component of the monomeric IgE effect on signaling might be related to the enhanced spontaneous aggregation at high receptor densities. At the other end of the spectrum, this model of receptor regulation predicts that there will be a steady state of approximately 5,000–10,000 receptors even in the complete or near absence of IgE [20]. Several experimental studies support this prediction [21]. Coupled with knowledge about the sensitivity of basophils to antigen-mediated stimulation, this fact has some influence on the ability to completely down-regulate the basophil/mast cell response during treatment with IgE-suppressing therapies and makes these therapies sensitive to modest changes in low levels of circulating IgE. The cytokine environment controlling constitutive synthesis of FcεRIα and FcεRIβ determine the maximum values of this low-end steady state (in vitro cultures of CD34-derived mast cells often show high synthetic rates and therefore 50,000–80,000 receptors even in the absence of IgE in culture).

While the evolutionary advantages of IgE are not really understood, the notion that IgE plays a role in parasite rejection provides a possible reason that high IgE levels are often found in patients with parasitic infections. It is possible to imagine that to counter the biological value of IgE, in rejecting parasitic infections the parasite induces nonspecific IgE generation as a biological buffer against specific IgE. If, during evolution, the host cells developed a way to up-regulate receptor to counter this defense of the parasite, one might find an explanation for the existence of the accumulating receptors. However, studies have shown that the relationship between IgE and receptor density is log-linear [22–24]. In other words, a tenfold increase in IgE leads to only a 2–3-fold increase in receptor. The cell cannot keep up with a parasite strategy that increases nonspecific IgE levels. But for atopy, this accumulation remains dangerous because each new specific IgE can still find space on a basophil and mast cell already populated with IgE. The typical basophil and mast cell can respond reasonably with fewer than 1,000 molecules of cell surface antigen-specific IgE (the median EC50 in the general population is approximately 2,000 [25, 26]), so that there is lots of room for many antigenic-specificities on a cell that is expressing 500,000 receptors. The median antigen-specific to total IgE ratio is approximately 1% for many types of antigens [27], indicating that a basophil or mast cell possesses 2,000–5,000 antigen-specific IgE molecules per cell, well past the normal EC50 for a response. There is population variance in the cell's sensitivity to stimulation; notably, there is a subpopulation of subjects whose basophils, for example, can respond strongly to less than 300 molecules of antigen-specific IgE [28]. The ability to modulate this variable, along with the ability to up-regulate receptor expression provides the basophil and mast cell two ways to combat a parasite's attempt to swamp the receptors with nonspecific IgE.

There is also hysteresis in the receptor–IgE relationship. Because IgE does not dissociate very quickly (endogenous IgE dissociates with a half-life of 8–10 days), an increase in IgE levels, even if temporary, leads to a sustained level of increased receptor and therefore IgE on the cell surface. Long after the circulating IgE is gone, the cell continues to "remember" the transient rise in IgE level. This characteristic has implications for IgE-modulating therapies.

The hysteresis has another consequence that relates to cell lifetimes [29]. If the cell lifetime is relatively short, compared to the lifetime of IgE bound to receptor, then the cell disappears long before IgE can dissociate. Any therapeutic maneuver that decreases IgE levels will stop up-regulation of the receptor, but cells that already had experienced up-regulation must live long enough for the IgE to dissociate in order to down-regulate the receptor. But if they have short lives, they will die and be replaced by cells that had not experienced the up-regulation due to the presence of IgE antibody. The consequence is that cells that live for short periods appear to down-regulate receptor much faster than cells that live for much longer. Of course, it is not that the short-lived cell is down-regulating receptor faster, it is simply dying and being replaced by another cell that did not up-regulate receptor. This model may provide an explanation for the observation that circulating basophils appear to down-regulate the receptor faster than skin mast cells [30]. Basophil half-lives are measured in hours and skin mast cells in weeks or months. Basophils may not be the only IgE-bearing cell that experiences rapid turnover, there are indications that intestinal mast cells cycle quickly. Such cells would also appear to quickly down-regulate receptor densities. One would predict that atopic conditions that are more dependent on basophils/gut mast cells (food allergy?) would be more quickly down-regulated following treatment with an IgE-suppressive therapy (e.g., omalizumab) than a condition dependent on skin mast cells (urticaria?).

The ability of IgE to up-regulate receptor expression (or lead to down-regulation if removed) leads to the good correlation between IgE receptor expression levels in circulating basophils and serum IgE titer. This correlation extends across the 7 log range of IgE concentrations, but it is not as strong a correlation as one would expect if this were the only parameter controlling receptor expression. Approximately 35% of the variance in log(IgE) accounts for the variance in receptor expression. The second parameter controlling expression may be the regulation of FcεRIβ levels although this has yet to be examined in detail. The regulation of both FcεRIα and FcεRIβ might be under the influence of cytokines [31]. As noted earlier, exposure of basophils to IL-3 has been shown to alter both mRNA and protein levels of FcεRIβ [11], and one study has noted that FcεRIα levels may also be under the influence of IL-3. In mast cells, IL-4 can be observed to alter receptor expression and studies using mast cells derived from nasal tissues indicate that IL-4 may directly influence the expression of FcεRIα; protein, mRNA, and cell surface FcεRIα were increased [32, 33] although IL-4 did not appear to increase mRNA for FcεRIβ. In other studies, IL-4 might also have an indirect effect on receptor expression by altering mast cell development [34–36]. It is not very clear whether FcεRIγ expression is another means to regulate cell surface expression of FcεRIα. The FcεRIγ subunit is used by a variety of immunoglobulin receptors and by newly described receptors like the leukocyte inhibitory receptors, so directed regulation of the presence of FcεRIα

would not be possible if the gamma subunit were being regulated. These issues lead to questions about the genetics underlying receptor expression.

Genetics of FcεRI Expression

The genomic organization of the three subunits of the IgE receptor are known in some detail, but the regulatory elements controlling transcription of the three subunits are not well known. Figure 2 shows the genomic organization and schematically shows some of the *trans*-acting factors and *cis*-elements that are thought to control transcription. For example, note that GATA-1 is considered a transcription factor for FcεRIα. This factor is also known to regulate a number of mast cell-associated genes [37, 38], so that its involvement in regulating FcεRIα makes sense. In the maturing mast cell, FcεRIα transcription occurs in concert with other mast cell-specific genes about the time that GATA-1 is becoming active. Co-expression of GATA-1 with either PU.1 or YY1 transcription factors dramatically enhances transcription of this gene in artificial cell models [39]. In some cell types, the PU.1 and GATA-1 transcription factors work to oppose each other's regulatory behaviors, so this cooperative behavior is a mast cell-specific characteristic. IL-4 has been shown to alter FcεRIα transcription in humans but not in mice. Therefore, it is interesting to note that 20 kilobase pairs upstream of the canonical exon 1 of FcεRIα lies an IL-4-sensitive promoter element in humans that is not found in mice [40]. Recent studies have identified Oct-1 as a transcription factor regulating the FcεRIβ promoter [41]. This transcription factor acts upstream of exon 1, but MZF-1 has been identified to suppress transcription [42] and this *trans*-acting element appears to act on a stretch lying between exons 3 and 4. A recent study has also suggested that the *trans*-acting factor FOG-1 suppresses the ability of GATA-1 to mediate FcεRIβ transcription [43].

Polymorphisms in these three genes have also been examined although there remains a great deal unknown about transcriptional control. With respect to the

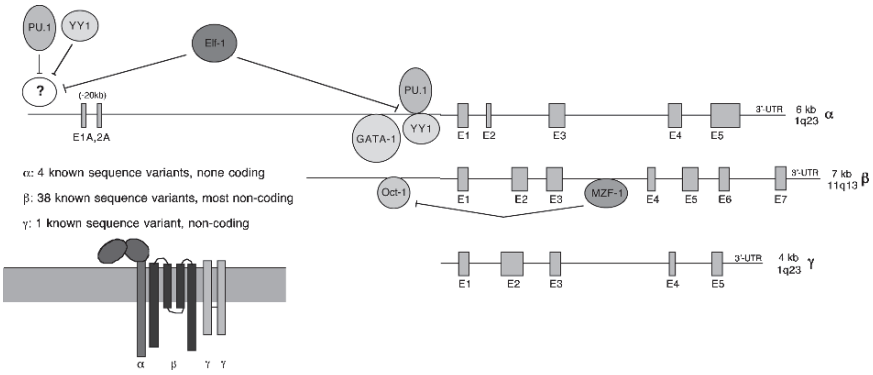

Fig. 2 Genomic organization and transcription factors for FcεRIα, FcεRIβ, and FcεRIγ

FcεRIα gene, only three SNPs have been identified and these occur in the upstream 5′ promoter region. One of these SNPs, located at −66 is a C/T exchange in which the T allele showed GATA-1 binding more tightly to this region, possibly because the T allele generates an additional GATA-1 binding site [44]. Promoter–reporter studies also find that transcriptional rates are enhanced with the T allele and markedly enhanced, relative to the C allele, when GATA-1 was overexpressed. This same allele has been found to be associated with higher IgE levels in asthmatic patients. In patients with atopic dermatitis, peripheral blood basophils showed the expected receptor expression-serum IgE correlation found in numerous other studies with the interesting caveat that the linear regression line, elevated in T/T subjects, was displaced to higher expression levels for equal serum IgE concentration.

The gene for FcεRIβ is located on chromosome 11q13, a region that has been associated in various studies with many aspects of atopic disease. Polymorphisms in the FcεRIβ gene have been associated with atopy, bronchial hyperresponsiveness, atopic dermatitis, total IgE levels, asthma, and allergic rhinitis. Therefore, there has been more intense scrutiny of this receptor subunit than the other two subunits. There are three notable coding region SNPs that alter amino acid composition of the protein. The I181L and V183L substitution SNPs have been studied in some detail for changes in FcεRIβ function of whole receptor expression and function. However, neither change has been found to alter the function, as best as it can be studied [45]. The third coding SNP, E237G, is more universally found and is particularly intriguing because it lies near the ITAM of FcεRIβ C-terminal cytoplasmic tail. However, studies of this change have also not revealed any functional differences from the wild-type subunit [45]. Given that this SNP has associations with a wide variety of atopic phenotypes [46], a search for linkage disequilibrium with another noncoding SNP was made. There are, indeed, two SNPs in the 5′ promoter region of this gene that appear to be in linkage disequilibrium and these SNPs appear to cause differences in gene transcriptional rates [46]. Notably, the −426T/C and −654C/T SNPs were tightly linked with +6940A/G SNP (which causes the E237G substitution) and the promoter carrying the minor allele demonstrated greater transcriptional activity in a reporter assay than the major allele. Electrophoretic mobility shift assays tentatively identified the transcriptional factor YY1 as showing preferential binding to the minor allele (−654T) and a combination of both minor alleles demonstrated even greater transcription in these reporter assays. There are 31 additional SNPs associated with the noncoding region of the FcεRIβ gene, but they have not been examined for an effect on gene expression [47].

FcεRII

The life cycle and functions of the low-affinity IgE receptor appear to be considerably more complex than FcεRI. FcεRII/CD23, in various forms, is expressed on many cell types (β cells, monocytes and macrophages, eosinophils, platelets, Langerhan's cells, and airway smooth muscle cells) and several active split

products are found in soluble form. It appears to possess cytokine-like properties as a soluble molecule and otherwise acts a receptor when anchored to the cell membrane. Aggregation of membrane-associated CD23 appears to provide an inhibitory signal down-regulating IgE generation while the presence of soluble CD23 up-regulates IgE generation in murine models. Some of the complexity also derives from the fact that it binds not only to IgE (with a relatively low affinity of K_a–10^7–10^8), but binds to CD21, CD11b, CD11c, and the vitronectin receptor. There are effects on IgE generation, which will be considered below, as well as effects such as inducing mediator secretion or oxidative burst that will not be considered here. Soluble CD23 levels and its appearance on selected cell types have been associated with allergic diseases and immunotherapy has been found to decrease CD23 expression [48].

Structure–Function Relationships

Unlike other immunoglobulin receptors, which belong to the Ig superfamily, CD23/FcεRII belongs to the animal C-type lectin family. Structurally, it has a very elongated extracellular domain comprised of a head-group, a "stalk" and a "tail" (see Fig. 3). The stalk region has the property of forming an α-helical

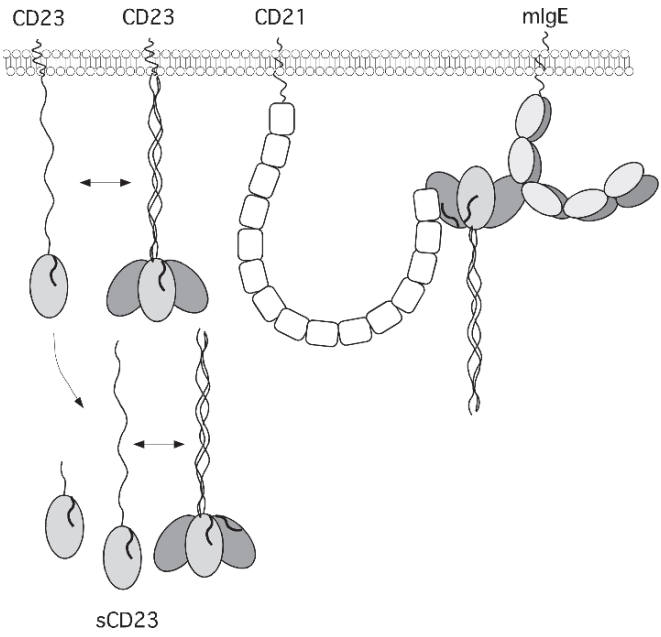

Fig. 3 Cartoon of cell surface FcεRII/CD23 and soluble CD23 structure and interaction with membrane IgE and CD21

coiled-coil or leucine zipper, which allows CD23 to self-associate into trimers and other higher oligomers. Two subtypes have been identified, with the FcεRIIa constitutively expressed on B cells and eosinophils and the FcεRIIb form being inducible on many of the other cells types where this protein is expressed [49]. The gene (on 19p13.3) is large with 11 exons and differences in the initiation site and RNA splicing account for the two forms, which differ only in a change on the cytoplasmic side of the receptor. Much of the complexity in the biology of CD23 derives from the fact that it can be cleaved from the cell surface by several different types of proteases, both endogenous and exogenous. For example, the metalloprotease, ADAM 10, is thought to be primarily responsible for cleavage [50], but other ADAM metalloproteases such as ADAM 8, 15, and 28 [51], and possibly 33 appear to have a role. Exogenous enzymes such as chymotrypsin and elastase can release the soluble form [52] and notably, the dust mite antigen DerP1 generates a smaller soluble form of CD23 [53, 54]. Each protease has preferred cleavage sites leading to the generation of a wide range of soluble molecules. However, it appears that a key element in understanding whether the soluble products have one property or another hinges on whether the leucine zipper stalk is retained in the soluble form [55]. Since this stalk region determines the ability of CD23 to form trimers rather than monomers, the binding properties of sCD23 differ depending on its presence or absence. For example, as noted earlier, CD23 can bind to proteins such as CD21; however, binding is limited to one cross-link between CD23 and CD21. In contrast, a multimeric CD23 might generate large aggregates of IgE and CD21. Recent studies suggest that it is this property that determines the biological activities of sCD23. It is known that cross-linking B cell surface IgM with CD21 leads to inhibition of Fas-induced apoptosis [56] and it is proposed that co-ligation of membrane IgE (which uses accessory molecules shared with membrane IgM [57]) with CD21 will generate the same response, thereby enhancing the generation of IgE antibody. Antibodies that bind to the same region of CD21 as sCD23 have been shown to enhance IgE synthesis, while antibodies that bind other regions do not [58], lending credence to the model described earlier. In contrast, derP1 generates a smaller sCD23 that is unable to form trimeric structures. Monomeric sCD23 has a much lower affinity for IgE (1 vs <0.1 μM, Kd) but it may nevertheless inhibit the interaction of the trimeric form causing a reduction in the enhancing signals generation by trimeric signaling complex.

It is evident from the above discussion that even at the level of B cell IgE synthesis, the regulatory mechanisms underlying CD23-dependent modulation are rather complex and not well understood. On one hand, soluble CD23 can co-ligate membrane IgE and CD21 to enhance cell survival and therefore up-regulate IgE synthesis by B cells, but in contrast, co-ligation of membrane CD23 with membrane IgD appears to decrease B cell proliferation. Monoclonal antibodies to different epitopes of CD23 can stimulate or inhibit cell growth. It is apparent that absence of CD23 reduces IgE-mediated presentation and therefore a decreased immune response [59]. This may occur because CD23 binds IgE–antigen complexes, allowing their internalization and processing through the endosomal

network responsible for loading peptides onto MHC II proteins for cell surface presentation to T cells.

Therapeutic Approaches Dependent on FcεRI and FcεRII

Today there are monoclonal antibodies being used in patients that bind CD23 (lumiliximab) or IgE (omalizumab/Xolair). The experience with omalizumab has demonstrated the efficacy of reducing free IgE levels as a way of controlling several diseases that have had a long association with atopy [60–62]. Sometimes, it has been surprising that there is more dependence on IgE than expected, such as the success of treating severe asthmatics with omalizumab [63]. However, these studies have also highlighted that this approach also fails in many patients so that there remains some missing pieces in our understanding of the role of IgE in these diseases. One of the clearly emerging characteristics is that IgE levels must be suppressed to less than 1% of the level found in a typical atopic patient [64]. It was noted earlier that this is likely the result of the high sensitivity of mast cells and basophils to stimulation with very few cell surface aggregates and the extremely high starting levels of cell surface IgE. Indeed, it is the down-regulation of FcεRI under conditions of low-circulating free IgE that probably allows this therapy to work at all. However, it is possible that a simple decrease in IgE is only managing part of the problem. A second observation derived from studies with omalizumab is that over the course of 1–2 years, there appears to be little change in the amount of IgE that the body is synthesizing. Total IgE levels remain quite high (although free IgE levels may be very low). It was anticipated, on the basis of experiments in mice [65], that a prolonged treatment with anti-IgE would eventually reduce the synthesis of IgE. Binding of omalizumab to IgE not only inhibits binding of IgE to FcεRI, but also to FcεRII. Therefore, one would expect some type of immune modulation that was dependent on CD23. In recent clinical trials of lumiliximab (anti-CD23), serum IgE levels decrease within days and for sustained periods, IgE levels were found to be approximately 25% of starting levels [66, 67]. This appears to be an inhibition of synthesis, unlike the situation with omalizumab. No changes in a variety of signs or symptoms indices were observed. Based on the experience with omalizumab, however, it is clear that by itself, this reduction in IgE level would be completely ineffectual in reducing the mast cell/basophil response. On the other hand, it is intriguing that it provides of way of reducing IgE distinct from simply creating a huge trap for the IgE that is otherwise continually synthesized. One other anticipated outcome of long-term suppression of IgE levels, be they free IgE or actual decreases in IgE, might be a dampening of the entire atopic state. In other words, without continual IgE-mediated activation of all the cells that display either FcεRI or FcεRII, the cytokine environment would shift away from one that perpetuates atopy. This has yet to be observed, at least in diseases such as asthma or rhinitis. It is possible that other atopic diseases, perhaps more dependent on this

perpetual cytokine environment rather than just the simple presence of IgE, might be more susceptible to therapies such as limiliximab or omalizumab.

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Superantigens and Allergic Disease

Mark S. Schubert

Introduction

Kappler, Marrack and colleagues were the first to describe and characterize the concept of superantigens in 1989 [1]. They found that staphylococcal enterotoxins, secreted proteins then known to be involved in staphylococcal food poisoning, were able to oligoclonally stimulate host CD4+ and CD8+ T cells to release massive amounts of pro-inflammatory cytokines. They described the mechanism of action of enterotoxins on T cells as dependent upon a binding interaction with T cell receptors (TCR) and major histocompatibility complex (MHC) class II molecules on professional antigen-presenting cells (APC). However, the conventional antigen recognition function of TCRs was bypassed. They coined the term "superantigen" because they found that several orders of magnitude more T cells could be strongly stimulated by enterotoxins compared to conventional antigen-specific T cell responses. Additional microbial superantigens have since been discovered and there has been a growing recognition of their potential roles in health and disease. Although initially found to be etiologic in staphylococcal food poisoning, superantigens are produced by a number of different microorganisms and are known to mediate various infectious syndromes including tissue necrosis, toxic shock, and sepsis [2–5]. Now, microbial superantigens have also been postulated to participate in the immunopathogenesis of specific chronic inflammatory diseases – most notably atopic dermatitis (AD) [6]. Evidence further suggests a role for superantigens in the immunopathology of chronic rhinosinusitis (CRS), chronic severe asthma, and some autoimmune diseases [7, 8]. An overview of microbes that produce superantigens and their potentially associated diseases is shown in Table 1.

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Table 1 Microbial superantigens and disease

Organism producing superantigen	Potential superantigen-caused or aggravated diseases
I. Bacterial	
Staphylococcus aureus	Food poisoning
	Toxic shock syndrome
	Kawasaki disease
	Experimental allergic encephalomyelitis
	Atopic dermatitis
	Psoriasis
	Chronic rhinosinusitis
	Chronic severe asthma
	Chronic obstructive pulmonary disease
Streptococcus pyogenes	Necrotizing fasciitis
	Toxic shock syndrome
	Rheumatic fever
	Psoriasis
Mycoplasma arthritidis	Arthritis
Mycobacterium tuberculosis	Tuberculosis
Yersinia	Reiter's syndrome
Pseudomonas fluorescens	Crohn's disease
Actinobacillus actinomycetemcomitans	Periodontal disease
II. Viral	
Mouse mammary tumor virus	Mouse mammary tumors
Mouse leukemia virus	Mouse AIDS
Human immunodeficiency virus	AIDS
Rabies virus	Rabies
III. Endogenous	
Human endogenous retroviruses	Insulin-dependent diabetes mellitus
	Multiple sclerosis
	B cell lymphoma
	Breast cancer
	Infectious mononucleosis
Protein Fv	Urticaria in viral hepatitis
IV. Unknown	Amyotrophic lateral sclerosis

Superantigen Interaction with Antigen Presenting Cells and T Cells

Superantigens stimulate massive T lymphocyte responses via simultaneous interactions with TCRs on responding T cells and human leukocyte antigen (HLA) MHC class II molecules on APC, such as dendritic cells (DC), Langerhans cells, and B cells. A number of microbes are currently known to produce superantigens that include *Streptococcus pyogenes*, *Staphylococcus aureus*, mycoplasma, and human endogenous retroviruses (HERVs) to name several [3, 5, 7, 9–12] (see Table 1). *S. aureus* alone makes over 20 distinct superantigens that include staphylococcal enterotoxin A (SEA), SEB, SEC, etc. [13].

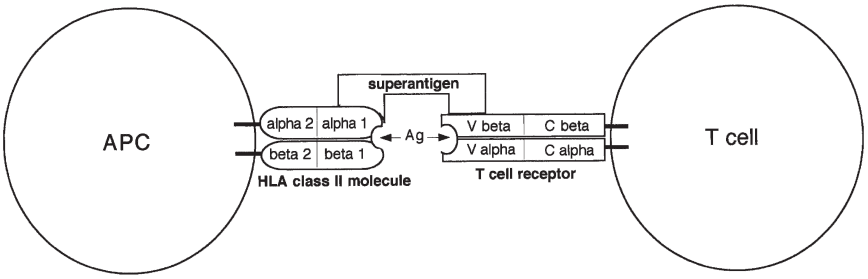

Fig. 1 T cell superantigen mechanism of action. APC antigen-presenting cell; Ag-peptide antigen-specific binding areas. Superantigens can also bind to beta 1 regions on HLA class II. From Ref. 7, with permission

Despite binding to both the MHC class II molecule on the APC and the TCR on the responding T cell, the defining feature of superantigens is that they mechanistically bypass peptide antigen-specific presentation by the MHC class II molecule and peptide antigen recognition by the TCR by forming a molecular bridge that results in a functional trimolecular MHC class II-superantigen-TCR complex [9, 14–17] (Fig. 1). Generally, superantigen binding is to the conserved regions on the sides of MHC class II and TCR molecules, away from their nominal peptide antigen-specific binding regions.

In this fashion, APCs are induced to strongly stimulate abnormally large numbers of T cells (5–30% of the total T cell population) without requirements for APC peptide antigen-presentation and conventional T cell antigen recognition. This is in contradistinction to frequencies of nominal antigen-responding T cells of around 0.01% [9, 15, 16]. However, unlike mitogens, there is a restricted heterogeneity of lymphocytes that can respond to a given superantigen. This is due to the structural binding requirements of superantigens for specific domains on the conserved regions of the TCR Vβ-chain away from the nominal peptide antigen-biding groove [1, 9, 15, 16].

The conventional alpha/beta TCR is a heterodimer made up of one α and one β chain. Each chain has a constant (C) and variable (V) region. The V regions of the α and β chains have variable and conserved domains. A given T cell superantigen requires the presence of specific conserved TCR Vβ domains on the responding TCR in order to bind properly. Different superantigens will bind to different Vβ domains, and may even bind to different motifs of the same Vβ [16, 17]. If the host has not inherited the specific TCR Vβ genes coding for the Vβ domains that a given superantigen requires for binding, then superantigen stimulation of T cells will be prevented. Therefore, the host's response to any given superantigen will be determined by their germline TCR Vβ genetics. The binding of superantigens to gamma/delta TCRs represents an addition to this rule, although they follow the same principles as their alpha/beta TCR counterparts [18].

There are about 60 TCR Vβ genes in the human repertoire, and each individual inherits a number of these. The reason that up to 30% of all T cells in an individual can be stimulated by a given superantigen is because most superantigens

are capable of binding to several different TCR Vβ families. [9, 15, 16]. For example, SEB binds to the human Vβ repertoire that includes Vβ 3.2, 6.4, 12, 13.2, 14, 15, 17, 20, and 22 [13]. SEB will therefore polyclonally activate all T cells that express these specific TCR Vβ families. This produces an "oligoclonal signature" of responding TCR Vβ-bearing T cells, including both CD4+ and CD8+ T cells, that immunologically identifies the presence of superantigen action [19]. Presence of superantigen-specific IgG or IgE will also identify significant exposure to the superantigen because superantigens can act as conventional antigens and are also capable of eliciting an antigen-specific immune response [7].

Superantigen facilitation of APC-T cell interaction induces T cell proliferation and cytokine release via activation of the same signal transduction pathways as conventional antigen-stimulation, but leads to enhanced T cell production of multiple T cell cytokines, such as tumor necrosis factor (TNF), interleukin-2 (IL-2), and interferon (IFN)-γ [10]. For example, injection of an SEA-SEB mix after primary immunization in the mouse enhances antigen-specific IgG titers up to fourfold [20]. Repeated or prolonged exposure of antigen-primed T cells to superantigen will further stimulate T cell proliferation and effector functions but naïve (non-antigen-primed) T cells of the same TCR Vβ specificity will undergo Fas/Fas ligand-mediated apoptosis with subsequent cell depletion [21, 22]. *S. aureus* enterotoxin-induced naïve T cell apoptosis supports further proliferation and functional amplification of the surviving superantigen-stimulated antigen-primed T cell responses by reducing local T cell competition for growth-supporting cytokines that are required by proliferating T cells.

The Role of MHC Class II in Superantigen Action

As compared to the TCR Vβ restriction of superantigen action, APC MHC class II binding by superantigen is permissive; any class II molecule isotype is capable of binding any superantigen [23]. However, there is a hierarchy of binding affinities that individual superantigens have for specific MHC class II isotypes that subsequently leads to more or less effective T cell stimulation [23]. In addition, the presence of specific MHC class II isotype polymorphisms (due to inheritance of allelic variants) on the APC leads to further modulation of T cell cytokine release by individual superantigens, resulting in increased or decreased T cell stimulation for the same superantigens [4, 24, 25]. For example, the superantigen streptococcal pyrogenic exotoxin A (SPE-A) was found to have a greater binding affinity for the HLA-DQA1*01 α chain than DQA1*03/05 α chains and led to increased T cell proliferation and cytokine release for the former [25]. Decreased T cell proliferative responses to SPE-A were seen when APC expressed DR1, DR4, or DR5 alleles [24]. The *Mycoplasma arthritidis* superantigen MAM elicited highest T cell responsiveness when APC bore HLA-DQA1*0103 and HLA-DQB1*0601 chains [26]. A clinical correlate of MHC class II-superantigen preference was the observation that patients with invasive group A streptococcal infections who had the DRB1*1501/DQB1*0602 haplotype had less

severe systemic inflammatory disease induced by the infection than those with other haplotypes [4]. Therefore, superantigen-mediated diseases depend upon the host's immunogenetics for the clinical outcome because of both TCR Vβ restriction and MHC class II haplotype preferences.

B Cell Superantigens

B memory cells are produced after cognate interaction of CD4+ T helper cells with naïve B cells. Cross-linking of the memory B cell receptors (BCR) will then stimulate the production of specific Ig. B cell superantigens bind to the conserved regions of the V domains of the heavy or light chains of the BCR similar to that seen with superantigen binding to TCRs [27, 28]. *S. aureus* protein A (SpA) is a B cell superantigen that binds to the V_H3 family of Ig germline V_H families. Because V_H3 is the largest (22 functional members) of the seven germline V_H families, roughly 50% of all B cells of an individual can be stimulated by SpA [27, 28]. Other B cell superantigens include the gp120 envelope glycoprotein of HIV-1 [29], protein L from *Peptostreptococcus magnus* [30] and the human liver protein Protein Fv. SEA and SED are both T cell and B cell superantigens [31].

Protein Fv, a liver protein, is an endogenous B cell superantigen found in stools of patients suffering from hepatitis A, B, C, and E [32, 33]. It binds to all immunoglobulin isotypes bearing V_H3, similar to SpA. Protein Fv is also a potent mast cell and basophil degranulator and secretagogue by virtue of its binding affinity to surface-bound IgE on these cells. It was found to be approximately 100 times more potent than anti-IgE in its ability to induce histamine release and IL-4 production from human basophils [34]. Because of its ability to bind IgE on Fc R1 + cells, causing release of histamine, pro-inflammatory mediators and Th2 cytokines, Protein Fv has also been called a "superallergen" [34]. It is likely to be responsible for the skin rashes and urticarial reactions common to viral hepatitis. For the same reasons, SpA is a superallergen and it has been found to effectively induce histamine release from human basophils and lung and skin mast cells [35, 36].

Superantigens and Mechanisms of Allergic Inflammation

Both T and B cell superantigens have the potential to be involved in allergic inflammation. The B cell superantigen Protein Fv described above is one example. The T cell superantigen *S. aureus* toxic shock syndrome toxin-1 (TSST-1) caused IgE isotype switching by normal human B cells in vitro [37]. The mechanism of action was found to be related to superantigen-induced T cell stimulation of IL-4 production and CD40 ligand expression, both of which promote IgE isotype switching. Keratinocytes, which became APCs upon stimulation with SEB-induced IFN-γ, participated with SEB to enhance dermatophagoides (Der p 1)-specific

T cell responses as measured by IL-4 production in vitro [38]. These findings suggest that T cell superantigens should be fully capable of amplifying disease-associated allergen-specific IgE. Additional support for this conclusion is discussed further in the specific disease subsections below.

Mandron et al. [39] have shown that human immature monocyte-derived DC will mature and activate upon exposure to SEB in vitro, leading to DC secretion of high levels of IL-2. The authors further found that these SEB-activated DC were capable of polarizing naïve T cells into the Th2 subset. The mechanism of SEB-induced maturation of DC may have been dependent upon direct SEB-induced stimulation of DC cell-surface Toll-like receptors (TLR). TLR are pattern-recognition receptors for conserved pathogen-associated molecular patterns. To test this, SEB was found to induce TLR2 signaling. The authors postulated that TLR2 signaling on DC via SEB exposure is fundamentally important in polarizing naïve T cells into the Th2 subset. This further allows superantigen-induced amplification of Th2 cells into key players in the generation of allergic inflammatory diseases such as AD.

The staphylococcal superantigens SEA, SEB, SEC, and TSST-1 can inhibit apoptosis of purified eosinophils in vitro [40]. This effect was shown to be at least partially mediated through up-regulation of the eosinophil survival cell-surface markers CD11b, CD45, CD54, and CD69. Eosinophil superoxide production was not found to be directly enhanced by superantigens, but was enhanced when eosinophils were initially exposed to superantigen and then incubated with eosinophil-activating cytokines such as IL-5. Taken together, *S. aureus* superantigens appear to both enhance eosinophil survival and increase cytokine-induced eosinophil activation after superantigen exposure. Eosinophils have also been shown to enhance SEB-induced generation of Th1 and Th2 cells in vitro [41], demonstrating that eosinophils not only play a key role as effector cells, but also may play an important role in amplifying superantigen-mediated T cell activation in allergic inflammation.

Epithelial cells can also actively participate in the amplification of superantigen-mediated allergic inflammation. For example, supernatants from human PBMC stimulated with SEB will induce the chemokines MCP-1 and RANTES from human epithelial cells in vitro, providing chemoattractants for T cells and monocytes [42]. Primary nasal turbinate epithelial cultures produce increased IL-6 in response to direct stimulation with SEB, potentially enhancing B cell differentiation [43]. Nasal epithelial cells also release IL-8, a neutrophil chemoattractant, upon exposure to SEA or SEB, particularly if primed with IFN-γ [44].

Severe chronic allergic inflammation as seen in chronic AD, CRS with nasal polyps, or chronic severe asthma often shows varying degrees of corticosteroid insensitivity [45, 46]. This is correlated with increased intracellular expression of the glucocorticoid (GR) β receptor, an inhibitor of GC action [45, 46]. Testing the hypothesis that chronic skin or respiratory mucosal superantigen exposure might play a role in the generation of GC insensitivity as a “virulence factor” in chronic allergic inflammation, incubation of normal PBMCs with SEB, SEE, or TSST-1was found to significantly antagonize the ability of dexamethasone to reduce T cell stimulation by mitogen [47]. Further, SEB was found to significantly increase Grβ expression in normal PBMCs in vitro. A human explant model of

nasal tissue found similar results, where ragweed allergen stimulation of nasal tissue from ragweed-allergic patients lost dexamethasone-induced IL-4 mRNA suppression, with concomitant up-regulation of the GRβ receptor in the presence of SEB [48, 49]. Therefore, superantigens are capable of inducing GC insensitivity by up-regulation of GRβ in both mitogen-activated and allergen-activated inflammatory models.

Superantigens and Allergic Rhinitis

Microbial superantigens have the potential to participate in the pathophysiology of allergic rhinitis as immunostimulants or even allergens, particularly those from *S. aureus* because of common nasal carriage and local chronic exposure. For example, Hofer et al. [50] isolated peripheral blood mononuclear cells (PBMC) from atopic patients and stimulated them with TSST-1. TSST-1 enhanced total IgE production from PBMC taken during the pollen season, but not during other times of the year. They also found that in cat, grass, and ragweed-allergic patients, TSST-1 increased allergen-specific IgE production but only in patients who were currently symptomatic. They also found that the concomitant expression of the co-stimulatory molecule B7-2, which would already be present on activated B cells during exogenous allergen stimulation in symptomatic patients, was required to allow T cell superantigen-induced augmentation of total and allergen-specific IgE production. B7-2 expression is known to correlate with APC survival and function [51].

Patients with perennial allergic rhinitis (PAR) to house dust mite were found to have higher symptom scores if colonized nasally with *S. aureus* [52]. Further, colonization with *S. aureus* strains that produced SEA, SEB, SEC, SED, and TSST-1 was found more frequently in the PAR patients as compared to nonallergic controls. PAR patients colonized with superantigen-producing *S. aureus* strains also had enhanced PBMC production of IL-4 and IL-5 in response to these superantigens in vitro. Although the authors did not find a significant difference in patient allergic symptom scores in PAR patients colonized with *S. aureus*-producing superantigens compared to nonproducers, only five superantigens were assayed. Since the study did not assay for all *S. aureus* superantigens, it is possible that other relevant *S. aureus* superantigens were locally present and active as immunostimulators in *S. aureus*-colonized PAR patients. For example, recent whole genome sequencing of methicillin-resistant *S. aureus* found evidence to suggest that the total potential heterogeneity of these superantigens may be as large as the genetic diversity of all TCR Vβ and MHC class II molecules combined [53].

Riechelmann and colleagues [54] further confirmed that nasal carriage of *S. aureus* in similar patients with PAR to dust mite was more common than nonallergic controls (68% carriage in PAR vs 22% in controls) and was significantly correlated with increased symptom scores and levels of total IgE, eosinophil cationic protein (ECP), and elastase in nasal secretions. Nasal *S. aureus* carriers with PAR were also found to have higher nasal IL-13/interferon (IFN)-γ ratios than noncarriers, which also

correlated with higher total IgE in nasal secretions. However, a recent study found no association of symptoms of allergic rhinitis in children with the presence or absence of specific IgE in serum to a mix of SEA, SEC, and TSST-1 [55]. Instead, they found that IgE to the superantigen mix was positively correlated with the presence of AD and/or wheezing. Interestingly, there was little difference in these findings when adjusted for the presence or absence of inhalant atopy [55].

Superantigens and Atopic Dermatitis

Several lines of evidence link the severity of AD to superantigen exposure from skin colonization with superantigen-producing strains of *S. aureus*. Over 90% of all patients with AD are colonized with *S. aureus* [6, 56]. Recent research suggests this high colonization rate is at least partly due to decreased expression in AD skin of the endogenous antimicrobial peptides cathelicidins and β-defensins [57]. The majority of these *S. aureus* isolates produce superantigens [58–60]. Lesional skin presence and density of superantigenic *S. aureus* strains correlate with AD severity [59, 60]. *S. aureus* superantigen-specific IgE is produced in the majority of AD patients [61] and correlates with disease severity [62, 63], particularly with titers of anti-SEB IgE. Direct application of SEB to either normal or AD patient intact human skin will induce an eczematous dermatitis [64, 65] that shows a selective accumulation of T cells bearing the SEB-reactive TCR Vβ repertoire specific for SEB [65]. A murine model of atopic dermatitis found that the application of topical allergen and SEB produced AD with a mixed Th1/Th2-CD8 + response with vigorous production of SEB and allergen-specific IgE and IgG2 [66]. Intradermal infiltrating T cells at sites of active human AD lesions exhibit shifts in TCR Vβ repertoires that exactly reflect the specific *S. aureus* superantigen profile found from lesional *S. aureus* cultures and superantigen analysis when compared to peripheral blood T cells [60]. All of these findings support an important role for *S. aureus* superantigens in the immunopathology and clinical severity of AD.

Although the predominant T cell functional phenotype in superantigen-activated AD appears to be within the Th2 compartment, evidence suggests both CD4 + and CD8 + T cells participate. The skin-homing cutaneous lymphocyte-associated antigen (CLA) + T cell subset identifies T cells that recirculate between the skin and peripheral blood [67]. Peripheral blood allergen-reactive T cells from patients with AD tend to be CLA+ [68]. Circulating CLA+ T cells in AD patients colonized with superantigen-secreting strains of *S. aureus* manifested significant superantigen-appropriate TCR Vβ repertoire-skewing of both CD4 + and CD8+ T cells [69]; this was not seen in either controls or patients with psoriasis. Further, AD patients' peripheral blood CLA + CD8 + T cells were found to be just as responsive as their CLA + CD4 + T cells to SEB-induced proliferation, and both subsets equally spontaneously released significant amounts of IL-4, IL-5, and IL-13 in vitro [70]. Both their CLA + CD4 + and CLA + CD8 + peripheral blood T cells stimulated IgE production in vitro from autologous B cells when compared to their CLA T cells.

Therefore, functional Th2 support for IgE production and eosinophil survival can be found from CD4 + and CD8 + T cells in patients with AD.

Neuber et al. [71] found that most of the infiltrating T cells in AD lesional skin were Vβ3 and Vβ12 (both known to be common TCR Vβ usage for SEB) and Vβ8. Anti-Vβ3 or Vβ8 monoclonal antibodies stimulated proliferation and IL-5 secretion of AD patients' PBMC over that seen with normal controls, further supporting a role for the Th2 phenotype in *S. aureus* superantigen-driven AD. Superantigen-induced Th2 predominance in AD is additionally supported by the findings that SEB stimulates IL-5 production from PBMC in patients with AD but not controls or asymptomatic atopics [72], and that *S. aureus* stimulation of PBMC from AD children results in higher proliferative responses and IL-4 production over that seen with nonatopic control patients [73]. A mouse model of atopic dermatitis generated by topical application of SEB evidenced a dermal infiltration with eosinophils and lymphocytes, and increased in situ mRNA expression and systemic (splenocytes) cytokine production of IL-4 but not IFN-γ [74]. Elevated serum levels of total and SEB-specific IgG1 and IgE were also found. Leung et al. reported that the majority of patients with AD had significant serum levels of specific IgE primarily to SEA, SEB, and/or TSST-1 [61]. The three superantigens also acted as allergens because they could directly degranulate autologous basophils in vitro, as well as IgE-stripped autologous basophils passively sensitized with superantigen-specific IgE.

Superantigens have been shown to have additional mechanisms of immunopathology in amplifying Th2-related effects in AD. For example, TSST-1 inhibited AD peripheral blood monocyte apoptosis in vitro via stimulation of GM-CSF production [75]. Zollner et al. found that skin culture positivity for *S. aureus* superantigen-producing strains correlated with up-regulation of the activation markers CD69 and HLA DR on peripheral blood T cells, and up-regulation of CLA on peripheral blood CD4 + T cells [59]. They also found that skin culture positivity for *S. aureus* superantigen-producing strains in AD inversely correlated with total serum IgE levels. The authors surmised that the inhibition of IgE production in vivo was related to high concentrations of bacterial superantigen exposure, because low concentrations were known to stimulate IgE production in vitro, but high concentrations were IgE-suppressive and likely due to strong stimulation of IFN-γ production [50,76]. An in vivo human-SCID mouse model confirmed inhibition of total serum IgE with high levels of exposure to SEB despite generation of epidermal inflammation and dermal T cell infiltration [77].

CD4 + CD25 + T regulatory (Treg) cells may also play an important role in the inflammatory biology of AD. Ou et al. have found a significant increase in these cells in peripheral blood in patients with AD compared to either asthmatic or nonatopic controls [78]. Patients with AD had the highest expression of CD4 + CD25 + Tregs within the peripheral blood CLA + T cell subset. Treg cells from AD, asthmatic, or nonatopic controls were all anergic to anti-CD3 stimulation, and actively suppressed autologous CD4 + CD25 T cell proliferation to anti-CD3, a known property of Treg cells. After in vitro exposure to SEB, Tregs from AD patients were no longer anergic to anti-CD3 stimulation, and lost their suppressive function in co-culture with autologous anti-CD3-stimulated CD4 + CD25 T cells. Therefore,

the normal immunosuppressive capacity of Tregs can be subverted in AD patients by exposure to SEB, further supporting the development and maintenance of the chronic inflammation seen in AD.

Superantigens and Asthma

Chronic persistent asthma, particularly poorly controlled asthma without obvious etiology, can be viewed as a chronic inflammatory disorder. Research into identification of antigen/allergen-specific triggers and other etiologic immunopathology remains central to our search for causations and ultimately the development of more effective and safer treatments. Several lines of evidence now support a role for superantigens in the immunopathogenesis of some cases of severe persistent asthma.

Hauk et al. [79] looked for a TCR Vβ oligoclonal signature in PBMC and bronchoalveolar lavage (BAL) T cells in poorly controlled asthmatics, well-controlled asthmatics, and normal controls. They found a higher percentage of Vβ8 + T cells in BAL from poorly controlled asthmatics when compared to the two other groups, and when compared to autologous PBMC. Several patients also had increased numbers of Vβ17 + T cells in BAL compared to autologous PBMC. Increased Vβ8 and Vβ17 TCR usage was found from within both CD4 + and CD8 + T cell subsets. Both Vβ8 and Vβ17 are known to have common superantigen-binding motifs [13]. These data are all consistent with the presence of T cell stimulation by an unidentified superantigen(s) within the lungs of poorly controlled asthmatics.

Herz et al. [80] tested the hypothesis in a mouse model that airways exposure to superantigen could generate asthma. Mice aspirated intranasal SEB or saline. SEB exposure generated airways hyperresponsiveness. SEB exposure also generated mucosal eosinophilic-lymphocytic-neutrophilic inflammation and elevated BAL levels of IL-4 and TNF-α, but not IFN-γ. A TCR Vβ analysis of BAL T cells showed airways T cell recruitment in both a superantigen-responsive and non-responsive manner. Therefore, airways exposure to superantigen was capable of generating an asthmatic response that mirrored human asthma in many ways.

Hellings et al. [81] extended these results by studying the effects of nasal aspiration and bronchial inhalation of SEB in an ovalbumin (OVA)-sensitized mouse model of asthma. SEB by either route of exposure enhanced the asthmatic response as measured by increased bronchial eosinophilic inflammation and increased bronchial expression of mRNA for IL-4, IL-5, IFN-γ, IL-12p40, eotaxin-1, and TGF-β. Bronchial SEB exposure also enhanced titers of serum OVA-specific IgE and total IgE. Therefore, airways exposure to *S. aureus* superantigen was shown to be capable of aggravating experimental allergic asthma.

Sixty-two percent of severe asthmatic patients tested were found to have specific IgE in serum to a mix of three *S. aureus* superantigens (SEA, SEC, and TSST-1) compared to 41% of mild asthmatics and 13% of non-asthmatic controls [82]. Of the 55 patients with either category of asthma, 31 had total serum IgE > 100 kU/ml, and of these, 21 (68%) had specific IgE to the *S. aureus* superantigen mix. Of 12

asthmatic patients who had total serum IgE > 500 kU/ml, 9 (75%) were IgE positive to the superantigen mix. Therefore, asthma tends to be correlated with the presence of specific *S. aureus* superantigen IgE, which further correlates with total serum IgE levels and asthma severity. Interestingly, elevated serum levels of specific IgE to the *S. aureus* mix were also found in a study of chronic obstructive pulmonary disease (COPD) patients [83]. COPD patients had higher specific IgE to the superantigen mix than smokers without COPD and normal controls.

Taken together, the association of asthma with markers for significant exposure to superantigens that correlates with disease severity, along with the evidence that superantigens can aggravate asthma in experimental models, suggests a role for superantigens in asthma immunopathogenesis.

Superantigens and Chronic Rhinosinusitis

The "superantigen hypothesis" [7] (Fig. 2) predicts that common forms of CRS (CRS with and without polyps, allergic fungal rhinosinusitis, eosinophilic mucin rhinosinusitis [EMRS], aspirin-sensitive CRS, atopic and nonatopic CRS) and related disorders can be unified immunopathologically in many patients by the action of exogenous and/or endogenous superantigens. Current evidence supports *S. aureus* as one major source for such superantigens [7, 84, 85].

Bachert et al. [84] found that 50% (10/20) of their patients with CRS with bilateral nasal polyps had significant levels of specific IgE to an SEA/SEB mix in nasal polyp tissue in situ. Interestingly, despite the finding that four of these ten CRS/polyp patients with in situ SEA/SEB-specific IgE tested negative to common aeroallergens on skin prick testing, they all were positive for aeroallergen-specific IgE in situ. Further, the presence of in situ IgE to the superantigen mix correlated with in situ levels of total polyclonal IgE, IL-5, eosinophil cationic protein (ECP), and soluble CD23 (the low affinity soluble IgE receptor whose presence is known to correlate

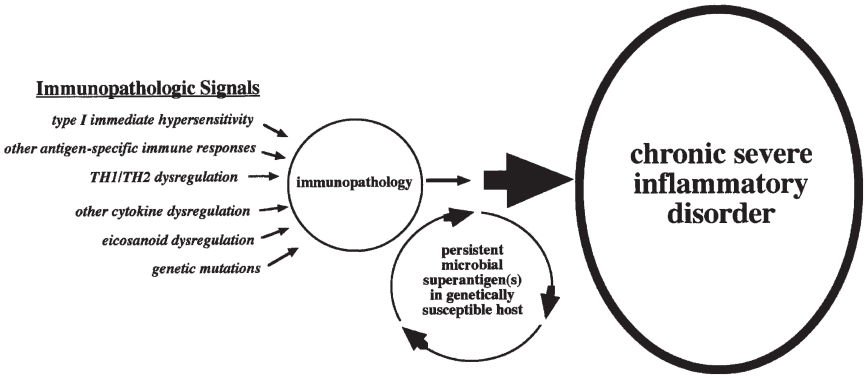

Fig. 2 Proposed model for superantigen-induced chronic inflammatory disorders. From ref. 7, with permission

with elevated IgE levels), but not IL-4, tryptase, or histamine. Cysteinyl leukotrienes in polyp tissue were also elevated in patients who were positive for specific IgE to the *S. aureus* superantigen mix compared to polyp tissue that was superantigen-IgE negative, irrespective of inhalant atopy by skin testing [86]. Therefore, the presence of specific IgE to *S. aureus* superantigens correlated entirely with the presence of polyp in situ inhalant atopy and markers for eosinophilic inflammation regardless of skin test results. This suggested that *S. aureus* superantigens are clinically relevant in CRS with nasal polyps as tissue-local amplifiers of both type I immediate hypersensitivity and eosinophilic-lymphocytic inflammation in general.

Tripathi et al. [85] found that 78% (18/23) of their CRS with polyps patients had specific IgE to either SEB or TSST-1 in serum, compared to none of their controls. There was no association seen between the presence or absence of superantigen-specific IgE and severity of rhinosinusitis assessed radiographically. Polyp in situ analysis for the presence of five different *S. aureus* superantigens found that 48% (14/29) of patients had the presence of at least one superantigen in polyp tissue, mostly SEB or TSST-1 [87]. Only 1 of 13 patients with CRS but without polyps was superantigen positive, and in this case it was from three different toxins. No controls were positive for any of the five *S. aureus* superantigens measured. Tissue eosinophil counts also correlated with the presence of superantigen, consistent with a role for *S. aureus* superantigens in tissue-allergic inflammation. A polyp tissue TCR Vβ analysis confirmed significant oligoclonal expansion and skewing of the Vβ repertoire over that seen with blood, all supporting the conclusion of a superantigen-effect within polyp tissue that is not seen in controls [88–90].

Bachert's group reported that *S. aureus* middle meatus colonization rates were roughly equivalent between controls and patients with CRS without polyps, but increased to 64% in CRS with polyps [91]. A subgroup analysis of CRS with polyps found *S. aureus* colonization rates of 67% in asthmatics, and 88% in patients with aspirin hypersensitivity. Polyp in situ rates of specific IgE positivity to an *S. aureus* superantigen mix of SEA, SEC, and TSST-1 correlated with the culture positivity rate, and was highest in the polyp subgroups with asthma or aspirin hypersensitivity [91–93]. Parallel correlations were found with in situ ECP and total IgE. A histologic study described lymphoid follicles and lymphoid accumulations within polyp tissue that strongly bound biotinylated SEA suggesting localized presence of SEA-specific antibody-producing B cells within these lymphoid areas [94]. Immunohistochemical staining has also recently demonstrated intraepithelial *S. aureus* and SEB in nasal polyp tissue [95]. These results all further support a role for *S. aureus* superantigens as immunostimulants in nasal polyps. Future research will further clarify the close relationship between *S. aureus* superantigens, CRS with nasal polyps and aspirin hypersensitivity. A possible relationship between CRS with aspiration of SEB and exacerbation of ulcerative colitis was also recently reported [96].

Schubert et al. [97] reported that the results of MHC class II genotyping in CRS and allergic fungal rhinosinusitis (AFRS) patients supported two roles for the class II molecule in these conditions – antigen presentation and preferential superantigen binding. They noted that both patient groups had an increased frequency of HLA-DQB1*03 alleles as a risk factor for disease. However, CRS patients presented with

any of the DQB1*03 allelic variants – DQB1*0301, *0302, *0303, *0304, and *0305, whereas AFRS patients presented primarily with DQB1*0301 or *0302. Therefore, they concluded that the role for HLA-DQ3 in AFRS and other types of CRS might include both antigen-specific peptide presentation (i.e., specific DQB1*03 alleles) and an additional function of the DQ3 molecule that utilized all DQ3 molecules without regard for peptide-presentation specificities (i.e., any DQB1*03 allele). The presence of both antigen specificity issues and preferential DQ3 molecule binding and presentation of a putative superantigen(s) were suggested as a possible explanation for this finding. A potential model for superantigen action in chronic inflammatory diseases is shown in Fig. 2.

Future Treatments for Superantigen-Related Diseases

New clinical approaches to treating superantigen-mediated inflammatory diseases in the near term will continue to focus on anti-inflammatory chemotherapy to target the types of inflammation involved. Inflammatory cell-surface receptors and adhesion molecules, pro-inflammatory cytokines, and intracellular signaling processes involved in inflammation are all viable therapeutic targets. Superantigen-specific therapy will depend in large measure on our ability to define the specific organisms and the superantigens involved. For example, vaccination against *S. aureus* and/or *S. aureus* superantigens is feasible [98–100]. A DRα-TCR Vβ bispecific chimera that prevents SEB binding with both MHC class II and the relevant TCR Vβ motif inhibited SEB-induced IL-2 synthesis and T cell proliferation [101]. A bioengineered soluble form of the TCR Vβ8.2 domain inhibited SEB-mediated T cell activation and neutralized SEB lethal activity in vivo in animal models [102]. A mutated SEA molecule without MHC class II binding ability blocked SEA-induced psoriasis in the SCID-hu mouse model, presumably by antagonizing SEA-TCR Vβ-binding [103]. A designed synthetic peptide that has homology to a part of the SEB molecule that is crucial for SEB biological effect protected mice against lethal challenge with SEB, inhibited SEB-induced IL-2, IFN-γ, and TNF-β mRNA synthesis, and led to rapid development of protective immunity from subsequent SEB challenges [104].

Conclusion

We are still in the early phases of a new era of investigation into the immunopathogenesis and treatment for chronic inflammatory diseases. Understanding of the interfaces between microbes and chronic inflammatory diseases promises to be a fruitful avenue for continued research. Since the landmark discovery of the superantigen mechanism [1], our understanding of the potential biological roles of superantigens has expanded from acute intoxication into chronic inflammation, including a possible role in autoimmune diseases. Multiple lines of evidence now support a central role for *S. aureus* superantigens in the immunopathogenesis of AD. Since

the seminal work identifying *S. aureus* superantigens as likely operatives in CRS [84], *S. aureus* has continued to be the primary choice for superantigen research in CRS, asthma, and AD. However, as predicted in the "superantigen hypothesis" [7], not only *S. aureus*, but additional microbes with superantigenic potential will likely be a focus of future investigations into the immunopathogenesis of chronic inflammatory respiratory tract diseases, as well as other chronic inflammatory diseases, including a possible role for the intriguing human endogenous retroviruses. Armed with a better understanding of disease immunopathogenesis, development of safer and more effective treatments for these potentially interrelated inflammatory diseases should be possible.

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Immune Complexes: Normal Physiology and Role in Disease

Michael M. Frank and C. Garren Hester

Introduction

The role of immune complexes in the induction of immunity, and in the development of tissue pathology has been a subject of intense study for over 100 years. In the late 1800s and the early 1900s, the fact that animals and man respond to antigenic stimulation with the formation of antibody was appreciated and a series of animal models of immunopathologic events were developed. These models still provide much of the basis of our understanding of the types of pathology that are caused by immune reactions. In addition, they provide much of our understanding of how antibody contributes to the host defense process. In 1902, systemic anaphylaxis was first reported [1]. We now know that this is usually caused by IgE antibody interacting with antigen and specific IgE receptors on mast cells, leading to the release of a variety of mediators. A different type of local immunopathologic reaction was discovered by the French investigator Maurice Arthus 1 year later, now called the Arthus reaction [2]. Arthus noted that when he injected antigen repeatedly into the skin of rabbits or later in guinea pigs, a local vasculitis developed that was characterized by tissue necrosis and local hemorrhage. On tissues sections one sees what is now termed leukocytoclastic vasculitis. There are neutrophils and neutrophil debris in the lesions and rupture of the vessel walls. He recognized that this reaction was mediated by antibody formed by the animal that interacts with the injected antigen. Other investigators noted that a type of antibody could be formed during the immune response that mediated lysis of certain types of cells and bacteria [3]. In this case, lysis required the interaction of the antibody bound to the cell or bacteria in addition to a heat-labile mediator system present in serum. The proteins of that mediator system are now given the collective name of complement.

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At about the same time a landmark series of discoveries was made by von Pirquet and Schick [4]. Following the work of Pasteur, immunization seemed to be the key to protection from infectious disease and the protective effect of specific antibody, made in response to a bacterium was recognized. By the early 1900s, injecting patients with antiserum made in an animal to combat infection was attempted and horse anti-diphtheria antiserum was developed as a treatment for diphtheria. It was used to treat thousands of children. It was noted that 5–8 days after the injection some of these children developed high fever, edema, proteinuria, and a urticarial eruption, which in most cases disappeared after a few days. It was generally believed that this was due to a toxic factor contaminating the horse serum. In a brilliant example of clinical investigation, Von Pirquet and Schick noted that children responded to the injection of the foreign serum protein with the formation of antibody to horse proteins. The timing of the illness corresponded with the formation of antibody to horse serum proteins. They reasoned that immune complexes were formed between the horse proteins and the antibody, and that these caused a wide variety of pathologic effects including albuminuria, edema formation, and skin rash, particularly urticaria, neutropenia, etc. This was the first suggestion that the products of the immune response could not only be beneficial, but could also cause disease in man, and they named the resulting illness serum sickness.

Animal Models of Immune Complex Diseases

With the advent of techniques for radiolabeling antigens and antibodies at the end of the Second World War, it became possible to study the metabolism of injected foreign proteins in experimental animals. In the first animal studies of serum sickness human serum albumin (HSA) or bovine serum albumin (BSA) was used to immunize rabbits [5]. It was discovered that as in man 5–8 days after antigen administration, IgG antibody was formed. The antibody could be detected because with its formation, the clearance of the foreign protein from the circulation was markedly accelerated. It was first assumed and later demonstrated that immune complexes formed and activated the animal's complement system causing a fall in serum complement [6]. The formation of immune complexes and the activation of complement were associated with the appearance of a wide variety of tissue pathology in experimental animals, which cleared as the immune complexes were removed from the circulation. These pathologic effects included vasculitis, carditis, glomerulonephritis, as well as rheumatologic and dermatologic manifestations of disease. This model of serum sickness was explored in great detail. The glomerulonephritis was found to be characterized by the deposition of IgG in the glomerulus, proteinuria, and swelling of endothelium, but there was little hematuria and few red cell casts. This was very similar to the horse serum-induced serum sickness in Worm man, which also showed marked proteinuria but few red cells and red cell casts. Neutrophil accumulation did not

appear to be essential for damage to occur and the role of complement in the induction was also not clear, since animals could be depleted of either and still develop pathology. Nevertheless, both neutrophils and complements were found deposited in the glomerular lesions. Arteritis was found in the coronary outflow tract and at the branching points of the great vessels. Here complement depletion clearly decreased the degree of pathologic damage.

In order to see what long-term pathology resulted from serum sickness, a chronic disease model was developed. In these experiments, the animals were given antigen repeatedly over many weeks. When the animals reached a point of antibody excess, where they simply cleared the injected antigen from the circulation, they showed no pathology. It was only when the animals achieved antigen excess with each antigen injection (i.e., more antigen available than antibody) and then cleared the antigen from the circulation that severe glomerulonephritis was observed. In fact proteinuria could become so severe in this model that some animals developed nephritic syndrome.

Biochemical Characteristics and Function of Immune Complexes

It was recognized that serum sickness in rabbits closely resembles systemic lupus erythematosus (SLE) in man and detailed studies of the behavior of the immune complexes formed in both the acute and the chronic model of serum sickness were performed in an attempt to understand the pathogenesis of SLE. It was found that pathology was observed when the complexes formed were at least 19S in sedimentation characteristics, that is, they were of large size [7]. Smaller-sized complexes were not associated with this pathology.

We have come to understand much about the function of antigens and antibodies over the years [8]. Nevertheless, there are many aspects of the process through which immune complex formation between specific antibodies and the microbes to which they are directed can on one hand defend the host but on the other hand can cause etc immunopathology that are still not clear. It is known that large-molecular weight antigens are more immunogenic than small-molecular weight antigens. Small, low-molecular weight chemicals are often attached to a large protein carrier providing “haptenic” groups to which antibodies and cellular immune responses may be directed. The chemical groupings on the surface of antigens, the degree of hydrophobicity, and the state of aggregation of the antigen are important determinants of the immune response, as is the duration of exposure and the number of times exposure occurs to that antigen. The route of immunization is also important with intravenous immunization often less immunogenic than subcutaneous or intradermal immunization. Some polysaccharides will also induce a strong antigenic response.

Characteristics of the antibody also are of importance in determining the behavior of the antigen–antibody complex. The valence of the immunoglobulin,

that is, the number of antibody-combining sites per molecule, is of importance. IgG, IgD, IgE, and monomeric IgA all have a valence of 2, two combining sites for antigen on each antibody molecule. IgM has an effective valence of 5. Therefore, in the case of IgM, low-affinity binding sites on each of the effective subunits binding to a multivalent antigen will be capable of causing agglutination, while this might not be true of IgG with similar affinity. Polymeric IgA has an effective valence of 4. The net charge of the antigen and antibody has also been shown to play an important role in determining their fate. Highly negatively charged or positively charged materials may bind to tissue and be fixed in place. For example, positively charged complexes tend to localize to the negatively charged basement membrane zone of the skin and renal glomerulus, where they are in a position to cause tissue damage. Basement membranes are, in general, negatively charged and are the sites of immunopathologic effects. The combining ratios of antigen and antibody are also found to be of critical importance. If antigen is mixed with antibody so that the antigen is in vast excess, the resulting complex tends to be soluble. For example, in the case of IgG, each of the IgG-combining sites is occupied by an antigen molecule and the complex has the formula IgG-Ag2, a small soluble complex. The same tends to be true in vast antibody excess, although the number of sites to which antibody can bind on the antigen is variable from antigen to antigen and therefore the size of the complex can vary. In the zone of equivalence, where neither is in excess, antibody and antigen molecules tend to form large cross-linked lattices that are far less soluble than in antigen or antibody excess. These large soluble lattices have the most biological activity in various assays like complement activation and are thought to be of the greatest importance in immune complex-related illnesses. It has been noted recently that the degree of sialation of IgG decreases as the immune response continues [9]. It is suggested that IgG with low levels of sialic acid is more pathologic and that those with higher levels of sialation serve a more protective function. The suggestion is made that the active material in IgG used therapeutically for treatment of patients with various autoimmune and rheumatologic diseases is only the heavily sialated protein.

Fate of Immune Complexes

Under most circumstances, immune complexes when formed in the circulation or which reach the circulation are removed rapidly by Kupffer cells within the liver sinusoids [10]. However, in autoimmune diseases they are found deposited in various organs where they cause local tissue pathology. Over the years, there have been many studies of the pathophysiology and clearance of immune complexes introduced into the circulation in attempts to understand the factors that lead to their being deposited in tissues [7]. It is astonishing that we still do not understand exactly how immune complexes exit the circulation to be deposited into tissues,

but some things are clear. Immune complexes upon formation are rapidly cleared by the liver and spleen. This clearance is due to their interaction with Fc receptors that recognize the Fc fragment of IgG present on phagocytes and particularly on the fixed phagocytes of the phagocyte system found in the liver and spleen. Facilitating the clearance of the complexes and their ingestion are complement receptors that exist on these same phagocytic cells.

Immune complexes bind to these fixed phagocytes by virtue of cell membrane complement or IgG Fc receptors and are rapidly phagocytosed and destroyed. The spleen serves as a secondary but critically important organ for removing these complexes from the circulation.

The ability of immune complexes to activate and bind complement proteins appears to play a multifaceted role in their removal from the circulation. C3 bound to the immune complex has multiple actions. The first product of C3 activation that binds to the complexes is C3b. C3b can facilitate phagocytosis by binding the immune complex to Kupffer cells by the complement receptors CD35 and the newly described CRIg in the liver as the blood passes through the liver sinusoids [11–13]. C3b also has the interesting property of allowing immune complexes to bind to the surface of erythrocytes which also have on their surface the C3b receptor, CD35. Although erythrocytes have a relatively few of these receptors per cell in the range of 900–1,500, they can still effectively bind immune complexes, that have bound C3b. The immune complexes with bound C3b adhere to the surface of the circulating erythrocyte where they are effectively removed from the circulation. The red cells pass through the sinusoids of the liver and spleen where lining macrophages remove the complexes and the red cells exit the liver and spleen complex free. They continue to have normal survival, although the removal of the complexes is associated with partial removal of the C3b membrane receptor, CD35. It is believed that in the absence of complement the immune complexes remain unbound in the circulation and are more likely to leave the circulation to be deposited in various organs. Complement binding also tends to break some of the bonds in the immune complex lattice, thereby solubilizing the immune complexes. Presumably this facilitates their clearance.

In spite of our learning a great deal about the behavior of injected immune complexes, we still do not understand all of the factors that govern their deposition into tissues. Almost all of the early experiments examined immune complexes formed in antigen excess, since these appeared to be most pathologic in rabbits. These experiments demonstrated that the rate of removal of immune complexes from the circulation was not influenced by depleting the animals of complement, and it was virtually impossible to cause immune complex deposition in organs like the kidney. To have an appreciable deposition it was essential to chemically modify the antibody so as to delay removal from the circulation. One method that proved effective was reduction and alkylation of the antibody used in formation of the complex [14]. This did inhibit complement activation and slowed the rate of clearance of the complexes from the circulation. Data was also presented, which suggested that an essential feature for tissue deposition was inflammatory mediator

release by local mast cells or basophils that, in turn, acted to open the tight junctions between the endothelial cells in the local vessels, allowing the immune complexes to escape [6].

Obviously, the amount of immune complex formed is an important para-meter determining the degree of immune complex deposited in tissue, since small amounts of immune complexes in the circulation are easily removed.

Many studies have addressed how immune complexes induce vasculitis and other forms of tissue injury. For example, the ability of immune complexes to interact and activate various mediator pathways to generate a host of inflammatory complement and kinin peptides is well known [15, 16]. Increasing evidence also indicates that endothelial cells are not passive bystanders in the evolution of immunologically mediated inflammation and tissue damage, as occurs in immune complex-mediated diseases. The ability of these cells to synthesize proinflammatory mediators such as interleukin-1 (IL-1), IL-6, IL-8, as well as proteolytic enzymes and tissue factor indicates their potential as active players in the inflammatory response. In addition, endothelial called cells adhesion synthesize and express a series of important cell-surface glycoproteins called cell adhesion molecules (CAMs). CAMs are cell-surface receptors that mediate cell–cell and cell–matrix interactions and play critical roles in diverse biologic processes, including inflammation, wound healing, and coagulation. At least five different CAM families exist: the integrins, immunoglobulin supergene family, selectins, adherins, and cartilage-link proteins. Endothelial cells express or can be induced to express CAMs from most of these families.

Some CAMs on endothelial cells mediate the attachment of leukocytes by binding to CAMs on the leukocytes. This “lock and key” phenomenon demonstrates specificity and is a highly regulated process. Thus, intercellular adhesion molecule-1 (ICAM-1) is present on endothelial cells and binds to the CAM leukocyte function-associated antigen-1 (LFA-1) on leukocytes. Furthermore, ICAM-1 expression on endothelial cells is increased by proinflammatory mediators, such as IL-1, tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and lipopolysaccharides (LPS), and can be up-regulated by complement during acute inflammatory lung injury [17]. These factors in turn lead to enhanced leukocyte binding and subsequent migration into tissue. Three CAMs – E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and P-selectin, are not normally expressed by endothelial cells, but their expression can be induced rapidly by proinflammatory mediators, IL-1 and TNF-α. Also IFN-γ up-regulates E-selectin and VCAM-1 and histamine up-regulates P-selectin. Because E-selectin and P-selectin preferentially bind polymorphonuclear neutrophil leukocytes (PMNs), these CAMs induce acute tissue inflammation. It has also become clear that the time course of CAM induction or up-regulation is an important aspect of the evolution of the character of the inflammatory cell infiltrate. E- and P-selectins are rapidly but transiently expressed on the surface of endothelial cells and preferentially bind PMNs, whereas the somewhat later appearance of VCAM-1 or the up-regulation of ICAM-1 may be more responsible for monocyte and lymphocyte binding [18].

Immune Complexes and Cytokine Release

Immune complex-mediated injury is associated with a number of inflammatory cytokines. Dermal vascular injury is highly dependent on IL-1β expression, and both IL-1β and TNF-α contribute to immune complex-induced injury in the lung [19]. Immune complexes also induce the release of IFN-γ and IL-2, and the release of IFN-γ is highly correlated with increased FcγRIIA expression on human monocytes and enhanced antigen presentation, as well as with increased synthesis of essential complement components [20, 21]. Complement opsonization of immune complexes in turn affects cytokine profiles, and inefficient opsonization is associated with increased IL-1β and IL-8 secretion from leukocytes. Complement C1q-bound immune complexes have also been shown to induce secretion of interleukin-8 (IL-8) from human umbilical vein endothelial cells (HUVECs) and from synoviocytes of patients with juvenile rheumatoid arthritis (JRA) [22]. The chemotactic complement fragment C5a has been widely studied and is associated with the production of IL-1β, TNF-α, and IL-8, as well as the sometimes anti-inflammatory IL-6 in human leukocytes.

Cytokine patterns characteristic are influenced by the immune complex antigen–antibody ratio [23, 24]. As the ratio approaches equivalence and antibody excess, a cytokine pattern shift from a Th1 or Th2 type immune responses has been reported. During this shift, IL-6 and IL-10 are released. This down-regulates the secretion of TNF-α and IL-1β, thus augmenting the stimulatory effects of proinflammatory cytokines on vascular permeability and neutrophil accumulation. In addition, interleukin-4 (IL-4) release has been found to down-regulate the IFN-γ̃-induced expression of FcγRIIA and up-regulate the expression of the inhibitory receptor, FcγRIIB2. An immune complex-induced shift from proinflammatory to anti-inflammatory cytokine release and the consequential modulatory effects on cellular expression of FcγRs may be important in attenuating immune complex-induced inflammation.

Effect of Antibody and Complement on the Survival of Erythrocytes

A number of more recent experiments in both animals and man have examined the effect of antibody and complement on the survival of erythrocytes or the fate of immune complexes in the circulation. In the case of erythrocytes, the antigens were proteins and lipopolysaccharides on the erythrocyte surface [25]. By labeling the erythrocytes with 51 chromium it was possible to follow their fate and determine the effect of antibody and complement. It is tempting to consider antibody and complement-coated red cells as large immune complexes, but it is clear that small antigen–antibody complexes, as might form between antibodies and individual proteins, may behave quite differently in the way they are handled. Nevertheless, there are important conclusions that come from erythrocyte studies.

The first experiments were performed with antibody- and complement-sensitized cells in guinea pigs. Using C4-deficient guinea pigs, it was possible to differentiate with precision the separate effects of antibody and complement. First, in the case of clearance of erythrocytes coated with IgM antibody, 1–200 molecules of IgM bound per red cell that can bind a similar number of C1 molecules and, thus can activate the classical complement pathway, can cause erythrocyte clearance. The cells were removed from the circulation by the Kupffer cells in the liver rather than by the spleen. Cleared cells were inefficiently phagocytosed and they remained adherent to the phagocyte surface. The C3 on the erythrocyte surface was degraded over time by complement control molecules causing the erythrocytes to be released from the liver. The released red cells had normal survival, although they were coated with complement degradation products. If systemic inflammatory cytokines were released, for example, by the injection of mycobacteria into the experimental animals, they activated the phagocytes with erythrocytes bound to receptors by their complement ligands and the erythrocytes were phagocytosed.

However, the clearance of IgG antibody-sensitized cells was quite different. Very few IgG-mediated complement fixing sites on the red cell surface were sufficient to signal the removal of red cells from the circulation. In the case of IgG, two IgG molecules side by side are required for clearance and, therefore, it required 1,000 or 2,000 molecules of IgG per cell before one complement fixing site was generated. IgG-sensitized cells with relatively few complement-activating sites were not cleared by the liver, but were cleared efficiently by the spleen. The liver receives about 50% of the cardiac output, and the spleen about 5%. Thus, one can assume that such cells pass through the liver without being recognized and are removed by the sinusoids of the red pulp of the spleen. As one increased the amount of IgG, one increased the amount of complement activation and at the same time increased the likelihood of interaction of the membrane-bound IgG with IgG Fc and complement receptors, causing a shift of clearance from the spleen to the liver. Unlike clearance of IgG-sensitized cells in which rapid clearance by the liver is followed by the release of the erythrocytes into the circulation, clearance by the spleen of IgG-sensitized cells was progressive; the cleared cells were never released back into the circulation but were phagocytosed.

Later studies extended these findings to clearance of erythrocytes in man [25, 26]. Here, it was possible to do only limited studies in complement-deficient human beings. Nevertheless, the overall conclusions of the guinea pig studies held true for man, with IgM cells being sequestered in the liver and released. IgG again caused splenic sequestration, but there was an important difference between the human and guinea pig studies with regard to the biological properties of the IgG. The IgG antibody used in these studies was anti-Rh antibody, which is known to activate complement poorly. It can be assumed that the splenic effects were mediated by splenic macrophage IgG Fc receptors and not by complement. Since these studies allowed for the separate evaluation of complement receptor and Fc receptor function, the clearance studies were extended to patients with a variety of diseases. It was found, for example, that patients with active SLE have a profound defect in their clearance of IgG-sensitized erythrocytes and it was suggested that patients

with SLE may have an Fc receptor defect that renders the clearance of IgG containing immune complexes inefficient, thereby contributing to disease activity.

Similar experiments on clearance in man have been performed with immune complexes consisting of hepatitis antigen and antibody or tetanus toxoid and antibody [27]. It was found that there are differences between the clearance of soluble complexes and red cells. Clearance again was the responsibility of phagocytes within the liver and the spleen. In general, clearance was progressive for these complexes, and they were not released back into the circulation. As discussed above, an observation in the red cell system was that patients with SLE have a defect in their ability to remove IgG-sensitized cells from the circulation. Patients with SLE have high amounts of circulating immune complexes that could block IgG Fc receptors and immune complex deposition in tissues. The complexes described above that were injected into humans were all complement-activating complexes. Nevertheless, a defect in splenic clearance was again described in patients with SLE. However, in SLE patients injected with complexes with IgG and complement in their composition, the liver compensated for the clearance defect in a way that did not occur with the antibody-sensitized red cells, and there was more rapid clearance by Kupffer cells of these complexes from the circulation. Thus, in patients there was a splenic defect compensated by hepatic increase in clearance.

IgG Fc Receptors and Their Function

In the last few years the IgG Fc receptors (FcγR) have been studied in extraordinary detail. With the advent of molecular cloning techniques, it became possible to clone and sequence each of these receptors and study their function in experimental animals [28–30]. The experimental animal findings could then be related to human Fc receptor function. The Fc receptors that have been studied in greatest detail are the receptors on phagocytes and lymphoid cells in the mouse. Here, there are four groups of receptors: FcγRI, FcγRII, FcγRIII, and FcγRIV. The FcγRI, II, and IV groups have been shown to be activating receptors; that is, interaction with immune complexes leads to cellular activation and often leads to phagocytosis. FcγRII in the mouse occurs only in the form of FcγRIIb and is an inhibitor of various cellular functions. In humans, FcγRII consists of a group of receptors with FcγRIIa and FcγRIIc being activating receptors and FcγRIIb having cellular inhibitory function. FcγRIIb is the only Fc receptor on human B cells and also occurs in man on a variety of other cells including follicular dendritic cells and macrophages. In general, the activating Fcγ receptors interact with immune complexes and bind an accessory molecule (γ chain), which is then phosphorylated to transmit their signal. FcγRIIb has an inhibitory motif on its intracellular cytoplasmic region and activates the inhibitory cascade directly. FcγRI is a high-affinity receptor having a Ka in the range of 1×10^{-9}. FcγRII and FcγRIII are low-affinity receptors having a Ka in the range of 10^{-7}. FcγRIV has midrange affinity with a Ka between FcγRI and FcγRII and III. FcγRI, II, and III in the mouse have been shown to be very similar in

function to FcRI, II, and III in human. FcγRIV in the mouse has been only recently defined, and presumably in time, one will find FcγRIV in human materials as well. In mouse and man high- and low-affinity IgE receptors have also been studied in great detail. Because these receptors are not thought to play a major role in immune complex physiology, they are not considered here in any detail.

The activating receptors use an accessory molecule called the γ chain, which allows their function. All but one of the receptors has a transmembrane domain assuring its firm attachment to the cellular membrane. A form of FcγRIII has a phosphatidyl inositol linkage, which allows rapid mobility in the cell membrane, while a second form of this receptor has a transmembrane domain as do the others. A total of eight genes have been identified for human Fc receptors for IgG: three for high-affinity receptor, Fcγ RI (FcγRIa, FcγRIb, and FcγRIc); and five for the low-affinity IgG receptors (FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa, and FcγRIIIb. The five low-affinity genes are clustered on human chromosome 1q23, while the three high-affinity genes are mapped to chromosome 1-2q. The activating receptors, usually working through an adaptor molecule has an ITAM immunoreceptor activation motif, which leads to cellular activation, while the inhibiting receptor FcγRIIb has an ITIM immunoreceptor-based inhibition motif, which leads to down-regulation. The activation motifs lead to phagocytosis, degranulation, cytolysis, cytokine up-regulation, and antigen presentation. The inhibitory motif down-regulates many of these same functions via triggering of an inhibitory intracellular pathway. This pathway has been defined in some detail and includes SH2-containing inositol phosphate (SHIP) as an inhibitory molecule disrupting ITAM activation and B cell receptor-mediated calcium influx.

Complement Receptors

The receptors important in the clearance of immune complexes recognize the complement fragments C3b or iC3b. C3b is the major fragment of C3 formed after the activation of C3 and the release of the anaphylatoxic fragment C3a [11, 12, 31]. Complement can be activated by immune complexes either by the lectin, classical, or alternative pathway, or most usually by the latter two pathways. In all cases C3b is the first C3 fragment formed. CD35 present on all phagocytes, B cells, and on most dendritic cells recognizes and binds to C3b. C3b can be degraded to iC3b. The B2 integrin receptors CD11b/CD18 and CD11c/CD18 present on phagocytes and dendritic cells but not on B cells recognize this degradation product of C3b, iC3b and mediate binding and phagocytosis. A newly defined hepatic complement receptor termed CRIg recognizes both C3b and iC3b, and also mediates adherence and phagocytosis [13]. The relative importance of all these receptors is still being defined, but it is fair to say that different experimental models of disease using either different antigen–antibody complexes or different animal models show a difference in the relative contribution of Fc receptors and complement receptors

to host defense and to the development of disease. If one generates immune complexes in serum at a ratio of antigen to antibody such that the complex formed is in mild antigen excess, these complexes will activate complement rapidly in vitro, but in vivo these immune complexes do not appear to require complement for their efficient removal from the circulation. On the other hand, there are circumstances where complement activation may be important in their clearance; for example, when the complexes are formed in antibody excess.

Many well-established mouse models of autoimmune disease have been studied after introducing a targeted gene deletion of activating or inhibitory receptors [30]. In mouse models of autoimmune disease, mice lacking the inhibitory receptor have had more severe glomerulonephritis, arthritis, and carditis. Animals missing some or all of the activating receptors have a decrease in clinical activity in these same areas. It is interesting that even anaphylaxis, not usually thought to be mediated by IgG Fc receptors FcγRb, is less severe in animals missing the activating receptors. Perhaps one reason for this is the recent demonstration that IgE antigen–antibody complexes are capable of interacting with the activating receptor FcγRIV leading to cell activation and its immunopathologic consequences.

The downstream intracellular consequences of activating these various receptors have been studied in considerable detail [28, 29]. In phagocytes, there appear to be a two phase activation processes. In the first phase, the src family of protein kinases is activated and, in turn, activate and cause tyrosine phosphorylation of cytoplasmic ITAM motifs. In the second phase, other kinase families such as syk are activated and bind the phosphorylated ITAM. Ultimately, this leads to a burst of intracellular-free calcium, which induces the release of inflammatory mediators including leukotrienes and prostaglandins, various hydrolases, and the transcription of cytokine genes. A polymorphism in FcγRIIIa has been identified in SLE patients, especially in patients with associated nephritis, and a polymorphism in FcγRIIIb has been linked to patients with susceptibility to rheumatoid arthritis [32]. Thus, it is possible that polymorphisms in these receptors and their efficiency in activation and inhibition are important in human disease as they clearly are in animal disease.

Physiology and Function of Immune Complexes

It is known that immune complexes are formed in normal individuals under a wide variety of circumstances, but they rarely cause disease. For example, someone who has a cold may have circulating antivirus antibody formed that can interact with the virus to form immune complexes that are then rapidly cleared from the circulation. Thus, the formation of immune complexes is part of everyday life. Moreover, it has been appreciated that natural antibody, the product of the cell termed B1a in mice and B1 in man, is generally present in the circulation and is capable of forming immune complexes [33–35]. The majority of the circulating immunoglobulins in normal people is comprised of this natural antibody. Much of it is IgM, but IgG and

IgA are formed as well. This antibody interacts usually with relatively low affinity with a wide variety of pathogens or other antigens, and acts as a first line of defense against infection. For the most part, natural antibody is the product of immunoglobulin genes in the germ-line configuration, that is, before the process of somatic mutation occurs, which leads to high-affinity antibody. In mice and presumably in man, these cells line the peritoneal cavity and are also present in a number of other locations. Natural antibody is often polyspecific in that it recognizes multiple antigens and often has self-reactivity. One of the basic properties of the adaptive immune system is that antibodies to self are formed and later the system deletes the clones of cells that produce these antibodies because they may be damaging the host. It is believed that any antigen that we encounter is likely to be introduced to the immune system in the form of immune complexes with antigen bound to natural antibody. For all of these reasons the presence of immune complexes in the circulation can be thought of as normal and we must go further to explain why in certain situations and in certain diseases they appear to cause tissue pathology.

Much of what happens in the immune response is determined by the organizational structure of secondary lymphoid tissues such as the spleen and the lymph node. The spleen will be used as an example. It contains the red pulp with open sinuses allowing phagocytosis and destruction of a wide variety of microorganisms and immune complexes that enter through the blood [34]. The white pulp is the portion of the spleen where the immunologic reactivity takes place and contains both T cell and B cell areas. The B cell areas are often in the form of follicles surrounded by T cell areas and red pulp. At the periphery of the follicle is the marginal sinus, and around the marginal sinus is the marginal zone. Within the follicle are developed germinal centers, where newly dividing, somatically mutating B cells are formed. These will become the plasma and memory cells responsible for the normal immune response. During the course of an immune response, antigens introduced into the bloodstream will within minutes come to the marginal zone of the spleen. Here, a specialized series of interactions take place leading to the formation of antibody within hours by B cells that reside in the marginal zone. Often this antibody is IgM. Thus, pathogens being introduced into the body first meet natural antibody, the product of cells that have not undergone somatic mutation that are already forming circulating antibody. This antibody can coat the pathogens. The pathogens are swept into the marginal sinuses in the marginal zone where B1 b cells (studies in mice) are capable of a rapid response with the elaboration of specific antibody within hours. Again, the specific antibody is not the product of somatically mutated cells. The cells that form this antibody have not had time to undergo mutation, but this antibody is responsible for important protection against infection. Antigen trapped by marginal zone B cells is then transferred to the follicle where the interaction of follicle dendritic cells and follicular B cells leads to the steps that completes the immune response. During these various steps, both in primary immune organs like the bone marrow and in secondary immune organs like the spleen, there are checkpoints at which cells with specificity for antibody for self are removed. It is this complex process, which is felt to be faulty in autoimmune diseases with circulating antibody to self-antigens.

Serum Sickness in Man

Some time ago, we had the opportunity to study prospectively serum sickness in man [36]. The occasion was studied to treat aplastic anemia in man with horse antithymic globulin. Like the horse serum that was used to treat diphtheria, horse antithymoglobulin was a foreign protein given to individuals in fair amount to control a specific disease. Unlike the serum given to children 100 years ago, this was highly purified immunoglobulin and thus was one serum protein. Nevertheless, it was a foreign protein and regularly produced serum sickness in the patients that received it. The patients that we studied had either a dose of the serum protein intramuscularly once a day for 10 days or had protein every other day for 2 weeks. It was a part of a trial to determine whether inhibition of the immune system would lead to recovery from aplastic anemia. It was found that about one third of patients recovered from aplastic anemia by virtue of this treatment, without the bone marrow transplantation that was planned when horse antithymoglobulin was introduced for the treatment of this disease. As mentioned, these patients regularly developed serum sickness following the introduction of the foreign protein. The foreign protein was given multiple times, and serum sickness set in a number of days after the last injection of the protein. These patients developed fever, edema, and proteinuria like the children of von Pirquet and Schick; however, they were more severe with immunologic disease and went on to develop arthralgias, blood in their stool, elevated liver enzymes, and an occasional true arthritis. Like the children of von Pirquet and Schick, these pathologic effects disappeared, as immune complexes were cleared from the circulation.

These patients developed urticaria at the time of the appearance of the circulating immune complexes like the patients of von Pirquet and Schick.

Like the rabbit model, these patients developed hypocomplimentemia at the time of circulating immune complexes, which disappeared when immune complexes were removed from the circulation. The prospective study validated in man the observations that had been made in the rabbit model in the 1950s, 1960s, and 1970s, making it likely that the pathologic processes noted in experimental animals mirrored those that took place in man.

Determining Immune Complex Levels in Blood

Although we understand that many diseases are associated with circulating immune complexes, it has not been highly useful clinically to determine immune complex levels in patients. Often we use a surrogate marker like the plasma level of complement C3 or C4 to determine whether immune complexes are likely to be present, because the measurement of immune complexes has not made much of a difference in therapy. Nevertheless, a number of assays for immune complexes have been developed, and they will be discussed briefly here [37]. Some simply

examine the physical presence of large insoluble immune complexes in the circulation. There are a number of these tests and examining serum for the presence of cryoglobulins is one such test. Large immune complexes tend to be less soluble in serum in the cold. Cryoglobulins are often antigen–antibody complexes composed of antibody and hepatitis C antigen. Incubating serum in the cold for several hours will often cause them to precipitate. This sort of test is often not very sensitive and not highly specific.

The C1q binding assay is an assay that measures the presence of the immune complexes by their capacity to bind a subunit of the first component of complement, C1q. Immune complexes present in the circulation and containing antibodies that activate the classical pathway can bind C1q. The assay looks for material in the circulation that can bind C1q. In practice, a sample of serum is taken from the patient who may have circulating immune complexes. C1q that has been radiolabeled or tagged by some other method is added to the serum in the presence of ethylenediaminetetraacetic acid (EDTA), which dissociates any intact C1 present on the complexes. The tagged C1q can replace the C1q present on the circulating complexes, and if the complexes are precipitated, such as done with polyethylene glycol, the tagged C1q will precipitate with the complexes. The labeled C1q added to normal serum remains in solution, because there are no immune complexes present. If immune complexes are present, the C1q will precipitate with the complexes. Appropriate controls are important, since C1q will bind to several other substances in blood, for example, free DNA.

A second very commonly used assay is the Raji cell assay [38]. Raji cells are a lymphoblastoid cell line from a patient with Burkitt's lymphoma. These cells have on their surface CD21, the receptor for the complement protein degradation fragment C3d. These cells do not have functional Fc receptors, which are normally present on lymphoid B cells. If immune complexes are present in the circulation and have bound complement, the complement goes through a series of reaction steps, which ultimately can lead to a portion of the complexes having C3d bound. Since there are no functional Fc γ receptors on Raji cells, immune complexes must bind to the Raji cell surface via C3d binding to CD21. If such binding occurs, the immune complexes can be detected with a tagged antibody to human IgG. Thus, a positive test infers the presence of complement bound to immune complexes through the level of C3 in the patient's serum. These two tests are used for research purposes, but are rarely used in everyday medical practice.

Conclusion

In summary, we have known that the formation of immune complexes can be associated with major organ pathology for over 100 years. The physiology of the complexes has been studied in man and animals and a great deal has been learned about the chemical properties of antigen and antibody that contributes

to the pathologic processes induced by the immune complexes. Nevertheless, there are a number of key issues that remain unresolved. These include a detailed understanding of the processes that lead to the immune complexes leaving the circulation to be deposited in tissues as well as the role of immune complex formation in the initial stages of the immune response. Hopefully, these issues will be resolved.

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What Is New About Eosinophil Activation in Asthma and Allergic Disease

Akira Kanda, Junichi Chihara, Monique Capron, and David Dombrowicz

Eosinophils and Asthma

This review will attempt to delineate the various effects of PGD_2 and its metabolites on allergic inflammation in general and on eosinophils in particular.

Indeed, eosinophils are key players in allergic diseases including asthma. Several studies have indicated that eosinophils not only act as effectors, but also as antigen presenting cell (APC). Duez et al. [1] have reported that lung draining lymph node eosinophils express MHC class II molecules and so may exert various roles according to their tissue location. Shi and collaborators also demonstrated that eosinophils can process inhaled antigens, traffic to regional lymph nodes, and act as APC to stimulate T cell responses [2]. However, the precise role of eosinophils in allergic asthma remains difficult to delineate. Two different lines of eosinophil-deficient mice have been generated. In PHIL mouse, an eosinophil-specific promoter drives expression of diphtheria toxin A [3]. In Δdbl-GATA mouse, absence of eosinophils caused a deletion of the high-affinity GATA-binding site in the GATA-1 promoter [4]. In both models, absence of eosinophils leads to decreased fibrosis. However, its impact on allergen-induced airway hyperresponsiveness (AHR) and mucus production is different.

PGD_2, Its Receptors and Metabolites

The lipid mediator prostaglandin D_2 (PGD_2) contributes to inflammation as well as homeostasis such as regulation of sleep, intraocular pressure, vasodilatation, and bronchoconstriction. PGD_2 is produced by activated mast cells and thereby has for a

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long time been associated with allergic diseases. Figure 1 shows the various metabolic pathways leading to and starting from PGD_2. Since high PGs concentrations have been detected at sites of acute inflammation [5, 6], biological activity of PGs including PGD_2 has been implicated for a long time in the pathogenesis of allergic diseases such as allergic asthma [7]. The functional role of PGD_2 in inflammatory reactions has dramatically progressed with the discovery of a second PGD_2 receptor [8], chemoattractant receptor-homologous molecule expressed on TH2 cells (CRTH2 [also as known DP2]), in addition to the already described D-prostanoid receptor 1 (DP1). Thus, PGD_2 binds both DP1 and CRTH2, which are seven transmembrane-type G protein-coupled receptors (GPCRs). Both receptors are expressed by several cell types within the immune system: DCs, Th2 cells, basophils, and eosinophils [9]. Interestingly, intracellular signaling pathways triggered by DP1 and CRTH2 activation are virtually antagonistic; DP1 activation leads to a G_s-mediated increase in intracellular cAMP and reduces calcium flux [10] while CRTH2, in contrast, is coupled to a G_i-type G protein and leads to inhibition of cAMP and increased intracellular calcium [8, 11]. However, a link between PGD_2 and inflammation is still controversial. Here, we first examine whether PGD_2 acts towards promotion or inhibition of allergic inflammation including eosinophilia. We will discuss the interactions between DP1 and CRTH2 on eosinophils.

PGD_2 is immediately metabolized into the cyclopentenone PGs of the J series, such as PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) in vivo [12, 13]. These PGD_2-derived prostaglandin J_s (PGJ_s) not only bind CRTH2, but also Peroxisome Proliferator-activated Receptors gamma (PPAR-γ), a ligand-activated transcription factor, a member of the nuclear hormone receptor superfamily, which includes receptors for steroids, thyroid hormone, vitamin D, and retinoic acid [14]. PPAR-γ is one of the three PPAR subtypes (α, β/δ, and γ). It is expressed in both murine and human eosinophils [15, 16], as well as in adipocytes, osteoblasts, endothelial cells, as well as on hematopoietic cells [17]. PPAR-γ negatively regulates inflammation [18, 19], acting on T lymphocytes [20], monocytes/macrophages [21], dendritic cells [22], and mast cells [23]. These evidences thus indicate that PGJ_s might play an anti-inflammatory role in part by acting on PPAR-γ expressed by eosinophils. Thus, the complete set of interactions of PGD_2 and its metabolites with PGD_2 receptors expressed by eosinophils, DP1, CRTH2, and PPAR-γ, represent a complex network. Therefore, understanding the effects of PGD_2 and PGD_2-derived PGJ_s on eosinophils will help to elucidate the mechanism of allergic inflammation.

We will finally discuss possible therapeutic strategies for asthma, focused on DP1, CRTH2, and PPAR-γ expressed by eosinophils.

Functional Role of PGD2 on Eosinophilic Inflammation

PGD_2 has, for a long time, been associated to inflammation including allergic diseases. Marsden et al. [24] first reported a functional link between PGD_2 and eosinophils by showing that intravenous infusion of PGD_2 in dogs increased the number of circulating eosinophils. Subsequent evidence demonstrated that PGD_2

caused accumulation of eosinophils in the tracheal lumen of dogs [25] and that PGD_2-activated human eosinophils displayed enhanced in vitro release of leukotriene C_4 (LTC_4), which is one of the central mediators of the asthmatic response [26]. On the other hand, Woodward et al. showed that BW 245C, a selective agonist for DP1 receptor, did not cause pathological effects in the conjunctiva, while PGD_2 increased conjunctival microvascular permeability, eosinophil infiltration, and goblet cell depletion [27]. The functional role of PGD_2 on inflammation remained unclear until early 2000s, when CRTH2 was identified as a receptor for PGD_2 (for affinity values see Fig. 1) [8]. Indeed, Hirai et al. [8] reported that PGD_2 selectively induces chemotaxis in Th2 lymphocytes, basophils, and eosinophils through CRTH2. At the same time, Monneret et al. also referred to CRTH2 as DP2 receptor and showed that eosinophil activation was neither induced by BW245C, nor inhibited by the potent DP1 receptor antagonist, BW868C. In contrast, PGD_2 stimulated actin polymerization, CD11b expression, L-selectin shedding, and chemotaxis of eosinophils, while BW868C inhibited cAMP accumulation in platelets [28].

DP1

DP1 receptor was initially identified as a PGD_2 receptor, involved in eosinophilic inflammation in a study using DP1-deficient mice (C57BL/6 background) in a model of allergic asthma. $DP1^{-/-}$ mice had a reduced AHR, attenuated eosinophil, and lymphocyte infiltration following allergen challenge [29]. In agreement with this study, a DP1 receptor antagonist, S-5751, dramatically inhibited not only early nasal responses, but also late responses such as mucosal plasma exudation and eosinophil infiltration [30] and compound 3i similarly suppressed allergic inflammatory responses such as the PGD_2-induced increase of microvascular permeability in vivo [31]. Substantial clinical study in patients with asthma using single-nucleotide polymorphisms (SNPs) supported that impaired DP1 expression was associated with a reduced risk of asthma [32].

In contrast, regarding the inhibitory effect of DP1 on inflammation, our group has reported that selective DP1 agonist (BW245C) reduces pathology including eosinophil infiltration, IL-5 production in the lung, and AHR in a mouse model of asthma in Balb/c mice [33]. Likewise, in a model of atopic dermatitis (AD) induced by epicutaneous sensitization with ovalbumin, DP1 activation by BW245C reduced skin inflammatory response: eosinophil and mast cell infiltration and epidermal thickening [34]. In another AD model using NC/Nga mice raised in conventional animal facility, DP1 activation decreased skin inflammation [35].

These apparently conflicting results on DP1 function in the control of Th2-associated inflammation might be due to differences between animal species or mouse strain and/or between experimental protocols used (genetic inactivation in the whole animal versus airway targeting by aerosol treatment with agonist/antagonist). Besides, timing of DP1 activation might be of particular importance in the subsequent development of local immune inflammatory response. Indeed, it has been shown that

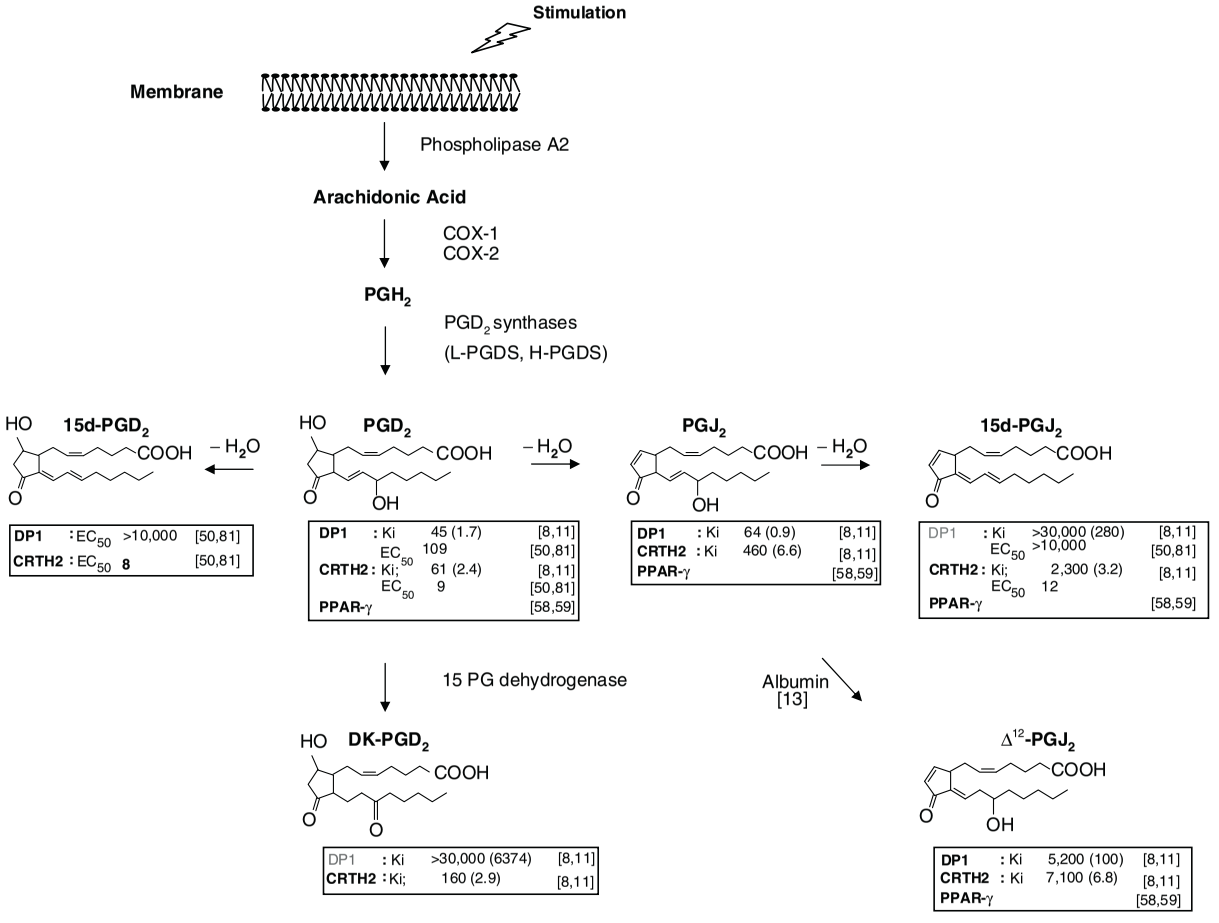
Stimulation
Membrane
Phospholipase A2
Arachidonic Acid
COX-1
COX-2
PGH2
PGD2 synthases
(L-PGDS, H-PGDS)
HO
15d-PGD2
COOH
O
– H2O
DP1 : EC50 >10,000 [50,81]
CRTH2 : EC50 8 [50,81]
HO
PGD2
COOH
OH
O
– H2O
DP1 : Ki 45 (1.7) [8,11]
EC50 109 [50,81]
CRTH2 : Ki; 61 (2.4) [8,11]
EC50 9 [50,81]
PPAR-γ [58,59]
PGJ2
COOH
OH
O
– H2O
DP1 : Ki 64 (0.9) [8,11]
CRTH2 : Ki 460 (6.6) [8,11]
PPAR-γ [58,59]
15d-PGJ2
COOH
O
DP1 : Ki >30,000 (280) [8,11]
EC50 >10,000 [50,81]
CRTH2 : Ki; 2,300 (3.2) [8,11]
EC50 12
PPAR-γ [58,59]
15 PG dehydrogenase
Albumin
[13]
HO
DK-PGD2
COOH
O
O
DP1 : Ki >30,000 (6374) [8,11]
CRTH2 : Ki; 160 (2.9) [8,11]
Δ12-PGJ2
COOH
OH
O
DP1 : Ki 5,200 (100) [8,11]
CRTH2 : Ki 7,100 (6.8) [8,11]
PPAR-γ [58,59]

Fig. 1 PGD_2 metabolites with structures and ligand selectivity
Prostaglandin D_2 (PGD_2) is derived from fatty acids through arachidonate metabolism. Arachidonic acid, released from membrane phospholipids by the action of phospholipases A_2 (PLA_2) after various stimuli, is converted into prostaglandin H_2 (PGH_2) by cyclooxygenases (COX) and then PGD_2 is generated from PGH_2 by two PGD synthases, known as lipocalin-type (L-PGDS) and hematopoietic PGD synthase (H-PGDS). Subsequently, PGD_2 is metabolized into 13,14-dihydro-15-keto-PGD_2 (DK-PGD_2) by 15 PG dehydrogenase, and 15-deoxy-$\Delta^{12,14}$-PGD_2 (15d-PGD_2) and PGJ_2 by dehydration. Then, PGJ_2 converts 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) by dehydration and Δ^{12}-PGJ_2 by -PGJ_2 in an albumin-dependent manner [13]. Bold in square frame: D-prostanoid receptor 1 (DP1), the chemoattractant receptor-homologous molecule expressed on T-helper-type-2 cells (CRTH2), and peroxisome proliferator-activated receptor-gamma (PPAR-γ) [58, 59] indicates selective ligand for individual PGDS and PGJ_s. *Ki* (nm) values and values in parentheses show the specific binding of [^{3}H] PGD_2 to human DP1 and CRTH2 in trasfected K562 [8] and HKS293 cells [11], respectively. EC_{50} (nm) values for DP1 and CRTH2 receptor indicate the effects of prostaglandins on camp levels in platelets and CD11b expression by eosinophils [50, 81], respectively

BW245C treatment during the sensitization phase reduces the ability of Ag-loaded DCs to locally activate Ag-specific T cells in AD [34]. Along the same lines, Hammad et al. have shown that DP1 activation inhibited murine asthma by actively suppressing DC function and by inducing $CD4^+$ $Foxp3^+$ regulatory T cells [36]. In another model, Hammad et al. have also shown that BW245C inhibited migration of intratracheal lung DC to draining lymph nodes following instillation of FITC-OVA [37]. Furthermore, intratracheal administration of BW245C did not induce eosinophilic inflammation in the lung following induction of systemic eosinophilia model by IL-5 injection, while PGD_2 induced such an effect [38]. Thus, most studies point towards an inhibitory role of DP1 on eosinophil-associated inflammation.

CRTH2

The functional role of CRTH2 in inflammation has been recently studied using two different strains of CRTH2-deficient mice and has, like for DP1 studies, led to controversial results. Chevalier et al. reported that $CRTH2^{-/-}$ mice (C57BL/6 background) displayed enhanced eosinophil recruitment into the lung compared with wild-type (WT) littermate mice [39]. In contrast, Satoh et al., using another strain of $CRTH2^{-/-}$ animals (Balb/c background), demonstrated decreased ear-swelling responses with reduced lymphocyte, eosinophil, and basophil, infiltration, and MDC and RANTES production in a model of chronic skin allergy [40]. Similar results were observed in wild-type (WT) mice treated with a hematopoietic PGD synthase inhibitor or a CRTH2 antagonist, such ramatroban [40]. These conflicting results on $CRTH2^{-/-}$ mice in allergic model might be due to differences in genetic background. Regarding pro-inflammatory effect of CRTH2, several papers have been published. Our group has reported that CRTH2 agonist, 13,14-dihydro-15-keto-PGD_2 (DK-PGD_2), increases inflammation and eosinophilia in both asthma and AD models [33]. Moreover, in the study of an IL-5-induced systemic eosinophila model, three CRTH2 agonists (DK-PGD_2, 11-deoxy-11-methylene-15-keto-PGD_2 [MK-PGD_2], and indomethacin [41]) promoted eosinophil infiltration, whereas CRTH2 antagonist, ramatroban, inhibited PGD_2 or DK-PGD_2-induced eosinophilia in bronchoalveolar lavage (BAL) [38]. Furthermore, clinical study in patients with asthma revealed that SNPs in CRTH2 gene were associated with a higher degree of bronchial hyperresponsiveness [42, 43]. Taken together, most evidences demonstrate that CRTH2 promotes eosinophilic inflammation.

Direct Functional Role of PGD_2 in Eosinophilic Inflammation In Vivo

Recently, evidences suggest that PGD_2 can directly promote eosinophilic inflammation in vivo, while PGs plays a protective role by inducing NGF and brain-derived neurotrophic factor (BDNF) production in astrocytes in vitro [44]. Following

allergen challenge, levels of Th2 cytokines are increased in lungs from prostaglandin D synthase transgenic mice and are accompanied by increased eosinophil and lymphocyte accumulation in BAL [45]. Moreover, in an experimental model of asthma that allowed for direct assessment of the role of PGD_2 in airway inflammation, allergen-sensitized mice when exposed to aerosolized PGD_2 displayed increased AHR and eosinophil, lymphocyte, and macrophage infiltration in BAL. PGD_2 asthma-promoting activity was mediated by induction of MDC synthesis by pulmonary epithelial cells [46]. Furthermore, mice sensitized by PGD_2 administration displayed enhanced LTC_4 production and formation of eosinophil lipid bodies, potential LTC_4-synthesizing organelles. These effects triggered by allergic challenge were prevented by pretreatment with HQL-79, an inhibitor of PGD synthase in vivo [47]. PGD_2 itself thus seems, in some instances, to promote allergic inflammation and eosinophilia in vivo. Of note, due to its very short half-life, direct effects of (unmetabolized) PGD_2 are extremely difficult to ascertain. Finally, PGD_2-derived PGJ_s exert anti-inflammatory through PPAR-γ activation (see Chapter 5).

Functional Role of DP1 and CRTH2 in Eosinophils

Expression of DP1 and CRTH2 by Eosinophils

Gervais et al. showed that human eosinophil express both DP1 and CRTH2 receptors [48], while Hirai et al. have shown that human eosinophils only expressed CRTH2 but no DP1 [8]. Regarding mouse eosinophils, our group has shown that eosinophils purified from IL-5 transgenic (Tg) appear to express greater amounts of DP1 mRNA compared with CRTH2 mRNA [33]. In contrast, Hirai et al. showed that mouse CRTH2 is predominantly expressed on eosinophils derived from another strain of IL-5 Tg mice [49]. So these discrepancies might be explained by differences in the animals models (IL-5 Tg mice) used for eosinophil purification (cadmium-induced metallothionein-driven ubiquitous expression versus CD2-driven T cell-specific expression in this study) and/or in cell purification protocol (positive versus negative selection in our case).

Bioactivity of DP1 and CRTH2 on Eosinophils In Vitro

PGD_2, in nanomolar (nM) concentrations, induces the selective eosinophil migration [8, 28] and LTC_4 production [26], and upregulates CD11b adhesion molecule on eosinophils [28]. Studies using selective antagonists and agonists for DP1 and CRTH2 have revealed the functional role of these receptors on eosinophils. CRTH2-selective agonists such as DK-PGD_2, 15d-PGD_2, and PGJ_2 but neither DP1-selective agonist (BW245C) nor PGD_2 induces eosinophil shape change, chemokinesis, degranulation, or CD11b up-regulation [28, 48, 50] (Fig. 2). Δ^{12}-PGJ_2, a CRTH2 agonist, markedly enhances eosinophil chemotactic response to

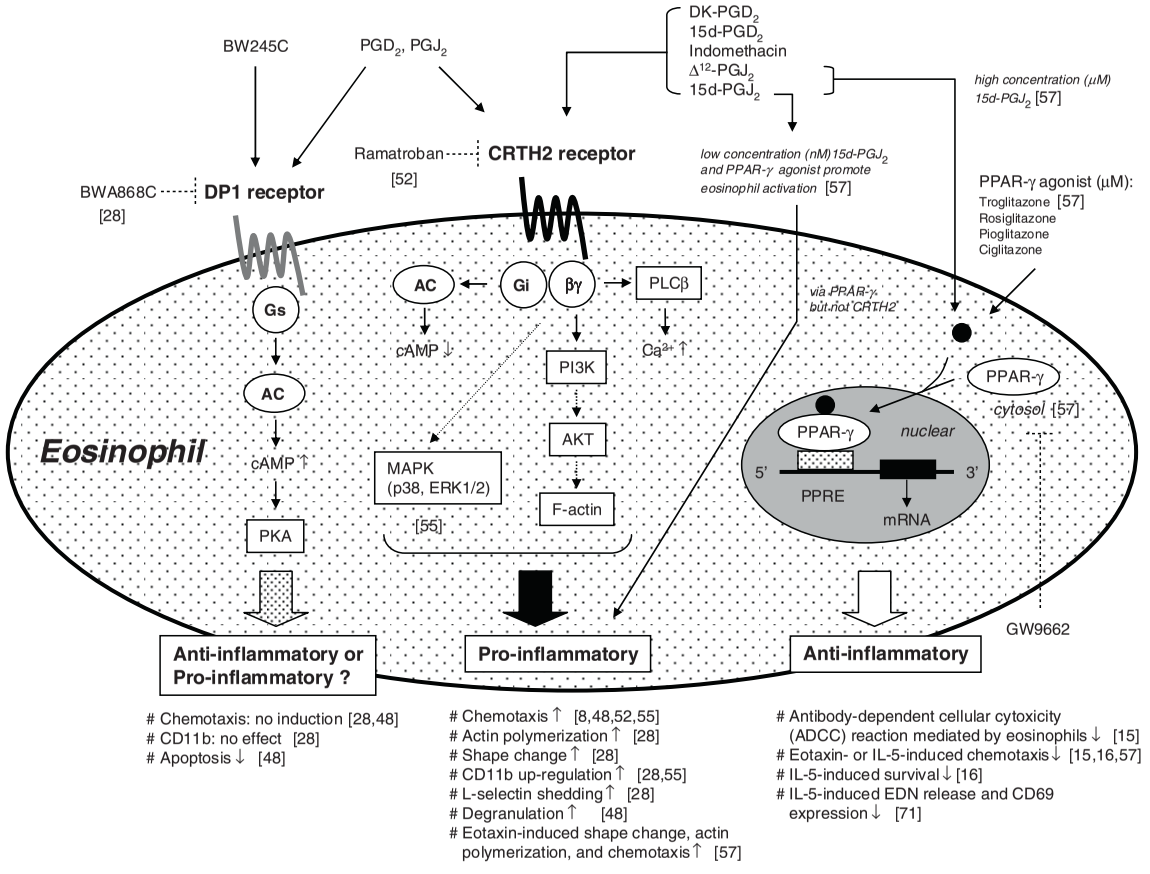

Fig. 2 Summary of the functional roles of PGD_2 and PGD_2-derived PGJ_s

eotaxin, and induces eosinophil release from bone marrow [51]. Along the same lines, ramatroban (Baynas, BAY u3405), a dual selective antagonist for CRTH2 and thromboxane A_2, reduces CRTH2-mediated chemotaxis [52]. Taken together, these data suggest that CRTH2 expressed by eosinophils promotes inflammation. On the other hand and very surprisingly, it has been reported that DP1 activation by BW245C (but not DK-PGD_2) delays the onset of apoptosis on eosinophils [48]. Moreover, blocking of DP1 receptor-mediated cAMP production by BWA868C, DP1 receptor antagonist, enhanced CD11b up-regulation by CRTH2 activation [28]. Thus, while CRTH2 unambiguously acts as an eosinophil activator, the direct effect of DP1 on eosinophils remains controversial.

Signal Transduction Through DP1 and CRTH2 in Eosinophils

It has been shown that PGD_2 itself induces increased intracellular calcium levels in eosinophils [28], while DP1 and CRTH2 activate eosinophils by distinct and antagonistic signal transduction pathways. Hirai et al. reported that DP-mediated calcium mobilization was blocked by $G\alpha_s$ inhibitor cholera toxin (CTX) but not by $G\alpha_i$ inhibitor pertussis toxin (PTX) and that PTX but not CTX inhibited CRTH2-mediated calcium mobilization, while no production of cAMP was observed following CRTH2 activation [41]. Thus, these results indicate that DP1 and CRTH2 are respectively coupled to the PTX-resistant receptor (Gαs protein) leading to increased intracellular levels of the secondary messenger cAMP [53], and to PTX-sensitive G protein-coupled receptor (Gαi protein) leading to an inhibition of cAMP production, calcium influx, and phosphorylation of inositol trisphosphate kinase (IP3K) [8, 41]. In general, eosinophil shape change induced by chemotactic factors is dependent on Gi protein-type receptors, phosphatidylinositol 3-kinase (PI3K) and MAP kinases such as p38 and ERK1/2 [54]. In keeping with this, CRTH2 activation on eosinophil seems to induce MAP kinase phosphorylation. However, Stubbs et al. demonstrated that PTX inhibited eotaxin-induced shape change, but not indomethacin-induced eosinophil shape change [55]. The p38 MAPK inhibitor (SB-202190) inhibited indomethacin-induced shape change and CD11b expression, but MEK inhibitor (U-0126) had no effect on indomethacin-induced eosinophil shape change and up-regulation of CD11b expression [55]. Moreover, Hirai's group subsequently reported that neither the p38 MAPK inhibitor, the MEK inhibitor, nor the PI3K inhibitor attenuated the effect of Δ^{12}-PGJ_2, a CRTH2 and PPAR-γ agonist, on promotion of eotaxin-induced chemotaxis and calcium influx or on Δ^{12}-PGJ-induced chemotaxis [51]. A recent paper demonstrated that PGD_2-induced chemotaxis through CRTH2 in Th2 cell depends on F-actin polymerization through PI3K-mediated Akt but not MAP kinase phosphorylation [56]. Signal transduction of DP1 and CRTH2 in eosinophil involves thus complex pathways. Indeed, potential involvement of MAP kinase pathway independently of

CRTH2 activation remains, however, a possibility while, CRTH2 agonist, 15d PGJ_2 (nM), exerts eotaxin-priming effect of eosinophil independently of MAP kinase and PPAR-γ [57].

Regulation of Eosinophil Function by PGD_2-Derived PGJ_s Through PPAR-γ

Functional Role of PGJ_2 in Physiological Concentration

PGD_2-derived 15d-PGJ_2 not only binds to CRTH2, but also to PPAR-γ [58, 59]. Several papers have reported that 15d-PGJ_2 exerts anti-inflammatory effects [60, 61]. Although 15d-PGJ_2 at micromolar (μM) concentration exerts anti-inflammatory effects through PPAR-γ activation [58] and IκB kinase inhibition [62], nM, i.e., physiological [60], concentrations up-regulate CD11b expression on eosinophil surface [50]. Regarding contradictory effect of 15d-PGJ_2, Zhang et al. demonstrated that low but not high concentrations of 15d-PGJ_2 did not suppress LPS-induced IL-8 gene expression in human monocytes via a ligand-specific and PPAR-γ-dependent pathway [63]. Moreover, 15d-PGJ_2 at low μM concentrations induce cytokine and reactive oxygen species (ROS) production, one of the exacerbating factors in the pathogenesis of airway inflammation. This indicates that 15d-PGJ_2 at μM concentrations does not act as physiologic anti-inflammatory mediator [64]. Furthermore, Kobayashi and Ueki et al. have reported that nanomolar concentrations of 15d-PGJ_2 enhance eotaxin-induced eosinophil chemotaxis, shape change, and actin polymerization in a dose-dependent manner. These priming effects are suppressed by a selective PPAR-γ antagonist and are not inhibited by signal transduction inhibitor such as MEK inhibitor (PD98059), p38 MAPK (SB203580), and NF-κB inhibitor (BAY11-7082) [57]. Thus, 15d-PGJ_2 appears to exert its concentration-dependent antagonistic effect with a delicate balance between PPAR-γ and CRTH2 activation.

Regulation of Eosinophilic Inflammation by PPARs

PPAR-γ expression in human asthmatic airways has been associated to early airway remodeling, based on enhanced proliferation and apoptosis of airway epithelial and submucosal cells, basal membrane (BM) thickness, and collagen deposition [65]. Recently, several evidences have indicated the regulatory role of PPAR-γ in allergic inflammation. Our group has demonstrated that aerosol delivery of PPAR-γ agonist, ciglitazone, to OVA-sensitized and OVA-challenged animals decreased AHR, inflammatory cell infiltration including eosinophils in BAL, cytokine (IL-4, IL-5, IL-6, and IL-13) production, GATA3 expression, and

serum anti-OVA IgE and IgG_1 concentrations. Furthermore, these effects were reversed by PPAR-γ antagonist, GW9662, and were in part due to a direct effect on eosinophils since eotaxin- or IL-5-induced eosinophil chemotaxis as well as eosinophil ADCC were inhibited by PPAR-γ agonists [15]. Moreover, subsequent study has revealed that ciglitazone administration by aerosols and/or gavages in mouse asthma model decreased the features of airway remodeling: AHR, mucus index, collagen deposition, BM thickness, and TGF-β production [66]. The reduction of PPAR-γ protein levels in the lung by ciglitazone and the reversion of this effect by then administration of a PPAR-γ antagonist (GW9662) suggests that ligand-induced PPAR-γ down modulation is associated to the reduction of inflammation [66]. Furthermore, using another PPAR-γ agonist, Trifilieff et al. have shown that PPAR-γ agonist (GI 262570) and a dual PPAR-α and PPAR-γ agonist (GW2331) inhibited allergen-induced eosinophil and lymphocyte infiltration on BAL [67]. In another murine asthma model using intratracheal transfer of OVA-pulsed dendritic cells (DCs), PPAR-γ agonist (rosiglitazone) inhibited DCs migration in the mediastinal lymph nodes, prevented eosinophilic inflammation and increased interferon (IFN)-γ and IL-10 production [68]. Furthermore, in a study using administration of a PPAR-γ-expressing adenovirus (AdPPAR-γ) or of a PPAR-γ agonist, authors observed reduced bronchial inflammation including eosinophilia, ROS production, and up-regulation of tensin homologue deleted on chromosome 10 [69, 70].

Bioactivity of PPAR-γ on Eosinophils

Troglitazone, a PPAR-γ agonist, reduces the IL-5-induced survival of eosinophils freshly purified from peripheral blood and inhibits eotaxin-induced eosinophil chemotaxis in a concentration-dependent manner [16] (Fig. 2). Subsequent experiments have demonstrated that troglitazone reduces IL-5-induced eosinophil-derived neurotoxin (EDN) release and CD69 surface expression [71]. Ward et al. reported that the proapoptotic effects of micromolar concentration of PPAR-γ natural ligand, 15d-PGJ_2, were caspase-dependent but not PPAR-γ-dependent, based on the finding that apoptosis was not induced by PPAR-γ agonist such as the thiazolidinediones (BRL49653 and ciglitazone) and not blocked by PPAR-γ antagonist (GW9662) [72]. Thus, these evidences suggest that the PPAR-γ mainly attenuates eotaxin- or IL-5-induced eosinophil activation. As mentioned earlier, Kobayashi and Ueki et al. demonstrated that low concentrations of PPAR-γ agonist, troglitazone, enhanced eotaxin-induced eosinophil chemotaxis and shape change. Interestingly, the authors found that actinomycin D, an inhibitor of gene transcription, did not inhibit this priming effect and that PPAR-γ is predominantly located in the cytosol of eosinophils (evidenced by immunofluorescence coupled with confocal microscopy) [57]. The authors suggested that either PPAR-γ exerts its effect by interaction with an eotaxin-induced cytosolic signaling pathway or through an unknown receptor-like steroid hormones [57].

New Eosinophilia-Targeted Anti-inflammatory Therapeutic Strategies Focused on PGD_2 and PPAR-γ Agonists

The current literature review suggests that PGD_2 receptor antagonist, especially CRTH2 antagonist, and PPAR-γ agonist represent promising eosinophil-related anti-inflammatory strategies. Regarding PGD_2 receptor antagonist, chemical synthesis of CRTH2 antagonists have been practically initiated since nonsteroidal anti-inflammatory drugs (NSAIDs) including indomethacin have been shown to display significant affinity for CRTH2 [41]. Furthermore, ramatroban (Baynas, BAY u3405), a thromboxane A_2 (TXA_2) antagonist marketed for allergic rhinitis has also been shown to acts as a CRTH2 antagonist on the basis of inhibition of PGD_2-induced migration of eosinophils [52]. To date, selective compounds have been developed for commercialization by pharmaceutical companies, for instance [73]; ramatroban analogues; aryl acetic acids; and nonacidic CRTH2 antagonists, mainly tetrahydroquinolines. On the basis of the initial observation that DP1 activation contributed to inflammation, it has been shown that DP1 antagonists, such as s-5751, reduce inflammation [30, 74]. Clinical trials are ongoing in Europe and the USA [9]. PPAR-γ agonists represent promising anti-eosinophilic/inflammatory with less side effects than steroids; however, thiazolidinediones not only display clinical anti-inflammatory effects, but also undesirable effects such as plasma volume increasing, weight gain, edema, and cardiac hypertrophy due to cardiac volume overload [75–78]. Several compounds have been developed aiming at clinical efficacy and limited side effects [79]. In particular, development of novel non-thiazolidinedione (nTZD) PPAR-γ agonists such as FK614 [80] is ongoing and is currently tested on patients with diabetic mellitus [79].

Conclusions and Outlook

In this review, we have summarized the role of PGD_2 as well as PGD_2-derived PGJ_s in eosinophil inflammation and the functional role of DP1, CRTH2, and PPAR-γ in this process. PGD_2 itself seems to promote allergic reaction including eosinophilic inflammation, although PGD_2-derived PGJ_2 bind PPAR-γ and exert an anti-inflammatory effect. These pro- and anti-inflammatory effects observed for nanomolar and micromolar PG concentrations mainly appear to be induced through CRTH2 and PPAR-γ activation, respectively, while effects of DP1 on eosinophilic inflammation are rather indirect and mediated through regulation of DC function. Thus, biological activity of PGD_2 and its metabolites depends on concentration and spatiotemporal regulation. Taken together, the delineation of the mechanism underlying the functional role of PGD_2 and its metabolites will contribute to elucidation of eosinophilic inflammation, and it may shed light on how to develop a new strategy to treat allergic disorders. Therefore, development of more selective commercial antagonists focused on CRTH2 antagonist and PPAR-γ on eosinophilic inflammation would be a major step forward.

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Mast Cell and Basophils: Interaction with IgE and Responses to Toll like Receptor Activators

Jean S. Marshall, Michael G. Brown, and Ruby Pawankar

Mast Cells in Host Defense and Innate Immunity

Mast cells have multiple roles, not only as effector cells in allergic disease, but also as sentinel cells in the regulation of early immune responses to infection and initiators of early acquired immune responses [1, 2]. These are fully established as cellular initiators of the symptoms of acute allergic disease and may have more complex immunoregulatory roles through IgE-mediated activation. There is also longstanding evidence, from nematode parasite infection models, that mast cell activation can contribute to host defense [3–6]. Over the past 11 years evidence has accumulated for a critical role for mast cells in host defense to a variety of bacterial pathogens – primarily as a result of enhanced neutrophil recruitment [7–10]. Mast cell production of TNF has been shown to be of particular importance to several of these processes [8, 9]. However, other multiple mast cell mediators, such as cytokines and proteases, are also of importance in regulating or initiating innate immune responses [11–13]. Mast cells have been shown to respond vigorously to fungal products in vitro [14, 15] and there is an increasing evidence of their important role in models of viral infection [16–18].

Bacterial, viral, and fungal infections have been demonstrated to be associated with the initiation or exacerbation of allergic symptoms. For example, *Staphylococcus aureus* infection in atopic dermatitis (AD) [19] as well as respiratory syncytial virus or other viral infection in asthma [20–22]. With this in mind, dual roles of mast cells and their mediators in allergic disease and host defense need to be carefully considered. Mast cell activation and production of mediators can occur through multiple mechanisms other than classical FcεRI cross-linking. In allergic disease, pathogen-associated mast cell activation may modify sensitization or alter the nature of disease expression. Understanding these processes could provide important opportunities for therapy.

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Mast Cell Expression of Innate Immune Receptors

Mast cells express a wide variety of receptors, which enable them to fulfill their role as sentinel cells in early host defense and participate in responses to secondary infections or antigen challenge (see Table 1). When considering these, it is important to distinguish between mast cells derived from different species or distinct anatomical sites, since there are wide variations between mast cell subsets in mediator content and responses to specific activators. It is not surprising that another aspect of such heterogeneity is the range of innate receptors expressed on cells from specific tissue sources or derived under particular culture conditions. Toll-like receptors (TLRs) are a major family of innate immune receptors that has received a great deal of attention in view of their pivotal role in host defense. These germ-line encoded pattern recognition receptors can be activated by a wide variety of pathogen products such as lipopolysaccharide (LPS), peptidoglycan (PGN), CpG motifs, and double-stranded RNA, and also by endogenous products produced during tissue injury. Their properties and signaling pathways have been studied in depth and are well reviewed elsewhere [23–26] (see Table 2). However, a number of other innate immune receptors, especially complement component receptors, lectin-like receptors for pathogen products, and adhesion molecules with known roles in innate immune activation are expressed by mast cells and can play an important role in responses to some types of infection [27–30].

TLR1, 2, and 6 have been shown to be expressed by mast cells from a variety of sources although in some cases expression is only observed at the mRNA level. TLR4 is widely expressed on murine mast cells [31–34] and has been described in human-cultured mast cells and *ex vivo* mast cells from some sites [35, 36], while TLR4 is absent from mast cells from other sites or in alternate conditions [37]. TLR5 has been described as absent from some human mast cells and mast cell lines [14] although reported in cultured peripheral blood stem cell-derived mast cells [38]. TLR3 expression has also been observed in both human and murine mast cells [38–40], while the presence of TLR7 and 9 appears to be highly dependent upon the location of mast cells. A good example of such mast cell source-specific variation in TLR expression within one species comes from studies of fetal mouse skin-derived mast cells (FSMCs) compared with murine bone marrow-derived murine mast cell (BMMC). FSMCs displayed a connective tissue mast cell phenotype based on granule contents and were found to express high levels of mRNA for TLR1, 2, and 7, a moderate level for TLR3, TLR4, and TLR6, and a low level for TLR9, but lacked TLR5 [39]. In contrast, BMMCs expressed high levels of TLR2 and TLR6, moderate levels of TLR4, and low levels of TLR1, TLR3, and TLR7, and lacked both TLR5 and TLR9.

In human, comparisons of neonatal foreskin mast cells with those derived from adult lung tissue have proved equally enlightening. Lung mast cells expressed high levels of TLR2, TLR3, moderate levels of TLR4, TLR7, and TLR10, and low levels of TLR5 and TLR9, and lacked TLR1, TLR6, and TLR8. In contrast, foreskin-derived mast cells expressed high levels of TLR3, moderate levels of TLR2, TLR4, and TLR9, and low levels of TLR5, TLR7, TLR10, and were deficient in TLR1, TLR6, and TLR8 [38]. In comparison to cultured human peripheral blood-derived mast cells [36], both of these tissue-derived mast cell preparations exhibited less TLR5, TLR7,

Table 1

Innate receptor type		Mast cell		Basophil	
		mouse	human	mouse	human
TLR1	mRNA	+	+	+	
	Protein				
TLR2	mRNA	+	+	+	+
	Protein		+		+
TLR3	mRNA	+	+		
	Protein				
TLR4	mRNA	+	+	+	+
	Protein	+	+		+
TLR5	mRNA		+		
	Protein				
TLR6	mRNA	+	+	+	
	Protein				
TLR7	mRNA	+	+		
	Protein				
TLR8	mRNA	+	+		
	Protein				
TLR9	mRNA	+	+		+
	Protein				
TLR10	mRNA		+		+
	Protein				
C1qR	mRNA				
	Protein	+			
C3aR	mRNA		+		
	Protein		+		
C5aR	mRNA				
	Protein		+		+
FimH R (CD48)	mRNA				
	Protein		+		
RNA helicase (RIG-I, Mda-5)	mRNA				
	Protein				
NOD1	mRNA				
	Protein				
NOD2	mRNA		+		
	Protein		+		
CD11b/CD18 (MAC-1)	mRNA				
	Protein	+			+
Dectin-1	mRNA				
	Protein		+		

References: Malaviya et al. (1994), Fureder et al. (1995), Ghebrehiwet et al. (1995), Rosenkranz et al. (1998), Malaviya et al. (1999), McCurdy et al. (2001, 2003), Applequist et al. (2002), Sabroe et al. (2002), Varadaradjalou et al. (2003), Kulka et al. (2004), Matsushima et al. (2004), Bonini et al. (2005), Inomata et al. (2005), Thangam et al. (2005), Florian et al. (2006), Komiya et al. (2006), Kulka and Metcalfe (2006), Kubo et al. (2007), Wu et al. (2007)

TLR9, and TLR10, with no TLR1, TLR6, and TLR8 mRNA. An additional study investigating the protein expression of TLR on a number of inflammation-associated immune effector cells, including mast cells, in both normal and allergic conjunctiva, indicated that while mast cells expressed both TLR2 and TLR4, they lacked any detectable TLR9 [41]. It has been suggested that mast cells from certain sites, such as the human intestine, may not express a range of functional TLRs at the protein level; this could represent adaptation of mast cells to these sites rich in microflora [42]. However, extended periods of *ex vivo* culture are required to obtain pure mast cells from this site, which could alter TLR expression.

Both pathogen products and the cytokine microenvironment have been demonstrated to profoundly alter TLR expression in other cell types and this is likely also true for mast cells.

In the context of ongoing disease, current evidence suggests that mast cells are capable of expressing a wide range of functional TLRs and thereby responding to multiple pathogen-associated signals. Analysis of protein level expression of several TLRs has been hampered by the lack of good monoclonal antibodies and by the primarily intracellular location of some TLRs making functional antibody-blocking studies difficult to interpret. However, from what we have learned, in other cell types even quite low levels of TLR expression can mediate substantial responses to certain pathogen products. Inflammatory cytokine and pathogen product exposure can also lead to alterations in the levels or type of TLR expression.

Mast Cell Responses to TLR 1, 2, 4, 5, and 6 Activators

TLR activators are found commonly in our environment, within our natural microbial flora and are a feature of most pathogens. In addition, substances produced during tissue damage can serve as TLR activators. Some examples of common TLR activators are provided in Table 2. Multiple studies using TLR-deficient animals have demonstrated the importance of this family of receptors in host defense against bacterial and

Table 2

Pathogen products	Receptor	References
PAM3CSK4 (triacylated LP)	TLR1	Marshall (2004), Miyake (2007)
PGN, zymosan, LTA	TLR2	Marshall (2004)
dsRNA	TLR3	Kulka et al. (2004), Matsushima et al. (2004), Kawai and Akira (2007)
LPS	TLR4	Malaviya and Georges (2002), Marshall (2004), Kawai and Akira (2007)
Flagellin	TLR5	McCurdy et al. (2001), Supajatura et al. (2001), Kulka et al. (2004), Kawai and Akira (2007)
MALP-2 (diacylated LP), PGN	TLR6	Kawai and Akira (2007), Miyake (2007)
ssRNA, R-848	TLR7	Dieboldo et al. (2004), Matsushima et al. (2004)
ssRNA, R-848	TLR8	Heil et al. (2004)
CpG	TLR9	Matsushima et al. (2004)
Unknown	TLR10	Hasan et al. (2005)

fungal infection. As part of their "sentinel cell" role, mast cells can respond very rapidly to activation with TLR ligands. A critical feature of mast cell responses to TLR activators is their selectivity. In both humans and mice, TLR activation usually leads to the production and release of only a subset of potential mast cell mediators and frequently occurs in the absence of degranulation [14, 36, 43]. Specific TLR interactions give rise to different mediator profiles, in keeping with a role for mast cell activation by pathogen products in defining the nature of the early immune and inflammatory response to infection. A number of co-receptors such as CD14 and dectin-1 can play an important role in the TLR-dependent mast cell responses and their activities may modify the mediator profile induced by TLR ligands.

Since the early 1990s we have been aware of the ability of rodent mast cells to respond to pathogen products such as LPS and give rise to selective cytokine production in the absence of degranulation [43]. More recent studies of human and rodent mast cells have confirmed a wide range of selective mast cell mediator responses to TLR activators and in several cases the role of specific TLRs have been confirmed using rodent gene deletion models or neutralizing antibodies [31, 32, 36, 44]. TLR2 and TLR4 on murine mast cells have been demonstrated to be critical for mast cell cytokine responses to LPS and bacterial PGN, respectively. There is some controversy surrounding the ability of bacterial PGN or TLR2-activating lipopeptides to induce mast cell degranulation, since there is little evidence to support a calcium signal being generated via TLR-mediated processes [45]. However, PGN is a very effective activator of complement via classical, alternate, and mannose-binding pathways, complement component receptors could lead to an effective degranulation response in vivo even in the absence of TLR2-mediated signals. Recent studies of murine responses to PGN support this concept [30]. Human-cultured mast cells have also been described to selectively respond to some TLR activators without degranulation under some conditions. McCurdy et al. [14] described the selective production of the cytokines IL-1β and granulocyte-macrophage colony-stimulating factor (GM-CSF) by cord blood-derived human-cultured mast cells in response to PGN, the bacterial lipopeptide analogue $Pam_3CysSer\ Lys_4$, and the yeast cell wall component zymosan. Both Zymosan and PGN were also able to induce the selective production of LTC_4 from such mast cells under conditions, which did not lead to any significant degranulation.

The ability of mast cells to produce leukotrienes in response to certain TLR activators has been established [14, 38]. However, while some of these responses are partially TLR2-dependent there is increasing evidence that they are not all mediated via TLR2 receptors, but can involve other members of a TLR2-containing signaling complex or alternate innate receptors. For example, the mast cell LTC_4 response to the yeast cell wall component Zymosan has been shown to be dependent on activation of the dectin-1 receptor [46]. This receptor activates Syk kinase [47, 48] and has the ability to induce a calcium signal, which together with TLR signaling, such as MAPK phosphorylation, provides an appropriate set of combined signals for the generation of LTC_4 by mast cells. Dectin-1 signaling has also been demonstrated to be important for lipid mediator production by macrophages in response to fungal products [49]. The dependence on co-receptors such as dectin-1 provides additional therapeutic opportunities to inhibit leukotriene generation selectively in response to fungal infection or environmental exposure.

Array studies have been performed using several TLR activators and both human and murine mast cells. These have revealed important differences in the range of mediators, particularly cytokines and chemokines, induced by TLR-mediated mast cell activation and those induced by FcεRI cross-linking [44, 50]. The data from such studies is extremely useful as a starting point for elucidating the biological role of mast cell TLR expression and function. In addition, to consider the range of mediators produced, it is also important to recognize that the mast cell can be a particularly rapid source of a number of critical cytokines and chemokines, either because they are found preformed or because the mast cell has specific mechanisms whereby protein level production can be rapidly initiated.

Mast Cell Responses to TLR 3, 7, 8, and 9

TLRs on innate effector cells such as mast cells help to differentiate between various types of pathogens, directing appropriate responses to a particular pathogen. Evidence in support of this concept is derived from a number of infection models and also from studies of the responses of mast cells to activation of receptors for DNA and RNA sequences associated with infection as compared with responses to bacterial and fungal cell wall components. The nucleic acid sequence receptors TLR3, TLR7, TLR8, and TLR9 have been shown to function primarily intracellularly associated with the endoplasmic reticulum and endosomes, and to signal primarily through similar pathways observed for other TLRs [26, 51].

Mast cell activation with polyI:C has been used as a model for examining TLR3 responses. Kulka et al. [36] revealed a potent and selective type 1 interferon response of human mast cells treated in this way that could be blocked by pretreatment of mast cells with an antibody to TLR3. This group also observed ablated type 1 interferon responses in BMMC derived from TLR3-deficient animals. This clearly demonstrates a role for TLR3 in the type 1 interferon response. However, it has been suggested that other aspects of the cellular response to polyI:C might be mediated by alternate cellular receptors for double-stranded RNA such as PKR and RIG-I. Treatment of mast cells with a number of viral preparations also gave rise to a type 1 interferon response. A number of other mediators have been demonstrated to be produced by human mast cells following viral infection. For example, dengue virus infection of mast cells is associated with the increased production of IL-1, IL-6, CCL3, CCL4, and most strikingly CCL5, but not CXCL8 [52, 53]. It is not yet known if these effects are TLR3-mediated.

Mast cell interactions with the extracellular matrix may also be altered via TLR activation. Following exposure to polyI:C human mast cell adhesion to vitronectin and fibronectin were reduced by an active TLR3-dependent process. In addition, mast cell responsiveness to IgE-mediated activation was inhibited. Such responses may enhance the localization of mast cells to sites of infection and help to focus the mast cell activities towards aiding antiviral responses [38]. In vivo studies of mast cell responses to TLR3 activation are limited, however Orinska et al. [40] observed

a mast cell-dependent recovery in CD8 T cells following polyI:C treatment or Newcastle disease virus infection, in experiments comparing mast cell-deficient mice with controls. In contrast, granulocyte recruitment to the peritoneal cavity occurred similarly in mast cell-containing and mast cell-deficient animals.

As previously indicated, murine skin-derived and bone marrow-derived mast cells have distinct profiles of TLR expression. Both inflammatory cytokine and chemokine secretions by these two mast cell types following stimulation with TLR ligands have been analyzed. TLR3, TLR7, and TLR9 stimulations of fetal skin-derived mast cells with polyI:C, R-848, and CpG respectively resulted in a dose-dependent production of TNF and IL-6. In contrast, BMMC response to these ligands was negligible or absent. Chemokine responses followed a similar pattern, with MIP-1α, MIP-2, and CCL5 produced by poly I:C, R848, and CpG-containing oligonucleotides, while similarly treated mBMMCs produced little of these chemokines [37].

In other studies, using the TLR7 activator imiquimod applied as a cream to the skin, it was shown that mast cells are essential for ensuing inflammatory response, including the mobilization of local Langerhans cell populations by mechanisms that involve mast cell-derived IL-1β and TNF [54]. The adjuvant effect of imiquimod in the generation of peptide-specific cytotoxic T-cell (CTL) responses was also shown to be mast cell-dependent.

There is some controversy surrounding the ability of mast cells to respond effectively to CpG-containing oligonucleotides. Early studies of BMMC demonstrated small but significant TNF and IL-6 responses [55], however, TLR9 dependence was not examined. More recent studies of human mast cells also noted modest production of IL-1β, IFN-α, and TNF in response to CpG-oligonucleotide treatment. Interestingly, CpG treatment in animal models of allergic disease has also revealed decreased mast cell accumulation, but it is not known if this is a direct effect on mast cells or is the result of a decreased severity of disease.

Evidence is emerging to suggest that certain pathogens have also acquired the ability to utilize TLRs to their benefit. A major example of this process has been described in mast cells treated with CpG-containing oligonucleotides. It has been demonstrated that the HIV virus is able to reactivate from latent infection in mast cells via stimulation through TLR9 [56]. It is not yet known if the mast cell also serves as a reservoir of other types of latent infection.

Interactions Between IgE and TLR Signaling

Since mast cells reside at classical mucosal surfaces and in the skin there are many situations in which mast cells might concurrently be in contact with allergens and TLR activators. Dual activation or modulation of Fc receptor expression by TLR activators could contribute to the regulation of allergic inflammation especially in the context of food allergies where large amounts of intestinal flora are present or atopic dermatitis when *S. aureus* colonization is common. Recently, Yoshioka

et al. [57] examined the ability of the TLR2 activator lipotechoic acid (LTA) to modulate FcεRI expression by pulmonary mast cells and a human mast cell line. They observed substantial down-regulation of FcεRI expression in response to LTA treatment and suggest that this could be employed as a method to control allergic disorders. However, the pro-inflammatory effects of TLR activators may limit the usefulness of this approach. Others have suggested a less beneficial role for TLR activators. Qiao et al. [45] observed that antigen cross-linking of FcεRI interacted synergistically with either TLR2 or TLR4 ligands to enhance production of pro-inflammatory cytokines in murine mast cell lines. Enhanced MAP kinase activation may have contributed to increased cytokine responses that occurred in the absence of enhanced degranulation or leukotriene generation. More recently, Fehrenbach et al. [58] examined the ability of both dipalmitoylated and tripalmitoylated TLR2 activators in enhancing IgE-mediated mast cell activation. They also observed substantial enhancement of cytokine production via a TLR2-dependent pathway.

Other opportunities for cross talk between IgE-mediated mast cell activation and TLR-mediated pathways are found when one considered the effects of released mediators in complex tissues. For example, Talreja et al. [59] observed that histamine induces TLR2 and 4 expression in endothelial cells suggesting that the pro-inflammatory effects of TLR activators at mucosal sites may be enhanced in the context of ongoing allergic disease. In animal studies, while some TLR activators (such as LPS [60]) have been demonstrated to inhibit the development or expression of allergic disease in a mast cell-dependent manner, other bacterial components such as PGN have been shown to enhance responses [61].

TLR Activation on Mast Cells Is Only Part of a Complex Integrated Innate Response

While a number of studies have identified key TLR-mediated processes with profound effects on the outcome of infection in rodent models, in most disease processes multiple TLRs and co-receptors are activated, in addition to a series of other innate immune responses that work in concert with TLR-mediated processes. For example, during viral infection, mast cells and other innate effector cells might respond to viral-derived double-stranded RNA through both TLR-dependent and TLR-independent mechanisms and also respond to specific viral proteins (e.g., F-protein of RSV) by activation of TLR4. In addition, local tissue damage could liberate substances such as fibronectin breakdown products that can further activate innate immunity in part, through mast cell activation [62].

For many TLR activators, TLRs are only one of a number of receptor systems that can be utilized for initiation of host defense. In vivo, there are often antibody responses to LPS and PGN that can contribute to mast cell activation either directly through Fc receptor cross-linking or indirectly through the activation of the classical complement pathway. Mast cells express multiple complement receptors including C3, C4 C3a, and C5a receptors, as well as the $\alpha_1\beta_2$ integrin receptor that serves as a receptor for activated C1q [28, 29]. These receptors can contribute substantially to

responses to active infection or pathogen products, but are often overlooked during in vitro studies of mast cell activation. Recent studies have demonstrated a critical role for complement in the mast cell-dependent early cell recruitment response to PGN [30]. In addition, mast cells express a number of lectin-like receptors that may contribute to host defense such as peptidoglycan recognition proteins and integrins such as MAC-1, which may contribute. NOD1 and NOD2 expressed by some mast cells might also contribute to responses to Gram-positive bacteria.

Mast Cell Activation by Pathogen Products: Effects on Acquired Immunity

Pathogen product activation by mast cells is thought to modulate acquired immune responses. Studies from Duke University [63] have demonstrated that mast cell activation by whole bacteria or other secretagogues is an important inducer of lymph node hypertrophy following infection. Such lymph node activation, which includes blockade of emigration of T cells from the node, increases the opportunity for antigen laden dendritic cells to come into contact with appropriate T cells and provides an appropriate microenvironment to support optimal antigen presentation and the development of an ensuing acquired immune response. This type of mast cell function may not be dependent primarily on TLR function. Jawdat et al. [64] noted that while *S. aureus* derived peptidoglycan injection into the ear-induced lymph node swelling by a mast cell-dependent mechanism this response was not inhibited in TLR2-, TLR4-, or MyD88-deficient mice.

Mast cell activation by either IgE/antigen or pathogen products also has the ability to mobilize dendritic cell populations and cause them to migrate to draining lymph nodes. This has been most effectively demonstrated in the skin where mast cell activation leads to Langerhans cell migration into the draining lymph node. While the role of mast cells in contact hypersensitivity responses remains highly controversial [65–68]. There appear to be some circumstances in which the mast cell can be pivotal for the development of an effective response. A number of T-cell responses have been shown to be mast cell-dependent by mechanisms which often require the mast cell as an early source of TNF. The role that mast cell activation by either TLRs or IgE have on allergic sensitization or effective responses to vaccination in humans remains to be determined.

Mast Cells in Allergic Diseases

Mast Cells in Allergic Rhinitis and Asthma

Allergic rhinitis (AR) is the most common allergic disease affecting up to 25–40% of the population. The pathophysiology of AR shares many similarities to allergic asthma and the two diseases are often considered manifestations of “one syndrome,

one airway, one disease" [69]. Mast cells constitutively reside in the nasal mucosa of normal subjects, but are not normally present in the nasal epithelium. Mast cells comprise of two phenotypes: tryptase (MCT)-positive and tryptase/chymase (MCTC)-positive [70]. While both MCT-positive and MCTC-positive mast cells reside within the subepithelium, the phenotypes of mast cells in the allergic nasal epithelium are predominantly of the MCTC [70–72]. Upon allergen exposure, mast cells migrate to, and proliferate within, the epithelium [73, 74]. Although Stem cell factor (SCF) is a strong mast cell chemoattractant and elevated in the nasal lavage fluid of patients with seasonal AR, the levels of SCF in the allergic nasal epithelium are relatively compared with the lamina propria. By contrast, the levels of RANTES (CCL5) are increased in the epithelium and may instead have a more prominent role in the intraepithelial migration of nasal mast cells (NMCs) as compared to SCF or Eotaxin (CCL11) [74]. A low SCF environment is known to decrease mast cell chymase expression, which may explain the selective accumulation of MCT in the nasal epithelium of AR [74, 75].

Mast cell degranulation is characterized by elevated levels of tryptase, histamine, LTB4, LTC4, and PGD2 in the nasal secretion of individuals with AR following nasal allergen challenge [76–79]. These mediators contribute to the characteristic early-phase symptoms of AR namely sneezing, pruritus, rhinorrhea, and nasal congestion. Histamine is a major mediator inducing vasodilation, increased vascular permeability, and increased glandular secretion. In addition, histamine acts on the sensory nerve endings of the trigeminal nerve to cause sneezing. However, more recently mast cells are found to be an important source of a variety of cytokines (TNF-alpha, IL-4, IL-5, IL-6, and IL-13) and mast cell activation results in orchestrating of the late-phase reaction, inducing the infiltration of eosinophils through the release of platelet-activating factor (PAF) and LTB4; and the upregulation of VCAM-1 expression on endothelial cells. The survival of eosinophils is promoted through the mast cell release of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5. Additionally, histamine upregulates RANTES (CCL5) and GM-CSF, while IL-4, IL-13, and TNF-α upregulate Eotaxin (CCL11) and TARC (CCL17), further contributing to the late-phase eosinophilic/T-cell infiltration. Clinically, this is displayed as an increase in nasal mucosal swelling with increased nasal airway resistance [74, 80]. As regard to nasal hyperresponsiveness it is the result of exaggerated neural reactivity, and the levels of nerve growth factor (NGF) in the nasal lavage fluids from patients with AR are markedly elevated and amplified through nasal allergen challenge [81, 82]. In addition, IL-4, IgE, and NGF increases the expression of FcεRI and c-Kit on mast cells [83, 84]. Furthermore, the FcεRI expression in NMC correlated well with the levels of serum IgE [83]. These findings taken in concert with the observations of Pastorello et al., who demonstrated a strong positive correlation between the levels of serum IgE and clinical symptoms, in symptomatic patients with allergic rhinoconjunctivitis [85], suggest a very important role for NMC in regulating chronic allergic inflammation. Moreover, mast cells can induce IgE synthesis in B cells and IL-4 and IgE can upregulate the FcεRI expression in mast cells. Several studies have demonstrated that local IgE synthesis occurs at sites of allergic inflammation [86–88]. Putting together all these

observations one can perceive a very important role for the mast cell in perpetuating allergic inflammation via the mast cell-IgE-FcεRI cascade [88].

As in allergic rhinitis, mast cells are primary effector cells in asthma and play crucial roles in the early-phase response, late-phase response, and chronic allergic inflammation [89–92]. Mast cell numbers are increased in the bronchial epithelium of atopic asthmatics [93]. Besides the epithelial compartment, mast cells are widely distributed in specific compartments of the asthmatic airways (i.e., the bronchoalveolar space [94]), beneath the basement membrane, in close proximity to blood vessels, near submucosal glands and in the ASM bundles [95]. In fact a close correlation between mast cell numbers in the bronchial smooth muscle of asthmatics and the degree of airway hyperresponsiveness to methacholine is well established [95].

Mast cells in asthmatics release a variety of mediators (histamine, cysteinyl leukotreine C4 (LTC4), PGD2, tryptase, and chymase), as well as cytokines and chemokines, which induce and sustain chronic inflammation in asthma by regulating the recruitment and function of T cells, macrophages, ASM, and epithelial cells. As mentioned earlier in this chapter increasing evidence suggests that histamine can regulate the activity and cytokine/chemokine release from other cells. Histamine induces lysosomal enzyme release and IL-6 and TNF-α production from human lung macrophages [96] and CysLTs exert a variety of responses on bronchial smooth muscle, human lung macrophages, mast cells, and peripheral blood leukocytes via the CysLTR1 and CysLTR2 [69–72].

Besides the above-mentioned mediators, mast cells in asthmatic airways express a wide spectrum of cytokines (e.g., IL-4, SCF, IL-5, IL-6, IL-8, IL-13, IL-16, transforming growth factor-beta [TGF-β], IL-25, TNF-α, and granulocyte-macrophage colony-stimulating factor [GM-CSF] [Pawankar R et al.]) [97]. IL-16 is chemotactic for CD4 + T cells and IL-25 can induce IL-4 and IL-13 gene expression, and this may contribute to mast cell–ASM interactions, as well as in amplifying allergic inflammation via the mast cell-IgE-FcεRI cascade [98]. IL-3, IL-5, and GM-CSF from mast cells can enhance histamine release and IL-4 synthesis in basophils, whereas IL-4 enhances the production of proinflammatory mediators (PGD2 and LTC4) and various cytokines by mast cells [99, 100]. Thus, Th2 (IL-4, IL-5, and IL-3) cytokines can induce changes in the biosynthetic pathways of mast cells at sites of allergic inflammation. SCF, a principal growth differentiating and chemotactic factor for human mast cells is expressed and released by lung mast cells [101]. Several mast cell mediators like histamine, tryptase, LTC4, TNF-α, and TGF-β1 also exhibit fibrogenic activity [102].

Mast cells that are treated with IL-4 express Toll-like receptor 4 (TLR4), which when activated by lipopolysaccharide (LPS) induces the release of Th2 cytokines [103]. Activation of mast cells via TLR2 and TLR3 selectively induce the release of leukotrienes, GM-CSF, and IL-1β [14, 36, 103]. Human-cultured mast cells also express TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, and TLR9 [36]. These findings highlight several novel mechanisms, whereby endogenous, bacterial, and viral proteins can activate human mast cells, thus providing the means by which viruses and bacteria can induce asthma exacerbations.

Structural alterations of the airways, defined as "tissue remodeling," account for a progressive and irreversible loss of lung function in chronic asthma. The positive correlation between the thickness of the tenascin and laminin layers and mast cell density in asthmatics suggests that mast cells may have a role in tissue remodeling [104]. Mast cells, which are closely associated with blood vessels and are increased at angiogenic sites, can contribute to various aspects of angiogenesis [105]. Mast cells synthesize and release various pro-angiogenic factors (histamine, tryptase, TGF-β, IL-8, and vascular endothelial growth factor [VEGF] and the production of VEGF by mast cells is increased by PGE2 and other cAMP-elevating agents) [106–108].

Implications of Mast Cells in Rhinitis and Asthma

The unique microlocalization of mast cells in specific nasal and lung tissue compartments, the ability of these cells to migrate to the epithelial compartment where they are easily activated by allergens or pathogens, the powerful effector repertoire, the recognition of their different microbial-related activating ligands, and their plasticity in response to various signals suggest that mast cells have a central role in allergic airway diseases like rhinitis and asthma. However, mast cells at different stages of maturation, might have different, or even, in some cases, protective roles in the appearance of the asthmatic phenotype. The extent to which each effector cell contributes toward each phase of allergic inflammation and tissue repair remains to be fully elucidated.

Mast Cells in Allergic Conjunctivitis

Ocular allergy occurs in 45% of the allergic population [109, 110]. The location of mast cells in close proximity to the external environment in the mucosa of the eye allows for exposure of these cells to allergen, thereby facilitating cross-linking of membrane-bound IgE, which leads to degranulation and release of inflammatory mediators. Although there are several types of ocular allergy, seasonal and perennial allergic conjunctivitis represent the majority of allergy cases [111]. In normal individuals, mast cells are abundant in the conjunctival stroma with an estimated 50 million cells residing at this environmental interface [112]. In symptomatic allergic patients, an increase in mast cells with evidence of degranulation is seen in conjunctival biopsies [113]. In addition to the increase in mast cells within the conjunctiva, the number of mast cells expressing IL-4 message is increased threefold in seasonal allergic conjunctivitis [114]. Further, use of a mast cell stabilizer (nedocromil sodium) reduces the amount of histamine and PGD2 by more than 70% after challenge, thereby supporting a major role for mast cells in allergic conjunctivitis [115]. In addition to common mast cell mediators such as histamine and cytokines, chemokines released from activated mast cells mediate late-phase

reactions by recruitment of additional inflammatory cells. Mast cells residing within the conjunctiva express CCR3 and the use of a CCR3 antagonist in a mouse model of allergic conjuctivitis ablated both the early- and late-phase reactions [116]. In this model, the CCR3 antagonist leads to mast cell stabilization and inhibition of immediate hypersensitivity, but also impaired neutrophil and eosinophil influx during the late-phase response [116].

Mast Cells in Atopic Dermatitis

Mast cells are increased in lesions of patients with atopic dermatitis (AD) [117, 118]. They reside in the papillary dermis and undergo migration through where they may influence keratinocyte activation and stimulation of endothelial growth with neoangiogenesis [119]. Although histamine has an established role in other atopic diseases, the effect of histamine in AD is questionable, given that levels are not increased compared with control subjects. Tryptase and activation of proteinase-activated receptor-2 (PAR-2) may contribute to the pruritus seen in AD, as tryptase is reported to be increased up to fourfold in AD patients and PAR-2 expression is markedly enhanced on primary afferent nerve fibers in skin biopsies from patients with AD [120]. On the other hand, chymase may play a role by weakening the skin barrier, in turn allowing an enhanced permeability to allergens and microbes [121]. An association between a promoter polymorphism (rs1800875) of the mast cell chymase gene (CMA-1) and AD has been reported [122]. Significantly elevated levels of total IgE are found in about 80% of patients with AD. Beyond traditional signaling through the FcεRI receptor on mast cells, a novel IgE-independent mast cell activation pathway has been proposed for AD involving CD30 and mast cells were shown to be the predominant CD30 ligand-positive cell in AD lesions and activation through CD30 induced a de novo. As in AR, mast cell–nerve interactions may also play a role in promoting inflammation in AD [123]. Contacts between mast cells and nerves are increased in both lesional and non-lesional samples of AD when compared with normal controls [124]. Inflammation appears to be mediated by neuropeptides such as substance P, calcitonin gene-related peptide, vasoactive intestinal peptide, and NGF [84, 125–127].

Mast Cells in Anaphylaxis

Anaphylaxis is an acute, severe, systemic reaction to a foreign stimulus that is often thought to be associated with mast cell activation. The strongest evidence of a role of mast cells in anaphylaxis is in that serum levels of tryptase, resulting mainly from mast cell degranulation, peaks 1–2 h following the onset of IgE-mediated anaphylaxis [128–130]. Besides, the classical IgE-dependent anaphylaxis can occur due to IgE-independent mechanisms namely through IgG and complement receptors

[131–134]. Although anaphylaxis is considered a systemic event, the presence and activation of mast cells in specific organs may play a critical role in the severity. Within the cardiac mast cells are located between myocardial fibers, around blood vessels and in the arterial intima, activation of these critically positioned mast cells may directly contribute to cardiopulmonary failure. PAF is thought to be a critical factor in the development of anaphylactic shock through its ability to induce hypotension and cardiac dysfunction [135–137]. The overall number of mast cells may also be relevant in anaphylaxis in that in those with recurrent anaphylaxis tend to have more dermal mast cells than those without anaphylaxis.

Closing Comments

The mast cell has therefore a central and versatile role in the pathogenesis of allergic diseases, and more recently has been shown to be involved in innate immunity in response to bacterial and parasitic infections [168]. Toll-like receptors and other innate immune receptors on mast cells may participate in effective host immunity and in the exacerbation of allergic inflammation at sites of infection. By better understanding these receptor systems and their function on mast cells we will obtain better tools with which the function of mast cells can be locally modified to enhance appropriate immune responses while limiting adverse inflammatory effects. In allergic disease, certain TLRs may hold promise as effective regulators of the development of allergic disease or as modifiers of ensuing mast cell-mediated responses. More in vivo animal studies and studies of human material are required to come to grips with the complexity of the multiple signals provided to local mast cells at sites of infection. As sentinel cells, the mast cell's range of mediator production and rapid selective responses are extremely important in dictating the nature of inflammatory and immune response. Harnessing these responses holds enormous promise as a strategy to modify local inflammatory responses and immune responses.

As our understanding of the various roles of mast cells in disease pathogenesis evolves, novel therapeutic targets will continue to be identified.

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T Cells in Allergic Disease

Catherine M. Hawrylowicz, Christopher Corrigan, and Alex Faith

Introduction

T cells play a central role in the orchestration of adaptive immune responses to self-antigens, pathogens and environmental antigens, including allergens. The two major T cell subsets present in the periphery are distinguished by the expression of CD4 or CD8 antigens on their cell surface. Through their antigen receptor complexes, they are able to recognise peptides derived from the sequence of antigenic proteins when these are presented by major histocompatibility (MHC) class I or II antigens [1] (APC). CD8 T cells recognise peptide antigens in the context of MHC class I antigens that are expressed by essentially all cells in the body, reflecting the functional importance of this T cell subset for the recognition and killing of cells expressing viral and tumour antigens. However, CD8 T cells are also recognised as an important source of cytokines with the capacity to exacerbate or ameliorate the allergic response dependent on the animal model of allergic airway disease being studied. CD4 T cells recognise peptide fragments bound to MHC class II antigens, which demonstrate a more restricted expression than MHC class I antigens. CD4 + T cells secrete cytokines that promote antibody production and the activation of effector cells and mechanisms associated with host defence. CD4 + T cells are further subdivided into Th1 cells that characteristically secrete the cytokine IFN-γ and are associated with protection against intracellular bacteria, as well as autoimmune disease [2]. Conversely, Th2 cells secrete IL-4, IL-5 and IL-13 cytokines that promote humoral responses and antibody production, and are important for host defence against helminths, but are also associated with allergic disease [2].

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T Cell Subsets in Allergic Disease

In the past 25 years, considerable evidence has accumulated for a central role for T cells, specifically Th2 cells and their cytokine products in asthma, rhinitis, food allergy and atopic dermatitis. Early studies demonstrated activation of CD4 + T cells in the peripheral blood and the infiltration of CD4 + T cells into mucosal sites of patients with allergic inflammation [3], as well as elevated expression of Th2 cytokines [4]. In allergy, IL-4, together with IL-13, is thought to be critical for promoting allergen-specific IgE production by B cells. IL-5 plays a central role in promoting eosinophil function, including recruitment, survival and activation. IL-13 promotes mucus production and airway hypersensitivity. Additionally, IL-9 is thought to be central for mast cell survival and function [5, 6]. A wealth of evidence supporting a role of these cytokines in promoting respiratory inflammation has also been generated in mouse models of allergic airway [7]. Despite these findings, the evidence for a role for T cells and their products in disease remains somewhat circumstantial and a discrepancy exists between descriptive studies of the nature of the inflammatory changes in the mucosa in humans and how precisely these are related to the clinical features of the disease. The failure of clinical trials of single anti-cytokine treatments to ameliorate clinical symptoms of disease again highlights the difficulties in studying human disease [8].

Signals that activate T cells in established allergic disease are likely to be diverse. While allergen exposure clearly exacerbates inflammation in atopic subjects, not all asthmatic patients are atopic. Even in atopic asthmatics it is unlikely that T cell activation is exclusively initiated by allergens. While T cell activation in asthma may occur initially in response to allergens is seems probable that additional factors such as susceptibility to respiratory viral infections [9] or exposure to air pollutants may act in those patients in whom disease persists to maintain chronic T call activation and thereby chronic disease.

A third CD4 T cell subset is the recently described Th17 population, which secretes the IL-17 family of cytokines. The potential involvement of Th17 in allergic disease is discussed later. Conversely, animal models and patient studies have raised awareness and interest in the therapeutic potential of populations of regulatory T cells, which suppress the allergic response.

Th1 and Th2 Cell Differentiation

The principal division of T cells is into $CD4^+$ and $CD8^+$ subsets. Over 20 years ago, it was observed that activated CD4 + T cells could be further classified into two polarised groups, Th1 and Th2, according to the ‘signature’ cytokines they produce (see Fig. 1). The process of differentiation of naïve CD4 + T cells to Th1 and Th2 T cells is influenced by a number of factors, but the most important determinant is probably that of interaction with antigen-presenting cells, and particularly dendritic cells (Fig. 1).

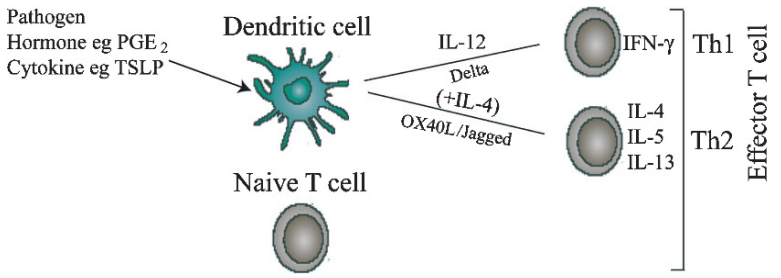

Fig. 1 Dendritic cells (DC) process allergens in the respiratory tract and receive additional environmental signals, which promote maturation and migration to draining lymph nodes. DC present allergenic peptides to naive T cells in the lymph node and dependent on environmental signals received can induce differentiation of Th1 or Th2 effector cells. DC matured under the influence of certain pathogens, hormones (e.g. PGE_2) or cytokines (e.g. TSLP) preferentially induce differentiation of Th2 cells. Expression by DC of co-stimulatory molecules (e.g. OX40L), Notch receptor ligands (e.g. Jagged) in addition to the cytokine IL-4 are all suggested to contribute to Th2 differentiation. In contrast, signalling by the Notch receptor ligand Delta and crucially, release by DC of the cytokine IL-12, promote Th1 differentiation

The outcome of T cell and dendritic cell interaction appears to depend on maturation signals received by the dendritic cells at a relatively immature stage [10].

Th1 differentiation occurs as a result of the production of the prototype Th1-inducing cytokine, IL-12, by dendritic cells during the process of presentation of antigenic peptides to naïve T cells [11]. IL-12-production is stimulated by direct interaction of immature dendritic cells with microbial products through the expression of pattern recognition receptors, such as the Toll-like receptors, that recognise 'pathogen-associated molecular patterns', or PAMPs, derived from micro-organisms [12]. While important for promoting Th1 responses, the presence of IL-12 appears to strongly impair the development of the Th2 lineage [11].

It is less clear what initiates Th2 cell differentiation. While IL-4 clearly supports Th2 development, there is no clear parallel to the APC-derived Th1-promoting properties of IL-12. Dendritic cells are necessary to drive Th2 differentiation and the involvement of a number of receptors has been proposed. For example, interaction of the Notch receptor with the Jagged ligand on dendritic cells has been suggested as a possible trigger for Th2 differentiation since it induces production of IL-4 by T cells [13]. Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine synthesised by epithelial cells. TSLP expression is increased in skin lesions of atopic dermatitis patients. Activation of dendritic cells by both Toll-like receptors and TSLP results in increased expression of MHC class II molecules as well as of T cell co-stimulatory molecules CD80, CD40 and CD86. However, TSLP in contrast to Toll-like receptors does not induce dendritic cells to produce IL-12 [14], but does induce expression of the TNF super family protein OX40 ligand (OX40L) on dendritic cells, which reportedly conveys a signal-promoting differentiation of Th2 cells [15]. The stimuli leading to the expression of TSLP are as yet incompletely characterised, but induction by environmental agents such as

allergens, pollutants and viral infections could provide an important link between environmental influences and induction and exacerbation of allergic diseases [16].

T Cell Memory

The adaptive immune system is characterised by the presence of immunological memory, which augments subsequent immune responses upon re-encounter with antigen. Expression of the CD45RO isotype on memory T cells distinguishes them from their naïve counterparts, which express CD45RA. Memory CD4 + T cells have been further classified into two distinct types based on the presence or absence of expression of the chemokine receptor CCR7. T central memory cells (T_{CM}) expressing CCR7 preferentially migrate to secondary lymphoid tissues, and proliferate upon antigen encounter, giving rise to a progeny of effector T cells. In contrast, effector memory T cells (T_{EM}), which lack CCR7 traffic directly to inflamed tissue and respond rapidly on activation, thereby exerting their effector functions in the tissues [17]. The mechanisms involved in maintenance of immunological memory are still not well understood, but are suggested to be dependent on the homeostatic cytokines, IL-7 and IL-15 [18]. Attempts to alter the cytokine profile of committed Th1 cells are reportedly less effective than those for naive T cells [19]. In contrast, several studies have demonstrated the functional plasticity of both in vitro-derived Th2 cells and bronchoalveolar T cells from atopic asthmatic patients, indicating their potential receptiveness to immunotherapeutic intervention, e.g. [20, 21].

14 Th17 T Cells

The IL-17 and IL-17R families comprise unique families of proteins [22]. The cytokines IL-17A and IL-17F are produced by a recently described functional subset of T cells distinct from Th1 and Th2 T cells called Th17 cells (Fig. 2) [22, 23]. The receptor for these cytokines, IL-17RA, is very widely expressed and IL-17 has been described to act on epithelial and endothelial cells, fibroblasts, osteoblasts and macrophages. Depending on the cellular target, IL-17 induces the production of colony-stimulating factors, CXC chemokines, metalloproteinases and IL-6. They thus have important activities in the recruitment and activation of neutrophils [24]. A high frequency of IL-17-producing cells have been detected in the gut and synovium of patients with inflammatory bowel disease and rheumatoid arthritis, respectively, indicating their importance in autoimmune disease [25, 26]. In contrast, IL-17A has been implicated in limiting allergic inflammation in a mouse ‘model’ of asthma by inhibiting the production of Th2-attracting chemokines such as eotaxin (CCL11) and TARC (CCL17) by dendritic cells. Its production in this model is in turn limited by the Th2 cytokine IL-4 [27]. Conversely, antigen-specific T cell sensitisation is reported to be impaired in IL-17-deficient mice and leads to the suppression of cellular and humoral allergic responses [28].

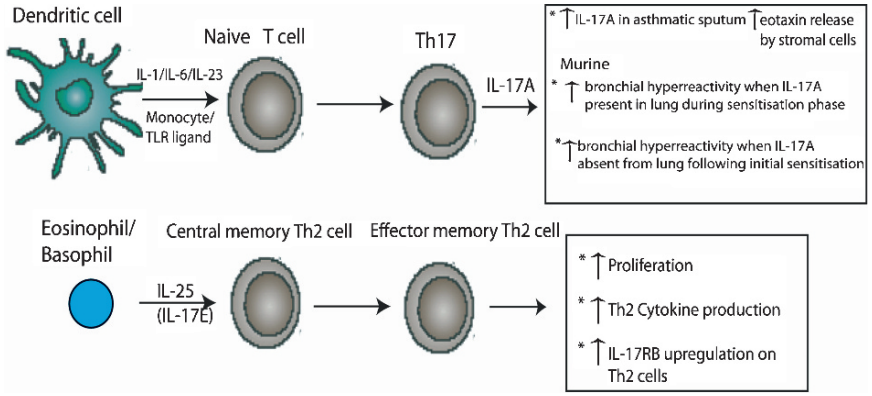

Fig. 2 The IL-17 family comprises a unique family of proteins (IL-17A–IL-17F). IL-17A, IL-17F and IL-22 are produced by the recently described T cell subset, Th17, which are functionally distinct from Th1 and Th2. The functions of IL-17A relevant to allergic disease are shown. IL-17E, also known as IL-25, is reportedly synthesised by keratinocytes, eosinophils and basophils, and amplifies Th2 responses, but can also induce epithelial hyperplasia, eosinophilia and mucus hypersecretion

In contrast, IL-25 (IL-17E), which is produced by keratinocytes and activated eosinophils and basophils, amplifies Th2 responses, reportedly through engagement with and expansion of T_{CM} cells expressing the IL-25 receptor [29]. These effects of IL-25 critically include sustained expression of GATA-3 and c-Maf, transcriptional regulators of Th2 differentiation reported to enhance allergic inflammation in animal models [30, 31]. IL-25 also acts on airways cells to induce epithelial cell hyperplasia, eosinophilia and mucus hypersecretion [32, 33], suggestive of a role in allergic inflammation. In vivo its expression appears most abundant in mucosal tissues [33]. The role of the IL-17 family is yet to be fully defined in allergic and asthmatic disease, but the capacity of different family members to variously modulate airway remodelling, allergic sensitisation, cellular recruitment and Th2 memory responses ensure continuing research interest in this arena.

T Cell Activation and Co-stimulatory Molecules

In addition to presentation of antigenic epitopes bound to MHC class II molecules, signalling via co-stimulatory molecules expressed on the surface of antigen-presenting cells such as dendritic cells modifies T cell function in a positive or negative fashion. Many of these molecules are constitutively expressed, but others are expressed de novo on T cell activation [34]. These latter comprise the positive co-stimulators ICOS, OX40, CD30, 4-BB and SLAM and the negative co-stimulators CTLA-4 and PD-1. Of the positive co-stimulators, ICOS, CD30, OX40 and possibly 4-1BB are particularly involved in the activation of Th2 effector cells [35].

In addition to driving Th2-type T cell responses ICOS substantially contributes to the induction of antibodies, including IgE, by B cells and is absolutely required for the generation of memory B cells [36]. However, ICOS also has a well-established role in production of the anti-inflammatory cytokine, IL-10, by CD4 + T cells. OX40 is particularly involved in regulating long-term T cell survival and the generation of T cell immunological memory [37] (see also discussion above). The roles of CD30 and 4-1BB are less well defined, but can result in Th2 T cell differentiation [37, 38]. The relative contributions of signalling via these various molecules to ongoing Th2 cytokine production and T cell activation in human allergy and asthma remain to be defined. The antigen-presenting cell and its environment appear clearly influence this. For example, co-stimulation through CD86 appears to be important in the function of alveolar macrophages in presenting allergens to T cells in atopic asthmatics [39], whereas, with respiratory tract dendritic cells and whole bronchial biopsy cultures, both CD80 and CD86 are implicated [40, 41]. There may, in some circumstances, be redundancy in signalling, requiring simultaneous blockade of several molecules. Blockade of ICOS and OX40 interactions with their ligands have theoretical attractions as therapeutic strategies in asthmatic and allergic inflammation, but their respective roles in IL-10 production and Th1 responses may limit their potential application in allergic disease. The targeted expression of negative co-stimulatory molecules to mucosal antigen-presenting cells may provide better opportunities for regulating allergic inflammation. For example, PDL-1 is postulated to both transmit a negative signal to activated T cells and to mediate the suppressor activity of CD4 + CD25 + regulatory T cells [42]. Strategies to enhance the latter function could have particular application in allergic disease.

Natural Killer T Cells

Thymus-derived natural killer (NK) T cells express conventional αβ T cell receptor chains as well as natural killer (NK)-specific markers such as NK1.1. In humans, a large majority of these cells (type I, invariant natural killer cells [iNKT] or classical NK T cells) express an invariant T cell receptor comprising the Vα24/Jα18 T cell receptor α-chain paired with a Vβ8 or Vβ2β-chain, hence their name 'invariant' NK T cells [43]. NK T cells are restricted by CD1d, a non-polymorphic MHC class I-like protein which binds to non-protein antigens such as glycolipids. CD1d is widely expressed in the body by mucosal epithelial cells, hepatocytes, T cells, B cells, macrophages and dendritic cells. Many inherited diseases that affect T cell development also affect NK T cells, such as X-linked severe combined immunodeficiency (mutation of CD132, the common γ-chain receptor of IL-2, IL-4, IL-7 and IL-15). NKT cells rapidly produce large quantities of the cytokines IL-2, IFN-γ, TNF-α and significantly, Th2 cytokines, following activation, due to an accumulation of cytokine-specific messenger RNA in the resting state [44]. CD1d knockout mice, which lack NK T cells, showed markedly attenuated airways hyperresponsiveness and inflammation following allergen sensitisation and challenge [45].

Activation of NK T cells exacerbated airways inflammation and bronchial hyperreactivity in this model. This effect was not observed in mice lacking CD1d.

Recent analyses of the expression of the invariant T cell receptor of human NK T cells have challenged the existing paradigm of the central role of conventional, CD4 + Th2 T cells in asthma pathogenesis. Studies using CD1d tetramers loaded with α-GalCer and reverse-transcriptase polymerase chain reaction (RT-PCR) reported that approximately 60% of the airways CD4 + cells in human asthmatic disease are NK T cells expressing the invariant Vα24/Jα18 receptor and not conventional, MHC class II-restricted T cells [46]. These cells exhibited a cytokine expression profile similar to that of conventional Th2 T cells. In contrast, NK T cells comprise <1% of the CD4 + population in human peripheral blood. These studies suggest that CD4 + NK T cells are selectively recruited to the asthmatic airways, possibly through CCR9 expression [47]. However, not all studies are in agreement with these findings [48–50]. Further studies are required to define the relative contributions of conventional, CD4 + Th2 T cells and NK T cells in the production of IL-4 and IL-13 in asthma and of the relative contribution of these cells to asthma pathogenesis in general. Analysis of differences in disease severity and technical approaches may help to unravel the various findings. It seems plausible that these cells will have complementary activities in disease pathogenesis, for example, at different times or stages in the disease process and/or in the recognition of different forms of antigen [51].

T Regulatory Cells

Epidemiological data in the last 40 years have shown a parallel rise in the prevalence of 'Th1-type' diseases such as diabetes and 'Th2-type' diseases such as asthma [52, 53]. Environmental influences, especially our decreased incidence and better control of infection, are thought to alter the development and function of regulatory pathways and are proposed to contribute to the increasing disease prevalence [53].

Two broad categories of T regulatory cells (Treg) have been described, 'natural' and 'adaptive' Treg (Fig. 3). 'Natural' Treg arise during the course of normal development of the immune system in the thymus, constitute less than 10% of circulating CD4 + T cells in healthy individuals and play a vital role in preventing autoimmunity and pathology caused by uncontrolled immune responses to infections [54–56]. However, cells with specificity for environmental antigens, including allergens, exist. Natural Treg are characterised by the constitutive, high expression of the alpha chain of the IL-2 receptor, CD25 (CD4 + $CD25^{high}$ Treg), but also express CTLA-4, CD45RO, CD38, CD62L, the glucocorticoid-induced tumour necrosis factor receptor gene (GITR) and neuropilin-1. None of these markers is specific to Treg, but are also expressed by effector T cells upon activation, making identification of these cells in patients difficult. CD4 + $CD25^{high}$ Treg proliferate poorly in vitro in response to conventional T cell activation stimuli and inhibit adaptive T cell responses in vitro through contact with or close proximity

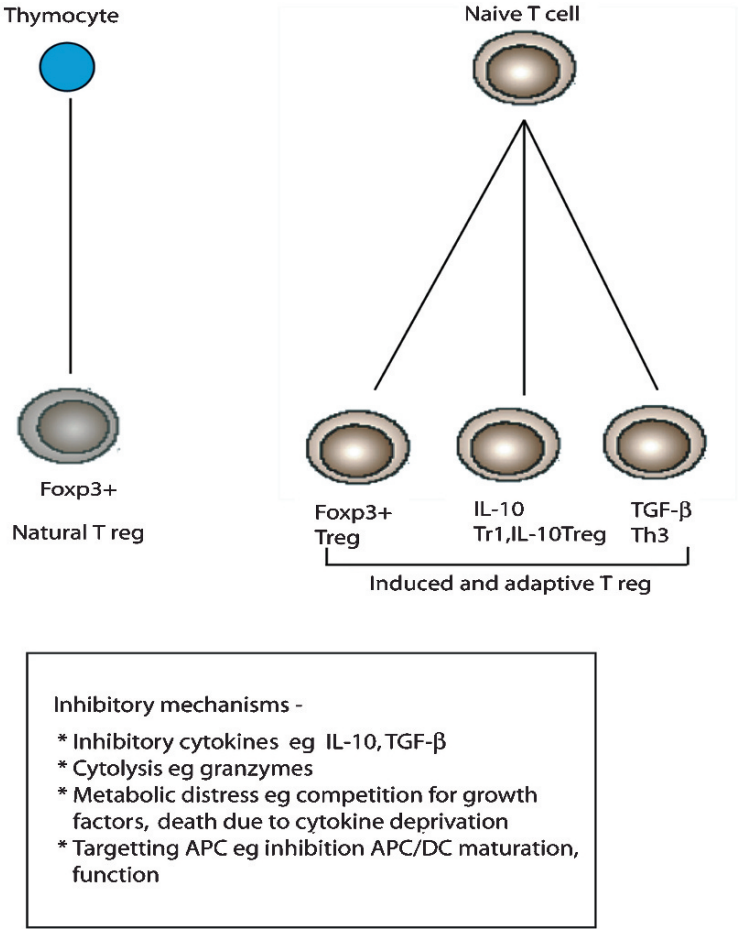

Fig. 3 CD3 + CD4 + regulatory T cell (Treg) populations. Natural Foxp3 + Treg are induced and selected in the thymus. Adaptive or inducible Foxp3+ and Foxp3− Treg populations are produced in the periphery and generally synthesise the inhibitory cytokines TGF-β and IL-10. These populations have been shown to inhibit immune responses through a variety of mechanisms in vitro and/or in vivo in different disease models

to T and/or APC (Fig. 3). Signalling through surface molecules such as CTLA-4, PD-1 and membrane TGFβ have been variously implicated, but competition for essential growth and survival factors and cytokine-deprivation-induced apoptosis are also likely to contribute [57]. Ligation of CTLA-4 on dendritic cells induces the enzyme indoleamine-2,3-dioxygenase (IDO), which starves T cells of tryptophan [58]. Inhibition by these cells in vivo, in different models of allergic airway disease occurs by a variety of inhibitory mechanisms, involving both cell contact and inhibitory cytokine-dependent suppressive pathways [6, 59].

The X-linked transcriptional regulator forkhead box P3 (FoxP3) is expressed in natural T regulatory cells and is thought to be critical for lineage specification and development [60]. Foxp3 + T cells with regulatory function can also be generated outside the thymus in the periphery, by pathways involving specialised APC, such as tolerogenic DC or lamina propria macrophages [61], and TGFβ [62]. Recent studies in the gut suggest APC-derived signals, such as retinoic acid [61, 63], promote this process. Pathways that facilitate the generation of Foxp3 + Treg at mucosal sites may explain the existence of allergen-specific CD4 + $CD25^{high}$ Treg populations.

Humans carrying loss-of-function mutations in FoxP3 develop the X-linked autoimmune and allergic dysregulation (XLAAD) or immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) and exhibit early onset, aggressive and fatal autoimmune disease. These individuals also suffer an increased incidence of severe eczema and food allergies [64]. Studies of the depletion of CD4 + $CD25^{high}$ Treg populations from peripheral blood of healthy subjects reveal robust Th2 responses to allergen in culture, e.g. [65]. Together these data imply a role for this population in prevention of Th2 responses to allergen in health and suggest that efficient natural Treg function is important to maintain normal immune homeostasis in health.

A second population of ‘adaptive’ or ‘induced’ Treg arise following encounter with antigen in the periphery, which secrete inhibitory cytokines such as IL-10 (Tr1, IL-10-Treg) and/or TGFβ (Th3) [6, 54, 66]. IL-10 is thought to be important in the control of allergic and asthmatic disease through its capacity to inhibit functions relevant to the disease process, including the production of pro-inflammatory cytokines, APC, mast cell and eosinophil function and T cell activation [6, 67]. IL-10 levels are elevated in health and decreased in inverse association with disease severity [6]. Therapies associated with the alleviation of disease symptoms are associated with the induction of IL-10 expression, including T cell expression, e.g. allergen immunotherapy and glucocorticoids (reviewed in [6]). The importance of TGFβ-secreting Treg is more difficult to ascertain in human disease, given its beneficial immunosuppressive functions versus the capacity of TGFβ to also promote fibrosis and structural remodelling in the airways [66]. Protocols described for the induction of adaptive IL-10-Treg or Tr1 include repetitive stimulation of T cells with immature dendritic cells [68], activation in the presence of cytokines such as IL-10, TGFβ and/or IFNα [69] or immunosuppressive agents such as 1,25-dihydroxyvitamin D3 and glucocorticoids [70].

The full importance of T regulatory cells in controlling the development and expression of allergic diseases is unclear [6, 71]. Their role in sensitisation to allergens in intrauterine or early life is still not fully defined. In patients with established disease, evidence for deficiency of allergen-specific natural T regulatory cells function has been reported in some [65], but not all [72] studies, but the mechanisms involved are undefined. The former study suggested that allergen-specific T regulatory cell activity was reduced in patients during the pollen season, although relevant mechanisms were not defined. These data may reflect defective Treg function and/or refractoriness of effector cells in an inflammatory environment [57, 73]. These studies do however highlight that external allergens are included in the antigenic

repertoire of natural Treg. In children increased numbers of CD4 + CD25^{high} bovine β-lactoglobulin-specific T cells were detected in the blood of those who had grown out of cow's milk allergy as compared with others who had not [74]. A recent study importantly provides evidence that in bronchial alveolar lavage fluid CD4 + CD25^{high} Treg numbers and function were impaired in pediatric asthmatics compared to non-asthmatic children with cough or control subjects. Inhaled corticosteroid treatment was associated with restoration of this response [75]. Similar data suggest a deficiency of allergen-responsive, IL-10-producing adaptive T regulatory cells in patients who develop sensitisation and symptoms in response to aeroallergens as compared with those who do not [76].

Therapeutic intervention, both non-specific and allergen-specific, also appears to be able to modify T regulatory cell activity. Inhaled or systemic glucocorticoid therapy of asthmatics is, for example, associated with elevated production of IL-10 in lung and blood cells [77, 78]. Glucocorticoid-mediated induction of IL-10-Treg [79] was demonstrated to be impaired in asthma patients who were clinically refractory to glucocorticoid therapy [80], implying a role for this pathway in their anti-asthma effects. Notably immunological manipulation by the active form of vitamin D, 1α25-dihydroxyvityamn D3 or calcitriol, both in vitro and following ingestion by steroid-refractory asthma patients, could enhance and restore steroid-mediated induction of IL-10 synthesis, suggesting potential strategies to overcome this serious condition [70, 81]. Glucocorticoids may also induce or enhance the Foxp3 + Treg compartment [75, 78].

Allergen immunotherapy also induces adaptive, allergen-specific T regulatory cells. Mechanistic studies have shown increases in allergen-specific, IL-10-producing IL-10-Treg or Tr1 and TGFβ-producing Tr3 type cells in the blood or airways following immunotherapy [82–84]. Furthermore, increased IL-10 production following allergen immunotherapy is not limited to T cells, but may also be observed in monocytes/macrophages and B cells [85]. These are all potential antigen-presenting cells and could conceivably directly suppress T effector cells or contribute to the induction of Treg populations in the host. Pursuing therapeutic induction of both natural and adaptive Treg as a means of long-term, directed and allergen-specific therapy for allergic diseases is an attractive therapeutic option and a wide range of 'add-on' therapies to conventional allergen immunotherapy are currently in various stages of investigation aimed at improving both safety and efficacy [6, 83, 84].

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Role of NKT Cells in the Regulation of Ongoing Type 2 Immune Response

Christelle Faveeuw, Thomas Roumier, Monique Capron, and David Dombrowicz

Introduction

Natural killer T (NKT) cells constitute a T cell subset that was initially defined by the co-expression of both T cell and NK cell markers, including the T cell antigen receptor (TCR) αβ and the C-type lectin NK1.1 (or CD161 in humans) [1]. Although NKT cells represent an heterogeneous population of innate/memory T lymphocytes that can be divided into at least three groups [1], most of them, in sharp contrast to conventional T lymphocytes, are reactive to the nonclassical class I antigen-presenting molecule CD1d and recognize glycolipid antigens rather than peptides [2, 3]. CD1d is found on several cell types that function as professional antigen-presenting cells (APCs), including dendritic cells (DCs) and B lymphocytes [4].

Many studies have shown a pivotal role of NKT cells in the maintenance of self-tolerance as well as in the regulation of immune responses associated with a broad range of diseases including autoimmunity, inflammatory and infectious diseases, and cancer [5]. In this article, we review recent progress on NKT cell role in regulatory mechanisms during Th2-related inflammation.

Definition, Distribution and General Properties of NKT Cells

In mice, the major subset of CD1d-restricted T cells expresses an invariant TCR α chain rearrangement (Vα14–Jα18) with a conserved CDR3 region, and they typically co-express Vβ8.2, Vβ2 or Vβ7. The homologous population of human

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NKT cells express a Vα24–Jα18 rearranged TCR α chain with a Vβ11-containing β chain [6, 7]. This population of cells, referred as "invariant NKT cells" (iNKT cells), is highly conserved in many aspects of its phenotype and functions in most mammals that have been studied to date. In mice, iNKT cells are found at the highest frequency in the liver (10–40% of lymphocytes); but are also present at lower frequencies in the thymus, spleen, lymph nodes, lung and blood [1]. Although iNKT cell distribution has not been extensively studied in humans, they are clearly less frequent in human liver (<1%) [8]. In mice, iNKT cells include at least two subsets, differing in their expression of CD4 ($CD4^+$ and $CD4^-$ $CD8^-$). In humans, some of these cells express CD8 [9].

iNKT cells recognize a limited number of synthetic or naturally occurring α-glycosylated sphingolipids associated to CD1d molecule [10]. The most efficient compound for activating iNKT cells in mice and humans is a synthetic glycolipid (originally derived from a marine sponge) known as α-galactosylceramide (αGC) [11]. The surrogate ligand αGC has been broadly used not only in various functional assays but also to generate a CD1d–αGC tetramer, specific for mouse and human iNKT cells, allowing a complete and quantitative description of this cell population. In rat, the efficient activation of iNKT cells requires presentation of αGC by syngeneic CD1d molecule [12]. Along with αGC, growing evidence suggest that microorganisms, including *Mycobacteria*, *Sphingomonas*, *Borrelia*, *Leishmania* and *Plasmodium* may also produce CD1d-restricted ligands capable of activating subpopulations of iNKT cells [13–17] (Fig. 1a). Recently, because iNKT are known to be auto-reactive cells, considerable attention has focused on self-ligands. Although controversial [18], one study suggests that isoglobotrihexosylceramide (iGb3), a lysosomal glycosphingolipid, represents one of the physiological endogenous ligands of iNKT [19].

Along with iNKT cells, other CD1d-restricted NKT cell subpopulations with more diverse TCRs (termed non-iNKT cells) have been described in mice and humans but these cells have been less well studied, mainly because of the absence of known specific markers [1, 6]. Nevertheless, some subpopulations of non-iNKT cells have been characterized. One of these non-iNKT cell populations expresses a Vα3.2 and Vβ9 TCR rearrangement and, although not representative of all non-iNKT cells, expresses most of the expected functional and surface phenotype characteristics of NKT cells such as the reactivity to CD1d in the absence of exogenous ligands, the activated cell surface phenotype and the profile of cytokines secreted upon activation [20]. One major difference between this subpopulation of non-iNKT cells and iNKT cells is their absence of reactivity to the potent iNKT cell activator, aGC. A sulfatide-reactive CD1d-restricted T cell population, distinct from the Vα3.2–Jα9-expressing non-iNKT cells, has also been characterized as part of the naïve T cell repertoire but also as a key actor in autoimmune demyelinating disease development [21]. In humans, CD1d-restricted non-iNKTs have also been described, some of them being reactive to CD1d–αGC tetramer [22].

Finally, NKT cells not restricted to the CD1d molecule have also been described. These NKT cells express a diverse repertoire of TCRs that are restricted by MHC class I and class II molecules [1, 6].

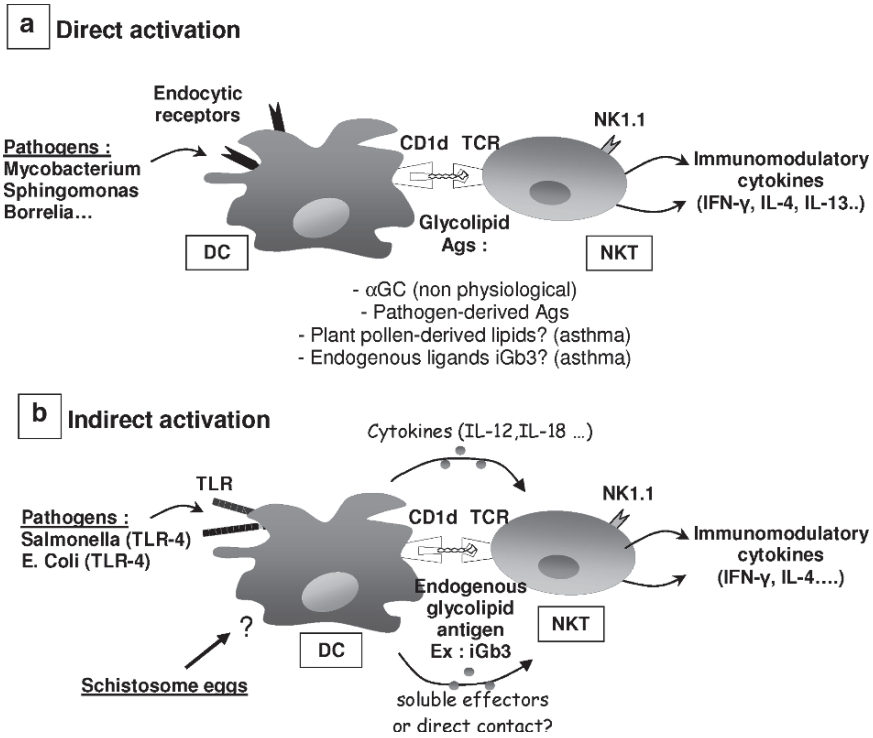

Fig. 1 Mechanisms by which iNKT cells become activated. (**a**) Direct activation. In this case, exogenous glycolipid AGs such as αGC, microbial-derived AGs or plant pollen-derived lipids (in asthma?). As well as endogenous AGs such as iGb3 (in asthma?). Induce iNKT cell activation by engaging their invariant TCR. (b) Indirect activation. Upon *S. typhimurium*/DC contact, activation of TLR-4 induces IL-12 secretion and generates CD1d-restricted endogenous AGs in DCs leading to iNKT cell activation. In response to *E. coli* LPS, DCs release IL-12 and IL-18, these cytokines being sufficient for iNKT cell activation. Finally, in response to schistosome eggs, TLR-mediated activation of DC is not involved in iNKT cell activation. The implicated receptors remain unknown. However, the recognition of endogenous antigen(s) presented by CD1d is necessary. In all cases, activation of iNKT cells shapes the acquired immune responses

NKT cells are part of the innate immune system and play a critical role in the control of the adaptive immune response. Most of our understanding on the role of NKT cells in the modulation of immune responses derived from the use of the surrogate ligand, αGC. After CD1d–αGC complex recognition by iNKT cells, they become rapidly activated and respond with vigorous production of type 1 (IFNγ) as well as type 2 (IL-4, IL-13) cytokines [5]. Much of their immunoregulatory function depends on the effects of their rapid cytokine burst that amplify and regulate adaptive immunity by influencing the function of dendritic cells (DCs), B and NK cells, as well as conventional $CD4^+$ and $CD8^+$ T lymphocytes [23, 24]. In some cases, iNKT cells are also capable to release immunosuppressive cytokines, such as IL-10 [25]. Thus, iNKT cells are relatively flexible and according to their interaction with CD1d-expressing APCs, the cytokines

present in the microenvironment as well as the nature of the ligand (affinity, doses), iNKT may modulate differently the ensuing immune response.

NKT Cells and Th2 Response During Allergic Disorders

Allergic Asthma

Allergic asthma is a major public health problem in the industrialized world. Asthma is a multifactorial respiratory disease involving both genetic and environmental factors, which, in combination, lead to the development of a strongly polarized type 2 response (IL-4, IL-5, IL-9 and IL-13) towards innocuous antigens, characterized by IgE production and tissue inflammation including eosinophilia. In airways, inflammation is accompanied by tissue remodeling (fibrosis) and mucus hypersecretion leading to airway hyperreactivity (AHR) [26]. Among effector cells, antigen-specific $CD4^+$ T lymphocytes have been shown to play a crucial role in the pathogenesis of bronchial asthma, mainly via their production of type 2 cytokines, although $CD4^+$ T lymphocytes also provide the necessary help for B cells and IgE production [27]. Besides conventional $CD4^+$ T lymphocytes, recent studies propose iNKT cells as key actors in the regulation of asthma development via their production of type 2 cytokines (Table 1). Indeed, several groups demonstrated that iNKT cells were essential for the development of AHR and other asthma-associated parameters [28–30]. In these models, CD1d-deficient mice, which lack all CD1d-restricted NKT cells, as well as Jα18-deficient mice, that lack specifically iNKT cells, failed to develop allergen-induced AHR, with a reduced IL-4 and IL-5 production in the bronchoalveolar lavages (BAL) and decreased circulating anti-OVA IgE and IL-5, following airway antigen challenge [28, 29]. Adoptive transfer of iNKT cells into Jα18-deficient mice just before allergen challenge, fully restored AHR. Moreover, wild type mice that were treated with anti-CD1d monoclonal antibody before the sensitization step, failed to develop AHR [28]. Finally, iNKT cell activation by αGC could also lead to asthma development [30–32]. Although the requirement for iNKT cells in the development of allergen-induced AHR has been established and confirmed by several groups, some studies using CD1d-deficient mice (129/Sv × C57Bl/6) or β2m-deficient mice, failed to show a significant role for CD1d-restricted T cell in a murine model of asthma [33–35]. This discrepancy in CD1d-deficient mice may be explained by differences in the analyzed parameters, since AHR, a cardinal feature of asthma that does not always correlate with airway eosinophilia, was not investigated in the initial study [35]. Moreover, differences in the mouse strains utilized could not be excluded. For β2m-deficient mice, the absence of $CD8^+$ T cells in these mice may cause immune dysregulation in several tissue compartments that affect airway response. Moreover, some studies showed that these mice still expressed some forms of CD1d molecule, suggesting that some residual CD1d-restricted NKT cells could still be involved in the development of airway inflammation [36]. Finally, in these studies, a role of CD1d-unrestricted NKT cells could not be excluded.

Table 1 NKT cells and allergic asthma. The role of NKT cell on asthma development is still a subject of debate, both in animal and human studies. To summarize, in animal models of asthma, initial studies using β2m–/– mice did not described any relationship between NKT and asthma. More recently, using Jα18–/– and CD1d–/– mice, it has been shown that NKT cells mainly secrete type 2 cytokines leading to the development of asthma (immunological and pathological parameters analyzed). Activation of iNKT cells by their powerful ligand αGC can either promote (via IL-4) or inhibit (via IFN-γ) asthma development depending on its mode of administration. In human studies, the role of NKT cells is still a matter of debate, some studies describing an increase of NKT cells in the lungs of asthmatic patients, the others claiming that NKT cells are not involved in asthma development (abbreviations: i.p.: intraperitoneal; i.v.: intravenous; i.n.: intranasal; β2m:β2 microglobulin; BALs: bronco alveolar lavages; AHR: airway hyper responsiveness)

Species	Effect	Activation	Models	Roles of NKT on asthma parameters	References
Mouse		By allergen	Jα18$^{-/-}$	↗ AHR	[27, 28, 29, 30]
			CD1d$^{-/-}$	↗ Th2 cytokines	
			Anti-CD1d treatment	↗ Eotaxin and airway eosinophilia	
			Adoptive transfer of iNKT	↗ Allergen-specific IgE	
	Pro	By αGC	i.v. injection	↗ AHR	[29, 30, 31]
			by challenge	↗ Th2 cytokines	
			solely or in combination	↗ IL5	
			with allergen	↗ Eotaxin and airway eosinophilia	
	Anti	By αGC	i.p. injection of αGC	↗ IFN-γ secretion	[47, 48, 49]
			i.v. injection of αGC	↗ Th1 polarization	
			i.n. injection of αGC	↘ Th2 cytokines	
			Jα18$^{-/-}$	↘ AHR	
			Adoptive transfer of iNKT	↘ Allergen-specific IgE	
			IFN-γ$^{-/-}$	↘ IL5 and airway eosinophilia	
			Anti-IFN-γ treatment	↘ VCAM-1 expression in lung vessels	
	No effect	By allergen	β2m$^{-/-}$	No consequence of NKT depletion	[32, 33, 34]
			CD1d$^{-/-}$		
			Anti-CD1d treatment		
			IL4$^{-/-}$		
	Pro	By allergen	Analysis of cell from:	Recruitment of CCR9^{+} iNKT in the airways	[36, 37, 39, 41, 43]
			Lung	Stimulation of Th2 polarized T cells in the airways	
			BAL		
			Sputum	↗ IL4 and IL13 secretion by T cells	
Human	No effect	By allergen	Analysis of cells from:		
			Lung biopsy	No modification of NKT cell number in asthmatic patients	[40, 42]
			Sputum	No correlation between NKT cell number and clinical status	
			Circulating cells		

In humans, the presence of iNKT cells in the airways of asthmatic asthma is still a matter of controversy (Table 1). An initial study reported an increased frequency of CCR9-expressing Va24 $^{+}$ iNKT cells in bronchi mucosa of asthmatic patients [37]. Recently, Umetsu's group showed that most of the $CD3^{+}$ $CD4^{+}$ cells present in the lungs of asthmatic patients were iNKT cells (60%), highlighting the potential pathogenic role of iNKT cells [38]. Furthermore, these pulmonary iNKT cells had a cytokine profile similar to Th2 cells, including expression of IL-4 and IL-13 but no IFNγ. These observations disagreed with several other studies where a very low proportion of CD1d-restricted cells in the BAL and sputum of asthmatic patients were detected (less than 1%) [39–44]. However, although very low, two studies showed that iNKT cells were significantly increased in the BAL and sputum of asthmatic children and severe asthmatic adults, respectively, compared to controls [40, 42, 44]. It is unlikely that these discrepant findings on iNKT frequencies in the BAL and/or sputum could be simply due to technical differences. Indeed, except for the recent report of Djukanovic's group where the authors used very strict flow cytometry approaches to identify iNKT cells [41], most of the studies presented above used similar approaches. One explanation would be that the high proportion of iNKT cells in airways of asthmatic patients observed by Umetsu's group might be associated with more severe asthma. Moreover, although the report of Thomas et al. showed a very low frequency of iNKT cells in the airways during asthma, the authors could not exclude an increase of iNKT cell number in asthmatic patients [43]. Further studies including a higher number of subjects would be necessary to provide an unambiguous answer. Biological significance of this small increase of iNKT in the pathogenesis of asthma remains to be determined. Although the frequency of iNKT cells in the BAL/sputum remains a matter of debate, their proportion in peripheral blood of patients with asthma remains unchanged (or slightly decreased) compared to non-asthmatic individuals [38, 45], indicating that the immunology of asthma is more accurately studied by the evaluation of cells in the lung rather than by examination of peripheral blood. This observation might also be true for other disorders involving NKT cells. The mechanisms involved in the specific recruitment and proliferation of these "Th2-like iNKT" cells in the lungs remain unclear, although these iNKT cells that home to the lungs could express a differential set of chemokine receptors.

The mechanisms by which iNKT cells modulate the development of allergic asthma might be multiple. iNKT cells may act directly as immunoregulatory cells, via the production of Th2 cytokines. Indeed, adoptive transfer of iNKT from mice deficient in both IL-4 and IL-13 into Jα18-deficient mice, failed to restore AHR, indicating that both cytokines are crucial for AHR [29]. For example, IL-13 is known to promote mucus production. Invariant NKT cell activation may also indirectly lead to the differentiation or impact on allergen-specific Th2 lymphocytes function, via production of IL-4 and IL-13. In humans, iNKT cells may also act either directly as an immunoregulatory cell, at least through IL-4 and/or IL-13 production, or indirectly by regulating Th2 cell functions. Invariant NKT cell products could also promote the recruitment of Th2 lymphocytes and effector (eosinophils) cells into the lungs and/or act directly on lung-resident cells to enhance severity

of the disease. Finally, although not directly shown in a murine model of asthma, reports have provided evidence for cross talk between iNKT cells and $CD4^+$ $CD25^+$ regulatory T cells [46]. Thus, iNKT cells could also modulate quantitatively and qualitatively regulatory T cell function leading to severe asthma.

The mechanisms by which iNKT cells activate during allergic asthma remain elusive. One hypothesis would be that self-glycolipids such as iGb3, which may be exposed in the lungs as a result of pulmonary inflammation, could activate iNKT cells leading to airway inflammation and asthma (Fig. 1a). Alternatively, exogenous glycolipids, such as those from inhaled plant pollens, may activate iNKT cells in the lungs and cause asthma. Recently, Umetsu's group demonstrated that the specific and direct activation of iNKT cells by exogenous glycolipid antigens, αGC and PS30, a *Sphingomonas*-derived glycolipid, resulted in a rapid AHR and airway inflammation independent of conventional $CD4^+$ lymphocytes, eosinophils or B cells [32] indicating that iNKT cells could be potent effector cells following direct activation by exogenous glycolipids from microorganisms or perhaps by plant pollens that enter the lungs. In humans, it has been showed that iNKT cells respond to lipids from cypress-tree pollen [47].

Besides the pathogenic role of iNKT cells in allergic asthma development, recent studies strongly suggest that iNKT cells, in certain circumstances, may be beneficial. Indeed, several studies now demonstrated that iNKT cell activation by αGC, whose administration suppresses tumour growth and autoimmune pathologies in various experimental models, inhibited allergic airway inflammation, in an IFNγ-dependent manner [48–50]. Although these studies differ regarding some of the parameters affected by the treatment as well as the mechanism involved, it appears that iNKT cells might exert antagonist effects on the outcome of asthma, at least in mouse experimental models. The nature and the strength of the triggering signal might account for these contrasting results. In experiments based on the comparison between NKT-deficient and wild type mice and those demonstrating the essential role of iNKT in the development of antigen-induced hypersensitivity, iNKT cell triggering is indirect. It is suspected that sensitization by a protein antigen (ovalbumin) induces the release, by an unknown mechanism, of an endogenous glycolipid that activates iNKT cells to produce IL-4 and IL-13. By contrast, αGC induces a direct activation of iNKT cells, which leads to a significant IFNγ production by iNKT cells, and subsequently by NK, DC and other cell types leading to pathology decrease. In humans, whether or not iNKT cells participate in asthma is still an open question and thus it seems premature to conclude that αGC might be used as a drug against asthma.

Allergic Skin Inflammation

Atopic dermatitis (AD) is a pruritic skin chronic inflammation of unknown origin, most often affecting infants and young adults, whose prevalence increases in industrialized countries. Infants affected by AD are predisposed to develop other atopic diseases (asthma, allergic rhinitis) [51]. Similar to asthma, AD is a Th2-driven

inflammation characterized by eosinophilia, elevated serum IgE and local Th2 cytokine production, principally IL-4 and IL-13 [52]. The role of NKT cells has recently been addressed in the development of this allergic disorder. The authors developed a murine model of allergic skin inflammation by epicutaneous sensitization with ovalbumin, this model displaying characteristics similar to human AD [53]. In contrast to allergic asthma, iNKT cells are dispensable for the development of allergic skin inflammation. Indeed, CD1d-deficient mice developed an intact skin inflammation with an IgG_1, and IgE antibody responses comparable to those observed in wild type mice. The presence of very low number of iNKT cells in the skin of wild type mice suggest that iNKT cells do not migrate in the skin. Interestingly, the authors documented an increased iNKT cells' number in the BAL of the same epicutaneous sensitized wild type mice after intranasal challenge with antigen. The airway inflammation observed in wild type mice is abolished in epicutaneous sensitized CD1d-deficient mice. The development of a normal Th2 response in CD1d-deficient mice after epicutaneous sensitization of ovalbumin corroborated previous data showing that antigen-induced Th2 responses and antigen-specific Th2 cells develop and respond normally in CD1d-deficient mice [54]. However, during allergic asthma, these Th2 responses per se seem to be insufficient for the induction of AHR, iNKT being crucial in the process.

In humans, two studies reported a decreased number of iNKT in peripheral blood, mainly $CD4^-$ $CD8^-$ iNKT, of AD patients [55, 56]. On the contrary, one study reported an increased proportion of $CD4^+$ iNKT cells in peripheral blood of AD patients, and this increase was related to IL-4 production and IgE levels [57]. These apparent discrepancies might reflect differences in the cohorts of AD patients examined. These observations should also be taken with caution as examination of peripheral blood does not reflect the local immune response as shown for allergic asthma. Examination of skin lesions would confirm or refute that iNKT cells are dispensable in AD development. The role of αGC in this allergic disorder has not yet been examined.

NKT Cells and Th2 Response During Helminth Infection

Infections by parasites that cause malaria, schistosomiasis, African trypanosomiasis, leishmaniasis and lymphatic filariasis are a major worldwide health concern. Among them, helminth infections rank as a major cause of morbidity and suffering especially in the developing countries. A striking feature of parasitic helminths, including nematodes and trematodes, is that they trigger a strong Th2 response in their hosts characterized, as in allergic diseases, by high levels of Th2 cytokines (IL-4, IL-13) associated with abundant IgE production and eosinophilia. However, very few studies have been devoted to investigate the role of iNKT cells, and to a larger extent of CD1d-restricted T cells, during helminth infections.

Nematode Infections

The role of NKT cells during nematode infections remains largely unknown. Indeed, Balmer et al. initially reported the expansion of NKT cells (on the basis of CD3 and NK1.1 markers) in the spleen and draining lymph nodes of mice as early as 24 h following infection with the nematode *Brugia pahangi* [58]. The number of IL-4 producing NKT cells, mainly $CD4^-$ NKT, was also increased at this time point, indicating that this early IL-4 production could polarize the immune response towards a Th2 profile during infection. On the other hand, Koyama et al. observed that depletion of NK1.1-expressing cells, including NK cells and subpopulations of NKT cells, had no effect on the Th2 response development during the gastrointestinal nematode *Trichuris muris* infection [59]. In both cases, the experimental approaches remain very descriptive and further investigations would be necessary to conclude to a role of NKT cells in the development of these nematode infection. A complete characterization of NKT cells, mainly iNKT, using αGC/CD1d tetramer and the use of CD1d- and/or Jα18-deficient mice would be helpful.

Trematode Infections

Besides the very limited information available on the role of NKT cells during nematode infections, several studies, including some of our laboratory, strongly suggest that NKT cells are important in the ongoing Th2 response during murine schistosomiasis.

Schistosomiasis is a chronic parasitic disease caused by the extracellular parasite *Schistosoma*. In the case of *S. mansoni*, infection is initiated by transcutaneous penetration of cercariae. Following infection, parasites transform into schistosomula that reside in the skin for 3–7 days, afterwards they migrate, through the bloodstream, to the lungs (1–2 weeks) and then to the hepatic portal system, where the sexually differentiated male and female mate. A key feature of the immune response in *S. mansoni*-infected mice is the occurrence of a strong Th2 response triggered by parasite eggs that are gradually deposited in host tissues, as early as week 5 postinfection [60]. The mechanisms leading to the promotion of this Th2-dominated response are still obscure. We and others showed the crucial role of soluble egg antigen (SEA) glycoconjugates in the Th2 response induction, leading to the hypothesis that egg glycolipids, through a CD1d-dependent mode of antigen presentation, may be important in the development of a Th2 response during murine schistosomiasis [61, 62]. Our initial study clearly showed that Balb/c CD1d-deficient mice had a reduced Th2 response after egg-laying and developed a less marked fibrotic pathology compared to wild type mice. This observation was recently confirmed in the C57Bl/6 strain of mice [63]. Thus, our initial results underlined the possible involvement of CD1d-restricted T cells in the early immunological events leading to the generation of Th2 response associated to the pathol-

ogy. In agreement with our hypothesis, Zaccone et al. reported that the treatment of autoimmune-prone NOD mice with schistosome eggs increased the number of NKT cells and the induced Th2 response protect mice from diabetes development [64]. However, the use of Jα18-deficent mice devoid of iNKT cells, compared to wild type and CD1d-deficient mice, gave surprising results [63]. Indeed, during the acute stage of the disease, Jα18-deficient mice develop a decreased Th1 response whereas CD1d-deficient mice, as described above, develop a reduced Th2 response. Thus, during the acute phase of murine schistosomiasis, iNKT cells appear to provide help for Th1 cell differentiation whereas non-iNKT cells appear to promote that of Th2 cells, indicating that both iNKT and non-iNKT cell subsets play opposite, and perhaps complementary, functions on the Th1/Th2 balance during murine schistosomiasis.

The role of iNKT cells in promoting Th1 response during *Schistosoma* infection is however surprising. Indeed, we have recently reported that DCs sensitized with schistosome eggs polarize the immune response toward a Th2 direction in immunized WT, but not in Jα18-deficient mice suggesting that iNKT cells provide help for Th2 responses in this model [65]. These apparent conflicting results could be explained by differences in the organs analyzed, knowing that iNKT cell functions vary according to the organ. Moreover, iNKT cells functions also depend on the frequency of their TCR stimulation [66, 67]. Therefore, in our immunization model, primary and single stimulation of iNKT cells by egg-sensitized DCs may be efficient at promoting Th2 immunity whereas during infection, chronic activation of iNKT cells may rather polarize the immune response toward a Th1 direction. Recent observations also suggest that the type of CD1d-expressing APCs is crucial in dictating iNKT cell functions [68, 69].

The mechanisms by which iNKT and non-iNKT cells could modulate immune response during infection remain unclear. However, our group showed that both iNKT and non-iNKT become activated during the course of infection [63]. Moreover, by using a synchronous model of egg deposition in the liver, we demonstrated that iNKT rapidly (48 h) produce IFNγ, IL-4 but not IL-5 and IL-10. Whether or not this rapid cytokine production occurs in vivo during infection and could modulate the subsequent immune response remains an open question that needs further investigations. Moreover, attempts are also underway to understand how non-iNKT cells could modulate adaptive immune response and favour Th2 response during schistosomiasis.

The mechanisms of activation of iNKT and non-iNKT cells also remain unresolved. An increasing body of evidence suggests that DC/pathogen interactions, involving Toll-like receptors (TLR), may lead to a DC activation/maturation process that leads to iNKT cell activation (Fig. 1b): iNKT cells could be activated (1) by a combined synthesis of IL-12 and CD1d-restricted self-lipids by *Salmonella typhimurium* LPS-stimulated DC or (2) by IL-12/IL-18 cytokine production by *Escherichia coli* LPS-activated DC (for review [70]). Although TLR activation is the main pathway by which DCs become activated during infection, our recent data, using TLR- and MyD88-deficient mice, clearly show that in response to schistosome eggs, activating pathways triggered in DCs by TLRs are dispensable for iNKT cell activation,

indicating that other pattern-recognition receptors are involved [65] (Fig. 1b). Their identification requires further investigation. Moreover, our results support the hypothesis that endogenous glycolipid ligands, such as iGb3 or related products, are implicated in their activation. To emphasize this hypothesis, our group could not detect iNKT activating compounds in fractions obtained from schistosome egg total lipids, within the detection limit of our assay. Finally, in our experimental setting, CD1d-independent mechanisms, such as secretion of cytokine or membrane-bound factors that would modulate iNKT cell function, might also play a role. The mode of activation of non-iNKT cells remain largely unknown and investigations are now underway to identify their mechanisms of activation, which appear to be DC independent, according to our preliminary results.

Conclusions

NKT cells represent highly potent immunoregulatory cells with a conserved specificity making them very attractive targets for immunotherapy. However, there is still much to learn about these cells before manipulating them safely for clinical use, due to their diverse and also antagonistic behaviour and to the differences observed between murine models and human subjects (subsets, repertoire, etc.). Thus, the identification and characterization of the various molecules/signals that lead to NKT cell activation is obviously an important goal for future research. The immunological association between helminth infections and atopic diseases has raised strong interest in the mechanisms promoting Th2 response, among which NKT cells seem to play a crucial role at least in rodents. However, the mechanisms involved in their initial activation remain too elusive. Moreover, although some information is now available in humans, mainly in allergic asthma, these are still very limited, and further investigations on the involvement of NKT cells in human Th2-related diseases are necessary. These approaches in the human system would help to develop new therapeutic strategies to neutralize iNKT cell deleterious role in asthma, for instance by using αGC analogues that favour secretion of IFNγ by pulmonary NKT cells. In the case of helminthiasis, continuous progress in the research field of NKT cells and their glycolipid ligands is important to our ultimate understanding of the biological significance of the Th2 response that underlines these parasitic infections.

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CD8+ T Cells Play a Key Role in the Development of Allergic Lung Inflammation

Nobuaki Miyahara and Erwin W. Gelfand

Introduction

Asthma is characterized by persistent airway inflammation and airway hyperresponsiveness (AHR); T cells play a central role in orchestrating the disease processes through the release of various cytokines [1]. CD4+ T cells, especially Th2-type cells, which produce IL-4, IL-5, and IL-13, are considered pivotal in the development of AHR and eosinophilic inflammation [1–5]. In contrast to CD4+ T cells, there are relatively few studies that have focused on the involvement of CD8+ T cells in the development of AHR and airway inflammation. During an immune response, naive CD8+ T cells, primed to antigens presented in the context of major histocompatibility complex (MHC) class I molecules, expand and acquire effector functions. After the acute immune response has subsided, an increased frequency of antigen-specific memory T cells is maintained for years after the initial priming [6–8]. Naive CD8+ T cells represent a relatively homogeneous, small resting T-cell population with low CD44 expression that do not produce any cytokines other than low levels of interferon gamma (IFN-γ) or IL-2, but can be efficiently primed with antigen and cytokines to become primary effector cells [9]. Although CD8+ T cells are important effectors of cell-mediated immunity, their precise role in the pathogenesis of asthma is unclear. No role or even active suppression of allergic airway disease has been associated with CD8+ T cells [10, 11]. However, recent studies in both humans and rodents have demonstrated that in addition to CD4+ T cells, CD8+ T cells are involved in the induction of allergic airway dysfunction [12–18].

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CD8+ T Cells in Allergic Disease

Although the role of CD4+ T cells in orchestrating the development of airway inflammation and AHR has been emphasized [1–5], there is now broad support for the heterogeneity of CD8+ T cells and their contribution to allergic airway disease. CD8+ T cells may not act solely as potent cytotoxic effector cells. They can secrete Th2-type cytokines and promote B cell production of antibody [19, 20]. Studies in both humans and rodents indicate that CD8+ T cells may play an important role in allergic inflammation [12–24]. A recent study showed that following sensitization and allergen exposure, mice deficient in the CD8α chain developed less airway inflammation and AHR compared to wild-type (WT) mice [15]. This was associated with decreased levels of the Th2 cytokine IL-13 in bronchoalveolar lavage (BAL) fluid. Interestingly, CD8+ T cells isolated from the lungs of sensitized and challenged WT mice were shown to stain positively for IL-13, indicating that CD8+ T cells are a source of IL-13 in the lungs of allergic mice. This ability of CD8+ T cells to produce IL-13 has been previously reported in human T cell clones which were specific for Epstein–Barr virus (EBV) [25] and recently in patients with atopic dermatitis [21]. In atopic dermatitis patients, CD8+ T cells responded to a superantigen, releasing both IL-13 and IL-5: in fact, the CD8+ T cells were the major source of IL-13 in this in vitro culture system. Under other conditions, CD8+ cells have been shown to possess the capacity to differentiate into type-2 cytokine (Tc2)-producing cells and produce Th2 cytokines [26–28].

CD8+ T cells have the potential to secrete high levels of IL-4 when primed in the presence of IL-4 [8] and produce Th1 cytokines when stimulated by IL-12 [29]. CD8+ T cell polarization into Tc1 and Tc2 cells is not restricted to in vitro conditions but has also been observed in numerous infectious disease states in mice [29, 30] and humans [8, 31]. CD8+ T cells displaying Tc2 profiles have also been found in the lungs of asthmatic patients [31].

Cerwenka et al. [18] have shown that transfer of antigen-specific Tc2 cells into naive mice followed by antigen challenge leads to the development of airway eosinophilia and AHR and that CD8+ T cells respond to soluble antigen in the airways via the MHC class I pathway, thereby contributing to the inflammatory response in asthma. It has also been reported that IL-5-producing CD8+ T cells, when challenged via the airways with cognate peptide, induced a significant eosinophilia [27]. In addition, infection with influenza A virus leads to an increase in airway responsiveness and allergen-specific IgE production in mice exposed to aerosolized antigen [32]. Moreover, data from human studies highlight the positive correlation between respiratory tract infection and the development of allergic disease [33, 34]. Cumulatively, these observations and experimental data support the premise that CD8+ T cell activation can promote asthma either directly or indirectly.

In rats, Isogai et al. [17] demonstrated that ovalbumin (OVA)-primed αβ+ CD8+ T cells from cervical lymph nodes of rats expressed IL-4, and, when adoptively transferred into naive recipients before challenge, induced late airway responses when challenged with OVA. This was associated with eosinophilia in BAL fluid and increased numbers of IL-4 and IL-5 mRNA-expressing cells. These

proinflammatory effects were not seen after transfer of naive cells. These data indicate that primed αβ+ CD8+ T cells participate in airway responses to inhalational challenge with exogenous antigen and have proinflammatory effects.

Studying human samples, Cho et al. [13] demonstrated increased numbers of IL-4+/CD8+ T cells in the blood of atopic patients with mild asthma. Activated CD8+ T cell infiltration into peribronchial tissues has been associated with asthma deaths [23]. Similarly, increased cytokine production (IL-4 and IL-5) from sputum CD8+ cells has been shown in patients with asthma, and this was related to disease severity [14]. Impressively, a recent study showed that the outcome of asthma, as determined by the annual decrease in FEV_1, could be predicted by the bronchial CD8+ T cell infiltrate but not by the airway eosinophilia or reticular basement membrane layer thickness [25]. Together, these studies provide additional support for the role of CD8+ T cells in the development of AHR and airway inflammation.

CD8+ T Cell Heterogeneity

Recent focus has been on defining different subtypes of memory CD8+ T cells, especially following allergen priming. Antigen-experienced populations of memory CD8+ T cells can be distinguished by surface expression of the CD62 ligand (CD62L) and the chemokine receptor 7 (CCR7). Different functional and migratory properties have been recently assigned to subsets of CD8+ T cells [35–37]. Antigen-experienced central memory CD8+ T cells (T_{CM}) are $CD62L^{hi}CCR7^{hi}$ and home preferentially to lymph nodes. Effector memory CD8+ T cells (T_{EFF}) are $CD62L^{lo}CCR7^{lo}$ and traffic more efficiently to non-lymphoid tissues and to sites of tissue inflammation. In vitro, antigen-specific CD8+ T cells can be induced to differentiate to provide either of these subtypes. When cultured in the presence of IL-15, antigen-specific CD8+ T cells acquire the phenotypic and functional characteristics of CD8+ T_{CM}, whereas CD8+ T cells cultured in the presence of IL-2 show characteristics of CD8+ T_{EFF} [37, 38].

When transferred into sensitized CD8-deficient mice, T_{EFF} were found in increased numbers in the lungs following airway allergen challenge, unlike transferred T_{CM}, which migrated to lymph nodes [16]. CD8+ T_{EFF} produced IL-13 and transfer of T_{EFF} into sensitized, CD8-deficient mice prior to challenge fully reconstituted airway inflammation as well as AHR, whereas transfer of T_{CM} had no effect on airway inflammation or AHR. In addition, transfer of these allergen-specific T_{EFF} into sensitized WT mice further increased the levels of AHR and airway inflammation following airway allergen challenge. These findings identify the capacity of a specific subset of CD8+ T cells in the development of allergic airway disease.

Previous studies have shown that the migration of T_{EFF} can be initiated by mast cell activation and that this migratory response can be regulated by the leukotriene B_4 receptor 1 (BLT1) expressed on T_{EFF} under in vitro conditions [39]. Of interest, several years ago, it was shown that leukotriene B_4 (LTB_4) could bind specifically to a small proportion of human peripheral blood T cells [40], but these findings were never further explored. Although BLT1 mRNA is also detected in CD4+ T

cells (in fact, several subsets of CD4+ T cells express LTB_4 receptor mRNA (Th0, Th1, and Th2)) [41], subsets of antigen-experienced CD8+ T cells do show a differential induction of BLT1 expression with BLT1 expressed on T_{EFF} but not T_{CM} [42]. Genetic manipulation (deletion) of BLT1 expression on T_{EFF} or CD8+ T cells impaired their recruitment to the lung, and after transfer to CD8-deficient mice, they failed to reconstitute AHR, airway eosinophilia, BAL IL-13 levels, or mucus hyperproduction [43, 44], suggesting that BLT1 expression was essential for CD8+ T cell recruitment to the lung and their mediation of allergic airway responses. In more recent experiments examining IgE and the mast cell-dependent development of AHR and lung eosinophilia, treatment of the mice with a specific antagonist of the LTB_4 receptor prevented the development of AHR and inflammation [45], further supporting the essential role of this LTB_4 receptor in triggering allergic responses in the lung. These data identify a novel pathogenic cell in the development of AHR, eosinophilic airway inflammation, and mucus hyperproduction [46]. This CD8+/BLT1+/IL-13+ subset of T cells appears to play a unique and important role in the development of allergen-induced disease in the lung (Fig. 1).

In concert with these findings, we have explored this role of CD8+ T cells in asthma, translating recent findings in the mouse models to human asthma.

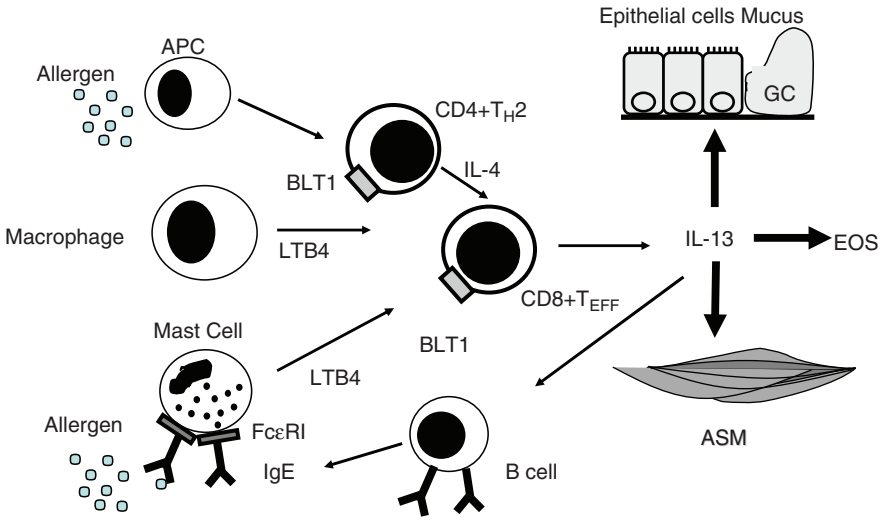

Fig. 1 Potential mechanisms of allergic airway disease in sensitized hosts following allergen exposure. Following allergen exposure, activation of mast cells via the high affinity Fc receptor for IgE (FcεRI) leads to mast cell activation, triggering release of mediators including lipid mediators such as LTB_4. LTB_4-BLT1 (leukotriene B_4 receptor 1) interaction leads to the recruitment/activation of allergen-specific effector CD8+ T cells (T_{EFF}) (likely following interaction with IL-4+ allergen-specific CD4+ type-2 helper T cells [Th2]). In the mast cell-independent pathway, allergen exposure leads to recruitment of effector T cells likely through LTB_4 generated by other cell types (e.g., macrophages). Effector T cells produce IL-13. IL-13 then acts on airway epithelial cells to induce goblet cell (GC) metaplasia, enhances airway eosinophilia, and acts on airway smooth muscle (ASM) to induce increased responsiveness. APC, antigen presenting cells

To determine whether an equivalent of these cells exists in human asthma, immunofluorescence staining of human airway tissue and BAL fluid cells from asthmatic subjects was performed. In these initial studies, CD8+/BLT1+/IL-13+ T cells were identified in BAL fluid specimens from asthmatic subjects and CD8+/BLT1+ T cells were detected in asthmatic lung tissue but not in lung tissue or BAL fluid cells from control subjects [47]. In a limited number of BAL fluid samples, lymphocytes reportedly represented 6–10% of total recovered cells, and 60% were CD3+; out of these, 20–30% were CD8+. Approximately 70–75% of the BAL CD8+ T cells coexpressed BLT1 and, after stimulation with phorbol myristate acetate and ionomycin, also stained for IL-13. CD8+ T cells were also prominent in the submucosa of asthmatic airway tissue, with approximately half of these tissue-infiltrating T cells expressing BLT1 (Fig. 2).

These findings identifying the critical role of these CD8+/BLT1+/IL-13+ cells in mice might be central to the human disease as well, especially in the maintenance-progression phase of asthma or in severe asthma. It is of interest to note that BAL LTB_4 levels correlate with asthma disease severity [48], and as noted, numbers of infiltrating bronchial CD8+ T cells correlate with disease severity [25], supporting a link between these previously assumed independent findings. IgE-stimulated mast cells might be an important source of LTB_4, necessary for the recruitment of CD8+/BLT1+/IL-13+ cells to the lungs of allergic hosts, as demonstrated in the animal models [45].

Suppressive Role of CD8+ T Cells

Viral infections characteristically elicit a CD8+ T cell lymphocytosis, dominated by cytolytic cells secreting IFN-γ, which in turn may lead to Th2 suppression [49, 50]. A number of studies have reported that CD8+ T cells play a protective role in allergic disease [51–53]; most of these studies utilized rat models. In the studies using rat models, depletion of CD8+ cells upregulated the late airway response, AHR, and airway inflammation [51, 52], and administration of antigen-primed CD8+ T cells suppressed these responses [53]. More recently, studies from the same laboratory have shown that αβ+ CD8+ T cells can mediate airway responses [17]. CD8+ γδ T cells suppressed these responses, and they proposed CD8+ γδ T cells as negative regulators of allergic airway responses [54]. Indeed, Brown Norway rats have relatively higher percentages of γδ T cells in the spleen and lymph nodes compared to mice [53]. However, the role for the CD8+ γδ T cells in rodents and humans has not been well defined and further investigation is required for full elucidation of the role of different CD8+ T cell populations in the downregulation of lung allergic responses.

This group also showed that depletion of resident CD8+ T cells enhanced CD4+ T cell-mediated late airway responses in rats [55], suggesting that resident naive CD8+ T cells may have a protective role against the development of allergic airway responses. On the other hand, activated viral-specific memory CD8+ T cells suppressed allergen-induced airway inflammation in a bystander manner [56].

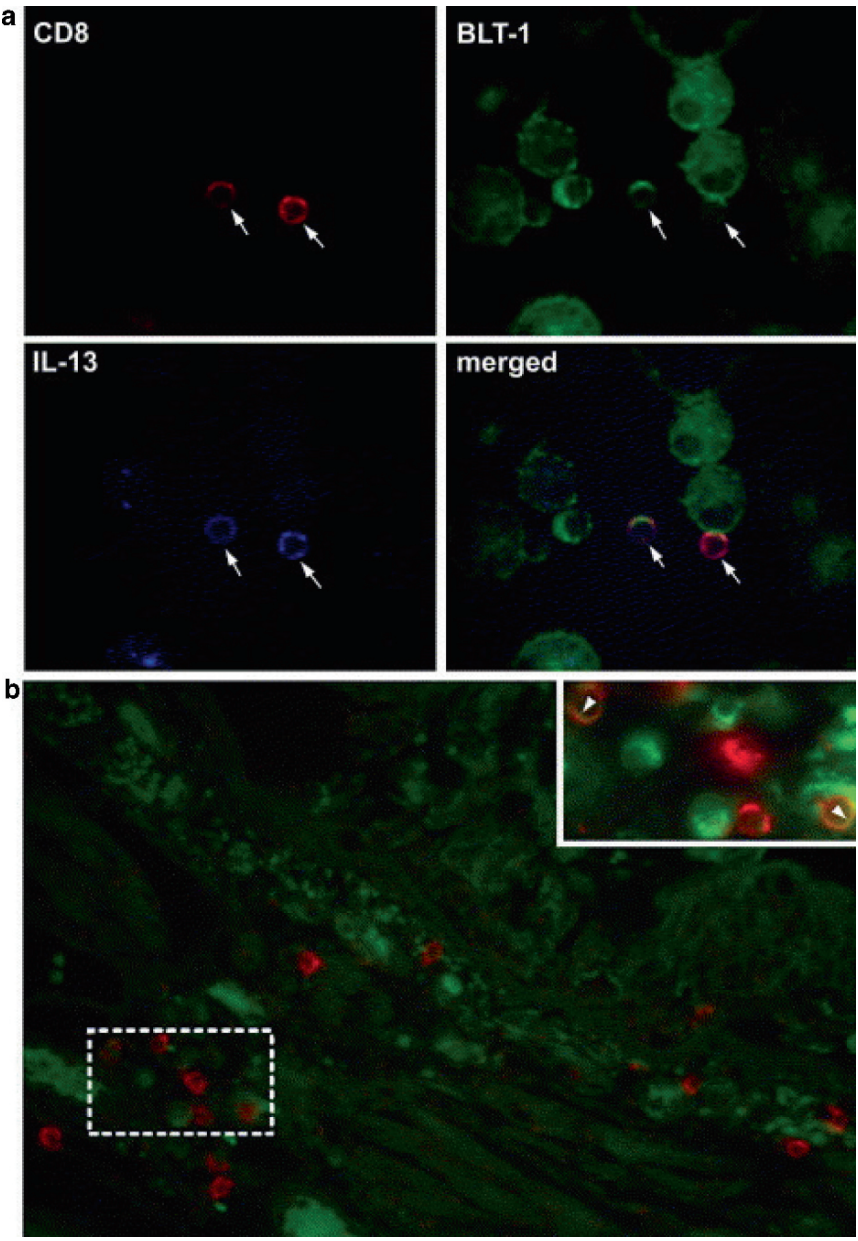

Fig. 2 Immunofluorescent detection of CD8+, BLT1+, and IL-13+ cells in BAL fluid (**a**) and tissue (**b**) of asthmatic subjects. Fresh BAL fluid cells were stimulated for 4 h in culture with phorbol 12-myristate 13-acetate (5 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (10 μg/ml). Cells were stained in suspension with mouse monoclonal antihuman CD8 antibody (dakocytomation, Carpinteria, CA) and then fixed with paraformaldehyde (4% in PBS, 10 min) and permeabilized with saponin (0.1% in staining buffer). Cells were then incubated with allophycocyanin

Therefore, in the absence of an environment where there is IL-4 or Th2-like responses, CD8+ T cells may follow a Tc1 pathway, secreting IFN-γ and suppressing allergic inflammation.

In a mice, heat-killed Listeria as an adjuvant given once with antigen prevented the development of AHR and airway inflammation in OVA-immunized BALB/c mice and significantly reduced airway eosinophilia and mucus production [57]. Moreover, the inhibitory effect on AHR was dependent on the presence of IL-12 and CD8+ T cells, was associated with an increase in IL-18 mRNA expression, and required close association between the heat-killed bacteria and the antigen [58]. T cell protective immune responses in the respiratory tract, and downmodulation of ongoing Th2-dominated responses, indicated that the use of heat-killed Listeria as an adjuvant for allergen immunotherapy might be clinically effective in the treatment of allergic asthma. Similarly, allergen-IL-18 fusion DNA protects against AHR through activation of CD8+ T cells and IFN-γ [58].

When examined together it appears that in some environments or experimental conditions, such as in the presence of viral infection or following administration of certain adjuvants, CD8+ T cells can suppress allergic airway inflammation. The different routes of sensitization, adjuvants, species differences, as well as differences in recipient status may all play a role in dictating the outcome mediated by antigen-primed CD8+ T cells. It is also possible that distinct subpopulations or different functional stages of αβ+/CD8+ T cells may exist, and, in response to specific environmental influences, either suppress Th2 responses or enhance Th2 responses by as yet unknown mechanisms. The different approaches and experimental designs may affect the polarization of distinct CD8+ T subpopulations, and, as a result, affect IgE or cytokine production, airway responsiveness, and airway inflammation in truly opposing ways [59].

Fig. 2 (continued) (APC)-conjugated rat antihuman IL-13 (Biolegend, San Diego, CA) and rabbit antihuman BLT1 (Cayman Chemicals, Ann Arbor, MI), followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit Ig (Jackson immunoresearch Laboratories, West Grove, PA). This anti-BLT1 antibody recognizes the C-terminus of the BLT1 receptor, which is located on the intracellular side of the plasma membrane. After washing, cells were incubated with alkaline phosphatase-conjugated goat anti-mouse Ig (dakocytomation), followed by incubation with fluorescent permanent red alkaline phosphatase substrate (dakocytomation), and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). For tissue staining, 5-μm sections of formalin-fixed and paraffin-embedded lung tissue specimens were permeabilized with 0.1% Triton X-100, incubated with rabbit anti-BLT1 and mouse anti-CD8 antibodies, washed with Tris-buffered saline, and incubated with FITC-conjugated goat anti-rabbit Ig and alkaline phosphatase-conjugated goat anti-mouse Ig, followed by washing and incubation with fluorescent permanent red alkaline phosphatase substrate and mounting as above. Staining was examined under a Leica DMRXA fluorescent microscope (Leica, Wetzlar, Germany) by using the following filters: Texas Red for CD8 (orange), FITC for BLT1 (green), and APC for IL-13 (blue). Arrows indicate the same cells stained for CD8, BLT1, and IL-13 (**a**). Arrowheads indicate BLT1 staining localized on the intracellular side of the membrane of CD8+ cells (**b**, inset representing the boxed area after Z-scan) (From Gelfand EW, Dakhama A (2006) CD8+ T lymphocytes and leukotriene B4: novel interactions in the persistence and progression of asthma. J Allergy Clin Immunol 117:577–582)

Activation of CD8+ T Cells

The pathway by which CD8 T cells are activated during allergen exposure is not clear. Exogenous proteins such as OVA are usually presented in association with MHC class II molecules and CD4+ T cells, and not MHC class I molecules which are required for the activation of CD8+ T cells. Hence, CD8+ T cells might not be expected to become activated following OVA challenge of sensitized mice. However, there are now several examples suggesting a "leak" between class I and class II pathways, at least in APCs [60–63], a process known as cross-priming, and first demonstrated in spleen APCs [60]. Some dendritic cells are specialized in processing exogenous antigens for presentation to CD8+ T cells. Recent studies indicate that a CD8+ population of dendritic cells is responsible for cross-priming of cytotoxic T cells in vivo [64]. It has also been reported that OVA-specific, MHC class I-restricted CD8+ T cells are inducible in vivo by OVA sensitization in conjunction with certain adjuvants [62, 63]. Indeed, we and others have shown that CD8+ T cells can be activated by exogenous antigen and contribute to allergen-induced AHR and eosinophilic inflammation in vivo [12, 15, 17, 65]. We have shown that in vitro-generated memory effector CD8+ T cells can respond to inhaled OVA and mediate allergic airway inflammation [16]. Cerwenka et al. [18] have also shown that in vitro-generated Tc2-type CD8+ T cells can respond to OVA, mediating AHR and inflammation in vivo. They have also shown that in vitro-generated Tc2-type cells respond to soluble antigens such as OVA in vitro and that OT1 cells whose T cell receptor (TCR) is specific for OVA peptide tend to polarize toward a Tc2 rather than a Tc1 phenotype when cultured with lower concentrations of OVA. When cultured with higher concentrations of OVA, they polarized toward Tc1-type cells producing IFN-γ. Under certain experimental conditions, such as during viral infection, CD8+ T cells triggered in the presence of Th2 cytokines can differentiate toward a Tc2 phenotype. As noted above, IL-5-producing CD8+ T cells, when challenged via the airways with cognate peptide or viruses such as respiratory syncytial virus (RSV) and influenza, were able to induce eosinophilic inflammation [27]. IL-4 produced during allergic responses may trigger a switch from a normally type-1-biased virus-specific T cell response toward type-2 responses [66, 67]. In mouse models of allergic airway inflammation, IL-4 production from CD4+ T cells may be essential to the polarization of CD8+ T cells toward type-2 responses, and IL-13 production in particular [67, 68].

Conclusion

In parallel to the demonstration of the heterogeneity of CD4+ T cell subsets and effector functions, there is now broad support for a similar heterogeneity among CD8+ T cells. This heterogeneity may be observed in distinct CD8+ T cell subsets or because of a plasticity exhibited in response to cues from the local environment. Studies in both rodents and humans indicate that CD8+ T cells, and specifically

IL-13-producing effector CD8+ T cells or Tc2 cells, contribute in significant ways to allergic airway responses. On the other hand, some studies also suggest that CD8+ T cells can exhibit suppressive effects on allergic airway inflammation, likely through IFN-γ production. The factors regulating differentiation of IL-13-producing "enhancing effector CD8+ T cells" or Tc2 cells on the one hand and "suppressive CD8+ T cells" on the other are beginning to be defined, and are essential for understanding the regulation and maintenance or curtailment of allergic lung inflammation and AHR. Further understanding of the role and control of these heterogeneous CD8+ T cell-mediated effector functions will reveal new therapeutic strategies for the treatment of bronchial asthma.

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Neutrophils and Their Mediators in Asthma and Allergic Disease

Kian Fan Chung

Introduction

The eosinophil has usually been associated with asthma and allergic diseases because it is a very characteristic feature of allergic inflammation. For many years, it has remained a focus of asthma research, while the neutrophil was very much considered a poor cousin of the allergic process. It is now clear that the neutrophil is very much part of the inflammatory milieu of allergic diseases and there is a growing focus on its role in these situations. Neutrophils are the cells recruited early during an acute inflammatory process often in response to bacterial infections, but in chronic allergic diseases such as in asthma, they are present in chronic stages even in the absence of any infections. This could reflect an increase in neutrophil trafficking from bone marrow to inflamed tissues. The significance of this remains unclear, and although there are many features of neutrophil activation that may contribute to airway inflammation and tissue damage in allergic diseases, there may equally be beneficial effects of neutrophil activation including inhibition of allergic inflammation, activation of the innate immunity, and aspects of tissue repair. The situation is not dissimilar with the initial hypotheses concerning the role of the eosinophil, when eosinophils were considered to have anti-inflammatory properties as they had the capacity to metabolise mediators of inflammation such as cysteinyl-leukotrienes, but later the role of the eosinophil enzymes such as major basic protein was considered to be pro-asthmatic by causing epithelial damage. More recently, a role for the eosinophil in the fibrosis of asthma has been put forward. The neutrophil may also possess these attributes and the precise role of the neutrophil in asthma and allergic disease still remains to be defined. The questions that arise concerning the neutrophil are as follows:

1. What are the mechanisms that are underlying the increase in neutrophils in asthma, and what is the relationship to eosinophilic inflammation?
2. What is the role of the neutrophil, if any, in asthma?

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3. Does the presence of the neutrophil indicate a different phenotypic form of asthma?
4. Is there any beneficial effect of targeting the neutrophil in asthma? Should we be targeting neutrophils in allergic inflammation?

Neutrophilic Inflammation in Asthma

The neutrophil is a prominent inflammatory cell in many different types of asthma so much so that it is often wondered whether it is a cell that is associated with only certain types of asthma. However, it is quite likely that the neutrophil like the eosinophil is intimately related to asthmatic inflammation. Bronchial allergen challenge leads to an early increase in neutrophils in the airways measured in bronchoalveolar lavage fluid [1–3] and also in the bronchial mucosa [4]. In this situation, the degree of infiltration in the airway mucosa of neutrophils correlates well with the number of eosinophils. The early neutrophilic response is also documented in allergen nasal challenges [5, 6].

Measurement of eosinophils and neutrophils in sputum induced by inhalation of hypertonic saline has been used as a marker of inflammation in asthma, but the caveat is that this does not truly reflect the cellular response measured in the airway mucosa. A significant proportion of patients with asthma do not demonstrate eosinophilic inflammation as assessed by this method. Eosinophilic inflammation was reported in 41% of asthmatics and non-eosinophilic in the remaining 59%, the latter group characterised by neutrophilic inflammation associated with high levels of CXCL8 (IL-8) in sputum, although myeloperoxidase (MPO) levels were elevated in both groups [7]. In another group of 93 asthmatic patients on inhaled corticosteroid therapy, neutrophilic inflammation was defined to be present in sputum when it exceeded 61% count and eosinophilic inflammation when eosinophil count was >1.01%. This study showed that 22% of patients had neutrophilia and 43% eosinophilia with 7% having both increased, but 33% had no increase in eosinophils or neutrophils. In this study, the degree of neutrophilia, but not eosinophilia, was inversely correlated with FEV_1 [8]. A similar pattern of sputum characteristics was reported in asthmatics children recovering at 2 weeks from an acute asthma episode with eosinophilia in 43%, combined eosinophilia and neutrophilia in 35%, and non-eosinophilia in 22% [9]. These studies introduce the concept that some asthma patients may solely have a neutrophilic-based asthma while others an eosinophilic-based asthma.

Increased neutrophilic inflammation of asthmatic airways has been documented in patients with persistent asthma suggesting that persistent asthma is characterised by neutrophilic inflammation [7, 10–13]. In a study that included all levels of severity of asthma [11], the degree of neutrophilic inflammation was the highest in patients labelled as corticosteroid-responsive asthma, patients who by definition needed oral prednisolone therapy to acquire control of asthma symptoms; these patients also had a degree of eosinophilic inflammation. Levels of IL-8 and eosinophil cationic protein

(ECP) in sputum of severe asthma patients were also the highest. An increase in neutrophils in bronchoalveolar lavage fluid (BAL) fluid and in both bronchial and transbronchial biopsies of patients with chronic steroid-dependent severe asthma has been reported [12]. While this study showed that there were lots of eosinophils in some biopsies and not in others, it reported no differences in neutrophil counts between the groups. When significant neutrophilia was found, it was associated with prominent infiltration with eosinophils, mast cells and CD3 T cells, and therefore making it difficult to determine whether the neutrophil is a real marker of severity. While this study suggested that the neutrophil may be a particular feature of severe asthma, it did not find groups of asthmatics with predominant neutrophilic inflammation alone. The studies that have used sputum to determine neutrophilic inflammation need to correlate their findings with airway neutrophils.

Neutrophilic Inflammation in Asthma Exacerbations

The presence of neutrophilic inflammation has been associated with worsening of asthma. The presence of increased neutrophils and IL-8 in sputum was associated with worsening of asthma following withdrawal of inhaled steroid therapy in asthma patients [14], while in other studies, the presence of eosinophils may also be such a marker [11, 15]. In children, the presence of both intraepithelial neutrophils and eosinophils was associated in exacerbations [16]. The neutrophil, in addition to the eosinophil, is also prominent in acute severe asthma. In 18 adults with acute severe asthma, high levels of activated neutrophils in sputum (75% of cells) were found in 10 of 18 samples and only 3 had more than 75% eosinophils [17]. Neutrophils were higher in the group that had described an upper respiratory tract infection compared to those that did not. Free neutrophil elastase levels were detectable [17]. In children, a mixture of eosinophils and neutrophils in sputum collected at 2 weeks after exacerbation was found, together with increased levels of ECP and MPO [18]. In patients with status asthmaticus on ventilatory support, high levels of neutrophils were found in bronchial lavage fluid, higher than what would be accounted for by being mechanically ventilated [19, 20]. These were associated with high levels of IL-8 together with eosinophilic inflammation and high levels of IL-5 and CCL5/RANTES (regulated upon activation, normal T-cell expressed and secreted) in bronchial lavage fluid. In patients who died of status asthmaticus, submucosal neutrophils were associated with sudden onset asthma, and were less abundant in cases of slow onset asthma, in whom eosinophils predominated [21].

Upper respiratory tract viral infections which are an important cause of exacerbations of asthma, may be an important cause of airway neutrophilia since in experimental rhinovirus infections, an increase in bronchial lavage neutrophils can be found at 96 h [22]. Likewise, exposure of asthmatic patients to the environmental pollutant ozone induces a neutrophilia together with raised levels of CXCL8/IL-8 in sputum [23]; the effects of diesel particle exhaust also induce more of a neutrophilic response in non-asthmatic subjects than in asthmatic subjects [24].

Neutrophil Activation in Asthma

There is quite good evidence that circulating blood neutrophils are in an activated state in asthma, possibly related to the allergic state (Fig. 1). This raises the issue as to whether this abnormality is already present from the bone marrow or there are activation factors in the circulating of asthma patients that could activate neutrophils. Several studies have noted that the circulating neutrophil produces more reactive oxygen species [25–28]. Whole blood chemiluminescence was increased on asthma patients with chronic airflow obstruction compared to those without airflow obstruction [27]; while another study finds that the ability of neutrophils to produce more superoxide anions on exposure to phorbol myristate acetate (PMA), f-met-leu-phe (FMLP) or calcium ionophore was related to the atopic status [28]. Fc gamma-receptor-mediated chemiluminescence was increased after allergen challenge in those patients with a dual early and late phase response [26]. There is an increased production of myeloperoxidase from neutrophils from asthmatic patients that correlated negatively with FEV_1 [29].

Neutrophils can be activated to release respiratory burst and MPO with specific allergen through activation of IgE receptors on neutrophils [30, 31]; similarly, neutrophils from allergic patients challenged with specific allergen led to a decrease in expression of the surface CD62L (L-selectin), associated with an increase in soluble L-selectin in supernatants indicating neutrophil activation [32]. Accumulation of neutrophils and eosinophils together with release of MPO, LTB4, ECP and histamine has been reported after allergen nasal challenge in allergic rhinitis patients [6], although an increase in MPO release was not reported in another study [33]. However, MPO release was reported in another allergen-specific conjunctival challenge [34].

It is interesting that immunotherapy has been reported to reduce the ability of neutrophils of allergic asthmatics to produce MPO [29]. One of the interesting recent observations related to this observation is the presence of high-affinity IgE receptors on the neutrophil from atopic asthmatic individuals [35] and monomeric IgE can delay spontaneous apoptosis of human neutrophils and cause release of IL-8 in vitro [36]. This may explain persistence of non-infectious neutrophilic inflammation in allergic asthma. Given these observations, a study of the effect of anti-IgE treatments on neutrophilic activation in asthma needs to be undertaken.

Increased expression of the Toll-like receptors for TLR2, TLR4 and CD14 are reported in sputum samples from asthma patients, as well as those for IL-8 and IL-1β, indicating activation of innate immune response possibly by the increased levels of endotoxin measured in the sputum samples, in the absence of any bacterial infection [37].

The neutrophil may play a significant role in severe asthma. The expression of the integrins, CD35 and CD11b, on neutrophils from patients with severe asthma was increased compared to those from mild asthma [38]. In bronchoalveolar lavage fluid cells from patients with severe asthma, the levels of active MMP-9 were increased and correlated with neutrophil numbers, suggesting that it was an important source of MMP-9 [39].

Mediators Associated with Neutrophil Recruitment and Activation

Neutrophil recruitment. The recruitment of neutrophils to the airways or to sites of allergic inflammation is likely through the production of a number of chemokines such as CXCL8 (IL-8), CXCL5 (ENA-78), GRO-α and CCL-3 (MIP-1α), and also of other lipid mediators such as leukotriene B4 and of C5a and GM-CSF. In the airways, the epithelium, airway smooth muscle cells and macrophages are important sources of IL-8, including neutrophils [40]. IL-8 has been reported in numerous studies in sputum samples and bronchoalveolar samples from patients with asthma. IL-8 is also the most potent activator of neutrophils and triggers the secretion of granular enzymes such as myeloperoxidase, elastase and MMPs [41]. IL-8 can also induce the production of LTB4 and oxygen radicals, and stimulate neutrophil phagocytosis. In terms of effects on airway smooth muscle, IL-8 can induce contraction of airway smooth muscle directly through the activation of calcium channels, and also can induce migration of airway smooth muscle cells [42] (Fig. 1).

Leukotriene B4 and lipoxins. LTB4 is an important chemoattractant and activator of neutrophils. It also inhibits neutrophil apoptosis [43]. Increases in LTB4 together with CysLTs have been reported in exhaled breath condensates of patients with asthma [44, 45]. LTB4 together with cysteinyl-leukotrienes (LTs) are increased in BAL fluid of patients with nocturnal asthma at 4 am, and levels correlated with either nocturnal fall in FEV_1. Zileuton reduced the levels of BAL fluid of LTB4 and urinary LTE4, with a trend for improvement in nocturnal FEV_1 [46]. Lipoxins (LXs) are also lipoxygenase-derived eicosanoids as leukotrienes but possess anti-inflammatory properties particularly on neutrophils. LXs inhibit neutrophil superoxide anion generation, chemotaxis, transmigration across endothelial and epithelial cells, and entry into inflamed tissues in mice [47]. LXA4 inhibit LTB4-induced chemotaxis, adhesion and transmigration [48]. This effect of LX may occur partly through the new intracellular signal transduction pathway of polyisoprenyl phosphate remodelling [49]. Interestingly, the production of the counter-regulatory LXA4 from whole blood of patients with severe asthma is impaired [50].

Cytokines. Neutrophils produce cytokines such as IL-9, TNFα and TGFβ, and express receptors for IL-8 and IL-9. IL-9 is of particular interest since it is a Th2 cytokine that has effects on inflammatory and structural cells in the airways. Interestingly, IL-9 through the activation of IL-9 receptors can induce the production and release of IL-8 by neutrophils. Higher levels of IL-9Rα receptors have been reported on neutrophils from asthmatics [51]. Thus, this may represent an autocrine effect of IL-9 that may be considered as a regulatory factor for activated neutrophils.

IL-17A. The IL-17 family of cytokines is made up of six homodimeric glycoproteins of which IL-17A, produced by CD4+ or CD8+ memory (CD45RO+ cells) termed Th17 [52]. When administered to rats and mouse airways, it causes neutrophil chemotaxis; it also activates neutrophils to release neutrophil elastase and myeloperoxidase. The most compelling evidence for a role for IL-17A in

neutrophil chemotaxis and activation comes from allergen challenge in mouse, where transcription of IL-17A occurs, paralleling the accumulation of neutrophils [53]. On the other hand, there is evidence that although IL-17 is required during antigen sensitisation to develop allergic asthma, it is a negative regulator of established allergic asthma, by down-modulating eotaxin (CCL11) and TARC (CCL17) [54]. In asthma patients, elevated concentrations of IL-17 were found in lungs and blood of allergic asthma patients and linked to severity of asthma [52, 55, 56]. Sputum IL-17A mRNA expression is reported to be increased and correlated with IL-8 mRNA levels and neutrophil sputum counts [57]. IL-17A can induce IL-8 release from airway smooth muscle cells [58].

Neutrophil serine proteases. Neutrophils contain granules that contain myeloperoxidase, defensins and serine proteases, comprising of cathepsin G, neutrophil elastase and proteinase 3. These serine proteases together with MPO are the main agents responsible for destroying bacteria [59]. Otherwise, the serine proteases are important regulators of non-infectious inflammation through activation of specific receptors and modulation of cytokines. The effects of neutrophil proteases are protected from degradation from the presence of extracellular protease inhibitors through the close contact of neutrophils with the extracellular matrix leading to creation of a microenvironment that excludes the large protease inhibitors. All neutrophil serine proteases can cause proteolysis of chemokines such as CXCL12 (SDF1α) or CCL3 (MIP-1α) resulting in loss of chemotactic activity of these chemokines [60, 61]; however, such N-terminal processing can lead, on the other hand, to an increase in affinity of the chemokines for their receptor, such as N-terminal truncation of CXCL8 (IL-8) and CXCL5 (ENA-78), which then have higher chemotactic activity towards neutrophils [62, 63]. Similarly, activation of a new chemokine that attracts antigen-presenting cells such as dendritic cells and macrophages, chemerin, is mediated by neutrophil elastase and cathepsin G [64]. Neutrophil elastase and cathepsin G degrade mature TNF, and all three serine proteases can inactivate IL-6 [65, 66].

Neutrophil elastase can induce the release of CXCL8 through signalling through TLR4, involving myeloid differentiation primary response gene 88 (MyD88), IL-1 receptor associated kinases (IRAKs) and TNF-receptor associated factor-6 (TRAF6) resulting in NF-kB activation in bronchial epithelial cells [67]. The mechanisms by which neutrophil elastase transduces signals through TLR4 is not known but this demonstrates how neutrophils could directly interact or stimulate the innate immune system. Neutrophil elastase has also been shown to stimulate the expression of TGFβ in airway smooth muscle involving TLR4 [68]. Neutrophil elastase mediates the induction of mucins such as MUC5AC through the activation of PKC and production of ROS [69], that in turn leads to release of soluble TGFα which in turn stimulates EGFR inducing the production of mucins [70, 71]. Neutrophil elastase also cleaves cell surface adhesion molecules such as ICAM-1, VCAM-1 and epithelial E-cadherin [72–74]. Neutrophil elastase can cause apoptosis of ASM cells [75].

In the context of asthma where both neutrophils and eosinophils coexist, perhaps one of the most important actions of neutrophil elastase is its ability to activate

eosinophils [76], which may explain the common finding of both neutrophilic and eosinophilic activation, particularly in severe asthma and in acute severe episodes.

Neutrophils in Airway Wall Remodelling

Raised sputum neutrophil counts were associated with irreversible loss in lung function in asthmatics, as measured by FEV_1 after completion of a 14-day course of prednisolone suggesting a link with airway-wall remodelling [77]. FEV_1 correlated with the degree of neutrophilia in sputum and bronchial biopsy specimens. Apart from the effect of neutrophils in causing inflammation and tissue damage, it is likely that neutrophils may play a role in tissue remodelling. Serine proteases neutrophil elastase and proteinase 3 degrades elastin leading to emphysema, which can rarely be a feature of asthma. Neutrophil elastase induces mucus cell secretion, and can also induce airway smooth muscle proliferation [78]. MMP-9 is the major MMP expressed in BAL fluid, sputum and bronchial biopsies from asthma patients [79, 39, 80]. MMP-9 is mainly produced by neutrophils in severe asthma [39]. Neutrophils are important sources of TGFβ, and neutrophils from patients with asthma release higher levels of the profibrotic growth factor than non-asthmatic individuals [79]. TGFβ expression in neutrophils and eosinophils is increased following allergen challenge [4]. In addition, neutrophil elastase can induce the expression of TGFβ from airway smooth muscle cells. TGFβ itself can increase airway smooth muscle proliferation and hypertrophy, and increase the content of α-smooth muscle action [81, 82]. Thus, one of the potential area where neutrophils may contribute is in its interaction with airway smooth muscle cells to induce airway smooth muscle proliferation and hypertrophy, and airway smooth muscle chemotaxis, with enhanced contractile responses, characteristic features of these cells in asthma (Fig. 1).

Effect of Asthma Therapies on Neutrophils

The role of neutrophils in asthma remains unclear. Previous studies in animal models of ozone-induced airway hyperresponsiveness supported a role for neutrophils in bronchial hyperresponsiveness [83]. In humans, there is only indirect evidence available. An LTB4 receptor antagonist, LY293111, which was found to be effective in guinea pig allergen challenge model in inhibiting lung neutrophilia [84], had no effect on the late phase response to allergen in mild asthmatics while reducing the neutrophilic response [85].

The effect of corticosteroids, one of the most efficacious inhibitors of asthmatic inflammation, could be used to determine the role of neutrophils. However, while corticosteroids reduce eosinophilic inflammation in asthmatic airways, they increase the number of neutrophils in tissue [86, 87] through inhibition apoptosis

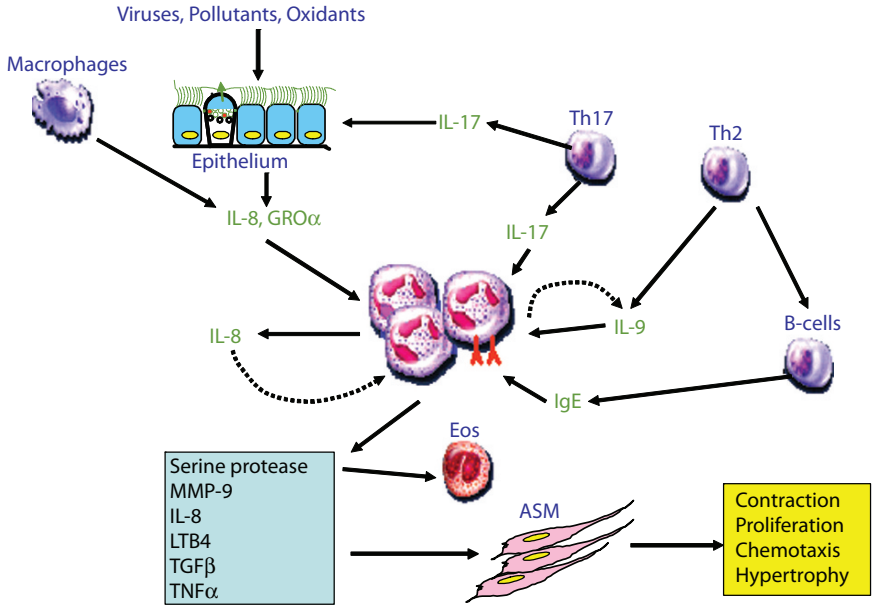

Fig. 1 Potential causes of neutrophil chemotaxis and activation in asthma and allergic diseases, and potential effects of neutrophil products on inflammatory and structural cells that may be lead to bronchoconstriction, bronchial hyperresponsiveness, inflammation and remodelling

of neutrophils, while augmenting apoptosis of eosinophils [88, 89]. In addition, although corticosteroids can inhibit IL-8 release from epithelial cells, treatment of asthmatics with oral corticosteroids increased the expression of IL-8 in airway epithelium, while decreased IL-8 in submucosal cells in bronchial biopsies [87]. This may explain also the persistence of neutrophils in asthma. Corticosteroid treatment, therefore, leads to an increase in the number of neutrophils in the lungs, and may confound the interpretation of neutrophils as a marker of severity of asthma. Another interpretation of the effect of corticosteroid in being unable to inhibit the number of neutrophils is that this may in effect contribute to the mechanisms of corticosteroid resistance seen in severe asthma.

Release of cytokines such as IL-8 and GROα from blood neutrophils can be inhibited by dexamethasone [90, 91], but lung neutrophils from cystic fibrosis patients appear to be totally resistant to dexamethasone in the LPS-release of IL-8 [91]. In severe asthma, there is certainly evidence of reduced response of the neutrophil to dexamethasone. In severe asthma circulating neutrophils, an increase in dexamethasone dosage had no effect on CD62L expression on neutrophils while its expression on eosinophils was concomitantly reduced; PMA-induced oxidative burst and IL-8 release induced by IL-1β, LPS or GM-CSF were all less inhibitable by dexamethasone [38]. Neutrophils that have migrated in response to IL-8 may subsequently cause eosinophils to accumulate into asthmatic airways [92]; this

would suggest a mechanism by which corticosteroids could paradoxically cause increased eosinophilic activation in severe asthma [11, 12]. BAL neutrophil-derived MMP-9 expression and activity were poorly inhibited by glucocorticoids [39]. The relative resistance of neutrophils to corticosteroid effects has been attributed to high levels of expression of the functionally inactive β-isoform of the glucocorticoid receptor, the synthesis of which is further upregulated on exposure of neutrophils to IL-8 [93].

Other asthma treatments have been reported to cause a reduction in neutrophils. Long-acting β2 agonists, salmeterol was shown to inhibit neutrophil numbers in bronchoalveolar lavage fluid and in the airways submucosa [94]. Whether this effect was related to an improvement in symptoms is not possible in this study. Similar results were obtained in a study of formoterol in mild asthma; this was accompanied by a reduction in IL-8 levels in sputum [95]. These studies suggest that a beneficial addition of long-acting β-agonists to topical corticosteroids is to keep the number of neutrophils in check.

Should Neutrophils Be Targeted in Asthma?

Despite the potential for the neutrophil to contribute to airway inflammation, tissue damage and airway remodelling in asthma, its exact contribution remains unknown and therefore whether neutrophils should be specifically targeted in this disease remains unclear. The neutrophil may have beneficial and deleterious effects in the airways of asthmatics, and interfering with such a delicate balance of effects may be a difficult act to control. Perhaps in certain types of asthma such as severe asthma or acute severe asthma, the neutrophil on balance may be more deleterious, but even in these situations suppressing neutrophil activities may lead to the increased risk of infections.

Macrolides are used as antibiotics to treat respiratory infections particularly with chlamydiae or mycoplasma, but also possess anti-inflammatory properties particularly in the inhibition of neutrophil recruitment and activation. Small changes in symptoms or surrogate markers of inflammation but not of lung function has been reported in some studies of asthma patients treated with macrolides [96–99]. In a study of acute severe asthma, telithromycin was shown to hasten the improvement in symptoms and lung function when added to usual treatment, although it was entirely clear whether this improvement could not be accounted for by the distribution of chlamydiae or mycoplasma infections in the groups [100]. Other current treatments under investigation in asthma therapy that may have an effect on neutrophil recruitment and activation under study include tumour necrosis factor-α blockers, phosphodiesterase 4 inhibitors [101], neutrophil elastase inhibitors and potential antioxidants [102]. Other treatments to block neutrophil chemotaxis are antagonists of CXCR1 and 2 to block the effect of IL-8 and anti-IL-17A [103].

COPD is the other inflammatory disease where the neutrophil also appears to be more prominent and inhibition of neutrophil recruitment and activation may

be considered to be important for therapeutic outcomes. However, despite the fact that the mechanisms of recruitment and activation of neutrophils and the resulting effects are different in COPD similar considerations as in asthma need to be taken into account regarding the advisability of inhibiting neutrophil inflammation and activation.

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Dendritic Cells, Macrophages and Monocytes in Allergic Disease

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Antigen-Presenting Cells

Dendritic cells, macrophages and monocytes are antigen-presenting cells (APC) and are critical for antigen-specific activation of T cells. Macrophages, in particular, are the source of many soluble mediators, which can enhance their own antigen-presenting function and that of dendritic cells. Changes in APC function, therefore, are likely to precede or parallel changes in T cell activation in the mucosa [1]. Indeed, animal models of allergy and studies of allergic patients demonstrate increased numbers of macrophages and dendritic cells (DC) at the mucosal surfaces of skin, gut and lung. Conversely, successful treatment has been demonstrated to correlate with decreased numbers of APC in the tissues [2].

APC can take up protein antigens into specialised endosome/lysosome compartments where antigens are processed into linear peptides and then presented to T cells in the context of major histocompatibility complex (MHC) class II molecules on the surface of the APC. Dendritic cells, however, are the principal or professional APC, and possess the unique ability to induce differentiation of naive T cells present in draining lymph nodes. DC are heterogeneous and several mouse and human mucosal DC subsets have been described, each with the potential to contribute to induction and regulation of the allergic response.

Dendritic Cells

(i) *Subsets of murine mucosal DC* Myeloid-DC, the principal DC subset detected at mucosal sites, form a contiguous network in the mucosal epithelium of the conducting airways, gut and skin. Myeloid-DC derive from a bone marrow progenitor, and enters the mucosa from the peripheral blood [3]. The principal

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mucosal DC subset in the lung and gut bears the adhesion marker, CD11b and high levels of MHC class II [4]. Myeloid-DC express C-lectin receptors including CD202 (mannose receptor) and CD205 indicating that these cells are specialised for antigen capture. A recent development has been the description of a novel myeloid-DC population located in the lung and gut mucosa, characterised by expression of the integrin, CD103 (alphaE) [5, 6]. It is significant that this CD103 + subset expresses tight junction proteins enabling the cells to migrate across epithelia for antigen capture, transport and presentation. CD103 + DC may also exert an immunosuppressive function in the mucosa [6]. In the skin a specialised DC, Langerhans cells, form a contiguous network in the epidermis, where they serve a sentinal role by sampling antigens migrating through the stratum corneum and respond to signals produced by the surrounding keratinocytes [1]. A population of CD11b + interstitial DC, phenotypically and structurally distinct from Langerhans cells, has been described in the dermis.

(ii) *Subsets of human mucosal DC* Two major DC populations, myeloid- and plasmacytoid-DC have been detected and are present at low frequency (<1%) in normal peripheral blood. Plasmacytoid-DC reportedly derive from a lymphoid progenitor and are located principally in the blood and lymphoid organs. Myeloid-DC, originally detected in the bronchoalveolar lavage (BAL) based on expression of the Langerhans cell marker, CD1a, [7] have now been detected in the nasal and bronchial mucosa [8, 9]. The development of new DC markers has identified CD1a + DC as a subset of CD1c + DC, the major DC population present in both the airways and peripheral blood [4]. Plasmacytoid-DC, expressing CD123 (IL-3α receptor) and the specific marker, BDCA-2, are present in low frequency in the normal healthy lung and nasal mucosa [8, 10] but may be attracted to the airways following allergen challenge. There have been few studies of human gut dendritic cells [11], but the characteristics of Langerhans cells and interstitial dermal DC from normal individuals and atopic dermatitis patients have been reported recently [12].

(iii) *Maturation of DC* Dendritic cells lining the mucosa bear an immature phenotype [13] characterised by the capacity for avid antigen sampling but little or no capacity to activate naive T cells present in draining lymph nodes. This immature phenotype is reflected in low levels of expression of the principal costimulatory molecules, CD40, CD80 and CD86. The full antigen-presenting capacity of DC is only apparent following receipt of maturation signals from microbial products, hormones and cytokines, which upregulate expression of costimulatory molecules, licence pro-inflammatory cytokine and chemokine production, and direct migration of DC from the mucosa to the draining lymph nodes [1]. Holt and colleagues have postulated that following allergenic challenge, airway-transiting memory CD4 + T cells, with which immature antigen-bearing DC form clusters, deliver cognate signals, which initiate the maturation process [14]. This does not, however, necessarily result in an immunogenic response. In normal individuals, responses to innocuous substances such as allergens result in tolerance induction in the draining

lymph nodes, perhaps through lack of fully effective costimulatory interactions, impaired cytokine production and subsequent death of antigen-bearing DC [15]. In contrast, where the full panoply of costimulatory molecule interactions and cytokine production enhances the life span of antigen-bearing DC, the outcome is likely to be clonal expansion and differentiation of antigen-specific naive T cells.

Dendritic Cells Exert Multiple Controls on the T Cell Response

(i) *DC determine the nature of the T cell response* Dendritic cells have the ability to link innate and adaptive immunity owing to their unique capacity to interpret and convey signals relayed by the tissue environment [16]. DC are thus able to determine the nature of the T cell response. Myeloid- and plasmacytoid-DC were originally reported to induce Th1 responses, characterised by production of IFN-γ, and Th2 responses, characterised by production of IL-4, IL-5 and IL-13, respectively [17]. Although DC lineage undoubtedly influences T cell responses, this model has been modified over the past few years. The capacity of DC to determine the nature of the immune response is generally believed to be due to their functional plasticity [18]. This is dependent on features such as maturation state and the nature and strength of the antigenic and environmental stimuli encountered by the DC. Thus, depending on these factors, myeloid- and plasmacytoid-DC have been shown to induce Th1, Th2 and tolerogenic responses.

(ii) *DC imprint specific tissue homing potential on naive T cells* It has now been established that DC, as well as determining the polarity, also imprint homing capacity on naive T cells. The evidence for this is strongest in T cells homing to the gut and skin [19]. Myeloid-DC induce upregulation of chemokine receptors such as CCR9 and CCR10, which direct migration to the gut and skin, respectively. The regulation of T cell recruitment to the lung appears to exhibit greater complexity. Recent studies of segmental allergen challenge in asthmatic patients have identified multiple chemokine receptors associated with T cell recruitment to the lung [20]. Several studies have, however, reported increases in the expression of the Th2-associated receptor, CCR4 and decreased or unaltered Th1-associated receptors, following segmental challenge [21, 22].

Myeloid-DC Induce Allergic Inflammation

(i) *Myeloid-DC traffic to mucosal surfaces and induce allergic inflammation* Animal models have demonstrated that myeloid-DC are necessary to generate airway inflammation and hyperresponsiveness in response to allergen challenge,

and may also play a decisive role in sustaining allergic disease [23]. Segmental allergen challenge in the bronchi of asthmatic patients has demonstrated that myeloid-DC precursors rapidly (3–6 h) infiltrate the bronchial mucosa [24]. Myeloid-DC precursors upregulate the mucosal DC marker, CCR6, permitting recruitment to mucosal sites in skin and lung in response to CCL20, released by epithelial cells. Myeloid-DC in the skin of atopic dermatitis patients reportedly upregulate chemokines such as CCL17, which attract Th2 cells [12].

Human myeloid-DC were originally isolated from the bronchoalveolar lavage (BAL) fluid of normal healthy individuals [7]. However, the frequency of myeloid-DC in BAL is low. The development of methods based on magnetic bead capture protocols has permitted us to isolate and purify CD1a + and CD1c + DC from the inferior nasal turbinates of patients with rhinitis [9]. Both CD1a + and CD1c + DC from patients with non-atopic rhinitis induced a weak Th2-biased response in mixed lymphocyte reactions (MLR). The DC expressed low levels of MHC class II molecules and did not secrete IL-12, the prototype Th1 inducing pro-inflammatory cytokine, features which characteristically promote Th2 responses [25]. In contrast, patient matched peripheral blood myeloid-DC, which expressed much higher levels of MHC class II, induced Th1 responses. Exposure of RTDC to GM-CSF, which is elevated in the airways of asthmatic patients [26] enhanced the Th2 response. The results, which support animal studies [23] indicate that in the steady state mucosal DC may limit potential damage to the delicate epithelial lining by induction of weak Th2 responses to innocuous airborne antigens.

(ii) *Thymic stromal lymphopoietin (TSLP)* Elevated levels of the epithelial cytokine, TSLP, have been detected in the skin of patients with atopic dermatitis and the asthmatic lung [27, 28]. TSLP, which has been proposed as a central mediator of allergic inflammation, reportedly induced maturation of peripheral blood myeloid-DC, and conditioned DC to promote differentiation of Th2 cells [27]. Recently, the production of TSLP by skin explant cultures was shown to be stimulated by a combination of pro-inflammatory cytokines such as TNF-α and Th2 cytokines [29]. This combination of cytokines could potentially initiate and/or sustain production of TSLP in the asthmatic lung. Immunointervention strategies to neutralise the priming effect of TSLP in the airways could have the potential advantage of being specifically directed to Th2 responses.

(iii) *Plasmacytoid-DC* These are the principal source of type I interferon and this attribute endows plasmacytoid-DC with a critical role in the induction of antiviral and anti-tumour CD4 + and CD8 + T cell responses [4]. Our studies have demonstrated that plasmacytoid-DC can be purified from human lung-draining lymph nodes, and induced to secrete type I interferon and promote Th1 and Tc1 responses following exposure to CpG oligodeoxynucleotides (ODN),

a synthetic ligand for the Toll-like receptor 9 (TLR9) [30]. Plasmacytoid-DC, however, in common with myeloid-DC, exhibit functional plasticity, as evidenced by their capacity to evoke allergen-specific Th2 responses through highly efficient uptake of allergen/IgE complexes bound to the high-affinity IgE receptor (FcεRI) [31].

Several recent murine studies have indicated that plasmacytoid-DC have the potential to induce tolerance to allergen challenge in the airways [16]. In a seminal study, depletion of plasmacytoid-DC was shown to exacerbate inflammation in the lung, and adoptive transfer of plasmacytoid-DC before sensitisation prevented the establishment of disease [32]. Moreover, CpG ODN have been shown to abrogate murine Th2 responses and airway inflammation, and to greatly reduce allergenicity compared to the effect of allergen alone when administered as a CpG-allergen conjugate to allergic patients [33]. The results demonstrate the potential of plasmacytoid-DC as a novel therapeutic target in allergic airway disease.

(iv) *The high-affinity FcεRI* The production of and response to IgE is central to the immunopathology of allergic disease. The high-affinity IgE receptor (FcεRI) is expressed by mast cells, basophils, macrophages and DC in the allergic airway [34]. Importantly, successful therapy for seasonal allergic rhinitis with a humanised anti-IgE antibody has been associated with decreased numbers and reduced expression of FcεRI bearing myeloid-DC in nasal biopsies from patients studied [35]. Functional studies demonstrated that peripheral blood myeloid-DC focused allergen/IgE complexes for 100–1,000-fold more efficient presentation to T cells compared to allergen alone [36]. These results imply that ligation of the FcεRI on DC results in a pro-inflammatory outcome. The nature of the T cell response may, however, depend on the lineage of the APC expressing the FcεRI.

The first evidence of this was obtained by Bieber and colleagues who demonstrated that ligation of the FcεRI on human monocytes induced production of the anti-inflammatory molecule, IL-10 and the tryptophan catabolising and immunoregulatory enzyme, indoleamine 2,3-dioxygenase (IDO) [37]. Upregulation of IDO in the murine RT was shown to suppress airway inflammation [38], and a strong correlation has been observed between serum IDO and IL-10 levels in asymptomatic atopic individuals during seasonal allergic exposure [39]. Ligation of the FcεRI has also been shown to inhibit type I interferon production and to induce production of IL-10 by peripheral blood plasmacytoid-DC [31]. These results indicate that ligation of the FcεRI may have tolerogenic as well as inflammatory potential. The functional role of the FcεRI on RTDC remains unclear, however, partly reflecting the absence of the receptor on mouse APC. Functional studies on human airway APC and the development of a transgenic mouse expressing the human FcεRI on airway APC will help to clarify the role of the FcεRI in allergic disease ([40] and D. Dombrowicz, 2007).

Dendritic Cells and the Induction of T Cells With Regulatory Function

(i) *CD4 + T cells with regulatory function (Treg)* Animal models of tolerance induction in the airways have indicated impaired proliferation of draining lymph node-derived effector T cells, combined with weak Th2 responses and lack of a sustained or augmented response on secondary stimulation [41]. The impaired Th2 response was originally ascribed to the induction of T cell anergy [42]. Evidence, however, has gradually accumulated from animal models and studies of non-atopic individuals of an active process of immune suppression, driven by allergen-specific T cells with regulatory functions [43–46]. Naturally occurring CD4 + CD25 + Treg and CD4 + T cells with IL-10-dependent regulatory function (IL-10Treg), derived in response to drugs or stimulation by DC, have been described and are postulated to regulate allergen-driven inflammation [41, 47]. For example, the frequency of allergen-specific IL-10Treg and CD4 + CD25 + Treg is reportedly reduced in the peripheral blood of allergic and allergic asthmatic individuals, respectively, compared to non-atopic controls [45, 48]. See chapter by Dr. C. Hawrylowicz.

(ii) *IL-10Treg are dependent on interaction with DC* Naturally occurring and IL-10Treg are dependent on interactions with DC. Human IL-10Treg were originally induced and expanded in vitro by several rounds of stimulation of naive CD4 + T cells with allogeneic, immature monocyte-derived DC [49]. IL-10Treg have also been induced in CD4 + and CD8 + T cell populations by previously exposing immature DC to IL-10 [50]. The latter cell populations have the potential therapeutic advantage that their regulatory function was observed to be antigen- and peptide-specific, and therefore did not inhibit third-party responses. Peripheral blood plasmacytoid-DC were also shown to induce IL-10Treg in both CD4 + and CD8 + T cell populations [51, 52]. Yong-Jun Liu and colleagues have suggested that induction of IL-10Treg was dependent on differential expression of inhibitory costimulatory molecules, for example, OX40L and ICOSL, by myeloid and plasmacytoid-DC [51].

Animal models have shown that tolerance to allergen challenge in the murine airways is dependent on the capacity of pulmonary DC to promote the induction of IL-10Treg [41]. Neutralisation of IL-10 production by DC or the adoptive transfer of DC in which the IL-10 gene had been deleted demonstrated that production of IL-10 by DC was essential to the induction of IL-10Treg [41]. Interactions between DC and Treg are reportedly bidirectional as IL-10Treg can downregulate activation of murine, airway mucosal DC by helper T cells [53].

The current evidence suggests that in normal individuals, a balance between inflammatory Th2 responses and IL-10Treg is maintained by the action of environmental signals such as CpG, prostacyclins [54] and IL-10 on DC (Fig. 1). Distortion of this equilibrium, due to excessive production by respiratory cells of pro-inflammatory cytokines such as TSLP, GM-CSF and IL-6 [55] may induce and/or amplify airway inflammation in individuals susceptible to allergic disease.

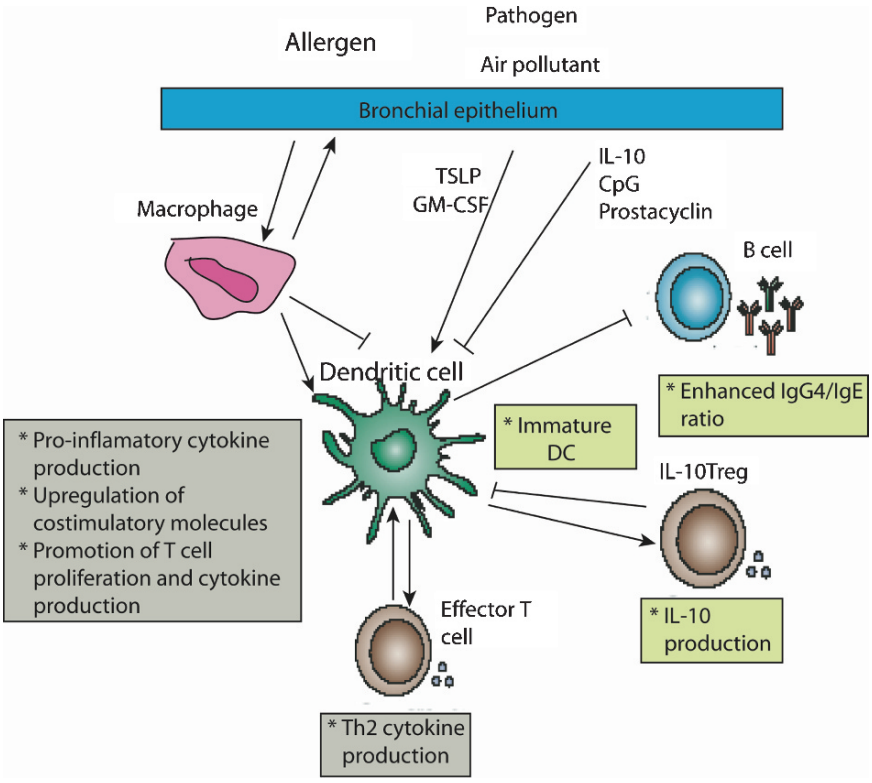

Fig. 1 In susceptible individuals, immature mucosal myeloid-DC acquire and process allergenic peptides, and receive maturation signals from microbial products and the bronchial epithelium. Dendritic cells then migrate to draining lymph nodes and induce differentiation of allergen-specific, naive CD4 + T cells to effector Th2 cells. Th2 cells enter the mucosa and transmit activating signals to myeloid-DC precursors transiting from the peripheral blood. The inflammatory process leading to IgE production is amplified by interaction among DC, AM and other respiratory cells. In healthy non-atopic individuals, in contrast, dendritic cells induce a tolerogenic response to allergen. This is achieved partly through homeostatic effects of AM and the bronchial epithelium on DC. Naturally occurring and IL-10Treg inhibit maturation of DC and effector function of Th2 cells. IL-10 promotes the secretion of anti-inflammatory IgG4 antibodies [45]. A dynamic multidirectional relationship between the cells of the respiratory mucosa thus determines the nature of the allergenic response

Monocytes/Macrophages

Macrophages are the most abundant immune cell in the airways and are derived from a bone marrow progenitor cell, which differentiates into cells of the monocyte/macrophage and granulocyte lineage [56]. Development of committed cells of the monocyte/macrophage lineage is regulated by the growth factors IL-3, GM-CSF and macrophage colony-stimulating factor (M-CSF). In the lung monocytes differentiate

into tissue macrophages, of which several distinct populations, including interstitial and alveolar macrophages (AM) have been described. AM share the characteristics and may derive from a CD14 + CD16 + peripheral blood monocyte subset [57]. AM are located in the air–tissue interface and are proposed to play a key role in maintaining the immune homeostasis of the lung through their potent phagocytic and microbicidal activity [58, 59]. In addition, depletion of AM in the murine lung has demonstrated that AM downregulate acquired immune responses [60]. AM depletion has been shown to enhance antigen presentation by dendritic cells [61]. These results from animal models are supported by studies of human AM demonstrating that AM from normal healthy individuals are weak antigen-presenting cells for T cell activation [62, 63].

Alveolar Macrophages and the Allergic Response

(i) *Toll-like receptors* Responses to environmental stimuli are mediated in large part by pattern recognition receptors such as Toll-like receptors (TLR), which transmit signals critical in disease processes [64]. TLR are type I transmembrane proteins expressed by haematopoieic and some stromal cells, which recognise conserved microbial structures and certain host molecules. Animal models indicate that efficient allergic sensitisation in the airways is dependent on synergy between allergen and low levels of microbial products such as the TLR4 ligand and bacterial lipopolysaccharide (LPS) [65]. Myeloid cells such as AM and DC, which express TLR4, are proposed to initiate responses to inhaled LPS and other microbial products resulting in enhanced leukocyte recruitment to the airways [66]. Sabroe and colleagues have postulated that ligation of TLR on AM may also sustain chronic inflammation in the airways through their capacity to secrete inflammatory cytokines and thus activate airway epithelium and smooth muscle [67].

(ii) *Inflammatory mediators* The capacity of alveolar macrophages to regulate T cell activation is mediated in large part by the synthesis and secretion of soluble mediators [56]. Although the capacity to synthesise these mediators is not unique to macrophages, their ability to synthesise many products in extremely large quantities is likely to exacerbate the inflammatory state. Soluble mediators include reactive oxygen metabolites and nitric oxide, lipids such as prostaglandins and leukotrienes, and pro-inflammatory cytokines. An important trigger for the production of these substances is ligation of the low-affinity IgE receptor, CD23, by allergen/IgE complexes.

(iii) *GM-CSF can activate AM for promotion of Th2 responses* A series of reports demonstrate that AM from allergic asthmatic patients lose their immunosuppressive phenotype and demonstrate enhanced capacity to activate autologous T cell proliferation and cytokine secretion [26, 62, 68]. Rodent studies have implicated GM-CSF in reversing the immunosuppressive phenotype of AM [69, 70]. We have recently demonstrated that the antigen-presenting function

of AM and monocytes from allergic asthmatic patients is upregulated on exposure to GM-CSF by a mechanism involving the release of leukotrienes [71]. AM and monocytes pretreated with GM-CSF significantly enhanced proliferation of autologous allergen-specific CD4 + T cells and production of Th2 cytokines. AM have been recently described as the forgotten cell in allergic inflammation [72]. New evidence of their potent pro-inflammatory effects in the airways may provide an impetus for further research on their interactions with other respiratory cells and the effect of novel immunotherapeutic strategies on their function.

Conclusion

A new conceptual framework of tolerance and immunity in allergy disease is emerging in which interactions between antigen-presenting cells and T cells are regulated by environmental stimuli at mucosal sites (Fig. 1). In the steady state immature dendritic cells receive and transmit tolerising signals, which tend to induce weak T cell responses. Concomitantly, immature myeloid- and plasmacytoid-DC induce and expand IL-10Treg, which in turn suppress allergic inflammation. Plasmacytoid-DC and alveolar macrophages also play a role by limiting activation of myeloid-DC. In allergen-driven inflammation the constraints on maturation of mucosal DC are released, and dendritic cells and alveolar macrophages receive activating signals leading to effector Th2 responses. In susceptible individuals the balance between Treg and Th2 responses may also be tilted owing to a lower frequency of Treg cells. Novel methods of immunointervention could be targeted to molecules identified as transmitting environmental signals, thus modifying the biology of antigen-presenting cells in chronic allergic disease.

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Function of Dendritic Cell Subsets in Allergic Disease

Bart N. Lambrecht and Hamida Hammad

Introduction

Allergic diseases, broadly divided clinically into allergic asthma, allergic rhinitis, and atopic dermatitis, are a significant cause of morbidity and mortality. Allergen-specific T helper type 2 (Th2) cells produce key cytokines like IL-4, IL-5, and IL-13 that regulate the synthesis of allergen-specific IgE and control tissue eosinophilic inflammation and remodeling of allergically inflamed tissues. Dendritic cells (DCs) were originally described by their capacity to efficiently process and present antigens and to prime naïve T cells [1]. It is increasingly clear that dendritic cells (DCs) are essential for inducing activation and differentiation of not only naïve but also effector CD4+ T and CD8+ T cells in response to allergens, and it has been well established that these cells play a pivotal role in the initiation and maintenance phase of airway inflammation in asthma and allergic rhinitis [2]. In this review, we highlight the recent discoveries in DC biology with special emphasis on mouse models of allergic diseases. Where possible, the applicability to the human situation and the therapeutic potential of novel findings will be discussed.

General Function of Dendritic Cells

Dendritic Cell Terminology

Over the last 3 decades, multiple DC subtypes have been defined, differing in phenotype, localization, and immune function [3]. Myeloid DCs, Langerhans cells (LCs), as well as natural type I interferon-producing cells (IPCs, also called plasmacytoid

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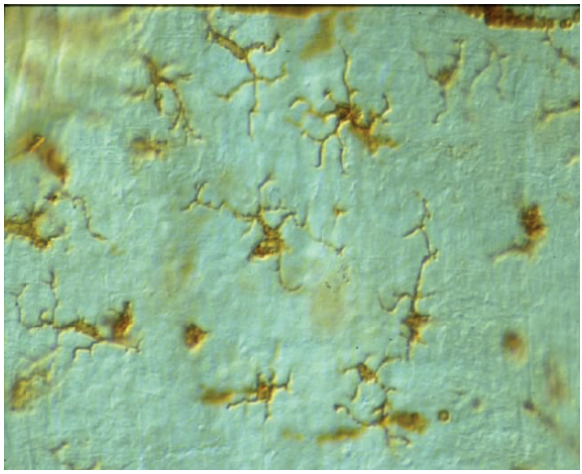

Fig. 1 MHCII positive mucosal dendritic cell network visualized by MHCII staining on a murine tracheal wholemount. Trachea was taken from a naïve unimmunized mouse

DCs [pDCs]) are part of the hematopoietic system and have a relatively short half-life in tissues. To maintain DC numbers in the tissues, there is a continuous renewal of DCs from hematopoietic precursors residing in the bone marrow or within the skin (for LCs in steady-state conditions [4]). A universal feature of DCs in tissues is their typical morphology with long dendrite-like extensions (hence their name) that can be beautifully demonstrated by staining for major histocompatibility complex (MHC) class II (see Fig. 1). Myeloid DCs in humans express markers shared with monocytes/macrophages such as CD33, CD4, and CD11c, whereas in mice they typically express CD11c and CD11b. In humans, pDCs were described in the bloodstream, lungs, and lymph nodes as lineage^{neg} CD11c^{lo} CD123+ BDCA2+ cells [3]. In the mouse, pDCs express specific markers (120G8, PDCA-1) as well as cell markers shared with myeloid DCs (MHCI and II, CD11c) and also with granulocytes (Gr1) and B cells (B220) [3]. Langerhans cells express CD1a, langerin and intracellularly demonstrate so-called Birbeck granules, tennis racket-shaped organelles.

Dendritic cells originate in the bone marrow from a CD34+ precursor and circulate in the bloodstream as a monocyte-like precursor before entering peripheral tissues [5, 6]. The exact nature of the precursor DC cell type is currently unknown (see Fig. 2), and could vary for myeloid versus plasmacytoid DC, inflammation versus steady state, or for lymphoid organ versus peripheral tissues [7, 8].

Antigen Uptake and Presentation

There are various ways by which antigen-presenting cells can acquire foreign antigen. A first mechanism is via receptor-mediated endocytosis. Immature DCs express a plethora of specialized cell receptors for patterns associated with foreign antigens, such as the C-type lectin carbohydrate receptors (langerin, DC-SIGN,

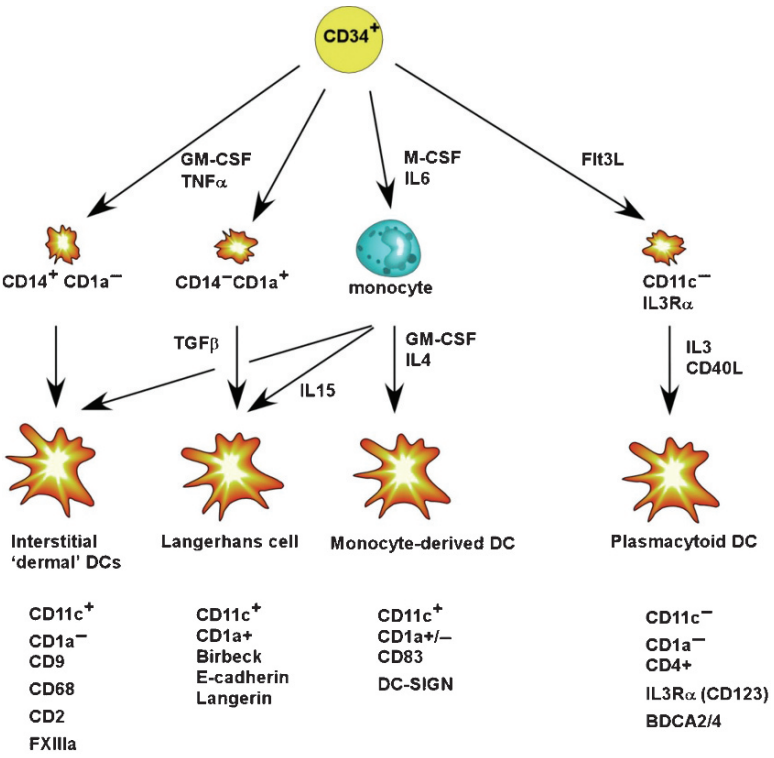

Fig. 2 Different origins and fate of DC subsets. All DCs originate from a CD34+ precursor in the bone marrow. These cells then further differentiate under the influence of various cytokines into Langerhans cells (LCs) of the skin, interstitial DCs of tissues, monocyte-derived DCs, or plasmacytoid DCs, each expressing specific markers. Certain cytokines like IL-6 and M-CSF inhibit DC development when precursors are continuously exposed to them

dectin, BDCA-2, macrophage mannose receptor and the unique carbohydrate receptor DEC-205) [9]. Lectin-receptor mediated uptake by DCs results in a ~100-fold more efficient presentation to T cells, as compared to antigens internalized via fluid phase [10]. Pollen starch granules were shown to bind to C-type lectin receptors on AMs and DCs, although internalization occurred only in macrophages [11]. Also, Pestel demonstrated that Der p 1 uptake into cultured DCs involves mannose receptor-mediated endocytosis, and that process is more efficient in DCs obtained from allergic donors [12]. In allergic individuals, DCs are furthermore loaded with allergen-specific IgE binding to the high-affinity IgE receptor (FcεRI), thus leading to efficient receptor-mediated endocytosis of the allergen [13].

A second mechanism of antigen uptake is constitutive macropinocytosis that involves the actin skeleton-driven engulfment of large amounts of fluid and solutes (~1 cell volume/h) by the ruffling membrane of the DC followed by concentration of soluble antigen in the endocytic compartment [14].

Third, immature LCs, cultured DCs, plasmacytoid DCs, and macrophages have been shown to phagocytose particulate antigens such as latex beads and even whole bacteria, as well as apoptotic cells, and this could be the dominant mechanism of uptake of particulate allergens [15, 16].

The extracellular antigens that are taken up by any of these mechanisms accumulate in the endocytic compartment, where they are loaded on newly synthesized and recycling MHC class II molecules but may also be transported into the cytosol, where they become accessible to the class I antigen presentation pathway, a process called cross-presentation. Recently, it has become evident that the mechanism of antigen uptake (receptor-mediated endocytosis verus pinocytosis) might target antigen specifically to either the cross-presentation pathway for CD8 cells or to the CD4 presentation pathway.

Dendritic Cell Activation

Dendritic cells originate in the bone marrow and circulate in the bloodstream as a monocyte-like precursor before entering peripheral tissues [5, 6]. Dendritic cell migration is a tightly regulated process in which many chemokines and other factors are involved (see Fig. 3). Myeloid DCs are attracted to peripheral tissues by a specific set of chemokines such as MIP3 (CCL20), MCP-1, and epithelial

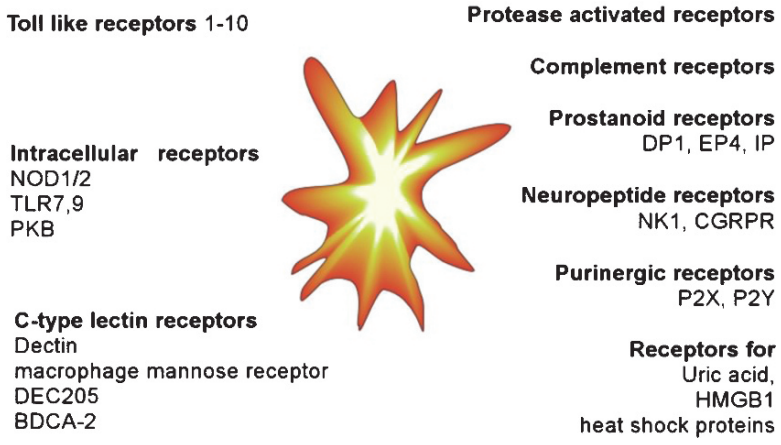

Fig. 3 Expression of "danger" receptors by dendritic cells. Dendritic cells express the ancient receptors of the innate immune system also expressed by macrophages, such as the Toll-like receptors (TLRs) and C-type lectin receptors. These receptors react to foreign pathogen-associated molecular patterns (PAMPs). In addition, DCs express numerous receptors for inflammatory mediators and necrotic cell debris, the so-called damage-associated molecular patterns (DAMPs). The exact receptors for uric acid, high-mobility group box 1 (HMGB1) protein, and heat shock proteins are not yet known

defensins acting on CCR2 and CCR6 [6, 17–19]. Plasmacytoid DCs respond preferentially to SDF1 (CXCL12) and CXCL9–11 and the newly described chemerin, a ligand for ChemR23 [20]. Once DCs extravasate, they form a network in the upper layers of the epithelium and lamina propria of the airways (see Fig. 1), gut, and skin. Here, DCs are said to be in an immature state, specialized for internalizing foreign antigens but not yet able to activate naïve T cells [21, 22]. The DC network serves a patrolling function, continuously scanning the environment for foreign antigens. The dendritic cell is endowed with numerous ancient receptors for foreign antigenic signature molecules such as bacterial cell walls, viral and bacterial DNA, and foreign sugar molecules (see Fig. 3). These so-called pathogen-associated molecular patterns (PAMPs) are recognized by Toll-like receptors (TLR1–11) and C-type lectin receptors, which are abundantly expressed on the surface of DCs [9]. The expression of various TLRs varies between DC subsets, particularly in human DCs. In human and mouse, pDCs preferentially express the TLR7 and TLR9. In contrast, in vitro generated conventional monocyte-derived DCs or ex vivo isolated mDCs express all TLRs except TLR9.

In addition to the direct molecular recognition of foreign antigenic structures, exposure to foreign antigens or necrotic cell death leads to tissue damage and this by itself can activate the DC system (Fig. 3). DCs express a plethora of receptors for these so-called damage-associated molecular patterns (DAMPs), including high-mobility group box 1 (HMGB1) protein, heat shock proteins, uric acid, adenosine triphosphate (ATP), complement cascade fragments, neuropeptides, prostaglandins, etc. Many of these compounds not only activate the already residing DCs but also attract new waves of cells to the periphery [23–25]. DC activation and maturation in the periphery can occur directly by ligation of DAMP or PAMP receptors and can occur indirectly through activation of the same receptors on the surrounding structural cells such as keratinocytes, epithelial cells, or fibroblasts [26].

Dendritic Cell Migration to the Draining Lymph Nodes

The recognition of danger (PAMPs or DAMPs) by peripheral dendritic cells dramatically alters the migration behavior of DCs and thus induces the surface expression of CCR7 on peripheral DCs (Fig. 4) [6]. The ligands for CCR7 are secondary lymphoid chemokine (SLC, now known as CCL21) and MIP3 (CCL19), which are expressed at the luminal side of afferent lymph vessels and by the T cell area of draining lymph nodes [27]. Another factor attracting DCs to the lymph node is the lipid mediator sphingosine-1-P. Blocking the S1P type receptor dramatically reduces the migration of lung DCs to the mediastinal LNs [28]. The responsiveness of CCR7 to CCL19 and CCL21 and the consequent lymph node migration of DCs are controlled by lipid mediators such as the leukotrienes and prostaglandins [29, 30]. In contrast to skin DCs, it was recently shown that lung DC migration is less dependent on the export of leukotriene B4 by the MDR [31]. For emigration

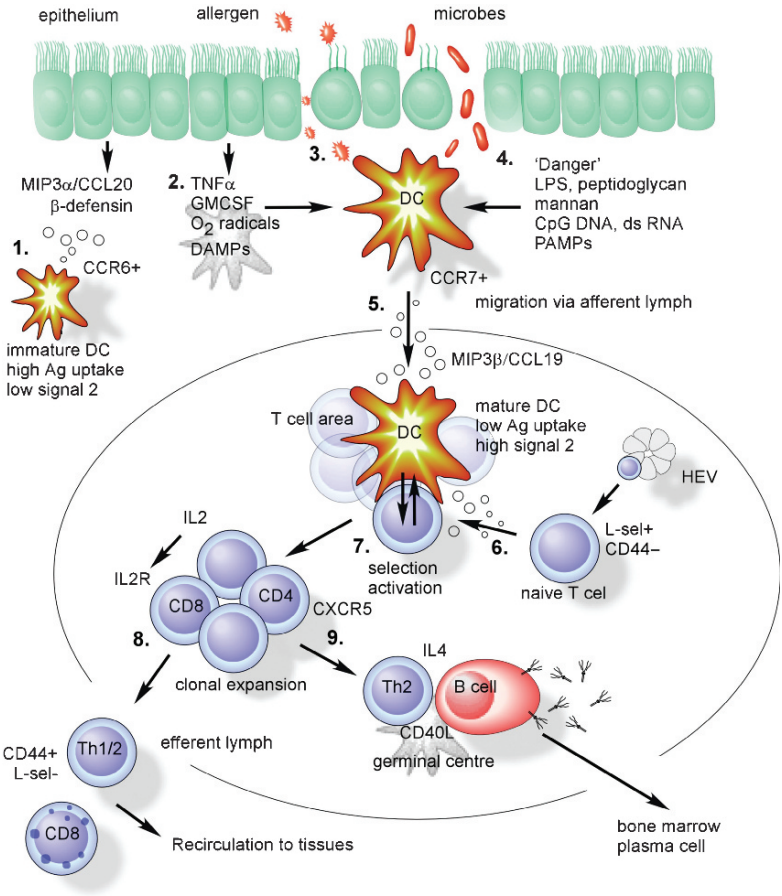

Fig. 4 Induction of the primary immune response by DCs. (1) Under baseline conditions and upon exposure to foreign antigens, epithelia produce macrophage inflammatory protein (MIP)3α (CCL20) and β-defensin to attract CCR6+ immature DCs from the bloodstream. (2) Resident cell types produce inflammatory mediators and growth factors that attract and activate the recently recruited DC. (3) DCs capture allergens and other foreign antigens such as bacteria and viruses. (4) DCs can discriminate between "dangerous" antigens, and non-pathogenic antigens such as self antigens and probably most allergens, by recognizing certain viral and bacterial patterns. (5) The recognition of infection and tissue damage upregulates the CCR7 and CXCR4 and DCs migrate to the T cell area of draining lymph nodes where the ligand MIP3β and SDF-1 are constitutively expressed. During this migration, DCs loose the capacity to take up antigen, but become strong stimulators of naïve T cells by their strong expression of costimulatory molecules (signal 2). (6) In the T cell area, DCs produce chemokines to attract naïve T cells that continuously leave the bloodstream via the high endothelial venules (HEV). (7) Naïve T cells are first arrested and then selected for antigen specificity. The recognition of the correct peptide–MHC induces the activation of naïve T cell, which will lead to further terminal differentiation of DC function. (8) The activation of T cells leads to autocrine production of IL-2 and to clonal expansion of Ag-specific CD4+ and CD8+ T cells. These cells differentiate into effector cells that leave the lymph node via the efferent lymphatics. These effector cells are poised to migrate to peripheral tissues, especially to inflamed areas. (9) Upon contact with DCs, some Ag-specific CD4+ T cells upregulate CXCR5 receptor and migrate to the B cell follicles of the draining lymph node. Here, they further interact with germinal center DCs to induce CD40L-dependent B cell immunoglobulin switching and affinity maturation (germinal centre reaction). Most high-affinity B cells go to the bone marrow to become Ig-producing plasma cells

of DCs from the skin, the CCR8 receptor for the chemokine CCL-1 (also known as I-309 in humans and TCA-3 in mice) acts in concert with CCR7 [32]. Whether this is also true for lung DC migration remains to be shown.

T Cell Activation by Dendritic Cells

By upregulating the lymph node homing chemokine receptors, DCs that have seen foreign antigen thus direct their interest to the regional draining LN T cell area where they interact with recirculating T cells and B cells [33]. Dendritic cells that have arrived in the lymph node undergo short-lived interactions with T cells in the paracortical region and during this initial antigen-independent event, individual T cells are scanned for antigen specificity. When antigen is being recognized, there is formation of a more long-term immunological synapse, leading to full-blown T cell activation, after which the T cell detaches, divides, and differentiates into an effector and possibly memory T cell. Dendritic cells also transport antigen without degrading it and thus offer intact protein to B cells at the interface between paracortex and B cell follicle [34, 35]. Dendritic cells that have reached the T cell area have lost the capacity to take up antigen, and now express a plethora of cell adhesion and surface molecules interacting with T cells, not previously expressed on peripheral-based DCs. This phenotype is called “mature dendritic cell” implying that functionally these cells are now fully adapted to induce effector responses from naïve resting T cells. DCs express the antigen on MHC molecules and provide so-called costimulatory molecules (CD80/CD86 family, TNF/TNFR family) together with cytokines to optimally expand and differentiate T cells for the particular job that needs to be done to clear the foreign antigen. Initially, T cells are stimulated in the draining lymph node, but after a few cell divisions they acquire effector potential [36], start expressing chemokine receptors for inflammatory chemokines expressed at sites of pathogen entry, and lose the expression of CD69, thus rendering them insensitive to the lymph node retention signal S1P [37].

Th Polarization by Dendritic Cells

Dendritic cells (DCs) are crucial in regulating the immune response by bridging innate and adaptive immunity. Signals from the type of antigen and the response of the innate immune system to it are translated by DCs into a signal that can be read by the cells of the adaptive immune response leading to an optimal response for a particular insult (see Fig. 5). Together, these signals consist of provision of a particular density of peptide–MHC, the expression of costimulatory or Th polarizing cell surface molecules, and the expression of soluble cytokines and chemokines that polarize T cells or enhance their survival. At the same time, DCs also control the function and expansion of regulatory T (Treg) cells that tightly control overzealous inflammatory T cell responses.

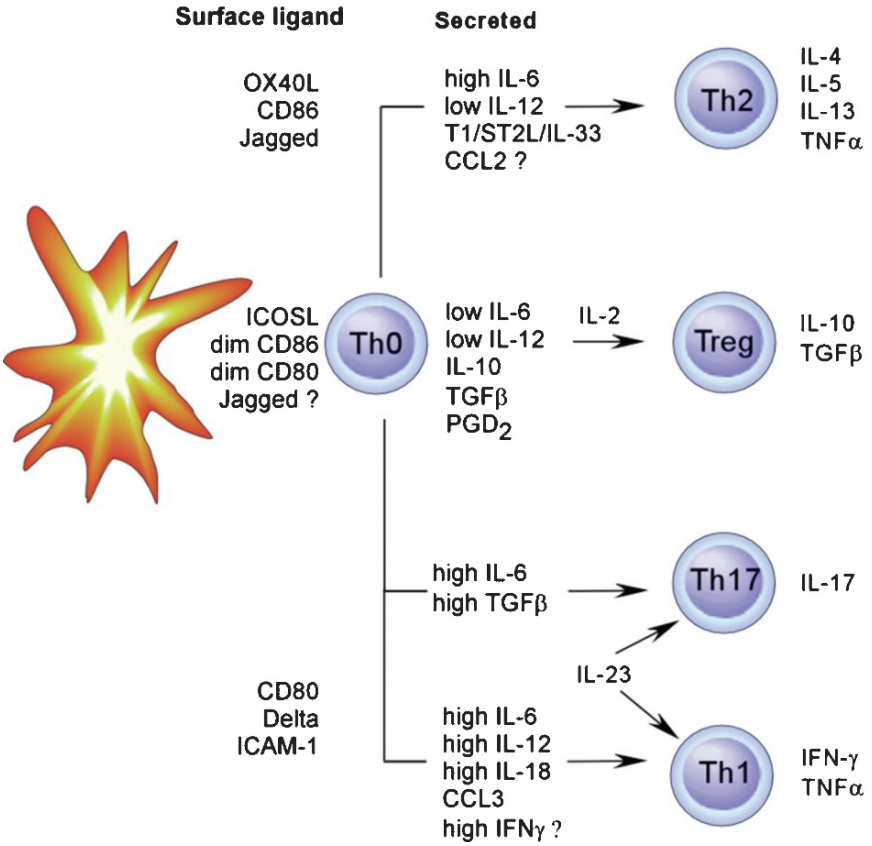

Fig. 5 T helper cell polarization by DCs. Depending on the type of antigen, the dose, the genetic background, and the tissue environment where antigen is first introduced, DCs can induce various types of Th response, tailor-made to protect the host, while avoiding autoimmunity. Often the response is extremely well balanced, to avoid tissue damage, while allowing clearance of the threat. The various cytokines and costimulatory molecules that favor a particular direction are indicated. See text for more explanation

Dendritic Cells in Allergic Asthma

Lung Dendritic Cell Subsets

It has long been established that the various lung compartments (conducting airways, lung parenchyma, alveolar compartment, pleura) contain numerous DCs, the precise lineage or origin of which have been poorly defined. Recently however, many groups have refined the ways in which lung DCs should be studied, both in mouse [38–41] and man [42, 43]. It is clear that different DC subsets can be found

in the lung, each with functional specialization. In the mouse, all of these express the integrin CD11c and subsets are further defined based on the expression of the myeloid marker CD11b, as well as anatomical location in the lung. The trachea and large conducting airways have a well-developed network of intraepithelial DCs, even in steady-state conditions. These cells in some way resemble skin Langerhans' cells, and have been shown to express langerin and CD103 while lacking expression of CD11b [40, 44]. In the submucosa of the conducting airways, CD103−CD11b+CD11c+ myeloid DCs can be found, particularly under conditions of inflammation, and these cells are particularly suited for priming and restimulating effector CD4 cells in the lung [44, 45]. The lung interstitium also contains CD11b+ and CD11b− DCs that access the alveolar lumen and migrate to the MLN [38, 41, 46]. In the nearby alveolar lumen, $CD11c^{hi}$ alveolar macrophages control the function of these interstitial DCs. Plasmacytoid DCs are CD11b−$CD11c^{int}$ cells expressing SiglecH and bone marrow stromal Ag-1 (recognized by the moAbs mPDCA-1 or 120G8), and are predominantly found in the lung interstitium [47].

How, where, and by which DC subset inhaled antigen is sampled from the airway lumen has been a matter of debate. Jahnsen et al. [48] demonstrated that, analogous to that reported in the gut, a subset of rat airway intraepithelial DC extend their processes into the airway lumen, providing a mechanism for continuous immune surveillance of the airway luminal surface in the absence of "danger" signals. In the mouse, CD103+CD11b− intraepithelial DCs express the tight junction proteins claudin-1, claudin-7, and zonula-2, allowing the sampling of airway luminal contents while keeping the epithelial barrier function intact [40]. This subset is also found in the alveolar septa and DCs lining the alveolar wall can take up inhaled harmless ovalbumin or bacterial anthrax spores by forming intraalveolar extensions and migrate to the mediastinal LN in a CCR7-dependent way [41, 44, 46]. It is still a matter of debate however whether the uptake and transport of inhaled antigen occurs exclusively by alveolar wall DCs, by intraepithelial DCs lining the large conducting airways, or by both [38, 41]. Another controversial issue is the location and extent by which plasmacytoid DCs take up inhaled antigen. Two reports describe that within 24–48 h following exposure of inhaled fluorescently labeled Ag almost 50–60% of pDCs are antigen positive [47, 49]. It remains to be demonstrated if pDCs take up antigen in the periphery of the lung and subsequently migrate, whether they get their antigen from another migratory DC [50], or whether they take up free afferent lymph while resident in the LN.

Outcome of Antigen Inhalation Depends on the Functional State of Myeloid and Plasmacytoid DCs

The usual outcome of inhalation of harmless protein antigen in the lungs is immunological tolerance. In true sense, this means that when the antigen is subsequently given to mice in an adjuvant setting (e.g. in combination with the Th2 adjuvant alum) it no longer induces an immunological response that leads to effector cells

causing inflammation [47, 51]. Inhalational tolerance is mediated in part by deletion of Ag-reactive T cells as well as induction and/or expansion of regulatory T cells in the mediastinal nodes [50–53]. Induction of tolerance to inhaled antigen is a function of lung DC subsets that migrate from the lung in a CCR7-dependent way [50].

Conventional lung DCs (either CD11b+ or CD11b−) are necessary for tolerance induction [50] but are also responsible for inducing Th2 sensitization, providing there is some form of activation (either LPS or TNFα) [54] leading to functional DC differentiation and their capacity to prime Th2 effector cells (see Fig. 6). In further support, Th2 sensitization can be induced by adoptive intratracheal transfer of GM-CSF-cultured bone marrow DCs, most closely resembling mature monocyte-derived CD11b+ DCs, but not by Flt3L-cultured bone marrow derived DCs that more resemble the immature steady-state DCs resident in the lymph nodes and spleen [47]. As activation of lung DCs is the common event leading to Th2 sensitization, it is likely that under homeostatic conditions, the degree of DC maturation is therefore constantly kept in check. One such pathway of tonic DC suppression seems to involve COX-2 derived prostaglandins or their metabolites, most likely derived from nearby alveolar macrophages [55]. Chimeric mice in which the PGD_2 receptor DP1 was selectively deleted on hematopoietic cells demonstrated spontaneous maturation of lung mDCs and subsequently, response to harmless antigen was greatly enhanced, suggesting tonic inhibition of DC function by PGD_2 in the lung [56]. When exposed to selective PGD_2 agonists, myeloid DCs induced the formation of $Foxp3^+$ Ag-specific Tregs that subsequently suppressed airway inflammation. A similar mechanism on DCs was found for stable PGI_2 analogues [57, 58].

When pDCs are depleted from the lungs, inhaled tolerance is abolished, and consequently pDC/mDC balance in the lung is tightly regulated, among others by the cytokine osteopontin as well as complement C5a [47, 49, 59, 60]. How exactly pDC depletion leads to sensitization is still unsolved, but in vitro and in vivo data suggest that pDCs directly suppress the potential of mDCs to generate effector T cells [47, 60]. Plasmacytoid DCs can also stimulate the formation of Treg cells, possibly in an ICOS-L-dependent way [47, 61]. Tregs expressing GITR could also induce the production of the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase through reverse signalling in pDCs [62]. In mice depleted of pDCs, there was endogenous release of extracellular ATP responsible for inducing mDC maturation and Th2 skewing potential. Th2 sensitization to inhaled ovalbumin (OVA) was abolished when ATP signalling was blocked using the broad spectrum P2X and P2Y receptor antagonist suramin. On the contrary, a non-degradable form of ATP was able to break inhalation tolerance to OVA [63]. How exactly purinergic receptor triggering on DCs promotes Th2 development is unclear at present, but could involve the formation of the inflammasome, a multiprotein complex that leads to activation of caspase 1 and processing and release of IL-1, IL-18, and possibly IL-33. The conditions regulating ATP release in the lungs will have to be studied more carefully before we can conclude how important the pathway of purinergic signalling is in sensitization to more common allergens, like house dust mite.

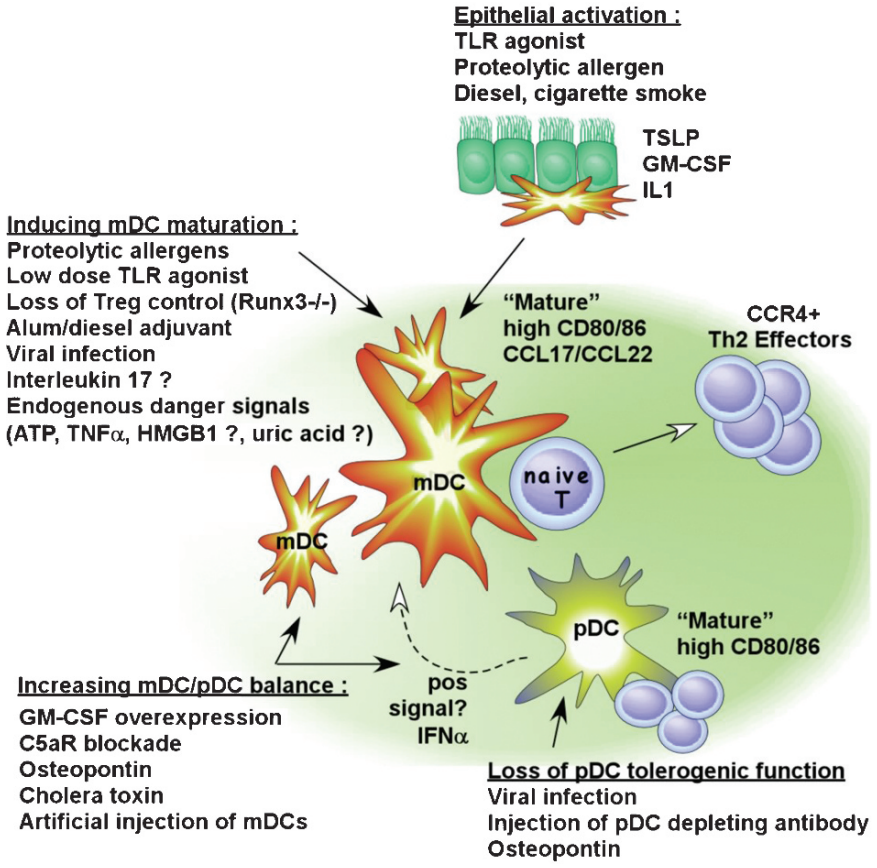

Fig. 6 Model of dendritic cell function during Th2 sensitization. Several known risk factors for atopy have been shown to interfere with DC function in the airways. Also, several experimental models have been developed that have seen sensitization to occur even after inhalation of harmless antigens to the lung, providing there is some form of DC activation. In these models, respiratory tolerance is broken. Some models have induced a shift in the pDC/mDC balance, and consequently mDCs induce priming because they are no longer suppressed by adequate numbers of pDCs. Activated mDCs also produce chemokines like CCL17 or CCL22 to further attract Th2 cells into the response. Some adjuvants induce proper activation of mDCs (yet not sufficient to induce IL-12) so that they now induce effector Th2 cells rather than regulatory T cells. Some stimuli, like concomitant viral infection, might have an additional effect by inducing maturation of pDCs and their production of IFNα. This is a known maturation stimulus for mDCs and in this way, these cells might even contribute to sensitization upon viral infections. Activation of epithelial cells by proteolytic allergens, virus infection, Toll-like receptor (TLR) ligands, or air pollutants is an indirect way of activating and polarizing the DC network, through release of thymic stromal lymphopoietin (TSLP) or granulocyte-macrophage colony stimulating factor (GM-CSF) or interleukin 1 (IL-1). The precise source and role of endogenous danger signals like ATP, tumor necrosis factor α (TNFα), high-mobility group box 1 (HMGB1), or uric acid is currently being investigated

Direct or Indirect Mechanisms of Th2 Sensitization to Inhaled Antigen

Induction of tolerance or immunity to inhaled antigen by DCs is tightly controlled by signals from alveolar macrophages, Tregs, NKT cells, complement activation, nervous system interactions, and epithelial activation. When studying the literature on particular substances that can break inhalation tolerance and induce Th2 priming, one needs to wonder therefore whether a stimulus acts directly on mDCs or pDCs or whether its effects are mediated indirectly through modification of any of the above interactions. For systemically administered TLR agonists, like endotoxin, the activation of DCs occurs mainly through direct recognition by TLR4 expressed on the DC, but in epithelia, the response could be clearly different [64]. As an example, low dose endotoxin was able to break inhalation tolerance to inhaled OVA by inducing mDC maturation [54]. These effects could be mediated directly via TLR and Myd88-depedent pathways in DCs, but could also be mediated via TLRs on bronchial epithelial cells [65]. Bronchial epithelial cells could produce chemokines as well as crucial growth and differentiation factors that subsequently attract, activate, and polarize lung DCs to prime Th2 responses. In this regard, the epithelial cytokines TSLP and GM-CSF might be crucial, as their overexpression in the lungs breaks inhalational tolerance [66, 67]. On the contrary, neutralization of these cytokines during priming regimens eliminates much of the adjuvant effects of diesel exhaust particles (DEPs) [68, 69] or pro-allergic effects of house dust mite [70]. Importantly, the production of these cytokines by bronchial epithelial cells in response to these triggers might be genetically regulated and this could be the explanation why some individuals become primed to inhaled antigen under the right environmental exposure [71]. Under some conditions, predictions about sensitizers or adjuvants can be made from in vitro experiments. Diesel exhaust particles (DEPs) induce maturation of human DCs indirectly, via promoting GM-CSF production in bronchial epithelial cells in vitro [69]. Another known sensitizer is cigarette smoke. When given concomitantly with harmless OVA, it induces Th2 responses, and this response is associated with enhanced DC maturation and migration [72]. DCs developed in a nicotinic environment fail to support the terminal development of effector memory Th1 cells due to their differential expression of costimulatory molecules and lack of IL-12 production [73]. As maternal cigarette smoking is a solid risk factor for becoming sensitized in early life, it is important to elucidate how it leads to DC activation (e.g. whether any indirect mechanisms acting via epithelial TLR4 contribute) as this might provide novel intervention strategies.

Function of Dendritic Cells in Established Asthma

Not only do DCs play a role in the primary immune response to inhaled allergens, they are also crucial during the effector phase of asthma (see Fig. 7). The number

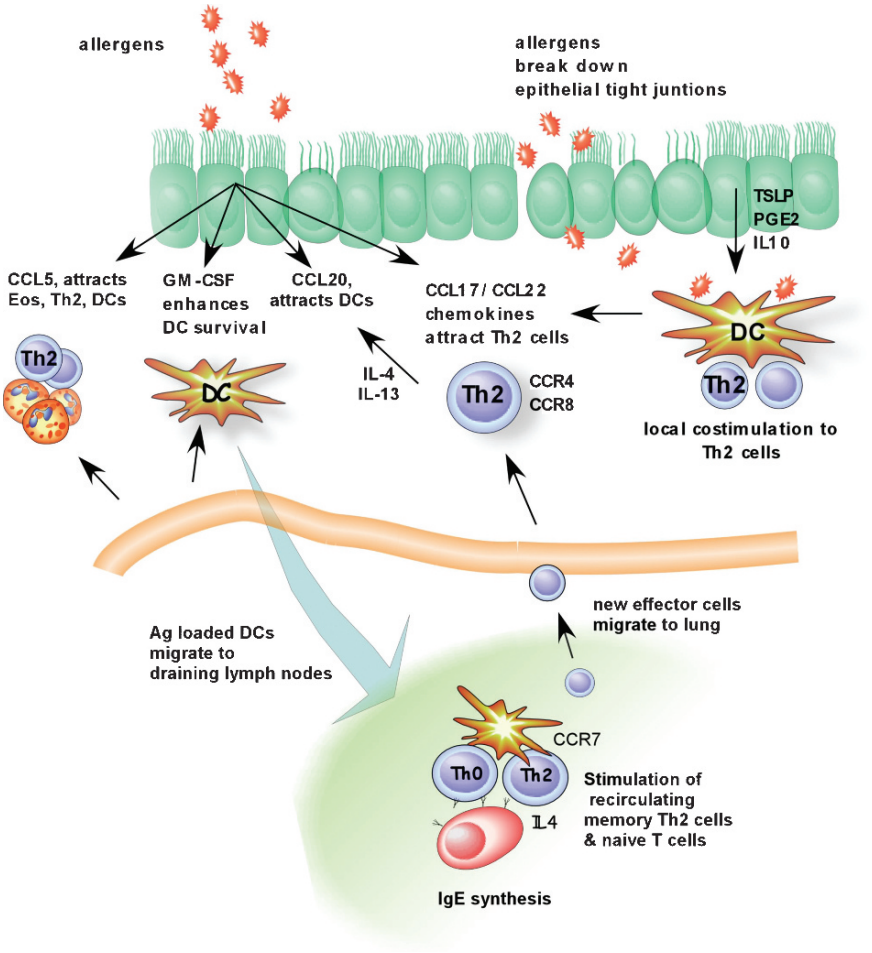

Fig. 7 Interaction between epithelial cells and dendritic cells during established inflammation. Allergens stimulate epithelial cells to release chemokines and growth factors for DCs, Th2 cells, and eosinophils. Thymic stromal lymphopoietin (TSLP) and GM-CSF are instrumental in inducing a Th2 prone phenotype in lung DCs. Epithelial cell tight junctions are opened up by protease activity of certain allergens like Der p 1 from house dust mite. In this way, allergens gain access to the DC extensions. The recruited DCs are also stimulated directly by allergen and produce even more chemokines for Th2 cells (TARC and mDC). Locally attracted Th2 cells interact with DCs in the airways, leading to local DC maturation and T cell costimulation of effector cytokine production. These activated Th2 cells eventually control the inflammatory process by activating eosinophils and mast cells and by feeding back on the epithelium and DCs. At the same time, DCs also migrate to the draining lymph nodes where they restimulate recirculating memory Th2 cells to become effector cells, and they recruit new cells into the response. In this way, effector cells are continuously replenished. DCs are also crucial for maintaining IgE synthesis, through their stimulation of interleukin 4-producing cells

of CD11b+ DCs is increased in the conducting airways and lung interstitium of sensitized and challenged mice during the acute phase of the response [45, 74, 75]. However, during the chronic phase of the pulmonary response, induced by prolonged exposure to a large number of aerosols, respiratory tolerance develops through unclear mechanisms. During this regulatory phase, the number of mDCs as well as their costimulatory molecules in the lungs steadily decreased, and this was associated with a reduction of BHR, possibly mediated by the action of Treg cells [51, 76, 77]. Inflammation, however, reappeared when mature inflammatory CD11b+ DCs were given [76].

The role of mDCs in the secondary immune response was further supported by the fact that their depletion at the time of allergen challenge abrogated all the features of asthma, including airway inflammation, goblet cell hyperplasia, and bronchial hyperresponsiveness [45]. Again the defect was restored by intratracheal injection of GM-CSF-cultured CD11b+CD11c+ mDCs, most closely resembling monocyte-derived "inflammatory DCs". It seems that "inflammatory" DCs are both necessary and sufficient for secondary immune responses to allergen. Upon allergen challenge, lung DCs upregulate the expression of CD40, CD80, CD86, ICOS-L, PD-L1, and PD-L2, particularly upon contact with Th2 cells [45, 47, 74, 75, 78]. Costimulatory molecules might be involved in activation of effector T cells in the tissues or in regulation of Treg activity. In allergen-challenged mice, DCs might also be a prominent source of the inflammatory chemokines CCL17 and CCL22 involved in attracting CCR4+ Th2 cells to the airways and in producing eosinophil-selective chemokines [60, 75]. A number of cytokines and innate immune response elements control the production of these chemokines. The pro-allergic cytokine TSLP induces the production of large amounts of CCL17 by mDCs, thus contributing to the recruitment of Th2 cells to the airways, explaining how it may act to enhance inflammation [66]. The complement factor C5a suppresses the production of CCL17 and CCL22 [60]. A similar effect was seen with the cytokine IL-17, explaining how it may suppress allergic inflammation when given during allergen challenge [79].

As the number and activation status of lung CD11b+ DCs during secondary challenge seems critical for controlling allergic inflammation, studying the factors that control recruitment to the lung during allergic inflammation will be important, as this might reveal therapeutic targets. In an elegant study, it was shown by Robays et al. [80] that CCR2 (and not CCR5 or CCR6) is crucial for releasing DC precursors from the bone marrow and attracting them into allergically inflamed lung. This was unexpected, as CCR6 is generally seen as the chemokine receptor attracting immature DCs into peripheral tissues.

In humans, allergen challenge leads to an accumulation of myeloid, but not plasmacytoid DCs to the airways of asthmatics, concomitantly with a reduction in circulating CD11c+ cells, showing that these cells are recruited from the bloodstream in response to allergen challenge [81, 82]. A recent report suggests that pDCs are also recruited into the bronchoalveolar lavage (BAL) fluid, but are poor APCs [82]. The exact role of plasmacytoid DCs in ongoing allergen-specific responses in asthma is currently unknown. It was shown that pDCs accumulate in the nose, but

not lungs, of allergen-challenged atopics [83]. When pDCs were pulsed with pollen allergens, they were as efficient as mDCs in inducing Th2 proliferation and effector function [84]. Others have suggested, as in the mouse, that pDCs might also confer protection against allergic responses [49].

Function of Dendritic Cells in Allergic Rhinitis

In allergic rhinitis (AR), CD4+ Th2 cells control inflammation by secreting Th2 cytokines but little is known how these cells are activated to cause disease. Elevated numbers of CD1a+ Langerhans' cells are present in the nasal mucosa of symptomatic grass pollen-sensitive AR patients, and these numbers further increase upon relevant allergen challenge to the nose [85–87]. In symptomatic AR patients, DCs bearing allergen-specific IgE in the nasal mucosa are present [88]. In AR, DCs had a more mature phenotype and were found in close approximation with T cells. Similarly, in a mouse model of ovalbumin-induced AR, CD11c+ DCs accumulated in areas of nasal eosinophilic inflammation and clustered with CD4+ T cells. In mice depleted from DCs, nasal OVA challenge in OVA-sensitized mice did not induce nasal eosinophilia, and did not boost OVA-specific IgE levels or Th2 cytokine production in the cervical lymph nodes. Conversely, when OVA-pulsed DCs were administered intranasally to sensitized mice, they strongly enhanced OVA-induced nasal eosinophilia and Th2 cytokine productions. These data in humans and mice suggest an essential role for nasal DCs in activation of effector Th2 function leading to allergic rhinitis and identify DCs as a novel target for therapeutic intervention [89]. In support, treatment of allergic rhinitis patients with intranasal corticosteroid therapy reduced dramatically the numbers of DCs in the nasal mucosa [90].

Function of Dendritic Cells in Atopic Dermatitis

AD is a chronic inflammatory skin disease that is characterized by eczematous lesions and is associated with elevated serum IgE levels, tissue, and blood eosinophilia.

In patients with AD, DCs highly express FcεRI, the high-affinity receptor for IgE [91]. Two FcεRI+ subsets of myeloid DCs have been identified in skin lesions of AD patients: (i) Langerhans cells expressing CD1a and birbeck granules found in the epidermis and (ii) inflammatory dendritic epidermal cells (IDECs) only found in inflamed skin [92, 93]. In AD, FcεRI+ LCs bearing the antigen migrate from the skin to the draining lymph nodes where they activate FcεRI-mediated Th2 immune responses. At the same time, LCs also present allergen-derived peptides locally to transiting T cells and induce a classic secondary immune response. Moreover, the aggregation of FcεRI on LCs stimulates them to release chemokines such as CCL17 and CCL22 and monocyte-attracting chemokines [94]. All these molecules

contribute to the recruitment of FcεRI^{hi} IDECs into the skin. IDECs are only found under inflammatory conditions, display high stimulatory capacities towards T cells, and serve as amplifiers of the allergic-inflammatory immune response. The stimulation of FcεRI on IDECs induces the release of IL-12 and IL-18 leading to the priming of Th1 cells, probably contributing to the Th1 response observed in the chronic phase of AD.

In addition to mDCs, pDCs have been found in increased numbers in the blood of AD patients and express FcεRI [91, 95]. pDC can process allergens by FcεRI-IgE and promote Th2 type immune responses. However, in contrast to LCs or IDECs, pDCs fail to accumulate in skin lesions of AD patients and seem to be retained in the bloodstream. Whether this is due to a lack of recruitment from the blood to the skin or to the high sensitivity of pDCs to proapoptotic signals present in AD skin remains unclear.

Dendritic Cells as Drug Targets in Allergic Diseases

If DCs are so crucial in mounting immune responses during ongoing inflammation in the lung, nose, and skin, then interfering with their function could constitute a novel form of treatment for allergic diseases. Additionally, pharmacological modification of DCs might fundamentally reset the balance of the allergic immune response in favor of regulatory T cells and thus lead to a more long-lasting effect on the natural course of allergic disease. Steroids are currently the cornerstones of anti-inflammatory treatment in allergic disease. Inhaled steroids reduce the number of lung and nose DCs in patients with allergic asthma and allergic rhinitis [96]. Steroids might also interfere with a GITRL-driven induction of the enzyme indoleamine 2,3-dioxygenase (IDO) in plasmacytoid DCs, thus broadly suppressing inflammation [62]. Recently, several other new molecules have surfaced that may alter DC function in allergic inflammation and thus treat disease. Many of these compounds were first discovered by their potential to interfere with DC-driven Th2 sensitization. The sphingosine-1-P analogue FTY720 is currently used in clinical trials for multiple sclerosis and transplant rejection. When given to the lungs of mice with established inflammation, it strongly reduced inflammation by suppressing the T cell stimulatory capacity and migratory behavior of lung DCs [28]. Also selective agonists of particular prostaglandin series receptors might suppress DC function. The DP1 agonist BW245C strongly suppressed airway inflammation and bronchial hyperreactivity when given to allergic mice by inhibiting the maturation of lung DCs [56]. A very similar mechanism was described for inhaled iloprost, a prostacyclin analogue acting on the IP receptor expressed by lung DCs [58]. Extracellular ATP might be released by platelets upon allergen challenge. Neutralization of ATP via administration of the enzyme apyrase or the broad spectrum P2 receptor antagonist suramin reduced all the cardinal features os asthma by interfering with DC function [63]. A specific small molecule compound (VAF347) that blocks the function of B cells and DCs was also shown to be effective in suppressing allergic airway inflammation in a mouse model of asthma [97].

Finally, specific inhibitors of *syk* kinase were shown to suppress DC function and cure established inflammation [98].

Conclusion

Our understanding of DC biology in the airways has grown considerably. The concept that different subtypes of DCs perform different functions not only during sensitization but also during established inflammation is a theme that will persist in the coming years. Slowly, therapeutic strategies are emerging from these basic studies on allergic diseases, which could one day reach clinical application. However, detailed knowledge of DC biology is still lacking.

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Epithelial Cell-Mesenchymal Interaction, Epithelial-Leukocyte Interaction and Epithelial Immune-Response Genes in Allergic Disease

Stephen T. Holgate

Asthma is an inflammatory and remodeling disorder of the conducting airways, which contract too much and too easily spontaneously and in response to a wide range of exogenous and endogenous stimuli. This airway hyperresponsiveness is accompanied by enhanced sensory irritability and increased mucus secretion. The different clinical expressions of asthma involve varying environmental factors that interact with the airways to excite both acute and chronic inflammation as well as the varying pathophysiological contributions that smooth muscle contraction, edema and remodeling contribute to disease expression (Fig. 1). Asthma is a heterogeneous disorder with multiple clinical sub-phenotypes that have different interactions with the environment, responses to treatment and outcomes. Overall, asthma is a good example of gene–environment interactions, although no single gene or environmental factor accounts for the disease. The great majority of asthma is associated with atopy but while over 40% of the population in the developed world is atopic, only 7–11% expresses this as asthma. In order to explain this paradox, in addition to allergy, there must be important factors that translate the atopic phenotype into the lower airways to manifest this as Th-2-type airway inflammation.

Asthma is a common inflammatory disorder of the airway that classically involve allergen driven Th-2 lymphocyte polarisation with coordinate production of IL-3, IL-4, IL-5, IL-9, GM-CSF, and IL-13 which are all encoded in a gene cluster on chromosome 5q 31–34 [1]. These cytokines drive the allergic inflammatory response through the recruitment and activation of T cells, eosinophils, basophils, and monocyte/macrophages. Interleukins-4, -9, and -13 in the presence of MHC-Class II-restricted allergen presentation by T cells and CD40 or OX40 costimulation causes enhanced IgE production with subsequent sensitization of mast cells and basophils through the binding of allergen-specific IgE to high-affinity receptors (FcεR1). In the case of nonallergic or intrinsic asthma, Th-2 pathways are dominant but IgE production is either localized to the airways [2, 3] or is no longer prominent. Many aspects of the inhaled environment influence the maturational direction of the subsequent T cell

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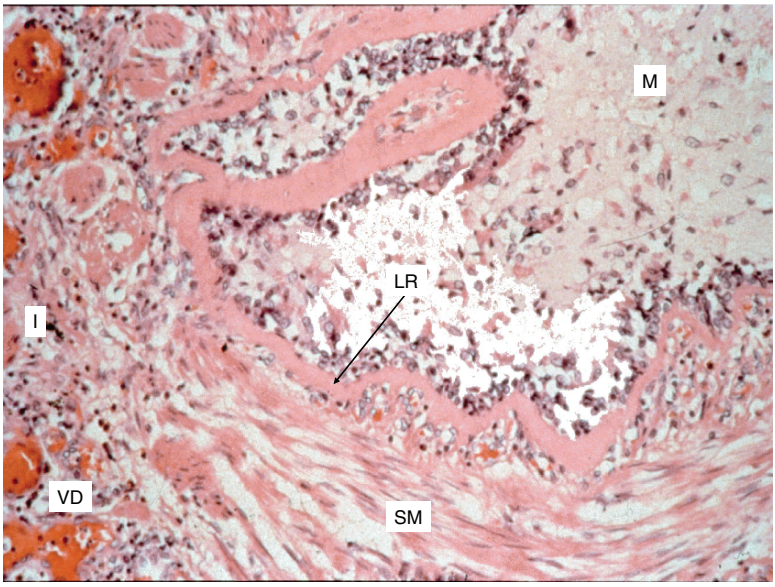

Fig. 1 Cross section of asthmatic airway showing widespread inflammation (I), smooth muscle increase (SM), vasodilatation (VD), epithelial disruption, matrix deposition in the lamina reticularis (LR) and obliteration of the lumen with mucus (M)

response by interacting with pattern recognition receptors on dendritic cells (DCs). Dust and airborne particulates containing cell wall and nucleic acid fragments of microorganisms interact with up to ten different Toll-like receptors (TLRs) to enhance and direct their response by in favor of Th-1, Th-2, Th-17, or Treg (regulatory T cell) subtypes, depending upon the composite stimulus. Precisely how activation of TLRs dictates T cell differentiation is not known, but altering the expression of different co-stimulatory molecules on DCs is likely to be important because these are intimately involved in directing the T cell response to TCR/MHC Class II signaling [4].

The inflammatory process is largely restricted to the conducting airways but as the disease becomes more severe and chronic the inflammatory infiltrate spreads both approximately and distally to include the small airways and in some cases adjacent alveoli [5]. The inflammatory response in the small airways appears to be predominantly outside the airway smooth muscle, whereas in the large airways inflammation of the submucosa dominates [6]. In addition to Th-2 type inflammation, asthma is characterized by features of epithelial stress and injury linked to tissue remodelling.

Epithelial Barrier in Asthma

Although initially considered purely in terms of barrier function, the epithelium is now seen as an important orchestrator of these diseases through the secretion of a wide range of cytokines, chemokines, and growth factors capable of supporting

both chronic inflammatory and remodelling responses. The identification of novel susceptibility genes for asthma and atopic dermatitis by positional cloning reveal that many are preferentially expressed in the epithelium (e.g., DPP10, GPRA, HLA-G, ETS-2 and -3, PCDH-1, filaggrin, and SPINK) [5, 7] supporting the idea that reduced epithelial barrier function and altered innate immunity are fundamental to the origin of these diseases. In addition to allergen exposure, a wide number of other environmental factors operating through the epithelium are associated with worsening asthma including exposure to chemicals, tobacco smoke and other pollutants, infectious agents, drugs, and even diet. As the barrier to the external environment, the bronchial epithelium is in a key position to translate these gene–environment interactions in asthma.

Many diseases are seriously exacerbated by, or may develop as a consequence of, loss of epithelial barrier function. A recent development has been the discovery of a number of genes encoded on chromosome 1 q, including filaggrin and the S100 proteins, that are involved in maintaining epithelial integrity in both the skin and the airways [8]. The association of genetic polymorphism of the pro-filaggrin gene on chromosome 1q21 with atopic dermatitis and asthma [9] is of considerable interest. Filaggrin, a key protein in facilitating epidermal differentiation and maintaining skin barrier function, is affected by loss-of-function mutations, which are strong predisposing factors for atopic dermatitis. Other diseases, such as food allergy, do not appear to have an underlying genetic cause, but appear to be driven by changes in gut epithelial permeability due to environmental stress [10].

In contrast to the skin, the physical barrier function of mucosal epithelial surfaces, including the airways and gastrointestinal tract, is dependent on cellular integrity and the coordinate expression and interaction of proteins in cell–cell junctional complexes, especially the tight junction (TJ) [11]. Tight junctions, situated at the subapical regions of polarized epithelial cells, selectively regulate the paracellular passage of molecules and ions, and restrict the lateral movement of molecules in the cell membrane. TJs comprise the integral membrane proteins claudins, occludin, and junctional adhesion molecules; cytoplasmic cytoskeletal linker proteins, ZO-1, ZO-2, and ZO-3; and associated signaling molecules and cell cycle regulators that control proliferation and differentiation [12]. Under normal circumstances the epithelium forms a highly regulated and almost impermeable barrier through the formation of tight junctions. These protein complexes at the apex of the columnar cells comprise a series of proteins that includes claudins and transmembrane adhesion proteins that connect adjacent cells. Structural integrity is also maintained through cell–cell and cell–extracellular matrix interactions that includes involvement of E-cadherin, desmosomes, and hemidesmosomes [13]. In vitro exposure of epithelial cells to the dust mite allergen Der p 1, a cysteine protease or inhalation exposure of ovalbumin in sensitized mice [14, 15] both cause airway epithelial TJ disruption.

Using differentiated epithelial cells in culture that have been brushed from normal and asthmatic airways, it has recently been shown that the permeability (leakiness) of the asthmatic epithelium is greatly increased, leading to greater access of inhaled allergens, pollutants, and other irritants to basal cells and the underlying airway

tissue [16, 17]. This increase in epithelial permeability has also been observed in vivo using inhaled radiolabeled probes, such as technetium^{99m}-labeled DTPA [18]. A reduction in the ability of the airway epithelium to exclude inhaled environmental agents may partly explain why certain atopic individuals go on to develop asthma whereas those with good barrier function do not. This loss of barrier function may reflect a broader abnormality in affecting other organs such as the skin, conjunctiva and gut that are foci for other atopic disorders [16].

The ability of growth factors such as epidermal growth factor (EGF) and keratinocyte growth factor (KGF) to restore barrier function after injury has been demonstrated in a number of in vitro and in vivo models [19, 20]. EGF has been shown not only to restore barrier function to cultured differentiated normal epithelial cells after tobacco smoke extract-induced injury, but also to enhance basal barrier function of asthma-derived cultures. It follows that locally applied (e.g., inhaled) growth factors may offer a potential new therapeutic approach for treating asthma by enhancing the ability of the epithelium to withstand environmental challenges. From a therapeutic perspective, topical application of EGF may maximize restoration of barrier function in asthma while minimizing unwanted side-effects such as increased proliferation. Studies evaluating the use of systemic KGF for treatment of oral mucositis have also shown that there is a marked effect of the growth factor on thickening of squamous epithelium [21], suggesting that route of administration may also be critical for KGF. Defects in epithelial barrier function have been observed in Crohn's disease and ulcerative colitis (IBD) [22, 23] with locally applied truncated form of EGF for 14 days inducing prolonged remission [24].

Impaired Virus Protection and Antioxidant Defense by the Epithelium in Asthma

A further example of an environmental injury targeting the airway epithelium in asthma is the effect of respiratory virus infections. It has long been known that common cold viruses such as rhinovirus are associated with exacerbations of asthma in both children and adults [25, 26]. Bronchial biopsy studies have shown that the airway epithelium is the preferential site for these viruses to enter the airway tissue [27]. Infection of asthmatic epithelial cells compared with normal epithelial cells in vitro has revealed that the former lack the ability to generate IFN-β and IFN-λ, cytokines essential for eliminating viruses partly through induction of apoptosis [28, 29]. In asthma the viruses continue to replicate until the epithelial cells are killed cytotoxically, leading to extensive virus shedding and infection of adjacent cells, as well as the release of mediators from the damaged cells (Fig. 2). By adding IFN-β back into the cell cultures, resistance to rhinovirus infection is restored [25], suggesting that this may be a new approach to the prevention and treatment of acute asthma exacerbations [30].

A second type of injury that targets the epithelium is air pollution, asthma worsening at times of air pollution episodes. A number of studies have shown

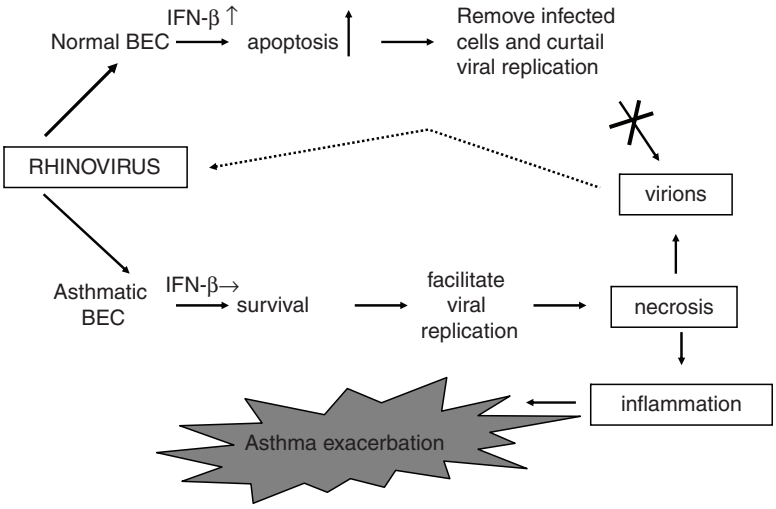

Fig. 2 Schematic representation of impaired innate immunity in asthma leading to virus-induced asthma exacerbation. The asthmatic epithelium fails to generate IFN-beta in response to virus infection resulting in increased viral replication and cytotoxic cell death rather than "quiet" removal of the infected cells by apoptosis

that antioxidant defenses mounted by the airway epithelium are markedly reduced in asthma associated with reduction in the antioxidant enzymes superoxide dismutase [31] and glutathione peroxidase [32, 33]. In not being able to defend itself adequately against oxidant damage, the airway epithelium is injured more easily [34]. The association of asthma with ozone and particle pollution episodes can be explained on this basis as well as the new data showing that air pollutant ozone and particle (PM_{10}) exposure impair childrens' lung growth [35, 36].

Pathological Evidence for Epithelial Damage in Asthma

The conducting airways contain an epithelium that is stratified, the upper layer containing a mixture of ciliated, goblet and Clara cells. Although at one time thought to be an artifact of sample handling [37], it is now clear in asthma that the epithelium is more fragile with easy loss of the columnar cells due to disruption of both tight junctions and desmosomal attachments [38–40].

Bronchial biopsies in anything but the mildest forms of asthma show areas of epithelial metaplasia and damage, thickening of the subepithelial basal lamina, increased number of myofibroblasts, and other evidence of airway remodeling such as hypertrophy and hyperplasia of airway smooth muscle, mucous gland hyperplasia, angiogenesis, and an altered deposition and composition of extracellular matrix proteins and proteoglycans [41]. These pathological features have been commonly

reported in asthma deaths (Fig. 1) and in bronchial biopsies from patients with asthma of varying severity, more recently similar findings have been found in the airways of children in relation to the onset of asthma [42–44]. Direct evidence for epithelial damage is found with upregulation of epidermal growth factor receptors (EGFRs) and features of impaired basal cell proliferation as shown by reduced expression of cell proliferation markers such as Ki67 and proliferating cell nuclear antigen (PCNA) as well as upregulation of the cell cyclin inhibitor, nuclear $p21^{waf}$, suggest that, as reported in adult asthma [45], the epithelium in childhood asthma is chronically injured and unable to repair properly [46, 47]. As referred to above, an important feature of the epithelium is its capacity to defend itself against oxidant injury [46, 48], an impairment of which may partly explain why asthmatic subjects are so sensitive to oxidant pollutants such as ozone, environmental tobacco smoke, and ambient air particulates [49].

In chronic asthma the number of goblet cells that secrete viscous mucus increases, with a parallel reduction in ciliated cells. Since mucus production is fundamental to the pathogenesis of chronic asthma, this metaplastic change in the airway epithelium is of great importance, particularly since it also spreads to the more peripheral airways which are normally devoid of goblet cells [50, 51]. The factors responsible for goblet cell metaplasia include the Th2 cytokines IL-4, IL-9, and IL-13 as well as TNF-α, which are all capable of directing differentiation to a mucus-secreting phenotype and interact by inducing EGFR-mediated goblet cell metaplasia through receptor transduction and induction of EGFR ligands such as HB-EGF and amphiregulin [52, 53] (Fig. 3). In asthma it is mucin 5 AC that dominates the mucin glycoproteins that are secreted and it is this mucin which interacts

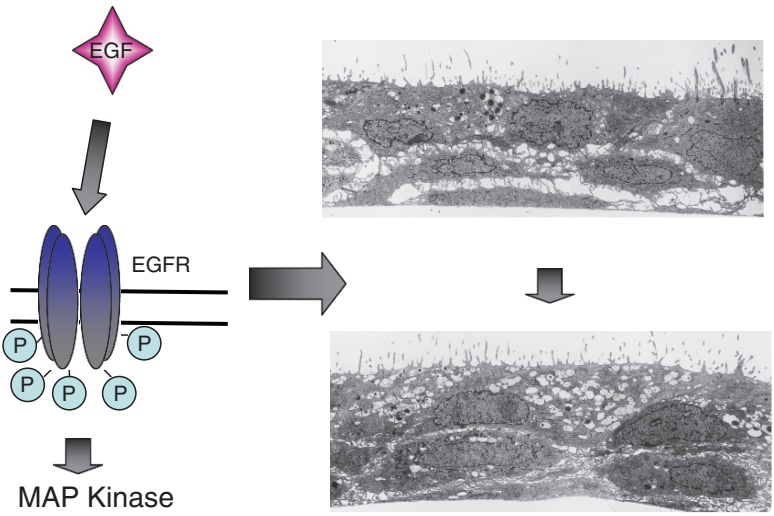

Fig. 3 Epidermal growth factor and related ligands interact with their tyrosine kinase receptors (EGFR) leading to receptor phosphorylation, activation of MAP kinase and induction of mucus-secreting goblet cells

with charged proteins such as eosinophil major basic protein and free DNA that is largely responsible for the unusual viscoelastic properties observed in asthmatic sputum that lead to difficulties in its expectoration. Interleukin-13 has been shown to regulate a chloride channel (GOB5; CLCA3), which is intimately involved in the regulation and secretion of mucin from goblet cells [54, 55]. However, the identification of multiple forms of GOB5 and its precise role in asthma has yet to be fully understood, though it does represent a therapeutic target. Mucous metaplasia can be viewed as an altered form of epithelial repair in response to injury.

Asthma as a Chronic Wound of the Airways

When damaged, the airway epithelium needs to repair but in asthma the repair process is compromised. As a consequence the airway epithelium enters into a chronic "wound scenario" with consequent production of a variety of cytokines and growth factors in an attempt to repair the "wound." As discussed above, one group of growth factors central to epithelial repair and its altered phenotype in asthma is epidermal growth factor (EGF) and related molecules (HB-EGF, amphiregulin) [56] that, by interacting through their tyrosine kinase receptors, promote repair. EGFR stimulation of the damaged epithelium also generates a mucus-secretory phenotype [57, 58] (Fig. 3) and an altered inflammatory response involving neutrophils [59], which are all characteristic of more chronic and severe asthma.

Other epithelial-derived factors include chemokines such as CXCL8 (IL-8), CCL17 (TARC), CCL22 (MDC) IL-25, (Il-17E), and IL-33 attracting neutrophils, mast cells, and eosinophils [60–62], as well as a range of growth factors including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF)-1, FGF-2, and TGF-β that are active on fibroblasts and smooth muscle [63]. The secretion of latent TGF-β and its activation by proteolytic enzymes are fundamental to the subsequent airway remodeling that accompanies all but the mildest asthma [64]. TGF-β itself is able to further impair epithelial repair responses while at the same time promoting the differentiation of fibroblasts into myofibroblasts [65] (Fig. 4). Bronchial biopsies from asthma have revealed that myofibroblasts are present in increased numbers in the subepithelial and submucosal region of asthmatic patients and increase in proportion to disease chronicity and severity [66–69]. The precise role of these cells in airway wall remodeling has yet to be proven, but it is highly likely that they do contribute significantly both by making good any damage caused to the epithelium through the deposition of matrix in the *lamina reticularis* of the basement membrane and their interactions with recruited inflammatory cells such as eosinophils and mast cells in sustaining the chronic inflammatory response.

One interesting idea that may partly account for the onset of asthma in genetically susceptible children is the fact that the airway epithelium is fundamentally abnormal both in its response to environmental injury and its repair. A recent study (COAST) in children born of asthmatic and atopic parents has shown that those who develop more persistent wheezing at the age of 4 years are those who had frequent symptomatic

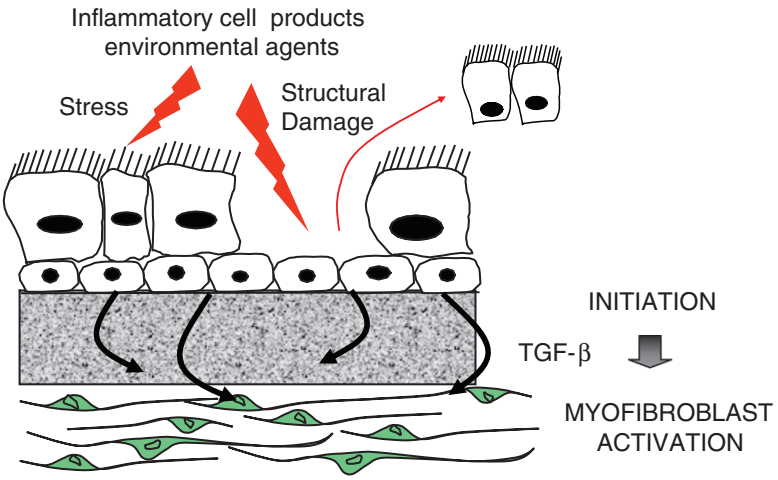

Fig. 4 Activation, damage and impaired repair of the asthmatic epithelium results in a chronic wound scenario with production of fibrogenic growth factors such as TGF-β that drives fibroblast differentiation to myofibroblasts and increased production of new matrix, an important component of airway wall remodelling

virus infections in early infancy, particularly rhinovirus [70]. Impaired innate immunity, possible due to a genetic defect in interferon production in response to virus infection or other defects in innate immunity, may initiate the chronic injury–repair cycle associated with the onset of chronic disease. Longitudinal cohort studies have also demonstrated that while atopy failed to predict persistent wheezing up to the age of 5 years, beyond this age those who persistently wheeze are the atopic children whereas those who lose their wheezing are the non-atopic children [71].

The Role of the Epithelium in Airway Wall Remodeling

Implicit in the chronic mucosal inflammatory response in asthma is the interaction between formed elements of the airways such as the epithelium, smooth muscle, vasculature, and nerves and the inhaled environment especially exposure to aeroallergens. Although difficult to define functionally, from a structural standpoint there is ample evidence that, beyond airway inflammation, changes to the formed elements of the airway contribute significantly to the pathophysiology of asthma [72, 73]. The most obvious change is in the airway smooth muscle, which not only increases in amount due to hypertrophy and hyperplasia, but as is also the case for mucus-secreting cells, also spreads both up and down the airways. In asthma the increase in muscle and its altered function most likely underlies an important component of hyperresponsiveness that characterizes this disease. However, intensive studies

have tried to identify cell and molecular abnormalities in the airway smooth muscle that may account for its abnormal behavior in this disease, but as yet there are no clear mechanisms that explain this [74]. Of particular concern is our ignorance over the relationship(s) between the increase in airways smooth muscle and its altered functions in asthma and the airways inflammatory response. Do both need to occur together at the start of asthma or are they entirely separate entities [75]?

In addition to changes to smooth muscle, asthma is characterized by the laying down of new matrix proteins including collagen fibers, increased proliferation of microvessels along with vascular leakage, and deposition of proteoglycans, with their ability to sequestrate water. In both children and adults, high-resolution computed tomography (HRCT) reveals an association between airway wall thickness and disease chronicity and severity that can be explained on the basis of remodeling [76, 77] correlated with airway hyperresponsiveness, suggesting that this thickening with deposition of matrix proteins may be a protective response against frequent smooth muscle contraction [78]. Thus, in patients with brittle asthma whom are highly hyperresponsive, the airway remodeling response is minimal, whereas in those with chronic disease who exhibit some degree of fixed airflow obstruction, remodeling is more prominent [79]. Remodeling as a series of interacting processes is complex. For example, while immunostaining for proteoglycans such as biglycan, lumican, versican, and decorin is increased in both moderate and severe asthma, with no differences in the amount present in the subepithelial layer, deposition of biglycan and lumican is significantly greater in the smooth muscle in moderate compared to severe asthma, suggesting a possible compensatory role [80].

Persistent airway inflammation is a key that contributes to airway wall remodeling, including the secretion of mediators and growth factors such as TGF-β1 from eosinophils. Kariyawasam et al. [81] have recently shown that, in those with both early and late asthmatic responses to allergen challenge, markers of airway wall remodeling such as tenascin, procollagens 1 and III, HSP-47 and α-smooth muscle actin (marker of myofibroblasts) were all elevated beyond the 7-day time point when the inflammatory response had resolved. However, as discussed above, epithelial injury, impaired repair, and the secretion of growth factors from this structure may also contribute to the ongoing repair response characteristic of remodeling. Since these changes have been described in the absence of eosinophil infiltration, it may be that epithelial injury and remodeling is a necessary precursor.

A Disintegrin and Metalloprotease (ADAM)33: A Remodelling Gene

One molecule that has emerged as involved in airway remodeling is ADAM33. This susceptibility gene for asthma on chromosome 20p13 was first identified by positional cloning [82] and has been replicated in a number of studies [83]. In being preferentially expressed in airway mesenchymal cells (fibroblasts and smooth muscle), ADAM33 has been shown to be involved in the pathogenesis of airway

hyperresponsiveness and decline in lung function over time [84, 85]. ADAM33 has metalloproteinase, fusagenic, adhesion, and intracellular signaling activities and exist in at least six alternatively spliced isoforms [86]. Although the full-length molecule (120 kDa) is expressed as a transmembrane protein, a 55 kDa soluble (sADAM33) form has also been identified whose levels in the airways increase in proportion to disease severity [87]. *ADAM33* polymorphism is also associated with impaired lung function early in life [88]. While *ADAM33* knockout mice fail to express a phenotype either in the absence or presence of antigen sensitization of the lung [89], human ADAM33 differs from that of the mouse in being expressed in at least six alternatively spliced forms which are highly expressed in primitive mesenchymal cells during human foetal lung development [90]. Of the many potential biological actions that ADAM33 possesses, its proteolytic activity, which is also present in the 55 kD soluble form, is likely to be important in generating growth factors that influence mesenchymal cell number and/or maturation [91].

In children and adults, angiogenesis is believed to be an important aspect of asthma pathogenesis. Increased expression of angiogenic mediators and their receptors has been correlated with disease severity and accelerated lung function decline [92–94]. The purified catalytic domain of ADAM33, but not its inactive mutant, causes rapid induction of endothelial cell differentiation in vitro, and neovascularization ex vivo and in vivo [95]. TGF-β2, a remodeling growth factor, enhances sADAM33 release from cells over-expressing full-length ADAM33 with the truncated form being biologically active. The discovery that sADAM33 promotes angiogenesis defines it as a tissue "remodeling gene" with potential to affect airflow obstruction and lung function independently of inflammation. As TGF-β2 enhances sADAM33 release, environmental factors that cause epithelial damage may synergize with *ADAM33* in asthma pathogenesis, resulting in a disease-related gain of function.

The Epithelial Mesenchymal Trophic Unit (EMTU) in Asthma Pathogenesis

In early life the central role that airway inflammation plays in the origin of asthma is being challenged with the discovery that, at its onset, asthma is associated with the presence of marked structural changes in the airways often in the relative absence of airway inflammation [96–98]. The process of "remodeling" of the airways as seen in adult asthma has many similarities with airway "modeling" observed during branching morphogenesis in the developing fetal lung and the structural changes seen early during the development of asthma in infancy [99] (Fig. 5). Similarities that exist between organ morphogenesis and wound healing have lead to a new concept of chronic inflammation, namely, one that is supported by structural components through activation of the epithelial-mesenchymal trophic unit (EMTU). Plopper [100] have reported that repeated exposure of infant rhesus monkeys to house dust mite allergen with or without ozone caused remodeling of the EMTU in the form of reduced airway number, epithelial cell hyperplasia, increased number

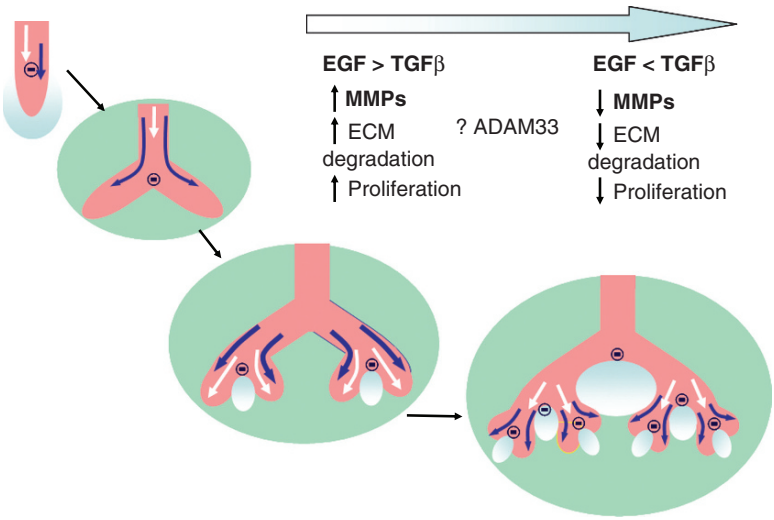

Fig. 5 Schematic representation of the reciprocal interaction between EGF and TGF-β in driving branching morphogenesis of foetal lungs. Secretion of EGF ligands promotes epithelial and mesenchymal cell proliferation linear growth whereas TGF-β initiates branching (Θ). MMPs, metalloproteases; ECM, extracellular matrix

of mucus-secreting cells, reorganization of airway vascular, and modified epithelial distribution of epithelial nerves, as well as altered immunity towards a Th-2 phenotype favoring the development of airways obstruction. The significance of these observations in nonhuman primates is strengthened by showing that these remodeling events are not influenced by corticosteroids, and are likely to be central to the long-term structural changes within the airways.

Thus, the tissue microenvironment provided by an activated EMTU has the capacity to provide the "soil" for the Th-2 related inflammation ("seed") to persist. The interdependency of structure and inflammation involving the epithelium, fibroblasts, myofibroblasts, smooth muscle, and their secreted matrix, microvasculature, and neural networks helps explain why chronic airway inflammation could persist at the more severe and chronic end of the asthma spectrum and for the incomplete response to anti-inflammatory treatments. A new paradigm for persistent asthma is that of a damaged epithelium which repairs incompletely. The consequence of this is a chronic wound scenario characterized by the secretion of a range of secondary growth factors by epithelial cells and underlying fibroblasts capable of driving structural changes linked to airway remodeling [101] (Figs. 4 and 6).

While there are many cytokines that are secreted by epithelial and mesenchymal cells that could support a Th-2 response, only recently has it been possible to tightly link epithelial injury with Th-2 type inflammation. Among the many products produced by the epithelium is thymic stromal lymphopoietin (TSLP), a 140 amino acid IL-7-like four helix bundle cytokine that was first isolated from a murine thymic stromal cell line and shown to support B cell development in the absence of IL-7 [102]. By interacting with the heterodimeric receptor IL-7Rα/TLSPR to

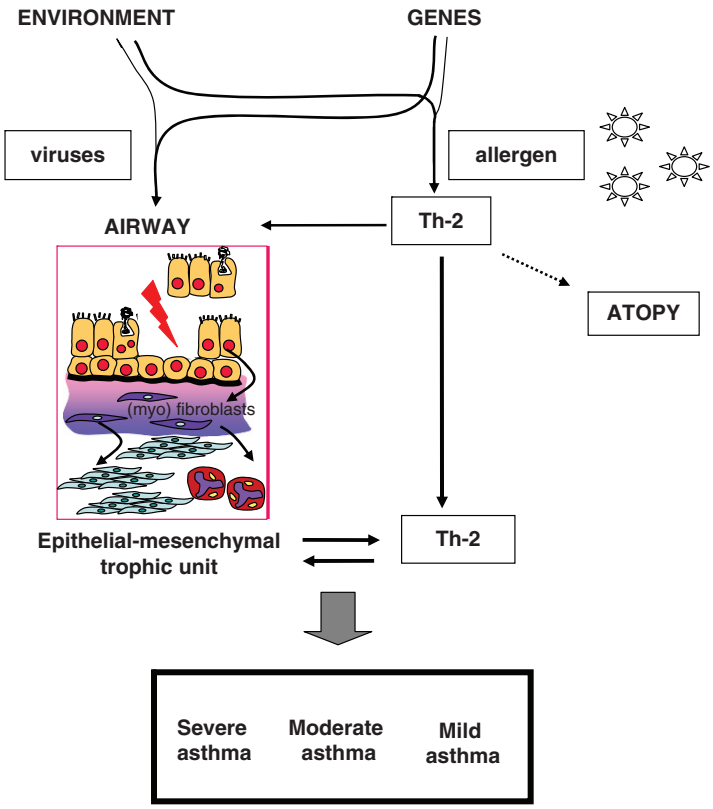

Fig. 6 Interactions between genetic and environmental factors in generating asthma sub-phenotypes involving Th-2-type immunological pathways and activation of the epithelial-mesenchymal trophic unit (EMTU)

initiate STAT-3 and STAT-5 phosphorylation [103], TSLP has recently been shown to produce a range of biological effects pertinent to allergic disease expressed at epithelial surfaces [104]. In addition to its thymic functions on the maturation of pre-B cells, TSLP activates dendritic cells by upregulating the co-stimulatory molecules OX40 and CD80/86 [105, 106] which, in the presence of the appropriate ligands, drives differentiation of $CD4^+$ T cells to a Th-2 phenotype [106]. Thus, TSLP produced by the activated epithelium might represent a critical factor that is able to initiate allergic responses at epithelial surfaces. In the case of asthma, TLSPR -/- mice failed to develop an antigen-specific Th-2 inflammatory response in the airways unless supplemented by wild type $CD4^+$ T cells [107]. In human asthma and AD, TSLP is markedly over-expressed in the airway epithelium when assessed at mRNA level or by immunohistochemistry [108]. With its ability to be induced by a wide variety of environmental insults and its capacity to generate a Th-2 environment in the airways with mechanistic relevance to asthma, TSLP is a highly plausible candidate for therapeutic intervention [109].

Concluding Comments

A crucial question that has not yet been answered is why in some people atopy expresses itself in one organ but not another. In the case of asthma and atopic dermatitis it is looking increasingly likely that the epithelium is critical in providing this localising stimulus. It is also likely that recognising that the formed elements of an organ help orchestrate the inflammatory and remodeling responses in diseases like asthma, they will also open the door to novel therapeutic targets and treatments for these chronic lifelong disorders.

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Role of Epidermal Growth Factor Receptor Cascade in Airway Epithelial Regulation of Mucus Production

Jay Nadel

Introduction

The epithelia form the interfaces between the internal milieu and the external environment. The main function of the respiratory system is to provide gas exchange. When animals migrated from sea to land during evolution, gills were exchanged for alveoli, thus increasing the surface area for gas exchange, and the lungs were relocated deep within the thorax. This new apparatus required a connection from the alveoli to the external environment. The airways provided this communication. Like the gastrointestinal epithelium and skin, the airway epithelium is exposed to a wide array of environmental invaders such as microbes (e.g., bacteria and viruses). The epithelium developed an early system for immediate responses to the "invader" as a first line of defense, which has been called "innate immune responses." The term "innate" refers to the fact that they do not require prior exposure to the invader (e.g., microbes) to respond. They have the capacity to intercept the microbial signals constitutively. The term "immune" refers to the fact that they are protective against infectious diseases.

Invading microbes take advantage of the epithelial protective system by entering the host airways and depositing on the epithelial surface. Normally, when inhaled bacteria deposit on the airway epithelium, a defensive cascade is stimulated to produce interleukin-8 (IL-8), which results in neutrophil recruitment to the airways [1], where the neutrophils can phagocytose the bacteria. Production of antimicrobial peptides [2] also assists in bacterial killing. The epithelium is stimulated to produce mucins, which entrap the neutrophils and the bacteria and are then cleared from the airways via cough [3] and mucociliary clearance [4]. This sequence of

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events usually occurs without symptoms. The interaction between the inhaled microbes and the epithelium is predicted to occur at the epithelial luminal surface. Therefore, this chapter focuses on airway epithelial *surface* signaling. Although here we focus on mucin production in developing the temporal sequence of discovery of the surface pathways involved in the signaling events, we also utilize novel information derived from studies of epithelia other than airways when they describe a significant newly discovered signaling event.

Many innate immune responses by the airway epithelium are modulated via activation of epidermal growth factor receptor (EGFR) activation. These include defensive responses such as mucin production [5], interleukin-8-induced neutrophil recruitment [1, 6], antibacterial peptide production [2, 7, 8], and wound repair [9–11].

In this chapter mucins, their sites of production, and their characterization will be described. Then the effects of mucous hypersecretion, which depend on the site in the airways that produce the mucus will be characterized. Thereafter, mucous hypersecretion in various airway diseases will be described. Because neutrophil infiltration frequently accompanies mucous hypersecretion in airway diseases, the discussion also briefly reviews epithelial recruitment of neutrophils. Because much recent progress has been made in the understanding of the epidermal growth factor receptor (EGFR) signaling pathways that results in mucin production and neutrophil infiltration, these pathways will be described in detail. Reactive oxygen species (ROS), which play important roles in epithelial surface signaling, are also reviewed.

Characteristics of Airway Mucus and Mucins

Mucus is produced and secreted into the airway lumen. It is a complex mixture of materials. Among these are secreted gel-forming mucins, which are high-molecular weight mucous glycoproteins that are key components of the mucous gel lining the airway epithelial surface. The mucous gel floats on a sol phase consisting of liquid that allows the cilia to move, propelling the gel toward the mouth. In airways, two mucins predominate, MUC5AC and MUC5B [12–15]. The sources of mucin production vary depending on their location: in large conducting airways, mucins are produced in both the surface epithelium (by "goblet" cells) and in submucosal glands (by "mucous tubules"). Small peripheral airways contain no glands; all mucins are produced by surface epithelial cells. In healthy individuals, the submucosal glands are small [16] and the surface epithelium contains few goblet cells [17, 18]. The thin mucous gel spreads on the epithelial surface, serving to protect the epithelial surface from inhaled invaders. Inhaled particulates are trapped in the mucus gel and are removed from the airways via cough [3] and mucociliary clearance [4] in the large conducting airways and via mucociliary clearance in peripheral airways. Mucus secreted by gland acini is transported via gland ducts, which terminate in the airway lumen. They are concentrated in numbers at bifurcations. Cough receptors are also concentrated at airway bifurcations [3], thus facilitating clearance of mucus secreted by glands.

Mucin production is normally scant. Inhalation of irritants results in innate immune responses, including mucin production, resulting in the killing and clearance

of the offending invader, usually without symptoms. In addition to microbes, airway epithelial surface signaling is stimulated by environmental irritants such as cigarette smoke [19] and its components [20], and by allergens [5], which mimic microbes in signaling innate immune responses.

Physiological Aspects of Mucous Hypersecretion

Chronic inflammatory airway diseases associated with mucous hypersecretion differ from one another pathophysiologically: allergic factors are involved in asthma, specific genetic abnormalities occur in cystic fibrosis, and COPD is closely linked to the inhalation of cigarette smoke. Thus, the responsible stimuli vary, but the responses are often similar.

The clinical manifestations of mucous hypersecretion depend on the anatomic location of the hypersecretory process. Hypersecretion of mucins by submucosal glands in proximal airways is predicted to be manifested by excessive sputum production (because glands can produce large volumes of sputum) and by cough (because of the colocalization of the cough receptors and the gland duct openings) in the large conducting airways, where cough greatly assists in clearance.

Unlike the large conducting airways, cough receptors are not present in peripheral airways, and consequently symptoms such as cough do not occur when peripheral airways are involved. In peripheral airways mucous hypersecretion may produce narrowing or obstruction of these airways more easily because of their small size and the fact that mucins that are secreted become hydrated and can expand more than 1,000-fold upon release and subsequent hydration [21]. In addition, with epithelial cell growth (e.g., with goblet cell proliferation), the epithelium occupies a larger percentage of the airway lumen compared to the epithelium of healthy subjects. Furthermore, airway smooth muscle contraction may be present, which can exaggerate the effects of secreted mucus on airway luminal narrowing.

In spite of the proclivity of small airways to become obstructed (so-called plugging), few clinical manifestations of peripheral airway obstruction may occur until extensive obstruction of the majority of the peripheral airways is present because individual small airways make only a small contribution to total airway resistance [22]. Thus, mucous obstruction of the majority of peripheral airways must occur before the resistive work of breathing is increased and the patient develops symptoms. Because symptoms of peripheral airway plugging do not occur early and because extensive plugging can be lethal, new methods of detection are required. For these reasons, the peripheral airways represent a relatively silent lung zone. In acute fatal asthma, mucous plugging is a major feature [23–26]. Similarly, lungs removed at the time of transplantation for cystic fibrosis were reported to contain plugs occupying >50% of the total luminal volume in approximately two thirds of the peripheral airways [18]. In COPD, Hogg et al. suggested that mucous plugging is associated with the progression of the disease [22]. All of these studies indicate the importance of mucous obstruction

of peripheral airways in severe, acute asthma and in other chronic inflammatory airway diseases. These manifestations are difficult to assess presently. This is an area ripe for further investigation. Also of interest is the relationship between mucous hypersecretion and bacterial colonization. While lower airways in healthy individuals are sterile [27], in diseases associated with mucous hypersecretion chronic bacterial colonization is common [28, 29]. The adherence of bacteria such as *Pseudomonas aeruginosa* probably plays an important role in the persistence of bacteria in the airways in chronic airway diseases associated with mucous hypersecretion.

Mucous Hypersecretion in Airway Diseases

In healthy human subjects, mucins are sparse in the airway epithelium [17, 30]. However, biopsies of the conducting airways in mild asthma show that mucin-containing goblet cells are increased in numbers, and patients with moderate asthma also have higher levels of airway mucins [17]. In asthma, overproduction of sputum is a common symptom, and sputum overproduction is exaggerated during exacerbations [31]. Interestingly, in patients with asthma, a history of sputum production is associated with an accelerated decrease in maximal expiratory airflow rates [32]. These findings suggest that mucous hypersecretion in the large conducting airways plays an important role in asthma pathophysiology. Mucous hypersecretion is profound in fatal asthma [26, 33]. Studies of mucous hypersecretion in peripheral airways are less common due to their relative inaccessibility. However, in morphologic studies of patients who died of acute asthma, plugging of small airways is common [26].

Mucin production is recognized to be involved in innate immune defenses by the host airway epithelium [34]. Normally, mucin production and secretion serve important roles in the clearance of inhaled irritants and microbes. In severe asthma, overproduction of innate immune responses is not limited to mucin production. The airway epithelium also responds to inhaled foreign particulates by producing interleukin-8 (IL-8), causing neutrophil infiltration [35]. Classically, eosinophil recruitment has been associated with asthma and with Th2 cell responses [36, 37]. However, special roles for neutrophils in severe and fatal asthma are suggested by the following: (a) fatal asthmatics preferentially show neutrophil presence in the airways [38]; (b) neutrophils, IL-8, and elastase are reported to be increased in lavage in subjects with status asthmaticus [39, 40]; (c) neutrophils predominate in the bronchial epithelium of severe asthmatics [41]; and (d) asthmatic sputum contains an increased number of neutrophils [42]. The increase in neutrophils in severe asthma can be interpreted as an excessive innate immune response of the airway epithelium. Products of neutrophils such as the serine protease, elastase, initiate signaling cascades in the airway epithelium, which may be important in various innate immune responses such as mucin production [43], cell proliferation, and wound healing [10].

Mechanisms of Cell Surface Signaling in Airway Epithelial Defense

Many authors have recognized that chronic airway diseases (e.g., asthma, cystic fibrosis, COPD, bronchiectasis) are associated with excessive production of mucus. The gel-forming mucins [12], large glycoproteins, are major contributors to human mucus. In spite of the recognition of their presence, understanding of mucin biology is a recent occurrence for various reasons. First, mucin studies were delayed by the difficulties of cloning the glycoproteins involved. Furthermore, many serious manifestations of mucin hypersecretion occur in peripheral airways, a relatively "silent zone," which did little to attract investigations. Finally, the mucin genes began to be cloned [44–47], and investigators reported that stimuli such as neutrophil elastase [48], tumor necrosis factor-α (TNF-α) [49], platelet-activating factor (PAF) [50], retinoic acid [51], ozone [52], acrolein [20], viruses [53], and bacterial products [54, 55] upregulate mucin production. Examination of the cellular pathways responsible for mucin production began with studies of intracellular signaling. Thus, Li et al. reported that the response to the Gram-negative bacteria *P. aeruginosa* involves a Ras-MAPK-pp90rsk pathway that is Src-dependent [56].

Because inhaled microorganisms and other intoxicants deposit on the surface of the airway epithelium, some investigators realized that the interception of signals from deposited microbes is likely to occur at the luminal surface of the epithelium. Recognition of pathogens is mediated by a set of germline-encoded receptors called pattern-recognition receptors, which recognize conserved molecular patterns shared by large groups of microorganisms such as Toll receptors. These are receptors found in *Drosophila* (fruit flies). They were discovered to be involved in antifungal immunity [57]. A homologous family of Toll receptors called "Toll-like receptors (TLRs)" was described in mammals [58].

The outer membranes of all Gram-negative bacteria contain the glycolipid lipopolysaccharide (LPS), which is responsible for many of their pathophysiological features. Poltorak et al. [59] reported that the effect of the Gram-negative bacterial product (LPS) signaling is due to TLR4. Subsequently, many Toll receptors have been described. In mammals, these receptors play essential roles in the recognition of microbial components. Ten mammalian TLRs are reported. Various TLRs exist in the airway epithelium. The associated signaling pathways may vary and they may result in different outcomes. Thus, different microbes may utilize different pathways, resulting in different host responses. Some TLRs are located on the luminal surface of airway (and other epithelial) cells. This facilitates their surface signaling.

These discoveries of TLRs provide a mechanism for microbes to bind to the host epithelium, but they may also explain how the host epithelium responds to the presence of the microbes. Binding of inhaled microbes to TLRs provides a mechanism for the microbes to adhere to the epithelium and thus invade. This binding also provides a logical "sensing device" by which the epithelium detects the presence of the microbes and thereupon initiates defensive responses.

The next step in discovering how the epithelium responds to stimuli was made in 1999 when Takeyama et al. reported that activation of the epithelial growth factor receptor (EGFR) plays a central role in epithelial cell surface signaling and in mucin production [5]. EGFR is a 170-kDa membrane glycoprotein that is expressed on the surface of various cells. In the airways, EGFR is expressed in the fetus, where it plays roles in proliferation, branching morphogenesis, and epithelial cell differentiation [60]. EGFR and its ligands are expressed in malignant lung tumors [61] and in asthma [62], but EGFR expression is sparse in healthy adult human airways [17, 63]. These findings suggest that EGFR upregulation plays important roles in epithelial cancer and in inflammation. Takeyama et al. [5] discovered that EGFR activation by its ligands (e.g., epidermal growth factor [EGF], transforming growth factor-α [TGF-α]) causes mucin production in human airway epithelial cell cultures in vitro and in rats in vivo, effects that were prevented by selective EGFR tyrosine kinase inhibitors. Furthermore, the authors reported that ovalbumin sensitization in rat airways causes similar induction of EGFR expression and activation and goblet cell production [5] via an EGFR signaling cascade. These findings implicate EGFR activation in airway epithelial cell production of mucins.

Following this discovery, investigators reported that many different stimuli cause mucin production via EGFR activation [64]. These investigations were performed in human airway epithelial cell lines, normal human bronchial epithelial (NHBE) cells, and in animals in vivo.

EGFR activation was originally shown to occur by the binding of an EGFR ligand to the EGF receptor. EGFR ligands (e.g., EGF, TGF-α, HB-EGF, betacellulin, amphiregulin, epiregulin) bind to and activate the receptor directly [65]. These ligands may be derived from inflammatory cells such as neutrophils [66], eosinophils [67, 68], mast cells [69], and macrophages [68, 70]. These migrating cells can release soluble EGFR ligands and thus promote EGFR activation in epithelial cells during close contact between migrating and epithelial cells. The exact roles and importance of these inflammatory cells in signaling EGFR activation in the airways need further investigation.

In addition to EGFR ligands from bone marrow-derived cells that migrate to the airways and release ligands that can activate EGFR, airway epithelial cells themselves produce EGFR ligands [71]. These ligands are synthesized as transmembrane precursors that can be cleaved by metalloproteases, releasing soluble active growth factors [72]. Epithelial cells contain G protein-coupled receptors (GPCRs) [73] that have been long recognized to be involved in intermediate metabolism. More recently, GPCRs have been recognized to be involved in cell signaling [74, 75] and to mediate cell growth and differentiation [76]. GPCRs include a wide variety of receptors including interleukin-8 (IL-8), lysophosphatidic acid, endothelin-1, thrombin [77], and P2Y [78]. In the original studies, stimuli that activate GPCRs [77, 79] were not found to release soluble growth factors to the culture medium, which led the investigators to conclude that these stimuli activated EGFR via a process that does not involve EGFR ligand binding, presumably via intracellular mechanisms. This has been called “ligand-independent EGFR phosphorylation.” However, the signaling process was reinvestigated by

Prenzel et al. in 1999, and they showed that GPCR activation releases EGFR ligands into the cell culture medium, suggesting that GPCR stimulation causes the cleavage of membrane-bound ligand, releasing it into the extracellular medium, where it binds to and activates EGFR [80]. Metalloproteases are known to cleave membrane-bound ligands [81]. When cells were pretreated with a general metalloprotease inhibitor (Batimastat), GPCR-induced EGFR ligand release, EGFR phosphorylation, and downstream cell proliferation were blocked [80]. These results implicate an unspecified metalloprotease in GPCR-induced EGFR activation.

The next important discovery in EGFR signaling was made by investigators studying the effects of the Gram-negative bacteria *P. aeruginosa* and its product, lipopolysaccharide (LPS). LPS was known to induce mucin production [55] via a Src-dependent MAP kinase pathway [56]. The surface signaling responsible for the induction of the bacterial signal to the downstream pathway was not yet explored. Subsequently, Kohri et al. [82] linked *Pseudomonas* bacterial supernatant-induced production of mucins by airway epithelial cells to EGFR activation (showing that EGFR tyrosine kinase phosphorylation led to downstream MAP kinase activation and subsequent mucin production). Using human neutrophil elastase (HNE) as another stimulus that induces mucin production in airway epithelium, these latter authors reported that elastase-induced mucin production occurs via cleavage of TGF-α (a "ligand-dependent" process). However, the metalloprotease responsible for the cleavage of the EGFR ligand was still a mystery.

The next advance in EGFR epithelial surface signaling was contributed by Shao et al. [83], who recognized that epithelial cells contain a membrane-bound metalloprotease (MMP) that was reported to be capable of cleaving membrane-bound molecules such as EGFR proligands [81]. The metalloprotease, tumor necrosis factor-α-converting enzyme (TACE), is a member of a disintegrin and metalloprotease (ADAM) family, which is a group of zinc-dependent transmembrane metalloproteases [84, 85].

Shao et al. [83] first confirmed that the Gram-negative bacteria *P. aeruginosa* supernatant (PA sup) and LPS increase mucin MUC5AC gene expression and protein production via an EGFR-dependent pathway. Then they studied the effect of an EGFR-neutralizing Ab, which blocks ligand-binding sites on EGFR and thus inhibits EGFR phosphorylation due to the cleavage, release, and subsequent binding of the released ligand(s) to EGFR. Pretreatment with an EGFR-neutralizing Ab inhibited PA sup- and LPS-induced mucin production, implicating ligand-dependent EGFR phosphorylation in the responses. Preincubation of the cells with a TGF-α-neutralizing Ab also prevented PA-induced mucin production, suggesting that TGF-α is an important EGFR ligand cleaved and released by this stimulus. The authors investigated the MMP responsible for the PA-induced EGFR cleavage. Preincubation with TAPI-1, a relatively selective inhibitor of the MMP TACE, and more specifically knockdown of TACE expression using TACE siRNA prevented the induced cascade leading to mucin production, implicating TACE in the shedding of the EGFR ligand, TGF-α, and subsequent mucin production [83]. These findings established the role of TACE in the autocrine activation of EGFR. Because of its

location on the luminal surface of airway epithelial cells, it is likely that TACE plays a major role in many other responses to EGFR activation, such as wound repair (see below), synthesis of other EGFR-dependent molecules, and cancer cell growth.

Roles of Reactive Oxygen Species in Airway Epithelial Defense

ROS are incriminated in cell damage associated with chronic inflammatory diseases [86]. However, ROS are also known to be involved in epithelial signaling. In 1996, ROS scavengers were reported to inhibit IL-8 production [87], although ROS-induced EGFR activation was not yet known. Likewise, some of the downstream signaling pathways (e.g., NF-κB) were known, but the relationship of ROS to these signaling cascades was still unknown. Recent studies have contributed to the understanding of the roles of ROS in airway epithelial cell signaling and mucin production. Thus, EGFR activation occurs with ROS (i.e., H202) stimulation [88, 89], and exogenous ROS (H202) is reported to cause EGFR and MAPK activation, resulting in mucin production [90]. These effects were inhibited by pretreatment with a selective EGFR tyrosine kinase inhibitor and by a selective MEK inhibitor, implicating an EGFR signaling pathway in the response [90]. These studies clearly implicate ROS in an EGFR-dependent signaling cascade, but they do not identify the locus and mechanism of ROS-induced EGFR signaling.

As mentioned above, the MMP TACE is implicated in mucin production via autocrine signaling in airway epithelial cells. Shao et al. [91] hypothesized that TACE activation could occur via ROS generation. Recently, dual oxidase-1 (DUOX1) was found in human airway epithelial cells and was shown to generate ROS [92, 93]. Shao et al. suggested that DUOX1 activates TACE via ROS generation, cleaving the EGFR ligand pro-TGF-α into soluble TGF-α, resulting in EGFR activation and mucin production [91]. Because human neutrophil elastase (HNE), a potent protease, was known to cause mucin production via EGFR ligand (TGF-α)-dependent EGFR activation [94], it was used as a stimulus. HNE-induced TACE activation, TGF-α release, and mucin expression, effects that were inhibited by ROS scavengers, implicating ROS in the responses. Inhibition of epithelial NADPH oxidase or knockdown of DUOX1 expression with small interfering RNA prevented HNE-induced ROS generation, TGF-α release and mucin expression. Furthermore, the PKC-delta/PKC-theta inhibitor rottlerin prevented the effects of HNE, suggesting that PKC-delta and PKC-theta are involved in DUOX1 activation. The authors concluded that DUOX1 plays a critical role in mucin expression via a PKC-delta/PKC-theta-DUOX1-ROS-TACE-pro-ligand-EGFR cascade [91].

As a component of a surface signaling pathway, DUOX1 may release ROS in a limited compartment adjacent to TACE. Such a localization could produce a signal (e.g., TACE activation) without epithelial cell damage. In fact, as a component of a defensive epithelial surface signaling cascade, DUOX1 could play a role in multiple epithelial responses, including promotion of wound healing and angiogenesis

(via VEGF production), interleukin-8 production and neutrophil recruitment, and antibacterial peptide production, as well as mucin production.

ROS liberated by inflammatory cells such as neutrophils recruited to sites of inflammation can also signal epithelial cells to produce mucins. Neutrophils are known to cause ROS when they are activated [95–97]. Thus, when neutrophils were isolated from blood and activated by FMLP and the neutrophil supernatant was added to human airway epithelial cells, the cells became EGFR tyrosine phosphorylated and increased mucin synthesis occurred, effects that were blocked by ROS scavengers and by selective EGFR tyrosine kinase inhibitors, implicating secreted neutrophil products in mucin synthesis [90]. Of course, neutrophils can produce large amounts of ROS that cause DNA damage and death of the epithelial cells [98, 99]. Thus, at low concentrations of released ROS, neutrophils can induce defensive responses in epithelial cells, while higher concentrations of released ROS can be destructive.

Epidermal Growth Factor and Its Ligands in Asthma and in Other Hypersecretory Diseases

Mucous hypersecretion plays important roles in multiple chronic inflammatory diseases including asthma [34], COPD [100], cystic fibrosis [18], and bronchiectasis [101]. Nevertheless, no current treatment is effective for the treatment of hypersecretory diseases. In cultured human airway epithelial cells, EGFR activation by multiple stimuli leads to mucin production. Furthermore, selective EGFR inhibitors prevent mucin production in these experimental models. These studies strongly suggest that EGFR cascades are responsible for mucin production. Drug trials evaluating the efficacy of therapy aimed at blocking EGFR activation in mucous hypersecretion remain to be tested. The clinical information available on the subject in disease is derived from morphometric studies of tissue removed from human lungs (e.g., airway biopsies, tissue removed at surgery or at the time of transplantation, or in postmortem studies). Anatomic studies indicate that in normal healthy airways, EGFR immunoreactivity is weak or absent [63, 102–104]. However, EGFR expression is increased in the airway epithelium of asthmatics [63, 102, 104], in individuals with COPD [105, 106], in individuals with cystic fibrosis [18, 107], and in smokers without evidence of COPD [105]. In asthmatics, there was a significant correlation between EGFR immunoreactivity and the area of MUC5AC mucin staining, suggesting a sequence of events by which EGFR activation is involved in mucin expression in asthmatic airway epithelium [63]. Puddicombe et al. commented that EGFR signaling is a key abnormality in asthma, and they have emphasized the roles of epithelial damage and repair in asthma [10].

In healthy human airways, EGFR ligands are expressed constitutively. These include TGF-α, EGFR, HB-EGF, amphiregulin, heregulin, and betacellulin [71, 106], and they can be upregulated in disease. In diseases such as asthma, cystic fibrosis, and COPD, usually only a single ligand has been studied [16, 18, 62,

71, 106–108]. EGFR ligands have related structures, and antibodies to individual ligands may cross-react. Generally, the role of individual EGFR ligands is not precisely known. Furthermore, various stimuli may activate EGFR via different ligands. In addition, as a result of the EGF receptor cascade, feedback signaling of the epithelial cells is likely to play critical roles in the complex activity of the cells. Finally, the status of the cells themselves is critical (e.g., the state of differentiation).

Possible Roles of Excessive Innate Immune Responses in the Pathogenesis of Chronic Inflammatory Airway Diseases

Innate immune airway epithelial defenses normally protect the host from inhaled environmental irritants. These responses occur with minimal symptoms or pathological effects. They involve the recruitment of leukocytes to kill the invader, the production of mucins to assist in their clearance, and the production of other responses that maintain a sterile environment and intact airway epithelium, with a minimum of inflammation. Airway diseases such as acute, severe asthma, COPD and exacerbations of COPD, cystic fibrosis, and bronchiectasis show pathologic responses of mucous hypersecretion and excessive neutrophil recruitment that resemble exaggerated manifestations of potent innate immune responses. Each disease includes different features, reflecting the differences in their pathophysiology. However, it is suggested that the exaggerated responses may reflect some common signaling pathways. Abnormal genetic factors could be involved in this potent signaling.

Summary and Future Perspectives

The airways serve as a conduit for inhaled air to be delivered to the alveoli, where the transfer of oxygen and carbon dioxide occur. Along with fresh air, the atmosphere contains microbes (e.g., bacteria, viruses) and other potentially damaging particulates (e.g., allergens, cigarette smoke) that are inhaled. When the “invaders” are inhaled and deposit on the epithelial surface, the epithelium responds, initiating an interaction between host and invader. In normal individuals, deposition of bacteria on the airway epithelium is followed by a defensive cascade, which results in bacterial killing and clearance of the offending organism, usually with minimal symptoms or pathologic effects. The signaling initiating these responses is located on the epithelial luminal surface, and this chapter has focused on this important signaling cascade. Figure 1 shows the important features of the EGFR surface signaling cascade responsible for these defensive responses.

The primary focus of this chapter is on mucins. These glycoproteins are important constituents of the airway mucosal defense system involved in the trapping and

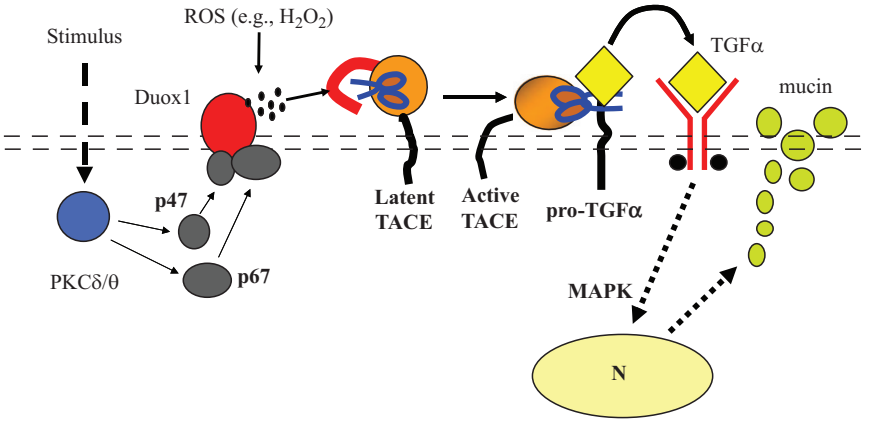

Fig. 1 Diagrammatic scheme of EGFR-dependent mucin production. This model includes Duox1, ROS, TACE activation, cleavage of EGFR proligand, and mucin production in airway epithelial cells. The stimulus activates PKC isoforms PKCδ and PKCθ, which recruit cytosolic components (e.g., p47phox and p67phox) to plasma membrane (double-dotted lines) to join Duox1 to form an active enzyme system for generating ROS (e.g., H_2O_2; represented by dark dots). ROS activate the latent form of TACE, which has an inhibitory prodomain (represented by a red line) covering its catalytic domain (represented by scissors), by removing the prodomain and exposing the catalytic domain to cleave pro-TGF-α (yellow diamond with a dark tail) into soluble TGF-α (yellow diamond without a tail), which binds to and activates EGFR, initiating MAPK signaling to the nucleus (arrow to nucleus), leading to mucin gene expression and mucin protein production (Modified from Shao et al., 2005 [92])

clearance of inhaled particulates. In chronic airway diseases, mucous hypersecretion plays an important role in the large conducting airways, resulting in cough and sputum production. In small airways, mucous hypersecretion leads to plugging. Because obstruction of these peripheral airways does not result in early symptoms, they exist as a "silent zone" in the lungs.

EGFR signaling plays an important role in mucous hypersecretion and neutrophil infiltration in experimental animals and in cellular models, but relevant clinical studies evaluating the efficacy of preventing these responses remain to be performed and evaluated.

Much remains to be learned. The EGFR cascade can signal a wide variety of outcomes, including cell migration and multiplication, and multiple events involving cell differentiation such as mucin, IL-8, and antimicrobial peptide production. How does an epithelial cell "decide" to produce one or another molecule? Or are all molecules generically produced concomitantly? Which aspects of the cascade vary to allow a specific outcome to occur? Innate immune responses are usually thought of as "early responses." Other responses occur later and include the recruitment of lymphocytes. How are surface epithelial signals involved in their recruitment into the airways? The studies reviewed here provide optimism for the future. They also acknowledge the limitations of present knowledge and the need for further innovation.

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Epithelial Cell Innate Responses to Rhinovirus Infection

Jean Kim and Robert Schleimer

Introduction

Rhinoviruses are a major cause of the common cold and play a significant role in the exacerbation of various diseases of the airways, including asthma, rhinosinusitis (CRS) and chronic obstructive pulmonary disease (COPD). In a normal person, they usually cause a self-limiting inflammatory response, mostly confined to the upper airways, and morbidity and mortality are minimal. In a patient with the above diseases, rhinovirus infection can cause a significant worsening of their preexisting disease and can cause life-threatening exacerbations. The purpose of this chapter is to briefly review the literature on the interaction of rhinoviruses with airway epithelium, the most important cellular target of rhinovirus infection, with the belief that improved knowledge of the molecular and cellular responses of the normal and diseased host with rhinovirus will lead to new opportunities to prevent exacerbations of airway diseases.

Epidemiology of Rhinoviral Infections

It is widely believed that human rhinoviral (HRV) infections play a key role in the development of airway diseases. Human rhinoviral infections are the primary cause of the common cold [1] and are the most prominent risk factor associated

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with exacerbations of asthma and COPD [2–4]. Viruses have been detected in 85% of children ages 9–11 who have exacerbations of asthma, and the most common virus detected was rhinovirus (66% of exacerbations) [3]. Upper respiratory viral infections are temporally associated with hospital admissions in both children and adults. Sixty-one percent of children ages 3 and up who were hospitalized for wheezing were found to have respiratory viruses. In adults, rhinovirus was detected by PCR in 25% of outpatient asthma exacerbations [2]. However, detection of the virus increased to 55% in a cohort of severe exacerbations of asthma requiring inpatient hospitalization [3]. In COPD patients, viral detection was positive in 56% and 64% of patients requiring hospitalization [4, 5]. In both adults and children, hospital admissions for asthma exacerbations are associated with the combination of viral infection and allergy [6, 7]. An association between upper respiratory viral infections and sinus disease has also been noted. Correlations have been noted between temporal patterns of insurance claims for upper respiratory viral infections (URI) and for CRS [8]. Not surprisingly, 45% of sinus brushings from patients with acute rhinosinusitis showed the presence of virus, as detected by real-time PCR, while only 3% of a corresponding control group were virus positive [9].

Life Cycle of Rhinovirus in the Airway Epithelium

Rhinoviruses belong to the Picornaviridae family of small non-enveloped viruses and contain a single-stranded positive sense RNA genome of ~7 kb. The genomic organization of human rhinovirus is similar to other types of Picornaviridae [10]. The 5′ end of the virus contains the internal ribosomal entry site (IRES) sequence that binds to the host translational machinery. The 3′ end contains a poly-A signal. The central portion of the RNA genome contains sequences coding for proteases required for inactivation of host cellular eLF-4F complex resulting in inhibition of translation of host cellular mRNA and initiation of translation of viral proteins. In addition, the central portion of the viral genome contains the sequence for the RNA polymerase and the Vpg protein that are required for synthesis of the negative strand RNA replication template. Viral replication occurs rapidly. Rhinoviral RNA and viral particles can be detected 5–10 h and 24 h after infection, respectively. Viral synthesis peaks within 48 h and declines by 72 h after infection of the human host. Since neutralizing antibodies are usually not produced until 72–96 h after infection, rhinovirus infection can often initially escape immune surveillance by the host.

To date, 102 types of rhinovirus have been identified. Characterization was established by serologic typing using a plaque assay in which absence of cross-reactivity of polyclonal antisera with established serotype defines a specific new serotype [11]. These strains are grouped into three classes based on their ability to bind three distinctly unrelated cell surface receptors. Ninety-one strains belong to the major group that binds to intracellular adhesion molecule-1 (ICAM-1). The minor group contains ten strains that bind to the very low density lipoprotein

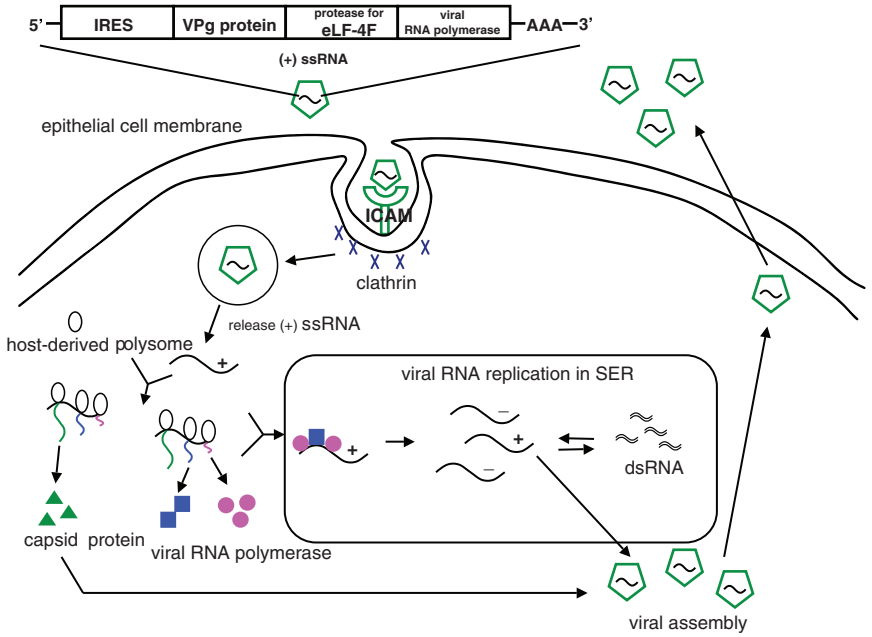

Fig. 1 Replication of rhinovirus within the epithelium**.** Vpg (virion genome), IRES (internal ribosomal entry site), AAA (poly-adenosine), elf-4F (eukaryotic protein synthesis initiation factor-isoform 4F), ICAM (intracellular adhesion molecule), and SER (smooth endoplasmic reticulum)

receptor (VLDL) [12–14]. A single strain of human rhinovirus-87 defines the third group which binds to sialoprotein as its receptor [15]. Each of these receptors plays roles in viral binding, uptake, and signal transduction processes.

Viral replication is confined to the cytoplasm of the epithelium [10]. Infection is initiated when the virion attaches to the specific receptor on the plasma membrane. The receptor functions to: (1) attach the virion to the plasma membrane and; (2) trigger a conformational change in the virion. This results in loss of viral protein, uncoating of the virus and delivery of the viral RNA genome across the plasma membrane and into the cytosol where viral translation can begin. For the minor group virus strain HRV-2, endocytosis of the virus is thought to occur in a clathrin-dependent manner [16]. Next the viral RNA confiscates host protein synthesizing machinery to synthesize its own polyproteins that function to replicate viral RNA. The incoming genomic plus sense viral RNA is copied to form a complementary minus strand, which serves as a template for synthesis of new plus stands, forming viral double-stranded RNA. At the same time, the viral RNA continues to translate viral capsid proteins in preparation for viral assembly. After viral assembly, the completed virions are released from the plasma membrane, completing the cycle. The precise details of the mechanisms controlling release of new viral particles are poorly understood.

Rhinovirus Ligation of ICAM

Recent work demonstrated that ICAM-1 ligation by HRV and phosphatidylinositol-3-kinase (PI-3-kinase) activation are important in early virus-induced signal transduction events. In endothelial cells, ICAM-1 crosslinking induces phosphorylation and activation of pp60 Src and pp125 RAK. The p85 regulatory subunit of PI-3-kinase serves as a substrate for both Src and FAK, implicating PI-3-kinase in the signal transduction by HRV engagement of ICAM-1 [17, 18]. Recent studies demonstrate that infection of 16 HBE cells with HRV-39 (major group) resulted in activation of PI-3-kinase [19]. Viral particles were shown in these studies to be co-clustered with membrane regions positive for PI-3-kinase. In addition, inhibition of PI-3-kinase resulted in blockage of internalization of radiolabeled HRV-39 and IL-8 expression, suggesting that IL-8 induction is dependent upon viral endocytosis, and not replication. This is also consistent with the observed time course in which IL-6, IL-8, and GM-CSF protein induction by HRV is detected in cultured monolayers on airway epithelial cells. That is, protein expression for these cytokines is detected much before viral replication is initiated, within 4 h after inoculation [20]. These data support the notion that the proinflammatory effects of rhinovirus infection may be due, in part, to early events involving viral entry into the epithelial cell, and not due to the effects of virus reproduction or of direct cytopathic effects of virus on the host cell.

Induction of ICAM-1 by HRV infection of both transformed and primary bronchial epithelial cells has been reported and may facilitate further viral entry and promote the adhesion, migration, and activation of inflammatory cells infiltrating the airways [21, 22]. In addition, exposure of the H292 human mucoepidermoid bronchial epithelial cell line to Th2 cytokines IL-4, IL-5, and IL-13 has been reported to induce ICAM-1 in vitro and the combination of these cytokines with HRV infection was additive [21]. However, in the widely studied BEAS2B human bronchial epithelial cell line, IL-4 by itself had no effect inducing ICAM-1 expression [23]. Thus HRV can potentially facilitate entry of itself into the epithelium, and Th2 cytokines in the environment may augment this process. Syk, an important immunoregulatory protein tyrosine kinase, is highly expressed by primary human bronchial epithelial cells and has been shown to be the immediate downstream signaling mediator activating the p38 MAPK pathway following ICAM-1 engagement by HRV-16 [24].

The Epithelial Cell in Rhinovirus Infections

That the airway epithelial cell is the major site of HRV infection has been shown both in vitro and in vivo. As noted above, rhinovirus is the major cause of the common cold and the most common pathogen associated with exacerbations of asthma, sinusitis, and COPD [8, 9, 25–27]. Although rhinovirus can infect monocytes,

macrophages, and fibroblasts, the major site of HRV infection is thought to be the airway epithelial cell. In contrast to events following infection of leukocytes and fibroblasts, the virus resides and replicates within the epithelial cell without compromising host cell viability [28]. Instead of exhibiting a cytotoxic response, the epithelial cell responds to infection by elaborating host defense and inflammatory mediators, which contribute to the recruitment and activation of inflammatory cells such as neutrophils, eosinophils, and T cells [20, 29, 30]. T cells recruited after the immediate antiviral response of the host epithelial cells are thought to be predominantly Th1 cells which elaborate Th1 type cytokines. However, since experimental infection of asthmatics with HRV-16 has been shown to induce the classic Th2 cytokines in sputum, there may be cross talk between the antiviral response and an ongoing allergic inflammatory response (see below) [31]. It is thought that the clinical symptoms of rhinovirus infection may be due primarily to induction of pro-inflammatory mediators, many of which are derived from epithelial cells. Table 1 summarizes mediators produced in response to HRV infection of airway epithelial cells in vitro and in vivo. Of note, most of the in vitro data were obtained using submerged cultures of undifferentiated human epithelial cells, either primary or transformed. As to whether the results obtained represent

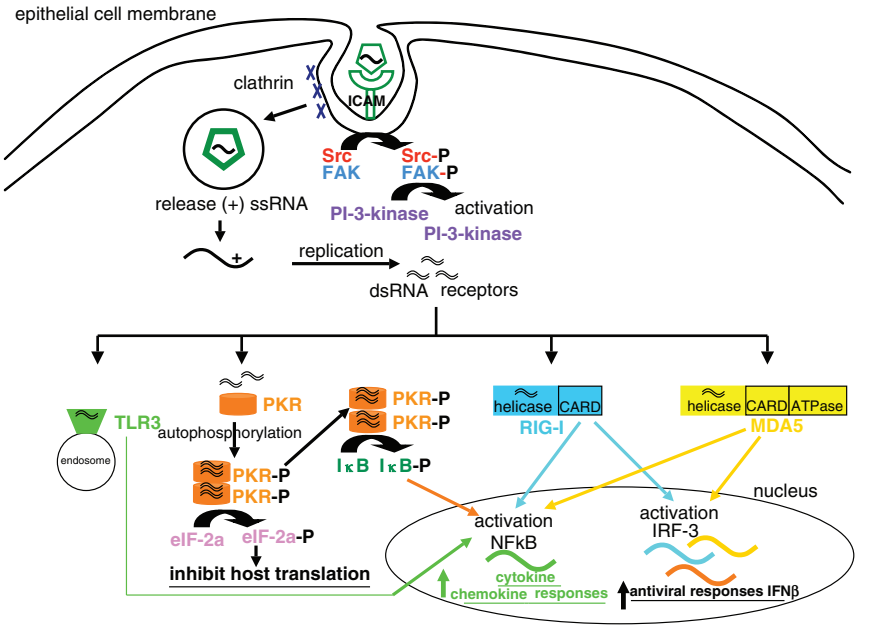

Fig. 2 Epithelial cell host responses to rhinovirus infection. Src (sarcoma family of nonreceptor protein tyrosine kinase), FAK (focal adhesion kinase), TLR3 (Toll-like receptor 3), PKR (Protein kinase R), RIG-I (retinoic inducible gene I), MDA5 (melanoma differentiation-associated gene 5), CARD (caspase recruitment domain), and IRF 3 (interferon regulatory factor-3)

Table 1 Inflammatory mediators induced by rhinovirus infection of airway epithelial cells

Mediator	Infected host type	References
Cytokines		
IL-1β	In vivo human	[36]
	Primary tracheal epithelial cells	[84]
IL-6	BEAS2B, primary human bronchial epithelial cells	[20, 78, 79]
IL-11	A549	[85]
GM-CSF	BEAS2B, primary human bronchial epithelial cells	[20, 79, 81]
TNFα	Primary human tracheal epithelial cells	[84]
ENA-78	BEAS2B	[86]
IFNβ	Primary human bronchial epithelial cells	[52]
IFNλ	Primary human bronchial epithelial cells, in vivo human	[53]
Chemokines		
IL-8 (CXCL8)	BEAS2B, primary human bronchial epithelial cells, in vivo human	[20, 79, 81]
IP-10 (CXCL10)	Primary human bronchial epithelial cells	[87]
RANTES (CCL5)	BEAS2B, primary human bronchial epithelial cells, in vivo human	[30, 88, 89]
Eotaxin 1 (CCL11)	BEAS2B, primary human bronchial epithelial cells, in vivo humans	[40–42]
Eotaxin 2 (CCL24)		
MCP-4	BEAS2B, primary human bronchial epithelial cells	[90]
MIP-1α (CCL3)	BEAS2B, in vivo human	[41, 89]
IL-1β	Primary human tracheal epithelial cells	[84]
TSLP	BEAS2B, primary human bronchial epithelial cells	[76]
Adhesion molecules		
ICAM-1	BEAS2B, A549, H292EC	[21, 22]
VCAM	A549, 16HBE, primary human bronchial epithelial cells	[82]
Enzymes		
NOS	Primary human bronchial epithelial cells	[54, 91]
5-LO	In vivo humans	[92]
COX	In vivo humans	[92]
Membrane receptors		
TLR3	BEAS2B	[50]
NK-R1	Primary human bronchial epithelial cells	[93]
Host defense molecules		
NO (nitric oxide)	Primary human bronchial epithelial cells	[29, 54]
HBD-2 (human beta defensin-2)		[58, 94]
MUC5AC	Primary human tracheal epithelial cells	[95]

infectious processes of phenotypically distinct differentiated airway epithelium remains to be determined. Epithelial differentiation induces many changes that can lead to resistance to infection, including improved barrier function, differentiation of mucus-secreting cells, formation of cilia, etc.

Although the epithelial cell is thought to be the main target of rhinovirus infection of the airways, other cells that express receptors for HRV and can take up or are known to be infected by HRV in vitro include monocytes [32–34] and macrophages [28, 35]. Rhinovirus-16 infection of human peripheral blood monocytes resulted in increased IFNα release and STAT1 phosphorylation, and subsequent induction of IP-10 [33, 34]. Infection of human alveolar macrophages with HRV-16 in vitro resulted in macrophage activation by a process that involved TNFα production and did not affect cell viability. However, in contrast to the case in epithelial cells, HRV does not replicate within monocytes and macrophages [28, 35]. Although cytokine elaboration by monocytes and macrophages in response to viral infection has been shown in vitro, this response has not been shown to occur in vivo.

There is a rapid and vigorous response to RV infection, including both innate and adaptive immune and inflammatory responses. Subjects with experimentally induced or naturally acquired colds demonstrated increased concentrations of the following mediators in their nasal secretions: kinins; cytokines IL-1β [36], IL-6, IL-11, TNF-a, GM-CSF, G-CSF; chemokines IL-8, MIP1a, eotaxin, eotaxin-1, and eotaxin-2; and RANTES (see Table 1 for references) [37, 38, 40–42]. These mediators have also been detected in sputum during natural or experimental HRV infections [39]. In one study, there was a direct correlation between the severity of symptom scores and levels of IL-6 and IL-8 [43]. Experimental rhinovirus infection of asthmatic human subjects results in symptoms of both upper respiratory infection and asthma exacerbation resulting in airway hyperresponsiveness [44]. Several studies suggest that virus-induced airway inflammation of the upper airways and the lower airways may be characterized by similar proinflammatory molecular events. It is now apparent that HRV can replicate in both the upper airways at optimal temperature of 33°C and in the lower airways at higher temperatures of 37°C. The differences in conditions have minimal effect on replication. After experimental nasal inoculation, HRV has been shown to be present and able to replicate in the lower airways, suggesting that upper respiratory viral infections have the potential to exacerbate asthma indirectly from upper airways inflammation or directly via infection of the lower airways [45].

Infectivity of HRV-16 has been characterized in both upper and lower airways. Unlike infection by other respiratory viruses, rhinovirus infects epithelial cells in a non-cytotoxic manner both in vivo and in vitro. During in vivo experimental HRV challenge, viral RNA was detected in the lower airway bronchial cells by RT-PCR and in situ hybridization, confirming infection of lower airways during upper airway inoculation [26, 46]. Mucosal biopsies of upper airways reveal little or no evidence of cytopathic effect on epithelium [47]. In situ hybridization of nasal biopsies revealed small clusters of intensely infected cells in the epithelial layer [48]. Similarly, immunohistochemical examination of bronchial biopsy tissue infected ex vivo demonstrated the presence of only a small subset of HRV infected cells in the epithelial layer [45]. In addition, exposure of epithelial cell monolayers derived from either the upper airway (adenoid) or the lower airway (bronchus) to HRV resulted in only 10% of cells showing presence of HRV by immunohistochemistry [45]. Taken together, these data indicate that HRV infectivity and

replication is limited to a small but significant number of epithelial cells in vitro and in vivo and that these cells remain viable for some period of time.

Rhinovirus Induction of Inflammatory Mediators

Infection of transformed or primary human airway epithelial cells with HRV results in production of proinflammatory cytokines and chemokines (see references in Table 1), including IL-6, IL-8 (CXCL8), IP-10 (CXCL10), IL-11, GM-CSF, and RANTES (CCL5); enzymes involved in the synthesis of proinflammatory mediators 5-LO and COX-2; and components of the innate immune system including human beta defensin-2 (HBD-2), the inducible form of nitric oxide synthase (iNOS) and Toll-like receptor 3 (TLR-3). The antimicrobial peptide HBD-2 is a chemoattractant for immature dendritic cells and memory T cells through interactions with the chemokine receptor CCR6 [49]. The pattern recognition receptor TLR3 is an intracellular receptor for viral dsRNA, product of HRV infection [50].

Recently, IL-17A, a pro-neutrophilic cytokine, has been shown to play a role in modulating HRV-16 modulation of inflammatory responses [51]. IL-17A and HRV-16 synergistically induced expression of the neutrophil chemoattractant IL-8 from primary bronchial epithelial cells (PBEC), and this process was shown to be dependent upon activation of p38 and p44/42 MAPK pathways. IL-8 promoter activation by the combination of IL-17A and HRV-16 was additive and this combination also led to stabilization of mRNA for IL-8, indicating that IL-17A may act post-transcriptionally to increase HRV-16 induction of IL-8. In addition, IL-17A also enhanced HRV-16-induced expression of HBD-2 from these cells by a process dependent upon p38 and JNK pathways. Interestingly, IL-17A exposure resulted in inhibition of HRV-16-induced RANTES expression. Thus IL-17A appears to promote and favor Th1 inflammatory conditions during rhinovirus infection.

In addition to the production of proinflammatory mediators following HRV infection of airway epithelial cells, the epithelial cell also responds with an antiviral response. Interferons function to limit spread of virus early in an infection. Type I interferons (IFNα/β) and type III interferons (IFNλs) are rapidly produced by epithelial cells in direct response to infection. Rhinovirus infection of PBEC has been shown to result in induction of IFNβ and IFNλ [52]. In contrast, human airway epithelial cells are not known to produce type II interferon (IFNγ), which is induced in T and NK cells in response to some TLR ligands or cytokines such as IL-12, IL-18, and IFNα/β. There is now growing literature suggesting that primary airway epithelial cells from asthmatic individuals behave phenotypically distinctly from those derived from non-asthmatic subjects. Bronchial epithelial cells obtained from asthmatic patients show impaired production of the antiviral cytokine IFNβ [52, 53]. In addition, viral RNA expression and late virus release into supernatants was increased 50- and 7-fold, respectively when virus was grown

on PBEC from asthmatics cells compared to PBEC from healthy controls. In the same studies, viral infection induced late cell lysis in asthmatic PBEC but not in normal PBEC [52]. More recently, the same group demonstrated that PBEC from asthmatics show deficient induction of type III interferons [or interferon-λs (IL-28A, IL-28B, and IL-29)] following rhinovirus exposure and that the extent of the deficiency was highly correlated with severity of rhinovirus-induced asthma exacerbations and virus load in experimentally infected human volunteers [53]. These results identify mechanisms of susceptibility to infection in PBEC from asthmatic subjects that are dependent upon the antiviral response elicited by HRV infection.

Nitric oxide has been shown to be a potent antiviral mediator produced in response to viral infection. In vitro exposure of cultured BEAS2B and PBEC infected with HRV-16 to exogenous NO resulted in inhibition of viral replication and rhinovirus-mediated induction of IL-6 and IL-8 [29]. This may be part of an epithelial antiviral response since both double-stranded RNA (dsRNA) and HRV-16 have been shown to induce iNOS expression in PBEC [54]. Induction of iNOS mRNA occurred as early as 4 h after exposure to dsRNA and iNOS protein was detected after 24 h of exposure. In addition, induction of iNOS by rhinovirus was dependent upon viral replication, as UV inactivated virus failed to induce iNOS mRNA. Furthermore, in vivo infection of human subjects during experimental HRV-16 resulted in induction of iNOS [54]. In addition, induction of exhaled NO in vivo was associated with induction of mRNA for iNOS in nasal epithelial cell scrapings and correlated inversely with symptom scores. This supports the concept that induction of iNOS and subsequently, NO, represents a protective host response to HRV infection [54].

Other mediators of innate immune responses triggered by HRV infection include expression of human beta defensin-2 (HBD-2). Defensins are cationic antimicrobial peptides characterized by three intramolecular disulfide bonds that determine their structural subclassification into either the alpha- or beta-defensin subfamily. Beta-defensins can have antibacterial activity whereas some alpha-defensins also have antiviral activity against enveloped viruses. Human beta-defensins are expressed by epithelial cells [55]. More recently, HBD-2 has been identified as a potent chemoattractant for immature dendritic cells and memory T cells by interaction with the chemokine receptor CCR6 [49, 56]. In addition, HBD-2 has been shown to induce TLR4 dependent activation of immature dendritic cells [57]. Infection of PBEC with HRV-16 or HRV-14 resulted in induction of HBD2 mRNA and protein [58]. This induction was dependent upon viral replication and involved activation of NFκB. In addition, in vivo experimental rhinovirus infection of normal human subjects resulted in induction of HBD2 mRNA expression as detected in nasal epithelial cell scrapings. These studies demonstrate in vitro and in vivo induction of HBD2 in human airway epithelial cells by rhinovirus infection [58]. These studies support the concept that HBD2 may play a key role in linking the innate and adaptive immune responses to HRV infection through its actions to recruit DC and other inflammatory cells.

Double-Stranded RNA as a Surrogate for HRV Infection

Recently, much attention has been focused on the effects of the potent immunomodulatory replication intermediate of HRV infection, double-stranded RNA (dsRNA). There are four known receptors for dsRNA: [1] Toll-like receptor 3 (TLR3), [2] cytosolic RNA-binding protein kinase R (PKR), and two RNA helicases; [3] retinoic acid inducible gene I (RIG-I); and [4] melanoma differentiation-associated gene 5 (MDA5) [59–62]. All four receptors ultimately transduce intracellular signals to activate NFκB and other transcription factors. All four receptors have been shown to be expressed in human airway epithelial cells [63–65].

Toll-like receptor 3 (TLR3) is a member of the family of innate immune-recognition receptors that recognizes dsRNA. Ligation of the receptor by poly IC was shown to induce activation of NFκB and the production of type I interferons [59]. TLR3 is constitutively expressed in human alveolar and bronchial epithelial cells [64, 66]. The subcellular localization of TLR3 is thought to be endosomal [67], and in A549 and BEAS2B airway epithelial cell lines, the receptor was found to be primarily intracellular, since flow cytometric detection by monoclonal antibody to TLR3 only occurred after permeabilization [66]. The intracellular localization of TLR3 in airway epithelial cells has also been supported by several laboratories [68, 69]. PKR is a kinase that phosphorylates the alpha subunit of eukaryotic initiation factor (eIF-2α) [70]. It is a serine/threonine kinase with two distinct kinase activities: (1) autophosphorylation, which results in activation, and (2) phosphorylation of eIF-2a, which results in inhibition of host cell protein synthesis. In the latent state, PKR exists in a monomeric form. PKR is activated in response to dsRNA of cellular, viral, or synthetic origin. Binding of two molecules of dsRNA to PKR results in dimerization into an active state. In addition to inhibition of eukaryotic translation, activation of PKR results in IκBα phosphorylation on serines 32 and 36 to promote degradation of IκBα and permit translocation of the transcription factor NFκB to the nucleus [70, 71]. RIG-I is an RNA helicase which binds dsRNA to induce antiviral responses in the host cell [65]. It contains a caspase recruitment domain that functions to transmit downstream signals to activate the transcription factors NFκB and IRF-3. Subsequent activation results in production of the type I interferons IFNα and IFNβ [60]. Of note, RIG-I itself is induced by IFNβ [62]. In a study using well-differentiated bronchial epithelial cells grown on an air-liquid interface, infection with HRV-16 in vitro resulted in induction of IFNβ [72]. In addition, blocking antibody to IFNβ and a JAK inhibitor resulted in inhibition of induction of HRV-16 sensitive gene expression. Furthermore, inhibition of PKR resulted in inhibition of IFNβ expression and increased viral production. MDA-5 is a cytosolic dsRNA dependent ATPase that also contains a caspase recruitment domain and an RNA helicase domain [61, 62]. MDA-5 is thought to function to promote IFNβ induced apoptosis and inhibit cell growth. Like RIG-I, MDA-5 is itself induced by IFNβ at the level of transcription. Recent studies showed that dsRNA upregulates the expression of inflammatory cytokines and chemokines in BEAS2B cells and human PBEC including RANTES, IP-10, and IL-8, through TLR3 [68]. Small

interfering RNA knockdown of TLR3 in BEAS2B cells resulted in decrease in mRNA expression of these chemokines, whereas dsRNA-dependent expression of RANTES, IP-10, and IL-8 cells was not dependent upon RIG-I, MDA-5, or PKR [73]. Thus TLR3 functions to transduce signals promoting a chemokine driven inflammatory response whereas the other dsRNA intracellular receptors RIG-I, MDA-5, and PKR may function in part to deliver an antiviral response in the host infected cell.

Double-stranded RNA has been used as a surrogate for HRV infection to examine mechanisms of airway epithelial activation. These studies assumed that the results specifically reflect the response of a host cell to viral replication. It is worth noting that, most, if not all, of the studies examining the effect of dsRNA on epithelial cells have been performed using the synthetic surrogate of the nascent rhinovirus replication intermediate, polyinosine-polycytidylic acid (poly IC), and not virally derived dsRNA. Therefore, as to whether poly IC mimics the effects of native viral dsRNA replication intermediate or that of rhinovirus itself remains to be determined. Expression of TLR3 is itself induced by poly IC [64, 66]. Several investigators have also shown that poly IC induces IL-8 and RANTES [64, 68, 74, 75]. Exposure of epithelial cells to poly IC resulted in induction of IL-8 and serum amyloid A protein and mRNA for MIP-3α, and GM-CSF [64]. In both BEAS2B and PBEC, dsRNA has also been shown to induce chemokines IP-10, RANTES, LARC, MIP1α, IL-8, GRO-α, and ENA-78; and cytokines IL1-β, GM-CSF, and IL-6; and the cell adhesion molecule ICAM-1 [68]. Small interfering RNA knockdown of TLR3 resulted in significant inhibition of mRNA levels of these mediators in response to poly IC. Confirmatory studies using HRV as the stimulus in vitro will be essential in imparting an understanding of intracellular mechanisms involved in HRV infection of airway epithelium.

More recently, dsRNA exposure has been shown to induce expression of thymic stromal lymphopoietin (TSLP) from human PBEC [76]. In humans, this newly identified cytokine functions to activate CD11c + myeloid dendritic cells by inducing expression of costimulatory molecules (CD40, CD80, and CD86) that subsequently induce Th2 cell differentiation. This induction was shown to be dependent upon TLR3, NFκB, and IFN regulatory factor 3 (IRF3), as siRNA knockdown of these signaling intermediates (as opposed to knockdown of PKR, RIG-I and MDA5) resulted in significant decrease in TSLP mRNA expression. In addition, HRV-16 infection of PBEC resulted in induction of mRNA for TSLP. Interestingly, TSLP induction was enhanced by co-exposure to the Th2 cytokine IL-4. These results suggest that rhinovirus infection of airway epithelial cells may promote Th2 inflammation by induction of TSLP through a TLR3-dependent mechanism. Recently, dsRNA, along with IFNβ, has also been shown to induce B cell-activating factor of TNF (BAFF) expression in both BEAS2B and PBEC [77]. BAFF is a newly identified cytokine implicated to function in B cell maturation, survival and T cell-independent and CD40-independent immunoglobulin class switching. BAFF induction in BEAS2B cells by dsRNA was dependent on the JAK-STAT pathway, as pretreatment with a pan-JAK inhibitor resulted in inhibition of BAFF induction. These results

indicate that induction of BAFF occurs by an autocrine/paracrine pathway involving IFNβ. Thus epithelial-derived BAFF induced by dsRNA may potentially contribute to class switch recombination and immunoglobulin synthesis by B cells in the airways.

Transcription Factor Activation by HRV Infection of Airway Epithelial Cells

Activation of the mitogen-activated protein kinase p38 (p38 MAPK) is a key early event in the activation of transcription factors involved in HRV induction of proinflammatory genes in both airway epithelial cells and in monocytes and macrophages [74]. Other transcription factors that appear to be activated by HRV infection of human bronchial epithelial cells include NFκB, AP-1, GATA, NF-IL6 (C/EBP), and interferon regulatory factors. As discussed above, induction of IL-6 and IL-8 by HRV has been shown to be mediated by NFκB [78, 79]. Other genes induced by NFκB include IL-1β, TNFα, GM-CSF, CCL3, CCL4, CCL5, CCL11, VCAM-1, ICAM-1, E-selectin, iNOS, COX-2, and HBD-2 [58, 80]. Mediators such as IL-6 and IL-8 were shown to require both NFκB and an additional transcription factor activated by HRV infection [79]. Treatment of BEAS2B cells and PBEC with inhibitors of NFκB resulted in inhibition of formation of NFκB promotor oligonucleotide complexes, but did not inhibit mRNA induction for IL-6 and IL-8, indicating that other transcription factors may be involved in transcriptional control of these genes [79]. Others have also shown that AP-1, in addition to NFκB, can also mediate HRV induction of IL-8 and GM-CSF [81]. Activation of GATA by HRV infection has been shown to induce VCAM-1 surface expression in the transformed airway epithelial cell lines A549 and 16HBE and in PBEC [82]. Rhinovirus induction of type I IFNβ and type II IFNλ would presumably signal through the JAK-STAT pathways and activate downstream interferon-regulatory factors, in addition to NFκB and AP-1. When dsRNA was used to stimulate BEAS2B cells, induction of IP-10, RANTES, LARC, IL8, GRO-α, IL1β, and GM-CSF promotor activity through activation of both NFκB and IRF occurred [83]. In this study, the transcription factor NFκB alone was shown to mediate the dsRNA induction of expression of ENA-78, IL-6, and ICAM-1, whereas the transcription factor IRF3 alone was also shown to mediate the expression of MIP-1α and IL-6.

Concluding Remarks

We have attempted to review the presently available literature describing studies on the roles of rhinoviruses in inflammatory and allergic diseases of the airways, with an emphasis on the molecular basis of the interaction of rhinoviruses with airways

epithelial cells. Though considerable information has been gained from numerous studies of this important topic, more information is still required to better design strategies to prevent RV infections and/or their sequelae.

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The Role of Platelets in Allergy

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Introduction

Over recent years, platelets have been the subject of much renewed interest and speculation. Their functional qualities have expanded from the better-known role in haemostasis and thrombosis [1] to an interesting and potentially highly significant role as inflammatory cells. Evidence has shown platelets to be essential elements of an integrated inflammatory response to both allergic and non-allergic stimuli, and have thus been suggested to play crucial roles in a wide range of diseases, including atherosclerosis [2], rheumatoid arthritis [3], tumour metastasis [4, 5], eczema [6], allergic rhinitis [7, 8] and asthma [9].

Despite being enucleated and substantially smaller than other cell types, with dimensions of approximately 0.5 × 3 μm, platelets contain genetic material in the form of messenger RNA (mRNA). This enables them to synthesise some platelet-specific proteins de novo, thus conferring a degree of independence from the uptake of proteins released by neighbouring cells. Their cytoplasmic granules enclose a vast variety of enzymes and mediators, including histamine, platelet factor 4 (PF4), serotonin (5-HT), as well as lipid mediators synthesised de novo, such as platelet activating factor (PAF) and arachidonic acid metabolites, all of which can be released upon platelet activation. It is now clear that a range of inflammatory stimuli can induce platelet activation, albeit with a potentially different functional response in addition to the classical signals that lead to thrombosis. The mechanisms underlying the activation of platelets by inflammatory stimuli are, in fact, often distinct from those leading to platelet aggregation. Importantly, studies have shown that platelets express a variety of adhesion molecules on their surface, which enables them to adhere not only to other platelets, but also to inflammatory cell

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types as well as metastatic tumour cells [4, 5] and bacteria [10, 11]. In this context, P-selectin (CD62P), a glycoprotein with a lectin-like domain stored in platelet α granules and released to the cell membrane upon platelet activation and degranulation, has been shown to be essential for platelet tethering to leukocytes and rolling on the vascular endothelium (via adhesion to its counterreceptor PSGL-1), while integrins such as $\alpha_2\beta_1$ (CD49b/CD29) and $\alpha_{IIb}\beta_3$ (or GPIIb–IIIa complex) are required for firm adhesion and subsequent diapedesis. Various elegant studies using physiologic flow or high shear conditions [12–17] and in vivo models [18, 19] have shown that platelet P-selectin is a necessary prerequisite that allows leukocytes to roll and subsequently attach to the vascular endothelium. This platelet-mediated process is thought to follow a similar multistep process as the adhesion of leukocytes to P-selectin-expressing, activated endothelium, as non-activated endothelium does not support leukocyte attachment [13]. However, activated platelets alone are able to stimulate the activation of leukocytes via binding of their P-selectin to PSGL-1, a process that triggers tyrosine kinase-dependent upregulation and conformational change to an active state of the leukocyte β_2 integrin Mac-1 (CD11b/CD18) [16]. The latter is then responsible for firm adhesion [17], possibly through interaction with fibrinogen presented by platelet $\alpha_{IIb}\beta_3$ [15]. Essentially, under physiologic flow conditions, platelets are thus able to activate leukocytes via an outside-in pathway, provided they are themselves activated and expressing P-selectin, and provided that Ca^{2+} and Mg^{2+} cations necessary for phosphorylation reactions are present in the extracellular environment [12].

All of the aforementioned features provide platelets with the machinery necessary to behave as fully functional inflammatory cells and, as such, they represent a potential target for novel anti-inflammatory drugs. Nevertheless, our understanding of the role of platelets is, as yet, incomplete. More research is required to better understand their potentially different function in conditions that are characterised by fundamentally distinct inflammatory responses. This chapter will discuss the evidence available to date suggesting a role for platelets as cells contributing to allergic inflammatory diseases.

Platelet Abnormalities in Patients with Allergy

There are now numerous observations in the literature showing altered platelet function in patients with allergic diseases. Allergic subjects have a reduced platelet survival time [20], prolonged bleeding time [21, 22] and platelets isolated from patients with asthma and allergy exhibit significantly reduced aggregation in vitro and ex vivo refractoriness to pro-aggregatory mediators such as collagen [21, 23], ADP [21, 24], PAF [23, 25], adrenaline [24] and arachidonic acid [21]. These findings are suggestive of so-called ‘exhausted platelets’ resulting from chronic in vivo stimulation. Interestingly, this reduced aggregation is not a feature common to all atopic diseases, as platelets derived from patients suffering from atopic dermatitis (AD) and nonatopic controls respond equally well to ADP, thrombin and

collagen [26]. However, other studies have found significantly elevated levels of lipid mediators derived from lipoxygenase metabolism of arachidonic acid released from the platelets of AD patients, compared to controls. In particular, release of the platelet-generated 12-HETE (12-hydroxyeicosatetraenoic acid) was twofold higher in unstimulated platelets and threefold higher in stimulated platelets derived from AD patients [27]. Other arachidonic acid-derived inflammatory mediators, such as LTB_4 and the cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4, in addition to the monohydroxylated eicosatetraenoic acids, such as 12-HETE, are thought to contribute to the inflammatory response as they can elicit plasma extravasation, and thus oedema formation, as well as leukocyte recruitment. The fact that platelets may well produce increased amounts of these mediators during an allergic inflammatory response suggests their involvement in this type and potentially other types of pathophysiological conditions.

Aside from studies on platelet responsiveness and platelet-release products, several groups have reported a modest thrombocytopenia in allergen-challenged asthmatics [28–30], a feature that links in well with studies indicating the sequestration of platelets in the bronchial vasculature of allergic patients following allergen challenge [31]. Increased numbers of platelet–leukocyte aggregates have also been observed in circulating blood of subjects with asthma during an allergic inflammatory response of the airway, as well as in lung tissue after histological analysis [32]. Nevertheless, the reports of reduced platelet survival time and sequestration in the lung circulation have been disputed by other groups who found no such differences between atopic and healthy patients [33, 34]. However, a recurrent observation is the abnormally high platelet activation found in symptomatic atopics with asthma [30, 35–38] or eczema/dermatitis syndrome [39], manifested as increased plasma or bronchoalveolar lavage (BAL) levels of the platelet-derived mediators β-thromboglobulin (β-TG) and PF-4 following antigen provocation. In addition, levels of the potent chemoattractant cytokines RANTES (CCL5, Regulated upon Activation, Normal T Expressed and presumably Secreted), TARC (CCL17, Thymus and Activation-Regulated Chemokine) and MDC (CCL22, Macrophage-Derived Chemokine), all contained within platelet α granules and released upon stimulation, are significantly higher in allergic patients compared to healthy controls [36, 40, 41]. This clearly suggests that activated platelets release chemokines that could contribute to the attraction of leukocytes, as well as Th1 and Th2 cells.

One of the most interesting findings that solidly links platelets to allergic disease, now confirmed by numerous studies, is the existence of high- and low-affinity IgE receptors (known as FcεRI and FcεRII, respectively) on the platelet membrane [42–45]. As a hallmark of allergy, IgE cross-linking by the antigen is an essential step in the cascade that ultimately leads to an allergic reaction. Importantly, the percentage of platelets bearing IgE receptors has been found to increase from 20% to 50% in both animals and patients that exhibit high levels of circulating IgE [42]. Separate studies have also shown that platelets from atopic subjects contain ten times as much IgE than control subjects within their α granules, therefore potentially acting as IgE storage pools that can be released upon platelet activation. Furthermore, these levels correlated with serum IgE levels, and additional

studies on the exocytosis of platelet IgE found that stimulation by PAF resulted in the release of 65% of the stored, intracellular IgE [46]. Activation of platelets via the high-affinity FcεRI induces the release of serotonin (5-HT) and RANTES [45, 46], suggesting that this mechanism of IgE-induced platelet activation could be a contributing factor in the inflammatory cascade. Thus, the existence of platelet IgE receptors and internal IgE storage pools provides additional support for the hypothesis that platelet activation may well occur via one of two distinct pathways: thrombotic and non-thrombotic (Fig. 1). In addition, it underscores the fact that platelets have an important role not only in non-allergic inflammation, but also in IgE-mediated disorders.

Experimental Evidence for a Role of Platelets in Allergy

Respiratory Tract Allergies

In the area of respiratory allergies, many in vivo studies have identified the accumulation of platelets within the lung during an acute reaction to an allergen [47, 48] or to certain inflammatory mediators [49, 50]. As early as 1977, Pinckard et al. observed platelet sequestration within the pulmonary vasculature during IgE-induced anaphylactic shock in the rabbit. Significantly, the lethal effects of anaphylactic shock were completely abrogated when the animals were depleted of their platelets before antigen challenge [47]. Pretolani et al. also showed, in the guinea pig, that intrathoracic platelet accumulation in response to antigen challenge is accompanied by thrombocytopenia in the venous circulation. Neither of these features could be modified by the administration of aspirin. However, the addition of PGI_2, a strong selective inhibitor of platelet activation, resulted in the abolition of pulmonary platelet entrapment and restored circulating platelet numbers to normal levels. The bronchoconstriction was, nevertheless, unchanged by the drug [48]. Interestingly, early data suggest the reverse to also be true; PAF-induced platelet accumulation in the lungs was shown not to be strictly associated with bronchoconstriction, as treatment with theophylline inhibited the increase in airway resistance without, however, affecting platelet accumulation in the lung vasculature [50]. Taken together, these studies suggest that the critical step in this reaction is the actual release of mediators from platelets, rather than their accumulation within lung venules. Indeed, a number of agents capable of inducing platelet activation, and thus causing the release of platelet mediators, will cause bronchoconstriction. Nevertheless, these data can also be interpreted as platelets not being directly involved in inducing bronchoconstriction but having a secondary role, potentially as chaperones to other inflammatory cells. As a strong inflammatory mediator, PAF, also known as acetyl glyceryl ether phosphorylcholine (AGEPC), induces acute bronchoconstriction accompanied by eosinophil infiltration, a major characteristic of allergic asthmatic responses. Elegant studies in the guinea pig have observed the

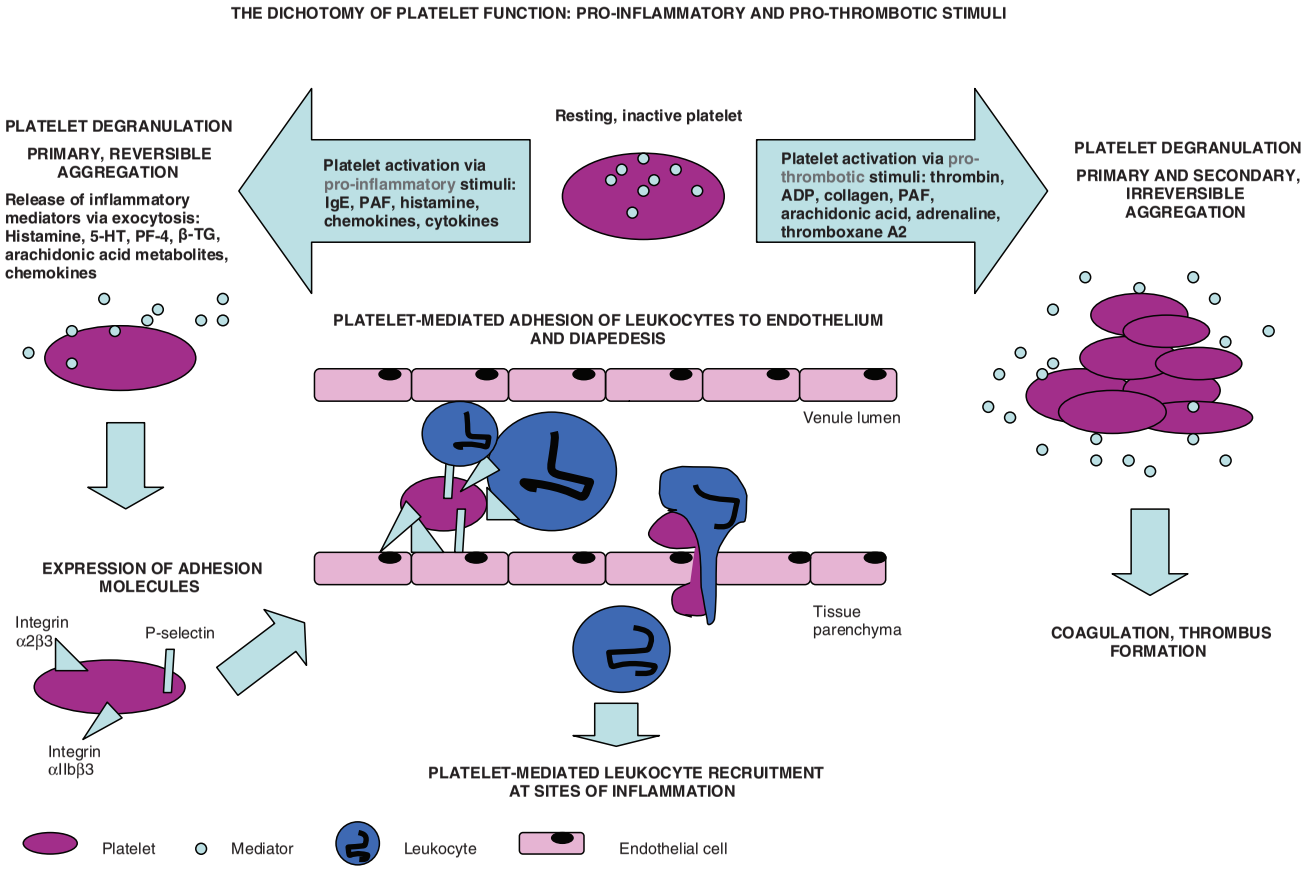

Fig. 1 Depending on whether they are stimulated by pro-inflammatory or pro-thrombotic stimuli, platelets can respond by activating and functioning in two different manners: as an inflammatory cell or as a clotting agent. Nevertheless, synergy between pro-inflammatory and pro-thrombotic stimuli does occur. The ultimate platelet response is therefore a result of the delicate balance between these stimuli

formation of platelet–neutrophil and platelet–platelet aggregates within alveolar capillaries following an intravenous PAF injection. In addition, through ultrastructural analysis, platelets were seen undergoing diapedesis towards the alveolar lumen. Subsequent administration of PGI_2 abolished both aggregation and diapedesis, but once again failed to modify the bronchoconstrictive response [51]. The administration of anti-platelet serum (APS), causing near total platelet depletion, to PAF or ovalbumin-sensitised guinea pigs greatly reduced eosinophilic infiltration, thus suggesting that platelets are involved and essential in the process of leukocyte recruitment [52]. Coyle et al. found a similar reduction in leukocyte numbers in the BAL of PAF-challenged [53] and ragweed extract-sensitised and challenged rabbits pretreated with APS [54]. In addition, studies in an ovalbumin mouse model of allergic lung inflammation have shown that thrombocytopenic animals exhibit a significantly reduced eosinophilic infiltration [55]. Interestingly, the transfusion of platelet-rich plasma (PRP) from healthy donor mice into thrombocytopenic allergic animals before allergen challenge restored the eosinophilia, in contrast to platelet-poor plasma (PPP). Functional and intact platelets are therefore required for the recruitment of eosinophils, as platelet-release products alone are unable to induce the response. As previously noted by Lellouch-Tubiana et al. in the guinea pig [51], platelet–eosinophil aggregates were also found in the peripheral circulation and lung parenchyma of allergen-sensitised and challenged mice [55] (Fig. 2). In the same murine model, platelets were also shown to be essential for the development of airway wall remodelling, a characteristic feature of chronic asthma [56]. It emerges that the presence of functional P-selectin on the platelet membrane is crucial to enable the formation of platelet–leukocyte aggregates. This interaction induces leukocyte activation, evident through upregulation of the adhesion molecule CD11b, and thus primes leukocyte recruitment. In fact, platelets from P-selectin knockout mice are unable to restore ovalbumin-induced eosinophil recruitment to the lungs in thrombocytopenic mice; nor are fixed, unstimulated platelets derived from normal donor mice [19]. The importance of this adhesion molecule is not limited to allergic disorders [58] and extends across animal species, including humans [59]. However, discussion of this equally interesting area of research is beyond the scope of this chapter.

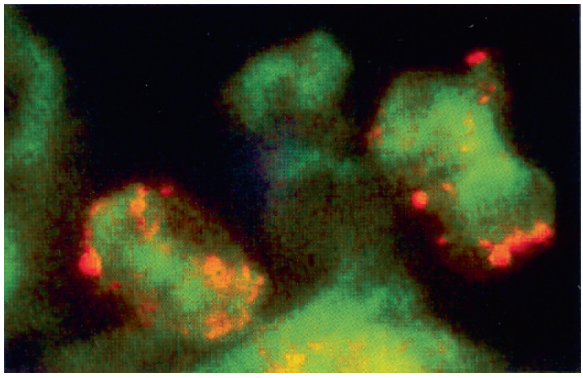

Fig. 2 Platelets (red, integrin $\alpha_{IIb}\beta$ positive) and eosinophils (green, major basic protein [MBP] positive) form adhesive conjugates in the lung parenchyma of allergen-sensitised and challenged mice (×1,000) (Adapted from [66])

Skin Allergies

In contrast to evidence on respiratory allergies, studies focusing on the potential role of platelets in other types of allergy have been sparse. Nevertheless, there have been reports, both clinical [26, 27, 39], as previously discussed, and experimental, suggesting that platelets are highly significant in the pathophysiology of atopic dermatitis (AD). This chronic inflammatory skin condition exhibits typical characteristics of allergy, i.e. elevated serum IgE levels and large infiltration of eosinophils and Th2-type cells. Readers are referred to the appropriate chapter in this encyclopedia discussing atopic dermatitis.

Watanabe et al. found increased expression of the PF-4 gene in the NOA (Naruto Research Institute Otsuka Atrichia) mouse, a model of AD, suggesting that this platelet-released chemokine, and therefore platelets themselves, could play a significant role in the development of this disorder [60]. Recent data provide strong evidence linking platelets to the development of chronic contact dermatitis, a Th2-dominant immune response, in mice. Depletion of more than 80% of circulating platelets resulted in the inhibition of leukocyte recruitment and significantly reduced ear swelling. Moreover, serum IgE levels were reduced in thrombocytopenic mice, as were levels of the chemokines MDC and TARC. These investigations also corroborate previous data on murine allergic lung inflammation by showing that blockade of P-selectin abolishes leukocyte recruitment into the skin. Interestingly, they also show that activated platelet supernatant, containing high levels of RANTES and TARC, is able to partly restore leukocyte infiltration, thus suggesting that both soluble platelet-release factors and P-selectin-bearing platelets are necessary to induce a full recruitment response [61].

Studies suggesting a contribution of platelets in skin allergies have also been undertaken in other species. Investigations in equine hypersensitivity to biting flies (termed ‘sweet itch’), a type of dermatitis, once again reaffirm the importance of PAF in allergic inflammation by showing that it mimics the effects of antigen challenge by inducing vascular permeability, oedema formation and leukocyte accumulation [62]. Because PAF has a strong stimulant activity on platelets, one may hypothesise that PAF-activated platelets are involved in the development of this highly pruritic condition, although to which extent remains unclear. Indeed, much of the experimental evidence linking platelets to the pathophysiology of AD is, in fact, indirect and circumstantial, deriving primarily from studies on inflammatory mediators also released by other cells. For instance, much evidence points towards an important role for TARC in AD. In the skin of patients, this chemokine was found to be released into the circulation and into the tissue by dendritic cells, T cells, epidermal keratinocytes [63] and venular endothelial cells [64], but no studies have undertaken to examine the specific role of platelet-released TARC. Moreover, the chemokine is a strong stimulus of platelet function [65], thus meaning that high serum levels of TARC could translate into high platelet activity. Therefore, in order to corroborate the clinical data [26, 27, 39], which clearly suggest a role for platelets in AD, more experimental studies need

to be directed towards the understanding of platelet and platelet-release factors involved in AD.

Conclusions

Platelets are acquiring a prominent role in many types of inflammatory diseases, not only because of the vast array of mediators they contain and release, but also because of their ability to function as inflammatory cells: reorganising their actin cytoskeleton enables them to modify their shape and this, together with the expression of adhesion molecules, allows them to adhere loosely or firmly to other cells, including the vascular endothelium, thus enabling them to undergo chemotaxis and diapedesis.

The accumulating evidence clearly points towards an important role for platelets in the amplification and perpetuation of an allergic inflammatory response, especially in the area of respiratory allergies, where the number of studies has been substantially greater. In the field of allergic asthma research, data emphasise the role of platelet adhesion molecules as well as platelet-release products in the development of the condition. On the other hand, studies of a role for platelets in other types of allergy are sparse. The fact that platelets have been shown to be essential elements of a non-allergic inflammatory response, as well as contributing factors in allergic disorders should spur on more research in this field. In any case, platelets have the potential to provide much-needed novel therapeutic targets to disorders such as asthma, where the existing drugs remain far from optimal. There are already indications that topical application of anti-platelet drugs, such as aspirin or clopidogrel, could provide an effective treatment for chronic allergic dermatitis [61], without the risk of causing excessive bleeding elsewhere due to the generalised inhibition of haemostasis. This is, in fact, the main problem encountered with existing anti-platelet therapies; these are targeted for the treatment of cardiovascular disorders, and are therefore not suitable for treatment of localised, inflammatory disorders. The aim for the future will be to develop a drug able to selectively target platelet–leukocyte interactions without, however, affecting the vital haemostatic functions of the platelet.

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Expression and Function of Siglec-8 in Human Eosinophils, Basophils, and Mast Cells[1]

Stephan von Gunten and Bruce S. Bochner

Introduction

Asthma and other forms of allergic inflammation involve selective tissue accumulation and activation of eosinophils, basophils, and mast cells [1]. High levels of chemotactic and anti-apoptotic molecules at the site of inflammation contribute to the accumulation of these cells by processes that involve selective migration and increased cell survival, the latter of which is of particular importance for the normally short-lived eosinophil [2, 3]. Upon activation, mast cells, eosinophils, and basophils release preformed mediators and granule proteins, newly generated lipid mediators and other proteins such as cytokines, contributing to the inflammatory processes that lead to the development of allergic diseases [1, 4]. All of these key pathomechanistic events are controlled, in part, by extracellular signals, including molecules on apposing cell surfaces that either enhance or suppress inflammatory responses [3, 5]. Such natural activators and suppressors of allergic effector cells may be exploited as targets for future therapeutic strategies to interrupt allergic inflammation. For instance, pharmacological antagonism of selectins, adhesion molecules on leukocytes which function as lectins that recognize glycans induced

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at sites of injury or inflammation, can have profound effects on cell-recruitment responses [6]. Recently, a new class of lectins, the so-called sialic acid-binding immunoglobulin (Ig)-like lectins (Siglecs), have received particular attention in light of their regulatory effects on inflammatory cells [7–11]. The focus of this chapter is on Siglec-8, a member of this receptor family, which in view of its expression and functional effects on mast cells, eosinophils and basophils, provides a potential therapeutic target for the treatment of allergic diseases [12, 13].

Characteristics of Siglec-8 and the CD33-Related Subgroup of Siglecs

Siglec-8, like the other members of the Siglec family, is a single pass transmembrane protein that belongs to the Ig superfamily. Siglecs are characterized by their sequence similarities and ability to bind sialic acid [7]. They contain a homologous N-terminal V-set Ig-like domain, followed by variable numbers of C2-set Ig domains, ranging from 1 in CD33 (Siglec-3) to 16 in sialoadhesin (Siglec-1). In humans, 13 members have been identified and are widely expressed on a variety of hematopoietic cells with the exception of myelin-associated glycoprotein (MAG/Siglec-4), which is expressed on oligodendrocytes and Schwann cells [8, 9]. Siglecs can be divided into two subgroups: the CD33-related Siglecs (hereafter abbreviated as CD33rSiglecs) and a second category that includes CD22 (Siglec-2) on B lymphocytes, sialoadhesin on macrophages and MAG. For CD33rSiglecs, sequence similarities are seen in the V-set Ig domain and two cytosolic tyrosine-based motifs. It was a CD33 homology screening of expressed sequence tag sequences from a human eosinophil cDNA library, generated from a patient of one of the authors (BSB) with idiopathic hypereosinophilic syndrome (HES), that led to the discovery of Siglec-8 (also named SAF-2 for sialoadhesin family member-2) [12, 14]. A subsequent analysis of human genomic DNA revealed the existence of an alternatively spliced form initially called Siglec-8L (L for long form), although now the term Siglec-8 refers to the full-length form and the original isoform lacking most of the cytoplasmic putative signaling domain is called Siglec-8 "short-form." Human eosinophils, basophils and mast cells express both isoforms, but while Siglec-8 is consistently expressed on these cells, the short form is only detected 65% of the time [15, 16]. Nevertheless, the selective expression of Siglec-8 on these cells has been independently confirmed using an unbiased comparative transcriptome approach [17]. Both isoforms contain an identical N-terminal lectin domain, two extracellular immunoglobulin-like domains, and a transmembrane region, but Siglec-8L possesses a much longer cytoplasmic tail (see Fig. 1). Siglec-8, but not the short form of Siglec-8, contains two cytosolic tyrosine-based motifs, which are characteristic for CD33rSiglecs, and are thought to be responsible for the inhibitory function of these receptors. The membrane-distal domain (EYSELK) is similar to a motif ((D/E)Y(S/T/A)E(I/V)(K/R)) found in CD150 (signaling lymphocyte activation motif LHYATL). The membrane-proximal domain has a consensus motif (LHYATL) that resembles a classical immunoreceptor tyrosine-based inhibitory motif (ITIM, specifically (I/L/V)xYxx(L/V)) found in

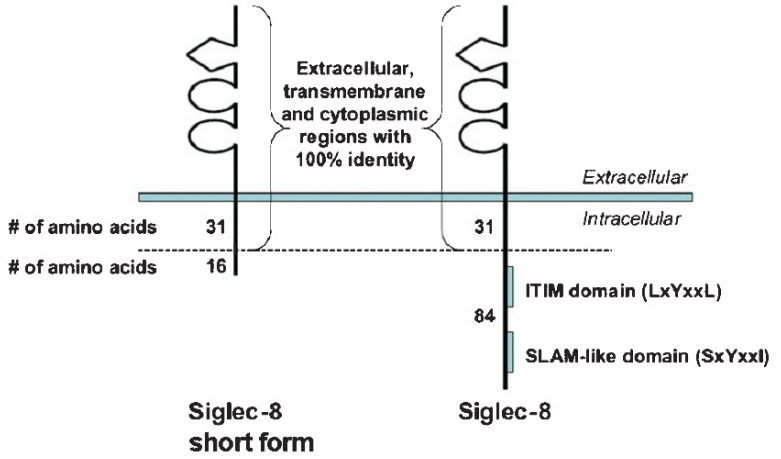

Fig. 1 Schematic representation of Siglec-8 and its short form. The predominantly expressed form of Siglec-8 contains an identical extracellular transmembrane, and a 31 amino acid long membrane-proximal cytoplasmic domain to that of the originally described Siglec-8 "short form." Siglec-8 has a longer cytoplasmic tail (an additional 84 amino acids instead of 16) possessing two tyrosine-based motifs, including a consensus immunoreceptor tyrosine-based inhibitory motif (ITIM) and another motif that resembles that seen in signaling lymphocyte activation molecule (SLAM) (CD150). Note that the scale of the intracellular domain is drawn larger than that of the extracellular domain for clarity (Reproduced with permission from [13])

many other inhibitory receptors on hematopoietic cells (e.g., killer cell Ig-like receptors, FcγRIIB, gp49B1). ITIMs, after ligation of the receptor, become phosphorylated and recruit inhibitory phosphatases such as Src homology domain 2 (SH2)-containing tyrosine phosphatase-1 (SHP-1), SHP-2 and SH2-containing inositol phosphatase (SHIP). The recruitment of these phosphatases may result in cell inhibition and even death [18–23]. For CD33rSiglecs both recruitment of SHP-1 [24–34] and inhibition of cell function have been shown to depend on the proximal ITIM motif [33, 35]. Other molecules that have been reported to associate with Siglecs at their cytoplasmic tail are plasma membrane calcium-ATPase [36], Grb2 [37–39], S100beta [40], phospholipase C-gamma [37, 41, 42], and the protein tyrosine kinases Syk [37, 42] and Fyn [43]. Both isoforms of Siglec-8 exist in human eosinophils, basophils and mast cells, but the presence of the two cytosolic tyrosine-based motifs in Siglec-8 suggests that this particular isoform is biologically functional. Further studies are needed to understand the biological function of each isoform.

Binding Properties of Human Siglec-8 and Its Functional Mouse Paralog Siglec-F

Evolutionarily, Siglecs represent a relatively new group of receptors that appeared once the sialic acid biosynthesis pathways had already been established [7]. In mammalians, sialic acids are abundantly expressed on secreted and cell-bound

glycoproteins and glycolipids. Although CD33rSiglecs all bind to various configurations of sialic acid-containing glycans, little is known about their exact physiological ligands. However, it is now appreciated that the binding specificity of a Siglec is determined by the terminal sialic acid, its linkage to the subterminal oligosaccharide, and the structure of the subsequent underlying sugars. The Siglecs seem to have tailored their binding properties to the evolutionary changes that occurred in their sialic acid-containing ligands [9, 44]. Hence, there are considerable intra- and interspecies differences among Siglecs in terms of binding specificity.

Specific lectin ligand identification has been facilitated by the development of carbohydrate array technology [45, 46]. In cooperation with The Consortium for Functional Glycomics (www.functionalglycomics.org) funded by the National Institutes of General Medical Sciences, hundreds of carbohydrate-based structures have been screened for their ability to bind human and mouse Siglecs (all data are eventually published online). Among human Siglec members, Siglec-8 exhibited a unique binding specificity for a single glycan, NeuAcα2-3(6-O-sulfo)Galβ1-4[Fucα1-3]GlcNAc, also referred to as 6′-sulfo-sLe^x because it consists of sialyl Lewis X with a sulphate ester at the 6-position of the galactose residue [47]. This is to be distinguished from the ligand for Siglec-9 (and coincidentally for L-selectin), namely 6-sulfo-sLe^x where the sulfate group is located on the GlcNac (see Fig. 2). Intriguingly, 6′-sulfo-sLe^x is recognized with equally high and unique specificity by mouse Siglec-F [48] that, like Siglec-8, is selectively expressed on eosinophils, but additionally found on alveolar macrophages [49, 50]. This was somewhat surprising as Siglec-F, based on sequence homology alone, was initially thought to be the mouse ortholog of human Siglec-5 [16]. Despite this, the sequence similarities of Siglec-8 and Siglec-F are considerable in conserved regions incorporating the putative ligand-binding domain. In light of the highly specific preference for the same glycan ligand 6′-sulfo-sLe^x, and its common selective expression on eosinophils, it is now generally thought that mouse Siglec-F and human Siglec-8 represent functionally convergent paralogs [48, 49, 51]. A Siglec-F mouse model might therefore provide a valuable and important means to study the role of Siglec-8 in eosinophil function and eosinophil-mediated diseases in vivo ([49, 50, 52] and see below).

In Vitro Studies of Siglec-8

To date, most data on the function of CD33rSiglecs have been generated in vitro. One experimental limitation of functional studies of CD33rSiglecs has been the lack of specific ligands. Therefore, such studies were typically performed using cross-linking strategies with Siglec-specific monoclonal antibodies. Using this approach in the first functional study on Siglec-8, published in 2003, we found that cross-linking of this receptor on eosinophils isolated from healthy donors induced rapid and marked apoptosis [53]. In the same study, experiments using the pan-caspase inhibitor z-VAD-fmk revealed caspase-dependence of Siglec-8-mediated death. The involvement

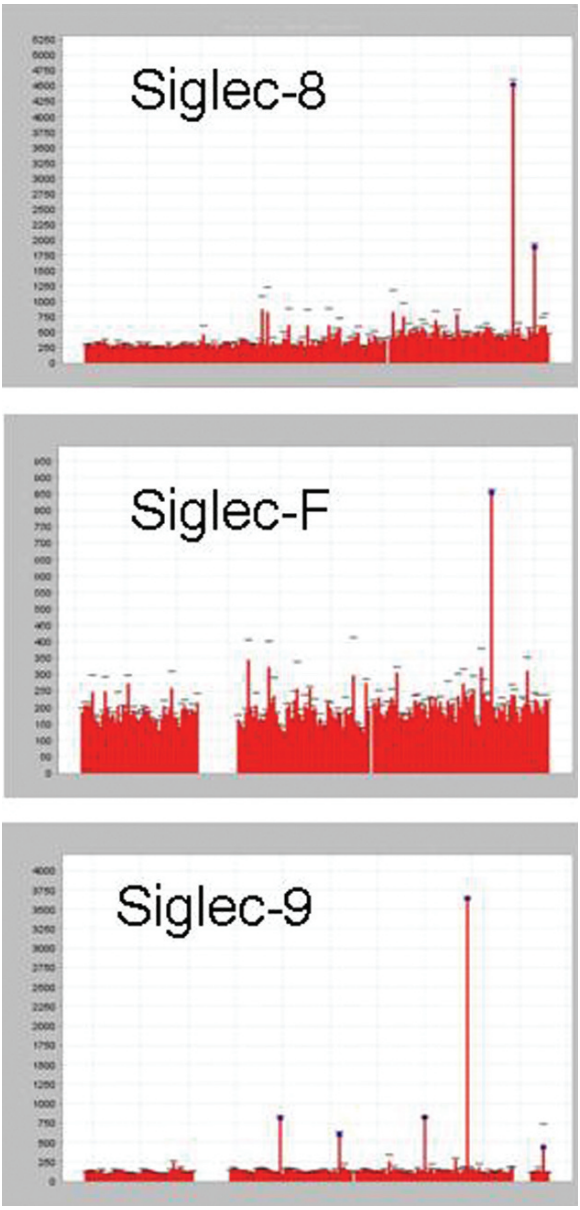

Fig. 2 Binding patterns of Siglec-8, Siglec-F, and Siglec-9 to their carbohydrate ligands. Siglec-8 and Siglec-F preferentially and selectively (major bar) bind 6′-sulfo-sLe^x. In contrast, Siglec-9 binds with high affinity (major bar) to the related molecule 6-sulfo-sLe^x (From www.functionalglycomics.org/glycomics/publicdata/selectedscreens.jsp)

of caspases was confirmed in subsequent studies showing cleavage of the signaling and effector caspase-3, caspase-8, and caspase-9 and inhibition of Siglec-8-mediated death by pharmacological inhibitors of caspases-8 and caspase-9 [54, 55]. Besides caspases, mitochondria appear to play a central role in the execution of Siglec-8-mediated death because cross-linking of Siglec-8 induced a marked reduction in

the mitochondrial membrane potential. Such loss of mitochondrial integrity with associated release of cytochrome *c,* is a characteristic feature of apoptosis [56].

Further evidence of the importance of mitochondria in Siglec-8-mediated death was provided by studies using pharmacological inhibitors of the respiratory chain. Complexes I and III of the electron transport chain are major sites for reactive oxygen species (ROS) production [57, 58]. ROS play an important role in the induction of apoptosis under both physiologic and pathologic conditions [59]. Rotenone and antimycin, inhibitors of the respiratory chain acting on complexes I and III respectively, prevented Siglec-8-induced apoptosis, but had no effect on spontaneous eosinophil apoptosis [55]. Further experiments with diphenyleneiodonium (DPI), a potent inhibitor of ROS production from various flavoenzymes including NADPH oxidase and mitochondria, confirmed the importance of ROS in Siglec-8-mediated death and showed that ROS involvement precedes mitochondrial injury [55]. Based on these results, a model has been proposed in which Siglec-8 cross-linking triggers the "intrinsic" stress-mediated apoptotic pathway through sequential ROS involvement, mitochondrial dysfunction, and caspase cleavage, which then leads to cellular execution [13].

It has also been found somewhat paradoxically that Siglec-8-induced eosinophil death is increased in the presence of proinflammatory cytokines such as IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (see Fig. 3) [53]. This was an intriguing and surprising finding as IL-5 and GM-CSF are well-known

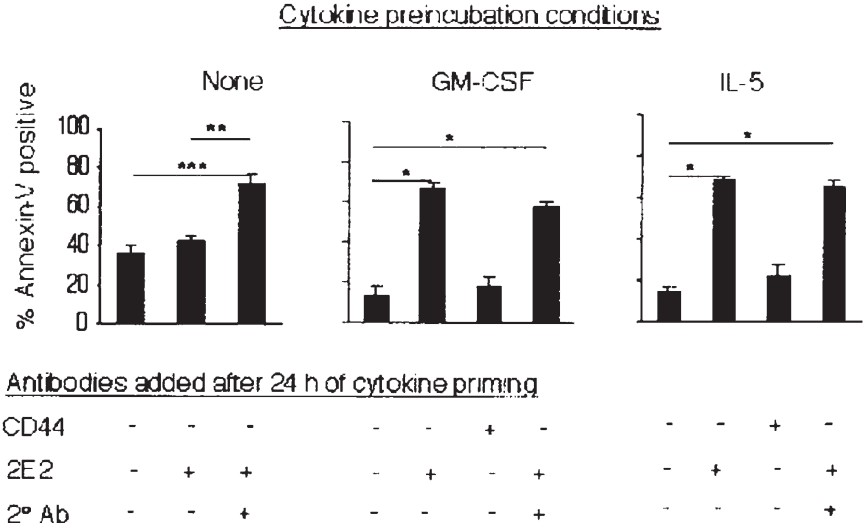

Fig. 3 IL-5 or GM-CSF priming enhances eosinophil apoptosis in response to Siglec-8 antibody. Eosinophils were preincubated with or without IL-5 or granulocyte-macrophage colony-stimulating factor (GM-CSF) (each at 30 ng/ml) for 24 h. Antibodies (2.5 µg/ml) were then added to the cultures, as indicated, and apoptosis was analyzed using annexin-V staining 24 h later. Data are presented as mean ± SEM from four experiments. $^{*}P < .0005$; $^{**}P < .005$; $^{***}P < .05$ (Reproduced from [53] with permission)

eosinophil survival factors that delay apoptosis of eosinophils by interference with molecular apoptotic pathways. Analogous to Siglec-8 in eosinophils, increased death was observed in normal neutrophils after stimulation of Siglec-9 in vitro with proinflammatory cytokines including GM-CSF, interferon-α (IFN-α), and IFN-γ, and in "inflammatory" neutrophils from patients suffering from rheumatoid arthritis and sepsis [60]. In the presence of cytokines, Siglec-9-induced death was ROS-dependent and involved both caspase-dependent and caspase-independent forms of neutrophil death characterized by an autophagy-like morphology. It has been concluded that Siglecs may act as a "safe-guard" mechanism that might overcome the survival effects of anti-apoptotic cytokines at the site of inflammation by the recruitment of alternative caspase-independent death pathways [10, 61]. Future studies with eosinophils are required to validate this concept.

Recently, intravenous immunoglobulin (IVIg) preparations have been shown to contain natural autoantibodies against Siglec-8 and Siglec-9 [61–63]. Intravenous immunoglobulin, used for replacement treatment in humoral immunodeficiencies, is increasingly appreciated as a potential therapy for the treatment of inflammatory diseases, especially when given at higher doses. One potential mechanism for the anti-inflammatory effects of IVIg is the cytotoxic effect mediated by naturally occurring autoantibodies directed against death receptors on immunocompetent cells. For instance, agonistic antibodies against the death receptor Fas (CD95) present in IVIg have been shown to help patients with toxic epidermal necrolysis by inducing apoptosis in neutrophils, lymphocytes, and monocytes in a caspase-dependent manner [64–67]. There is recent evidence that naturally occurring Siglec-8 autoantibodies are responsible for a dramatic acceleration of eosinophil death observed in vitro after IVIg incubation in the presence of IL-5, GM-CSF, and leptin or in cells derived from patients with hypereosinophilic syndrome (HES) [63]. The effect on HES eosinophils may be explained by prior in vivo exposure and priming in response to IL-5, which can be detected in the serum of many HES patients [68, 69]. Both the efficacy and potency of IVIg in cytokine-primed or HES eosinophils was increased and the cells died by the recruitment of caspase-dependent and caspase-independent death pathways, while the death of normal unactivated cells was exclusively caspase-dependent [63]. The role of Siglec-8 autoantibodies for the enhanced cytotoxicity of IVIg in the presence of cytokines was confirmed by subsequent studies using Siglec-8 autoantibody-depleted IVIg (see Fig. 4) and pharmacologic blocking experiments with a recombinant Siglec-8–Fc fusion protein. It appears that autoantibodies against Siglec-8 are most effective in the regulation of eosinophil survival in an inflammatory setting. The pathophysiologic or pharmacologic role of these autoantibodies in vivo remains to be determined, but this may explain the efficacy reported when high-dose IVIg has been used to treat patients with eosinophilic disorders. For example, in patients with Churg-Strauss syndrome and chronic urticaria, diseases characterized by tissue eosinophilia and skin mast cell activation, respectively, beneficial effects of IVIg treatment have been observed [70–72]. Siglec autoantibodiy titers might be useful predictors of IVIg efficacy in these conditions, and variations in these titers might explain the inconsistencies in the literature regarding efficacy. Although the

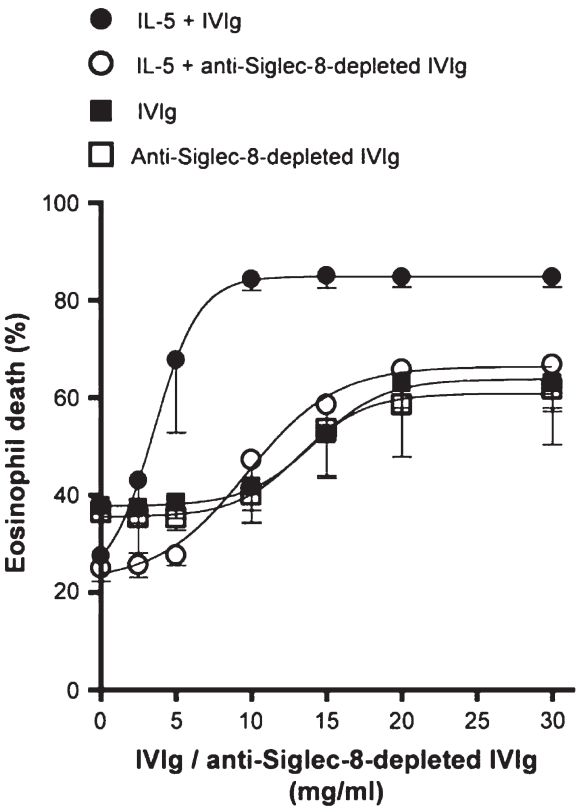

Fig. 4 Concentration–effect curves of total IVIg and anti-Siglec-8-depleted IVIg, respectively, in 24-h eosinophil cultures. In the absence of IL-5 pretreatment, both death efficacy and potency of anti-Siglec-8-depleted IVIg was similar to total IVIg. Depletion of anti-Siglec-8 autoantibodies, however, resulted in loss of IL-5-mediated increase of death efficacy and potency of IVIg ($n = 4$) (Reproduced from [63] with permission)

evidence about IVIg treatment of allergic diseases and asthma is incomplete, some studies have shown high-dose IVIg to be beneficial [73–75]. In these clinical studies it should be considered that the individual cytokine profile of patients may influence the efficacy of IVIg treatment, as some effector mechanisms of IVIg, such as Siglec-mediated immunoregulation, may depend on target cell priming. Future work should aim to identify those patients with eosinophilic and other inflammatory diseases that are most likely to benefit from IVIg treatment.

Although Siglec-8 is expressed on mast cells and basophils, few studies have focused on its biology on these cell types. Using mast cells cultured from human $CD34^+$ progenitor cells, Siglec-8 appears on the cell surface during maturation, with similar kinetics of appearance of other mast cell markers such as histamine and surface FcεRI [76]. Recent data suggest that in such culture-derived mast cells cross-linking of Siglec-8 by monoclonal antibodies does not induce apoptosis, but does inhibit FcεRI-dependent mediator release [77]. Similar results have been found in Siglec-7- or Siglec-9-transfected rat basophilic leukemia (RBL) cells, where Siglec cross-linking inhibits serotonin release [35]. Future studies are required to determine the mechanism of the inhibitory effect of Siglec-8 on mast cells, as well as the biological effects of this receptor on basophil function. It is important to note, however, that both these cell types also express CD33 (Siglec-3)

and the Siglecs most brightly expressed on human mast cells and basophils are Siglec-6 and Siglec-5, respectively [78].

Studies in Vivo

Siglec-F Biology

Mouse models have been employed to study the in vivo role of Siglec-8. As Siglec-8 is not expressed in mice, its closest functional paralog in the mouse, Siglec-F, has been used as a surrogate target. The common expression of Siglec-8 and its mouse paralog on eosinophils [16, 49–51] makes it possible to study the in vivo role of these molecules in allergic and hypereosinophilic disease models.

There is recent evidence that stimulation of mouse Siglec-F triggers eosinophil death in mice, analogous to its human paralog Siglec-8. It has been shown that cross-linking of the Siglec-F receptor by systemic administration of a specific monoclonal antibody results in the depletion of circulating eosinophils [50, 52]. Eosinophils isolated from IL-5 transgenic mice underwent increased death after incubation with antibodies against Siglec-F [49, 50]. Hence, it appears that the phenomenon of eosinophil death induction also holds true for Siglec-F, similar to the observations made with Siglec-8 in human studies on eosinophils in vitro. Together these findings support the hypothesis that immunoregulation by Siglecs is particularly effective in an inflammatory setting where granulocyte priming cytokine levels are enhanced [10].

To further explore this hypothesis, Siglec-F-deficient mice and wild-type mice were sensitized and challenged with ovalbumin (OVA), as a model for allergic inflammation [49]. Although these mice had normal numbers of blood and tissue eosinophils under baseline conditions, allergen-challenged Siglec-F-null mice show increased lung eosinophil infiltration, enhanced bone marrow and blood eosinophilia, delayed resolution of lung eosinophilia, and reduced peribronchial eosinophil apoptosis. A trend towards enhanced airways hyperreactivity was also observed. Similar preliminary results were also reported using the classical ovalbumin (OVA) mouse model of asthma following administration of Siglec-F antibodies [52]. These data strongly support the proposed negative feedback role for Siglec-F and predict a regulatory role for human Siglec-8 in the pathogenesis of human eosinophil-mediated disorders.

Natural Tissue Ligands for Siglec-8 and Siglec-F

Tissue expression and prevalence of the Siglec-F ligand may significantly affect its inhibitory and death-promoting activity, and determine the outcome of an inflammatory process at a particular anatomic site. While it is known that both mouse Siglec-F and its human counterpart Siglec-8 recognize the glycan 6′-sulfo-sLe^x

(see above), little is known about location and identity of the physiological ligands carrying this sialoside. Immunohistochemical investigations probing tissue sections of mice with recombinant Siglec-F Ig fusion protein [49, 79] and Siglec-8 Ig fusion protein [79] localized potential ligands for these receptors to airway epithelial cells. Based on morphometric analysis of the bronchial epithelial staining intensity, it has been concluded that Siglec-F ligands throughout the lung are upregulated following OVA sensitization and challenge [49]. Modestly increased expression of Siglec-F on peripheral, bone marrow, or spleen eosinophils was detected after OVA challenge, although levels of Siglec-8 on eosinophils from various types of patients, following eosinophil activation in vitro, and on lung-derived eosinophils, do not change (unpublished observations). Based on the epithelium-associated expression of Siglec-F ligands, the occurrence of sulfated sLe^x structure and the propensity to alter glycosylation and sulfation patterns in inflammatory conditions, bronchial mucins have been suggested as candidate ligands for Siglec-F and Siglec-8 [49]. Further studies are underway to definitively identify the nature of the physiological ligands of Siglec-F and Siglec-8.

Another approach to determine the tissue localization of the ligands might be to examine the expression of critical enzymes required for the synthesis of the requisite glycan ligand 6′-sulfo-sLe^x. Synthesis of 6′-sulfo-sLe^x requires sulfation at the 6-position of the galactose, and the presence of this sulfate group is critical for binding to Siglec-8 or Siglec-F (see Fig. 5) [47, 48]. This sulfation step is likely to occur in the Golgi apparatus of the cell by the enzyme keratin sulphate galactose 6-*O*-sulfotransferase (KSGal6ST), also referred to as carbohydrate sulfotransferase-1 (CHST-1), which is known to sulfate galactose residues linked

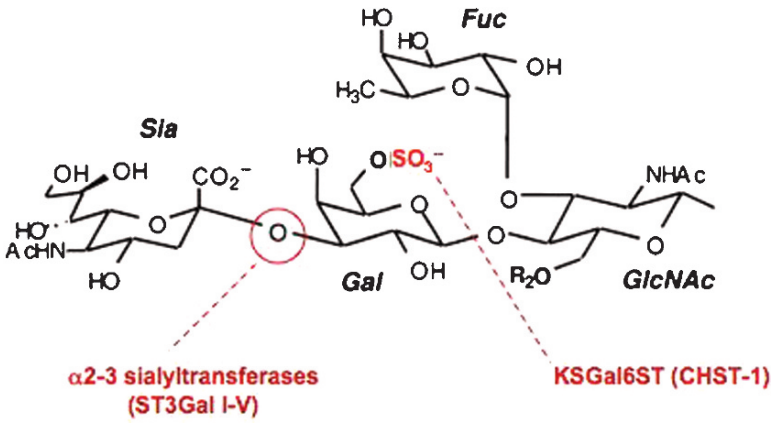

Fig. 5 Structure of NeuAcα2-3(6-O-sulfo)Galβ1-4[Fucα1-3]GlcNAc also called 6′-sulfo-sLe^x, the glycan ligand for Siglec-8 and Siglec-F. Enzymes required for its biosynthesis are shown. The enzyme keratin sulfate galactose 6-*O*-sulfotransferase (KSGal6ST), also referred to as carbohydrate sulfotransferase-1 (CHST-1), mediates the sulfation at the 6-position of the galactose, probably in the Golgi apparatus of the cell. Alpha2,3-sialyltransferases (likely ST3Gal I–V) add the terminal sialic acid to the subterminal galactose

to *N*-acetyl glucosamine. Other enzymes required for the biosynthesis are α2,3-sialyltransferases that add the terminal sialic acid to the subterminal galactose. Ongoing studies using immunohistochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR) should determine the tissue localization of these enzymes and whether the expression levels of these enzymes are differentially regulated during eosinophilic inflammation. Furthermore, strategies using mice lacking enzymes required for the biosynthesis of 6′-sulfo-sLe^x might provide an in vivo model to study the functional effects of ligand depletion on Siglec-F biology.

Future in vivo studies elucidating mechanisms involved in Siglec-F downstream signaling may serve to confirm the biological relevance of data achieved for Siglec-8 in vitro. As mentioned above, inhibitory phosphatases are felt to play an important role in Siglec-associated signaling pathways. For instance, SHIP-1-deficient mice spontaneously exhibit progressive and severe pulmonary inflammation with accumulation of macrophages, lymphocytes, neutrophils, and eosinophils; mucous hyperplasia; airway epithelial hypertrophy; and subepithelial fibrosis, accompanied by exaggerated production of TH2 cytokines and chemokines [80]. A similar approach was used to implicate SHP-1 in controlling allergic pulmonary inflammation [81]. This raises the question of whether some of these pathological features, such as the persistent eosinophilia, are due to the SHIP-1 or SHP-1 deficiency inactivating the Siglec-F apoptosis pathway in recruited eosinophils. In planned studies, NADPH oxidase-deficient mice will be used to directly investigate the involvement of ROS in Siglec-F signaling. In earlier work, it has been shown that neutrophils from human subjects with chronic granulomatous disease (CGD) that genetically lack NADPH oxidase activity fail to undergo Siglec-9-induced neutrophil apoptosis, suggesting that ROS are indispensable signaling and effector molecules [60]. Future in vivo studies, discussed below, may be helpful not only to understand the biology of Siglec-F and Siglec-8, but also to test potential therapeutic strategies that target these receptors.

Summary and Conclusions

Taken together, in vitro studies provide functional evidence that Siglec-8 acts as an inducer of death or as a secretory inhibitory receptor depending on the cell type. Biologically, cell death may be the most efficient way to regulate the persistence of eosinophils at the site of inflammation, while inhibition of mediator release may be a more relevant means to control the effector functions of tissue mast cells. The increased potency and efficacy to trigger cell death in the presence of cytokines observed in vitro, at least for eosinophils, contributes to the notion that Siglec-8 provides a “safe guard” mechanism to control allergic responses [10]. Our current knowledge about the biology of Siglec-8 supports the notion that this receptor may become the target of future therapeutic strategies. The increased efficacy and potency to transmit death signals into eosinophils in a cytokine-rich microenvironment and the inhibitory effects on mast cell activation may be part of a natural

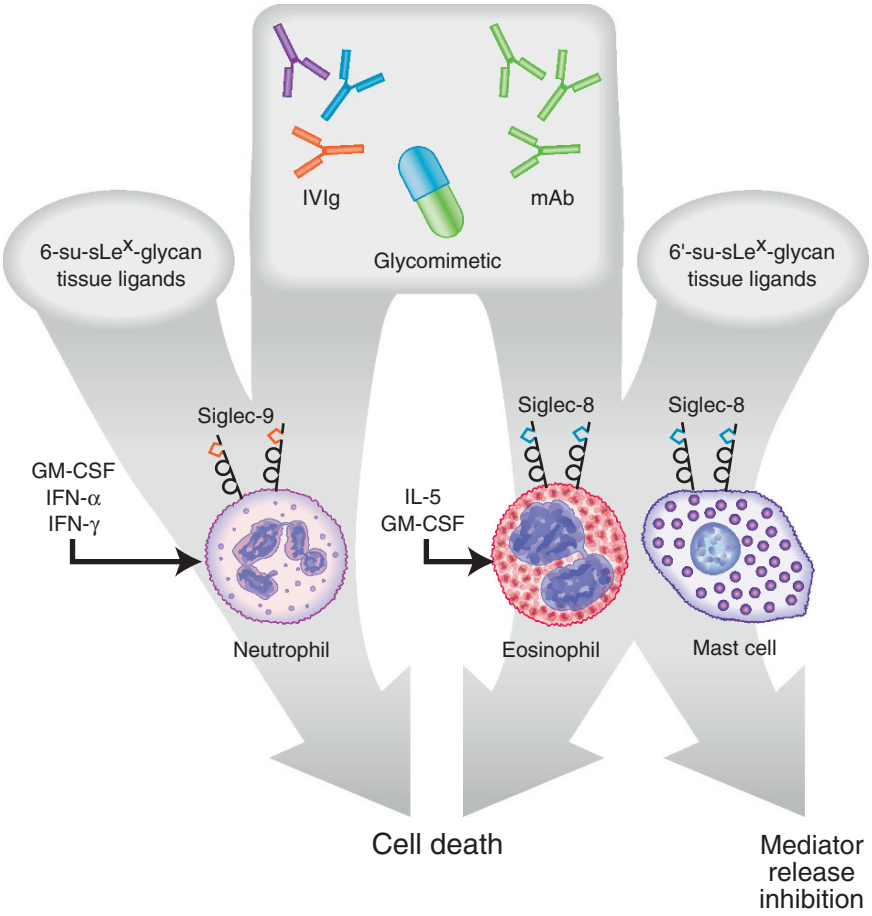

Fig. 6 Proposed natural and therapeutic pathways for negative regulation of neutrophils, eosinophils and mast cells by Siglec-8 and Siglec-9. Engagement of Siglec-8 by tissue ligands containing the glycan structures 6′-sulfo-sLe^x may induce cell death in eosinophils and inhibit the secretion of inflammatory mediators by mast cells. Similarly, engagement of Siglec-9 on neutrophils by tissue ligands expressing the related but different glycan, 6-sulfo-sLe^x, may facilitate death in these cells. In the presence of the indicated cytokines at the site of inflammation, Siglec-mediated cell death of eosinophils and neutrophils is enhanced due to priming effects. This natural anti-inflammatory system might be pharmacologically exploited using agonistic Siglec cross-linking therapeutics, such as intravenous immunoglobulin (IVIg), monoclonal antibodies or glycomimetics (Art by Jacqueline Schaffer)

feedback system that may be pharmacologically exploited (see Fig. 6). Potential pharmacological strategies to trigger Siglec-8 may include IVIg or monoclonal antibodies. Biomarkers are required for such a distinction and may include functional assays that determine the in vitro response of patient eosinophils to Siglec engagement. Given the potential risks, costs, and inherent scarcity of IVIg, careful

consideration of the indications for and administration of IVIg is warranted [73]. An alternative approach to consider is to specifically target Siglec-8 by administration of a synthetic molecule that imitates the natural ligand, 6′-sulfo-sLe^x. Such molecules are generally referred to as glycomimetics and have the additional theoretical advantage of possibly being applied topically or inhaled. Regardless of therapeutic strategy employed, the specificity of Siglec-8 expression on key effector cells of the allergic response make this molecule an attractive target for inhibiting or reducing the numbers of these cells in conditions where they contribute to disease pathophysiology.

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Effects of Nitric Oxide on Mast Cells: Production, Functions, and Mechanisms of Action

Tae Chul Moon, Yokananth Sekar, and A. Dean Befus

Introduction

Mast cells (MC) are widely distributed in extravascular areas throughout the body, especially at surfaces that interface with the external environment. They originate from $CD34^+$ progenitor cells in bone marrow [1]. Following their recruitment into tissues they mature and express diverse biological functions under the influence of microenvironmental signals, locally derived growth factors and cytokines. Activated MC play important roles in allergic and inflammatory responses through secretion of a wide array of inflammatory mediators [2]. Thus they have long been implicated in anaphylaxis and other allergic disorders by virtue of their ability to be activated through FcεRI-bound antigen-specific IgE. Recently MC have become increasingly recognized for their roles in innate and adaptive immune responses [2, 3].

MC are highly heterogeneous depending on local microenvironmental factors that alter gene expression and cell function. Discrete MC subtypes have been classified based on their serine protease expression in rodents (mucosal MC [MMC] and connective tissue MC [CTMC]) and humans (MC_T [contain tryptase but not chymase], MC_{TC} [contain both tryptase and chymase], and MC_C [contain chymase but not tryptase]). In this chapter, we review the information on nitric oxide synthase (NOS) expression and nitric oxide (NO) production by MC, as well as the effects of NO on regulation of MC phenotype and function (Fig. 1).

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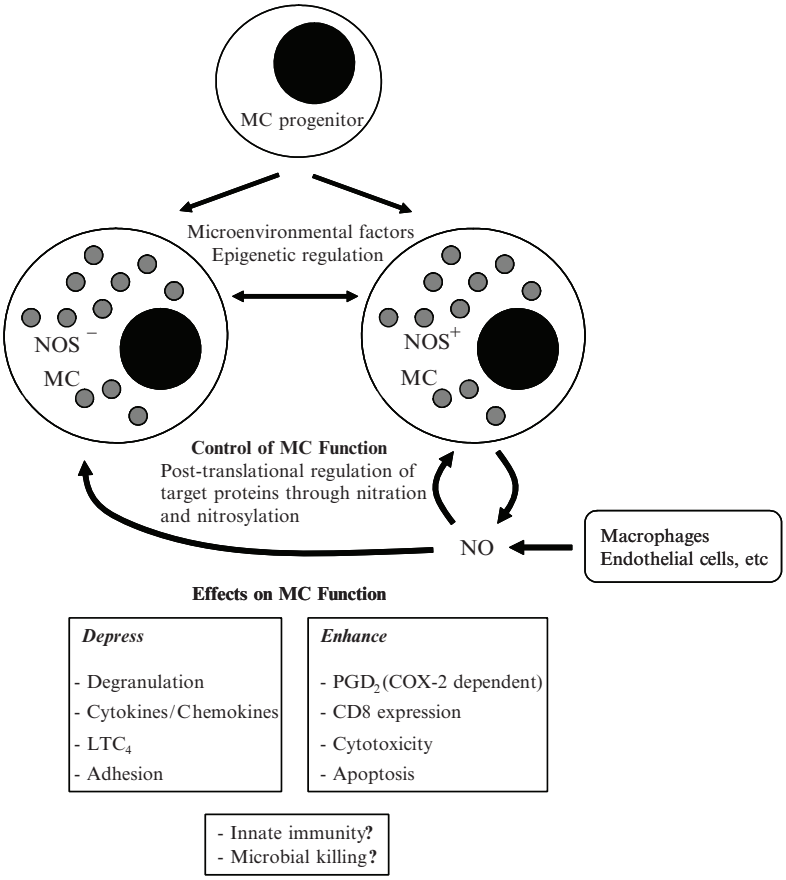

Fig. 1 Conceptual model of NOS expression in mast cells and effects of nitric oxide on mast cell function

Nitric Oxide and Nitric Oxide Synthases

NO is a gas and free radical with a half-life of a few seconds. One of its biological activities, namely vascular smooth muscle relaxation, leads to an early name for the unidentified factor, endothelium-derived relaxing factor (EDRF). This small diatomic molecule can rapidly diffuse across cell membranes, and can regulate critical cellular functions. It also has been reported as a mediator of cellular damage in a range of conditions [4]. NO is synthesized from L-arginine by NOS, together with several cofactors. There are three NOS isoforms, with their genes located on different chromosomes, NOS 1 (neuronal, nNOS), NOS 2 (inducible, iNOS), and NOS 3 (endothelial, eNOS). NO synthesized by different NOS isozymes can

elicit distinct physiological and pathological functions according to the location, microenvironmental factors, and concentration.

NOS 1, the first NOS to be cloned and purified, was originally found in neurons and NO generated by this isozyme has a role as a neurotransmitter. The inducible isozyme, NOS 2 has been found in many cells including activated macrophages and epithelial cells. NO produced by NOS 2 has an effect in host defense as an antimicrobial, antiviral, antiparasite, and antitumor factor, but it can also be involved in pathophysiology of asthma, arthritis, multiple sclerosis, colitis, psoriasis, neurodegenerative diseases, tumor development, transplant rejection, or septic shock [5]. NOS 3 was first found in endothelium and the major function of NO synthesized by endothelial cells involves regulation of blood flow and blood pressure through vasodilation. However, all the NOS isozymes can be expressed in a wide range of cell types including leucocytes and function in homeostasis.

The expression of specific NOS isozymes in different tissues can be regulated by epigenetic mechanisms. DNA methylation and histone modifications represent the major epigenetic mechanisms implicated in the regulation of gene transcription in mammals. DNA methylation and histone acetylation can play important roles in control of transcription and expression of NOS 3 in endothelial cells. Moreover, histone deacetylation by histone deacetylase (HDAC) 1 is involved in repression of NOS 3 expression in cells that do not normally express NOS 3. The constitutively repressed NOS 3 gene can be activated for expression by HDAC inhibitor, trichostatin A (TSA) [6]. The inducibility of NOS 2 gene varies among species and between different tissues within the same species. For example, primary human endothelial cells and vascular smooth muscle cells are resistant to NOS 2 induction, whereas certain human cell types such as A549 pulmonary adenocarcinoma cells are capable of NOS 2 expression. Differences in the methylation status of the NOS 2 promoter are responsible for this observed difference in inducibility of the NOS 2 gene [7]. Histone deacetylation can enhance induction of the NOS 2 gene [8]. Unfortunately, there is no information about epigenetic control of NOS expression in MC (see below).

Nitric Oxide Synthase Expression and Nitric Oxide Production by Mast Cells

NOS isozymes have been identified from various mast cell sources using RT-PCR, western blotting, immunostaining, or confocal microscopy (Table 1, see [5] for details). All three isoforms of NOS have been detected in rat and human MC. However, NOS 2 and NOS 3, but not NOS 1, have been found in mouse MC. NOS 1 was expressed in 30% of human intestinal MC and colocalized with tumor necrosis factor (TNF) in granules of human nasal mucosa MC. Although NOS 1 and NOS 3 have been regarded as constitutive NOS, their expression can be upregulated in certain conditions. In HMC-1, NOS 1 expression was increased when the cells were treated with IFN-γ. As with NOS 1, NOS 3 was upregulated by IFN-γ in RBL-2H3, rat cultured MC, and HMC-1, and by cerebral ischemia in

Table 1 Expression of NOS in mast cells (See [5] for details)

NOS		MC	Conditions/treatment
NOS 1 (nNOS)	Rat	PMC	Constitutive
	Human	Skin MC	Constitutive
		Nasal mucosa MC	Constitutive
		HMC-1	Constitutive, IFN-γ
		IMMC	Constitutive
NOS 2 (iNOS)	Mouse	P815	L-arginine/TNF
		Uterus MC	Ovariectomy, pregnancy
		BMMC	FcεRI cross-linking, TNF/IFN-γ
		Skeletal muscle MC	IFN-γ, TNF, ischemia/reperfusion
	Rat	Angiogenic pedicle MC	Post-cauterization
		PMC	FcεRI cross-linking, IFN-γ, TNF, anti-CD8, constitutive
		Skeletal muscle MC	Ischemia/reperfusion
	Human	Peribiliary MC	Liver cirrhosis
		HMC-1	PMA + A23187
NOS 3 (eNOS)	Mouse	BMMC	Constitutive
	Rat	Neurohypophyseal MC	Cerebral ischemia
		RBL-2H3	Constitutive, IFN-γ
		PMC	Constitutive
		BMMC	Constitutive
		IMMC	Constitutive
		RCMC	Constitutive, IFN-γ
	Human	Skin MC	Constitutive
		HMC-1	Constitutive, IFN-γ
		LAD2	Constitutive
		CBMC	Constitutive
		IMMC	Constitutive

BMMC, bone marrow-derived cultured mast cell; CBMC, cord blood-derived mast cell; IMMC, intestinal mucosal mast cell; RCMC, rat cultured mast cell; PMC, peritoneal mast cell

neurohypophyseal mastocytes. NOS 2 is inducible and can produce large amounts of NO in response to pregnancy, ischaemia/reperfusion injury of skeletal muscle, angiogenesis, antibody against CD8, and liver cirrhosis as well as by several inflammatory stimuli such as IFN-γ, IL-1β, and cross-linking of IgE in MC. FcεRI-mediated MC activation can induce NO generation through NOS 2 expression (see [5] for details). Bidri et al. reported that mouse bone marrow-derived MC (BMMC) cultured with IL-3 for 3 weeks express NOS 2 mRNA when activated by IgE cross-linking. Furthermore, BMMC cultured with SCF for an additional 2 weeks express some phenotypic characteristics of CTMC and express NOS 2 mRNA, and this is increased when they are activated by IgE cross-linking [9].

NOS expression and NO generation in MC can be regulated by certain cytokines through autocrine as well as paracrine loops (Fig. 1). TNF, a cytokine that can be preformed and stored in some MC and also de novo synthesized following MC activation, induces NO production and NOS 2 expression in rat peritoneal MC and the mouse P815 mastocytoma cell line. IL-1β enhances NO generation

when rat peritoneal MC are activated by calcium ionophore and IL-1β can also inhibit release of platelet-activating factor (PAF) from peritoneal MC through an NO-dependent mechanism. IL-10 inhibits both constitutive and antigen-induced NO production by rat peritoneal MC. IL-4 inhibits NO production and enhances anti-IgE-mediated MC degranulation in peritoneal cells. However, IFN-γ overcomes the IL-4-mediated enhancement of serotonin release and suppression of NO production. It was thus suggested that IL-4 and IFN-γ reciprocally regulate MC degranulation through their effects on NO production by monocyte/macrophages in the MC-containing peritoneal cell preparation (see [5] for details). However, in spite of many studies on NOS expression and substantial evidence of NO production by MC, controversy exists about the generation of NO by MC.

Controversy About Nitric Oxide Production by Mast Cells

The IFN-γ-induced inhibitory effect of NO on IgE-dependent MC degranulation was exhibited in mixed cell populations but not in highly enriched rodent MC in the hands of some workers [10, 11]. Moreover, recently it was reported that rodent and human mast cells produce intracellular reactive oxygen species (ROS), but do not generate intracellular NO or express NOS 2 protein (NOS 1 and NOS 3 were not studied) [12]. These results are not consistent with our findings or those of others (see Table 1). Initial studies in the early 1990s used impure (85–90%) rat peritoneal MC, and NO may have been generated in large part by contaminating cells. It is difficult to rule out the possibility that low levels of NOS gene expression and NO production attributable to MC are not due to production by contaminating macrophages in cell populations isolated from the peritoneal cavity or tissues. However, using live cell confocal analysis we reported that IgE/Ag and IFN-γ induced production of intracellular NO in rat peritoneal MC, and that alterations in NOS activity via availability of the cofactor tetrahydrobiopterin (BH_4) may be critical to production of NO by some MC (Fig. 1) [13, 14]. In situ RT-PCR of NOS expression in individual MC also supports our conclusion that MC express NOS and produce NO [13]. Moreover, constitutive expression of NOS 1 and NOS 3 has been reported in human mast cell lines and skin-derived human MC [15]. A significant increase in NO production was demonstrated in individual rat peritoneal MC, and human HMC-1 and LAD2 using confocal analysis after stimulation with A23187 or by IgE cross-linking [14, 15].

Possible Resolution for Controversy

It is well known that there are marked differences among the techniques used and types of MC in different studies. For example, Swindle et al. [12] used rat peritoneal MC from Brown Norway (BN) rats, while many of the studies by others used Sprague Dawley (SD) rats. Indeed BN rats are good models for allergy because of the high IgE levels and strong allergic responses observed in them. Such responses might be related to low NO

production by MC from BN rats, since endogenous NO plays a role as an inhibitor of MC allergic responses. We have preliminary results that there are rat strain differences in NO production and NOS expression by MC, after IFN-γ stimulation. MC from BN rats are poor producers of NO when compared to SD-derived peritoneal MC (Muñoz S, 2005).

The main issue in the controversy about NOS expression and NO production by MC is the purity of MC population used (Table 1). Given that it is impossible to fully purify in vivo derived MC, methods using single cells, such as in situ RT-PCR or confocal microscopy, are helpful to confirm whether MC express NOS and produce NO (see above). Moreover, given that morphological, biochemical, and functional heterogeneity have been found in MC, there may be heterogeneity in NOS expression in MC subpopulations. Microenvironmental factors and epigenetic regulation may alter NOS expression in MC (Fig. 1). The findings that repressed NOS 3 gene can be activated for expression by HDAC inhibitor, trichostatin A (TSA) [6] and differences in the methylation status of the NOS 2 promoter can be related to the inducibility of the NOS 2 gene [7], demonstrate epigenetic control on the expression of NOS among different microenvironments (Fig. 1). Furthermore, there are elegant epigenetic effects on T cell differentiation [16, 17] and MC gene expression [18–20]. Enhanced knowledge of epigenetic effects on MC differentiation and function will provide clues to understand MC phenotypes and function. Future studies must more fully characterize mechanisms underlying the effects of microenvironmental factors or strain-associated differences in NO production and the potential relevance of such differences.

Role of Nitric Oxide in Mast Cells

Mast Cell Degranulation

It is well accepted that NO inhibits MC degranulation (Fig. 1). NO donors, such as sodium nitroprusside (SNP), spermine-NO, and 3-morpholinosydnonimine (SIN-1) reduced histamine release from guinea pig and rat cardiac MC [21, 22], rat peritoneal MC [23, 24], mouse BMMC [25], and attenuated MC degranulation in the mesentery after ischemia/reperfusion in the rat [26]. Spermine-NO prevented MC-dependent neutrophil adhesion, PAF-induced leukocyte adhesion, and albumin leakage [27]. Nicorandil, a potassium channel opener and NO donor inhibited the release of TNF and β-hexosaminidase from RBL-2H3 cells [28]. IFN-γ induced NO production in peritoneal cell populations, which in turn inhibited IgE-mediated secretory function of MC [10, 29]. Liao et al. suggested that the protective effect of NO against the deleterious effects of oxidized LDL may be related to its ability to prevent MC degranulation [30], and Kawabata et al. suggested that NO plays a protective role in the PAR-1-mediated increased vascular permeability accompanied by edema formation in the rat hindpaw, an inflammatory event mediated predominantly by MC degranulation [31].

NOS inhibitors such as L-NAME and NG'-dimethyl-L-arginine (endogenous vascular endothelium-derived NOS inhibitor) increased RMCP II (rat MC protease II) activity in plasma of L-NAME-treated animals [32], and also elicited MC degranulation, enhanced leukocyte adherence, emigration, platelet–leukocyte aggregation, and albumin leakage [33–37]. Unfortunately, the mechanisms underlying how NO depresses MC degranulation are unknown.

Mast Cell Cytokine and Chemokine Production

There is increasing evidence that NO regulates cytokine and chemokine production by MC (Fig. 1). Coleman et al. [11, 38] showed that pretreatment with the NO donor, SNOG blocked IgE/Ag-induced expression of IL-4, IL-6, and TNF mRNA in RBL-2H3 and mouse BMMC. SNOG inhibited phosphorylation of phospholipase Cγ1 and the AP-1 transcription factor c-Jun, and abrogated DNA-binding activity of the nuclear AP-1 proteins Fos and Jun. In addition, incubation of HMC-1 with IFN-γ inhibited expression and production of CCL1 and IL-8 and this effect was eliminated by pretreatment with L-NAME [39].

Thus, NO blocks expression of some cytokines and chemokines in MC. However, given the ability of MC to synthesize and release many kinds of cytokines and chemokines, it is likely that NO may affect these mediators in selective ways, depending on the stimuli and other microenvironmental factors.

Mast Cell Lipid Mediator Production

NO also affects production of eicosanoids by MC (Fig. 1). When activated by cross-linkage of their high-affinity IgE receptors or by other stimuli, MC generate LTC_4, one of the cys (cysteinyl)-LTs, and small amounts of LTB_4 through the 5-LO (5-lipoxygenase) pathway. MC can also produce PGD_2 through the COX (cyclooxygenase) pathway. These lipid mediators were initially recognized for their bronchoconstricting and vasoactive properties, but are known to participate in host defense, inflammation, and allergic diseases through diverse activities such as effector cell trafficking, antigen presentation, immune cell activation, and fibrosis [40, 41].

The inhibitory effect of NO on LT synthesis has been shown in cultured alveolar macrophages as well as MC. In alveolar macrophages, LPS suppresses LT synthesis mediated in part by induction of NOS 2 and NO production. Peroxynitrite ($ONOO^-$), formed from NO and O_2^-, suppresses 5-LO activity by nitration and S-nitrosylation of recombinant 5-LO [42]. Moreover, 5-LO and NOS 3 can translocate to the nucleus and can be colocalized in HMC-1 and LAD2 MC lines following calcium ionophore A23187 and IgE/anti-IgE stimulation respectively. This is accompanied by calcium-dependent phosphorylation of NOS 3, known to increase

its activity. The NO donor, SNOG inhibited, whereas the NOS inhibitor, L-NAME potentiated LT release, suggesting that endogenously produced NO can regulate LT production by MC [15].

There is evidence that NO regulates PG synthesis by altering COX enzymatic activity in many cell types, although there are some differences among cell types [43]. Some reports suggest that NO upregulates COX-2 protein expression and its activity in macrophages [44, 45]. This may occur by reducing O_2^-, which is involved in autoinactivation of COX, or by increasing catalytic activity of COX through nitrosylation of cysteine residues in its catalytic domain. However, others reported that NO downregulates PG synthesis, in some cases, via inhibition of COX-2 expression in vascular endothelial cells [46], rat Kupffer cells [47], and J774 macrophage cells [48]. In MC, our recent observations show that the NO-donor, SNOG augmented COX-2 protein expression and increased COX-2-dependent PGD_2 generation in mouse BMMC by increasing COX-2 mRNA stability [49]. The effect of NO on the COX pathway seems to differ according to the cell type studied and the nature and intensity of stimuli. Further research on the role of NO in the regulation of the COX pathway is warranted.

Mechanism of Action of Nitric Oxide on Mast Cell Activities

Posttranslational Modifications of Proteins by Nitric Oxide

One common mechanism through which NO mediates its effect in vivo is by activation of soluble guanyl cyclase (sGC) by nitrosylation of its heme group. NO can diffuse from its site of synthesis to where it will activate sGC thereby increasing cGMP levels, which in turn activate cGMP-dependent kinases and modulate intracellular calcium levels. However, in many studies it has been shown that NO can also exert its effects on MC through cGMP-independent pathways [15]. At a posttranslational level NO acts to modify protein structure and function through several mechanisms including nitration and nitrosylation (Fig. 1) [50].

Nitration: NO combines with superoxide (O_2^-) to generate peroxynitrite ($ONOO^-$), which is more reactive than either of the parent molecules. Peroxynitrite is a potent oxidizing molecule which mediates tyrosine nitration of various proteins, a covalent modification resulting from the addition of a nitro ($-NO_2$) group to the aromatic ring of tyrosine [4]. This posttranslational modification can have profound effects on protein structure and function, generating novel antigenic epitopes, changing catalytic activity of enzymes, altering cytoskeletal organization, and impairing cell signal transduction [51]. The possible role of NO and $ONOO^-$ in health and in the pathogenesis of different diseases has been explored [4]. Developments in proteomics technology have revealed that nitration is a highly selective process, limited to specific tyrosine residues on only some proteins [4].

The mechanisms that impart specificity of nitration to selected tyrosine residues are poorly known, but this specificity suggests that there are exquisite controls on these events. HDAC2, an important enzyme in gene expression, can be nitrated, which in turn augments inflammation. Thus, epigenetic control of gene expression may be influenced by this critical posttranslational nitration [52, 53].

In spite of various studies demonstrating production of NO by MC, there are no studies that have identified potential targets for protein tyrosine nitration in MC. Antigen/antibody activation of guinea pig lung MC results in generation of $ONOO^-$, which in turn modulates the release of inflammatory mediators from the MC [54]. In a hypothesis-generating proteomic approach, we have identified aldolase, a common glycolytic enzyme as one of the targets for protein tyrosine nitration in human MC (Sekar Y et al., submitted for publication, 2008). We are currently characterizing the pathways involved in nitration of aldolase and its significance in controlling MC phenotype and function.

Nitrosylation: Nitrosylation refers to the binding of an NO group to a transition metal or cysteine residue, which is a dynamic, reversible posttranslational modification that plays a central role in NO-mediated signaling. The specificity of the changes in protein function associated with NO involves *S*-nitrosylation and its reversibility, and precise domains in the target protein that are modified by *S*-nitrosylation [55].

Given that NO downregulates multiple MC functions, it may be a prominent regulatory mechanism in the cell. In our earlier studies on the effects of NO on MC we established that the activity of calpain, an enzyme involved in integrin clustering, was inhibited, probably by nitrosylation, which in turn inhibited MC adhesion to fibronectin, an important interaction that facilitates mediator secretion (Fig. 1) [56]. We are working toward identification of other nitrosylated proteins involved in the control of MC function by NO.

Epigenetic Regulation of Target Gene Expression by Nitric Oxide

Epigenetics is defined as mitotically and meiotically heritable changes in gene expression that are not due to changes in the DNA sequence itself [57]. These changes may be induced spontaneously, in response to environmental factors, or in response to the presence of a particular allele. There are two major areas of epigenetics that have been studied: DNA methylation and histone modifications. Acetylation of histones by histone acetyltransferase (HAT) is one of major modifications that activates gene expression. However, HDAC suppresses gene transcription and returns histone to its condensed basal state by removing acetyl groups from hyperacetylated histones. There is evidence that NO epigenetically regulates expression of some target genes through reduction of HDAC2 activity by nitration of tyrosine residues [52, 53]. Although direct evidence of epigenetic regulation of gene expression by NO in MC is absent, inactivation of HDAC2 by NO suggests many possibilities to explain the inhibitory mechanism of NO on MC cytokine and

chemokine production, as well as the regulation of NOS expression in MC noted above (Fig. 1).

Future Directions

There is increasing evidence that NO regulates MC activities, although the extent to which MC themselves express NOS and produce NO is still controversial. As shown in Fig. 1, we postulate that there are MC subpopulations that can be distinguished by their expression of NOS or not. Furthermore, we postulate that the expression of NOS in MC can be regulated epigenetically or by other means following exposure to various microenvironmental factors. NOS-positive and NOS-negative MC phenotypes may be reversibly changed by local factors. Both exogenous NO from adjacent cells, such as macrophage and endothelial cells, and endogenous NO produced by NOS positive MC likely regulate MC functions in many ways, including altering DNA-binding activity of transcription factors, posttranslational modifications of target proteins and epigenetic regulation of target gene expression. However, further study is still needed to understand the precise roles and mechanisms of action of NO in the regulation of MC function, including in innate immunity. Single-cell analysis such as in situ RT-PCR or confocal microscopy, and epigenetic studies will be helpful to test these postulates about NOS expression and NO production by MC and the effects of NO on MC activities. A more complete understanding of the fundamental mechanisms of NOS expression in MC and the NO-mediated modulation of MC function will provide critical information to help clarify the functions of this cell in normal physiology and pathophysiology, and identify meaningful therapeutic strategies for allergic and other inflammatory diseases.

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Histamine and Its Receptors

Martin K. Church

Introduction

Histamine has been recognised for almost a century to be a mediator of acute allergic responses. β-Imidazolylethylamine was first synthesised in 1907 by Windaus and Vogt [1] and was later named *histamine* (from the Greek *histos* = tissue) because of its ubiquitous presence in animal tissues, particularly in mast cells. In classic pharmacological studies, Sir Henry Dale demonstrated the potent bronchoconstrictor and vasodilator activity of histamine when injected intravenously into animals [2]. In the same laboratory 13 years later, it was noted that many of the symptoms of antigen injection into sensitised animals could be reproduced by histamine and it was, therefore, considered to be a humoral mediator of the acute allergic response [3]. With the description of the wheal-and-flare response in human skin, Thomas Lewis further expanded on the vascular actions of histamine [4]. However, it was not until 1953 that histamine present in human skin was localised to mast cells of the dermis [5].

Histamine is synthesised in the Golgi apparatus of mast cells and basophils by decarboxylation of its precursor amino acid, histidine, under the influence of histidine decarboxylase. It is stored in ionic association with the acidic residues of the glycosaminoglycan (GAG) side chains of heparin or related proteoglycan [6]. Once in the extracellular environment, histamine is metabolised rapidly (t½ ~1 min) by either of two enzymatic pathways, by ring methylation by histamine-*N*-methyltransferase (HMT) or by oxidative deamination by diamine oxidase (DAO), the dominant route of metabolism depending on the tissue. HMT is a ubiquitous enzyme, which is regarded as the key enzyme for histamine metabolism in the bronchial epithelium and nasal mucosa [7, 8]. Because HMT is an intracellular enzyme and histamine is a charged molecule, which enters the intracellular space with difficulty, a facilitated uptake mechanism, known historically as ‘uptake 2’ is

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necessary. This is performed by a group of organic cationic transporters (OCTs), predominantly OCT-2 and extraneuronal monoamine transporter (EMT), sometimes known erroneously as OCT-3 [9]. The reaction product of HMT-catalysed histamine metabolism, *N*-methylhistamine, is excreted by the kidney and may be measured in the urine as an index of endogenous histamine release [10]. A proportion of the methylated product is oxidised further by monoamine oxidase and excreted as methylimidazole acetic acid. In mammals, diamine oxidase expression is restricted to specific tissues, the highest activities being in the intestine, placenta and kidney, where it is thought to be responsible for blocking the transport of extracellular histamine from these organs into the circulation [11]. Although diamine oxidase is usually found associated with the plasma membrane, it is a soluble protein and is thought to be released into the extracellular environment upon cell stimulation [12] allowing it to act as either a cell-associated or cell-free enzyme. Diamine oxidase oxidises histamine to imidazole acetic acid. This intermediate undergoes condensation with phosphoribosyl diphosphate followed by dephosphorylation forming the terminal metabolite, riboside-*N*-3-imidazole acetic acid.

Mast cells isolated from human lung, skin, lymphoid tissue and small intestine contain 3–8 pg of histamine per cell [13–15]. Histamine is secreted spontaneously at low levels by mast cells, the resting level in the skin being approximately 5 nM [16], somewhat higher than those of 0.5–2 nM found in the plasma. In normal individuals, urinary histamine clearance is around 10 μg/24 h, while in mastocytosis it may exceed 150 μg/24 h [17].

G Protein-Coupled Receptors

In humans, there are four subtypes of histamine receptors, H_1, H_2, H_3 and H_4, all encoded on different genes [18]. All histamine receptors identified so far are members of the superfamily of G protein-coupled receptors (GPCR). This superfamily represents at least 500 individual membrane proteins that share a common structural motif of seven-transmembrane (TM) α-helical domains, numbered TM I–VII, arranged in a circular fashion [19–21] (Fig. 1). All GPCRs have specific receptor site, usually within the central core of the cylinder formed by the transmembrane α-helical domains. These sites confer ligand specificity, ligands ranging from photons, Ca^{2+} ions and small organic molecules to complex polypeptide hormones [22]. The common functional characteristic of all GPCRs is their intracellular signal mediation, activation of a Gαβγ heterotrimer of the cytosolic G protein complex (Fig. 1) [19].

The intracellular mechanisms of signal transduction by GPCRs is the subject of an excellent review by McCudden and colleagues in 2005 [23]. In this review, guanine nucleotide-binding proteins or ‘G proteins’ are referred to as cellular ‘switches’, which alternate between a GDP-bound off state and a GTP-bound on state (Fig. 2). In the inactive state, the Gα subunit binds GDP and is closely associated with the Gβγ heterodimer, the resultant trimeric complex being closely associated with the cytosolic domain of the GPCR. In this state, Gβ facilitates the coupling of Gα to the receptor and also acts as a guanine nucleotide dissociation inhibitor (GDI) for

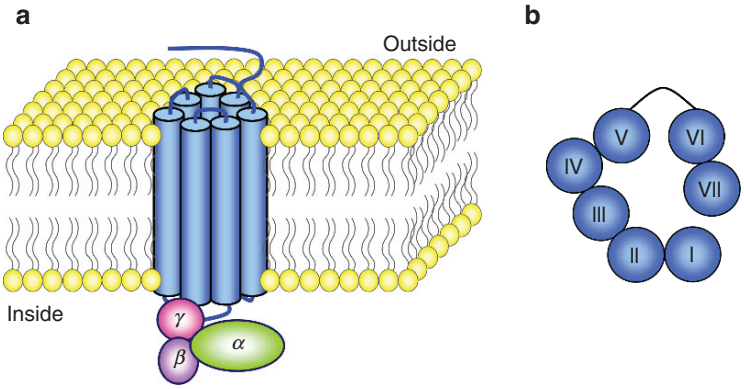

Fig. 1 Diagram of a GPRC within a membrane. (**a**) The Gγβα complex can be seen associated with the intracellular part of the receptor complex. (**b**) Numbering of the transmembrane (TM) domains of a GPCR. The inside and outside of the cell are labelled in this figure. This orientation is consistent in all figures

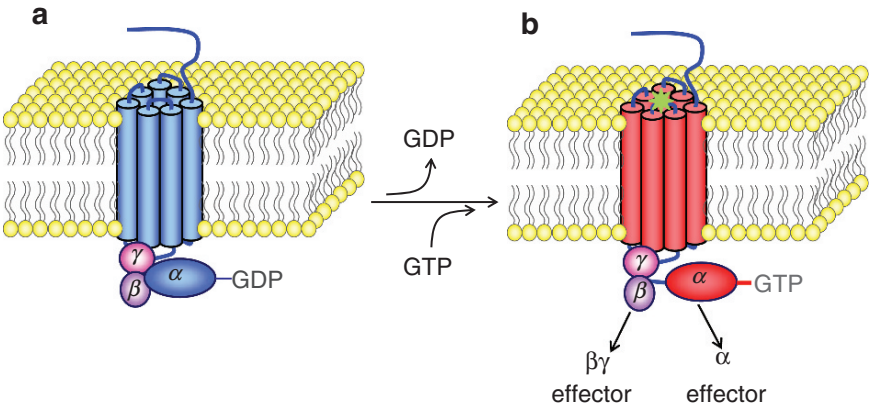

Fig. 2 Standard model of the activation of a GPCR. In the absence of ligand, the Gα subunit is GDP-bound and closely associated with α γβ the Gγβ heterodimer. The Gα-GDP/Gγβ heterotrimer interacts with the cytosolic loops of a seven-transmembrane-domain G protein-coupled receptor (GPCR). Gγβ facilitates the coupling of Gα to receptor and also acts as a guanine nucleotide dissociation inhibitor (GDI) for Gα-GDP, slowing the spontaneous exchange of GDP for GTP. Ligand binding (green star) stimulates guanine nucleotide exchange factors to induce a conformational change in the Gα subunit, allowing it to exchange GTP for GDP. Gγβ dissociates from Gα-GTP, and both Gα-GTP and Gγβ may then signal to their respective effectors. The cycle returns to the basal state when Gα hydrolyses the gamma-phosphate moiety of GTP, a reaction that is augmented by GTPase-accelerating proteins (gaps) such as the regulator of G protein signalling (RGS) proteins

Gα-GDP, slowing the spontaneous exchange of GDP for GTP. Activation of the receptor occurs by binding to a site on the GPCR specific for each ligand. In the case of the histamine H_1-receptor, histamine cross-links a site on transmembrane domain III containing an aspartate residue with one on transmembrane domain V containing

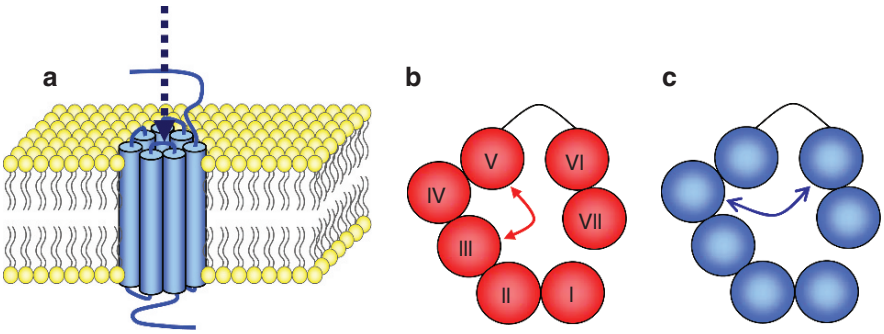

Fig. 3 The binding of histamine and cetirizine to the H_1-receptor. (**a**) The ligand-binding site for GPCR is within the transmembrane (TM) domains. (**b**) Histamine links TMS III and V to stabilise the receptor in the active state. (**c**) Cetirizine, an H_1-antihistamine, links TMS IV and VI to stabilise the receptor in the inactive state

lysine and asparagine residues (Fig. 3) [24]. This induces a conformational change in the GPCR, which causes the rapid dissociation of the Gαβγ trimer from the receptor [25], an event which stimulates the Gα subunit to exchange GDP for GTP and separate from the Gβγ subunit, allowing both subunits to signal to their respective effectors. The cycle returns rapidly to the basal state by the hydrolysis of the Gα GTP to GDP, a reaction that is augmented by GTPase-accelerating proteins (GAPs) such as the regulator of G protein-signalling (RGS) proteins.

In the human genome, there are 16 Gα genes, which encode for 23 known Gα proteins giving wide diversity to GPCRs. While these are normally classified by their structure, these proteins may also be divided into four major classes according to their cellular targets: Gαs, which stimulates adenylyl cyclase to generate cyclic AMP; Gαi, which inhibits adenylyl cyclase and thus opposes the action of Gαs; Gαq, which activate phosphoinositide-specific phospholipase C (PI-PLC) isoenzymes; and Gα12/13, which can regulate the small G-associated protein, RhoA.

In addition to a large number of Gα proteins, there are five known human Gβ and 12 human Gγ subunit genes resulting in at least 60 potential combinations of Gβγ dimers. The Gβγ dimer was once thought only to facilitate coupling of Gαβγ heterotrimers to GPCRs and act as a Gα inhibitor. However, it is now known that Gβγ subunits are free to activate a large number of their own effectors, including the regulation of K^+ channels, Ca^{2+} channels, adenylyl cyclase and a variety of kinases.

The Concept of Inverse Agonism

The classical concept of interactions of competitive agonists and antagonists with receptors was formulated by Ariëns in 1964 in his book *Molecular Pharmacology* [26] (Fig. 4). This theory stated that an *agonist* must have both *affinity* to combine with the receptor and *efficacy* to stimulate the receptor. A ‘full agonist’ was defined

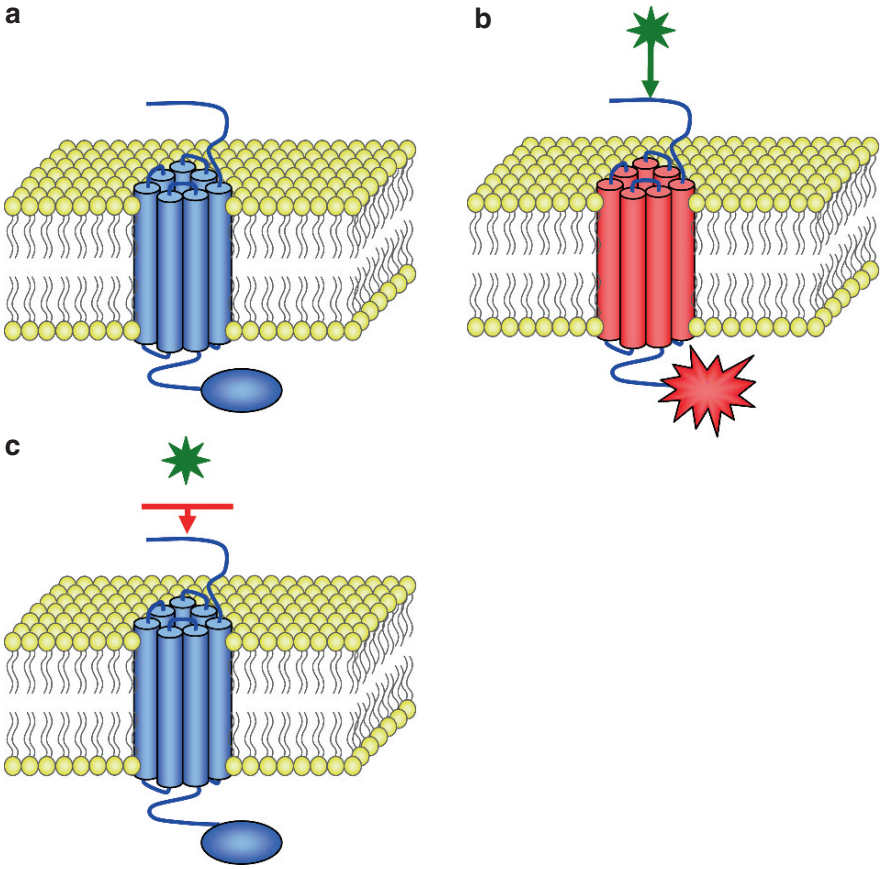

Fig. 4 The classical concept of interactions of competitive agonists and antagonists with receptors. (**a**) An unstimulated receptor where the Gαβγ complex is in the inactive state (blue oval). (**b**) An activated receptor where an agonist (green star) interacts with the ligand-binding site leading to the activation of the Gαβγ complex (red star). (**c**) A 'blocked' receptor where an antagonist (red line) has interacted with the ligand-binding site and prevented the agonist from binding. The Gαβγ complex is in the inactive state (blue oval)

as a compound that had maximal efficacy at its receptor and was given an intrinsic activity = 1. To explain the observation that not every agonist induced the same maximum effect at its receptor, the term 'partial agonist' was introduced for weaker agonists with an intrinsic activity of less than 1. In this scheme, an 'antagonist' was described as a compound with *affinity* for the receptor and but *no efficacy* to stimulate it. By definition antagonists possess an intrinsic activity of 0.

Thus, using this concept, an antagonist prevented receptor stimulation by binding to a receptor thereby preventing the subsequent binding of an agonist (Fig. 4). In retrospect, it is remarkable that the concepts of receptor stimulation and its blockade were developed over a period of around 75 years using only the measurement of responses of isolated tissues to pharmacological agents. It is only recently

with the advent of techniques for routine cell culture and molecular biology that we have begun to realise the inadequacies of these concepts.

With the introduction of the molecular biology of GPCRs in 1986 [27], it became clear that the single-state model described above was not correct. Instead, we should visualise the receptor as a *two-state model* [28] (Fig. 5a). In this model,

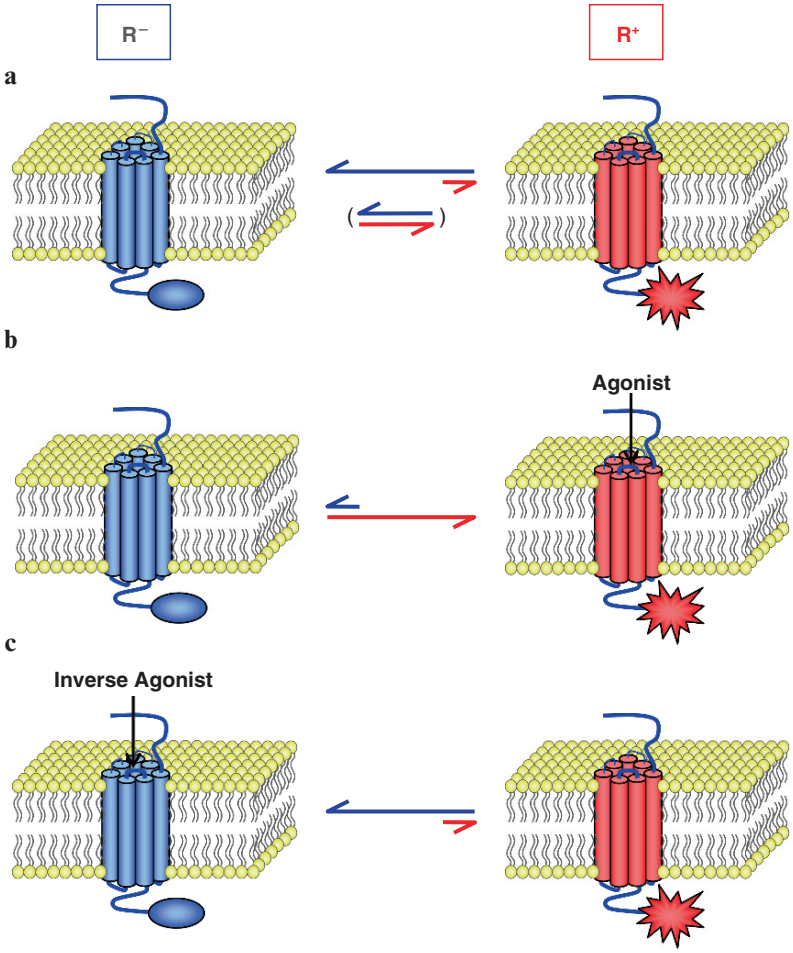

Fig. 5 Simplified two-state model of GPCR activation. In this model the two isomeric forms of the receptor, the inactive state $\mathbf{R}^-$ and the active state ($\mathbf{R}^*$) are in equilibrium (blue and red arrows, respectively). (**a**) In the resting state the equilibrium is usually in favour of the inactive $\mathbf{R}^-$ configuration. However, if the receptor shows constitutive expression, the equilibrium will shift partially to the active $\mathbf{R}^+$ configuration (*arrows* on parentheses). (**b**) Binding of an agonist stabilises the receptor in the active $\mathbf{R}^*$ configuration and swings the equilibrium in that direction to increase receptor signalling. (**c**) Binding of an inverse agonist stabilises the receptor in the inactive $\mathbf{R}^-$ configuration and swings the equilibrium in that direction to decrease receptor signalling. In this model, neutral antagonists have equal affinity for both $\mathbf{R}^-$ and $\mathbf{R}^+$ isoforms of the receptor and, therefore, do not affect the equilibrium between the two states

an equilibrium exists between the receptor isoforms, the inactive $\mathbf{R}^-$ state and the active $\mathbf{R}^+$ state [29, 30]. When viewed from the intracellular space, the transmembrane (TM) domains I–VII of the GPCR are arranged in a clockwise fashion. The conformational switch from $\mathbf{R}^-$ to $\mathbf{R}^+$, which is highly conserved among GPCRs from different families, involves rotation of TM III relative to TM VI [31]. *Full agonists* induce optimal stabilisation of the $\mathbf{R}^+$ state of the GPCR causing the equilibrium to shift maximally towards $\mathbf{R}^+$ (Fig. 5b). The conformational change involved in the isomerisation of $\mathbf{R}^-$ to $\mathbf{R}^+$ enables the GPCR to promote the dissociation of GDP from G proteins, the initial and rate-limiting step in the G protein cycle [32]. Full agonists are very efficient at increasing the basal GDP/GTP exchange rate of the $G\alpha$ subunit of the receptor complex and, thereby, stimulating the downstream events of receptor stimulation. *Partial agonists* are less efficient than full agonists at stabilizing the $\mathbf{R}^+$ state and, therefore, increase GDP/GTP exchange less efficiently. In contrast to, *full inverse agonists* induce optimal stabilisation of the $\mathbf{R}^-$ state of the GPCR, causing the equilibrium to shift maximally towards $\mathbf{R}^-$ and reducing basal GDP/GTP exchange (Fig. 5c). *Partial inverse agonists* have similar effects, but are less efficient than full inverse agonists. *Neutral antagonists* do not alter the equilibrium between $\mathbf{R}^-$ and $\mathbf{R}^+$ and do not change basal G protein activity, but they block both the inhibitory effects of inverse agonists and the stimulatory effects of agonists.

Before explaining the concept of constitutive activity, it is pertinent to highlight the differences between agonist and inverse agonist binding to the receptors. Histamine H_1-inverse agonists bind to different sites on the receptor compared with histamine. For example, in contrast to histamine binds which cross-links TM III and V to activate the receptor, the inverse agonists, cetirizine and acrivastine, cross-link amino acids on TM IV and VI to stabilise the receptor in the inactive form (Fig. 3). Also, the binding times are quite different. For example, the dissociation half-time for levocetirizine, the eutomer of cetirizine, is 142 min [33] compared to a few microseconds for histamine. Thus, if a receptor is constitutively active, the long duration of binding facilitates its reversal by inverse agonists.

The Concept of Constitutive Activity

While it is well established that GPCRs respond to stimulation by extracellular ligands, the concept that they may remain in the 'switched on' state in the absence of ligand stimulation, i.e. in a constitutive or spontaneous manner, is relatively new and potentially important for the understanding of some disease processes [34–36].

The first evidence for constitutive activity of GPCRs was obtained for the δ-opioid receptor [37] and the β_2-adrenoceptor [38]. Since that time, more than 60 wild-type GPCRs and several disease-causing GPCR mutants have been found to exhibit constitutive activity [34].

Smit and colleagues [36] have recently reviewed the known disease-causing GPCR mutants and concluded that single-point mutations of GPCR genes may

cause structural changes in the transcribed receptors that may increase or decrease their constitutive activity. For example, normal parathyroid hormone-related peptide (PHRP) does not express constitutive activity. However, two-point mutations have been reported to be associated with a high level of constitutive activity of the mutant receptor resulting in Jansen-type metaphyseal chondrodysplasia, a rare disorder that is typically characterised by severe growth plate abnormalities that lead to short-limbed dwarfism [39]. Conversely, growth hormone secretagogue receptor-1a (GHSR-1a) naturally possesses a high level of constitutive activity. However, two mis-sense mutations have been reported that selectively reduce the constitutive activity of the GHSR while preserving its ligand responsiveness. These mutations are associated with short stature due to growth failure [40].

In addition to the human GPCR variants described above, a relatively novel and intriguing class of GPCRs, encoded by herpes viruses, exhibit marked constitutive activity. The herpes- and poxviruses encode more than 40 GPCRs, most of them displaying homology to chemokine receptors known to be implicated in the regulation of the immune response. Although the roles of these viral-encoded receptors have not been fully defined, they are believed to subvert the immune system and to contribute to virus-induced pathogenesis (reviewed in [36]).

Wild-type GPCRs, i.e. those found in the most common phenotypes in the natural population, are the subject of a review by Seifert and Wenzel-Seifert [34]. Included in the tabulated list of all the known wild-type GPCRs, which have been shown to exhibit constitutive activity are three reports of constitutive activity in H_1-receptors, three in H_2-receptors, seven in H_3-receptors and two in H_4-receptors. Perhaps the best explored of these is the H_1-receptor-dependent constitutive expression of the transcription factor NF-κB [41]. Interestingly, their data suggest that both Gαq/11 and Gβγ subunits play a role in the agonist-induced H_1-receptor-mediated activation of NF-κB, but that constitutive NF-κB activation by the H_1-receptor is primarily mediated through Gβγ-subunits.

Oligomerisation, Domain Swapping and Receptor Cooperativity

In almost all diagrams of GPCRs they are depicted as single units. This has led to the common belief that they function as discrete monomeric units. However, this is not usually the case. GPCRs are gregarious by nature and readily form dimers and often higher-order oligomers. When forming dimers, GPCRs may form either homodimers or heterodimers, the latter leading potentially to cooperativity. As this new and complex field is largely the domain of molecular biologists and computer simulation scientists, interested readers are directed to a series of recent reviews for further information [42–45]. However, two examples involving histamine receptors are given below (Fig. 6).

The first example involves the co-expression of the Gi/o-coupled human 5-hydroxytryptamine receptor-1B ($5\text{-}HT_{1B}R$) and the Gq/11-coupled human

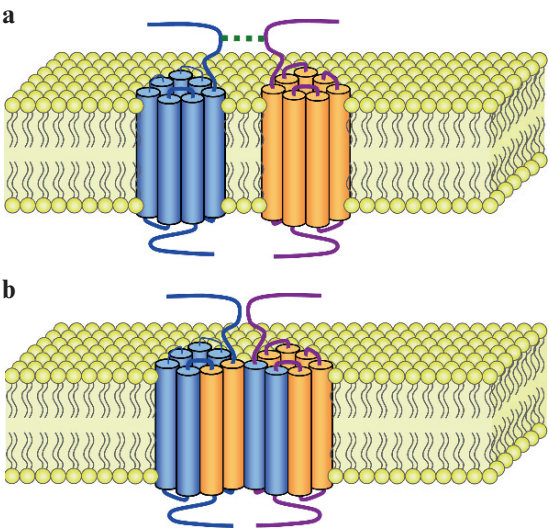

Fig. 6 Dimerisation and domain swapping in GPCRs. (**a**) A simple heterodimer where the two GPCRs are held together by electrostatic bonding (green dotted line) between the extracellular domain of TM I. (**b**) A domain swapped heterodimer where the GPCRs have 'swapped' domains VI and VII

H_1-histamine receptor (H_1R) [46] (Fig. 6a). Co-expression resulted in an overall increase in agonist-independent signalling, which was augmented by 5-$HT_{1B}R$ agonists and inhibited by a selective inverse 5-$HT_{1B}R$ agonist. Furthermore, inverse H_1R agonists inhibited constitutively H_1R-mediated as well as 5-$HT_{1B}R$ agonist-induced signalling in cells co-expressing both receptors. This phenomenon is not solely a characteristic of 5-$HT_{1B}R$ and H_1R receptors, but it is also evident with many other GPCRs ranging from neurotransmitters to cytokines [46]. Such cross-talk is not surprising as, in vivo, cells are co-stimulated simultaneously by a wide variety of agonists and must be able to respond to them all in a coordinated manner.

The second example involves domain swapping in the human histamine H_1-receptor. Bakker and colleagues [47] investigated the potential dimerisation of the wild-type human H_1R in the presence and absence of two mutated H_1R (Fig. 6b). The results demonstrated the presence of both monomeric and homodimeric H_1R together dimers in which there was reciprocal exchange of transmembrane domains 6 and 7 between the receptors present in the dimer. These data suggest that domain swapping between heteromeric GPCRs may occur but its clinical relevance is, as yet, unclear.

Are Inverse Agonism, Constitutive Expression and Receptor Dimerisation Clinically Relevant?

Whenever a new concept is introduced, there is a period of enthusiasm followed by a period of doubt and finally a levelling out of its significance at a realistic level. With inverse agonism, constitutive expression and receptor dimerisation we are

clearly in the first phase, enthusiasm. If constitutive activity of GPCRs is clinically important, particularly in disease settings, then there is a desperate need for the pharmaceutical industry to develop potent and specific ligands and inverse agonists. This is well recognised and research in this area is blossoming [48–51]. We have yet to go through the period of doubt before we really know the clinical relevance of these novel concepts.

Histamine Receptors

The Histamine H_1-Receptor

Most of the clinical symptoms of allergic disease result from H_1-histamine receptor stimulation. In the nose, H_1-antihistamines reduce the symptoms of rhinorrhoea, itching, sneezing and oedema, the last being one component of nasal blockage [52]. The major component of nasal blockage, dilatation of venous capacitance vessels resulting from nasal inflammation, is less amenable to H_1-antihistamine therapy [52]. In the eye, stimulation of H_1-histamine receptors is responsible for the majority of the primary symptoms of seasonal allergic conjunctivitis, namely, lacrimation, reddening, itching and chemosis [53]. In the airways, stimulation of H_1-histamine receptors contributes to the contraction of bronchial smooth muscle and stimulation of mucus production. However, the more dominant role of the leukotrienes in producing these symptoms means that H_1-antihistamines are minimally effective in reducing the symptoms of asthma [54]. In the skin, histamine H_1-receptor-mediated effects include contraction of post-capillary vein endothelial cells to cause a wheal, and sensory nerve stimulation to cause pruritus and a widespread neurogenic flare in which neuropeptides, particularly calcitonin gene-related peptide (CGRP), are the final mediators of the vasodilatation [55].

The Histamine H_1-Receptor and Inflammation

H_1-receptor stimulation may also activate the transcription factor NF-κB [41, 56, 57] (Fig. 7). NK-κB is a key pro-inflammatory cytokine, which is elevated in asthma [58] where it is involved in the production of cytokines, including TNFα, and IL-8, and adhesion molecules such as E-selectin, ICAM-1 and VCAM-1 [59]. As both the H_1-receptor and up-regulation of cytokines and adhesion proteins by NF-κB are known to be involved in allergic conditions [60], it is attractive to speculate that in such disorders the coupling of the H_1-receptor to the NF-κB pathway is of physiological importance [61]. Indeed, the reduction of NF-κB activation by H_1-antihistamines [61–63] may well explain their long-term effects against allergic inflammation and nasal blockage [64–66].

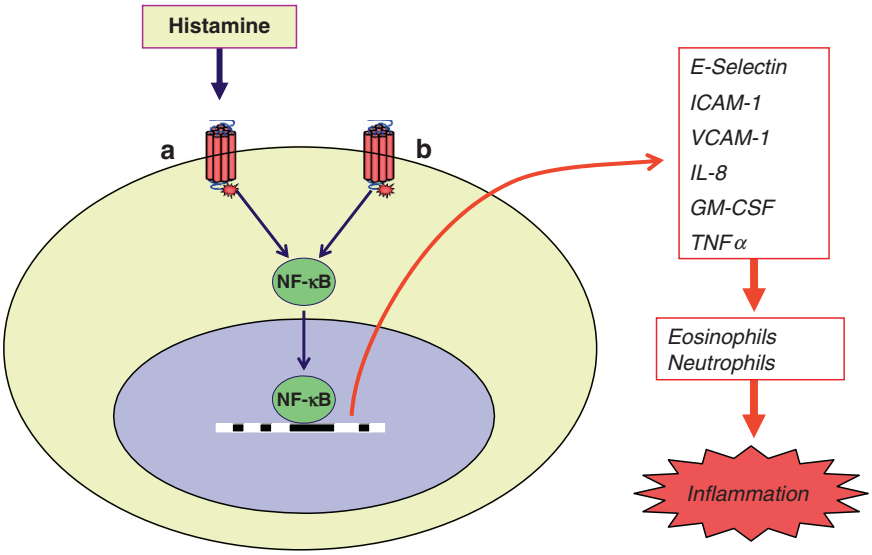

Fig. 7 H_1-receptor activation of the transcription factor NF-κB. Two histamine H_1-receptors are shown activating NF-κB, the left-hand one (**a**) being stimulated by histamine and the right-hand one (**b**) having constitutive expression in the absence of histamine. Activated NF-κB is transported to the cell nucleus where it stimulates the transcription of pro-inflammatory adhesion molecules and cytokines

The Paradox of H_1-Antihistamines and Bradykinin

Bradykinin, a nonapeptide formed by the enzymatic actions of kallikrein on extracellular kininogen, plays a central role in the production of inflammation and pain. It is of particular interest in dermatology because of its ability to cause local oedema and increased blood flow in the skin and because activation of kinin pathways are believed to be involved in angioedema, which occurs in some patients with urticaria, particularly those with hereditary angioedema and C1 esterase deficiency.

When injected intradermally, bradykinin produces a wheal-and-flare response which is, in appearance, very similar to that produced by histamine and histamine-releasing agents. However, assessment of extravascular histamine concentrations by dermal microdialysis showed that bradykinin released negligible quantities of histamine, certainly not sufficient to cause the observed wheal-and-flare response [16, 67]. These results are consistent with in vitro studies, which report that human skin mast cells do not release histamine in response to bradykinin [15, 68] and with the observation that the cutaneous sensation following the intradermal injection of bradykinin, a relatively long-lasting 'burning' sensation, is quite different from that of histamine, suggesting a different mechanism of action [16, 67].

Even though bradykinin releases negligible quantities of histamine, H_1-antihistamines such as mepyramine [69], chlorpheniramine [70], terfenadine [71] and cetirizine [72, 73] are all potent inhibitors of the response. The ability of H_1-antihistamines

to inhibit wheal-and-flare responses to inflammatory mediators, which induce small or negligible amounts of histamine is not unique to bradykinin, having been reported also for methacholine [74, 75] and platelet-activating factor (PAF) [76, 77]. Interestingly, H_1-antihistamines do not block bradykinin-induced responses in the nose [78] and only weakly reverse them in the lung [79].

The mechanism(s) by which H_1-antihistamines abrogate bradykinin-induced responses in the skin is not clear. It is tempting to speculate, therefore, that heterodimerization of histamine H_1-receptors for bradykinin B_2-receptors, M_3-receptors for methacholine and PAF-R for platelet-activating factor may be responsible for the ability of H_1-antihistamines to inhibit the effects of all four stimuli. Indeed, it has already been reported that the bradykinin B_2-receptor has the capability of forming both homodimers and heterodimers, which lead to changes in its responsiveness upon stimulation [80]. However, no direct experimental evidence is currently available to support this hypothesis.

Central Nervous Effects Mediated by the Histamine H_1-Receptor

Histamine is sometimes referred to as a 'waking amine' in that it is significantly increased during awake or light periods during which time it plays a neuroregulatory role. In laboratory animals, specific H_1-receptor agonists increase wakefulness while specific H_1-antihistamines produce opposite effects [81]. Clearly, the ability of H_1-antihistamines to cause sedation is of great clinical importance and is dealt with in the chapter on antihistamines.

Molecular Aspects and Intracellular Signal Transduction

Histamine H_1-receptors cloned from different species show a wide diversity of structure [82–84]. For example, the third intracellular domain of the guinea pig H_1-receptor, the predicted binding site for the GTP-binding protein, showed only 50% identity with those of the rat [84], explaining the diversity of histamine action between these species. However, within each species, there appears to be only a single receptor protein, the human histamine H_1-receptor gene on chromosome 3p25 [85, 86] encoding for a 487 amino acid protein with a molecular mass of 55.8 kDa [85, 87]. Furthermore, the absence of introns in the H_1-receptor gene indicates that a single mRNA will be transcribed with no splice variants [87].

The histamine H_1-receptor belongs to the Gq/11 subtype of GPCR and is a so-called Ca^{2+} mobilizing receptor [46] (Fig. 8). Mobilisation of the Gqα subunit following activation of the H_1-receptor stimulates membrane-associated phospholipase Cβ (PLCβ) to catalyse the hydrolysis of the membrane-associated inositide phospholipid, phosphatidyl 4,5-biphosphate, to form inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). The IP_3 binds to ligand-gated Ca^{2+} channels on

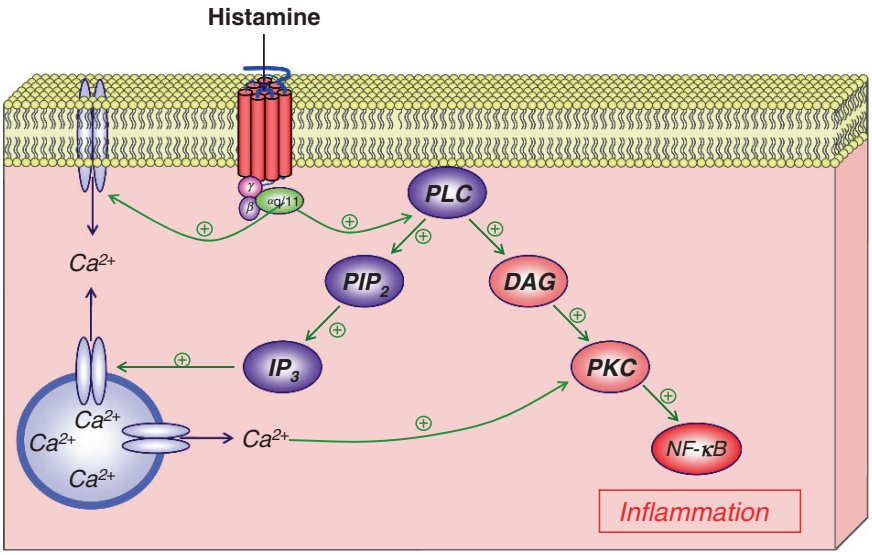

Fig. 8 Activation–secretion coupling of the histamine H_1-receptor. Mobilisation of the Gαq subunit stimulates membrane-associated phospholipase C (PLC) to catalyse the hydrolysis of the membrane-associated inositide phospholipid, phosphatidyl 4,5-biphosphate, (PIP_2) to form inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). The IP_3 binds to ligand-gated Ca^{2+} channels on internal membranes, leading to an influx of calcium ions and production of the physiological response. Activation of protein kinase C (PKC) by DAG in the presence of calcium leads to NF-κB activation

internal membranes, leading to an influx of calcium ions into the cytoplasm where they bind to the calcium modulatory protein, calmodulin. The subsequent activation of calmodulin-dependent kinase leads to production of the physiological response [18, 88, 89].

In addition to stimulating the classical effects, histamine H_1-receptor-mediated effects, histamine acting through the H_1-receptor may also induce the transcription of NK-κB as mentioned above (Fig. 8). Biochemically, this involves the activation of PKC by calcium and diacylglycerol and the stimulation of NF-κB transcription. Recently, Bakker et al. [41] explored the H_1-receptor-mediated activation NF-κB in COS-7 cells transfected with the human H_1-receptor. They showed that both Gαq/11 and Gβγ subunits play a role in agonist-mediated NF-κB activation, but that constitutive NF-κB activation by the H_1-receptor is mediated primarily through Gβγ subunits.

The Histamine H_2-Receptor

The histamine H_2-receptor is a Gs-coupled GPCR, which modulates cell function by elevating cyclic AMP. It was initially defined pharmacologically by Sir James

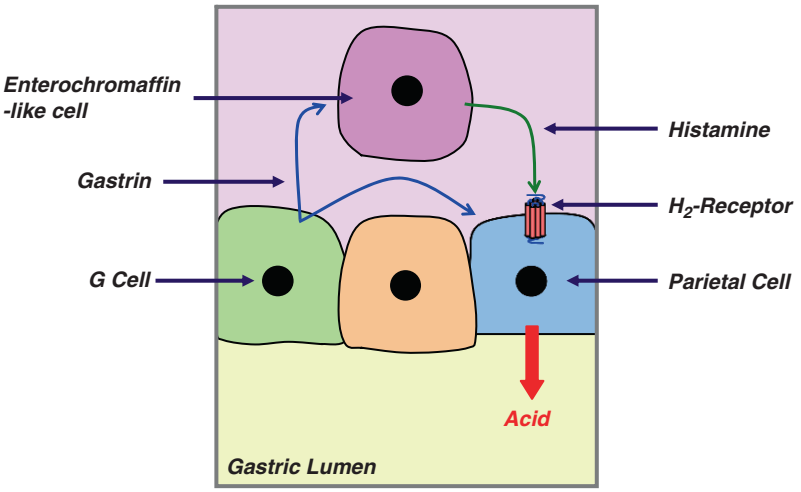

Fig. 9 Stimulation of gastric acid release from the gastric parietal cell stimulated by histamine acting at the histamine H_2-receptor

Black and colleagues [90] following the synthesis of the 'antagonists' burimamide and cimetidine. Subsequently, H_2-receptor antagonists became blockbuster drugs for the control of gastric acid secretion in the treatment of gastric and duodenal ulcers and Zollinger–Ellison syndrome (Fig. 9). More recently, they have been largely superseded by proton pump inhibitors.

The use of highly selective H_2-receptor agonists, such as 4-(*S*)-methylhistamine, dimaprit, amthamine and impromidine, and antagonists such as ranitidine, tiotidine and famotidine, has suggested a wider spectrum of biological actions regulated by this receptor. Possible physiological actions included in a review by Del Valle and Gantz [91] are relaxation of airway and vascular smooth muscle; regulation of chronotropic and inotropic effects in right atrial and ventricular muscle respectively; inhibition of basophil chemotactic responsiveness; inhibition of mitogen-mediated immunocyte proliferation via induction of suppressor T cells; and differentiation of promyelocytic leukemic cells to mature granulocytes.

For allergists, probably the most interesting H_2-receptor-mediated effects are those on the immune system. It has been demonstrated [92] that histamine enhances Th1-type responses by acting on the H_1-receptor, which is predominantly expressed in the Th1 cells, whereas histamine suppresses both Th responses by acting on the H_2-receptor. By these and other mechanisms, histamine interferes with the peripheral tolerance induced during specific immunotherapy. By actions on the H_2-receptor, histamine induces the production of IL-10 by dendritic cells, induces IL-10 production but suppresses IL-4 and IL-13 production by Th2 cells, and enhances the suppressive activity of transforming growth factor β on T cells [93]. These observations suggest that the H_2-receptor might participate in peripheral tolerance or active suppression of inflammatory-immune responses [93]. This is supported by the finding that premedication with the H_1-antihistamine, terfenadine,

during rush immunotherapy with honeybee venom, enhanced long-term immune protection [94].

The immunoregulatory effects of histamine on antigen-presenting cells, such as dendritic cells and monocytes, have been reviewed recently [95]. In monocytes stimulated with Toll-like receptor-triggering bacterial products, histamine, acting through histamine H_2-receptor stimulation inhibits the production of pro-inflammatory IL-1-like activity, TNFα, IL-12 and IL-18, but enhances IL-10 secretion. Histamine H_2-receptor stimulation also down-regulates CD14 expression on human monocytes through the regulation of ICAM-1 and B7.1 expression, leading to the reduction of innate immune responses stimulated by lipopolysaccharide. In maturing dendritic cells, histamine enhances intracellular cyclic AMP levels and stimulates IL-10 secretion, while inhibiting the production of IL-12. Finally, it has been demonstrated recently that histamine H_2-receptor stimulation reduces monocytes apoptosis, thus prolonging their life span and their ability to infiltrate to the site of inflammation. This process has been suggested to contribute to the establishment of chronic allergic disorders, such as atopic dermatitis [96].

Molecular Aspects and Intracellular Signal Transduction

Although it was initially defined pharmacologically in 1972, it was not until nearly 20 years later that the gene for the H_2-receptor, encoded on chromosome 5q, was cloned [97]. Although long and short splice variants of the resultant protein have been isolated, they appear to have similar binding with ligands and similar constitutive activity [98].

The H_2-receptor was initially defined pharmacologically as a Gs-coupled GPCR which modulates cell function by stimulating adenylyl cyclase. However, subsequent studies with the cloned H_2-receptor have shown that it can also couple to phosphoinositide second messenger systems via a Gq subunit [99] (Fig. 10). Further studies demonstrated that there is differential coupling between the Gs and Gq subunits and the second and third intra-cytoplasmic loops of the GPCR [100]. Dual coupling of H_2-receptors to Gs and Gq in cardiac myocytes is suggested to represent a novel mechanism to augment positive inotropic effects by simultaneous activation of two different signalling pathways via one receptor, the activation of the Gs-stimulated cyclic AMP–PKA pathway to promote Ca^{2+} influx through phosphorylation of L-type Ca^{2+} channels together with the Gq-stimulated increase in phosphoinositide turnover and Ca^{2+} release from intracellular stores [101].

The Histamine H_3-Receptor

The histamine H_3-receptor is a Gi/o-coupled GPCR, which serves primarily as a presynaptic receptor for histamine on nerves and is expressed almost exclusively

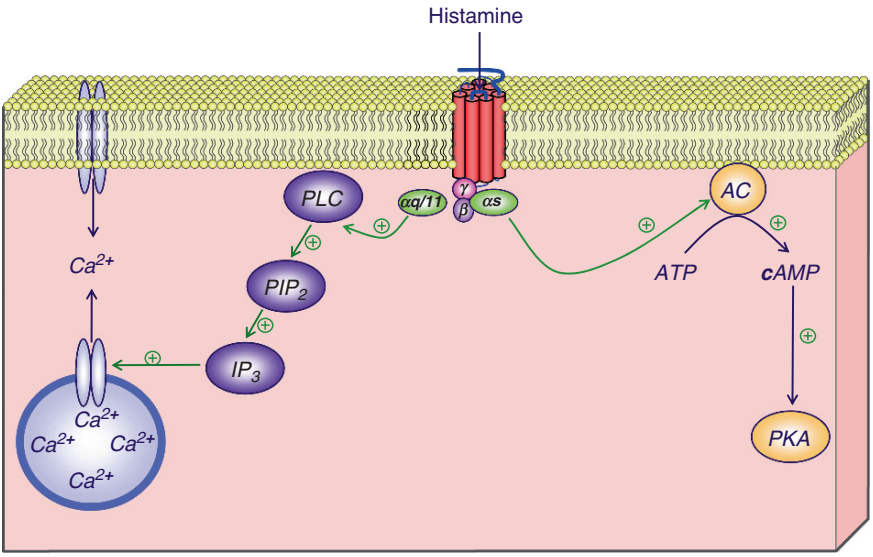

Fig. 10 Activation–secretion coupling of the histamine H_2-receptor Mobilisation of the Gαs subunit stimulates the synthesis of cyclic AMP by membrane-associated adenylyl cyclase (AC) leading to increased activation of protein kinase A (PKA) and production of the physiological response. Also, mobilisation of the Gαq/11 subunit stimulates PLC to form PIP_2 and IP_3 and mobilise calcium as seen in the H_1-receptor

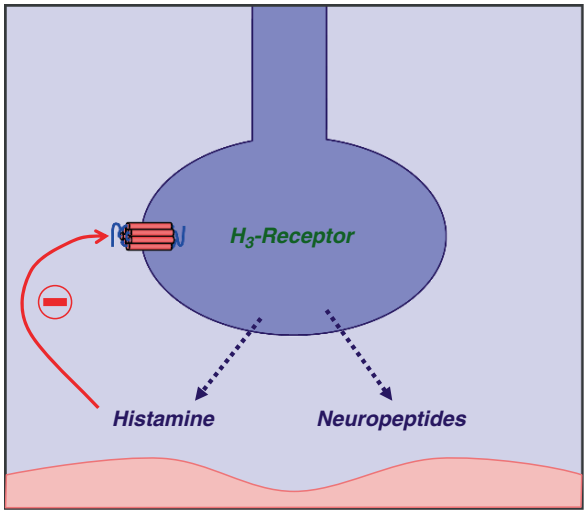

Fig. 11 The histamine H_3-receptor acting as an inhibitory presynaptic receptor for histamine on a nerve

in the brain (Fig. 11). The presence of H_3 receptors in the brain was suggested in 1983 by Jean-Charles Schwartz and his co-workers [102] while investigating the ability of histamine to inhibit its own neuronal synthesis and release from depolarised slices of rat cortex via presynaptic feedback mechanisms. The existence of

the H_3-receptor was confirmed pharmacologically in 1987 following the synthesis of the agonist *R*-α-methylhistamine and the antagonist, thioperamide [103] and cloned in 1999 [104]. Autoradiographic showed that the H3R is primarily in the brain, predominantly in basal ganglia, hippocampus and cortical areas – the parts of the brain that are associated with cognition [105].

Histamine H_3-receptor ligands and antagonists have been the subject of intense research within the pharmaceutical industry over the last decade. Several reviews [106–108] have examined the properties of H_3-receptor antagonists and inverse agonists and suggest that the most promising areas of research are in narcolepsy, cognitive disorders, Alzheimer's disease, obesity, attention impairment and neuropathic pain.

Molecular Aspects and Intracellular Signal Transduction

The human histamine H_3-receptor is a Gi/o-coupled GPCR encoded on chromosome 20 q. The H_3-receptor gene has been reported to contain three exons and two introns [109, 110]. The presence of introns within a gene allows alternative splicing of its products. Indeed, at least 20 isoforms of the human H_3-receptor have been identified to date [111], all of which have the potential of different organ disposition and signal transduction capabilities. Preliminary information on organ disposition indicates that the full length receptor (hH3R-445) is found almost exclusively in the brain, particularly in the thalamus, caudate nucleus, putamen and cerebellum with a lower signal in the amygdala and a faint signal in the substantia nigra, hippocampus and cerebral cortex. No signal was observed in the corpus callosum, spinal cord or in peripheral tissues [109]. In contrast, splice variants hH3R-329 and hH3R-326 showed a high level of expression in the amygdala, substantia nigra, cerebral cortex and hypothalamus, while hH3R-373/365 isoforms are expressed at a high level in the stomach and the hypothalamus [109, 112].

The activation–secretion coupling of the H_3-receptor (Fig. 12) has been the subject of a recent review by Bongers and colleagues [111].

- The primary function of this Gαi/o-coupled GPCR is the inhibition of adenylyl cyclase, which causes a decrease in intracellular cyclic AMP and a subsequent reduction of protein kinase A (PKA) activity. This pathway shows considerably constitutive activity, which may be inhibited by H_3-receptor inverse agonists.
- The H_3-receptor-mediated activation of phospholipase A_2 is also dependent on Gαi/o-proteins. This pathway, which has high constitutive activity, leads to the release of arachidonic acid, which has been suggested to be important in the H_3-receptor-mediated relaxation of the guinea pig epithelium [113].
- Besides H_3-receptor-mediated signalling through Gαi/o-proteins, Gβγ subunits are known to activate signal transduction pathways such as the MAP kinase pathway. MAP kinases are known to have pronounced effects on cellular growth, differentiation and survival, as well as to be important in neuronal plasticity and memory processes [114].

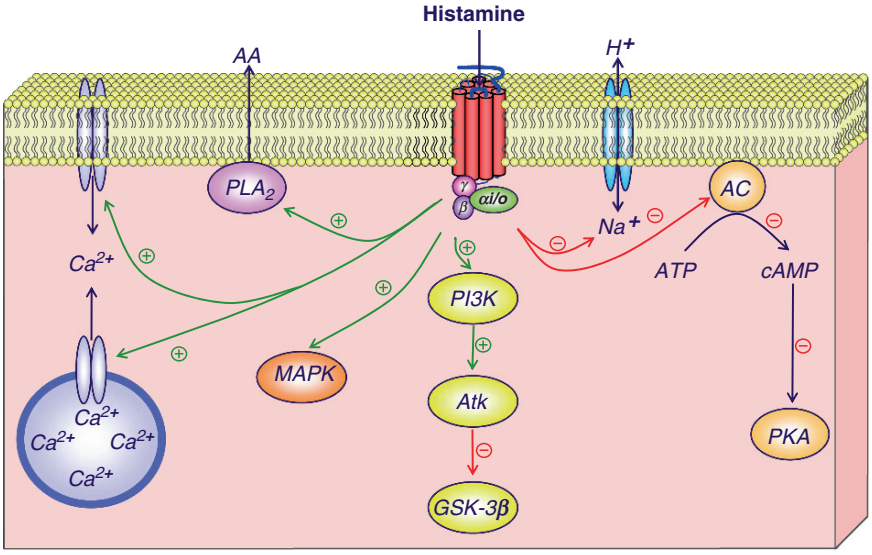

Fig. 12 Activation–secretion coupling of the histamine H_3-receptor The primary function of this Gαi/o subunit is the inhibition of adenylyl cyclase (AC) to cause a decrease in intracellular cyclic AMP and a subsequent reduction of protein kinase A (PKA) activity. The H_3-receptor-mediated activation of phospholipase A_2 (PLA_2) leading to the release of arachidonic acid (AA) is also dependent on Gαi/o-proteins. H_3-receptor-mediated signalling through Gβγ-subunits activates the MAP kinase (MAPK) pathway, Akt/GSK-3β kinases and calcium mobilisation

- Akt/GSK-3β kinases have also been shown to be activated by the H_3-receptor, again an activation pathway, which shows high constitutive activity. In the central nervous system (CNS), the Akt/GSK-3b axis plays a prominent role in brain function and has been implicated in neuronal migration, protection against neuronal apoptosis and is believed to be altered in Alzheimer's disease, neurological disorders and schizophrenia.
- H_3-receptor activation reduces the K^+-induced mobilization of intracellular calcium. This signal transduction mechanism has been linked to inhibitory effect of the H_3-receptor on norepinephrine exocytosis in cardiac synaptosomes [115].
- Activation of the H_3-receptor has been shown to diminish neuronal Na^+ H^+ exchanger activity. Inhibition of this exchanger is essential for the restoration of intracellular physiological pH and preventing acidification during ischemia. It is by this mechanism that H_3-receptor ligands inhibit the excessive release of norepinephrine and the precipitation of cardiac arrhythmias during protracted myocardial ischemia [116].

While the studies of the above pathways have contributed greatly to our knowledge of activation–secretion coupling of the H_3-receptor what is not yet clear is whether they are stimulated preferentially by different isoforms and what is the extent of their clinical relevance.

The Histamine H_4-Receptor

Unlike other histamine receptors, the gene for the H_4-receptor was discovered using knowledge of the human genome and sequence information of the H_3-receptor [117, 118]. Conclusive demonstration of which cell types express the H_4-receptor has been difficult because of its low level of expression and the fact that its expression appears to be controlled by inflammatory stimuli [119]. However, H_4-receptor expression has been shown in the bone marrow and spleen, and on eosinophils and mast cells [120–122].

Ligand-binding studies have shown similarities between the H_3- and H_4-receptors in the binding of the agonist, R-(alpha)-methylhistamine [117] and other H_3-agonists and antagonists, albeit with a different rank order of affinity/potency than at the H_3-receptor [120]. One study suggests that H_4-receptors do not bind conventional H_1- and H_2-antihistamines such as diphenhydramine, loratadine, ranitidine and cimetidine [120]. However, competition-binding studies have shown that some H_1, H_2 and H_3 ligands also show binding at H_4-receptors [123]. The highest affinities were for the tricyclic antidepressants, amitriptyline and chlorpromazine, which also have a high affinity for the H_1-receptor. The other H_1 ligands, which displayed high affinity for H_4-receptors are promethazine, doxepin, indicated for depressive illness, particularly where sedation is required and pruritus in eczema, and cinnarizine, which is indicated for motion sickness and vestibular disorders, such as vertigo, tinnitus, nausea and vomiting in Ménière's disease. The other compounds, which displayed binding were imetit (H_3-selective agonist), imaprit (H_2-selective agonist), mianserin (H_1- and H_2-antagonist), cyproheptadine (nonselective histamine/serotonin antagonist) and clozapine (an atypical antipsychotic drug with high affinity for a large number of receptors) [123].

Functional studies of the H_4-receptor are in their infancy. However, this receptor has been suggested it to be involved in eosinophil chemotaxis and shape change [124–126], in mast cell chemotaxis [121] and in neutrophil chemotaxis by stimulating LTB_4 production [127]. In vivo studies with H_4-receptor-deficient mice and mice treated with H_4-receptor antagonists showed decreased allergic lung inflammation, with decreases in infiltrating lung eosinophils and lymphocytes and decreases in Th2 responses [128]. Ex vivo restimulation of T cells from those animals showed reductions in IL-4, IL-5, IL-13, IL-6 and IL-17 levels. The authors postulate that H_4-receptor blockade on dendritic cells leads to decreases in cytokine and chemokine production and limits their ability to induce Th2 responses.

The expression of histamine H_4-receptors in human synovial cells obtained from patients suffering from rheumatoid arthritis [129, 130], in inflammatory bowel disease [131], in nasal polyposis [132] and in the human placenta in diabetes-complicated pregnancy [133] suggest a widespread role for this receptor in inflammation. Furthermore, its expression on mast cells and eosinophils suggests that the histamine H_4-receptor may represent a therapeutic target for the regulation of immune function, particularly with respect to allergy and asthma.

Molecular Aspects and Intracellular Signal Transduction

The histamine H_4-receptor gene is encoded on chromosome 18q11.2 [117, 123, 134] and has a similar structure to that of the H_3-receptor gene, having three exons and two introns [119]. At the protein level, the human H_4-receptor has a sequence identity of 54% in the transmembrane domains and an overall sequence identity of 31% compared with the H_3-receptor [135]. Only recently have splice variants been reported and that is only in the patent literature [136].

The H_4-receptor is coupled mainly to Gi/o proteins stimulation of which leads to a pertussis-toxin-sensitive decrease in the production of cyclic AMP and the inhibition of downstream events such as cyclic AMP responsive element-binding protein (CREB)-dependent gene transcription [135] (Fig. 13). As with most Gi/o-coupled GPCRs, H_4-receptor activation increases [^{35}S]GTPγS binding [137]. The observation of high basal levels of [^{35}S]GTPγS in cells transfected with H_4-receptors indicates that the H_4-receptor is constitutively active [135]. This is confirmed by the ability of the inverse agonist, thioperamide, to decrease the basal binding of [^{35}S] GTPγS to H_4-receptors in the absence of ligand [137].

Histamine-mediated activation of endogenous H_4-receptors in mast cells results in a clear Ca^{2+} response, which is sensitive to both pertussis toxin and the phospholipase C inhibitor U73122 [121]. These observations indicate that phospholipase C is

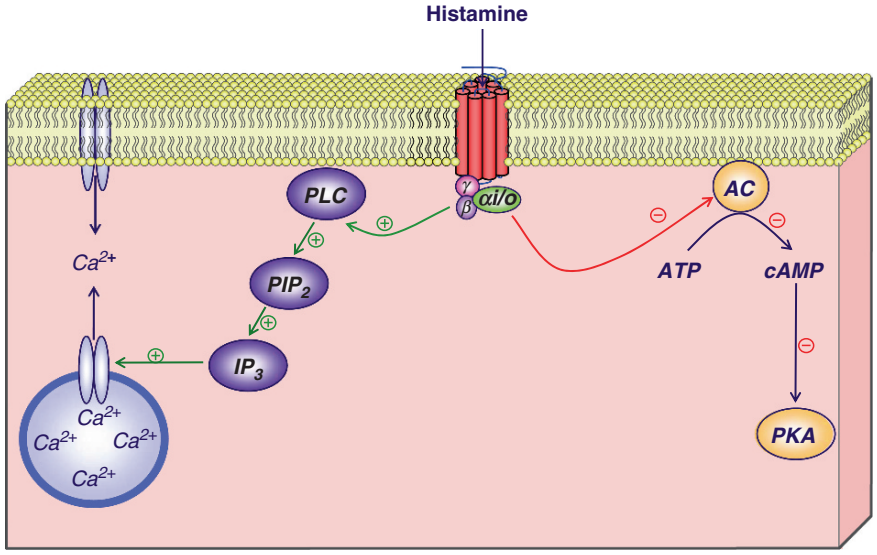

Fig. 13 Activation–secretion coupling of the histamine H4$_3$-receptor Like the H_3-receptor the H_4-receptor is coupled mainly to Gi/o proteins, stimulation of which leads to a decrease in the production of cyclic AMP. Also, the Gβγ subunits that dissociate from Gi/o proteins following H_4-receptor stimulation in mast cells stimulate the PLC/PIP_2/IP_3 pathway to raise intracellular calcium

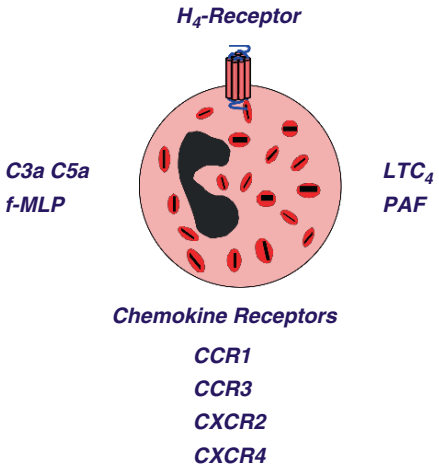

Fig. 14 Receptors linked to eosinophil activation

activated via Gβγ subunits that dissociate from Gi/o proteins following H_4-receptor stimulation in mast cells [135]. This Ca^{2+} response, which is likely to be linked to chemotaxis, has also been demonstrated in eosinophils [125].

While the functional histamine H_4-receptors have been demonstrated on mast cells and eosinophils, their effects on cell activation and their clinical importance compared with cytokines and chemokines are as yet unknown (Fig. 14).

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Adenosine: Its Contribution to Our Understanding in Airway Inflammation

Riccardo Polosa

Foreword

The purine nucleoside adenosine is normally present at low concentration in the extracellular compartment to regulate a wide array of physiologic and immune responses including anti-inflammatory actions [1, 2]. Nonetheless, chronically increased levels of adenosine in the airways may be detrimental. Current research in adenosine deaminase (ADA)-deficient mice indicates that elevated levels of adenosine evoke strong inflammatory responses in the lung with associated features of airway remodeling, tissue fibrosis, and emphysema-like lesions [3–5]. The consequential implication is that an excess of adenosine in the lung parenchyma is likely to dictate a pathogenic mechanisms for the initiation and maintenance of a chronic inflammatory response of the airways such as that characterizing asthma and COPD. However, the contribution of adenosine to the initiation and persistence of this inflammatory response can be quite unpredictable since it may be dictated by changes in the pattern expression, function, and affinity of the four known adenosine receptors subtypes.

Consistent with the hypothesis of adenosine playing an important role in the pathogenesis of chronic inflammatory disorders of the airways, elevated levels of adenosine are found in chronically inflamed airways as they have been detected both in bronchoalveolar lavage (BAL) fluids and exhaled breath condensates (EBC) from patients with asthma [6, 7]. Furthermore, aerosol adenosine exposure induces a dose-related bronchoconstriction in patients with asthma and COPD, but not in normal controls [8, 9].

Taken together, these observations suggest that adenosine signaling could regulate important features of chronic inflammatory disorders of the airways. Selective agonist or antagonist for adenosine receptor subtypes can therefore be used to assess novel therapies for asthma and COPD. In addition, recent work has

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emphasized that bronchoprovocation with adenosine can also be exploited in the clinical and research setting as a useful diagnostic tool for discriminating asthma from COPD and as an accurate biomarker to monitor airway inflammation and corticosteroids requirements in asthma management [10, 11].

Early Findings

A recognized role for adenosine in pulmonary diseases was first described in the late 1970s when Holgate et al. [12] observed that adenosine and related synthetic analogues had the ability of increasing IgE-dependent mediator release from rodent mast cells. A few years later, adenosine was tested by inhalation in man and it was shown to be a powerful bronchoconstrictor of asthmatic, but not of normal airways [8]. Further work showed that both allergic and nonallergic asthmatics responded in a similar way and that the effect was also reproduced with adenosine 5´-monophosphate (AMP) and ADP [13]. As AMP was more soluble than adenosine most of the future inhalation challenge work was conducted using this nucleotide.

Most of the subsequent work with adenosine challenge in asthma was directed at elucidating its mechanism(s) of action by pharmacological dissection in vivo. The main conclusion stemming from these studies was that adenosine could cause bronchoconstriction in asthma indirectly via mast cell activation (Fig. 1).

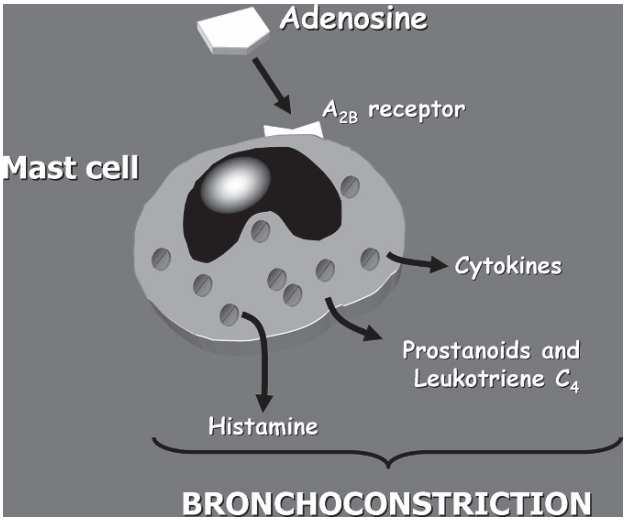

Fig. 1 Mechanisms for adenosine-induced bronchoconstriction. Stimulation of specific adenosine A2B receptors by adenosine activates airway mast cells to release pro-inflammatory mediators, some of which (especially histamine, prostaglandins, and leukotrienes) act as potent bronchoconstrictors in vivo (Modified from [48])

As a matter of fact, AMP provocation of asthmatic airways in vivo was associated with a rise in circulating histamine levels [14] and the immediate bronchoconstriction provoked by inhaled AMP was efficiently antagonised by inhibiting the effects of individual mast cell mediators using selective histamine H1 receptor antagonists [15, 16], cysteinyl leukotriene receptor 1 (*cys*LT1) antagonists [17], and inhibition of cyclooxygenase 1 and 2 [18–20]. Furthermore, premedication with the mast cell stabilizing drugs, sodium cromoglycate, nedocromil sodium and, more recently, andolast, has been shown to powerfully inhibit AMP-induced bronchoconstriction [21–23]. Likewise, the inhibition of the adenosine response by loop diuretics administered by inhalation [24, 25] could also be in relation to their known effects on chloride channels on mast cells to reduce their threshold for activation [26].

Besides the evidence based on simple pharmacological dissection work, more direct evidence that mediators released from airway mast cells are critical for adenosine-induced responses has come from our work in which direct instillation of AMP into asthmatic bronchi [27] or into the nose of patients with allergic rhinitis [28, 29], which resulted in significant increases in the concentration of histamine, tryptase, and prostaglandin D2 (PGD2) in their lavage fluid.

Adenosine as a Diagnostic Tool for Airway Inflammation

For clinical and research purposes, airway responsiveness is commonly assessed by bronchial provocation testing with inhaled methacholine or histamine. However, the bronchoconstrictive stimulus AMP has been recently proposed as a useful diagnostic test for asthma by virtue of its superior specificity and sensitivity.

The current opinion is that airway hyperresponsiveness (AHR) to inhaled AMP is closely related to allergic airway inflammation, whereas AHR to methacholine appears to be a function of the airway caliber. AHR to AMP, but not to methacholine, markedly correlates with both sputum eosinophilia [30, 31], and the measured level of exhaled nitric oxide (eNO) [32, 33], two well-known markers of airway inflammation. Taken together these initial studies have underscored the view that responsiveness to AMP may be used to detect inflammatory changes in the asthmatic airways. A further series of clinical studies have substantiated the view that adenosine provocation is a sensitive inflammatory marker. Bronchoprovocation with AMP appears to be so responsive that might help to identify those latent conditions characterized by subclinical airway inflammation as reflected by studies in non-asthmatic subjects with rhinitis [30] and in adolescents with allergic asthma in complete clinical remission [34]. In both conditions, AHR to inhaled AMP reflects this state of inflammation as shown by the significant inverse correlation between tissue eosinophilia and the PC20 value of AMP (the provocative concentration of AMP that causes a 20% fall in the forced expiratory volume in 1 s), but not of methacholine. Various investigations of allergen avoidance at high altitude have demonstrated a pronounced improvement in responsiveness to AMP, but not

to methacholine or histamine, corroborating the notion that allergen avoidance reduces airway inflammation [35, 36]. Furthermore, the exquisite sensitivity of AMP has been also exploited to assess the nonsteroidal anti-inflammatory potential of several therapeutic agents, including allergen immunotherapy, the leukotriene receptor antagonist montelukast, and the humanized monoclonal anti-IgE antibody Omalizumab [37–39].

Perhaps the most important feature of AMP challenge stands in its greater ability, compared with other surrogate markers of asthmatic inflammation, to promptly discriminate changes in airway reactivity in response to ICS. For example, Van den Berge et al. [40] observed that, compared with the PC20 value of methacholine, the PC20 value of AMP better reflects the reduction in airway inflammation following regular treatment with ICS. More recently, we have examined in more detail the time course of change in AHR to both methacholine and AMP, and in sputum cell counts before, during, and after withdrawal of therapy with the ICS, budesonide [41]. Our findings demonstrated that AMP detected inflammatory changes in the asthmatic airways already at the first week of treatment with budesonide, whereas changes in AHR to methacholine and in terms of eosinophil and epithelial cell counts could be observed only by the forth week of treatment with budesonide. The rapid airway response of AMP to the effect of ICS has been the subject matter of recent work that has detected important reductions in AHR to AMP (but not to histamine) occurring already within 2 h of a single inhalation of ICS [42, 43]. Although the mechanisms that make the response to AMP so sensitive to ICS in asthma remain to be explored, this unique feature of adenosine airway responsiveness could be of great value in asthma management [44]. For example, it would be valuable to monitor corticosteroids requirements in asthma, to establish the appropriate dose needed to control airway inflammation, or to predict safe dose reductions of ICS as advocated in the recent work of Prieto et al. [45].

Adenosine as an Inflammatory Mediator of the Airways

In addition to its role as a diagnostic tool in asthma, adenosine has recently received much attention with regard to its involvement in the pathogenic mechanisms for the initiation and maintenance of a chronic inflammatory response of the airways including asthma and COPD. Most of the evidences have been accumulated from the work in mice with genetic deletion of adenosine deaminase (ADA) gene and with overexpression of IL-13 cytokines indicating that elevated levels of adenosine evoke strong inflammatory responses in the lung with associated features of airway remodeling, tissue fibrosis, and emphysema-like lesions [3–5]. This work shows that when adenosine is generated in large amounts (especially during inflammation) it has the ability of upregulating the expression of molecules that can in turn enhance inflammation and sustain progression, thus providing a compelling mechanism for adenosine-mediated amplification pathway for chronic airway inflammation and remodeling (Fig. 2).

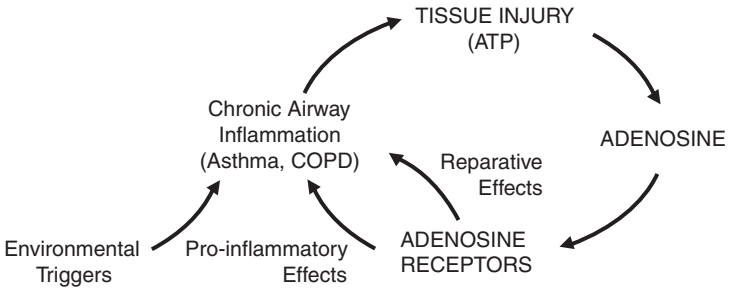

Fig. 2 Proposed model for adenosine-mediated amplification pathway for chronic airway inflammation. Several triggers and cellular mediators lead to the development of asthma and chronic obstructive pulmonary disease (COPD). The resulting inflammation and tissue damage in the lung can create a hypoxic environment conducive to the formation of extracellular adenosine. Although normal levels of extracellular adenosine might serve important roles in the resolution of inflammation or tissue repair by activation of high-affinity adenosine receptors, elevated levels of adenosine may lead to the exacerbation of lung inflammation and damage through the activation of low-affinity adenosine receptors such as the A2B receptor

Bleomycin-induced pulmonary fibrosis models have been recently used to assess the contribution of adenosine in the development of pulmonary diseases. In this animal model, bleomycin exposure causes intense pulmonary inflammation and fibrosis; this is associated with elevated adenosine levels in the lungs of mice exposed to bleomycin as a consequence of enhanced ecto-5´-nucleotidase (CD73) activity [46]. This observation indicates that CD73 is implicated in augmentation of adenosine production in response to a specific inflammatory stimuli such as bleomycin. An elegant extension of their work in a model of double knockout for ecto-5´-nucleotidase ($CD73^{-/-}$), they showed that the bleomycin-dependent severe pulmonary inflammation and fibrosis was still present despite low levels of adenosine. Furthermore, intranasal instillation of exogenous ecto-5´-nucleotidase in $CD73^{-/-}$ mice restored adenosine accumulation and appeared to decrease pulmonary inflammation and fibrosis in response to bleomycin challenge.

The implication from the data obtained from these murine models is that adenosine appears to exert a protective role from inflammatory process in the lung. This has been recently confirmed in Balb/c and C57BL/6 mice [5]. Stimulation with IL-4 in Balb/c IL-4 elicited modest eosinophilic inflammation and mild airway fibrosis, while in C57BL/6 mice, IL-4 caused profound tissue eosinophilia, airway fibrosis, emphysematous alveolar destruction, and premature death. This difference was not attributed to different Th2 or Th1 cytokines secretion profile, but to differences in adenosine metabolism with increased adenosine levels and to enhanced expression of A1, A2B, and A3 adenosine receptor subtypes in C57BL/6 animals. Treatment with ADA reduced the inflammation, fibrosis, and emphysematous destruction and improved the survival of C57BL/6 animals.

Additional evidence for adenosine as a key modulator of inflammatory processes in chronic airway inflammation come from a study on differential gene expression in association with lung inflammation/damage using adenosine deaminase

(ADA)-deficient mice [47]. Out of 1,176 genes studied on the panel array, the expression intensity of several genes was consistently altered compared to wild-type animals; 93 were upregulated and 29 were downregulated. In general, adenosine accumulation was instrumental to upregulation of several pro-inflammatory gene products, such as urokinase-type plasminogen activator (uPA) and its receptor (CD87), the costimulatory molecule B7.2 (important regulator of the development of murine allergic asthma), and several adhesion molecules including intercellular adhesion molecule-1 (ICAM-1), P-selectin, and leukocyte adhesion protein-1 (LFA-1) (all involved in eosinophils trafficking). Furthermore, PEG-ADA treatment in ADA-deficient mice restored adenosine levels, but most importantly this was associated with a significant reduction of these gene overexpressions.

Further evidence linking adenosine signaling to chronic inflammatory airway diseases is provided by the finding in purified cultured cells systems. Several inflammatory and resident cell types known to play an important role in the pathogenesis, progression, and exacerbation of chronic inflammatory airway diseases, constitutively express adenosine receptors and their activation through stimulation of these receptors leads to pertinent effects associated with asthma and COPD phenotypes (reviewed in [48]). Although the contribution of adenosine to the initiation and persistence of the inflammatory response appears to be quite unpredictable depending on the pattern of expression, function, and affinity of the four known adenosine receptors subtypes, it seems that adenosine exerts predominant anti-inflammatory and protective effects in response to tissue injury by activation of high-affinity adenosine receptors such as the A2A receptor [49]. However, elevated levels of adenosine may lead to the exacerbation of lung inflammation and damage through the activation of low-affinity adenosine receptors such as the A2B receptor. Additionally, adenosine receptors may be upregulated by inflammatory cytokines, including TNFα [50].

In the following paragraphs, it is summarized an overview about the role of adenosine signaling in major cell types involved in the pathogenesis, progression, and exacerbation of asthma and COPD.

Neutrophils

Neutrophils have a significant role in the pathogenesis of COPD and are known to be associated with severe chronic asthma [51, 52]. Neutrophils are known to express all four adenosine receptor subtypes and in the course of inflammatory activation are capable of releasing adenosine. Hence, the effector functions of inflammatory neutrophils may thus be subject to autocrine and paracrine control by endogenous adenosine.

The effects of adenosine on neutrophil recruitment from the circulation appear to be bidirectional depending on its extracellular concentration. Enhancement of neutrophil adhesion to the vascular endothelium by stimulation of A1 receptors has been shown at submicromolar concentrations, whereas inhibition of adhesion

occurs at micromolar levels [53–55], which is thought to be mediated by A2A and A2B receptors expressed by neutrophils [56, 57]. Thus, it is likely that adenosine's inhibitory effects probably prevail under inflammatory and hypoxic conditions during which extracellular adenosine levels rise markedly.

After adhesion, neutrophils migrate away from the vascular endothelium up to a gradient of chemoattractants produced in inflamed tissues. Directed migration (i.e., chemotaxis) of neutrophils is promoted by nanomolar concentrations of adenosine through activation of neutrophil A1 receptors [58]. Adenosine appears to have no effect on chemoattractant-induced neutrophil chemotaxis at higher concentrations (i.e., micromolar) [59].

Once activated, neutrophils contribute to the regulation of inflammation by releasing a variety of inflammatory mediators [60]. Conversely, adenosine is also capable of inhibiting the generation of several inflammatory mediators by neutrophils, including LTB_4 (via a decreased A2A receptor-mediated mechanism) [61, 62], TNFα, macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-3α, and MIP-2α (via activation of A2A receptors) [63, 64]. Thus, by inhibiting the production of several pro-inflammatory mediators, adenosine signaling might counteract exuberant neutrophil activation. The role of neutrophils during inflammatory processes is terminated by their apoptosis. High micromolar concentrations of adenosine have been shown to induce apoptosis of HL-60 cells through stimulation of A3 receptors [65], whereas A2A receptor agonists appear to delay the onset of neutrophil apoptosis [66, 67].

Monocytes/Macrophages

Mononuclear phagocytes are innate immune cells that reside in the bloodstream as monocytes or in various tissues as macrophages. In contrast to neutrophils, which principally contribute to acute inflammatory responses, monocytes/macrophages are a major component of chronic inflammatory responses. Alveolar macrophages are strongly implicated in the inflammatory response and tissue injury of COPD by secreting several inflammatory products in response to cigarette smoke and other stimuli [68].

All four adenosine receptors are expressed by both monocytes and macrophages, with receptor expression and function being dependent on their inflammatory activation. For instance, A2A receptor function in human THP-1 monocytes was shown to be upregulated by IL-1 and TNFα, but to be downregulated by IFNγ [69], where in LPS-stimulated macrophages an increase in both A2A and A2B receptor expression was observed [70, 71]. These data seem to suggest that anti-inflammatory A2 receptors are gradually being upregulated following activation of mononuclear phagocytes, possibly as an auto-regulatory mechanism limiting inflammatory processes and prompting resolution.

Overall, adenosine appears to prevent excessive accumulation of monocytes/macrophages in inflamed tissues by inhibiting chemotactic responses [72]. High

micromolar concentrations of adenosine have been shown to inhibit LPS-induced production of the inflammatory chemokine CCL3 by macrophages via A3 receptor activation [73]. Furthermore, direct production of chemokines (i.e., CXCL8) by monocytes is also shown to be inhibited by adenosine [74].

Adenosine may be involved in the regulation of NF-κB activity in monocytes/macrophages, even though its precise role appears to be a matter of debate. Adenosine at high micromolar concentrations inhibited TNFα-induced NF-κB activation in human KBM-5 monocytic cells via activation of A2 receptors [74]. The pathway leading to NF-κB activation may differ in relation to the specific activating agents (e.g., TNFα vs LPS). Indeed, several reports suggest that LPS-mediated activation of NF-κB in monocytes/macrophages is not affected by adenosine [75–77]. A3 receptor stimulation by adenyl carbocyclic nucleoside analogues has been shown to inhibit LPS-induced nuclear translocation of NF-κB in both human and mouse macrophages [78]. Therefore, adenosine might be also activating a negative feedback regulation of pro-inflammatory mediator synthesis during inflammatory responses via inhibition of transcription factors, such as NF-κB.

The effect of adenosine on the ability of monocytes/macrophages to release pro-inflammatory or anti-inflammatory mediators has been investigated in several studies. Adenosine is a well-known inhibitor of the potent pro-inflammatory cytokine TNFα in activated monocytes/macrophages, an effect mediated through activation of multiple adenosine receptor subtypes [75, 79]. Adenosine also inhibited LPS-mediated production of the immunomodulatory cytokine IFNγ in macrophages via stimulation of A3 receptors [80]. Although adenosine does not affect IL-1β production by human and mouse macrophages [81], other studies have shown that adenosine receptor agonists inhibited IL-1β production by immunostimulated human monocytes [82]. Lastly, adenosine increases IL-10 production by both human monocytes/macrophages, through activation of A2A and, as recently shown, A2B receptors [71, 83].

Lymphocytes

There is considerable evidence to support a role of T cells in asthma (mainly CD4 + Th2 cells) [84] and COPD (mainly CD8 + T cells) [85]. Both CD4+ and CD8+ cells constitutively express A2A, A2B, and A3 receptors, whereas little or no A1 receptors are detected on these cells [86, 87]. Furthermore, expression of A2B receptors has been demonstrated to be upregulated following activation of human peripheral CD4 + and CD8 + T cells [88].

Antigen-triggered TCR signaling in naive Th cells induces production of IL-2 and upregulation of cell-surface IL-2 receptors (CD25). Adenosine, at low micromolar concentrations (5–10 μM), appears to inhibit IL-2 and CD25 dependent activation and proliferation of antigen-specific CD4 + and CD8 + T lymphocytes, primarily via stimulation of high-affinity A2A receptors [89, 90]. Although the

role of A2A receptors during inflammatory responses when high micromolar concentrations (50–200 μM) of adenosine accumulate within the inflamed tissues has been little investigated, A2B receptors subtype plays a role in lymphocyte deactivation as it was shown to inhibit TNFα-induced activation of NF-κB in Jurkat T cells [75, 91].

Adenosine modulates cytokine production by activated T cells, but the overall effect on lymphocyte-driven inflammatory responses remains undetermined. TCR-mediated activation of CD4 + cells induced rapid upregulation of functional A2A receptors with inhibition of IFNγ release by these cells [92]. However, another recent study showed that A2A receptor stimulation inhibited IL-2 secretion, but not IFNγ secretion, by a mixed population of effector CD4 + and CD8 + cells [93]. Furthermore, IL-10 secretion (a well-known anti-inflammatory cytokine), but not secretion of IL-4, by a mixed population of effector CD4 + and CD8 + cells was also moderately inhibited through A2A receptor stimulation.

Mast Cells and Eosinophils

Mast cells and eosinophils are strongly implicated in the inflammatory response and tissue injury of a number of chronic inflammatory conditions of the airways, predominantly in asthma, by secreting several inflammatory products in response to a number of immunologic stimuli [94, 95].

Marquardt et al. [96] were the first to suggest that adenosine may potentiate stimulated release of inflammatory mediators (i.e., histamine) from murine mast cells in vitro. Further evidence quoted for the enhancement of mediator release by adenosine came from the work in human lung mast cell. Adenosine markedly enhances the release of both preformed and newly formed inflammatory mediators (especially prostaglandins) from human lung mast cells obtained by enzymatic dispersion [97, 98] and by bronchoalveolar lavage [99]. More direct evidence that histamines released from airway mast cells are critical for adenosine-induced responses has come from our work in which direct instillation of adenosine into asthmatic bronchi [27] or into the nose of patients with allergic rhinitis [28, 29] resulted in significant increases in the concentration of histamine, tryptase, and prostaglandin D2 (PGD2) in their lavage fluid.

Adenosine Receptors Subtypes

Accurate information on the functional role of a specific adenosine receptor subtype is central to our understanding of the role of adenosine and its receptors in airway inflammation. Extracellular adenosine elicits its pro- and anti-inflammatory effects by interacting with four cell-surface G protein-coupled receptors designated as A1, A2A, A2B, and A3 adenosine receptors [100]. All four adenosine receptor

subtypes are expressed in the lung and in inflammatory cells involved in asthma. Ability of adenosine to activate these four receptor subtypes varies. In many tissues, A1 and A2A receptors are present in relatively high amounts and can be activated by the physiological levels of adenosine and thus mediate the stimulant action of adenosine. On the other hand, A2B and A3 receptors appear to have relatively lower affinities and/or receptor reserves for adenosine and require higher concentrations of adenosine for their activation. However, the tissue adenosine levels in many pathophysiological conditions are raised to sufficiently high levels to activate the A2B and A3 receptors.

A1 Adenosine Receptor

The evidence that the A1 receptor is involved in asthma stems from experimental work in rabbits rendered allergic by immunization protocols with allergen; these animals exhibited a bronchoconstrictor response to adenosine that was attenuated by pretreatment with A1 receptor blockers [101, 102]. A confirmation of the role of the A1 receptor in this response was obtained in a rabbit model engineered with an antisense oligodeoxynucleotide targeted against the initiation codon of the A1 receptor mRNA. Animals selectively depleted of their A1 receptors in this way manifested reduction of the bronchoconstrictor response to adenosine and, significantly, to the early response to allergen [103]. However, the relevance of these observations to human asthma can be questioned due to the fundamental mechanistic difference between bronchoconstriction in the rabbit, which is due to activation of A1 receptors on the bronchial smooth muscle, and that in man, which appears to be dependent on activation of mast cells who do not normally express the A1 receptor [104]. Besides, human smooth muscle cells (HSMCs) are known to express only traces of adenosine A1 receptors [105].

If in the rabbit A1 receptor activation appears to mediate bronchoconstriction, in ADA-deficient mice genetic removal of the functional A1 receptor gene leads to enhanced pulmonary inflammation and alveolar destruction, thus indicating that the A1 receptors may serve a protective role in the regulation of pulmonary disorders in which adenosine levels are elevated [106]. However, a putative anti-inflammatory role for A1 receptor signaling in ADA-deficient mice is not consistent with pharmacological studies with adenosine A1 receptor antagonist, which have been shown to be beneficial in attenuating ischemia reperfusion [107] and endotoxin-induced [108] lung injury.

A2A Adenosine Receptor

In the airways, A2A receptors are present on some structural cells and on all major immunoinflammatory cells that have been implicated in asthma. Both human bronchial smooth muscle cells and human lung fibroblasts have been shown to express

A2A receptors [105, 109], but the level of A2A receptor expression appears to be low and in both cell types these receptors do not appear to be functionally active using cyclic AMP accumulation as a functional readout. It is well known that stimulation of A2A receptors abates neutrophil adherence to the endothelium [69], prevents upregulation of integrin expression on neutrophils stimulated with formyl-Met-Leu-Phe [110], and inhibits degranulation of activated neutrophils and monocytes [111, 112].

Therefore, administration of an A2A receptor agonist might exhibit anti-inflammatory potential when neutrophil/monocyte-mediated tissue injury is strongly implicated. Activation of T lymphocytes, which plays a key role in the recruitment of leukocytes to the lung in clinical asthma, is also suppressed by A2A receptor activation [113]. Thus, there are a multitude of mechanisms by which activation at A2A receptors could result in suppression of asthmatic inflammation.

Consistent with the hypothesis that A2A receptor activation could result in suppression of asthmatic inflammation, the selective A2A receptor agonist CGS 21680 proved to have considerable anti-inflammatory activity in a murine model of allergic asthma [114]. The relevance of the pharmacological activation of A2A receptors as a key mechanism limiting inflammation is that compounds such as theophylline, which also block A2A receptors at clinical concentrations, could be pro-inflammatory and hence less effective as therapeutic agents.

The A2B Adenosine Receptor

Functional human adenosine A2B receptors have been identified in the smooth muscle cells [105], lung fibroblasts [109], endothelial cells [115, 116], bronchial epithelium [117], and mast cells [118]. Activation of A2B receptors in the human mast cell line HMC-1 augments IL-8 release per se and potentiates phorbol 12-myristate 13-acetate (PMA)-induced secretion of IL-8 [119]. These findings have been subsequently confirmed by pharmacological studies in vitro describing that selective A2B receptor antagonists potently suppress the activation and degranulation of human mast cells induced by adenosine [120]. In addition, adenosine A2B receptor signaling upregulates Th2 cytokines in mast cells and promotes IgE synthesis by B lymphocytes [121]. Human B lymphocytes cocultured with NECA-stimulated HMC-1 produced very high levels of IgE, whereas lymphocytes cocultured with nonstimulated HMC-1 produced no IgE suggesting that adenosine-dependent responses are involved in the amplification of the allergic inflammatory responses associated with asthma.

The case for A2B receptor signaling in the pathophysiology of asthma receives further support from pharmacological studies of enprophylline, a methylxanthine structurally related to theophylline. It was shown that enprofylline is a selective antagonist for the A2B receptors, whereas theophylline has similar binding affinities for A1, A2A, and A2B receptors. Importantly, the therapeutic concentrations of theophylline and enprophylline are in the range of their affinities for A2B receptors.

These findings support the notion that the A2B receptor might be the therapeutic target for the long-term clinical benefit achieved with relatively low doses of theophylline [120, 122]. There is now evidence that a specific functional antagonism at A2B receptors level is also producing anti-inflammatory effects. In ADA-deficient mice, treatment of an A2B antagonist, CVT-6883, inhibited pulmonary inflammation as determined by the number of inflammatory cells in BALF, as well as the expression of pro-inflammatory cytokines and chemokines [123]. In addition, A2B antagonism inhibited pulmonary fibrosis revealed by reductions of collagen deposition and accumulation of myofibroblasts in the lung of ADA-deficient mice. Treatment with CVT-6883 also reduced pulmonary inflammation and airway remodeling in bleomycin-induced pulmonary fibrosis mice [123]. Altogether, these findings provide strong support that A2B antagonists might be promising therapeutic agents in the treatment of pulmonary diseases such as asthma, COPD, and pulmonary fibrosis.

The A3 Adenosine Receptor

Although the role of the A3 receptor in the pathogenesis of chronic inflammatory airways diseases remains controversial, there is some evidence for a functional role of A3 receptor in mast cell activation. The A3 receptor knockout mouse appears to exhibit altered mast cell functions. In contrast, to bone marrow mast cells obtained from wild-type mouse, adenosine could no longer potentiate antigen-induced mediator release from A3 receptor knockout mouse mast cells [124]. Likewise, adenosine failed to induce histamine release from lung mast cells obtained from A3 knockout mice [125]. Interestingly, in this strain of mice (C57BL/6), adenosine-induced airway hyperresponsiveness was markedly reduced, but not completely blunted in the A3 knockout mice, suggesting the existence of both A3-dependent and A3-independent mechanisms in rodents.

In man there is no evidence of A3 receptor protein in lung mast cells, but rather relatively high density of functionally active A3 receptors are expressed in human eosinophils [126]. Transcript levels for the A3 receptor are elevated in lung biopsies of patients with asthma or COPD [127] and appears to be involved in the inhibition of eosinophil chemotaxis when stimulated [127, 128]. Furthermore, inhibition of important pro-inflammatory functions of human eosinophils by the selective A3 receptor agonist, IBMECA has been reported [129, 130]. Since, asthmatic inflammation is characterized by extensive infiltration of the airways by activated eosinophils [131], it is possible that the elevated adenosine concentrations associated with asthma would contribute to inhibition of eosinophil activation through stimulation of A3 receptors. In contrast, in their effort of dissecting out specific signaling pathways involved in adenosine-mediated pulmonary inflammation and airway remodeling in ADA-deficient mice, Young et al. [132] have recently demonstrated that mice treated with the selective A3 receptor antagonists MRS 1523 resulted in a marked attenuation of pulmonary inflammation, reduced eosinophil infiltration into the airways, and decreased airway mucus production.

Concluding Remarks

It has been 20 years since the first demonstration that adenosine is a bronchoconstrictor in asthmatics. Since then, a large body of literature has accumulated to provide the basis for a new asthma therapy as well as a diagnostic test for asthma and COPD. While all four adenosine receptors have been found in association with lung tissues and appear to be finely modulated during chronic inflammatory responses, a critical role of A2B antagonists in pulmonary inflammation, fibrosis, and airway remodeling have been confirmed in several animal models. The notion that adenosine receptors are widely distributed in different organs and that elevated levels of adenosine are present in chronically inflamed airways emphasize the importance of adenosine as a central therapeutic target. Recognition of the potential role of adenosine receptor signaling in the pathogenesis of chronic airway inflammatory diseases advocates the principle that modulating adenosine receptor signaling could translate into clinical benefit for patients with chronic airway diseases.

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Airway Smooth Muscle Dysfunction in Asthma

Maria B. Sukkar and Kian Fan Chung

Introduction

Asthma is characterised by intermittent airflow obstruction with excessive bronchoconstriction and bronchial hyperresponsiveness (BHR). Since the airway smooth muscle (ASM) is the main contractile cell of the airways, it is clear that the ASM plays a major role in the genesis of these abnormalities. Airflow obstruction does not usually occur in non-asthmatics, thus one would presume that the ASM is abnormally contractile in asthma and there is now evidence that the abnormality of the contractile apparatus can be demonstrated in the asthmatic ASM itself. Therapies to relieve airflow obstruction promptly such as the inhalation of β_2-adrenergic agonists are directly aimed at the ASM. However, over the last 15 years, with improved techniques for culturing ASM cells from the airways and studying ASM in bronchial biopsies, there has been a greater understanding of the capabilities of the ASM cell. Far from being just a contractile cell, the ASM possesses synthetic properties capable of producing inflammatory mediators, growth factors and proteases, thus potentially extending the range and involvement of this cell in airway inflammation and remodeling. The ASM cell can respond to a range of cytokines released by other cells including T-helper type 2 (Th2) cells, which are the orchestrators of the allergic inflammatory response. The ASM cell in asthma is also likely to be subjected to alterations in its proliferative and synthetic phenotype within this inflammatory milieu, not solely due to the effects of other cells or inflammatory mediators, but also secondary to its interactions with its own extracellular matrix. This brings in the concept that the ASM itself may govern its phenotypic properties to a certain extent. We will review the abnormalities of the ASM in asthma and assess its potential role as a synthetic cell and its interactions with the extracellular matrix and inflammatory cells (summarised in Fig. 1 and Table 2).

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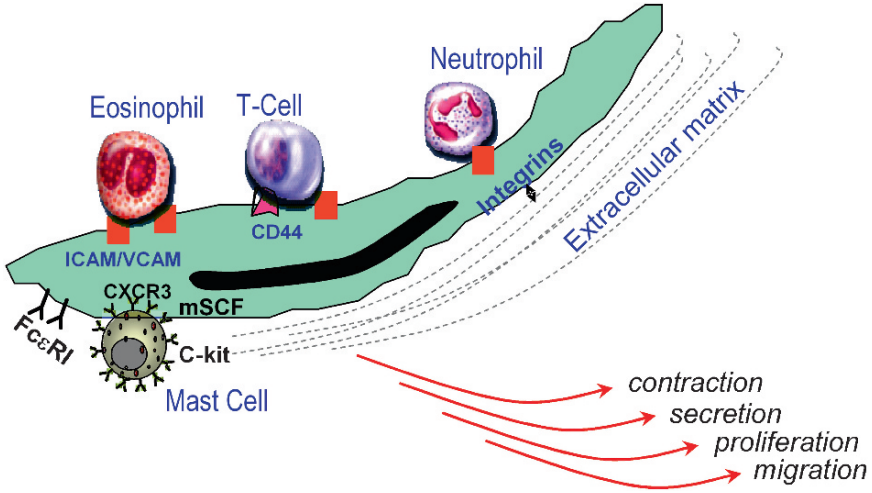

Fig. 1 Schematic diagram showing interactions between ASM, inflammatory cells and extracellular matrix that may lead to phenotypic changes in the contractile, secretory, proliferative and migratory properties of ASM in asthma

Role of ASM

Is there a particular physiological role for the ASM cell? This is a continuing debate amongst physiologists as this area remains contentious and unclear [1, 2]. There have been propositions that the ASM is needed to match ventilation/perfusion relationships, to stabilise and stiffen the airways or to assist in mucus propulsion and clearance, but the evidence for these roles is lacking. The opposite view is that the ASM is vestigial, representing the 'appendix' of the lungs, which can be disposed of without any harm [3]. One technique of 'removing' ASM is that of bronchial thermoplasty, which applies heat energy intraluminally to medium-sized airways leading to removal of the ASM cell layer without apparent long-term effects on the airways of dogs [4]. When this technique was applied to the airways of asthmatic patients, there was an improvement in bronchial hyperresponsive (BHR) [5]. This provided the first direct proof that ASM cells are important for BHR in asthma. Bronchial thermoplasty in patients with moderately severe asthma has led to improvement in asthma control [6], suggesting that ASM cells have no useful physiological function, but rather, contribute to airway dysfunction in disease.

ASM Contractile Responses

BHR, which is the increased contraction of the airways of asthmatic patients in response to inhaled constrictor agents or other stimuli such as allergens, exercise or cold air, can be divided into an increased sensitivity, with a leftward shift and a maxi-

mal response which is exaggerated in asthmatics [7]. In non-asthmatics, a maximum airway narrowing can be achieved with high concentrations of bronchoconstrictor agonist while, in asthmatics, this 'plateau' response is not reached with increasing concentrations. If the maximum response is ever reached in some asthmatics, this may represent a very high level of narrowing that could lead to a fatal attack of asphyxia which is likely to represent total airway closure. It has been proposed that asthmatics have lost a braking mechanism that prevents this excessive narrowing, as occurs in non-asthmatics. The increase in maximal airway narrowing may be due to several factors affecting the ASM, including an increase in ASM mass, an increase in the mechanical properties of non-contractile components of the lung and parenchyma, an uncoupling of ASM from the lung parenchymal recoil, and the lack of ability of the ASM to stretch leading to airway wall stiffening [8, 9].

The ASM in asthma may generate more force in vitro, given that ASM hyperplasia and hypertrophy may be present [10, 11]. Few studies report an increase in maximal isometric contractile responses to constrictor agonists of asthmatic airways [12, 13], whilst others report no changes [14]. More recently, ASM cells grown from asthmatic airway biopsies in collagen gels were found to contract more to constrictor agonists when compared to ASM cells from non-asthmatics [15]. Increased maximum shortening capacity and velocity of single human bronchial smooth muscle cells from asthmatics have been reported [16]; these were associated with an increase in smooth muscle myosin light chain kinase activity, which may lead to enhanced phosphorylation of myosin light chain, allowing actomyosin ATP to be activated by actin, leading to cross-bridge cycling [17]. The contractile response of ASM needs to be examined in its natural environment. For instance, a reduced load on ASM due to uncoupling between ASM and surrounding parenchymal tissues as a result of inflammation and oedema may lead to greater force generation and airway luminal narrowing [18, 19]. Paradoxically, a deep inspiration in asthmatics is ineffective in inducing bronchodilation after bronchoconstriction has been induced, as occurs in non-asthmatics [20]. This effect in asthmatics has been attributed to the loss of interdependence caused by inflammation and oedema that could uncouple the airways from the parenchyma such that the effect of deep inspiration in opening up the airways is no longer effective. This concept is supported by the improvement in bronchodilation following deep inspiration after a course of high dose prednisone in asthma [21]. Thus, excessive airway narrowing and BHR in asthma is not only the result of intrinsic abnormalities of ASM, but also results from the structure and mechanical properties of the airway wall [9].

ASM Hypertrophy and Hyperplasia

The increase in the amount of ASM in the airways appears to be related to the degree of severity of asthma [11, 22, 23]. Both hyperplasia and hypertrophy of ASM cells are present in asthma [10]. Benayoun et al. showed that there was only hypertrophy of ASM cells in severe asthmatics compared to mild/moderate asthmatics, with an increase in myosin light chain kinase content [11]. The increase in proliferative

activity of ASM cells from asthmatic patients observed under culture conditions [24] suggest that this phenotype is ingrained since it is present with repeated passages. There is however limited and conflicting data regarding proliferation of ASM cells in situ. Benayoun et al. could not detect ASM proliferation in airway biopsy tissue from asthmatic patients by using staining for Ki67, a nuclear antigen expressed by proliferating cells [11]. On the other hand, hyperplasia rather than hypertrophy has been demonstrated in mild-moderate asthma [23]. It is possible that proliferation of ASM cells may occur over a prolonged period of time, possibly even starting in childhood [25], thus active proliferation of ASM cells at a snapshot in time may be a rare event not readily detectable by cellular proliferation markers.

The possibility that the ASM is in a proliferative state is quite plausible since proliferation of ASM cells under culture conditions can be induced by a range of growth factors [26]. In addition, studies in animal models of chronic allergen exposures indicate that the ASM can be made to become hyperplastic [27, 28]. Animal models have shed some light as to the mediators that may be involved in this hyperplastic process in ASM cells, which includes cysteinyl leukotrienes, endothelin-1, IL-11, TGF-β and the Th2 cytokines IL-4, IL-5, IL-13 [29–36].

Although ASM proliferation has yet to be convincingly demonstrated in human asthmatic tissues, its increased proliferative activity in culture may have a molecular basis. Asthmatic ASM cells in culture lacked the anti-proliferative isoform of the CCAAT-enhancer binding protein-α (cEBPα) [37]. cEBP-α is an important regulator of the cell cycle inhibitor $p21^{waf/cip1}$, and therefore a deficiency results in increased proliferation. Lack of cEBP-α may also lead to enhanced expression of myosin light chain kinase. In addition, this may also explain the lack of effect of corticosteroids in inhibiting proliferation of ASM cells from asthmatics, while corticosteroids are usually effective in non-asthmatic ASM cells [38]. Increased proliferation may be the result of increased activity of TGF-β in asthmatic ASM cells [39, 40].

ASM Migration

Another possibility underlying the hyperplasia of ASM cells in asthma is that there is recruitment of ASM cells or ASM cell precursors such as fibrocytes or myofibroblasts to the ASM bundle [41]. Therefore, migration of mesenchymal cells from the blood compartment and from the sub-epithelial space to the muscle bundle is necessary. After allergen challenge of allergic asthmatic subjects, an increase in myofibroblasts has been observed in the airways submucosa, and this has led to the suggestion that the myofibroblasts may be migrating and accumulating within the bundle of ASM cells [42, 43]. The other possibility is that proliferation of myofibroblasts in the region of the subepithelial sub-basement membrane may lead to a new population of ASM cells in close proximity to the airway epithelium, which has been observed in severe asthma [11]. These ASM cells may then migrate to the deeper muscle bundles.

ASM cell migration may be induced in vitro by various cytokines and growth factors including IL-1β, TGF-α/β, platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) [44, 45]. Th2 cytokines such as IL-4, IL-5 and IL-13 do not have direct chemotactic activity for ASM cells but IL-13 augments ASM migration towards PDGF [46]. Products of arachidonic acid metabolism including prostaglandins, leukotrienes and lipoxins have also been implicated in ASM migration [45–48]. Of the lipid mediators studied, Prostaglandin D_2 (PGD_2) is the only one with direct chemotactic activity, mediating its effects via activation of DP_2/CRTh2 receptors expressed on ASM cells [46]. Urokinase and its receptor urokinase-type plasminogen activator (uPAR) have also been shown to regulate pro-migratory responses in human ASM cells [49–52].

ASM cells express several chemokine receptors and activation of these receptors by their respective ligands, including CCR3 which is activated by CCL11 (eotaxin) [53], CCR7 which is activated by CCL19 (MIP-3β) [54] and CXCR1 and CXCR2 which are activated by CXCL8 (IL-8) [55], also leads to ASM migration in vitro. ASM cells from asthmatic subjects express greater levels of CCR3 and CCL11 compared with non-asthmatic ASM cells [53, 56] suggesting that the CCR3–CCL11 chemokine axis may be an important mechanism underlying ASM migration in asthma. CCL19 is expressed by ASM cells in patients with severe asthma but not patients with mild/moderate asthma and whilst $CCL19^+$ cells are observed within the bundle of ASM in mild/moderate asthmatics, there are no $CCL19^+$ cells within the bundle of ASM in non-asthmatic patients. Increased numbers of $CCL19^+$ cells are also observed within the bronchial submucosa of mild, moderate and severe asthmatics compared with normal controls [54]. Together, these observations indicate that CCL19, produced by the ASM itself or other cells (particularly mast cells) that accumulate within and around the bundle of ASM may also be involved in driving ASM migration.

ASM Cytokine and Chemokine Synthesis

Evidence pertaining to the secretory function of ASM cells is largely based on cell culture studies where an extensive capacity for these cells to release a large number of cyokines and chemokines has been demonstrated. However, expression of cytokines and chemokines has also been demonstrated within the smooth muscle layer in bronchial biopsy tissues, indicating the capacity for these cells to secrete cytokines and chemokines in situ. Indeed, increased expression of CCL11 [57], CCL19 [54], CXCL10 (IP-10) [58], CX3CL1 (fractalkine) [59] and TGF-β [40, 60] is observed within the ASM layer in bronchial biopsy tissue from asthmatics compared with healthy subjects. Moreover, ASM expression of CCL11 and CXCL8 is greater in patients with severe asthma compared with those with moderate asthma [22]. ASM cells in situ also express CCL5 (Rantes), but there is no difference in the level of expression between healthy subjects and mild asthmatics [61], nor between mild and severe asthmatics [22]. In vitro, cytokine and chemokine

synthesis by ASM cells is largely inducible by inflammatory cytokines such as IL-1β and TNF-α [57, 62–77], but it is also under the control of immunoregulatory cytokines such as IFN-γ [72, 78, 79], IL-4, IL-9, IL-13 [62, 63, 71, 80–87], IL-17A [88–93] and TGF-β [71, 78, 85, 94] (Table 1).

Chemokines released by ASM cells may lead to recruitment and accumulation of inflammatory cells within the airway wall and within the ASM bundle itself [59, 60, 95]. CCL5, CCL11, CXCL1 and GM-CSF released from cytokine-activated ASM cells have been shown to promote eosinophil and neutrophil chemotaxis [57, 62, 76] and eosinophil survival [96].

The microlocalisation of mast cells within smooth muscle bundles is a key factor in the development of BHR, altered airway mechanics and airway remodeling in asthma [95, 97, 98]. Accumulation of mast cells within the ASM bundle has been attributed to the secretion of mast-cell chemotactic factors by the ASM itself. Indeed, CXCL10 produced by asthmatic ASM cells is associated with an enhanced chemotactic activity for human lung mast cells, and neutralising antibodies directed against CXCL10 or CXCR3, the dominant chemokine receptor expressed on mast cells that localise within ASM bundles in asthmatic patients, inhibits almost 80% of this chemotactic activity [58]. Mast cell infiltration of the smooth muscle layer in asthmatic biopsies is also associated with increased ASM expression of CX3CL1 and the neuropeptide vasoactive intestinal peptide (VIP). Since mast cells express the CX3CL1 receptor, CX3CR1 and generation of CX3CL1 by ASM cells promotes chemotaxis of mast cells primed with VIP, the CX3CL1/CX3CR1 axis may be another important mechanism underlying accumulation of mast cells within

Table 1 Regulation of cytokine and chemokine synthesis in ASM cells in vitro

Cytokines and chemokines induced by IL-1-β and TNF-α	• GM-CSF, G-CSF, IL-6, LIF, TSLP • CCL2, CCL5, CCL11, CXCL1, CXCL5, CXCL8, CXCL10, NAP-2
Regulation of ASM cytokine and chemokine synthesis by the Th1 cytokine IFN-α	• IFN-γ synergises with TNF-α in production of CX3CL1 and CXCL10 • IFN-γ inhibits TNF-α induced IL-6, CCL5 and CXCL8
Regulation of ASM cytokine and chemokine synthesis by the Th2 cytokines IL-4, IL-9 and IL-13	• IL-4, IL-9 and IL-13 induce CCL11 release and synergise with IL-1β to augment CCL11 release • IL-4 and IL-13 induce eotaxin-3 release • IL-13 increases CCL2, CCL27 and IFN-β gene expression and inhibits CXCL8 gene expression • IL-4 and IL-13 synergise with TNF-α to augment CCL17 release • IL-4 and IL-13 inhibit IL-1β-induced CCL5 release • IL-4 and IL-13 inhibit IFN-γ/TNF-α dependent CCL5 and CXCL8 release
Regulation of ASM cytokine and chemokine synthesis by the Th17 cytokine IL-17A	• IL-17A induces CCL11 and CXCL8 release • IL-17A augments IL-1β and TNF-α dependent IL-6, CCL11 and CXCL8 release

Table 2 Abnormalities of ASM in situ and in cultured cells

ASM abnormality	ASM in situ	ASM cells in culture
Hyperresponsiveness	• Increased contraction; Impaired relaxation	• Increased contraction; Impaired relaxation
Increased muscle bulk	• Hypertrophy and hyperplasia	• Increased proliferation that is resistant to corticosteroids
Altered ECM synthesis	• Increased lumican and biglycan synthesis	• Increased perlecan, fibronectin, collagen I synthesis • Decreased laminin α1, collagen IV synthesis
Increased production/ activity of inflammatory mediators and growth factors	• Increased CCL11, CCL19, CXCL8, CXCL10, CX3CL1, TGF-β, CTGF; • Increased IL-9Rα, IL-17BR, CCR3	• Increased CCL11, CXCL10
Decreased production of anti-inflammatory and broncho-protective mediators		• Decreased stimulated-induction of COX-2 and PGE_2
Heightened immune function	• Increased FcεRII/CD23 expression	• Increased FcεRII/CD23 expression • Increased stimulated-expression of CD40 and OX40L • Increased OX40L-mediated IL-6 release • Increased viral-stimulated IL-6 and CXCL8 release

the smooth muscle layer [59, 78]. Interestingly, culture supernatants from non-asthmatic ASM cells activated with Th1 or Th2 cytokine mixtures inhibit the mast cell chemotactic activity of similarly activated asthmatic ASM cells, suggesting that asthmatic ASM cells may lack an inhibitory factor(s) that results in excessive mast cell migration in asthmatic inflammation [99]. Once recruited to the ASM bundle, mast cells may become activated and thereby lead to a perpetuating process of mast cell recruitment and activation within the ASM bundle [60, 100, 101].

ASM Prostanoid Synthesis

In addition to synthesising cytokines and chemokines, ASM cells are also a rich source of prostanoids. PGE_2 is one of the major prostanoids produced by ASM and has been the most widely studied [102–108]. PGE_2 has a number of broncho-protective effects in human airways including the inhibition of proliferation and migration of ASM cells [45, 47, 109] and the inhibition of bronchoconstriction [110]. PGE_2 also acts to limit ASM-inflammatory responses by inhibiting

production of GM-CSF, CCL5 and connective tissue growth factor (CTGF), the expression of cell adhesion and co-stimulatory molecules such as ICAM-1, VCAM-1 and CD40, and the adhesion of activated T-lymphocytes in cytokine-stimulated ASM cells [70, 111–115]. On the other hand, PGE_2 also acts to promote cytokine-mediated induction of IL-6 and G-CSF [73, 116] and the angiogenic factor vascular endothelial-derived growth factor (VEGF) [115, 117–119]. Interestingly, in asthmatic ASM cells, trypsin- and bradykinin-mediated induction of COX-2 and PGE_2 is reduced compared with that in non-asthmatic ASM cells. In addition, impaired PGE_2 synthesis was observed in asthmatic ASM cells under proliferating conditions [107]. The reduced capacity for asthmatic ASM cells to synthesise PGE_2 may suggest a compensatory mechanism to limit the production of certain cytokines and angiogenic factors. However, because PGE_2 has anti-inflammatory, anti-proliferative and anti-migratory effects in ASM cells, the reduced capacity for asthmatic ASM cells to synthesis PGE_2 may also potentially contribute to increased muscle bulk and ASM-mediated inflammation in asthma.

Role of ASM as an Immune-Regulatory Organ

Emerging evidence over the past 15 years indicates that ASM cells may directly access cells of the adaptive immune system by virtue of their capacity to express a host of cell adhesion and co-stimulatory molecules. Cellular interactions between ASM cells and T-lymphocytes lead to reciprocal activation of both cell types and may potentially be an important mechanism linking immune-mediated inflammation and abnormal ASM function in asthma. The discovery that ASM cells also express functional MHC class II molecules and IgE receptors suggest that ASM cells may also elicit activation of adaptive immune mechanisms. More recently, ASM cells have been shown to express functional Toll-like receptors (TLRs), pattern-recognition receptors that mediate innate and adaptive immune responses to infectious pathogens. Since infections of the respiratory tract by various pathogens, including bacteria, viruses and fungi, are the major cause of disease exacerbation in asthma, activation of TLRs in ASM by microbial products may be a possible mechanism of disease exacerbation. In the following sections we will present the evidence pertaining to the expression and function of immune receptors and cell surface molecules in ASM cells.

IgE Receptors

Gounni and colleagues [120] demonstrated expression of the high-affinity IgE receptor (FcεRI) within the bundle of ASM in bronchial biopsy tissue from asthmatic subjects and in cultured ASM cells. In contrast, Hakonarson and colleagues [121, 122] did not detect FcεRI expression in ASM tissue isolated from either

asthmatic or non-asthmatic patients, nor in cultured ASM cells. These investigators, however, did find increased expression of the low-affinity IgE receptor (FcεRII/CD23) in ASM tissue isolated from asthmatic patients compared with non-asthmatic patients, and also detected its expression in cultured cells. ASM cells can be 'sensitised' (meaning they can be induced to express FcεRI and CD23) when incubated with IgE or atopic asthmatic serum containing IgE-immune complexes [120–122]. Expression of CD23 is also induced in response to GM-CSF or IL-4, but not other Th2-type cytokines such as IL-5 and IL-13 [123]. Activation of both the high and low-affinity IgE receptors has been associated with pro-inflammatory and hyper-contractile responses in ASM cells in vitro [120–122].

Cell Adhesion, Co-stimulatory and MHC Class II Molecules

ASM cells express cell adhesion molecules (CAMs) such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), which facilitate their interaction with a number of inflammatory cell types, including T-lymphocytes, eosinophils and neutrophils [75, 114, 124–128]. In addition to CAMs, ASM cells also express a number of co-stimulatory molecules. The co-stimulatory molecules CD40 and OX40 ligand (OX40L) are constitutively expressed in ASM, and their expression is further up-regulated following stimulation with inflammatory cytokines such as IFN-γ or TNF-α [114, 129, 130]. Importantly, however, following stimulation with TNF-α, asthmatic ASM cells express greater levels of CD40 and OX40L compared with non-asthmatic ASM cells. Ligation of CD40 and OX40L in ASM by their respective counter-ligands, CD40L and OX40, activates signal-transduction pathways leading to generation of inflammatory cytokines such as IL-6 [129, 130]. Moreover, ligation of OX40L in asthmatic ASM cells leads to greater levels of IL-6 production compared with non-asthmatic cells [130]. In concert with CAMs, co-stimulatory molecules serve to facilitate cellular interactions between ASM cells and inflammatory cells [126].

Contact-dependent interactions between ASM cells and activated T cells have been implicated in ASM proliferation and hyperresponsiveness in asthma. This was first demonstrated in vitro by Lazaar and colleagues who showed that adhesion of activated T cells to ASM cells was associated with increased proliferation of ASM cells [124]. In in vivo studies, adoptive transfer of $CD4^+$ T cells from sensitised rats leads to increased ASM bulk in naïve recipients following repeated antigen challenge. In this animal model, adoptively transferred T cells were localised to the ASM bundle and the increase in muscle bulk was attributed to T cell mediated inhibition of apoptosis and increased proliferation of ASM cells. Moreover, in complimentary in vitro studies, increased proliferation of ASM cells isolated from sensitised rats was observed when these cells were co-cultured with activated $CD4^+$ T cells [28]. Hakonarson and colleagues showed that incubation of rabbit tracheal segments with activated human T cells leads to altered airway responsiveness, indicated by enhanced acetylcholine-induced contraction and impaired β-adrenergic-mediated

relaxation. Alterations in ASM responsiveness were attributed to induction of IL-5 and IL-1β release [131] and intercellular co-ligation of ICAM-1 and the co-stimulatory molecules CD40 and CD86 [126], cytokines and surface molecules which were shown to be inducibly expressed in human ASM cells following adhesion of activated T cells to the ASM [125, 126, 131].

In addition to CAMs and co-stimulatory molecules discussed above, adherence of activated T-lymphocytes to ASM also induces expression of MHC class II molecules on the ASM, an effect that is mediated by IFN-γ secreted by activated T-lymphocytes [125]. Inducible expression of MHC class II in ASM indicates that ASM cells may potentially function as non-professional antigen-presenting cells, however, despite this, Lazaar and colleagues showed that ASM cells pre-treated with IFN-γ could not induce proliferation nor expression of the activation marker CD25 in co-cultured T cells, indicating that ASM cells could not present alloantigen [125]. Recently, however, it was shown that ASM cells were capable of presenting the superantigen (SAg), staphylococcal enterotoxin, via their MHC class II molecules to resting $CD4^+$ T cells, thereby eliciting T cell activation and ASM hyperresponsiveness [128].

Toll-Like Receptors

Toll-like receptors (TLRs) recognise a diverse range of microbial-derived lipid, protein and nucleic acid structures known as micro-organism associated molecular patterns (MAMPs) [132]. All ten of the currently known human TLRs (TLR1-10) have been demonstrated in human ASM cells in culture. ASM expression of TLRs 2, 3 and 4 is regulated by inflammatory cytokines such as IL-1β, TNF-α and IFN-γ [133].

Exposure of ASM cells to the TLR2 ligand lipoteichoic acid (LTA) induces calcium mobilisation and activates ERK1/2 signaling pathways [134] whilst exposure to LPS, a TLR4 ligand which is the major cell wall component of gram-negative bacteria, release several cytokines including IL-6, IL-8 and CCL11 [133, 135, 136].

The viral replicative intermediate dsRNA, which is a ligand for TLR3, is a potent inducer of ASM mediator release, inducing abundant expression of IL-6, IL-8, CCL11, CCL5 and CXCL10 [133, 137, 138]. It also induces expression of ICAM-1, the cell surface receptor for rhinovirus [137] as well as that of its own receptor (TLR3) and other TLRs (TLR2 and TLR4) [133]. Infection of cultured human ASM cells with respiratory viruses such as respiratory syncytial virus (RSV) or rhinovirus leads to induction of ASM cytokine and chemokine synthesis [94, 139]; however, it is not known whether this is mediated via activation of viral sensing TLRs. Evidence that rhinovirus infected ASM cells from asthmatic patients have an enhanced capacity to synthesise IL-6 and IL-8 compared to cells from non-asthmatic subjects possibly indicates up-regulation of TLR expression and function in asthmatic ASM [139].

Activation of TLRs in ASM cells may also facilitate ASM-inflammatory cell interactions and thereby lead to amplification of inflammatory responses in both cell

types. This is indicated by studies where addition of TLR2, TLR4, TLR7 or TLR8 ligands to ASM cells in co-culture with peripheral blood mononuclear cells (PBMCs) leads to greater release of IL-6, CXCL-8 and CCL2 compared to TLR-activation of either cell type alone [135, 137]. LPS and dsRNA may also promote ASM-inflammatory cell interactions via induction of cell adhesion molecules [127, 137].

In addition to promoting ASM-inflammatory responses, activation of TLRs in ASM may alter ASM responsiveness to contractile and relaxant agonists, and thereby contribute to induction of BHR in asthma. This is indicated by studies which show that dsRNA and LPS potentiate agonist-induced contractile responses whilst limiting agonist-mediated relaxant responses of tracheal segments in vitro [136, 140]. BHR secondary to respiratory viral infection in asthma may also possibly be due to TLR-mediated changes in ASM responsiveness. Rhinovirus, a respiratory virus highly implicated in the pathogenesis of asthma, leads to increased contractility and impaired relaxation of rabbit tracheal tissue in vitro [141]. Furthermore, infection of cultured human ASM cells with respiratory syncytial virus (RSV), another respiratory virus linked to the pathogenesis of asthma, leads to inhibition of β-adrenergic responses, as demonstrated by inhibition of agonist-induced cAMP formation and decreased β2-adrenergic receptor density [142]. Whether virus-induced changes in ASM responsiveness are mediated by viral sensing TLRs such as TLR3, TLR4, TLR7 or TLR8, or other intracellular viral recognition proteins remains to be determined.

ASM and the Extracellular Matrix (ECM)

The quantity and composition of the extracellular matrix are altered in the airways of asthmatic patients [143, 144]. Increased deposition of the proteoglycans lumican, biglycan and versican is observed within the sub-epithelial layer in moderate asthmatic patients compared normal controls, and whilst there is no difference in sub-epithelial expression of these proteoglycans in moderate and severe asthmatics, there is increased expression of lumican and biglycan within the ASM layer in *moderate* asthmatics compared with *severe* asthmatics [145–147]. In contrast, the expression of the proteoglycan decorin is decreased in patients with mild asthma compared with normal controls [146]. Changes in the composition of the ECM also include increased deposition of type I, III and V collagens, fibronectin, tenascin and most laminin subunits, and decreased deposition of collagen IV and elastin [148–154]. Increased matrix turnover in asthmatic patients is also indicated by the presence of increased levels of fibronectin and hyaluronan in bronchoalveolar fluid [155, 156].

An important aspect of ASM cells is their capacity to regulate and interact with their surrounding extracellular matrix. ASM cells in culture have the capacity to produce many ECM proteins including collagens, fibronectin, elastin, laminins and the proteoglycans decorin, perlecan and versican when exposed to serum or growth factors such as TGF-β, epidermal growth factor (EGF), CTGF and

VEGF [157–163]. Cytokines such as GM-CSF have also been shown to induce ASM collagen I and fibronectin synthesis [164]. ASM cells from asthmatics appear to produce more fibronectin, perlecan and collagen I and less laminin α1 and collagen IV when compared to non-asthmatic cells [56, 165]. This pattern of ECM secretion reflects, to some extent, the altered composition of ECM in asthmatic airways, indicating that altered regulation of ECM synthesis in ASM cells may underlie abnormalities of the ECM within the airway wall. Enhanced ECM production may be the result of increased activity of TGF-β in asthmatic ASM cells. TGF-β induces the expression of growth factors such as CTGF and VEGF, which may in turn act on the muscle to induce expression of collagen I and fibronectin [39, 40, 115, 162, 163, 166].

The extracellular matrix surrounding the ASM can influence the function of the ASM cell itself, including its attachment, migration, proliferation, apoptosis, contractile response and cytokine production, thus, altered regulation of ASM cell ECM synthesis, may in turn, lead to altered ASM function. This is supported by studies showing that ECM prepared from asthmatic cells leads to increased proliferation and CCL11 production in non-asthmatic cells [56, 165]. Increased production of fibronectin and collagen type I by asthmatic ASM cells may explain ASM modulation away from a contractile phenotype in asthma, as ASM cells grown on fibronectin or collagen type I show enhanced proliferative, secretory and migratory responses compared to cells grown on plastic [56, 167–171]. Fibronectin and collagen type I enhancement of ASM proliferative and secretory responses are mediated via their β1-integrin receptors expressed on ASM cells [169, 170].

Fibronectin and collagen type I also lead to decreased expression of ASM contractile proteins and attenuation of ASM contractile responses in vitro [167, 171], thereby suggesting that modulation towards a proliferative, secretory and migratory phenotype is associated with a co-comittant loss of contractile function. In contrast to fibronectin and collagen type I, ASM cells grown on laminin display features of a more contractile phenotype [167, 168, 171]. Moreover, endogenous expression of laminin-2 (consisting of the laminin subunits α2, β1 and γ1) and laminin-binding integrins is required for ASM maturation to the contractile phenotype in vitro [172, 173]. Thus, altered regulation of laminin-dependent processes in asthmatic ASM cells, possibly indicated by reduced secretion of the laminin α1 subunit [165], may also contribute to altered ASM function in asthma.

Another very important function of the ECM surrounding ASM is that it provides a very strong survival signal to these cells. Indeed, ligation of the common integrin heterodimer α5β1 by various ECM components, including fibronectin, collagens type I and IV, and laminin, prevents apoptosis of ASM cells in vitro [174]. Degradation of the smooth muscle pericellular matrix by chymase, a protease released by activated mast cells, leads to inhibition of ASM growth [175], whilst matrix degradation by neutrophil-derived proteases, including elastase, cathepsin G, and proteinase 3 induces ASM apoptosis [176]. Mast cells accumulate within the ASM bundle in asthma [97, 100], whilst neutrophils are prominent cells in severe asthma [177–179], thus, these proteases may serve to

counteract growth signals to the ASM and thereby curtail the increase in muscle bulk in asthma.

ASM Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes that play a major role in matrix turnover, remodeling and angiogenesis [180]. Human ASM cells constitutively express pro-MMP-2, MMP-3 and MT1-MMP [181]. MMP-3 is usually bound to ASM-derived matrix, consistent with the staining for MMP-3 in the submucosal matrix of patients with chronic asthma [182]. IL-1β activates human ASM cells to secrete MMP-9 [183] and MMP-12 [184]. Increased expression of the disintegrin-metalloproteases ADAM-33 and ADAM-8 in asthmatic ASM has been reported [185] whilst the metalloprotease domain of ADAM-15 has an inhibitory effect on ASM migration induced by PDGF [186]. ASM cells also produce tissue inhibitor of metalloproteinase (TIMP) particularly TIMP-1 and TIMP-2, which counteract the proteolytic activity of secreted MMPs [181]. Whether there is a shift in the MMP/TIMP balance in ASM cells in airway disease remains to be determined.

There is evidence to indicate that MMPs may contribute to airway wall remodelling by modulating ASM proliferation and migration. The autocrine production of MMP-2 by ASM cells may regulate ASM proliferative and migratory responses [187, 188]. MMPs regulate ASM proliferation by causing the release of immobilised growth factors such as TGF-β [189]. In addition, MMPs may degrade insulin-like growth factor binding proteins causing the release of insulin-growth factor (IGF) [190]. IGF-II is released from ASM cells and induces ASM proliferation [191].

The cells in the lung are constantly exposed to forces of stretch and relaxation and excessive stretch in airway disease due to excessive airway narrowing may potentially contribute to airway wall remodelling by promoting ASM cell proliferation and migration. Mechanical strain applied to human ASM cells in culture increases their proliferation and migration by inducing expression of extracellular MMP inducer which leads to the subsequent release and activation of MMP-1, -2, -3 and MT1-MMP [192].

ASM Phenotypic Changes

With these changes noted in ASM cells from asthmatic patients, the major question in ASM biology is whether the ASM cell can switch phenotype between a hypercontractile, synthetic or proliferative state (Fig. 1 and Table 1). Under culture conditions, synthetic cells with a low density of contractile proteins and high content of cytoskeletal proteins are in a proliferative state and may lose their

contractile ability [193]. They may be producing more inflammatory mediators and extracellular matrix proteins. On the other hand, hypercontractile cells have been obtained in canine trachealis smooth muscle cells cultured after prolonged withdrawal of serum [194]. The content of smooth muscle myosin light chain kinase was increased 30-fold and there was faster and greater shortening in response to contractile agents. This is reminiscent of the changes these investigators observed in asthmatic ASM cells [16], although the hypertrophy of ASM cells in mild-to-moderate asthma is not a uniform observation, and in one study using gene analysis, no differences in ASM phenotypic markers was reported [23]. In a chronic allergen-exposed rat model, an increase in maximal contractile response was observed in the presence of significant reduction of ASM contractile proteins [195], and alterations in the contractile apparatus is more likely to underlie this increased contractility, associated with actin-myosin remodelling rather than with quantity of contractile proteins [196].

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Genetic Variation in Cytokines, Asthma, and Atopy: The Role of IL-4/IL-13 Pathway Polymorphisms

Michaela Schedel and Michael Kabesch

Introduction

Cytokines belong to a group of hematopoetic molecules which are particularly important, mediating both the innate and adaptive immune responses. Due to their central role in all kinds of immune responses, cytokines are also involved in the pathogenesis of asthma, a major chronic inflammatory disease of the airways [1, 2]. All cytokines serve the cell-to-cell communication. Therefore, it is difficult to dissect the effect of individual cytokines in a disease as the simultaneous release of other cytokines may overlap in function. The signaling of cytokines is mediated through specific receptors, which are mainly expressed on the surface of inflammatory cells. Subsequently, cascades of intracellular signaling may regulate the expression of several genes and their transcription factors involved in inflammatory responses. In turn, other cytokines may be produced, the number of surface receptors for other molecules may be increased or the effect of the respective cytokine itself may be suppressed.

To our current understanding, there are five groups of cytokines which may be involved in the development of atopic diseases: (1) lymphokines (IL-2, IL-3, IL-4, IL-5, IL-13, IL-17), (2) pro-inflammatory cytokines (IL-1, TNF [tumor necrosis factor], IL-6, GM-CSF), (3) anti-inflammatory cytokines (IL-10, IFN-γ, IL-12, IL-18), (4) chemokines (RANTES, MCP1-4, eotaxin, IL-8) and (5) growth factors (PDGF, TGF-β) [2]. An alternative classification is based on the distinct function of cytokines in the proliferation and functioning of different subsets of T cells: T helper cells type 1 (T_H1 cells, IFN-γ, IL-12), T_H2 cells (IL-4, IL-5, IL-13), T_H17 cells (IL-6, IL-17) and $T_{regulatory}$ cells (IL-10, TGF-β), respectively.

Numerous cytokine signaling pathways play an important role in the pathogenesis of atopic diseases. Hence, genetic variations in cytokines were intensively studied within the past years, as cytokines were determined putative susceptibility genes

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for asthma and atopy. Of all cytokine pathways involved in asthma, the IL-4/IL-13 cytokine pathway is one of the best characterized. Its activation mainly regulates the switching of immunoglobulin (Ig) production from IgM to IgE, which is a crucial step for the development of an atopic immune response. Additionally, the IL-4/IL-13 pathway is also important for many other features found in asthma and atopy, such as airway remodeling and smooth muscle contraction. In the following chapter, we will discuss the role of genetic variants in cytokine genes in this pathway as an example for many others for which not nearly as much data have yet been published.

The Activation of the IL-4/IL-13 Pathway Induces Class-Switching to IgE

Both cytokines IL-4 and IL-13 signal through this common pathway inducing IgE production by B cells [3, 4] (Fig. 1). On the surface of T cells, IL-4 binds to its receptor composed of IL-4 receptor α (IL-4Rα) chain and the common γ chain. In contrast, IL-13 binds to its own specific receptor subunit IL-13Rα1 chain, to which IL-4 cannot bind, and additionally to the IL-4Rα chain [5]. After the binding of IL-4 and IL-13 the oligomerization of these receptors is induced. Subsequently, the activated Janus tyrosine kinases (Jak) initiate the phosphorylation of the intracellular molecule signal transducer and activator of transcription 6 (STAT6). Once

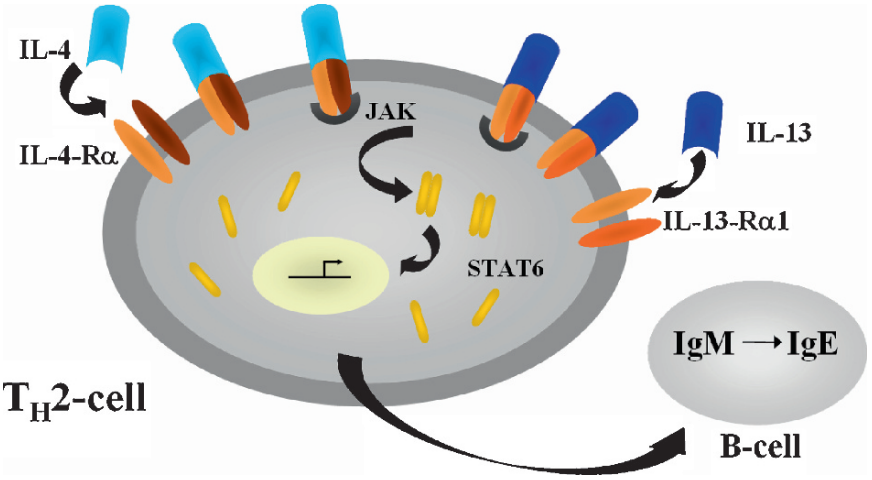

Fig. 1 IL-4 and IL-13 bind to a shared receptor on the surface of T_H2 cells inducing the activation of Janus tyrosine kinases (Jak). The phosphorylation of the intracellular molecule STAT6 through the activated Jaks leads to a homodimerization of STAT6, which translocate to the nucleus and bind to specific regions of IL-4- and IL-13-inducible genes. Thus, crucial target genes for the synthesis of IgE are activated

phosphorylated, STAT6 homodimers translocate into the nucleus. Thus, STAT6 binds to IL-4/IL-13 responsive regulatory gene regions leading to the class-switch to IgE in B cells.

Both cytokines IL-4 and IL-13 are located on chromosome 5q31 in close vicinity to each other. IL-4 and IL-13 are primarily produced by T_H2-polarized cells but natural killer cells [6] and eosinophils [7, 8] are also an important source. IL-4 is additionally synthesized by B cells [9].

IL-4 and IL-13 are both crucial for the IgE regulation, but they exert further functions some of which are overlapping, including T_H2 differentiation and the T_H2 development as well as differentiation of B cells and monocytes [10]. It was shown that mice lacking IL-4 or IL-13 had a significant impaired induction of the T_H2 differentiation [11, 12]. Interestingly, only double knockout mice led to a complete abrogation of eosinophil infiltration, IgE production, IL-5 secretion and differentiation to a T_H1-like phenotype [13]. Thus, the expression of either IL-4 or IL-13 may be sufficient to induce a T_H2 response, but their concomitant release seems to exert an additive effect on the immune response against allergens.

Furthermore, the crucial role of both cytokines in the pathogenesis of atopic diseases has been well documented: in IL-4-deficient mice, the eosinophilic inflammation as well as the induction of bronchial hyperresponsiveness (BHR) were absent [14]. In addition, antigen challenge of IL-4 knockout mice resulted in a substantial attenuation of IL-5 secretion [15]. IL-13-deficient mice were unable to develop BHR even though airway inflammation and high levels of IL-4 and IL-5 were detectable [16]. However, the induction of BHR was restored after administration of recombinant IL-13 to these mice indicating that IL-13 is also necessary for BHR. In contrast to IL-4 knockout mice, only IL-13-deficient mice failed to generate goblet cells which are responsible for mucus overproduction in asthma [17]. Hence, IL-13 may be more relevant for mucus hypersecretion and subepithelial fibrosis.

Both cytokines IL-4 and IL-13 mediate their signal through the IL-4 receptor α (IL-4Rα), which is also known to play a crucial role in allergic inflammation. The IL-4Rα expression has been observed on hematopoetic as well as non-hematopoetic cells [18]. In *IL-4Rα* knockout mice, the IgE production was diminished and the differentiation to T_H2 cell was impaired [19]. Mice lacking *IL-4Rα* were also not able to develop BHR, to induce mucus production and airway inflammation [20]. Furthermore, it was previously suggested that the goblet cell metaplasia and the mucus production after allergic inflammation strongly depend on the signal transduction through IL-4Rα [21].

In contrast to IL-4Rα, little is known about the function of the IL-13 receptor α chain. It was shown that IL-13 but not IL-4 can signal through IL-13Rα [5] with only weak binding on its own. However, in the presence of both IL-13Rα and IL-4Rα a substantial binding affinity is induced [22]. Recent findings support the hypothesis that IL-13Rα1 may influence the IL-13 reactivity of eosinophils as IL-13 and IL-4 decreased the mRNA and protein expression of IL-13Rα1 on eosinophils, while IFN-γ, TNF-α and TGF-β enhanced detectable IL-13Rα1 [23].

There is evidence that both receptors IL-4Rα and IL-13Rα involve the activation of STAT6 for the downstream signaling of the IL-4/IL-13 pathway. Hence, STAT6 may also be an important candidate gene to mediate atopic diseases. STAT6 is ubiquitously expressed in all cell types, but its expression may be augmented in B and T cells after cell stimulation [24].

The importance of *STAT6* in T_H2 differentiation was shown as *STAT6*-deficient mice were not capable of differentiating naive T cells into T_H2 cells [25–27]. Furthermore, *STAT6*-deficient mice were not able to undergo class-switching to IgE although detectable levels of IgG, IgA and IgM were present [25, 26]. Accordingly, these mice did not contain IgE-producing B cells [26]. In addition, *in vitro STAT6*-deficient T cells had a significantly impaired proliferative response to IL-4 stimulation.

STAT6 is not only relevant for T_H2 differentiation and IgE regulation. It is assumed that the JAK-STAT signaling pathway is involved in many of the physiologic events that are deregulated in asthma: analyzing the immunochemistry of STAT6 revealed that patients with atopic and non-atopic asthma significantly develop more STAT6 immunoactive cells than healthy controls [28]. However, peripheral blood monocytes (PBMC) from asthmatic and allergic patients did not display significant differences in the level of STAT6 activity relative to healthy controls [29].

The Relation Between IL-4 SNPs and Allergic Inflammation

Based on linkage studies, a susceptibility locus for allergic inflammation and IgE synthesis was attributed to a region on the short arm of chromosome 5 (5q31-33) where both genes *IL-4* and *IL-13* are located [30–32]. Association studies followed to identify putative polymorphisms responsible for the observed linkage signal.

Rosenwasser and coworkers first described a polymorphism located in the promoter region of the *IL-4* gene (C-589T) to be associated with elevated IgE levels in atopic asthmatics [33]. Significant associations with this *IL-4* promoter polymorphism were observed in a number of studies, but to other atopic phenotypes including asthma [34, 35], atopic dermatitis [36] and bronchial hyperresponsiveness [37]. However, these results could not be replicated in other populations of different ethnicities [38–41].

In 2003 a systematic screening for genetic variants in the complete *IL-4* gene was performed in 33 unrelated German subjects [42]. In total, 14 novel polymorphisms, all located in the intronic region of the gene, were identified and two previously reported SNPs were confirmed. In this Caucasian population, the polymorphisms within the *IL-4* gene revealed two tightly linked clusters. Five SNPs were genotyped in a study of 1,120 German children. The strongest association with asthma was observed with the promoter polymorphism C-589T. In accordance, two additional genotyped SNPs were significantly associated with asthma due to their high linkage

to the promoter polymorphism C-589T. A positive association between three SNPs (C-589T, C-33T and VE6523) and total IgE levels was described after stratification for positive skin-prick test. Functional experiments have been performed to identify the putative regulatory relevance of the proximal IL-4 promoter polymorphism C-589T [43]. These data showed alterations in the binding of a transcription factor and changes in the *IL-4* gene expression depending on the genotype of the SNP C-589T. It is postulated that these effects are caused by a modulated binding of the transcription factor nuclear factor of activated T cells (NFAT) [44].

The relation between genetic alterations in the *IL-4* gene and the regulation of IgE was further investigated by Basehore and coworkers in three populations of diverse ethnicities: Caucasians, African Americans and Hispanics [45]. In the Caucasian population, nine SNPs were significantly associated with total serum IgE levels, whereas five were also associated with asthma. One polymorphism (G3017T) located in intron 2 of the *IL-4* gene seemed to be the most consistent SNP over all three populations. Furthermore, associations of G3017T with total serum IgE levels in the African American as well as in the Hispanic population were described. This polymorphism was additionally associated with asthma in Hispanic subjects. Performing haplotype analyses, the distribution and frequency of haplotype combinations were different across the three tested populations. Among the Caucasian and the African American population different haplotypes were associated with elevated total IgE levels. No significant relations between haplotypes and asthma have been observed.

IL-13 Polymorphisms Influence Atopic Diseases

As both cytokines IL-4 and IL-13 are important in the activation of the IL-4/IL-13 pathway, the putative role of genetic variants in the *IL-13* gene has also been investigated. In a mutation screening of the *IL-13* gene including all exons, introns and 1,676 bp of the promoter region, seven polymorphisms were identified [46]: two SNPs were located in the *IL-13* promoter, one in intron 3 and one in exon 4, leading to a predicted amino acid change from arginine to glutamine (Arg130Gln), and three in the 3′UTR. The observed polymorphisms were genotyped in three different populations, all of Caucasian origin. Overall, the strongest effect was observed between the exonic SNP (G2044A) and elevated total serum IgE levels consistently in all three independent populations. Within the German population in this study, two *IL-13* promoter polymorphisms (A-1512C and C-1112T) showed the most significant association to the same phenotype. However, as all seven polymorphisms within *IL-13* are in high-linkage disequilibrium with each other, it is difficult to dissect which SNP is causal for the observed association signal.

The polymorphism G2044A in exon 4 and the promoter polymorphism C-1112T have been extensively studied in further association analyses to confirm their putative relevance on the development of atopic diseases. A significant association

for G2044A with asthma [47, 48], the regulation of total serum IgE [49–51] and atopic dermatitis [49] have been described among different ethnicities. The variation C-1112T in the promoter region of *IL-13* has been shown to be involved in the IgE regulation in another German population [39] as well as in the development of asthma [52, 53], BHR and skin-test responsiveness in a Dutch population [53]. In addition, Heinzmann *et al.* described a novel coding variant (Gln110Arg) in exon 4 of the *IL-13* gene leading to an amino acid change [47], whereas Gln110 was significantly more frequent in asthmatic children compared to non-atopic individuals in British and Japanese individuals. Accordingly, in homozygous carriers of Gln110 significantly higher levels of IL-13 were detected. *In silico* analysis had initially predicted that the presence of the wild-type allele of Gln110Arg may have an impact on the binding of IL-13 to its receptor, which may subsequently enhance the IL-13 signaling.

Thus, functional studies were performed to better understand the regulatory influence of the genetic variants G2044A and C-1112T. As the polymorphism G2044A is located in exon 4, it was hypothesized that the alteration may modify the structure and the affinity of the IL-13 protein to its receptor. However, it was demonstrated that the affinity of the recombinant IL-13 protein containing either the wild-type or polymorphic allele of G2044A to the IL-13Rα1 was almost unaffected, but seemed to be relevant for the affinity of IL-13 to IL-13Rα2 [54]: IL-13 protein carrying the polymorphic allele showed a lower affinity to IL-13Rα2 causing less clearance of *IL-13*. Consequently, IL-13 levels in the serum of asthmatic individuals carrying the polymorphic A allele were elevated in comparison to individuals with the wild-type allele. These data indicated that the exonic alteration in *IL-13* may be important in the genesis of allergic diseases through the upregulation of IL-13 levels. Recent data supported the functional relevance of the *IL-13* SNP G2044A: IL-13 protein isolated from primary cells expressing the polymorphic A allele was significantly more active in inducing STAT6 phosphorylation and CD23 expression in monocytes and IgE switching in B cells [55].

To further analyze the mechanism of how the associated *IL-13* promoter polymorphism C-1112T may influence allergic diseases, the role of this promoter SNP was investigated in different T cell subtypes by Cameron and coworkers [56]. In gene expression experiments, it was shown that the polymorphic T allele of the SNP enhanced the promoter activity of IL-13 in primary human and murine $CD4^+$ T_H2 cells. This effect may be explained by observed changes in the binding properties of transcription factors (STAT6, Oct-1 and NFAT2) depending on the genotype. However, this outcome seemed to be strongly influenced through the nuclear environment, as a reciprocal effect of the transcriptional activity was seen in nonpolarized $CD4^+$ T cells and in the Jurkat T cell line accompanied by a modulated DNA–protein binding interaction. To support the hypothesis that SNP C-1112T may be involved in the regulation of atopic diseases, IL-13 secretion was compared between groups either expressing the wild-type C or the polymorphic T allele: a significant increase of IL-13 in the serum was observed in homozygous carriers of the T allele [56]. Hence, a regulatory influence of the promoter polymorphism C-1112T on *IL-13* expression has been established.

The Complex Picture of IL-4Rα SNPs and the Genetic Predisposition to Allergic Diseases

The central role of *IL-4Rα* in regulating IgE production encouraged numerous groups to investigate the influence of *IL-4Rα* polymorphisms on the receptor signaling and, hence, on atopic diseases. In the *IL-4Rα* gene, 14 SNPs have so far been identified, all of which are located in the coding region [57–59]. Out of these SNPs, eight polymorphisms are predicted to lead to an amino acid change, but association data for any of the reported *IL-4Rα* SNPs are more or less contradictory. While some studies observed a relation between SNPs in the *IL-4Rα* gene and the genetic predisposition to asthma [59–61], atopic disease [58, 62] and IgE levels [63–65], others could not find an association with any atopic phenotype [66–69].

A study carried out by Ober and coworkers in three populations of different ethnicities gave evidence that different single SNPs were associated with atopy and asthma *per se*. Performing haplotype analyses, the association signal with asthma became stronger, but still different haplotypes among these populations were responsible for the observed effects [59]. Comparable results were seen in two other populations [58, 61]. In a study of German children, where the initial analysis did not show statistically significant association, only in haplotype analyses a positive association with elevated total serum IgE was demonstrated [70].

Functional studies were performed to gain information on the influence of *IL-4Rα* polymorphisms in relation to the development of atopic diseases. In mouse and human cell lines, it was shown that the wild-type allele of I50V significantly upregulated the receptor activity of IL-4Rα after IL-4 stimulation in contrast to the polymorphic allele [71]. Hence, an augmented STAT6 activity was detectable, subsequently leading to increased cell proliferation. Additionally, I50 induced higher expression of CD23 and promoted IgE production [72].

The SNP Q576R was also investigated in functional experiments. 576R was associated with IgE regulation and induced expression of higher CD23 levels after IL-4 stimulation [58]. It was argued that changes in the binding specificity of a tyrosine residue in close vicinity to Q576R may explain these observations. However, no effect on CD23 expression was observed by Risma *et al.* when the function of 576R was investigated in more detail [73]. Nevertheless, the combination of the polymorphic alleles of two SNPs (I75V–Q576R) induced expression of IL-4Rα with enhanced sensitivity to IL-4.

Previously, it has been shown that genetic variations may also alter splice sites of a gene, leading to alternative splicing products [74–76]. A comparable mechanism may be postulated for SNPs and splice variants of *IL-4Rα* which may correlate with the development of atopic diseases [77, 78]. Through alternative splicing, two proteins of *IL-4Rα* have been identified: membrane-bound IL4-Rα is coded by exons 3–7 (extracellular domain), exon 9 (transmembrane domain) and exons 10–12 (intracellular domain), whereas soluble IL-4Rα product includes only exons 3–8 [79]. In a Swedish Caucasian population of adults, soluble IL-4Rα was reported to be increased in asthmatics compared to controls [78]. In the same population,

an association between atopic asthma and an IL-4Rα haplotype containing three polymorphisms (C-3223T, Q551R and I50V) was described. Within a further subgroup, lower levels of soluble IL-4Rα were measured when the less frequent alleles of these SNPs were present.

Bergin *et al.* investigated the effect of a haplotype of four SNPs within the alternatively spliced exon 8 (relevant for soluble IL-4Rα) of the *IL-4R* gene in more detail [77]. Hence, *in vitro* expression of minigene constructs comprising exons 7–9 (including exon 8) was compared between haplotype groups. It was indicated that the minigene expressed about 100 times less of exon 8+ gene transcript (comparable to soluble IL-4Rα) when the minor haplotype was present compared to the major haplotype. These data were confirmed by analysis of mRNA expression in peripheral blood mononuclear cells of asthma patients and controls, as lower expression levels of soluble IL-4Rα were detected in those individuals with the minor haplotype.

Furthermore, the question of whether SNPs in the *IL-4Rα* gene may be an indicator for the severity of asthma symptoms was addressed. In a prospective cohort study, asthma patients carrying the polymorphic allele of Q576R had a significantly increased risk for asthma susceptibility [80]. In addition, a correlation between homozygous individuals of 576R with asthma severity was observed. Previously, Wenzel and coworkers analyzed whether polymorphisms in *IL-4Rα* may influence asthma exacerbation, lung function and tissue inflammation (indicated by overall mast cell numbers and IgE^+ expression on mast cells) [81]. These data indicated that homozygous carriers of the polymorphic allele for two SNPs (E375A and Q551R) were significantly associated with a history of asthma exacerbation, increased overall numbers of mast cells and IgE^+ expressing mast cells. However, as the picture of genetic variants within the *IL-4Rα* gene is very complex, and at present still somewhat contradictory, further studies will be needed to elucidate their regulatory function in the development of atopic disease.

IL-13Rα1 Polymorphisms May Have an Effect on Atopy

As it was shown that the concomitant presence of *IL-13Rα1* and *IL-4Rα* can induce a substantial binding affinity of IL-13, not only SNPs in *IL-4Rα* but also those in the *IL-13Rα1* gene may be relevant for the regulation of immune responses. Screening the coding region of *IL-13Rα1*, which is located on the X chromosome, three SNPs were identified, all of which led to a silent mutation [47]. An association study with the most frequent polymorphism A1398G was performed in two populations of British and Japanese individuals. SNP A1398G was associated with high levels of serum IgE, although, only in the British population. As the *IL-13Rα1* gene is located in the X chromosome, sex-dependent differences between both populations and atopic phenotypes were investigated in more detail. These data indicated that the observed effect was mainly driven by males, as the association with elevated IgE levels was much stronger in this subpopulation of British individuals. In contrast, no significant effect was observed with asthma.

Comparable results were detected in a Japanese study population of children [82]. The polymorphism A1398G revealed higher total IgE levels in children with asthma carrying the heterozygous or homozygous polymorphic allele (G allele) in contrast to the control population. However, no effect was observed between the SNP and susceptibility for asthma. These data indicate that polymorphism A1398G may be more relevant in the regulation of IgE than for asthma susceptibility.

Genetic Variants in the STAT6 Gene Contribute to the Development of Allergic Disorders

It is worth noting that *STAT6* is an important bottleneck in the IL-4/IL-13 signal transduction as both receptors *IL-4Rα* and *IL-13Rα1* are known to operate through *STAT6*. Initial genetic studies of *STAT6* identified a GT dinucleotide repeat located in the first exon of *STAT6*. Its presence was investigated in three independent populations of Asian and Caucasian descent [83–85]. Among these populations of different ethnicity, deviating numbers of GT-repeats were described (Japanese: 13–16 [84], German and Swedish: 13–17 [83], Americans: 12–17 [85], British: 13–18 [85]).

In the association study of Duetsch *et al.*, 16 GT-repeats were significantly associated with an increase in the total number of eosinophils [83]. In contrast, subjects in the British study population with 16 GT-repeats were protected against the development of asthma, whereas carriers of 13 GT-repeats showed an inverse association with asthma [85]. Individuals with 13 GT-repeats furthermore had higher levels of total IgE elevated in vitro transcriptional activity in three different cell lines [85]. Thus, it was assumed that the number of GT-repeats may be a marker for differences in the promoter activity of the *STAT6* gene.

In 2002, Duetsch and coworkers systematically screened the *STAT6* gene to identify further genetic variants which may influence the regulation of the gene [83]. In total, 16 noncoding polymorphisms were genotyped. A significant association with single SNPs was described between total IgE levels and three *STAT6* polymorphisms located in intronic regions of the gene (introns 2, 17 and 18) and one SNP in the 3′UTR in a Caucasian population of asthma sib-pairs. Furthermore, for one polymorphism located in the 3′UTR a significant association with bronchial challenge was described. Testing the correlation of *STAT6* haplotypes with atopic diseases, no significant effect was observed. Potentially, this was due to the small sample size of the study population [83].

Genotyping six *STAT6* polymorphisms in a population of German children, a significant association between a high-risk haplotype and total serum IgE levels was described [86]. This effect was mainly driven by two polymorphisms located in intron 2 (C2892T) and the 3′UTR (T12888C) of the gene which confirmed the initial report of Duetsch *et al.* [83]. In addition, the association between polymorphism C2892T in intron 2 of the *STAT6* gene and elevated total serum IgE levels has been replicated in a third independent study population [87]. Weidinger and coworkers reported a similar effect between increased levels of total serum IgE and

the polymorphism C2892T in a cross-sectional cohort study of 1,407 German adults [87]. Thus, the association of SNP C2892T was found independently and repeatedly in three different studies comprising children as well as adults. These data indicate that C2892T may not only be relevant for childhood asthma, but may also be involved in the development of atopic diseases in adulthood. A potential role in the regulation of *STAT6* expression may be assumed for intron 2 harboring C2892T, as this region was also shown to be fairly conserved among apes and primates [86]. Furthermore, *in silico* analyses suggested that SNP C2892T is located in the center of a NF-κB transcription factor binding site potentially explaining the association between the polymorphism C2892T and increased risk for elevated total serum IgE.

Gene-by-Gene Interaction Analyses of the IL-4/IL-13 Pathway

Consistent with the fact that IL-4 and IL-13 share common functions and the same activation pathway (including *IL-4Rα*, *IL-13Rα* and *STAT6*), the question as to whether polymorphisms within these genes may interact with each other was addressed. Hence, it was important to study polymorphisms of each IL-4/IL-13 pathway gene not only separately but also in combination. As atopic diseases seem to be regulated by multiple genes, it was suggested that the presence of a single SNP in only one of the genes involved in the IL-4/IL-13 pathway may not be sufficient to result in profound changes in IgE regulation or asthma susceptibility. Therefore, gene-by-gene interactions may modify the effects of SNPs in the IL-4/IL-13 signaling pathway: the quite small functional changes on the respective gene observed for numerous SNPs of the major pathway genes may lead only in combination to significant effects and subsequently to the clinically detection of the disease.

In 2002, Howard *et al.* performed the first published gene-by-gene interaction analyses in this pathway in Dutch families, although in a rather small study population for a study of multiple interaction terms ($N = 233$) [88]. SNPs C-1112T in *IL-13* and S478P in *IL-Rα* were selected for the analyses as they revealed the strongest association results in single SNP analyses [53, 88]. A significant gene-by-gene interaction between the IL-13 promoter SNP C-1112T and S478P in *IL-4RA* with asthma susceptibility as well as increased levels of total serum IgE levels were observed. Yet, the interaction of these polymorphisms was stronger for the development of asthma: individuals with the common allele of S478P and the minor allele for C-1112T showed a fivefold increased risk for the development of asthma compared to individuals who did not carry the respective genotypes.

The genetic interaction between other IL-4/IL-13 pathway SNPs was further addressed by Heinzmann and coworkers in a population of British ($N = 150$ cases, $N = 150$ controls) and Japanese ($N = 100$ cases, $N = 100$ controls) subjects [47]. In total, four polymorphisms including *IL-4* (C-589T), *IL-13* (Gln110Arg), *IL-4Rα* (Ile50Val, Arg551Gln) and *IL-13Rα1* (A1398G) were selected for gene-by-gene

interaction analyses. However, no significant effect between these polymorphisms and the development of asthma and atopy was observed.

The nature of interactions of SNPs of the four major genes (*IL-4*, *IL-13*, *IL-4Rα* and *STAT6*) of the IL-4/IL-13 pathway has recently been studied for asthma and atopy in more detail in a population of 1,120 German school children [89]. Only common polymorphisms which have previously been described to be associated with atopic diseases [42, 46, 57, 83, 90] and of putative functional relevance were included in the analyses: SNP C-589T in the proximal promoter region of IL-4 was chosen for this analysis, as it alters the binding of a transcription factor [41]. For *IL-13* the promoter polymorphism C-1112T was selected, as it may alter the binding of transcription factors and influences the gene expression of *IL-13* [56]. In the *IL-4Rα* gene, SNP A148G was included in the analyses, as it alters an amino acid in the extracellular part of the receptor (I50V) which leads to an increased downstream activation of *STAT6* and increased IgE production in B cells [58]. In *STAT6*, the polymorphism C2892T located in a potential NF-κB-dependent highly conserved regulatory element was studied [86]. According to its biological function, the effects of SNPs in the IL-4/IL-13 pathway on two main outcomes were studied: the regulation of serum IgE levels and the development of childhood asthma.

These data demonstrated that the risk for elevated serum IgE levels increased in a stepwise introduction of SNPs from the maximum single effect of 1.7-fold for *STAT6* C2892T to a maximum of 18-fold for the triplet interaction between *IL-13* C-1112T, *IL-4Rα* A148G and *IL-4* C-589T (Fig. 2). An even stronger outcome was observed on asthma susceptibility whereas the maximum observed effect of any single SNP was 1.6 (*IL-13* C-1112T), which increased to 26-fold for the interaction between *STAT6* C2892T, *IL-13* C-1112T and *IL-4* C-589T (Fig. 3). These data suggested that an interaction between different polymorphisms in the IL-4/IL-13

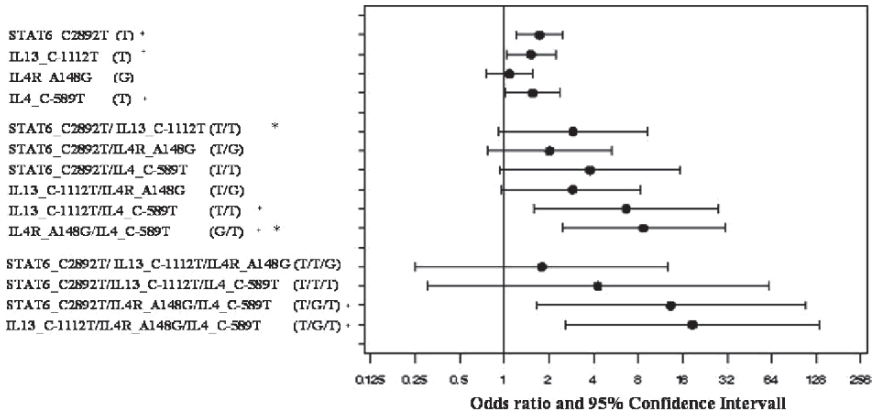

Fig. 2 Combination of the polymorphic alleles of IL-4/IL-13 pathway SNPs increases the risk to develop elevated IgE levels (90th percentile). In the upper panel, effects of single SNPs are shown. In the middle panel, haplotype pairs, and in the lower panel, haplotype triplets are presented. Significant associations (marked +) and haplotypes from genotype combinations showing statistically significant epistatic interactions (marked *) [103] (From Elsevier. With permission)

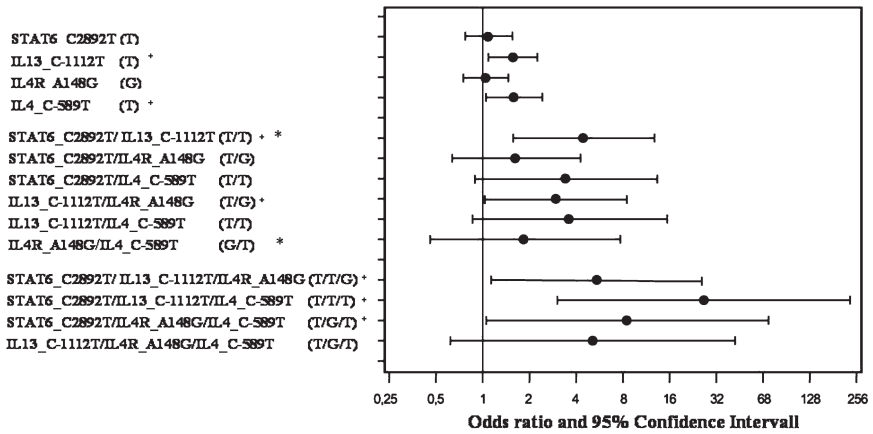

Fig. 3 Combination of the polymorphic alleles of IL-4/IL-13 pathway SNPs increases the risk to develop asthma. In the upper panel, effects of single SNPs are shown. In the middle panel, haplotype pairs, and in the lower panel, haplotype triplets are presented. Significant associations (marked +) and haplotypes from genotype combinations showing statistically significant epistatic interactions (marked *) [103] (From Elsevier. With permission)

pathway comprising *IL-4*, *IL-13*, *IL-4Rα* and *STAT6* significantly influences the genetic control of serum IgE levels and the development of asthma. Out of the four SNPs tested, *STAT6* C2892T and *IL-13* C-1112T were overrepresented in those extended haplotype combinations conferring the greatest risk to develop elevated IgE and/or asthma. This may indicate that these two polymorphisms are key in the deviation of the IL-4/IL-13 pathway while other SNPs in the IL-4/IL-13 pathway may rather facilitate a modifier function.

These gene-by-gene interactions in common diseases with a non-mendelian genetic trait may help to better understand their complex nature. As IgE switching is fundamental for the human immunity and survival, the regulation of IgE is delicate and in part redundant, which may be a common observation in biologically important pathways. Thus, it can be deduced that a certain amount of genetic variance (maybe in combination with certain environmental factors) is necessary in these pathways to overwhelm the system and to result in a profound, clinically measurable expression of a disease. However, statistical interaction alone does not prove biological interaction but only hints to it. It may therefore be necessary to establish biological interaction between these SNPs in the IL-4/IL-13 pathway in functional assays *in vitro* and *in vivo*.

The observations on gene-by-gene interactions in the IL-4/IL-13 pathway may however have implications of practical clinical relevance. A combined analysis of SNPs in the IL-4/IL-13 pathway may prove valuable as a predictor of atopy and asthma in children. Even though the prevalence of children carrying SNPs in three or more genes of the pathway may be low, the extremely elevated risk to develop asthma in carriers may predict the disease in those individuals. On the other hand, children who do not show any alterations in the pathway seem to be protected

against the exaggerated production of serum IgE. How valuable SNPs in the IL-4/IL-13 pathway are as disease predictors, and if they could be used as screening tools for the development of asthma in childhood, still remain to be tested and further replication studies are necessary.

Indisputably, many further mechanisms involving different cytokine networks may contribute to the development of asthma and atopy. These include, for example, polymorphisms in the *tumor necrosis factor* (TNF)-α, IL-10 and IL-5. In the *TNF-α* gene, which is a pro-inflammatory cytokine, the promoter polymorphism G-308A in particular has been shown to be associated with asthma [91–94], elevated bronchial hyperresponsiveness [95] and childhood wheezing [96, 97]. Polymorphisms in the anti-inflammatory *IL-10* gene and their relation to the development of asthma have also been investigated within the past years. Hence, different promoter polymorphisms have been shown to be positively associated with asthma in Caucasian children [98] as well as in a population from North India [99]. Furthermore, it was demonstrated that different genetic combinations of *IL-10* promoter polymorphisms may affect the secretion of *IL-10* [100]. In addition, a polymorphism in the T_H2 cytokine IL-5 (located within the 5q31 gene cluster in proximity to *IL-4* and *IL-13*) has been reported to be associated with spirometric markers of asthma severity in Korean children but not with asthma susceptibility *per se* [101].

In pathways where cytokines may play a role for the development of asthma and atopy, gene-by-gene as well as gene-by-environment interactions may play a crucial role as we have reviewed in detail for the IL-4/IL-13 pathway. Similar studies have been performed now for other pathways. Thus, the interaction between different genes relevant for the regulation of eosinophilic inflammation has recently been analyzed in a more systematic approach [102]. Eosinophilia is known to be crucial for asthma, as a relation between chronic asthma and eosinophilic migration, maturation and activation has been observed. Hence, polymorphisms with a putative functional relevance in different eosinophilia-regulating factors (*GM-CSF*, *IL-3* and *IL-5*), the respective receptors (*CSF2Rb* and IL-5R) and an enhancer and regulatory element (for *GM-CSF*) were studied together. In that study, the protective effect of the polymorphic T allele in a promoter polymorphism of the *IL-5* gene increased depending on a second polymorphism located in a further regulatory element.

Conclusion

Cytokines have been proven to be valuable candidate genes for asthma and atopy, contributing significantly to the genetic susceptibility of these disorders. The genetic effects observed may be subtle when investigated in isolation. Much more relevant information can be retrieved when these polymorphisms are analyzed in the setting of their respective pathways as demonstrated for the IL-4/IL-13 pathway. While gene-by-gene interaction studies, e.g., within the IL-4/IL-13 pathway and the eosinophilia regulation pathway, have now been performed on a population genetics level, functional studies of these effects are progressing slowly. Little

information on the effects of single SNPs within these genes is so far available [43, 56, 60, 73], let alone data in the role of polymorphisms on gene-by-gene interaction on a biological and functional level. However, only when this research is complete will we be able to fully understand how cytokine polymorphisms influence the development of asthma and allergy.

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Allergy and the Bone Marrow: Transmigration Pathways of Hemopoietic Progenitor Cells from the Bone Marrow

Roma Sehmi, Jennifer V. Thomson, and Adriana Catalli

General Introduction

Allergic airway diseases are a leading cause of morbidity and mortality both in Canada and globally, and as such, highlight the pressing need for effective pharmacotherapy to adequately control these conditions. Asthma is a chronic inflammatory disease characterized by variable bronchial obstruction, airway hyperresponsiveness (AHR) and infiltration of the airways by activated pro-inflammatory cells, predominantly eosinophils implicated as prime effector cells of the pulmonary pathology of asthma. Novel anti-eosinophil therapies such as interleukin-5 (IL-5) blocking antibodies have proven largely unsuccessful in ameliorating critical symptoms of asthma including AHR, thus emphasizing the need to understand the broader scope of the asthmatic response and the multifactorial nature of this disease [1].

It is now recognized that systemic processes involving the bone marrow (BM) are also features of the inflammatory pathology of atopic asthma. Signals generated at the site of allergen (Ag) challenge trigger the increased turnover and traffic of hemopoietic progenitor cells (PC) from the BM to the lungs via the peripheral blood (PB), where in situ differentiation in the presence of locally elaborated cytokines provides an ongoing source of effector cells during the allergic inflammatory response (Fig. 1) [2–4]. A brisk BM response, where IL-5-driven eosinophilopoiesis was detected as early as 5 h post-Ag inhalation challenge in both atopic subjects [5, 6] and sensitized mouse models [7, 8], indicates a 'real-time' role of BM-derived PC in airway inflammation. It is therefore important to have an understanding of how directional cues, i.e. cytokines/chemokines, orchestrate the traffic of PC in atopic diseases such as asthma. While activation of hemopoietic events within the bone marrow and traffic to sites of inflammation in allergic

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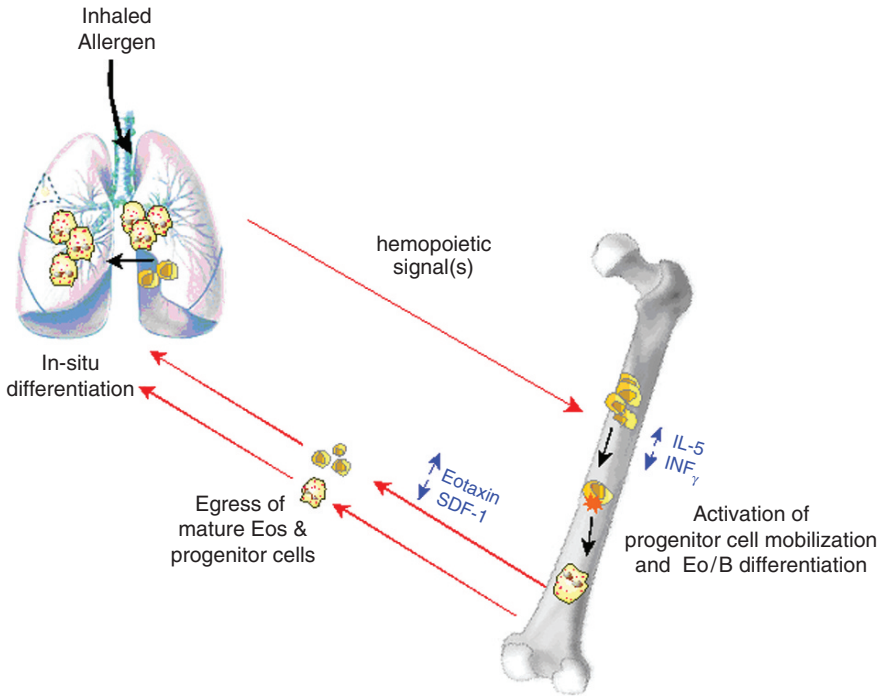

Fig. 1 Schematic representation of activation of hemopoietic processes within the bone marrow of atopic subjects. Allergen inhalation challenge in asthmatics triggers the production of signals that activate specific hemopoietic pathways in the bone marrow resulting in either eosinophil-lineage commitment of progenitors, which mature within the bone marrow, or mobilization of primitive progenitors to the site of allergen challenge. Once at the site of allergic inflammation in situ differentiation of the progenitors in the presence of locally elaborated cytokine growth factors could provide an ongoing source of inflammatory effector cells or structural cells, thus contributing to ongoing tissue remodelling changes

diseases have been described in detail elsewhere [4], this chapter will focus on chemokine-mediated control of pC egress from the BM in allergic inflammatory responses in asthma subjects.

Evidence of PC Traffic in Allergic Inflammation

Until recently hemopoiesis was thought to be restricted to major extravascular compartments such as the BM, where proliferation and differentiation give rise to immature and mature PB cells that pass into the circulation at a basal rate under normal conditions or are selectively mobilized in increased numbers during conditions of stress, tissue injury or inflammation. The recent detection of low levels of primitive PC circulating in the PB under normal conditions that increase

in response to stress, tissue injury or inflammatory stimuli has initiated the concept of stem cell plasticity [9]. This hypothesis suggests that primitive PC can circulate within the PB and home back to the BM and/or to the 'involved' organ/tissue under the influence of locally elaborated chemokines. The cytokine microenvironment of the target organ dictates the differentiation of PC towards pro-inflammatory cells or structural cells thus contributing to tissue repair or remodelling. This has spawned an interest in factors that control PC traffic in various diseases [10].

In the context of allergic inflammation, the focus to date has been on the role of hemopoietic PC in the development of eosinophilic airway inflammation. Increased levels of BM and PB $CD34^+$ cells have been shown to be a feature of atopic asthma [11, 12]. It has been shown also that $CD34^+$ cells can co-express the low-affinity binding sites for Interleukin-5 receptor α-subunit (IL-5Rα) and suggested that $CD34^+$ IL-5Rα^+ cells may be the phenotype of the earliest eosinophil-lineage committed progenitor [13]. Selective increases in numbers of $CD34^+$ IL-5Rα^+ cells in the BM associated with increases in IL-5-responsive eosinophil/basophil colony forming units (Eo/B-CFU) in atopic asthmatics who develop an airway eosinophilia, and increased methacholine AHR 24 h post-Ag challenge have been reported. Thus highlighting a link between increased production of eosinophil-lineage committed PC within the BM and the onset and maintenance of an allergic inflammatory response with associated airway dysfunction [13, 14]. These observations coupled with previous findings of increased levels of Eo/B-CFU in PB of atopics during an acute asthma exacerbation suggest that changes in PB PC are reflective of changes in hemopoietic processes within the BM [14–17]; the similarities between the two compartments are likely due to PC migration from the BM in response to an allergic inflammatory response.

Recently, $CD34^+$ cells with the capacity to undergo IL-5 stimulated proliferation and differentiation into Eo/B-CFU have been extracted from nasal polyp tissue [18], nasal biopsies from rhinitics [19, 20], and lung tissue from ovalbumin (OVA) sensitized mice [7]. In a mouse model of asthma and in atopic asthmatics, it was shown that post-Ag inhalation, i.e., significant increases in $CD34^+$ and $CD34^+$ IL-5Rα^+ PC were detected within the lung tissue and induced sputum samples, respectively [6, 7]. The absence of Ag-induced increases in $CD34^+$ cell numbers in an ex vivo model, where sensitized mouse lungs were challenged with OVA in the absence of a peripheral circulation, indicates the likelihood of PC influx to the lung as opposed to a proliferative response by tissue resident stem cells [7]. That BM PC migrate to tissue sites of inflammation is further supported by studies in which irradiated male mice were infused with green fluorescent protein (GFP) transfected $CD34^+$ BM cells and following inhalation challenge with bleomycin, GFP^+ cells were detected in the lung tissue [21]. Although these studies have not formally been performed in a mouse model of allergic inflammation, earlier studies using Bromodeoxy Uridine (BrdU) to label actively proliferating cells within the BM undergoing DNA synthesis showed increased numbers of $BrdU^+$ cells entering the PB and the bronchoalveolar lavage fluid (BAL) 24 h after Ag-inhalation challenge in sensitized animal models [22, 23]. The detection of greater numbers of both $CD34^+$ cells and $CD34^+$ IL-5RαmRNA$^+$ cells in bronchial biopsies from

atopic asthmatics compared with normal subjects and the observation of a correlation between $CD34^+$ IL-5RαmRNA$^+$ cell numbers and asthma severity as judged by airway calibre [24] raise the possibility that control of $CD34^+$ cell egress from the BM and control of local differentiation and expansion of $CD34^+$ IL-5Rα^+ cells within lung tissue may both be prerequisites for reduction of eosinophilic inflammation in the airway in asthma. Clearly, understanding the signal(s) and molecular mechanisms that regulate the traffic of progenitors from systemic sites such as the BM after local airway Ag-challenge requires further investigation and may provide novel therapeutic targets for the more effective treatment of asthma.

Transmigration Pathways of PC from BM – General

Hemopoiesis in the BM is restricted to the extravascular space, where stromal cells surround dense cords of hemopoietic cells. The stromal cells produce extracellular matrix (ECM), primarily comprised of glycoproteins such as fibronectin. ECM is thought to regulate adhesion, differentiation and migrational responses of PC [25]. The movement of PC from the BM is proposed to be a multi-step process involving changes in specific pro-adhesive interactions with the BM microenvironment, increased motility, migration through the hemopoietic compartment and transmigration across the BM sinus endothelium [25]. The key players mediating this process being cyokines/chemokines, potent proteases and adhesion molecules [26, 27]. A molecular basis for this sequence of events has been proposed based on information arising from clinical transplantation studies where factors such as granulocyte colony stimulating factor (G-CSF) and IL-8 have been used to mobilize large numbers of progenitors to the PB for harvesting [28, 29].

It is currently postulated that mobilization of progenitors could potentially occur by at least two general mechanisms. The mobilizing stimulus may trigger (i) phenotypic changes in the PC leading to enhanced migration into the intravascular space or (ii) changes in the BM microenvironment altering anchorage and thus retention signals for PC [30, 31]. The former model predicts that the phenotypes of mobilized PB progenitors versus progenitors within the BM under steady-state conditions should be different. In fact, several studies have consistently detected differences between these two groups in relation to adhesion molecule expression. In particular, lower levels of the β1 integrins (very late antigen-4 [VLA-4; α4ß1, CD49d/CD29], and VLA-5 [α5β1, CD49e/CD29] and β2 integrins (leukocyte function associated antigen [LFA-1; CD11a/CD18]) have been detected on mobilized progenitors in PB compared to steady-state BM [27, 32, 33]. More importantly, reports of a reduced functional state of VLA-4 on mobilized PC indicate that this phenotype promotes transmigration to PB [34]. In addition, the fact that inducible ablation of α4 integrin in adult mice leads to hematopoietic egress reinforces the concept that VLA-4 is involved in the firm retention of PC within the BM [35]. The major ligands for β1 integrins are fibronectin and vascular cell adhesion molecule (VCAM)-1 expressed by stromal

cells and vascular endothelial cells, whereas β2 integrins adhere primarily to intracellular adhesion molecule (ICAM)-1 expressed by endothelial cells [35]. Studies in mice deficient in β1 or β2 or in E- and P-selectins have shown that disruption of α4β1 functioning caused the greatest impact in progenitor cell egress from the BM [36–38].

Stromal cell-derived factor-1 (SDF-1), a CXC chemokine, is a potent chemoattractant for $CD34^+$ progenitors [39, 40]. Initially thought to have a critical role in the homing of PC from the foetal liver to the BM in vivo [41], the interaction of SDF-1 and its mutually exclusive receptor, CXCR4, is now proposed to regulate the release of PC from the BM [10]. For example, it has been shown that G-CSF-mobilized PB $CD34^+$ cells are less responsive to SDF-1-induced migration across endothelial cell monolayers compared with their BM counterparts [39], the refractory response to SDF-1, indicating prior exposure in the BM during mobilization. Additionally in animal studies, interference of SDF-1/CXCR4 coupling, either through the use of CXCR4 blocking antibodies [42] or changing the SDF-1 gradient between BM and PB, has been shown to promote egress of PC from the BM [43, 44]. It is now known that SDF-1 modulates the firm adhesive interactions of PC to ECM components, such as fibronectin, by the selective activation of LFA-1/ICAM-1 and VLA-4/VCAM-1 interactions.

Interestingly, SDF-1 has been shown to stimulate eosinophil migration to levels comparable to eotaxin, in vitro [45]. The expression of CXCR4 on eosinophils was upregulated by Th1-related cytokines, interferon (INF)-γ and tumour necrosis factor (TNF)-α, and down-modulated by Th2-related cytokines, IL-4 and IL-5. Based on these findings, it has been postulated that rather than playing a role in eosinophil recruitment in allergic inflammation, a predominantly Th2-dominant state, CXCR4 may affect the mobilizable pool by holding eosinophils within the BM. The Th-2 dominant state may favour the liberation of BM eosinophils through down-modulation of CXCR4. Desensitization of signalling through CXCR4 has also been shown to occur by pre-exposure to eosinophil active chemokines such as RANTES and MCP-1, which bind CCR5 and produce heterologous down-modulation of signalling [46]. Changes within the BM microenvironment, namely production of proteases including granule proteins, metalloproteases and tissue inhibitors of metalloproteases, have been shown to not only to cleave VCAM-1 expressed by stromal cells, but also to reduce bioactive levels of SDF-1 [10].

Transmigration Pathways of PC from BM in Allergic Inflammation

Eotaxin, a potent eosinophil chemoattractant, is thought to act locally at the site of inflammation to recruit eosinophils into the tissue [47, 48]. Increased levels of eotaxin have been detected in sputum and blood plasma levels of atopics [49, 50], in asthmatics post-Ag [51] and following acute asthma exacerbations [52], implicating it as a critical modulator of eosinophilia in allergic inflammation. A systemic

effect of eotaxin has been indicated where intravenous administration of eotaxin in mice resulted in increased myelopoiesis in the BM which was ablated when the mice were pretreated with anti-eotaxin antibody [53, 54]. In addition, using an in situ perfusion system of guinea pig femoral bone, eotaxin infusion into the arterial supply stimulated the rapid and selective release of mature eosinophils and eosinophil colony forming PC into the draining vein [55]. In contrast, intravenous infusion of IL-5 stimulated the delayed and sustained release of mature eosinophils but not eosinophil progenitors. In asthmatics treated with anti-IL5 intravenously, there was a significant attenuation of eosinophils in BM and PB but no changes in $CD34^+$ and $CD34^+$ IL-5Rα^+ cells in these compartments and no significant effect on Ag-induced airway function [56, 57]. This treatment significantly reduced $CD34^+$ IL-5Rα^+ cell numbers in lung biopsy tissue indicating that IL-5 may be important in in situ eosinophilopoiesis but not the mobilization of progenitors in allergic inflammatory responses [58]. Based on this, we have investigated the role of various chemokines in modulating the egress of progenitors from the BM during an allergic inflammatory response in atopic asthmatics.

To investigate the role of eotaxin and SDF-1 in the egress of BM PC, fluctuations in CXCR4 and CCR3 expression on BM and PB $CD34^+$ PC in atopic asthmatics were assessed post Ag-challenge [59, 60]. In order to correlate changes in progenitor cell populations with major symptoms of asthma, two groups of asthmatics were studied: early responder (ER) asthmatics who only develop an early bronchoconstrictor response to Ag-challenge with no significant airway eosinophilia or AHR, and dual responder (DR) asthmatics who develop both an early and late bronchoconstrictor response to Ag-challenge, where the late response is associated with a significant airway eosinophilia and methacholine AHR. We found that BM $CD34^+$ $CCR3^+$ cell numbers were significantly increased in DR asthmatics, 24 h post-Ag compared to pre-Ag levels. In contrast, a significant attenuation of BM $CD34^+$ $CCR3^+$ 24 h post-Ag compared to pre-Ag levels was detected in ER asthmatics [59]. Using an in vitro transwell migration assay, we showed that ligation of the CCR3 receptor resulted in a migratory response by BM $CD34^+$ cells to eotaxin and that this response was enhanced by pre-incubation with IL-5, which itself did not stimulate $CD34^+$ cell migration. Our findings to date indicate that while upregulation of IL-5Rα expression on BM $CD34^+$ cells during allergic inflammation may favour eosinophilopoiesis, the upregulation of CCR3 on $CD34^+$ cells favours increased migrational responsiveness to eotaxin, thus facilitating the egress of PC from the BM.

The SDF-1/CXCR4 axis has been described as providing a tissue retention signal for PC [10]. In agreement with this, Dorman et al. study showed that CXCR4 is expressed at higher levels on BM $CD34^+$ cells from normals compared with atopic asthmatics [61]. Related to this, PC from normal subjects showed a greater migrational responsiveness to SDF-1 than PC from atopic asthmatic subjects, in vitro. A significant positive correlation between the level of expression of the CXCR4 on $CD34^+$ cells and the migrational responsiveness to a sub-optimal dose of SDF-1 suggests that the magnitude of functional responsiveness to SDF-1 is related, albeit weakly, to the level of expression of CXCR4. Twenty-four hours

after Ag challenge in asthmatics who developed a sputum eosinophilia and AHR (i.e. DR but not ER asthmatics), we observed that BM $CD34^+$ cells downregulated the intensity of CXCR4 expression and had a reduced migrational response to SDF-1, in vitro. In addition, significantly lower levels of SDF-1 were detected in BM serum [61]. In contrast, increased numbers of $CD34^+$ $CXCR4^+$ were detected in PB 5–24 h post-Ag in DR asthmatics only. Taken together, these data suggest that following Ag-challenge in asthmatics a downregulation in CXCR4 expression on BM $CD34^+$ cells associated with a *hyporesponsiveness* to SDF-1 may act to reduce the tethering forces within the BM, thus facilitating the egress of PC from the BM in response to chemokines such as eotaxin. Although it remains unclear as to what may stimulate the downregulation of CXCR4 on $CD34^+$ cells, preliminary data from our group suggest that this may be orchestrated by eosinophilopoietic cytokines such as IL-5, which is increased within the BM in atopic subjects in allergic inflammatory responses [5, 62]. In addition, we have found that $CD34^+$ cells from atopic subjects may have a greater sensitivity to the CXCR4 down-regulating effects of IL-5 compared to PC from normal non-atopics (Thomson J, personal communication).

Transplantation and apheresis studies have shown that modulation of adhesive interactions of $CD34^+$ cells with BM stromal elements may play a critical role in the mobilization of PC [36, 63, 64]. In the context of allergic disorders we have investigated fluctuations in expression of β1 and β2 integrins on $CD34^+$ cells. In DR asthmatics we observed a significant decline in the expression of the β1 integrin, VLA-4 (CD49d), 24 h post Ag-challenge on BM derived $CD34^+$ cells and as early as 6 h post-challenge on PB derived $CD34^+$ cells [65]. This VLA-4 downregulation on PB PC was maintained for up to 4 weeks following Ag exposure, suggesting that these cells may be maintained in circulation rather than homing back to the BM as a means for a swift future response to Ag, or for involvement in ongoing remodelling changes within the lung [66]. In the same subject population, a delayed reduction in the expression of β1 integrin, VLA-5 (CD49e), on PB PC was also noted, whereas the expression levels of β2 integrin Mac-1 remained unchanged up to 96 h following Ag-challenge. As previous studies in mice indicated that disruption of VLA-4 function had the greatest impact on progenitor cell egress compared with other β1 or β2 integrins [36], it is perhaps of little surprise that we observed more prominent modulation of VLA-4 on $CD34^+$ cells following Ag-challenge in atopic asthmatics.

Since SDF-1 induces firm adhesion and transendothelial migration of human $CD34^+$ cells through VLA-5/fibronectin, VLA-4/VCAM-1 and LFA-1/ICAM-1 interactions [41, 67], we examined the changes in adhesive responses of $CD34^+$ cells following Ag-challenge. We observed that SDF-1-induced adhesion of BM PC to fibronectin was significantly attenuated 24 h post Ag-challenge, temporally corresponding to, and likely resulting from, the decline in BM PC β1 integrin expression. This is in agreement with our findings that both VLA-4 and VLA-5 (VLA4 > VLA5) are required to mediate SDF-1 stimulated adherence of $CD34^+$ cells to fibronectin [68].

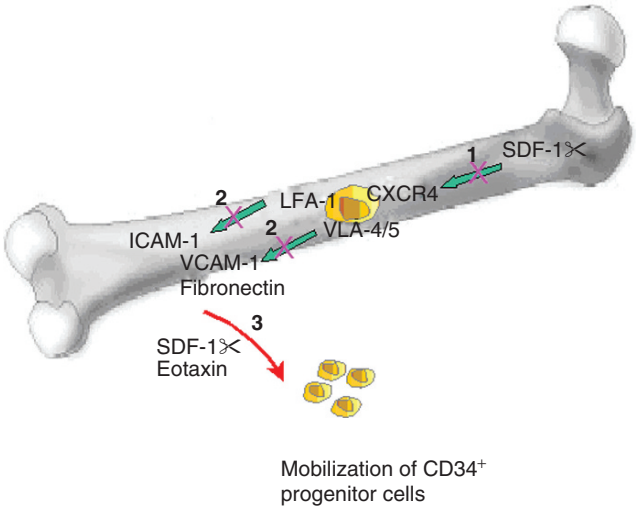

Fig. 2 Mobilization of progenitor cells from the bone marrow. It is proposed that following allergen exposure in atopic subjects (1) downregulation of CXCR4 expression on $CD34^+$ cells and (2) reduction in β1 (VLA4/5) mediated adhesive interactions with the microenvironment of the bone marrow facilitates (3) the egress of progenitor cells to the peripheral circulation along positive chemotactic gradients to chemokines such as eotaxin

In summary, we propose that post Ag-challenge in atopic subjects, increased levels of IL-5 within the bone marrow, stimulate the downregulation of CXCR4 expression on $CD34^+$ cells resulting in PC hyporesponsiveness to SDF-1 and the resultant downstream events of VLA-4 and VLA-5 downregulation. Reduced SDF-1 mediated tethering forces within the BM may thus facilitate the egress PC to the PB orchestrated by positive chemotactic gradients to chemokines such as eotaxin (Fig. 2).

Conclusion

We have presented a considerable body of evidence indicating that exposure to Ag in atopic subjects stimulates changes within the microenvironment of the BM, which may promote the mobilization of progenitor cells to the peripheral circulation. Of relevance is the role that PC play in the pathogenesis of allergic diseases. It remains unclear as to whether PC that home to the site of allergic inflammation contribute solely to the local generation of pro-inflammatory cells or whether PC can differentiate into tissue structural cells that may contribute to tissue remodelling changes observed in chronic inflammatory conditions. It is therefore of importance to understand the factors that control the traffic of PC from the BM so that modulation of this traffic may help to further our understanding of these cells in

the allergic process and provide future targets for the effective control of allergic diseases such as asthma.

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Hemopoietic Mechanisms in Allergic Rhinitis and Asthma

Gail M. Gauvreau and Roma Sehmi

Introduction

There is mounting evidence that atopic diseases such as allergic rhinitis and atopic asthma are driven by local tissue inflammatory responses, as well as having a systemic component. An important systemic mechanism to consider is the activation of hemopoietic processes within the bone marrow (Fig. 1). In response to signals generated from the upper airway after exposure to sensitizing allergens, progenitor cells are directed to the mucosa of the respiratory tract. Once in the airways, hemopoietic cells contribute to both the acute inflammatory response and the tissue remodeling changes related to chronic allergic airway diseases.

Hemopoietic progenitor cell (HPC) differentiation and maturation in adults has traditionally been thought to be restricted to the microenvironment of the bone marrow. However, a novel view has emerged in recent years according to which at least some HPC present in tissue may be recruited from the bone marrow, trafficking through the peripheral circulation, and into mucosal tissues, becoming part of a regenerative and/or inflammatory process at "distal" tissue sites [1]. The trafficking of HPC suggests a form of cell plasticity whereby primitive cells have the potential not only to give rise to mature blood cells while resident within the bone marrow, but can themselves egress from the bone marrow and home to specific organs under the orchestrated control of specific chemokines and cytokines. Once within the tissue, the fate of these HPC is determined by locally elaborated growth factors that permit a process termed "in-situ hemopoiesis" [2–5]. We provide herein

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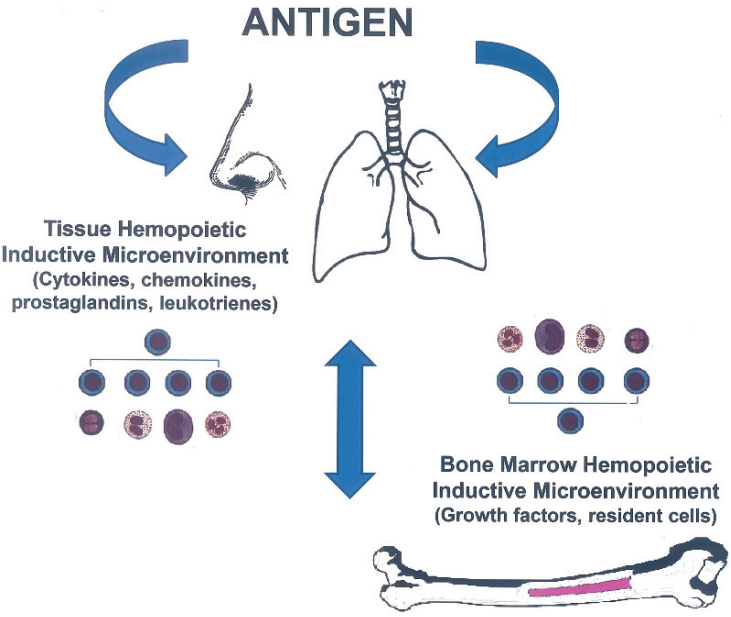

Fig. 1 Hemopoietic inductive microenvironments are located in bone marrow and tissue. Progenitor cells proliferate and differentiate in the bone marrow in response to growth factors and signals from marrow-resident cells. Upon antigen stimulation of airways, progenitor cells in tissue proliferate and differentiate in response IgE-mediated signals

an overview of the role of systemic and in-situ hemopoiesis, as part of the already complex scenario of allergic inflammation within the upper and lower airways, and highlight the benefit of controlling this component of the inflammatory process for optimal treatment of allergic airway diseases.

Enumeration of Hemopoietic Progenitor Cells

To date several techniques have been utilized to follow the fluctuations in numbers of hemopoietic progenitor cells (HPC) in various compartments of the body including the bone marrow, blood, and mucosal tissue either during periods of seasonal allergen exposure or following controlled laboratory exposure to allergen. This has provided a body of evidence supporting a role of systemic hemopoietic processes in allergic inflammatory diseases such as rhinitis and asthma.

Clonogenic Assays of Hemopoietic Progenitors

The earliest techniques used to assess the role of hemopoietic processes in allergic inflammation were colony-forming assays performed by plating progenitor-rich populations into semisolid liquid culture for 14 days. In clonogenic assays, progenitor cells, defined as colony-forming units (CFUs) because of their ability to generate

clonally derived aggregates of daughter cells in vitro, can be detected and classified based on distinct morphological features as either eosinophils and basophils (Eo/B)-CFU or as granulocyte-macrophage (GM)-CFU by light microscopy [6, 7]. The coincidence of eosinophils and basophils in the same colony indicate a common precursor and shared pathway of differentiation [8]. The commitment of this progenitor to the Eo/B lineage is regulate by a triad of pro-inflammatory cytokines: IL-3, IL-5, and granulocyte-macrophage colony stimulating factor (GM-CSF) [9, 10]; IL-5 in particular, acting on the myeloid-committed progenitor to induce specific differentiation along the Eo/B lineage [11, 12] whilst IL-3 and GM-CSF act to expand more primitive progenitor cell populations [13].

Phenotypic Analyses of Progenitor Cells

Enumeration of HPC using clonogenic assays, though useful in determining the engraftment potential of donor samples for clinical transplantation to myeloablated recipients, has several drawbacks. These include the length of time for colony growth (approximately 14 days) and potential for contamination risking the loss of useful information from valuable clinical samples. Over the last decade, accurate enumeration of HPC has been determined by fluorescence activating cell sorting (FACS) using a protocol that employs multiparametric gating analyses using 3–4 color cytofluorometry [14].

Early lymphohemopoietic progenitor cells are primarily identified by high levels of surface expression of CD34, a 115 KD monomeric, transmembrane, O-sialylated phosphoprotein that is highly glycosylated [15–17]. The expression of CD34 is recognized as a stage-specific antigen which when used in combination with small size and low granularity determinants, can accurately enumerate HPC in cord blood, peripheral blood, bone marrow, and more recently, lung tissue and sputum by flow cytometric methodologies [18–20]. The CD34 antigen is also expressed on tissue structural cells, including fibroblasts and vascular endothelial cells, and thus caution should be exercised when enumerating HPC in paraffin embedded tissue blocks by single stain immuno-cytochemistry [14, 16]. Double-staining techniques where CD34-immunopositive cells are co-stained with CD133, a marker expressed by primitive stem/progenitor cells and retinoblastoma [21], may provide more accurate estimates of HPC within human tissue.

The cloning of the full length and truncated forms of human and murine CD34 cDNAs, has failed to establish a function for the CD34 antigen in early hemopoeisis [22, 23]. Initial studies suggested that expression of CD34 maintains stem cells in G0 phase [17]. However, this was brought into question when reports indicated that the most quiescent and primitive hemopoietic stem cells are $Lin^{-}CD34^{-}$ [24–26] and that CD34 expression can be induced by ex-vivo coculture with stem cell factor to produce cells with a greater clonogenic capacity [27]. The general consensus now is that as opposed to "emergency" hemopoiesis seen in infection and inflammation, conditions of "normal" hemopoiesis down-regulate CD34 expression on HPC [28]. Other studies have proposed properties as an adhesion ligand for L-selectin which

is now known to be restricted to the sulfated glycoform of CD34 restricted to high vein endothelial cells in lymph nodes [29, 30] and not to the glycoform of the CD34 molecule expressed on HPC [31]. A role for CD34 on HPC in orchestrating cell adhesion to stromal components as well as cell aggregation both mediated by β2 integrins has been proposed [32]. However, the ligand for CD34 and the intracellular interacting protein mechanisms remain to be elucidated.

With relevance to allergic inflammation, expression of CD34 has recently been described on immature but not mature eosinophils [33]. However, its role in the maturation and migrational responses of these granulocytes remains to be clarified.

Further phenotype analyses, have shown that $CD34^+$ HPC cells can co-express the low-affinity binding sites for an eosinophil-specific cytokine, interleukin-5 (IL-5 receptor alpha-subunit; IL-5Rα) and suggested that $CD34^+$ IL-5Rα^+ cells may be the phenotype of the earliest eosinophil-lineage-committed progenitor [19]. Recent studies focusing on the expression of nuclear transcription factors have shown that expression of IL-5Rα on $CD34^+$ cells is preceded by activation of the GATA-1 reporter gene [34]. Transcription factors have been shown to be key determinants in the orchestration of myeloid identity and differentiation fates. Most transcription factors show cell-lineage-restricted and stage-restricted-expression patterns. Therefore, understanding factors that control granulocyte transcription factors such as C/EBPβ [35, 36] and GATA-1 [35] may provide useful information related to controlling eosinophil-lineage commitment particularly in situations of "emergency" granulopoiesis as seen in allergic inflammatory responses.

In studies of $CD34^+$ HPC induced to differentiate to Eo/B lineage from human cord blood samples, the appearance of immunoglobulin E receptor one (IgER1) has been reported [37]. Figueroa et al. have shown that cysteinyl leukotriene receptor 1 (CysLT1 R) is expressed on $CD34^+$ HPC [38]. Information on the mechanism of action of various receptor antagonists (discussed later in this chapter) suggests targeting of both mature inflammatory cells and their progenitors, providing a systemic explanation for these therapies.

The Role of Inflammatory Cell Progenitors in Allergic Rhinitis

The majority of hemopoietic activity takes place in the bone marrow under the influence of resident marrow stromal cells and accessory cells and/or their products. This makes up the complex network of the hemopoietic inductive microenvironment which is crucial for providing signals necessary for maintenance of populations of progenitors at varying stages of lineage commitment.

Currently, many hemopoietic cytokines and growth factors have been identified which exert their effects via binding specific receptors on the cell surface [4, 39]. Various cytokines participate in hemopoiesis, with SCF and IL-6 acting very early in the hemopoietic lineage hierarchy [40], while other cytokines act at later stages

in the development of progenitors. Our own work has demonstrated that binding of cytokines, including IL-3, IL-5, and GM-CSF, promotes differentiation of committed progenitors towards an eosinophilic/basophilic lineage [41]. Cytokine receptors, therefore, can regulate hemopoietic cells by influencing cell proliferation, differentiation, and survival [39, 42, 43]. Progenitor cells also express receptors that bind chemokines, including eotaxin, MIP-1-alpha and SDF-1, which have been implicated in the regulation of progenitor proliferation and movement [44, 45]. Since hemopoietic cells develop under the influence of their surroundings, the hemopoietic inductive microenvironment in the bone marrow, circulation, or tissue, is crucial in directing and modulating hemopoiesis: changes in composition of the hemopoietic inductive microenvironment can lead to the development of hematological disorders or to characteristic inflammatory responses [46]. For example, increased expression of Th2 cytokines (particularly IL-5) in individuals with atopy may promote differentiation of lineage-committed, eosinophil/basophil progenitors [47].

Following natural allergen exposure or laboratory-controlled allergen-challenge preferential increases in pro-inflammatory cells namely eosinophils and basophils within the blood and airway tissue occur as a consequence of sequential multistep recruitment mechanisms [48–50]. These involve dynamic changes in inter- and intracellular adhesion forces and chemotactic gradients causing the release of mature cells from storage pools in hemopoietic compartments such as the bone marrow and ultimate directional homing to the site of inflammation. Events related to the traffic and tissue-specific differentiation of precursors of these mature inflammatory cells constitutes an additional mechanism underlying the systemic response to allergen.

Studies in adult subjects have shown that, compared to non-atopic controls, significantly greater numbers of Eo/B-CFUs are detected in the peripheral blood of asymptomatic subjects with various allergic airway disorders, including rhinitis, nasal polyposis, and asthma [51]. Furthermore, in response to inhaled allergen in atopic asthmatics relevant fluctuations in blood and bone marrow Eo/B-CFU associated with the development of blood and airway eosinophilia has been found [50–52]. Similar fluctuations in blood Eo/B-CFU were found in allergic rhinitics following natural seasonal exposure to ragweed supporting the view that HPC contribute to the unique inflammatory infiltrate associated with allergic inflammation [53, 54]. Of note at the onset of the pollen season, a sensitized atopic individual has an initial rise in circulating Eo/B-CFU, followed by a significant decline to below baseline levels at the peak of the season and a gradual return to baseline levels after the end of season. A reduction in the numbers of circulating Eo/B-CFU in symptomatic allergic rhinitics at the peak of the ragweed pollen season was the first indication that progenitor cells may be home to tissue during the inflammatory process [53, 54].

Inflamed tissue from patients with allergic rhinitis and nasal polyposis produce hemopoietic cytokines that promote the differentiation and maturation of Eo/B-CFU and mast cell progenitors [5, 55–60]. Furthermore, mononuclear cells extracted from nasal polyp tissue have the potential to produce Eo/B-CFU

in response to IL-5 supporting the concept of *in-situ* hemopoiesis whereby locally elaborated growth factors can drive the differentiation and maturation of HPCs [5, 61]. Autocrine production of growth factors in Eo/B colony cells grown from blood of allergic asthmatic subjects, suggests that cytokine expression by differentiating progenitors may provide an additional stimulus to enhance differentiation *in situ* [62, 63].

Similar lines of evidence have been presented by phenotypic analyses of HPCs. Increased numbers of blood and bone marrow $CD34^+$ cells numbers were detected in atopic subjects compared to non-atopic controls; where the progenitor cells showed increased responsiveness to IL-5 in the atopic subjects [18]. The observation that increased levels of bone marrow $CD34^+$ IL-5Rα^+ cells is a unique feature of atopic airway disease [64] and that selective increases in bone marrow $CD34^+$ IL-5Rα^+ cells and IL-5-stimulated Eo/B-CFU occur in atopic asthmatics who develop an airway eosinophilia and increased methacholine airway responsiveness 24 h post-Ag challenge [19, 52] highlights a link between increased production of eosinophil-lineage committed progenitors within the bone marrow and the onset and maintenance of an allergic inflammatory response [18, 65]. Similar antigen-driven increases in $CD34^+$ IL-5Rα^+ cell numbers and resultant enhancement of IL-5-dependent eosinophilopoiesis have become evident in the bone marrow after allergen inhalation in sensitized mice providing evidence that the increase in eosinophil production is occurring as a result of the expansion of the relevant eosinophil progenitor population in the bone marrow and not strictly from an increase in the release of preformed mature eosinophils from holding pools within the body [66–70].

Wilson et al. studied allergic rhinitic subjects with pre-existent sputum eosinophilia in whom systemic, hemopoietic process may be expected to be responsive to, and upregulated by, allergen challenge in the nose. This study reported that a single allergen nasal challenge provoked increases in nasal symptoms and eosinophilia [71]; however, no changes were detected in the numbers of Eo/B-CFU 24 h post-allergen exposure in contrast to the mouse models referred to below and to previous data in seasonal allergic rhinitis. As discussed by the authors, the lack of relevant fluctuations in progenitor cell populations within the blood may have reflected lack of adequate powering of the study. In addition, based on recent data acquired in models of experimental allergic rhinitis and airway eosinophilia, the time course of blood sampling may have missed the peak of Eo/B-CFU changes in response to allergen: kinetic studies in the mouse model of pure allergic rhinitis have shown changes in bone marrow eosinophilopoiesis *as early as 2 h* after allergen challenge, indicating that progenitor cell fluctuations may occur more rapidly than originally postulated [72, 73].

That HPC may traffic from the bone marrow to the site of inflammation (discussed in detail elsewhere in this book) is highlighted by findings that greater numbers of CD34 immunopositive/IL5Rα $mRNA^+$ cells are detected in lung biopsies from atopic asthmatics compared to normal controls and that these cell numbers correlated the degree of airway dysfunction [74]. In addition, *ex vivo* allergen challenge of nasal explant tissue from ragweed-sensitive allergic rhinitics demonstrated the emergence of major basic protein (MBP)-positive cells with the associated

attenuation in CD34-immunopositive cells within 6–12 h post-allergen challenge [75]. The authors also showed that this process of *in-situ eosinophilopoiesis* was IL-5-driven. Furthermore, in mouse models of allergen-induced airway eosinophilia, increased numbers of IL-5-responsive Eo/B-CFU could be grown from lung-extracted progenitors following allergen-challenge, compared with saline-challenge [73]. Although the trafficking of progenitors to sites of inflammation has not been fully elucidated, Wood et al. have previously used Bromodeoxy Uridine (BrdU) to label actively proliferating cells undergoing DNA synthesis within the BM in a canine model of AHR [76]. In this model, increased numbers of $BrdU^+$ cells entered the PB and the bronchoalveolar lavage fluid (BAL) 24 h after an Ag inhalation challenge that provoked bronchial hyperresponsiveness. Analyses of CD34 and BrdU co-expression was not assessed in this study to verify if any of the BrdU-positive cells in the BAL were primitive progenitors. However, more recent studies in mice have shown that BrdU-positive cells present in the BAL and lung tissue following allergen challenge express CD34, and that the lung homing of these cells is eotaxin-2 dependent [33, 77, 78].

In a model of pure allergic rhinitis, Saito et al. have shown that sensitized- and allergen-challenged mice developed significant nasal symptoms, hyperresponsiveness, and nasal mucosal changes, such as increases in levels of eosinophils; basophils; and $CD4^+$, $IL4^+$, and $IL\text{-}5^+$ cells [69]. The bone marrow in these animals showed significant increases in $CD34^+$ $IL\text{-}5R\alpha^+$ cells and mature eosinophils and basophils, indicating that even in isolated allergic inflammation in the nose, bone marrow progenitor cell production is up-regulated, presumably by way of mediators from the inflamed organ acting distally.

Communication Between the Nose and Bone Marrow

Studies that have assessed changes in cytokines levels within the bone marrow of sensitized mice or atopic asthmatics following allergen exposure have detected increases in levels of IL-5 consistent with the kinetics of eosinophil-lineage commitment [68, 79]. Closer analyses of the cell-associated cytokine production within the bone marrow have shown that $CD34^+$ cells and T cells produce IL-5 during the course of the allergic inflammatory response. Additionally, increased numbers of $CD3^+$ cells expressing mRNA for IL-5 have been detected in the bone marrow of asthmatics who develop early and late phase bronchoconstriction, airway inflammation, and hyperresponsiveness (dual responder asthmatics) compared to those who only develop early phase bronchoconstriction (early responder asthmatics) [80]. Dorman et al. have also noted a delayed increase in interferon gamma ($INF\gamma$) in the marrow supernatants of dual responder asthmatics 24–24 h post-allergen challenge [81]. It is therefore conceivable, though not proven, that activated T cells from allergic tissue may home to the bone marrow, releasing cytokines such as IL-5 and IL-9 that may locally orchestrate activation of hemopoietic events during an allergic inflammatory response [77, 82].

Mouse models of allergic rhinitis have highlighted the multifactorial nature of this inflammatory disease, and the findings directly implicate the role of hemopoietic processes in allergic airways disease. The up-regulation of myeloid progenitors in the bone marrow after airway allergen challenge [69, 76], and their trafficking to the airways from the marrow in several animal models of either upper or lower airways inflammation have been demonstrated [83–85]. The resultant blood, nasal, and/or pulmonary eosinophilia in these models can be blocked by antibodies to IL-5 [86], or by deletion of the gene encoding IL-5 [81, 82]; eotaxin is also critical in this process [19, 65]. Rather than demonstrating a complete ablation of the allergic inflammatory response in the nose, IL-5-deficient mice develop *delayed* nasal symptoms and basophilic, rather than eosinophilic, inflammation within the nasal mucosa [69, 83]. Bone marrow cells taken from these IL-5 deficient mice showed an intact clonogenic response to IL-3 and GM-CSF in methylcellulose cultures suggesting biological redundancy among IL-3, IL-5, and GM-CSF and provide alternative systemic mechanisms for induction of eosinophilic responses in the absence of one of these cytokines, e.g., IL-5 [83, 85]. This observation maybe of direct relevance to clinical situations of refractory chronic eosinophilic rhinosinusitis, which can persist even after multiple therapeutic attempts.

Phenotypic analyses of progenitor cells using CD34 together with antibodies to cytokine and chemokine receptors has provided insight into the contribution of systemic and tissue related-hemopoietic processes in the development of allergic inflammatory responses. Described in detail elsewhere within this book, studies have shown that whereas up-regulation of IL-5Rα on $CD34^+$ cells within the bone marrow promotes increased eosinophilopoiesis [19], the up-regulation of the eotaxin receptor, CCR3, on $CD34^+$ cells may favor increased traffic of progenitor cells from the bone marrow to the peripheral circulation [87]. Chemokines may therefore orchestrate the egress of progenitor cells from the bone marrow microenvironment and the homing of progenitor cells to tissue sites of inflammation. Another important chemokines is stromal cell-derived factor-1 (SDF-1), which is a potent progenitor cell chemoattractant and plays a role in stem cell homing during embryogenesis [88].

Recent studies provide further insight into the role of progenitor cells in allergic rhinitis, and specifically to events that may be occurring in the tissue in response to systemic perturbations. Using nasal biopsy tissue from allergic rhinitics, immunopositive CD34 cells were enumerated and changes in numbers of these cells in relation to natural allergen exposure were assessed. The authors reported increased numbers of immunopositive CD34 cells and immunopositive CD34 cells co-expressing CXCR4, as well as eosinophils expressing CD34, following single allergen challenge in nasal biopsies [89]. An increase in local SDF-1 was proposed to play a role in recruiting progenitor cells into the inflamed tissue. Interestingly, this study showed in subjects that were pre-treated with fluticasone compared with placebo there was a significant reduction in immunopositive CD34 cells and immunopositive CD34 cells co-expressing CXCR4, as well as eosinophils expressing CD34 following allergen exposure. This is contrary to a previous study with nasal polyp tissue in which topical steroid pretreatment significantly increased

$CD34^+$ cell numbers but reduced eosinophilia indicating the down-modulating effect of topical corticosteroids on in-situ eosinophilopoiesis [61]. It remains unclear as to whether the disparity between these two studies is related to the dose of the inhaled steroid that was used.

Strategic Targets for Treatment

Two related, but distinct, mechanisms by which HPC could contribute to the processes of allergic inflammation are: one initiated by hemopoietic events in the bone marrow, and the other by hemopoietic events in target tissues. These eosinopoietic mechanisms are associated with the process of allergic inflammation, and as such, may provide potential targets for antiallergic therapy as will be discussed below.

Corticosteroids

Corticosteroids are still accepted as the "gold standard" for the treatment of allergic inflammatory diseases like asthma [90–92]. Due to the systemic bioavailability, the anti-inflammatory actions of corticosteroids can potentially extend beyond the environment of the airways, to exert additional *beneficial* systemic effects by acting upon hemopoietic mechanisms that originate in the bone marrow. For the most part, treatment with inhaled corticosteroids in vivo has been shown to have a suppressive effect on circulating eosinophil progenitors. A study involving a controlled, stepwise withdrawal of inhaled corticosteroids resulted in an immediate increase in Eo/B progenitors assayed in peripheral blood; reconstitution of therapeutic steroid treatment restored progenitor levels to baseline [93]. One week of treatment with inhaled corticosteroid was reported to significantly attenuate allergen-induced levels of circulating Eo/B CFU after whole lung inhalation challenge, further supporting the efficacy of corticosteroids on progenitors in peripheral blood [63].

Examination of bone marrow responses to corticosteroid treatment in vitro or in vivo has yielded inconsistent results. In canine studies using allergen-induced bronchial hyperresponsiveness as a model of allergic inflammation, inhaled corticosteroids prevent allergen-induced increases in bone marrow myeloid progenitors [90]. In humans, allergen inhalation challenge of atopic asthmatics results in an increase in the number of $CD34^+$ cells, an increase in IL-5Rα expression on these cells, as well as an increase in vitro in the number of Eo/B CFU [18, 19]. In contrast, pre-treatment with inhaled budesonide at 400 μg twice daily for 1 week was not able to attenuate these allergen-induced changes; nonetheless, this short-term pretreatment was able to reduce baseline numbers of bone marrow Eo/B CFU [65].

The significance of these findings is that inhaled corticosteroids are able to attenuate the levels of allergen-induced eosinophils and their progenitors in peripheral

blood and, to some extent, in bone marrow; this has engendered the hypothesis that topical corticosteroids, via regulation of tissue cytokine expression, exert *indirect* suppressive effects on the differentiative pathway of eosinophil progenitors, thus exhibiting a "beneficial" systemic effect.

Despite what appear to be beneficial, suppressive systemic effects of corticosteroids when administered in vivo, paradoxical stimulatory effects of steroids in vitro have been observed. Early studies utilizing bone marrow cultures indicated that at physiological and pharmacological concentrations, hydrocortisone was able to *stimulate* eosinopoiesis [94]. Using a more refined colony assay developed over the last decade, Dorman et al. have shown, in cultures of bone marrow non-adherent mononuclear cells isolated from atopic asthmatics, that budesonide at concentrations of 10^{-7} M and 10^{-8} M actually causes an *increase* in the number of IL-5-responsive Eo/B CFU [95]. In support of these data, further analysis by our group has shown that budesonide, at a concentration of 10^{-8} M, causes a significant *increase* in IL-5-responsive Eo/B CFU in cultures of purified $CD34^+$ progenitor cells isolated from bone marrow or cord blood [96]. Others have shown, using a murine model, that glucocorticoids indeed enhance bone marrow eosinopoiesis in both normal and allergic mice, as assayed using semisolid and liquid cultures [97]. This latter group also demonstrated that pretreatment of these mice with dexamethasone for 24 h in vivo resulted in *increased* progenitor responses to both GM-CSF and IL-5 ex vivo. These studies collectively put forward the contrary notion of a direct, stimulatory effect of corticosteroids on eosinophil progenitors. Whether corticosteroids work to prevent apoptosis of primitive progenitor cells or selectively up-regulate lineage-specific receptors through increased production of transcription factors thus enhancing the eosinophilopoietic response remains to be clarified.

Alternatively, results from tissue analyses of steroid pretreated tissue showing increased $CD34^+$ cell numbers suggests that withdrawal of steroid treatment may remove the breaking mechanism thus providing increased numbers of locally available pro-inflammatory precursor cells ready to respond to the production of growth factors during an allergic inflammatory event. This may play a role in the rebound effect that is often seen when steroid treatment is withdrawn in allergic rhinitics.

Anti-Leukotrienes

With the increasing recognition of the role of leukotrienes as important inflammatory mediators, novel therapies targeting leukotrienes have been developed. Pharmacologically, there are two ways of inhibiting the actions of leukotrienes: blocking the 5-lipoxygenase pathway, by targeting the 5-lipoxygenase enzyme or its activating protein, 5-lipoxygenase activating protein (FLAP) and thus inhibiting synthesis; or, specifically blocking the interaction of cysteinyl leukotrienes (CysLT) with their receptors (CysLT1R) using competitive antagonists [98]. Both of these

types of compounds have been shown to inhibit eosinophil survival by inducing apoptosis [99].

Pathologically, CysLTs contribute to changing airway function by contracting airway smooth muscle [100], increasing vascular permeability [48], stimulating mucous secretion, and decreasing mucociliary clearance [101], and are capable of recruiting eosinophils into the airways [102]. Cysteinyl leukotrienes have also been shown to induce chemotaxis and endothelial migration of bone marrow-derived $CD34^+$ progenitor cells, acting through the CysLT1 receptor [103].

Significant increases in LTB4-stimulated colony formation of granulocyte-macrophage progenitors (CFU-GM) from human bone marrow was demonstrated in early work by Claesson et al. [104]. It was hypothesized that LTB4 either directly acts to increase proliferation or acts indirectly, stimulating other factors that contribute to increased colony formation. Work by Miller et al. [105] further supported these results by showing that LTC4 and LTD4 were essential intermediates in colony stimulating factor (CSF)-stimulated CFU-GM derived from human bone marrow. Furthermore, Stenke et al. [106] provided evidence that LTB4 and LTC4 possess stimulatory effects on GM-CSF-induced human myeloid progenitor cell growth. Conversely, LTB4 and LTC4 were shown to cause a reduction in both granulocyte-macrophage (CFU-GM) as well as erythroid (CFU-E and BFU-E) colony formation, and this effect was reversed with the addition of nordihydroguaiaretic (NDGA), a leukotriene synthesis inhibitor [107]. Although conflicting data exist, it is becoming apparent that the CysLTs can indeed exert stimulatory effects on bone marrow hematopoiesis.

The effects of cysteinyl leukotrienes on bone marrow and peripheral blood eosinophil progenitors have been examined. It has been shown that LTD4, but not LTE4, causes a significant increase in peripheral blood Eo/B CFU in the presence of GM-CSF in vitro [108]. LTD4 exerts stimulatory effects only in the presence of a growth factor, GM-CSF; this effect is partially abrogated by the addition in vitro of montelukast, a CysLT1 receptor antagonist. Given the recent discovery of CysLT1 receptor expression on $CD34^+$ cells [38, 103], the inhibition of the observed stimulatory effect of LTD4 by montelukast may be mediated by antagonism of leukotriene signaling via the CysLT1 receptor on progenitor cells.

Possible direct effects of leukotrienes on progenitors via the CysLT1 receptor have also been examined in a murine model of experimental allergic rhinitis [109]. Investigation of in vivo montelukast treatment (1- or 2-week duration) on eosinophil progenitors in sensitized mice, revealed that there was a significant reduction in IL-5-responsive murine bone marrow Eo/B CFU ex vivo. It was also found that there was a significant suppression of eosinophil maturation by montelukast, as indicated by lower numbers of mature eosinophils per CFU, in the presence of rmIL-5 in vitro. This result showed that montelukast, through inhibition of the CysLT1 receptor, acts to down-regulate Eo/B lineage commitment and/or maturation of existing, committed progenitors. These studies strongly suggest that, in addition to effects on mature eosinophils, montelukast may exert an anti-inflammatory effect by inhibiting the differentiation of eosinophils.

Anti-IL-5

Eosinophils have been shown to play an important role in allergic inflammation and their effects are regulated in large part by the Th2 cytokine, IL-5, which has been shown to stimulate the growth and differentiation of eosinophils [110] as well as promoting and prolonging eosinophil survival by inhibiting apoptosis [111]. In addition, IL-5 has been shown to regulate eosinophil migrational responses, adhesion molecule expression [112], degranulation [113], cytokine synthesis [114], and production of other pro-inflammatory mediators such as oxygen radicals [115] and leukotriene [116]. When delivered to asthmatic subjects by inhalation, IL-5 has been shown to modulate eosinophil progenitors in the airway mucosa and bone marrow [117]. Therefore, because IL-5 has been shown to modulate various functions of eosinophils, it has been proposed as an excellent therapeutic target in allergic inflammation.

In a murine model of asthma, it has been found that administration of an antibody directed at IL-5 completely inhibits eosinophil infiltration into the airways [118, 119]. Additional evidence, using IL-5-deficient mice, has shown that after allergen challenge these mice do not develop airway inflammation or airway hyperreactivity [120]. Other animal models have shown that airway eosinophilia and increased bronchial hyperreactivity do not occur in guinea pigs [121] or monkeys [122] pretreated with anti-IL-5 monoclonal antibody prior to allergen challenge. However, a study in humans showed that treatment with a blocking monoclonal antibody directed against IL-5 resulted in a significant reduction in allergen-induced eosinophilia, without an effect on the late asthmatic response or airway hyperreactivity in atopic asthmatic subjects [123]. It is important to note that this latter study is methodologically problematic, as outlined by O'Byrne et al. [124]. Subsequent reports of anti-IL-5 treatment in mild atopic asthmatic subjects, however, have demonstrated a reduction of airway eosinophils, arrest of bone marrow eosinophil maturation, and decrease in eosinophil progenitors in the bronchial mucosa [125, 126].

We have very recently sought to clarify the role of IL-5 in regulating eosinophilia and bone marrow progenitor responses in allergic inflammation. Using our experimental model of murine allergic rhinitis, the effect of IL-5 deficiency on bone marrow function was assessed [83]. IL-5-deficient mice displayed an absence of eosinophilia in the nasal mucosa and the bone marrow, significantly lower numbers of IL-5 responsive Eo/B CFU and maturing CFU eosinophils, as well as reduced expression of IL-5Rα on bone marrow-derived $CD34^+$ $CD45^+$ progenitor cells. These results indicate that, during the process of experimental allergic rhinitis in the mouse, the presence of IL-5 is required in the bone marrow microenvironment for normal eosinopoiesis to occur in response to specific antigen sensitization. The fact that responses to IL-3 and GM-CSF were normal indicates that there are redundant cytokine mechanisms that can compensate for IL-5 deficiency. These findings highlight the multifactorial nature of allergic inflammation and indicate that combined as opposed to single-line therapies maybe more effective in the treatment of diseases such as asthma.

Although the results yielded from animal models regarding the role of IL-5 in allergic inflammation are promising, long-term clinical studies in humans are required to more fully understand the effects of anti-IL-5 treatment in allergic disease.

Chemokine Receptor Antagonists

Clearly an understanding of the signal(s) and molecular mechanisms that orchestrate the traffic of progenitors (i) from the BM during and allergic inflammatory response; and (ii) into specific tissue sites, after local Ag-challenge will provide an increased understanding of the fundamentals of eosinophilic inflammation in allergic diseases and may provide novel therapeutic targets for the treatment of asthma.

As mentioned earlier there is growing evidence of the presence of primitive progenitor cells at the tissue sites of allergic inflammation. Although several studies have provided evidence of the potential ability of resident progenitor cells to differentiate locally, there is little direct evidence of the traffic of progenitors from the bone marrow to the tissue. Animal models show influx of newly formed cells. In human studies the role of eotaxin in stimulating progenitor cell egress from the bone marrow has been put forward.

Antihistamines

The use of antihistamines is a common treatment for atopic diseases [127]. For the most part, there has been no consistent evidence to indicate that antihistamines provide therapeutic benefit in asthma, as reviewed in a meta-analysis of clinical trials [128], although some individual studies have shown positive effects [127]. However, it has been proposed that the new generation of antihistamines may in fact regulate certain effector functions of eosinophils, which include the inhibition of eosinophil chemotaxis and recruitment into the airways after allergen exposure [129, 130].

The effects of one of these new antihistamines, desloratadine, on eosinophil progenitor responses was recently assessed in a double-blind, placebo-controlled study of subjects with symptomatic, seasonal allergic rhinitis during a ragweed pollen season [54]. The expected fall in the number of circulating Eo/B progenitors from baseline (out of season) to peak season was blunted by desloratadine. These results indicate that desloratadine may act to suppress the differentiation of Eo/B progenitors in vivo through mechanisms that may directly or indirectly relate to antagonism of H1 histamine receptors.

Conclusion

We have reviewed the evidence implicating the role of the eosinophil progenitor in the processes of allergic inflammation. Current therapeutic regimens that include corticosteroids and anti-leukotrienes appear to exert variable effects on systemic hematopoietic mechanisms while other therapies, such as anti-IL-5 and antihistamines, require further study to elucidate their systemic therapeutic effects. The systemic nature of allergic diseases such as asthma has now been well established,

and therefore, the development of treatments that target the hematopoietic response may provide additional therapeutic benefit.

The activation of eosinophilopoietic processes within hemopoietic compartments, and, importantly, at mucosal tissue sites during an allergic inflammatory response provide novel targets for the treatment of allergy as a systemic process and disease. The clinical limitations of monotherapy with topical corticosteroids, oral cysteinyl leukotriene antagonists, and cytokine antagonists such as antibodies to IL-5, among others, point out that suppression of hemopoietic contributions to allergic inflammation may constitute a prerequisite for full control of allergic inflammation and disease. This chapter emphasizes the importance of hemopoietic processes in the development of allergic rhinitis, and, by extension, in its optimal control.

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Part II
Allergy and the Nervous System

Neuroanatomy of the Airways

John Widdicombe

Introduction

All the airways, from nares to alveoli, are supplied with sensory and motor parasympathetic and sympathetic nerves, with the possible exception of the alveoli which have not been shown to have a motor innervation. The sensory nerves relate signals mainly from the mucosa, which cause sensation and set up various reflexes. Some sensory nerves may have a role in neurogenic inflammation, which is important in allergic reactions. The motor nerves act mainly on blood vessels and mucus-secreting glands and, in the lower airways, also on tracheobronchial smooth muscle. In view of the extensive literature on this subject, this chapter through lack of space is bound to be rather superficial. It will deal with all regions except the olfactory zone and the larynx, pharynx and mouth. These areas have highly specialized innervations in relation to their particular functions, and are probably not so important in allergy as are the other parts of the respiratory tract. The central nervous coordination of afferents and efferents will also be omitted for reasons of space.

The Nose

Sensory Innervation

The sensory nerves of the nose are supplied via the ophthalmic and maxillary divisions of the trigeminal nerves. The nasociliary branch of the ophthalmic division gives rise to the anterior and posterior ethmoidal nerves that supply the upper and anterior areas of the lateral walls and septum of the nose. The maxillary division supplies the posterior and inferior areas. More detailed neuroanatomy is given in Refs. [1–4].

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Sensory fibres include nonmyelinated (C) and most categories of myelinated (A) fibres. Nonmyelinated terminals can be found in the walls of arterioles and veins, and in gland acini (Fig. 1). They also extend up to and into the epithelium. The terminals contain material which can be stained for neuropeptides such as substance P (SP), calcitonin gene-related peptide (CGRP), neurokinin A (NKA), gastrin-releasing peptide (GRP) and nerve growth factor (NGF) [5–10]. These substances are thought to mediate neurogenic inflammation. They are released after allergen or hypertonic saline challenge in animal (including human) nose. On the membranes of the nasal neural sensors, the capsaicin receptor (TRPV1) has been identified [11], but the extensive analysis of sensory membrane receptors carried out for the lower airways has not been extended to the nose.

The trigeminal ganglia contain the cell bodies of the nasal afferent fibres, and in particular the rostro-medial part of the ganglia that gives rise to the ophthalmic branch of the trigeminal nerve [2, 3]. The origin of the maxillary branch also contains nasal sensory cell bodies. In cats and rats, the afferents project to the superficial laminae of the subnucleus caudalis and to the subnucleus interpolaris, where nociceptive inputs are also processed [3]. Neuropeptides are found in the C-fibre nerve terminals in the brain, where their release is thought to mediate synaptic transmission.

The roles of individual sensory fibre groups have not been established in detail, unlike those for the bronchi and lungs; the main reason for this is that ‘single-fibre’

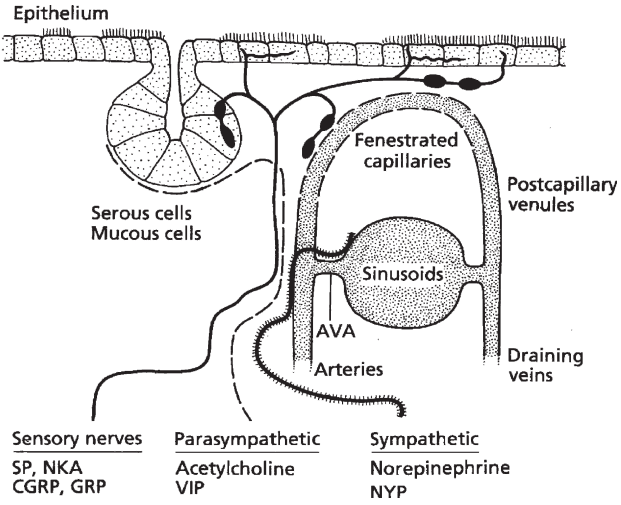

Fig. 1 Schematic representation of human nasal mucosa. Arterial vessels carry blood to arteriovenous anastomoses (AVAs), which regulate blood flow into venous sinusoids, and a superficial plexus of periglandular and subepithelial fenestrated capillaries. Sensory, parasympathetic and sympathetic neurons innervate the vessels and glands, while sensory neurons also innervate the epithelium. Sensory neurons have neurosecretory varicosities, which release acetylcholine and tachykinins such as substance P (SP), neurokinin A (NKA), gastrin-releasing peptide (GRP) and calcitonin gene-related peptide (CGRP) during the axon response. Motor nerves release noradrenaline and neuropeptide Y (NPY) (Reproduced from [53]. With permission)

recording from nasal nerves or ganglia is much more difficult than it is from the vagi and its ganglia. However, action potential recording studies have been done [12]. Most studies have concentrated on C-fibres and their possible roles in inflammation and responses to irritants. Sensations mediated by the nerves include touch, pain, cold, itch and airflow; airflow and agents such as menthol probably act via cold sensors. Itch and even pain can be caused by irritants such as ammonia, sulphur dioxide, capsaicin, histamine and hyperosmolar solutions. 'Extra-nasal' reflex responses that can be elicited include sneeze [13], apnoea, bradycardia, laryngeal closure and bronchoconstriction or dilation. Apart from sneezing, these responses are very similar to those seen with the diving reflex, normally elicited from the skin outside the nose [14]. Intranasal reflex responses include rhinorrhoea and nasal vasodilation with sinus congestion and airflow limitation. A stimulus located to one side of the nose produces a response on the opposite side, a cross-over effect [13].

Hypersensitivity is outside the scope of this chapter but is well established for the nose [2, 5, 15–18]; its mechanism is presumably related to the plasticity of the afferent sensory pathways, peripherally and centrally [19], which has been extensively studied for the lower airways.

Motor Innervation: Parasympathetic

The origin of the parasympathetic supply to the nasal mucosa (including the nasopharynx and the nasal sinuses) is in the facial nuclei of the brainstem and the superior salivatory nuclei. The emerging fibres run in the superficial petrosal and the vidian nerves and synapse in the sphenopalatine ganglia [1–3]. Postganglionic fibres reach the nasal mucosa via the posterior nasal nerves, and innervate arteries, arteriovenous anastomoses (AVAs), veins and mucous glands. The pre-postganglionic transmitter is acetylcholine (ACh), and the postganglionic transmitters are acetylcholine, vasoactive intestinal polypeptide (VIP), peptide histidine methionine (PHM), peptide histidine valine (PHV) and secretoneurin. Of the muscarinic receptors, the most abundant is M3, found on glands, arteries and veins, but M1, M2, M4 and M5 are also present [20]. VIP receptors are found on glands, arterioles and epithelium, and secretoneurin has multiple leukocyte trafficking and angiogenetic effects. The postganglionic parasympathetic nerves also contain nitric oxide (NO) synthase [21], which may have an action on various nasal blood vessels [21].

The parasympathetic innervation, when activated, releases glandular secretions rich in mucoglycoproteins, lactoferrin, lysozyme, secretory leukoprotease inhibitor, neutral endopeptidase and secretory IgA; these arise both from submucosal glands and from the lateral and anterior nasal glands [2, 3]. The response can be mimicked by ACh and blocked by atropine. A parasympathetic vasodilation also occurs, which is atropine-resistant and may be due to the release of vasoactive intestinal polypeptide (VIP).

Motor Innervation: Sympathetic

The sympathetic innervation of the nose has preganglionic fibres arising from the thoracolumbar region of the spinal cord, passing into the vagosympathetic trunks in the neck, and relaying in the superior cervical ganglia [1–3]. Postganglionic fibres run in the deep petrosal nerves, which join the greater petrosal nerves to form the vidian nerves. Thus, the vidian nerves contain both sympathetic and parasympathetic motor fibres. Some sympathetic fibres also reach the nose via the carotid plexuses through branches of the trigeminal nerves. The sympathetic motor fibres supply primarily the nasal vasculature, although some fibres may also go to the nasal secretory glands. The predominant action of the nerves is to cause vasoconstriction, including emptying of the venous sinuses, which increases the patency of the nasal airway by decongestion [2, 3, 22].

The released noradrenaline acts on α_1- and α_2-adrenoceptors on the vascular smooth muscle, increasing vascular resistance and decreasing venous sinusoidal volume and airflow resistance [21, 22]. β_1- and β_2-adrenoceptors are also present on the vasculature, and lead to vasodilation. They may also promote glandular secretion. Neuropeptide Y (NPY) is also present in the sympathetic nerve terminals, especially in those going to the arterioles and AV-anastomoses. It may also facilitate leukocyte vascular adhesion [23].

The Tracheobronchial Tree and Lungs

Unlike the nose, the innervation of the tracheobronchial (TB) tree and lungs is readily available to surgical exploration (especially the vagus nerves) and physiological study. On the motor side, its vasculature differs in that there are no muscular-walled venous sinuses, and both the bronchial and the pulmonary vasculatures have different aspects of control. While there are prominent venous sinuses in the larger airways of some species (e.g. sheep) [24], the sinus walls lack smooth muscle, although nervously mediated congestion can be shown [25, 26], and there is no reason to believe that their congestion has an appreciable effect on airflow resistance. The main difference between the innervations of the nose and the TB tree is that the latter contains abundant airway contractile smooth muscle, with distinctive reflex and motor controls.

Sensory Innervation

The TB tree and lungs contain many different neural sensors; they can be broadly divided into two groups: those with myelinated (A) nerve fibres and those with nonmyelinated (C) fibres. The former comprise slowly adapting pulmonary stretch receptors (SARs), rapidly adapting receptors (RARs), Aδ-nociceptors and ‘cough

receptors'. The C-fibre receptors can be subdivided into pulmonary and bronchial, according to their site, and recently a subdivision has been made based on membrane properties.

SARs. As their name implies, these adapt slowly to a maintained inflation of the lungs [27, 28]. They are found mainly in airway smooth muscle, and appear as 'flower-spray endings' [29]. Originally, each ending was thought to be discrete, but recent 3-D analysis has shown that several endings may connect to a single vagal afferent fibre, and that some of the endings may be outside the smooth muscle in the submucosa [30]. They have afferent fibres in the Aβ–δ range, with conduction velocities in the 5–68 m s^{-1} range, and are distributed mainly unilaterally in the vagus nerves. They seem to be attached to the smooth muscle so that its contraction sensitizes or stimulates the ending and enhances the reflex they provoke [27, 28]. They are all stimulated by lung inflations, but some also respond to deflation; their discharge is regular. They have been subdivided into types I and II [31]. The former are found mainly in the trachea and larger bronchi and their frequency of discharge plateaus at about 10 cm H_2O inflation pressure; the latter are mainly intrapulmonary, have higher volume thresholds, only reach maximum discharge frequency at high inflation pressures and often also respond to deflation of the lungs. It has not been shown whether the two types of SARs have different reflex actions. In general, SARs are irresponsive to chemical stimuli, apart from veratrum alkaloids, which are strong stimuli.

The SARs immunoreact to Na^+/K^+-ATPase and calretinin, but not to CGRP or SP [32]. The way in which stretch activates the receptors is thought to be by mechanical opening of stretch-activated cation channels, which in turn sets up a generator potential, which then if adequate leads to an action potential [33]. This process has yet to be experimentally confirmed for the SARs, and is analysed in detail elsewhere [30].

The main reflex from the SARs is the Hering–Breuer inflation reflex, the inhibition of inspiration and the initiation of expiration produced by lung inflation and conducted by the vagus nerves [27, 28]. Thus, the reflex controls the pattern of breathing. Other reflexes from SARs include tachycardia, bronchodilation and possibly hypotension. It may cause or contribute to sinus arrhythmia.

RARs. These adapt rapidly, often within 1 s, to maintained inflation or deflation of the lungs [34, 35]. They have myelinated afferent nerve fibres in the vagus nerves and in the Aγ–δ range, and an irregular pattern of discharge. Most are stimulated both by lung inflations and deflations and, unlike many of the SARs, have no spontaneous activity during the expiratory pause; some are active during the inspiratory phase of quiet breathing. There is some uncertainty as to their morphology. Early evidence indicated that they were free nerve endings lying in and under the airway epithelium, but more recent studies suggest that they are flower-spray endings under the epithelium and perhaps connected to other sensors supplied by the same vagal nerve fibre [30]. Their terminals include those running to mucosal blood vessels. In the tracheal mucosa, in vitro studies show a total receptor field of about 1 mm diameter. The RARs are concentrated at the points of branching of the larger airways, where they would be subject to impact by inhaled materials [34, 35].

Histological identification of mucosal receptor type is often difficult, but RARs, unlike the C-fibre sensors, seem to contain no neuropeptides [32]. The cell bodies of RARs are found in the nodose ganglia.

In addition to their response to airway volume change, RARs are sensitive to mechanical probing of the epithelium and therefore presumably to inhaled foreign bodies. They are also stimulated by a variety of chemicals, including cigarette smoke, sulphur dioxide, ammonia, volatile anaesthetics and hypo- and hyperosmolar solutions [34, 35]. Some of these agents may act on acid-sensing ion channels (ASICs) and other stimuli by opening Na^+ channels [33]. In in vitro preparations they are not activated by capsaicin, or by inflammatory mediators such as histamine, prostaglandins, bradykinin and platelet activating factor (PAF). However, in vivo these agents may stimulate RARs by an indirect action. Thus, the RARs are sensitized or stimulated by underlying smooth muscle contraction, mucosal vasodilatation and mucus secretion, which occur during airways inflammation [34, 35].

Much evidence points to cough as a reflex response to stimulation of RARs [36], and perhaps the expiration reflex (a brief strong expiratory effort not preceded by an inspiration) [34, 35]. They have been shown to cause the deep inspirations of augmented breaths, and rapid breathing due to shortened expiratory pauses. How these diverse respiratory responses can be induced from a single category of receptors is not clear, and perhaps there are subgroups of RARs, or other types of sensor are also involved. Other reflex actions from RARs include airway smooth muscle contraction, mucosal vasodilatation and secretion from submucosal glands [34, 35]. As indicated above, these last responses may themselves sensitize or activate the RARs. Cardiovascular reflexes from the RARs are less well established, but probably include hypertension and tachycardia. Laryngeal constriction is a further reflex from RARs, and may occur even in the absence of cough.

Aδ-nociceptors. The sensors resemble RARs in some ways, in particular adapting rather rapidly to mechanical stimuli, but differ in others [32, 36]. They have slower conduction velocities, adapt less rapidly to mechanical stimuli and do not respond to airway volume changes. They have cell bodies in both the jugular and nodose ganglia. They do not contain SP or other tachykinins [32]. They are stimulated by punctate touch, acids, capsaicin, hypertonic solutions and bradykinin. Their morphology, central connections and reflex actions are unknown, but may be related to cough.

'*Cough receptors*'. Until recently, RARs were assumed to mediate the cough reflex and to be the only sensor in the lungs to do so. However, 'cough receptors' have now been identified in the guinea pig airways, which are a group distinct from the A- and Aδ-receptors [32, 36]. They have myelinated afferent nerve fibres in the δ-range, and their terminals are arranged circumferentially around the airway mucosa between the smooth muscle and the epithelium. They do not contain SP or other neuropeptides and their membranes do not have TRVP1 receptors. They are sensitive to punctate stimuli, but not to airway distension, and are activated by acid but not by capsaicin, bradykinin, histamine, methacholine, leukotrienes or 5-hydroxytryptamine.

The evidence that 'cough receptors' cause cough is indirect, and the relative roles of RARs, Aδ-receptors and 'cough receptors' (and indeed C-fibre receptors) in producing cough are being hotly debated.

C-fibre receptors. Seventy-five per cent of TB tree afferent fibres are nonmyelinated (C), with cell bodies in the jugular and nodose ganglia [37, 38]. A few of the C-fibre afferents run in the spinal sympathetic nerves with cell bodies in the dorsal root ganglia. They were first identified by Paintal in 1955 [39] who called them juxta-pulmonary capillary receptors, and later type-J receptors. He concluded that they lay in the alveolar walls; indeed, the only sensory supplies in the alveoli are C-fibre sensors. Coleridge and Coleridge [40] showed that there were distinct pulmonary and bronchial C-fibre sensors, the difference based on their separate blood supply by the pulmonary and bronchial circulations, and by their relative sensitivities to various chemicals and inflammatory mediators and to mechanical stimulation. A more recent subdivision is based on the observation that the nodose ganglionic afferents have different membrane receptors and sensitivities to various agents compared to the jugular afferents [41]. Morphologically, the C-fibre endings spread out widely in the mucosal tissues; they contain various tachykinins such as SP, CGRP and NK1 [37, 38]. They are responsible for neurogenic inflammation in the airways [42]. They exhibit 'plasticity': their tachykinin contents and membrane receptor properties are changed in chronic bronchopulmonary conditions, which will enhance inflammatory responses [43].

One group of C-fibre sensors is sensitive to chemicals including 5-HT, adenosine compounds, capsaicin, nicotine, ozone, acrolein, ammonia, cigarette smoke, prostaglandins and volatile anaesthetics; in other words, there are polymodal. The second group responds only to capsaicin, bradykinin and hypertonic solutions, of the agents tested [36]. The corresponding membrane receptors are found in each of these groups; for example, the first have TRPV1 and nicotine/ACh receptors, and the second have TRPV1 and B_2 receptors [32]. The C-fibre sensors are stimulated by large inflations and deflations of the lungs, but are relatively insensitive to mechanical stimuli compared with SARs, RARs and Aδ-nociceptors [34, 35]. They are stimulated by pulmonary microemboli, presumably by the release of inflammatory mediators. The pulmonary C-fibre sensors are stimulated by increases in pulmonary blood flow, and it has been suggested that this may be an inhibitory influence on breathing in severe exercise, although there is also evidence against this view.

Reflexly, the C-fibre sensors cause apnoea and rapid shallow breathing [34, 35]. Their role in cough is uncertain. There are claims that they cause cough, but also experimental evidence that they inhibit cough [36]. Recent studies suggest that they may play a modulating or permissive role in some forms of cough. They inhibit spinal reflexes and have profound reflex effects on the autonomic nervous system; these include bronchoconstriction, laryngoconstriction, vasodilatation (including in the bronchial and nasal circulations) with resultant hypotension, tachycardia and airway mucus secretion [37, 38]. They are probably responsible for the sensation of irritation or tightness associated with lung irritation, and felt especially in the laryngeal and tracheal areas.

Neuroepithelial bodies (NEBs). These are collections of neuroendocrine cells found in the epithelium of the lower airway. They have a complex innervation, possibly consisting of up to five neurons [44]. The afferent fibres include myelinated ones to the jugular and nodose ganglia, and nonmyelinated ones to the dorsal root ganglia.

The latter contain CGRP and SP. The nerves ramify among the cells of the NEBs and the axons may subserve local reflex connections to peripheral ganglia, as well as provide the basis for local axon reflexes. The NEBs are thought to respond to hypoxia and, by release of amines, peptides and possibly ACh and purines, to act on local airway smooth muscle and vascular beds [44]. Any reflex actions of their vagal and spinal afferent pathways do not seem to have been established.

Motor innervation. Motor nerves control the tone of airway smooth muscle, tracheobronchial vascular muscle, pulmonary vascular muscle and mucus-secreting glands [45–48] (Fig. 2). Both parasympathetic and sympathetic systems may supply all these tissues. The parasympathetic supply is via the vagus nerves, and their subdivisions are the superior and recurrent laryngeal nerves and the pulmonary nerves. They synapse in peripheral neural ganglia in the submucosa of the airways. The sympathetic supply arises from the ventral spinal roots, synapses in the ganglia of the sympathetic trunk, and reaches the airways via the pulmonary nerves and, for the larynx, via the recurrent and superior laryngeal nerve.

Airway smooth muscle. The nerve density of the smooth muscle remains fairly constant from trachea to bronchioles, but the types of fibres based on their inclusions vary greatly (see below). The parasympathetic nerves are of two types. Cholinergic nerves containing choline acetyltransferase are found in high densities throughout

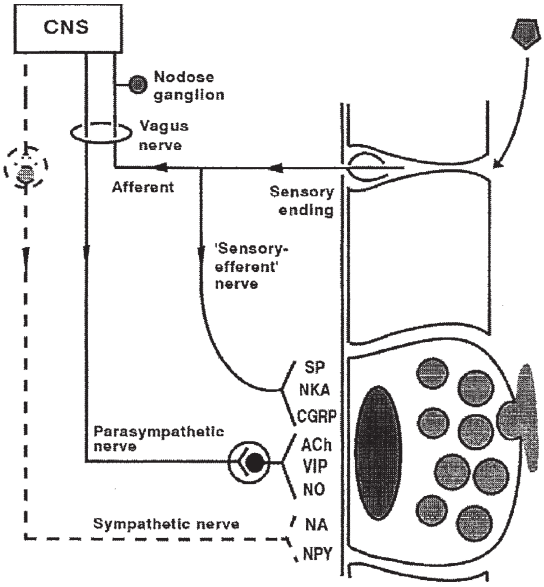

Fig. 2 Innervation of airway-secreting cells (simplified model). Parasympathetic nerves act via acetylcholine (ACh), vasoactive intestinal polypeptide (VIP) and nitric oxide (NO). Sympathetic nerves act via noradrenaline (NA) and neuropeptide Y (NPY). Sensory nerves mediate neurogenic inflammation via substance P (SP), neurokinin A (NKA) and calcitonin gene-related peptide (CGRP), and also set up central nervous system (CNS) reflexes (Reproduced from [47]. With permission)

the airway smooth muscle, and presumably represent the main contractile control of the muscle [45, 46]. When these nerves are stimulated, small airways can collapse and the larger ones constrict. The action is via M1 receptors on the smooth muscle membrane. M2 receptors are also present, and can antagonize bronchoconstriction [20]. There is muscle tone in healthy lungs at rest and, since this is blocked by drugs such as atropine or ipratropium, it is thought that the predominant motor control of the muscle is the vagal parasympathetic cholinergic supply.

There are also parasympathetic nerves that are relaxant. These run mainly to the larger bronchi and the trachea [45, 46]. They release the dilator VIP, and also possibly NO (formed from arginine by NO synthase), VIP and galanin. These substances are unlikely to be coreleased with ACh, but come from a different set of nerves.

The sympathetic nerves are bronchodilator or muscle-relaxant, with noradrenaline as the transmitter. NPY may be a cotransmitter, although it has only weak action on airway smooth muscle. Their action is mediated by β_2-adrenoceptors, and is mimicked by β-agonists such as salbutamol. Sympathetic constrictor responses have also been described, and are thought to be due to the action of noradrenaline on α-receptors on the smooth muscle membrane, but these responses have not been seen with human airways.

Sensory nerves containing SP, NKA and CGRP have been found in airway smooth muscle, especially in the guinea pig [45]. The first two agents are thought to underlie neurogenic inflammation, part of which includes smooth muscle contraction. The complex innervation of airway smooth muscle has been studied mainly with in vitro preparations, and mainly in rodents such as guinea pigs and rats [45]. The extension of these results to humans in vivo is bound to be somewhat speculative.

Mucus-secreting glands. Abundant research shows that the main motor innervation of airway submucosal glands is vagal and parasympathetic, with acetylcholine as the transmitter [47, 48]. Other possible innervations have been less studied. NO and VIP inhibit secretion, and VIP receptors have been identified on gland nerves and cells, but a neural pathway based on these transmitters has not been established. Similarly, α-receptor stimulants can augment or modulate secretion, but the role of a sympathetic nervous pathway has not been established. Similarly, there are clues that the glands may be involved in neurogenic inflammation, since they are stimulated by SP and NKA, but not significantly by CGRP, but such a mechanism has not been confirmed.

Airway vasculature. Both sympathetic and parasympathetic nerves supply the TB vasculature [49–52]. Electrical stimulation of the sympathetic nerves to the airways reduces blood flow (vasoconstriction), an effect mimicked by noradrenaline and blocked by α-adrenoceptor antagonists. β-adrenoceptor agonists increase blood flow. Vagal stimulation increases blood flow (vasodilatation) by a double mechanism. Part of the response is atropine-sensitive and mimicked by injections of ACh, and is thus cholinergic; part remains after atropine and is probably due to release of VIP or NPY, both of which vasodilate the vasculature.

The trachea of some species, such as the sheep, has conspicuous venous sinuses that differ from those in the nose in that they lack smooth muscle in their walls [24].

Vasoconstrictor drugs such as noradrenaline shrink the mucosa and vasodilator drugs thicken it by up to 300 μm [25, 26]; nervous control of this variable has not been tested, but its existence seems plausible.

Pulmonary vasculature. As with other motor systems in the airways, the pulmonary vasculature has both a parasympathetic and a sympathetic supply [53–56]. This is conspicuous in the pulmonary arteries but is anatomically almost absent in the arterioles. Vagal stimulation causes dilatation by a cholinergic mechanism; thus, the vasodilation is blocked by atropinic drugs. Sympathetic stimulation causes vasoconstriction, blocked by α-adrenoceptor antagonists. In healthy humans there seems to be very little resting tone, but vasoconstrictor tone can be greatly enhanced in conditions such as stress and in various diseases.

Conclusions

Both the nose and the TB tree have multiple sensory innervations, and multiple motor outputs to the various effector tissues seen in each region. The sensors have functions appropriate to the region, e.g. sneeze for the nose and cough for the TB tree. The motor pathways affect vasculature and secretory tissues in both zones, and in the case of the lungs, airway smooth muscle. While most experimental studies have been with rodents, enough human investigations have been performed to suggest that the general pattern seen in rodents also applies to humans.

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Biology of Neurotrophins, Neuropeptides, and Muscarinic Receptors in Asthma

Sanchaita Sonar and Harald Renz

Biology of Neurotrophins in Asthma

Neurotrophins (NTs) are a family of homologous peptides, similar in receptor utility and activity. They were originally identified for their critical role in promoting neuronal survival, growth, and differentiation [1, 2]. The functions of neurotrophins have grown and extended to other areas and neurotrophins are now also considered prime mediators of immunological disorders including asthma. The classical neurotrophin is the nerve growth factor (NGF) and the family also includes the brain-derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin-4/5. NGF was the first neurotrophin to be discovered 50 years ago by Levi-Montalcini and coworkers [1]. Neurotrophin levels like NGF and BDNF are increased in serum, bronchoalveolar lavage, tissues, nasal washings, and tears of patients with allergic diseases. Neurotrophins and their receptors are present in inflammatory cells that infiltrate asthmatic lung and NGF has also been identified as a survival factor for eosinophils [3]. Furthermore, neurotrophins like NGF has been shown to modulate immune cells by promoting survival, proliferation, and differentiation of lymphocytes and mast cells, stimulating mast cell degranulation, enhancing survival of eosinophils, monocytes, and neutrophils, and also inducing release of mediators from T cells, macrophages, basophils, and eosinophils [4–6].

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Neurotrophins and Their Receptors

All of the neurotrophins are synthesized as precursors and are processed either inside the cell by furin or pro-hormone convertases, or extracellularly by plasmin or the matrix metalloproteinases (MMPs) MMP3 or MMP7 to produce mature neurotrophins. Pro-NGF, pro-NT-3, and pro-NT-4 are packaged into constitutive vesicles before secretion; pro-BDNF, however, is mainly packaged into regulated secretory pathway vesicles, processed and secreted in an activity-dependent manner [7, 8]. The structures of the mature proteins have been elucidated by X-ray crystallography and they each appear very similar in conformation. Neurotrophins signal via two types of receptors, the receptor tyrosine kinases (Trk) and the $p75^{NTR}$, a member of the TNF receptor/Fas/CD40 superfamily. The Trk receptors (Fig. 1) with a molecular weight of 140–145 kD are specific receptors of neurotrophins with NGF preferentially binding to TrkA, BDNF to TrkB, NT-3 with TrkC, and NT-4/5 overlaps with BDNF for the TrkB. The $p75^{NTR}$ is a glycoprotein with a molecular weight of 75 kD that binds all mature neurotrophins, however with lower affinity and unique kinetics [2, 9, 10]. The $p75^{NTR}$ is also capable of enhancing the affinity of NGF to TrkA and increases receptor specificity for its ligand, NGF. Immunohistochemical studies in lung have identified the expression patterns of all NTs and their receptors.

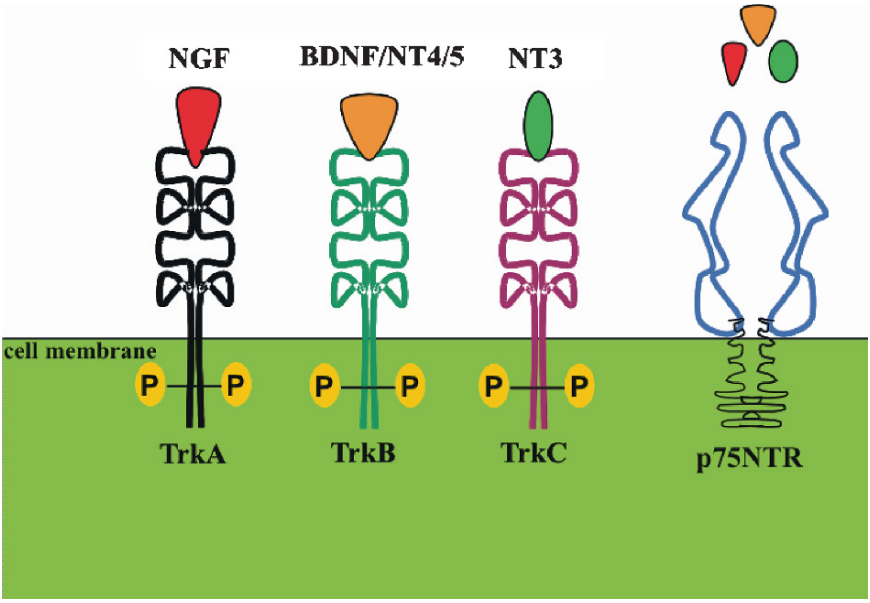

Fig. 1 Neurotrophin receptors. Neurotrophins signal via the receptor tyrosine kinases (Trk) and the $p75^{ntr}$. NGF preferentially binds to TrkA, BDNF to TrkB, NT-3 with TrkC and NT-4/5 overlaps with BDNF for the TrkB receptor. The $p75^{ntr}$ binds all mature neurotrophins, however with lower affinity and unique kinetics

Neurotrophins in Asthma and Allergic Diseases

NGF is the most prominently investigated neurotrophin in allergic and asthmatic studies. Neurotrophin levels are low under physiologic conditions; however, their levels increase significantly in allergic diseases like asthma, urticaria, and atopic dermatitis [11–13]. Increased NGF and BDNF levels have been shown to correlate positively with disease severity in the case of atopic dermatitis. Higher levels of neurotrophins like NGF, BDNF, and NT-3 have been shown to increase in allergic asthma and their amounts also increase upon segmental allergen provocation [14]. Allergen challenge seems to be a strong signal for drastic increases in neurotrophin levels. However, levels are higher 24 h after allergen challenge as compared to 4 h post-challenge [15]. Upon treatment with corticosteroids, the levels of neurotrophins have been found to decline. A recent report also found increased neurotrophins in a fungal experimental model of allergic asthma. *Penicillium chrysogenum* extract (PCE)-exposed mice had dose-dependent increases in NGF, NT-3, and NT-4 in both bronchoalveolar lavage fluid (BALF) and an elevation in positive immunohistochemical staining for NGF in the airway epithelium and smooth muscle cells (SMCs), in addition to infiltrated cells such as mononuclear cells, eosinophils, and macrophages [16].

Production and Regulation of Neurotrophins in Asthma

A wide range of cells that actively participate in allergic immune responses produce neurotrophins and/or their receptors. These include both the inflammatory cells that mediate inflammation and the structural cells that respond to its environment (Figs. 2 and 3). Synthesis of neurotrophins like NGF and BDNF by structural cells like the airway epithelium has been demonstrated. In vitro studies with human lung cells in culture have identified bronchial epithelial cells [17, 18], primary fibroblasts [19], and bronchial smooth muscle cells [20] to express NGF. There are also reports of synthesis of other NTs like BDNF and NT-3 from structural cells like rat thoracic aortic smooth muscle cells [21] and human keratinocytes which express NT-4/5 [22]. Consistent with the in vitro studies, in vivo studies corroborate very well with the expression of neurotrophins by structural cells in the airways. Immunolabeling of bronchial biopsies from healthy patients without asthma show considerable staining of NGF in epithelial cells, bronchial smooth muscle cells, and fibroblast [23]. Immunohistochemistry performed in mouse airways in our laboratory show epithelial cells lining the airways as potential sources of NTs like NGF, BDNF, and NT-3 (Figs. 4 and 5). During asthma, induced by challenge to ovalbumin (OVA), the expression of NGF, BDNF, and NT-3 is upregulated in structural as well as the inflammatory cells as seen in the lung sections of control (PBS) and challenged mice (OVA) (Fig. 3). Inflammatory cells like eosinophils express the TrkA and TrkB receptors responsive to NGF and BDNF, respectively and coculture with airway epithelial cells results in an enhanced epithelial neurotrophin production

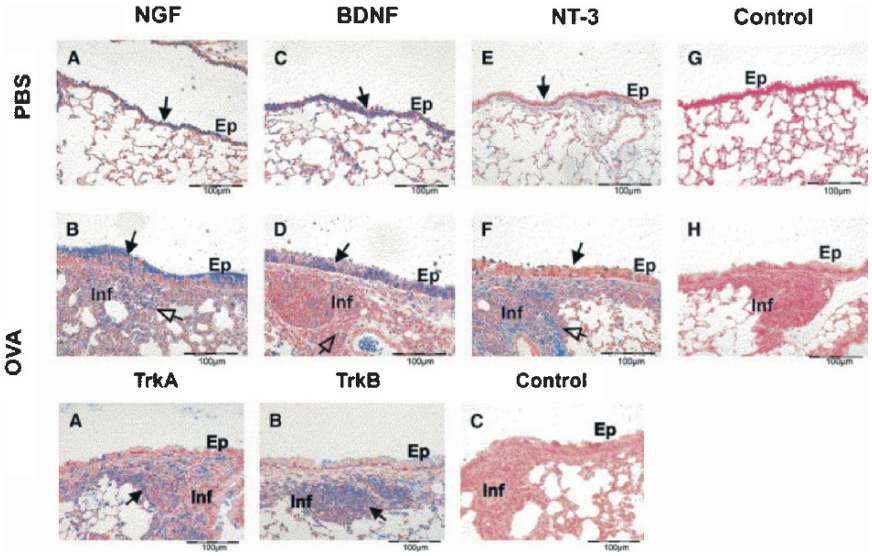

Fig. 2 Immunohistochemical staining of NGF, BDNF, and NT-3 in a murine model of experimental asthma, induced by ovalbumin (OVA). The figure represents lung sections of PBS control mice *(PBS)* and OVA-sensitized mice *(OVA)* stained for NGF (**a** and **b**), BDNF (**c** and **d**), and NT-3 (**e** and **f**). Rabbit IgG was used as a control antibody (**g** and **h**). *Ep*, Bronchial epithelium; *Inf*, inflammatory infiltrate. Original magnification 200×. Epithelial cells constitutively express NTs in the airway and the expression increases during inflammation where the inflammatory infiltrate also produce increased NTs during asthma [3]

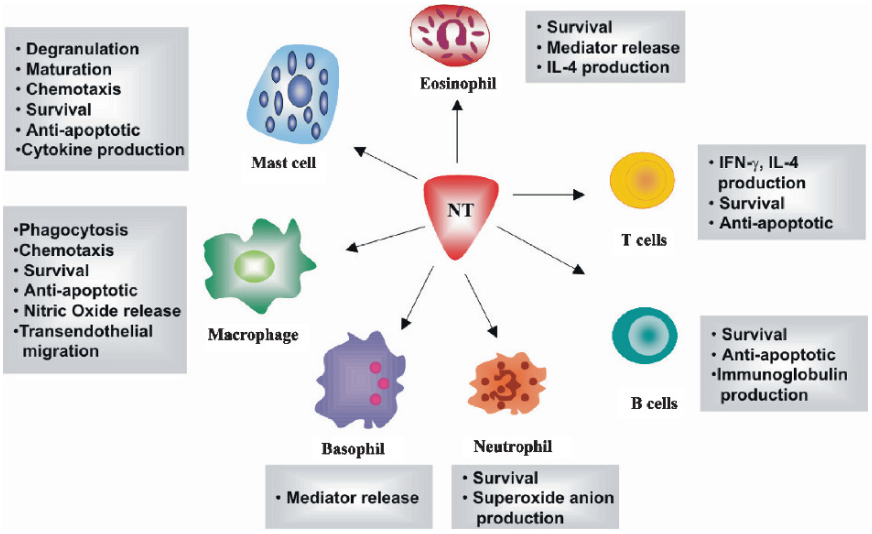

Fig. 3 The inflammatory cell sources of NTs and their effects during allergic inflammation

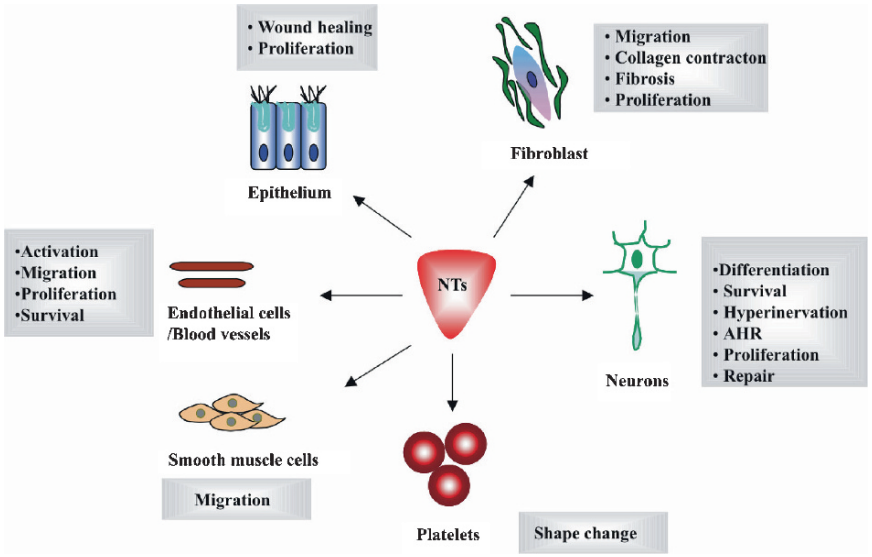

Fig. 4 The structural cell sources of NTs and their effects during allergy and inflammation

which in turn enhances eosinophil survival [3]. Other factors that lead to an induction in neurotrophin levels by structural cells are cytokines. Pro-inflammatory cytokines, such as IL-1β and tumor necrosis factor-α (TNF-α), or transforming growth factor-β (TGF-β), increase the synthesis of NGF by airway structural cells. Recent studies have also suggested NGF capable of directly stimulating structural cells. NGF has been shown to induce contraction and migration of human pulmonary fibroblasts [24, 25] and vascular smooth muscle cells. The presence of specific receptors for neurotrophins in structural cells like TrkA and $p75^{NTR}$ further suggest an autocrine effect that could be useful for the cells in pathological conditions. NGF production has been shown to increase in mast cells upon cross-linkage of the high-affinity IgE receptors [26].

Modulation of Inflammation by Neurotrophins in Asthma

Several lines of evidence suggest that neurotrophins may play a role in the development of inflammation. High neurotrophin levels have been found in patients with severe allergic asthma. Neurotrophins like NGF have been shown to be increased in serum, BALF, nasal washings, and tears of subjects with allergic diseases. Among the inflammatory cells, neurotrophins are produced, stored, and secreted by mast cells, eosinophils, and CD4 + T cells. NGF has been shown to cause a significant dose-dependent eosinophil peroxidase release, while not affecting eosinophil viability [27]. NGF has a priming effect on anti-IgE histamine release from circulating human basophils and induces a selective release of cytotoxic mediators from

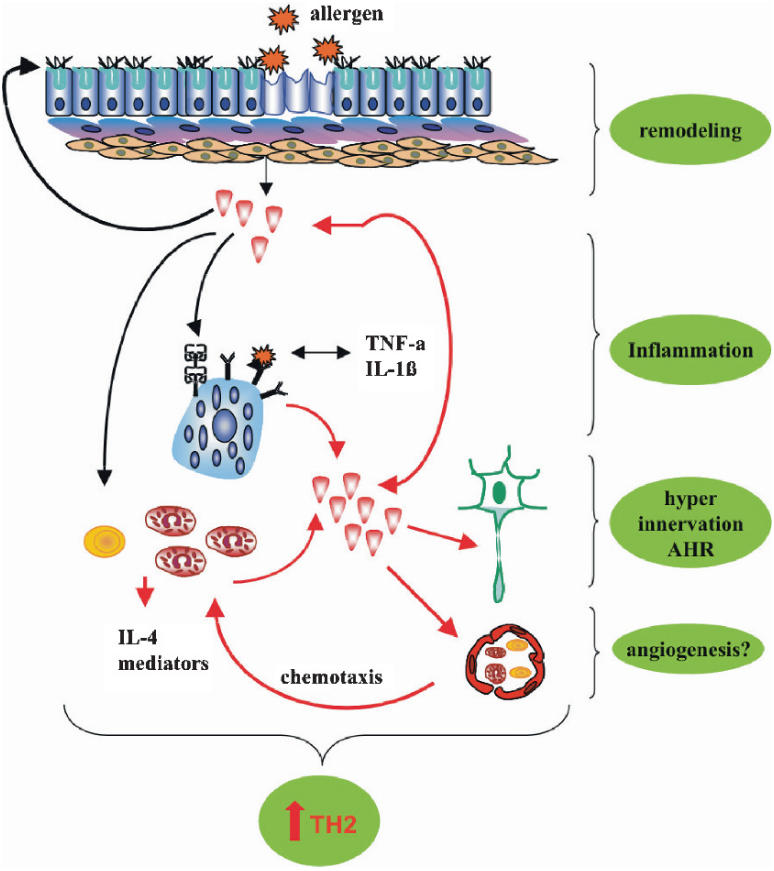

Fig. 5 The following figure shows an overall scheme how the NTs could function during inflammation in asthma. Its release by structural cells like the epithelium, smooth muscle cells (SMCs), and fibroblasts could serve as an initial signal for the upregulation of its receptors in an autocrine as well as a paracrine way for the incoming inflammatory cells. This could activate mast cells, and release mediators and inflammatory cytokines. This, in turn would lead to more production of NTs in the inflamed lung allowing the NTs to modulate many processes like airway remodeling, hyperinnervation, AHR, angiogenesis (?) and an increased Th2 phenotype, exacerbating the symptoms of asthma

peripheral blood eosinophils. Therefore, besides production of neurotrophins, most cells directly involved in inflammation also express the specific receptors, therefore allowing an autocrine as well as a paracrine function for these cytokines. Besides functioning as an inflammatory cytokines, neurotrophins can also be induced by other pro-inflammatory cytokines, which are present at high levels in the airways of patients with asthma. This could serve as a potential source of the elevated levels of neurotrophin synthesis observed during asthma. A brief overview of events following allergen induced asthma and the sources and effects of neurotrophins (NTs) therein are illustrated in Fig. 5. NTs and their receptors expressed by structural and inflammatory cells during asthma have been linked to affect hallmark features of

asthma like airway remodelling, inflammation and AHR. These events lead to an augmentation of the TH2 response typical of asthma and help sustain the disease. Besides asthma, neurotrophins are known to be elevated in various other inflammatory conditions [28–30].

Neurotrophins and AHR

The increased neurotrophin levels in serum and BALF of patients with asthma also coincides with airway hyperreactivity (AHR). The first report of the involvement of NGF in airway hyperreactivity came from experiments in a murine model of allergic asthma. The development of airway hyperreactivity as assessed by electrical field stimulation of trachea segments of allergen-sensitized mice was prevented by anti-NGF treatment [31]. Nerve growth factor has also shown to participate in the airway hyperresponsiveness induced by IL-1β. IL-1β increased the release of NGF from human-isolated bronchi in vitro and the AHR induced by IL-1β was abolished by blocking with anti-human NGF antibody [32]. NGF regulates substance P (SP) production and stimulation with NGF induces a significant increase in SP responsiveness. Blocking the signal transduction of TrkA inhibits the development of AHR, and also prevents the increase in SP in the nodose ganglia and lung tissue completely [33]. Interestingly airway eosinophilia, AHR, and elevations in serum IgE and IL-4 and IL-5 in BALF were completely abolished in $p75^{NTR}$ gene-deficient mice after antigen provocation. The antigen-induced production of interferon (IFN)-gamma and nerve growth factor (NGF) were not altered by depletion of $p75^{NTR}$ gene and the authors suggested that the p75 gene deficiency disrupts allergic airway inflammation and AHR in mice by interfering with type 2 helper T (Th2) cell responses [34]. Experiments from mice overexpressing NGF in the clara cells of the lung (CCSP-NGF-tg) revealed that the CCSP-NGF mice were more sensitive than normal mice to capsaicin-induced increases in respiratory system resistance probably due to increased sensory innervation leading to modulation of airway function [35]. In these NGFtg mice, the tachykinin-containing sensory fibers and sympathetic fibers were increased around the airways and this could be one reason for the increased sensitivity observed. In another study by de Vries, a similar observation was made where intravenously applied NGF in guinea pigs induced a 200% increase in histamine-induced bronchoconstriction. This effect was blocked with neurokinin-1 receptor antagonist. It was suggested that NGF could therefore affect airway hyperreactivity by a rapid tachykinin release [36].

Neuropeptides in Asthma

Tachykinins and calcitonin gene-related peptide (CGRP) are the main neuropeptides in the airway sensory nerves. In addition, the airway sensory nerves contain several other neuropeptides that might participate in neurogenic inflammation. These include

galanin, cholecystokinin octapeptide, and enkephalins. The recently described peptide secretoneurin, derived from secretogranin II (chromogranin C), is also localized to sensory nerves and is a potent attractant of eosinophils [37], but its role in asthma has not yet been explored. The mammalian tachykinins are also called neurokinins. At present, five mammalian tachykinins have been identified: substance P (SP), neurokinin A (NKA), neurokinin B (NKB), and the N-terminally extended forms of NKA, neuropeptide K (NPK), and neuropeptide c (NPc). Tachykinins (SP, NKA, and NKB) have been considered as a group of neuropeptides due to their widespread distribution in the central and the peripheral nervous system (capsaicin-sensitive primary afferent neurons and capsaicin-insensitive intrinsic neurons). However, they have quite often been reported in other non-neuronal structures [38, 39]. The biological activity of tachykinins depends on their interaction with three specific receptors: the tachykinin NK1, NK2, and NK3 receptor [40, 41]. The tachykinin receptor NK1 shows the highest affinity for substance P, NK2 receptor for neurokinin A, and the NK3 receptor for neurokinin B [42]. However, all tachykinins can act as full agonists on all the three different receptors, but with lower affinities. Tachykinins are subject to degradation by at least two enzymes, angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP) [43]. The tachykinins SP and NKA have various effects that could contribute to the changes observed in the airways of patients with asthma and chronic obstructive pulmonary disease (COPD). These include smooth muscle contraction, submucosal gland secretion, vasodilatation, increase in vascular permeability, stimulation of cholinergic nerves, mast cells, B- and T-lymphocytes, macrophages, chemo-attraction of eosinophils and neutrophils, and the vascular adhesion of neutrophils. SP has been shown to be increased in atopic individuals with grass pollen allergy as well as after intrasegmental allergy provocation. Patients with asthma and with chronic bronchitis have a significantly higher concentration of sputum SP than normal subjects, which also correlates with the index of airway obstruction [44]. SP and NKA have been reported to have an in vivo bronchoconstricter effect exclusively in asthmatic patients [45–47]. However, in another study it was found that NKA causes bronchoconstriction in normal persons although asthmatics were more sensitive than normal subjects [48, 49]. Inhalation of SP was found to enhance the maximal airway narrowing to metacholine in patients with asthma [50]. SP is also capable of stimulating mucus secretion from submucosal glands in human airways and is a potent stimulant of goblet cells in guinea pig airways via the activation of NK1 receptors.

Expression of Tachykinins

A large amount of functional data demonstrates the expression of tachykinin NK1 and NK2 receptors in the airways. Also functional evidence for the presence of tachykinin NK3 receptors exists in human airways [51, 52]. A number of studies have demonstrated the presence of SP, NKA, and CGRP in human airways. Nerve fibers containing SP-like immunoreactivity have been described in the larynx, trachea, in and around bronchi, bronchioles, the more distal airways, and occasionally extending into the alveoli. Numerous NKA-like immunoreactive nerves are present around intrinsic

neurones of local bronchial ganglia and within the bronchial smooth muscle layer [53]. CGRP has been localized using immunocytochemistry in airway neuroendocrine cells, tracheal serous cells, and in terminals of C-afferent non-adrenergic non-cholinergic (NANC) fibers [54, 55]. Tachykinins have been shown to be released into the airways after exposure to allergen or ozone. A significantly large amount of SP was found in the bronchoalveolar lavage fluid (BALF) of atopic subjects [56]. Intrasegmental provocation with allergen also induced a significant increase in BALF SP levels. In another study NKA was recovered from BALF and was found to be increased in asthmatic patients 4 h after inhalation challenge with house-dust mite [57].

Inflammation

Sensory nerves containing neuropeptides are sparse in human airways; however, much data has accumulated over the role of inflammatory cells such as eosinophils, macrophages, lymphocytes, and dendritic cells that can produce the tachykinins SP and NKA. Such an infiltration of cells could further stimulate airway sensory nerves to express several neuropeptides, which might contribute to the inflammatory response. Mediator release from mast cells has been shown to be induced by SP, CGRP, neurotensin, and neuropeptide Y [58, 59]. SP, NKA, and CGRP have been shown to induce histamine release from airway mast cells obtained by BAL from non-atopic controls and from patients with cough-variant asthma [60]. Human macrophages are able to produce SP and its receptor [61]. SP stimulates macrophage phagocytic and chemotactic capacity, as well as increased macrophage IL-1, IL-6, TNF-α, superoxide anion, prostaglandin E, and thromboxane production [62–64]. Furthermore, airway macrophages collected from ovalbumin-sensitized guinea pigs display an enhanced responsiveness to tachykinins [65]. Both human and murine eosinophils are capable of producing SP and are able to induce the release of eosinophil cationic protein and superoxide anion by human eosinophils [66]. SP can also prime eosinophils for chemotaxis by other agents such as platelet-activating factor, leukotriene B, and interleukin-5 [67, 68]. SP activates neutrophils [69, 70] and has been shown to stimulate human polymorphonuclear cells to release superoxide anion [70], IL-8. It also mediates increased antibody-dependent cell-mediated cytotoxicity [71], adherence, and chemotaxis of human neutrophils [72–74]. In addition, it plays a role in airway remodelling as it can induce proliferation of smooth muscle in vitro and can induce proliferation of fibroblasts. Tachykinin NK receptors are expressed on human and murine lymphocytes [75, 76] and enhance the production of IL-2 by human T-lymphocytes [77] and modulates immunoglobulin production [78–80]. CGRP inhibits human peripheral blood mononuclear cell proliferation, in part through the release of IL-10 [81]. In antigen-stimulated non-adherent splenocytes and helper T cell clones CGRP inhibits IFN-γ production, but has no effect on IL-4 production [82]. Substance P had no effect on IFN-γ production, however, it enhanced IL-4 production consistently [82]. Since asthma is predominantly a Th2-driven immune response, neuropeptides might play a role in influencing the direction of this response and modulate airway pathology.

Neuropeptides and AHR

SP aerosol exposure to guinea pigs has been reported to elicit airway hyperresponsiveness to bronchoconstrictor agents [83–86]. The bronchoconstrictor effect of exogenous SP was first reported in 1977 in guinea pigs and cats. Since then, SP was shown to cause a dose-dependent bronchoconstriction in various animal species, both in vitro as in vivo [87–89]. SP contracts human bronchi and bronchioli, but is less potent in its actions than histamine or acetylcholine [88, 90]. NKA instead is a more potent constrictor and is, on molar base, 2–3 orders of magnitude more potent than histamine or acetylcholine. NKB does not exert a contractile activity on human airways [91]. Tachykinins have been found to be involved in antigen-induced bronchoconstriction and enhanced bronchial responsiveness in various animal models. Several studies have reported that exposure of guinea pigs to an aerosol of either capsaicin- or SP-elicited airway hyperresponsiveness to bronchoconstrictor agents. Intra-airway application of SP has been shown to induce airway hyperresponsiveness in guinea pig, which was not associated with changes in the number of eosinophils or eosinophil activation status in vivo, suggesting that SP does not mediate its effect by activation of eosinophils [92]. Furthermore, it has been shown that capsaicin-induced excitatory NANC neuropeptide depletion resulted in a complete inhibition of the ovalbumin-induced airway hyperresponsiveness in guinea pigs [93, 94], while the presence of eosinophils was still evident in these animals after allergen challenge. Similar findings were obtained after a viral infection [95]. Furthermore in the same line, IL-5-induced airway hyperresponsiveness to histamine, but not the eosinophilia, was completely blocked by pretreatment of animals with a tachykinin NK receptor antagonist.

Muscarinic Receptors in Asthma

Asthma is a disorder associated with increased activity of the parasympathetic nervous system, increased basal tone, and increased bronchoconstriction. The use of anticholinergic drugs inhibits increased tone and decreases airway hyperreactivity in patients with asthma and COPD. The parasympathetic nerves are the principal nerves that provide autonomic innervation to the airways (Fig. 6). All the effects of the parasympathetic nerves are mediated by muscarinic receptors located on airway smooth muscle, submucosal glands, blood vessels [96], and nerves [97]. The parasympathetic nerves maintain airway tone [98–100], contract airway smooth muscle [98], stimulate mucus secretion from submucosal glands [101, 102], and dilate blood vessels [103]. Acetylcholine is the prime parasympathetic neurotransmitter in the airways responsible for inducing airway smooth muscle contraction, bronchoconstriction, and mucus secretion. The release of acetylcholine from the parasympathetic nerves is controlled by muscarinic auto-receptors located on the nerves. Acetylcholine acts on both muscarinic and nicotinic receptors and the release of acetylcholine and its effects on the muscarinic receptors mediates hyperresponsiveness and also mucus production in the airways during inflammatory disorders like asthma and COPD. Asthma and chronic

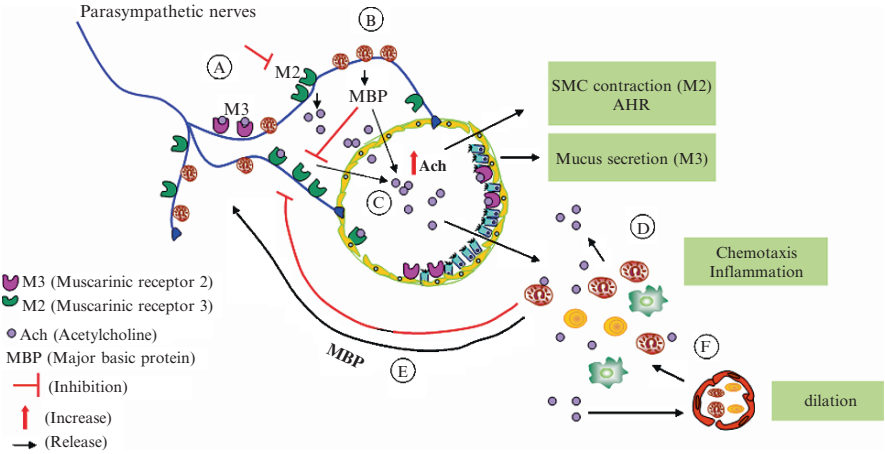

Fig. 6 The parasympathetic nerves provide autonomic innervation to the airways and its effects are mediated by muscarinic receptors located on nerves, structural cells, blood vessels, and inflammatory cells. The excessive release of acetylcholine is responsible for inducing airway smooth muscle contraction, bronchoconstriction, mainly via the M2 muscarinic receptors, and mucus secretion via the M3 (**A**, **C**, **D**) during inflammatory disorders like asthma and COPD. Eosinophils are present along and within the nerve bundles, ganglia, and along the nerve fibers in the smooth muscle. Eosinophils release proteins like major basic protein (MBP) that can potentially block the M2 muscarinic receptor thereby inhibiting agonist binding (**B**, **E**). The inhibitory M2 receptors are dysfunctional in asthma. Decreased neuronal M2 receptor function increases release of acetylcholine from the nerves (**A–C**). Loss of the M2 receptor function causes hyperresponsiveness via increased release of acetylcholine and increased contraction of smooth muscle. The M3 receptor mediates smooth muscle contraction (**C**). The increased acetylcholine is also suspected to mediate chemotaxis of inflammatoty cells in the airways (**D**, **F**)

obstructive pulmonary disease (COPD) are associated with increased parasympathetic tone. Figure 6 illustrates the release of acetylcholine and its modulation during asthma and the regulation of the muscarinic receptors. Among the five subtypes of muscarinic receptors (M1–M5), the M1, M2, and M3 exert physiological effects in the airways. Basal airway tone is increased in patients with asthma. The muscarinic receptor regulation of airway smooth muscle tone is asthma and COPD via increased smooth muscle contraction and due to increased release of acetylcholine.

Expression of Muscarinic Receptors in the Lung

Autoradiographic mapping studies have indicated that muscarinic receptors are predominantly localized to airway smooth muscle, vascular endothelium, submucosal gland cells, and neuronal structures, although in some species, including humans, there is also localization to alveolar walls [104–106]. Ml-receptor mRNA has shown to be localized in alveolar walls and receptor mapping studies also indicate their localization in submucosal glands of the larger airways in humans. M2 receptor has been detected

in human airway smooth muscle cells and they are predominantly expressed compared to others [107, 108]. Binding studies in guinea pig and human lung membranes indicate the presence of M3 receptors. Autoradiographic studies have demonstrated M3 receptors in airway smooth muscle of large and small human airways and also the submucosal glands in human airways. M3 receptors are also expressed on airway epithelial cells and have been found in human nasal biopsies. They are also localized to endothelial cells of the bronchial circulation and presumably mediate the vasodilator response to cholinergic stimulation of the proximal airways. The M3 muscarinic receptors mediate mucus, water, and electrolyte secretion [109–111] and is the primary subtype responsible for bronchial and smooth muscle contraction. This has also been shown in the M3 receptor knockout mice where both methacholine and vagally induced bronchoconstriction are lost [112]. The M2 receptor has been indicated to mediate a less-prominent role in peripheral airway smooth muscle contraction. The M1 receptors appear to contribute to the regulation of water and electrolyte secretion [109–111]. In asthma, the expression of the M3 muscarinic receptors on airway smooth muscle is not altered by antigen challenge or viral infection, the M2 muscarinic receptors on parasympathetic nerves is, however, decreased. Decreased neuronal M2 muscarinic receptor function increases the concentration of acetylcholine released onto the smooth muscle, and results in an enhanced bronchoconstriction to vagal nerve stimulation.

Airway Smooth Muscle Contraction

Besides the parasympathetic nervous system, acetylcholine is also released from non-neuronal cells like bronchial epithelium and inflammatory cells [113]. Within the smooth muscle, the greatest density of muscarinic receptors is in the lower trachea with more M2 than M3 receptors in some species [96, 114, 115]. However, it is the M3 receptor that mediates smooth muscle contraction. The M3 muscarinic receptor has the capacity to activate multiple signaling pathways in various cell types [116], and the activation of phospholipase C (PLC) via intermediary heterotrimeric G protein Gq is the predominant pathway that brings about ASM contraction [117, 118]. The inhibitory M2 receptors are dysfunctional in asthma. Decreased neuronal M2 receptor function increases release of acetylcholine from the nerves. Loss of the M2 receptor function causes hyperresponsiveness via increased release of acetylcholine and increased contraction of smooth muscle (Fig. 6).

Mucus Secretion

Mucus secretion, a pathological feature of asthma contributing to airflow limitation is under cholinergic control. The M3 receptor is the primary mucus-secreting receptor and acetylcholine via the muscarinic receptors is responsible for mucus secretion [119]. Animal models have revealed that repeated administration of

muscarinic agonists like pilocarpine and methacholine can promote goblet cell hyperplasia and mucus gland hypertrophy [119]. Mucus production is largely vagally mediated and airway inflammation leading to increased acetylcholine therefore regulates mucus secretion.

Inflammation

Increasing evidence suggests the presence of acetylcholine and its synthesizing enzyme (ChAT) in inflammatory cells like eosinophils, lymphocytes, macrophages, mast cells, and neutrophils. Besides inflammatory cells, the primary structural cells involved in asthma, the epithelial, endothelial, and smooth muscle cells also localize them, which may serve as chemotactic release factors for inflammatory cells. Bronchial epithelial cells have been reported to release eosinophil, monocyte, and neutrophil chemotactic factors by acetylcholine and also the release of GM-CSF via nicotinic receptors [120]. Furthermore, muscarinic receptors are also expressed in several inflammatory cell types [121]. Elevated levels of acetylcholine have been seen in skin biopsies of atopic dermatitis patients. All five types of muscarinic receptors, although with individual variability have been detected in lymphocytes. Muscarinic agonists lead to increase in cytosolic Ca^{2+} in T and B cells in response to atropine. Another muscarinic agonist, oxotremorine, increases the expression of c-fos mRNA, key to the transcription of IL-4, IL-5, and GM-CSF, and which in turn activates c-fos transcription. This may provide one mechanism of perpetuating the asthmatic inflammatory process [122–125]. Also phytohemaglutinin, a T cell activator, has been reported to increase ChAT mRNA and muscarinic M5 expression in stimulated mononuclear leukocytes. T cells are prime players for eliciting a predominant Th2 type of response in asthma. Furthermore, both infiltrating and structural cells play a key role in mediating the outcome of asthma. Therefore, more studies in this aspect would be a key to understand the role of non-neuronal acetylcholine and the muscarinic receptors in immunological diseases like asthma. A study among smokers and patients with COPD revealed a regulated expression of muscarinic receptors on macrophages and neutrophils [126]. Such a study in asthmatic patients would reveal if muscarinic receptors are also regulated in specific inflammatory cells.

Airway Hyperreactivity and Muscarinic Receptors

Airway hyperresponsiveness, characteristic of asthma is thought to result from increased contraction of the airway smooth muscle. The excessive release of acetylcholine results in increased hyperresponsiveness due to excessive contraction of the airway smooth muscle. Airway hyperreactivity is clearly associated with loss of neuronal M2 muscarinic receptor function. Neuronal M2 muscarinic receptors

no longer respond to muscarinic agonists in antigen-challenged guinea pigs [127]. Protection or restoration of M2 muscarinic receptor function prevents or acutely reverses airway hyperreactivity, respectively. Respiratory viral infections further increase baseline airway resistance and bronchial reactivity in asthmatics. These decreases in airway function can be prolonged and often persist beyond the period of clinical illness [128]. The mechanisms by which viruses induce bronchoconstriction and airway hyperreactivity are not fully understood, but may, in part, be due to activation of the parasympathetic nervous system. Bronchial hyperreactivity induced by respiratory viral infections in normal subjects is blocked by atropine [129, 130], indicating an increase in cholinergic activation after viral infection. Animal models of asthma confirm that increased parasympathetic drive underlies airway hyperreactivity. Antigen challenge of sensitized animals causes an immediate bronchoconstriction followed by hyperreactivity to stimuli such as electrical stimulation of the vagus nerves or histamine [127]. Vagotomy or vagal blockade abolishes antigen-induced hyperreactivity [131–134]. Loss of M2 muscarinic receptor function in antigen-challenged guinea pigs is mediated by eosinophils. Depletion of eosinophils with an antibody to IL-5 [135] or inhibition of eosinophil migration into lungs with an antibody to very late activation antigen-4 [136] prevents antigen-induced M2 muscarinic receptor dysfunction and hyperreactivity. The eosinophils adhere to the nerves via an interaction between CD11/18 on the eosinophils and intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 on nerves [137]. Glucocorticosteroids protect M2 receptor function and prevent airway hyperreactivity, not by inhibiting the influx of eosinophils to the lung, but by preventing eosinophils from associating with nerves [138]. Since M3 receptor expression and functions are not changed on the airway smooth muscle of patients with asthma, hyperresponsiveness could be as a result of increased release of acetylcholine from the vagus nerves.

Activated Eosinophils

Eosinophils are the prime inflammatory cells in asthma and are recruited to the airway nerves in patients dying of acute asthma. They are present along and within the nerve bundles, ganglia, and along the nerve fibers in the smooth muscle [139]. They release preformed proteins, like eosinophil cationic protein, eosinophil peroxidase, major basic protein (MBP). Such positively charged proteins can potentially block the M2 muscarinic receptor by binding to an allosteric site and thereby inhibiting agonist binding [140]. Eosinophil MBP is a selective, allosteric antagonist for M2 muscarinic receptors and has been shown to selectively displace labeled antagonist from the M2 muscarinic receptors in a dose-dependent manner [141]. Airway dysfunction and hyperreactivity in antigen-challenged guinea pigs can be prevented by depleting eosinophils prior to antigen challenge by anti-IL-5 or even eosinophil migration with antibodies to the very late antigen-4. Airway dysfunction and hyperreactivity are prevented by blockade of MBP despite infiltration of eosinophils in

the lung [142] and reversed by the polyanionic molecule, heparin [143]. The effect of heparin on M2 receptor function was tested in antigen-challenged animals and the administration of heparin acutely restored M2 receptor function in antigen-challenged guinea pigs and rats [143]. Therefore, loss of neuronal M2 muscarinic receptors is potentially due to MBP released from eosinophils. Tachykinin antagonists are also known to protect the function of the M2 receptors and prevent airway hyperreactivity in antigen-challenged guinea pigs [139], but they most likely do so by inhibiting eosinophil degranulation [142] rather than by inhibiting eosinophil migration into the lungs or nerves or via a direct effect of tachykinins on the parasympathetic nerves.

Conclusion

Accumulating evidence suggests a strong role for neurotrophins like NGF in the pathogenesis of asthma. NGF has been repeatedly shown to be upregulated in allergic diseases and the local NGF upregulation is detected in the airways of asthmatics, where it contributes to airway inflammation and bronchial airway hyperresponsiveness. NGF, as a target has a possibility to be modulated by anti-inflammatory agents in the pathogenesis of asthma. In line with this, glucocorticoids have been shown to be effective in decreasing NGF secretion from airway cells. A selective antagonist for TrkA is therefore underway for development in several laboratories. The TrkAd5 is a small globular protein that binds NGF with pico-molar affinity and therefore may be used therapeutically to sequester NGF. The effects of TrkAd5 were investigated in a number of preclinical models of inflammatory pain and also asthma [144]. Similarly, a number of tachykinin receptor antagonists have been described to downregulate typical symptoms of asthma like bronchoconstriction [145], antigen-induced plasma extravasation [146], bronchial hyperresponsiveness [147], eosinophils, expression of both Th1 and Th2 cytokines, and late airway responses. At present not much is known whether tachykinins and their receptors play a role in allergen-induced changes in human airways. However, a few NK1 antagonists have been tested in asthma. The first clinical study of an NK2 tachykinin receptor antagonist in asthmatics was reported recently by Van Schoor et al. [148]. Acetylcholine, the prime parasympathetic neurotransmitter in the airways is responsible for inducing airway smooth muscle contraction, bronchoconstriction, and mucus secretion in asthma. The release of acetylcholine, and its receptors and airway function are modulated in asthma and COPD, suggesting that the effects of acetylcholine could contribute significantly to the pathophysiology of these obstructive airways diseases. Recent clinical and experimental findings support this hypothesis, suggesting that anticholinergics have the potential to reduce airway and lung function decline in addition to its effects as a bronchodilator.

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Neural Regulation of the Immune Response

Paul Forsythe and John Bienenstock

Introduction

The nervous and immune systems are the major adaptive systems of the body and are charged with maintaining homeostasis. It is now established that these two systems collaborate through intricate bidirectional interactions to facilitate the fine control required to maintain the complex dynamic equilibrium of the internal milieu [1–3]. Disruption of these regulatory systems can result in immunodeficiency, which can lead to infection and cancer, or an excessive immune response causing disorders such as rheumatoid arthritis, Crohn's disease, atherosclerosis, diabetes, Alzheimer's disease, multiple sclerosis, and asthma [1, 4].

There are two major pathways by which the CNS acts peripherally to regulate the immune system: the first is the hormonal response, mainly through the hypothalamic–pituitary–adrenal (HPA) axis, but also involving the hypothalamic–pituitary–gonadal (HPG), the hypothalamic–pituitary–thyroid (HPT), and the hypothalamic–growth-hormone axes [5–8].

The HPA axis is activated during many bacterial and viral infections, resulting in the release of corticotrophin releasing hormone (CRH) from the hypothalamus, which stimulates the expression and release of adrenocorticotropic hormone (ACTH) from the pituitary, inducing the secretion of corticosteroids from the adrenal cortex. Both CRH and ACTH can modulate immune cells directly although their major in vivo effects are exerted through interaction with other hormones and components of the immune system. Glucocorticoids are the main effectors of the neuroendocrine system and, through the glucocorticoid receptors, have multiple effects on immune cells [9–12]. Glucocorticoids are essential for normal immune function. In particular, they modulate the proliferation and survival of T cells and cause

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a shift in cytokine production from a Th1 to Th2 profile [11, 12]. There is also evidence that the HPA axis plays a major role in stress-induced suppression of host defenses [13].

The second major pathway through which the CNS can direct the immune response is the autonomic nervous system (ANS) [1, 14, 15]. While neuroendocrine mechanisms act systemically for sustained regulation of inflammation and in particular to protect against the deleterious effects of the pro-inflammatory mediators, neural regulation, in contrast, is fast-acting, discrete and localized in tissues where injury or infection typically originates. Thus, the nervous system is ideally suited to check immune activation at the crucial early stages of an emerging response. In addition, neural regulation of biological functions is short-lived, allowing for fine control of the immune response. After a short refractory period, responding cells can resume function as required in the absence of further neural input. This allows for precise control of immune cells and permits local inflammatory responses to be mobilized during persisting threat or infection.

The nervous system is composed of sensory systems (which detect the state of the body and organs) and motor systems (which transmit signals to the body and organs). While the somatic motor system controls voluntary movements, the autonomic motor system controls visceral body functions and innervates glands. The autonomic nervous system has two principal divisions, the sympathetic (noradrenergic) and parasympathetic (cholinergic) systems. These systems originate in the CNS with cell bodies in the brain stem and spinal cord, and can act in synergy or in opposition to mediate basic physiological responses.

All divisions of the nervous system (sympathetic, parasympathetic, and sensory) utilize intimate associations with inflammatory cells to regulate immune responses. Such associations have been documented in lymphoid organs and in tertiary sites such as the skin and mucosal surfaces [16–19]. During adaptive immune responses, signals from the brain are transmitted back to the periphery, primarily via activation of the sympathetic nervous system (SNS) (and HPA) [1], while in the case of peripheral inflammation, the vagus (parasympathetic) nerve transmits signals from the brain to the periphery via the neurotransmitter acetylcholine [20, 21]. In addition, peripheral sensory nerves regulate immunity locally, at sites of inflammation, through neuropeptides such as substance P (SP), calcitonin gene-related peptide (CGRP), and vasoactive intestinal polypeptide (VIP) [22, 23]. What follows is an overview of immunoregulation by the major components of the ANS and the implications of such control mechanisms in health and disease.

Sympathetic Control of Immune Responses

Sympathetic nerve fibers are found in the parenchyma of primary and secondary lymphoid organs innervating the bone marrow, thymus, splenic white pulp, and lymph nodes [16, 17]. Within the secondary lymphoid tissues, sympathetic nerve endings are found primarily in areas adjacent to CD4+ T cells, and in the vicinity

of macrophages and B cells within the marginal sinus [17, 24]. In vivo studies in which the SNS is ablated by chemical sympathectomy or interrupted surgically by cutting sympathetic nerve innervation of lymphoid organs indicate that the SNS has an important role in regulating immunity at a regional level.

Sympathetic regulation of immune responses occurs principally through the interaction of noradrenaline and adrenaline with two principal receptor classes, the α- and β-adrenoreceptors (AR). α-AR are subdivided into subtypes 1, 2, and 3, whereas β-AR are subdivided into types 1 and 2, each of which comprise additional subtypes [25, 26]. Immune cells are equipped to respond to adrenergic signaling from the network of nerve terminals within the primary and secondary lymphoid organs as, with the notable exception of T helper (h) 2 cells, virtually all lymphoid cells express AR [14, 27–29].

While noradrenaline (NA) is the principal mediator of sympathetic postganglionic neurons, the sympathetic varicosities store additional mediators including adrenaline, neuropeptide Y (NPY), β-endorphin, and adenosine triphosphate (ATP) [30]. These transmitters are co-released and while each can individually alter immune cell function, they also act synergistically or antagonistically to modulate immune responses.

Sympathetic Control of Adaptive Immunity

Response to Antigen

Introduction of antigen is associated with the release of norepinephrine from the sympathetic nerve terminals located within lymphoid tissue. However, while systemic infection, lipopolysaccharide (LPS), or infectious microorganisms increase the rate of NA release in the spleen during the first 15–25 min of exposure [31, 32], the antigen-specific release of NA takes between 9 and 18 h following immunization [33]. This suggests that antigen does not directly activate the SNS, but requires uptake, processing, and presentation to activate immune cells to produce cytokines. These cytokines then communicate with the brain or peripheral nervous system, which in turn activate the SNS to release NA that in turn regulates the activity to the immune cells. Evidence that the brain is involved in regulating NA release following immune challenge is indicated by the fact that the increase in sympathetic nerve activity following antigen exposure is partially prevented by pharmacologically blocking nerve transmission from the brain to the periphery [33].

T Cell Regulation

One of the early indications that sympathetic signaling may modulate T cell function was provided by Madden et al., who demonstrated that the contact sensitivity

response is decreased in mice that are depleted of NA either prior to or following sensitization, when compared to nondepleted mice [34]. The decreased response was due to a decrease in T cell reactivity, suggesting that NA was needed for the development and/or progression of the T helper (Th) 1 cell-mediated immune response. However, when two different strains of mice, C57Bl/6J (Th1 cell-slanted strain) and Balb/c (Th2 cell-slanted strain), were depleted of NA and immunized 2 days later with the T cell-dependent antigen KLH (keyhole limpet hemocyanin), splenic cells from both strains of mice produced more Th1 and Th2 cytokines [35], suggesting that NA exerts a suppressive effect on Th1/Th2 cell development and/or progression.

The release of Interferon-γ (IFN-γ) by Th1 cells is critical for maintaining our resistance to infectious organisms as well as for controlling susceptibility/resistance to autoimmune disease development and progression. NA-deficient mice are more susceptible to the Th1-promoting pathogens, *Listeria monocytogenes*, and mycobacterium tuberculosis and produce significantly less IFN-γ, suggesting that NA participates in the optimal protective Th1-mediated immune response in vivo [36]. In vitro studies demonstrated that NA exerts an effect during naive T cell differentiation that increases the amount of IFN secreted by the Th1 cells that develop [37]. Exposure of Th1 cells to NA or a β2AR-selective agonist before their activation has been shown to decrease both IL-2 and IFN-γ production, while stimulation either at the time of or after cell activation appeared to be either without effect or induced an increase in IFN-γ, respectively [37, 38]. Studies have also shown that mice deficient in the Y1 NPY receptor are resistant to dextran sulfate sodium (DSS)-induced colitis [39] and develop reduced delayed-type hypersensitivity (DTH) to methylated bovine serum albumin [40], which are two models of Th1-mediated inflammation. The altered phenotypes were associated with decreased levels of IFN-γ, indicating a role for Y1 signaling in Th1 induction. Taken together, these studies suggest that SNS exerts an effect on early naive CD4+ T cell development into a Th1 cell, the commitment to becoming a Th1 cell, and/or the amount of IFN-γ secreted by the effector Th1 cell.

In vitro exposure of human peripheral blood mononuclear cells (PBMC) to NA or a β2-AR agonist induces a decrease in IFN-γ production, but an increase in (Interleukin-4) IL-4 and IL-10 [41], thus suggesting that NA can cause a shift to a Th2 cytokine environment. Given that murine Th2 cells do not appear to express the β2-AR, it is unlikely that the SNS influences Th2 cells directly, but may modulate a Th2 response through a combination of controlling Th2 development from naive T cells and indirectly through stimulation of β2-AR on macrophages or dendritic cells (DC). In support of this, it has been shown that mice receiving thermal burn injury have increased plasma NA levels, which is associated with increased chemokine production by macrophages, which in turn promotes effector cells to predominate a Th2-like response [42, 43]. In addition to stimulating their expected cytokine patterns, NPY has also been shown to induce the secretion of IFN-γ by Th2 cells and IL-4 by Th1 cells. However, when these cells were stimulated in the presence of the specific antigen and syngeneic APC, NPY elevated IL-4 in Th2 cells and inhibited IFN-γ in Th1 cells [44]. Thus, NPY induces a Th2 shift under in vitro conditions that mimic physiological circumstances of antigen stimulation.

B Cell Regulation

NA exerts an enhancing effect on B cell antibody production when B cells and Th cells are exposed to Th cell-dependent antigens [45, 46]. One mechanism for this enhancement may involve a β2-AR-induced increase in the frequency of B cells differentiating into Ab-secreting cells. The differential effect of NA on Th1 and Th2 cytokine production may also play an important role in modulating B cell function. IFN-γ-producing Th1 cells induce B cells to produce immunoglobulin G (IgG)2a (in humans, IgG1), whereas IL-4-producing Th2 cells induce B cells to produce IgE and IgG1 (in humans, IgG4) [47]. Thus, in studies by Sanders et al. [46], the inhibition of IFN-γ production by Th1 cells induced by the β2-AR agonist terbutaline was associated with subsequent suppressed IgG2a production by mouse B cells. In addition, the β-AR agonists, salbutamol and fenoterol, potentiate IL-4-induced IgE production by human PBMC [48], while Wheway et al. [40] observed that Y1-deficient mice had reduced numbers of B cells and a defect in isotype switching to IgG2a normally associated with Th1 responses. The enhancement of antibody production, specifically of IgE by β-AR agonists [48], is suggestive that the SNS can act to inhibit cellular immunity and while potentiating humoral responses.

Sympathetic Control of Innate Immunity

While much research on the SNS modulation of immune responses has focused on the effects on adaptive immunity and antibody production as outlined above, less is known about adrenergic effects on innate immunity.

Activation of the SNS that occurs following stressful stimuli, such as exercise and psychological stress, is associated with decreased natural killer (NK) cell activity and occurs through a mechanism involving modification of NK cell receptor ligation to target cells, blockade of NK cytokine secretion required for NK maturation, and inhibition of target-induced activation of the cytoxic mechanisms [49]. NA inhibits phagocytosis and the release of lysosomal enzymes by neutrophils [50]. Furthermore, the superoxide generation and formation of oxygen radicals that play an important microbicidal role are both suppressed at by adrenaline, an effect prevented by β2-AR blockade [51, 52].

In vitro, NA mediates its immunosuppressive effects on DC and monocytes by inhibiting the production of pro-inflammatory cytokines, including TNF, IL-1, IL-6, and IL-12, while upregulating the production of anti-inflammatory cytokines such as IL-10 [53]. Given that the phagocytic capabilities of DC are predominantly dedicated to acquiring antigen, the demonstration that DC from NPY Y1 receptor (Y1R)-deficient mice display decreased phagocytosis [40] suggests that antigen presentation in the absence of the Y1R is blunted. As DC operate at the crossroads between innate and adaptive immune functions and are inducers of adaptive immunity, it has been proposed that the effects of the SNS on DC might also contribute

to the clearance of pathogens and modulation of the type and strength of the adaptive response. However, as depletion of noradrenaline has been shown to reduce resistance to some bacterial infections, the role of SNS responses in bacterial host defense remains to be determined.

Comment

It is clear that the sympathetic nervous system constitutes a major integrative and regulatory pathway between the brain and the immune system. At local sites of action the SNS has the potential to exert pro- or anti-inflammatory effects. For example, in an adjuvant-induced model of arthritis, β-agonists were found to be pro-inflammatory in the early asymptomatic stage, but anti-inflammatory in the later symptomatic phase [54]. Chemical sympathectomy had similar time-dependent effects in antigen-induced arthritis [55]. The seemingly contradictory effects of sympathetic signaling may be influenced by several factors, such as the presence or absence of antigen, the nature of antigen and/or the relative expression of particular receptor subtypes on the surface of immune cells (e.g., β2- versus α2-adrenergic receptors), the stage of activation/differentiation of the cell, or the presence or absence of a particular receptor (e.g., β2-ARs on Th1 but not on Th2 cells). Given the importance of such factors, further studies are required to precisely map the distribution and type of adrenoreceptor expressed on lymphoid cells and to understand their coupling to intracellular pathways according to their stage of maturation, differentiation, and tissue localization. Such studies may provide new pharmacologic tools that allow for precise tailoring of inflammatory responses.

Peptidergic Nerves

The peripheral sensory nerves that are involved in pain, touch, and temperature perception regulate inflammation locally through the release of neuropeptides. Peripheral neuropeptides that are known to regulate inflammation include SP, CGRP, and VIP. It has long been accepted in clinical dermatology and gastroenterology that peptide mediators released from cutaneous c-fibers and enteric nervous system respectively give rise to neurogenic inflammation and underlie a range of conditions, including dermatitis, psoriasis, eczema, Crohn's disease, and colitis. The stimulation of peripheral nerves by innate immune mediators results in the characteristic features of local inflammation, including vasodilation, vascular leakiness, edema, and pain. Collateral c-fiber axons provide an efferent route for inflammatory cues to spread laterally from afferent terminals in the form of a local reflexive arc, termed the "axon reflex."

Neurogenic inflammation is a major component of allergic reactions both in the airways [56] and in the skin [57]. c-fibers are intimately associated with mast cells throughout the body and stimulate the release of histamine, further aggravating inflammatory conditions. Antagonists to these neuropeptides generally reduce inflammation at inflamed sites, while cutting peripheral nerves tends to reduce inflammation that is distal to the site of the nerve lesion.

Neuropeptides such as VIP, cholecystokinin (CCK), SP, and CGRP are also found in nerve terminals innervating primary and secondary lymphoid organs [58]. Upon neuropeptidergic stimulation and without any additional stimulatory molecule, CGRP stimulates Th1-nonactivated T cells to secrete IL-4, a typical Th2 cytokine, while SP stimulates IL-4 in antigen-activated Th1 cells [59]. Such findings imply that neuropeptides can cause specific subsets of T lymphocyte to alter their cytokine secretion.

Substance P

Substance P is an 11 amino acid peptide released by peripheral nerves that mediate pain perception. SP binds with highest affinity to the G-protein-coupled neurokinin-1 (NK1) receptor [60]. In addition, SP can also activate cell signaling through receptor-independent activation of G-proteins [61]. SP is generally pro-inflammatory and stimulates secretion of TNF, IL-1, IL-2, and IL-6 from macrophages and T lymphocytes in vitro [62, 63]. In addition, SP has been shown to stimulate atypical cytokine release from Th cells lines, inducing IFN-γ secretion by a Th2 cell line and in combination with antigen induces IL-4 release from Th1 cells [59]. Furthermore, SP induces the release of inflammatory mediators from mast cells, including histamine and serotonin [19, 64, 65], and therefore contributes to vascular leakiness and edema at sites of inflammation. Furthermore, SP receptor antagonists have been shown to be effective as anti-inflammatory agents [66, 67]. SP also appears to have a stimulatory effect on immunoglobulin secretion when coactivated with other factors [68–71].

Vasoactive Intestinal Peptide

Vasoactive intestinal peptide (VIP) is a 28 amino acid peptide that exerts its action on cells through two G-protein-coupled receptors, VPAC1 and VPAC2 [72]. VIP inhibits the production of pro-inflammatory cytokines, such as IL-2, IL-6, IL-12, and TNF, by PBMCs, monocytes, and T cells [73–77] and stimulates macrophage production of the anti-inflammatory cytokine IL-10 [74]. VIP also enhances the ability of macrophages and immature dendritic cells to stimulate Th2 cells through an increase in expression of the co-stimulatory molecule B7.2 (CD86) [75]. However, LPS-activated macrophages and dendritic cells express less B7.1 and B7.2 following exposure to VIP, resulting in decreased co-stimulatory ability of these APC [78, 79]. VIP can also increase production of regulatory T cells (Tregs). VIP treated DC-induced

IL-10 and TGFβ, producing Tregs in vitro [80], while in vivo, injecting TCR transgenic mice with VIP leads to increased production of Foxp3 positive Tregs [81, 82]. The effect of VIP on immunoglobulin production by B cells is a prime example of how the effects of neuropeptides can differ depending on dose, tissue site, and co-stimulatory signals. VIP can also control IgA synthesis in the GALT, increasing IgA while decreasing IgG levels in isolated human intestinal lamina propria cells [83]. This effect appears dose-dependent as continuous infusion of rats with high doses of VIP for 96 h decreased IgA-producing cells, but not IgG- and IgM-producing cells in the lamina propria [84]. VIP in combination with CD40, but not VIP or CD40 alone, stimulates IgA production by human IgD B cells and resting B cells, without affecting IgM, IgG, and IgE levels [85]. In pre-B cells derived from bone marrow, VIP with CD40 slightly increases IgA and IgM, but not IgG levels [86]. This response is further enhanced by IL-7 but blocked by VIP antagonist. These effects may be organ-specific as VIP increases IgA, but not IgM levels in cells isolated from mesenteric nodes and the spleen, while reducing IgA and increasing IgM levels in concavalin A-stimulated mononuclear cells of Peyer's Patches [68].

Calcitonin Gene-Related Peptide

CGRP is a 37 amino acid neuropeptide that mediates its effects through G-protein-coupled receptors and is expressed predominantly in sensory nerve fibers. CGRP has been reported to both increase and suppress cytokine production by T cells and peripheral blood mononuclear cells (PBMC) [87, 88], suppressing IL-2 production from T cells in vitro, but stimulating IL-6 and TNF release from human PBMCs after LPS administration in vitro. CGRP inhibits the concavalin A- and phytohemagglutinin-induced proliferation of murine nodal and splenic T lymphocytes dose-dependently [89] and at concentrations as low as 10^{11} M inhibits pre-B cell colony formation and their surface immunoglobulin expression through IL-7 [90]. Granstein and colleagues demonstrated that CGRP inhibited antigen presentation by Langerhans cells in human epidermis [91], and subsequently demonstrated that CGRP played a role in the regulation of cutaneous immunity and could inhibit delayed-type hypersensitivity (DTH) in mice [92]. CGRP also has direct effects on DC, inhibiting their activation, resulting in reduced expression of MHC class II and co-stimulatory molecules, and reduced production of IL-12, thereby impairing their ability to activate T cells [93].

Neuropeptides and Airway Inflammation

Neuropeptides, in particular SP, have been demonstrated to play an important role in mediating airway inflammation and are suggested to be involved in the pathogenesis of asthma.

In the lung, sensory nerve fibers are strategically placed just below the epithelial surface so that any change in the bronchial environment may stimulate the release of the neuropeptides. The bronchial associated lymphoid tissue (BALT) plays an important role in seeding the airways with IgA-producing B cells, thereby playing an important role in airway mucosal immunity [94]. A common feature of BALT is innervation by a variety of nerve fibers including those that contain tackykinins, CGRP, catecholamines, NPY, and various opioids [95, 96]. There is little known about the effect of nerve stimulation on IgA production of the airways, but in the gastrointestinal system, evidence supports a role for neuroregulation of IgA production. SP and somatostatin enhance IgA production while catecholamines reduce it [97]. The airway epithelium contains numerous dendritic cells as well as a rich supply of afferent fibers. The apposition of nerve fibers and dendritic cells suggests the possibility that the nervous system may regulate antigen presentation and other aspects of dendritic cell function [98]. Neuronal activation can lead to the degranulation of mast cells and the influx of neutrophils, thereby recruiting components of innate immunity to the area [99–101].

During respiratory syncytial virus (RSV) infection, stimulation of these nerves causes a marked increase in airway vascular permeability over that in pathogen-free rats and results in an increase in overall inflammatory status. These changes appear to be mediated by the NK1 receptor, the expression of which is greatly increased by RSV [102, 103]. There is also a high level of expression of the NK1 receptor on T lymphocyte subpopulations within the bronchial-associated lymphoid tissue of RSV-infected rats. As a consequence, stimulation of the sensory nerves by any airborne irritant has the potential of causing a new inflammatory cycle mediated by NK1 receptor-expressing T lymphocytes attracted into the airways and activated by SP [102, 103]. This mechanism may establish important neuroimmune interactions, which undergo long-term dysregulation after RSV infection and predispose to airway inflammation and hyperreactivity. Furthermore, while substance P levels have been demonstrated to be increased in the airway of RSV-infected rats, CGRP levels were decreased and treatment with exogenous CGRP inhibited the development of airway inflammation and AHR in RSV-infected animals. These results suggest that an imbalance in sensory neuropeptides plays some role in RSV-induced airway dysfunction [104]. A recent study demonstrated that CGRP is markedly upregulated in the airways of atopic asthmatics challenged by inhalation of allergen-derived T cell peptides and suggested this neuropeptide is involved in the late-phase asthmatic response [105].

Parasympathetic Control (the Cholinergic Anti-inflammatory Pathway)

While investigations of neural control of the immune response have traditionally focused on the SNS and sensory nerves, more recent work by Tracey and colleagues [106–110] has highlighted the role of the parasympathetic pathway in immunoregu-

lation. Vagal fibers contain sensory and motor components that control diverse organ functions such as heart rate and digestion. In addition to these classic physiological functions, it is now clear that the vagus constitutes another hard-wired connection between the nervous and immune systems and functions as an anti-inflammatory mechanism in systemic and local inflammation.

Experimental activation of the cholinergic anti-inflammatory pathway by direct electrical stimulation of the efferent vagus nerve inhibits the synthesis of TNF in liver, spleen, and heart, and attenuates serum concentrations of TNF during endotoxemia [106, 110], while in contrast, vagotomy significantly exacerbates TNF responses to inflammatory stimuli and sensitizes animals to the lethal effects of endotoxin. Stimulation of either the right or the left cervical vagus nerves protects against the development of hypotension and inhibits serum TNF responses to ischemia reperfusion [107], while in a standardized carrageenan-induced model of experimental murine, arthritis vagus nerve stimulation inhibits the inflammatory response and suppresses the development of paw swelling, indicating that the cholinergic anti-inflammatory pathway can inhibit systemic localized inflammation specifically [111].

Experiments with α7 nicotinic acetylcholine receptor (α7nAChR) knockout mice revealed that in the absence of the α7nAChR, vagus nerve stimulation was ineffective at preventing TNF release in response to endotoxin, indicating that the α7 subunit is key to communication between the cholinergic nervous system and the immune system [110].

The α7nAChR is expressed on non-neuronal cells including macrophages, endothelial cells, dendritic cells, keratinocytes, and lymphocytes [112–114]. Exposure of human macrophages, but not peripheral blood monocytes, to nicotine or acetylcholine inhibits the synthesis of proinflammatory cytokines (TNF, IL-1, and IL-18) but not anti-inflammatory cytokines (such as IL-10). Macrophages also have a prominent role in mediating intestinal inflammation, and a recent study has identified these cells as the main target of the anti-inflammatory function of the vagus nerve in a murine model of inflammatory bowel disease [115]. While macrophages have been identified as the major source of TNF during endotoxemia, dendritic cells, endothelial cells, and lymphocytes also synthesize and release pro-inflammatory cytokines and are substantial contributors to the innate immune activation underlying inflammatory responses. In keeping with this, vagus nerves stimulation and cholinergic agonists significantly block endothelial cell activation and leukocyte recruitment during inflammation.

Recently, a functional connection between the vagus nerve anti-inflammatory activity and the spleen was identified as vagus nerve stimulation fails to inhibit TNF production in splenectomized animals during lethal endotoxemia [116]. Furthermore, administration of nicotine that mimics vagus nerve stimulation increases proinflammatory cytokine production and lethality from polymicrobial sepsis in splenectomized mice, indicating that the spleen is critical to the protective response of the cholinergic pathway [116].

Interestingly, Luyer et al. [117] have demonstrated that dietary fat has a role in modulating the cholinergic anti-inflammatory pathway. They found that administration of high-fat diet reduced circulating levels of TNF and IL-6 in rats subjected to hemorrhagic shock, a manipulation known to activate high blood cytokine levels. When this experiment was repeated in animals that had undergone vagotomy, the high-fat diet failed to decrease TNF and IL-6 as did the administration of nicotinic

receptor antagonists. This study also revealed a mechanistic role for cholecystokinin (CCK), a neuropeptide released after consumption of dietary fat that triggers several digestive functions including stimulation of gall bladder contraction and exocrine pancreas secretion, and activation of afferent vagus nerve signals that induce satiety. It has been suggested that this mechanism may play a role in helping suppress local inflammatory responses to commensal microbes. There is also evidence in both acute and chronic models of inflammatory bowel disease that the vagus signaling through the α7nAChR exerts a tonic anti-inflammatory effect on the intestine [118].

The discovery of the cholinergic anti-inflammatory pathway offers intriguing new possibilities for controlling inflammatory conditions. Surgically implanted vagus nerve stimulators have been successfully used in the treatment of epilepsy and vagus nerve stimulation is an approved treatment for depression [119]. Therefore it has been suggested that devices designed to specifically stimulate efferent vagal nerve fibers could be beneficial in the treatment of inflammatory disorders [21]. Available information on the distribution and function of the α7 subunit suggests that it can be selectively activated without altering ganglionic transmission or sympathetic activation. Thus, the α7nAChR also represents a promising target for pharmacological suppression of inflammation and studies using specific agonists have supported the suitability of such an approach.

Classical Conditioning of Immune Responses

Classical conditioning can be regarded as a form of learning, establishing the temporal or causal relationship between external and internal stimuli to allow for the appropriate preparatory set of responses before biologically significant events occur. Interest in conditioning of the immune response was sparked in part by the discovery by Ader and Cohen that an antibody response to antigen could be reenlisted in rats via a Pavlovian conditioning paradigm [120]. Since this key study, the conditionability of both cellular and humoral immune responses in laboratory animals has been well documented [121–126].

The experimental conditioning paradigm employs the pairing of a novel stimulus (conditioned stimulus, CS) with an immunomodulating drug (unconditioned stimulus, UCS). Upon reexposure to the CS alone, immune functioning is altered in a similar manner to that which occurs following actual drug administration. Whilst a number of different stimuli have been used as the CS, the most commonly utilized is the novel tasting saccharine solution. In addition to immunological effects, conditioned paradigms are associated with behavioral changes where animals learn to avoid the conditioned stimuli previously paired with the noxious or illness-inducing effects [122, 127].

Several reports have documented that the conditioned effects on immune functions are independent of the elevation in corticosterone levels [128, 129] and evidence suggests that during conditioning of immune responses, the CNS utilizes the close appositions between sympathetic nerve terminals and both lymphocytes and macrophages within lymphoid tissue, in particular the spleen [130, 131]. Studies have demonstrated that reduction of splenic NA following surgical denervation of the

spleen does not affect avoidance behavior indicating acquisition of the CS–UCS association, but completely abrogates the efferent phase of the response, i.e., conditioned reduction in splenocyte proliferation and IL-2 production [131]. While conditioned immunosuppression has been described in humans, a role for either the SNS or HPA axis in this response is unclear. Furthermore, nothing is known about the potential contribution of parasympathetic signaling and the cholinergic anti-inflammatory pathway to conditioned immune responses of either rodents or humans.

Classical conditioning phenomena have also been proposed to play a significant role in the pathogenesis of stress-induced allergic diseases, including asthma. Studies have shown that histamine, one of the most important bronchoconstrictive mediators, is released not only by the direct action of allergens, but also in response to a learned association in guinea pigs [124, 132, 133]. Furthermore, it has been shown that in immunized rats, secretion of mediators by mast cells can be conditioned using audiovisual cues [134]. In humans, the nasal tryptase release following dust mite exposure can be conditioned using novel taste CS [135]. Conversely, in a study of asthmatic children [136], a β_2 agonist bronchodilator inhalation (salbutamol) was paired with a vanilla odor twice a day for 15 days. Control subjects received only the bronchodilator or unpaired exposures to the odor. In conditioned children, reexposure to the odor alone increased pulmonary function. It was also reported that children accustomed to inhaler therapy showed a significant increase in pulmonary function in response to a placebo inhaler.

It has been suggested that one way in which conditioned responses may be of benefit is in combination with drug therapy with the aim of reducing the dose of medication required. However, in recent years there appears to have been limited progress in this potentially important area and it is clear that a greater understanding of the principles behind this form of learning is required before any potential clinical applications of behaviorally conditioned immunosuppression can be identified. In this regard, a recent study [137] utilizing cyclosporin A as an UCS and saccharin as a CS in rats demonstrated, by lesioning brain regions, that the insular cortex is essential to acquire and evoke this conditioned response of the immune system. In contrast, the amygdala seems to mediate the input of visceral information necessary at the acquisition time, whereas the ventromedial nucleus of the hypothalamus appears to participate within the output pathway to the immune system necessary to evoke the behavioral conditioned immune response.

Stress and Immunity

The term allostasis meaning literally “reestablishing stability through change” was coined to describe the short-term activation of the neuroendocrine and autonomic nervous systems to promote adaptation and survival during periods when the body is challenged physically or psychologically [138]. During allostasis, physiological systems operate at a higher or lower level than during “normal” homeostasis; providing allostatic responses are shut off when they are no longer needed; the body

is able to adapt to and survive the immediate challenge without suffering long-term consequences. However, if the same response systems are activated over a longer period of time or remain active when no longer needed, these adaptive changes lead to other consequences including receptor desensitization and tissue damage that may precipitate or exacerbate disease processes. This has been termed "allostatic load," and it refers to the price the tissue or organ pays for an overactive or inefficiently managed allostatic response.

In the periphery, the role of controlling adaptive stress response and minimizing allostatic load is shared primarily by the systemic sympathetic and adrenomedullary system (SNS) and the HPA axis (Fig. 1). At rest, these systems maintain homeostasis as major regulators of fuel metabolism, heart rate, blood vessel tone, and thermogenesis. When homeostasis is disturbed or threatened by internal or external challenges, both the SNS and HPA axis become activated, resulting in

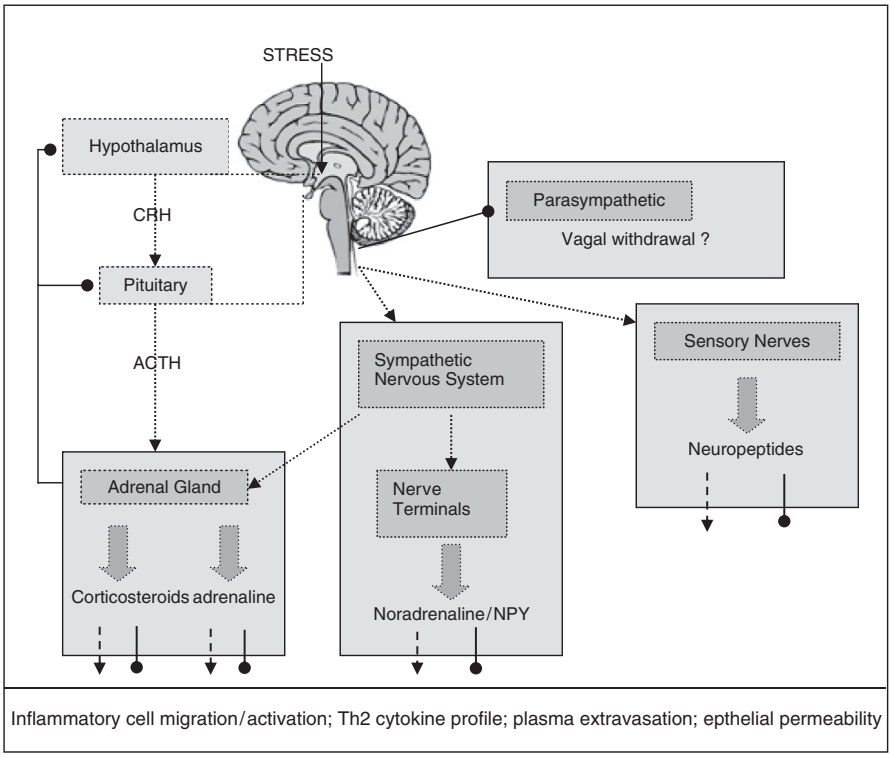

Fig. 1 The stress response (arrow indicates stimulation, circle indicates inhibition). Stress perceived by the brain leads to the activation of the HPA axis and sympathetic nervous system, which ultimately produces corticosteroids and noradrenaline, respectively. Additionally, stress can induce the release of neuropeptides from sensory nerves and may lead to vagal withdrawal and loss of the cholinergic anti-inflammatory pathway. Depending on the duration and intensity of stress exposure these responses exert numerous positive or negative effects on the body. See text for more detail on effects of individual mediators

increased peripheral levels of catecholamine and glucocorticoids that act in concert to keep the steady state of the internal milieu. Any immune challenge that threatens the stability of the internal milieu can be regarded as a stressor.

There is a substantial volume of literature demonstrating that psychological stress may influence inflammatory and immune cell trafficking, proliferation, and function, including cytokine and inflammatory mediator production [139–141]. It is generally considered that stressful experiences suppress the ability of the immune system to respond to challenge and thus increase susceptibility to infectious and neoplastic disease. However, while immune responses are blunted in humans experiencing excessive physical stress and in caregivers exposed to psychological stress, there is also evidence that stress can enhance immune function, suggesting a complex relationship between stress and the immune system [142].

Chronic stress promotes the development of glucocorticoid resistance, which has been associated with increased pro-inflammatory cytokine production and which may also release the SNS from inhibitory control, further promoting inflammatory activation, while less is known about the role of the parasympathetic nervous system in mediating stress-induced immunomodulation. The existence of the cholinergic anti-inflammatory pathways suggests that vagal withdrawal in response to stress might also promote inflammation [143]. Stress and depression have both been associated with reduced heart rate variability, while decreased vagal tone, as manifested by reduced heart rate variability, has been associated with increased inflammatory markers in women with coronary artery disease [144].

Chronic clinical stress has been shown to play an important role in disease, usually resulting in exacerbation of symptoms, primarily in inflammatory diseases. Mental stress, anxiety, and anger have been found to be positively correlated with cardiac ischemia, periodontal disease, inflammatory bowel disease (IBD), and it is hypothesized to play major roles in autoimmune diseases such as Graves' disease, multiple sclerosis, rheumatoid arthritis, and others [145–151].

Stress and Asthma

In contrast to other inflammatory disorders such as IBD and atopic dermatitis, good clinical evidence regarding the effect of stress on asthma is still relatively sparse. Studies in humans show the significant role that stress plays in stimulating asthma exacerbations, including negative correlations with important physiological markers of airway function. Asthmatic students who are writing exams have sputum samples with higher eosinophil inflammation and IL-5 as compared to other students and other times of the year [152], whereas adolescent asthmatics undertaking a stressful computer puzzle test have increased breathlessness [153]. Other studies are not as conclusive. One paper found no significant relationship between daily life stress and serum IgE levels or bronchial hyperresponsiveness [154]. Studies have shown that stress-induced changes in physiological parameters of breathing may be similar in asthmatic and non-asthmatic children, but these changes may have higher clinical significance in asthmatics due to their higher baseline airway resistance [155].

Animal studies have supported the predictions from the observational human studies on the effects of stress on asthma. A number of groups have demonstrated that chronic stress leads to increased airway hyperresponsiveness and/or increased BAL leukocytes in mouse models of asthma [156–158]. The effects of long-term stress on airway inflammation have also been replicated in rat and guinea pig models [159, 160].

While the HPA axis is an important stress response system, evidence from animal models indicate that corticosteroids are not directly involved in the effects of long-term stress on airway inflammation. Joachim et al. [156] have shown that the long-term stress mechanism likely involves the role of the NK1 receptor, which is present in the lung and submucosal glands and in addition is on mast cells, lymphocytes, and macrophages. Furthermore, use of a highly specific inhibitor of the NK1 receptor results in ablation of long-term stress-induced effects in the guinea pig lung. Recently, Okuyama et al. [158] demonstrated that the exacerbation of airway inflammation induced by chronic stress is abolished in μ opioid receptor-deficient mice, indicating there may also be an important role for opioids in mediating the immunoregulatory effects of chronic stress in the airway.

Conclusion

The CNS and immune system communicate through multiple neuronal pathways, allowing the brain to monitor the immune status and actively respond to challenges in a rapid discreet and threat-specific manner. Increased knowledge of the interactions between the brain and the immune system holds considerable promise for expanding our understanding of the mechanisms underlying health and disease. Future challenges will include determining how the brain coordinates this response and precisely how the components of the nervous system interact to modulate immune cells both in host defense and in contributing to immune and inflammatory disorders. In addition, psychoneuroimmunological research is helping to uncover the role emotions and stress play in this equation and is increasing our awareness of the clinical significance of psychosocially induced changes in immune function. Defining the molecular mechanisms underpinning psychologically conditioned and stress-induced immune responses may not only provide us with novel pharmacologic therapies, but also identify the potential for behavioral modification strategies in the treatment of immune and inflammatory disorders.

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Neuroregulation of Mucosal Vasculature

Alfredo Chetta, Giovanna Pisi, and Dario Olivieri

Introduction

The nose as well as the bronchial airways have a very well-developed network of microvessels placed beneath the epithelium. In the past, these microvessels were considered as simple, essential conduits for nutrients. However, there is increasing evidence that they may have several highly significant roles in normal homeostasis as well as in inflammatory airway processes [1–2]. Viable functions of the airway microvasculature include supplying oxygen and nutrients, temperature regulation and humidification of inspired air, as well as being the gateway for the immune response to inspired organisms and antigens [1]. Additionally, the mucosal vessels can act as ports of entry for inflammatory cells [2]. An increase in bronchial mucosal circulation, which may occur in asthma, is also believed to reduce luminal diameter by engorgement or oedema of the airway wall, thereby causing airflow obstruction [3].

The mucosal vessels are regulated by efferent and afferent autonomic nerves. In response to the conditions of the inspired air, the sensory nerves recruit appropriate reflexes, which in turn can induce different vascular processes, such as vasodilatation, vasoconstriction, plasma extravasation, and exudation. In addition to the classic cholinergic and adrenergic innervation, neural mechanisms which are not blocked by cholinergic or adrenergic antagonists are present in the airways and are known as the nonadrenergic noncholinergic (NANC) system [4]. This chapter deals with the interactions of vessels and nerves within the airway mucosa under healthy conditions and in inflammatory diseases.

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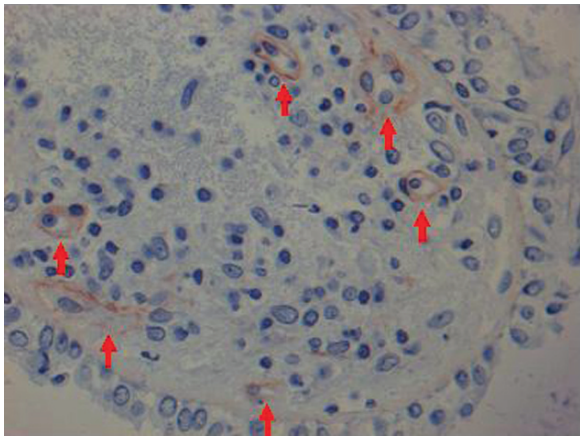

Fig. 1 The microphotograph shows immunostaining for collagen IV to identify blood microvessels in the lamina propria of a normal subject. The arrows indicate the vessels. Light microscopy was performed at 400× (personal observation, unpublished)

Vascular Structure

The mucosa of the nose and the tracheobronchial tree is highly vascular (Fig. 1), but the microvascularity qualitatively differs in these two districts of the respiratory tract. These differences depend on the various functions of the nose and the tracheobronchial tree.

Nasal Mucosa

Afferent arteries from the maxillary, ophthalmic, and facial arteries lead to arterioles, arteriovenous anastomoses (AVAs), capillaries, postcapillary venules, venous sinusoids, and muscular veins that drain the sinusoids [5]. There is a copious subepithelial capillary network, and a deeper erectile cavernous plexus. Venous drainage passes into the orbit, cavernous sinus and pterygoid plexus with many anastomoses with veins in the face, palate, pharynx, and superficial veins in the brain [6].

Arteries supplying the subepithelial zone are derived from periosteal or perichondral vessels that rise towards the surface through the zone of the cavernous plexus. Some branches of these arteries form the cavernous plexus by their AVAs. Near the surface, the stem arteries give rise to arterioles that end in a capillary network in the subepithelial zone and around the glandular tissue. An extensive system of AVAs is evident in the nose and may take at least half of the nasal blood flow. This system, which is not present in the lower airways, is presumably concerned with temperature control and air conditioning [7].

In the nose, the subepithelial capillaries run parallel to the epithelium and are fenestrated, likely facilitating extravasation of plasma proteins [8]. The rate of plasma exudation may be controlled by arteriolar tone. Unlike other mucosal vascular systems [9], the pressure gradient across the walls of the fenestrated capillaries

is small. Vasodilatation of arterioles will increase capillary blood flow and capillary vascular pressure, leading to an increase in plasma transudation. Extravasated plasma contains a variety of chemical mediators that may lead to local tissue inflammation and damage. Conversely, a bulky flow of plasma water and macromolecules across fenestrated capillaries provides a rapid and efficient mechanism to flush offending stimuli from the nasal mucosa.

The nose has also a prominent, collapsible plexus of venules, sinuses or sinusoids present deep in the glandular zone that constitute a capacitance system [10]. Venous sinusoidal filling with mucosal swelling leads to thickening of the septum and turbinates, thereby causing airflow obstruction. Vascular dilatation of these structures may contribute to the sensation of nasal congestion. Lymphatic vessels are present in the interstitial region and their role in the clearance of interstitial fluid has not been established.

Tracheo-Bronchial Mucosa

The bronchial circulation is still considered to be a separate circuit from the pulmonary circulation of blood flow through the lungs. However, there is some anatomical overlap between the regions which both circulations supply and there are a few points of direct connection, e.g., in the precapillary region and the deep bronchial veins draining to pulmonary veins. Total bronchial blood flow accounts for only a very small fraction of cardiac output – about 1% – but it supplies a visceral area whose defence mechanism must be rapidly mobilized to defend the respiratory exchange region. The best known function of the bronchial circulation (also called airway circulation) [11, 12] is to provide oxygen and nutrients to the airways down to the levels of the terminal bronchioles, nerves, glands, lymph nodes, and pulmonary vessels. It also plays a role in immune response and host defence, water balance, airway metabolism, and thermoregulatory functions [1]. Additionally, the mucosal vessels may also affect the uptake, distribution and removal of chemical substances released within the airway wall (e.g., mediators from nerves, epithelium, or inflammatory cells) or delivered onto the airway surface via the lumen (e.g., bronchodilator aerosols). Moreover, the bronchial microcirculation can act as a port of entry for inflammatory cells, via upregulation of cell adhesion glycoproteins [2]. Bronchial blood flow is increased by several factors such as hyperventilation, changes in the temperature of inspired air and in the osmolarity of the fluid lining the airways, and by the presence of air pollutants [13]. Changes in bronchial microvasculature are present in chronic inflammatory airway diseases and may contribute to airway remodelling in asthma [3]. Currently, it is assumed that the bronchial microcirculation in asthma may be involved via at least three different mechanisms: angiogenesis, dilatation, and permeability [3].

Bronchial arteries arise from the thoracic aorta during the 9th to 12th weeks of gestation. In about 40% of people, a single bronchial artery is present in each lung. The right bronchial artery may arise directly from the aorta or from an intercostal,

subclavian, or internal thoracic artery. On the left, about 70% of individuals have two or more bronchial arteries, generally arising directly from the aorta. The bronchial arteries supply the distal trachea and carina and ramify within the walls of the bronchi and intrapulmonary airways. The branches constitute a plexus in the peribronchial space, and small arterioles penetrate the muscular wall of the airway to form a submucosal plexus [1].

The peribronchial and submucosal plexuses go along the airways down to the level of the terminal bronchioles, where they give off capillaries that communicate with pulmonary capillaries and veins through coiled anastomoses. Presumably, these anastomoses control the pressure gradient from bronchial to pulmonary arteries. Under normal conditions the bronchial to pulmonary communications are closed, but they can be open when the pulmonary arterial circulation is acutely obstructed or in chronic diseases, such as bronchiectasis and congenital heart disease.

Ultrastructural studies show that bronchial capillaries are lined by continuous nonfenestrated endothelium [14]. Comparison with the pulmonary microvasculature reveals that bronchial capillaries have thicker endothelium and more cytofibrils and pericytes. These characteristics permit rapid hypertrophy, regeneration, and angiogenesis in response to injury. Venous drainage of intrapulmonary airways (70% of the total) is via pulmonary veins into the left atrium, causing a small degree of venous admixture. True bronchial veins drain blood from hilar structures and extrapulmonary airways into the right atrium.

The lower airways have also a system of capacitance vessels, which are far less extensive than in the nose, and are also species-dependent. The physiological advantages of capacitance vessels in the tracheobronchial mucosa are difficult to understand. Possibly the capacitance vessels provide a source of heat or water that would lessen cooling and evaporation during hyperventilation and they are thought to be the cause of exercise-induced bronchoconstriction. Another important action of the capacitance system could be to reduce the compliance of the airway walls caused by the congestion of the bronchial mucosa [10].

Innervation

In the airways, the autonomic neural control mechanisms are very complex and include cholinergic and adrenergic innervation, as well as the NANC system. The latter was originally considered to be an anatomically separate nervous system, but now it is clear that at least certain NANC neural effects are mediated by the release of neurotransmitters from classic cholinergic or adrenergic nerves.

Parasympathetic and Sympathetic Nervous Systems

Parasympathetic nerves are crucial for normal respiratory function, since they control glandular secretion in the upper and lower airways and bronchoconstriction

in the lower airways [4]. Acetylcholine (ACH) is the major neurotransmitter of cholinergic nerves. ACH acts by binding to muscarinic (M) receptors, of which at least three subtypes can pharmacologically be recognized in the human lung and in the human nasal mucosa [15]. Excitatory M_1 receptors are present in airway parasympathetic ganglia and may facilitate neurotransmission, which is mediated via nicotinic receptors [16]. Conversely, M_2 receptors act as negative feedback autoregulators and are located on postganglionic nerve terminals in human central airways and subsegmental and terminal bronchi. Accordingly, they may inhibit the release of ACH, thus reducing the stimulation of postjunctional M_3 receptors, which in turn constrict airway smooth muscle [16]. M_1 receptors are also placed in airway glands and alveolar walls, while M_3 receptors are found on airway glands [16] (Table 1). Muscarinic receptors regulate human nasal glandular secretion and vascular tone by contributing to parasympathetic vasodilatation and thereby inducing nasal congestion. In particular, M_1 receptors regulate serous and mucous glands of the nasal mucosa, while M_2 receptors apparently do not exist in the nose [16]. Lastly, M_3 receptors are diffuse in the nose and are found on blood vessels, as well as in both serous and mucous nasal glands [16] (Table 1).

The sympathetic or adrenergic nervous system is less conspicuous than the parasympathetic nervous system in human airways [4]. Sympathetic nerves induce vasoconstriction and reduce mucosal thickness [17]. Noradrenaline and neuropeptide Y (NPY) are the main neurotransmitters of the sympathetic nervous system [18]. Noradrenaline can activate α- and β-adrenergic receptors on target cells in the airways. The complete distribution of α-adrenergic receptor subtypes in nasal and bronchial mucosa has not yet been fully described. The α_1-adrenergic receptors mediate smooth muscle contraction and are relatively sparse in human airways [19]. Prejunctional α_2-adrenergic receptors act as autoreceptors and may inhibit noradrenaline and NPY release from adrenergic nerves [20]. In clinical practice, the α_2-adrenoceptor agonists are commonly used as topical nasal decongestants, since they can reduce nasal blood flow and lead to mucosal shrinkage [21]. The β-adrenergic receptors mediate bronchorelaxation and are widely distributed in human lungs [22]. Autoradiography has revealed that they are distributed with dense labelling over airway epithelium, the alveolar walls, and submucosal glands, while a lower density of grains is found over airway and vascular smooth muscle [22]. The β-adrenoceptor agonists are mild vasodilators [23] and have no effect on vascular secretory processes in human nasal mucosa [24].

Table 1 Location of muscarinic receptors in human lung and in human nose

Location	M_1	M_2	M_3
Autonomic ganglia	+	0	0
Airway glands	+	0	0
Airway smooth muscle	0	+	+
Nasal glands	+	0	+
Nasal blood vessels	0	0	+

M_1 = muscarinic subtype receptor 1, M_2 = muscarinic subtype receptor 2, M_3 = muscarinic subtype receptor 3, + = present, 0 = not present

Beside noradrenaline, adrenergic nerves contain NPY, which is part of the excitatory NANC (e-NANC) nervous system. NPY, a 36-amino acid peptide, is a cotransmitter with noradrenaline and may amplify its effects [25]. In addition, NPY may act as a potent vasoconstrictor in some vascular beds [25]. In animal models, exogenous NPY was able to mimic the effect of sympathetic stimulation by attenuating subsequent parasympathetically evoked vasodilatation of the nasal mucosa [26]. In the nasal mucosa, sympathetic tone, generated by rapidly acting short-lasting noradrenalin and slow-onset long-lasting NPY, likely contributes to the normal cycle of periodic coordinated unilateral mucosal swelling with nasal obstruction followed by mucosal shrinkage and mucus secretion [27]. Damage to this system, as in Horner's syndrome and in other nonallergic rhinopathies, may lead to the absence of this regulatory mechanism and the onset of congestion and oedema.

The NANC System

The NANC system constitutes a very heterogeneous population of nerves and is classically divided into inhibitory and excitatory (i-NANC, e-NANC) systems. Of note, the terms inhibitory and excitatory apply to airway smooth muscle, but the neurotransmitters also act on other targets such as blood vessels, glands, and epithelium, where individual actions may be opposing, thus the nomenclature may be sometimes misleading [28].

The i-NANC system is the only neural bronchodilator pathway in human airways. Anatomically, the i-NANC system is co-localized with ACH in the parasympathetic nerves [29]. Neurotransmitters of the i-NANC system include nitric oxide (NO) and neuropeptides, such as vasoactive intestinal peptide (VIP), peptide histidine methionine (PHM), and pituitary adenylate cyclase-activating peptide. NO is a major intercellular messenger molecule and is considered to be a major neurotransmitter of i-NANC nerves in human airways [30]. The ubiquitous presence of NO in vascular endothelium, in the epithelium and in epithelial sensory nerves emphasizes the importance of NO as a mucosal mediator, though its complex role is not yet completely clear [28]. NO has potent smooth muscle-relaxing properties and vasodilator effects. An increased NO production in the airways may result in hyperaemia, plasma exudation, and mucus secretion [31]. VIP is a 28-amino acid peptide and acts by binding to VIP receptors [32], which are localized in pulmonary vascular smooth muscle, airway smooth muscle, airway epithelium, and submucosal glands [33]. In addition to bronchodilating effects [34], VIP can regulate mucus secretion [35] and is a potent vasodilator [36].

The e-NANC system is located in a subpopulation of nonmyelinated sensory C fibres [37]. C fibres may be stimulated both by exogenous substances, such as capsaicin or cigarette smoke, and by endogenous ones, such as histamine, bradykinin, and prostaglandins [37]. When stimulated, the C fibres transmit information to the central nervous system, where reflex responses may be evoked. In addition, the

stimulation of these nerve endings may result in an axon local reflex with antidromic conduction down afferent nerve collaterals and release of sensory neuropeptides into the airway microenvironment [38]. In the airways, sensory neuropeptides act on bronchial smooth muscle, the mucosal vasculature, and submucosal glands to promote airflow obstruction, hyperaemia, microvascular hyperpermeability, and mucus hypersecretion [39]. Proinflammatory effects of these peptides also promote the recruitment, adherence, and activation of inflammatory cells [39].

Neurogenic Inflammation

The scenario subsequent to the release of neuropeptides from the peripheral endings of sensory nerves via an axon reflex is known as neurogenic inflammation (Fig. 2), which has a potential importance in airway diseases. The most studied neuropeptides of sensory nerves are the tachykinins, substance P (SP), neurokinin A (NKA), and the calcitonin gene-related peptide (CGRP). Additionally, secretoneurin has been found to be a neurotransmitter of sensory nerves [40].

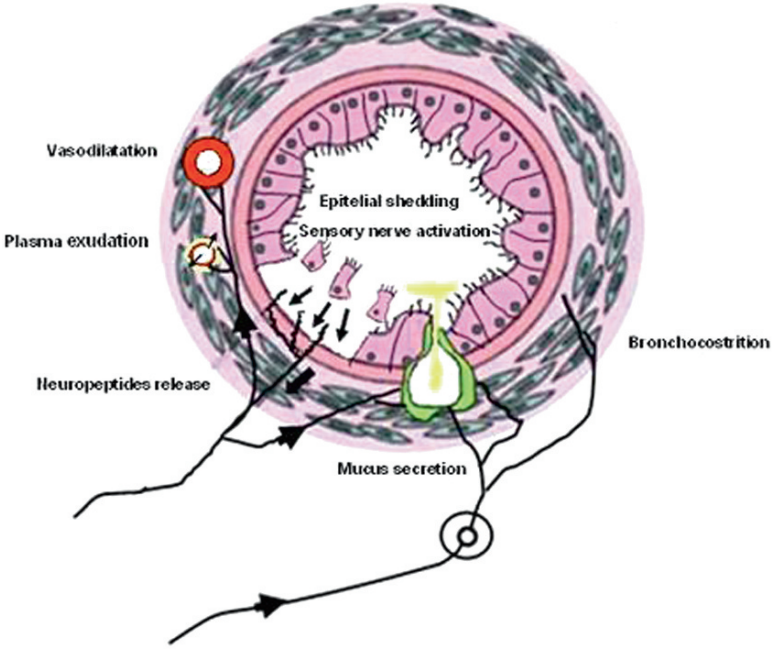

Fig. 2 Schematic representation of the neurogenic inflammation in the airways. For details, see the text

Airway Tachykinins

Tachykinins are known to bind to three different G protein-coupled receptors, so-called neurokinin receptors (NK). More precisely, SP acts via the NK_1 receptors, NKA acts via NK_2, and neurokinin B (NKB) acts via NK_3 receptors [41]. NK_1 receptors are predominantly localized in the airway epithelium, submucosal glands, and vessels, while NK_2 receptors are mainly expressed on airway smooth muscle [41]. NK_3 receptors have only recently been considered in studies on airway regulation, and based on functional and immunohistochemical analyses, they have been found to be expressed by airway parasympathetic ganglia [42].

SP stimulates mucus secretion from submucosal glands in human airways via activation of NK1 receptors [43]. In addition, NK_1 receptors mediate the increased plasma exudation and vasodilator response to tachykinins [43]. In an animal model, the administration of NK_1 receptor antagonists reduced plasma extravasation in airways [44]. NKA can potently constrict human airway smooth muscle via NK_2 receptors with significantly greater potency in small airways [45]. Tachykinins may also interact with inflammatory and immune cells, although the pathophysiological significance remains to be clarified. Furthermore, substance P can stimulate proliferation of blood vessels and may therefore be involved in the new vessel formation that is found in asthmatic airways [43]. There is little evidence that airway nerves express NKB; however, SP and NKA are considered to be full agonists at NK_3 receptors and may activate them [42]. Upon activation, NK_3 receptors evoke depolarization of airway parasympathetic ganglia neurons and facilitate synaptic transmission [42]. Accordingly, this may potentiate airway parasympathetic reflexes.

Tachykinins are metabolized by at least two enzymes, angiotensin-converting enzyme (ACE) and neural endopeptidase (NEP), which is a key enzyme for degrading tachykinins in the airways. Accordingly, the activity of NEP can affect the responsiveness to tachykinins within the airways. The mechanical removal of the epithelium decreases the activity of NEP. It has also been shown that a variety of factors, relevant to airway diseases, including viral infections, allergen exposure, inhalation of cigarette smoke, and other respiratory irritants, are able to reduce NEP activity, thus enhancing the effects of tachykinins within the airways [46].

Calcitonin Gene-Related Peptide (CGRP)

CGRP is a 37-amino acid peptide which is expressed and co-localized together with tachykinins in sensory nerve fibres, innervating both upper and lower airways [47]. CGRP receptors are widely distributed in human airways. A dense labelling is found in bronchial and pulmonary blood vessels and in alveolar walls, while sparse labelling is present in regions of airway smooth muscle and the epithelium of large airways, and no labelling in submucosal glands [48]. The most prominent effect of CGRP in the airways is a long-lasting vasodilatation [49]. Although CGRP does

not directly affect airway microvasculature leakage, by increasing blood flow, it is a key factor in enhancing plasma protein extravasation in postcapillary venules caused by other mediators, such as SP [49]. A further pulmonary effect of CGRP is the induction of eosinophil migration [49]. CGRP may be subject to degradation by several enzymes expressed in the human airways; however, the exact cleavage pathways have not been established.

Neurotrophins

In addition to neuropeptides, neurotrophins (NTs) may further interact with sensory neurons, thereby propagating neurogenic inflammation within human airways [50]. The NTs comprise a family of peptides which induce neuronal development and differentiation, regulate apoptotic mechanisms, and influence synaptic function of the central and peripheral nervous systems [51]. The most prominent members of the NT family are nerve growth factor (NGF), brain-derived neurotrophic factor, NT-3 and NT-4/5 [50]. The traditional cellular sources of NTs under physiological conditions are mainly nerve-associated cells, such as glia cells, Schwann cells, or fibroblasts and neurons themselves [52]. Additionally, NGF can be produced by inflammatory cells, such as mast cells [53] or lymphocytes [54]. Under airway inflammatory processes, NTs may potently affect peripheral nerves by inducing the "neuronal plasticity," which consists in quantitative and/or qualitative changes of the functional activity and capacity of peripheral neurons [50]. Examples include increased production of neuropeptides and tachykinins, increased receptor expression, increases in the number of nerves producing neuropeptides and tachykinins, and lowering of the firing threshold of nerves [50]. In parallel, NTs also may exhibit profound effects on immune cells residing in airway and lung tissue, thereby sparking off a vicious circle [50].

Pharmacological Implications

Sensory neuropeptides have various effects that could contribute to the changes in airway walls that are observed in asthma and chronic obstructive pulmonary disease (COPD). Accordingly, the pharmacological strategies which interfere with the action of sensory neuropeptides within the airways could be clinically relevant. These strategies include drugs which can prevent either the activation of sensory nerves or the release of neuropeptides. Cromones may have direct effects on airway C fibres and this might contribute to their anti-asthma effect [55]. In addition, in asthmatic patients, inhaled furosemide inhibits metabisulphite-induced bronchoconstriction, which is believed to act via neural mechanisms [56]. Opioids effectively inhibit the release of neuropeptides; however, an inhaled μ-opioid agonist was ineffective at inhibiting metabisulphite-induced bronchoconstriction [57]. Another

alternative approach to modulate the action of sensory neuropeptides consists in the tachykinin receptor antagonists, which, up to now, have given disappointing results [58]. Further studies with more potent tachykinin receptor antagonists could provide new therapeutic options to treat chronic inflammatory airway diseases.

Closing Remarks

A profuse microvasculature is present in the airway mucosa, though it may qualitatively differ from the nose to the bronchial airways because of the different functions of these two districts of airway tract. An intricate network of efferent and afferent autonomic nerves, including cholinergic and adrenergic nerves and the NANC system, regulates the mucosal airway vessels. The nerve/vessel interplay is very complex and not yet completely clarified. In response to inspired air conditions, the sensory nerves recruit appropriate reflexes, which can induce different vascular processes, such as vasodilatation, vasoconstriction, plasma extravasation, and exudation. Additionally, the stimulation of C fibres may result in an axon local reflex with antidromic conduction down afferent nerve collaterals and release of sensory neuropeptides, which in turn may act on the mucosal vasculature to promote vasodilatation and microvascular leakage. In addition to the neuropeptides, NTs may further interact with sensory neurons, thereby propagating neurogenic inflammation within human airways. The neurogenic inflammation may play a key role in allergic diseases, such as asthma, as well as in COPD, a smoking-related disease. Accordingly, the pharmacological modulation of neurogenic inflammation may represent an important therapeutic approach to chronic inflammatory airway diseases.

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Part III
Allergy and Mucosal Immunology

Mucosal Immunity: from Allergy to Coeliac Disease

Per Brandtzaeg

Introduction

Adverse reactions to food can affect many organs, thereby causing a perplexing variety of symptoms. According to a position paper from the European Association of Allergology and Clinical Immunology (EAACI) in 1995, which was revised in 2001, there are two main entities of food reactions, toxic and non-toxic; the latter comprises both immune-mediated (food allergy) and non-immune-mediated pathogenic mechanisms [1, 2].

The immunological, or truly allergic, food reactions can be divided into IgE-mediated (Type I hypersensitive) or non-IgE-mediated conditions, which mechanistically may be further classified as Type III (IgG or IgM immune complexes) or Type IV (delayed-type) hypersensitivity [3]. According to this classification, also coeliac disease (gluten-sensitive enteropathy) can be considered an 'allergic' disorder because the immunopathology of the duodenal/jejunal lesion is driven by Type IV and perhaps also Type III hypersensitivity reactions [4]. Notably, even a large proportion (~50%) of patients suffering from classical food allergy appear to have a non-IgE-mediated, delayed-type immune reaction [5–7]. However, it is important to be aware of the fact that the terminal effector mechanisms operating in the mucosal lesions are difficult to distinguish by available clinical tests [8–11].

To avoid hypersensitivity in the extensive and vulnerable mucosae, they are protected by specialized anti-inflammatory immune defences including secretory IgA (SIgA) antibodies and hyporesponsiveness to innocuous substances, particularly dietary antigens and components of commensal bacteria (Fig. 1). Nevertheless, the induction of mucosal immunity depends on exogenous stimuli and the neonatal period is critical to this end. Both the mucosal barrier with its reinforcement by SIgA and the immunoregulatory network require successful adaptation after birth. In most cases, mucosal immune homeostasis is remarkably successful in view of the fact that

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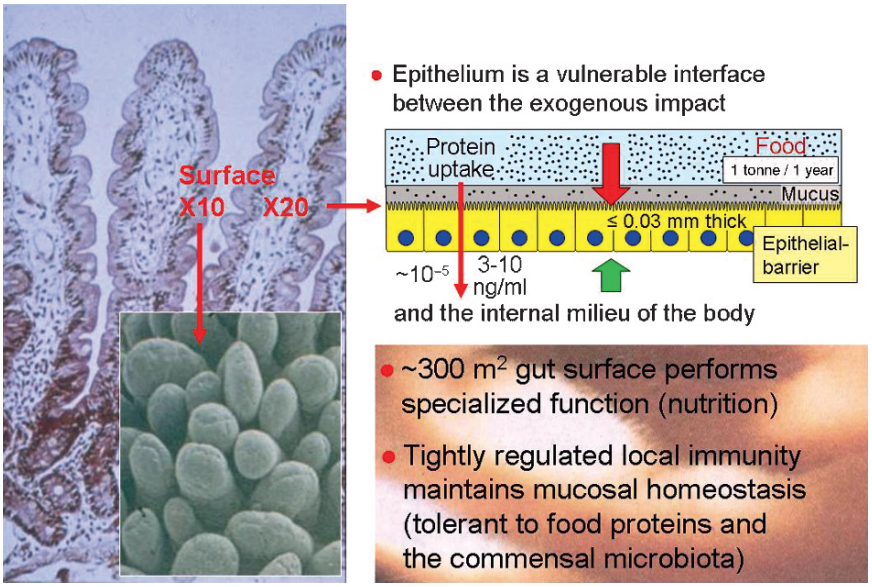

Fig. 1 Importance of the epithelial barrier function in the human gut. The two left panels depict histological section and scanning electron microscopy of normal small intestine, while the right panel illustrates at the top the monolayered epithelium with its microvilli ('brushborder'). The background below is taken from an endoscopic picture of the upper duodenum. The total luminal surface area of the 6–7 m long adult human gut (~300 m^2) is increased by folds (not shown), villi (×10) and microvilli (×20). The magnitude of food protein uptake in the normal small intestine is indicated

a tonne of food may pass through the gut of an adult every year. Classical food allergy, as well as coeliac disease, reflects lack of such homeostasis – either due to immaturity of the mucosal barrier function and retarded immunological development in infancy, or persistently imbalanced immunoregulatory mechanisms later on – associated with abrogation of tolerance against innocuous antigens.

Mucosal Immune Regulation

Two Layers of Anti-inflammatory Defences

Numerous genes are involved in the regulation of innate and adaptive immunity. It appears that human immunogenetics has evolved to identify 'danger' under the pressure of a 'dirty environment' and natural food – the hunting-gathering period being quite extensive compared with modern hygienic life. In the evolutionary process the mucosal immune system has generated two layers of anti-inflammatory mechanisms: (i) immune exclusion performed by SIgA antibodies to control epithelial colonisation of micro-organisms and inhibit penetration of potentially

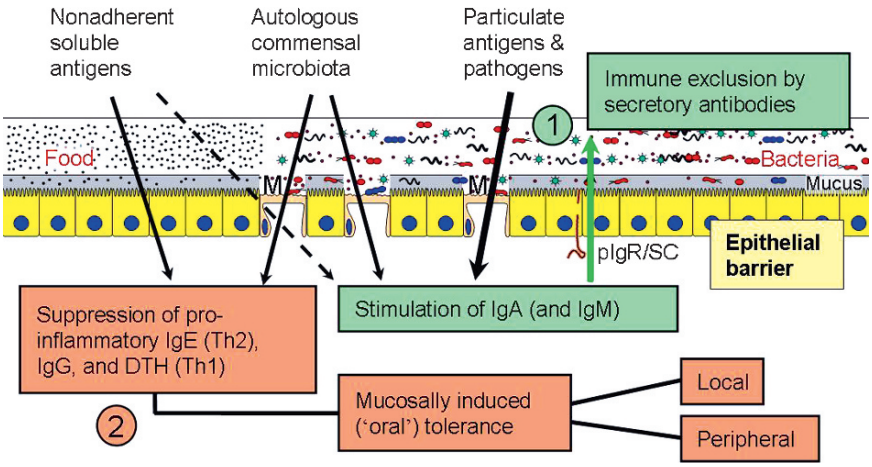

Fig. 2 Two major anti-inflammatory immune mechanisms operating in the normal gut. (1) Immune exclusion limits epithelial colonization of microorganisms and inhibits penetration of harmful foreign material. This first line of protection is principally mediated by secretory antibodies of the IgA (and IgM) class in cooperation with various non-specific innate defences (not shown). Secretory immunity is preferentially stimulated by pathogens and other particulate antigens sampled by thin M cells (M) in the domes of gut-associated lymphoid tissue. Locally produced dimeric IgA (and pentameric IgM) is actively transported to the lumen by the polymeric Ig receptor (pIgR), also called membrane secretory component (SC). (2) Penetrating dietary antigens and components of the commensal microbiota stimulate secretory immunity as well, but not to the same extent as pathogens (graded arrows). Such innocuous antigens, instead, preferentially induce suppression of proinflammatory humoral immune responses (Th2 cytokine-dependent IgE antibodies and IgG) as well as Th1 cytokine-dependent delayed-type hypersensitivity (DTH). This homeostatic regulation of the Th1/Th2 balance induced via the gut is collectively termed 'oral tolerance'; it exerts down-regulatory effects both locally and in the periphery

dangerous substances; and (ii) immunosuppression to avoid local and peripheral hypersensitivity to innocuous antigens (Fig. 2). When induced via the gut, immune-mediated homeostasis is referred to as 'oral tolerance' [12]. Together, these mechanisms explain why overt and persistent food allergy, with the exception of coeliac disease, is relatively rare [5]. Similar down-regulatory mechanisms apparently operate against antigens from the commensal microbiota [13–15].

Mucosally induced tolerance appears be a rather robust adaptive immune function in view of the fact that there is a substantial intestinal uptake of intact dietary antigens after meals (Fig. 1) – estimated to be in the range of 10^{-5} of the intake and reaching a level of 3–10 ng/ml in the circulation [3]. The neonatal period is particularly critical, however, with regard to the mucosal barrier function and priming for allergic disease [16]. The epithelial tightness and the immunoregulatory network remain fragile for a variable period after birth [17, 18]. Importantly, the postnatal development of mucosal immune homeostasis depends on the establishment of a balanced indigenous microbiota as well as adequate timing and the dose of dietary antigens when first introduced [15, 19, 20]. The effect of commensal bacteria on mucosa-associated lymphoid tissue is strikingly

revealed in experimental animals colonised with a conventional indigenous microbiota after being reared in a germ-free state [21, 22].

Secretory Antibodies and Epithelial Barrier Function

The normal intestinal mucosa of an adult contains at least 80% of the body's activated B cells – terminally differentiated mainly to IgA-producing plasmablasts and plasma cells (PCs) [23]. Most mucosal PCs produce dimeric IgA [24] which, along with pentameric IgM that likewise contains a polypeptide called 'joining (J) chain' [25], can be actively exported by secretory epithelia [23, 26]. This J chain-dependent external transport is mediated by the polymeric Ig receptor (pIgR), also known as membrane secretory component, or SC [27, 28]. Immune exclusion is performed mainly by SIgA, and to a lesser extent secretory IgM (SIgM), in cooperation with innate non-specific defences (Fig. 2). In newborns and subjects with selective IgA deficiency, SIgM antibodies are of much greater importance than in healthy adults [17].

IgA-producing PCs are generally undetectable in the mucosa before 10 days of age, but thereafter a rapid increase takes place, although IgM-producing PCs often remain predominant up to 1 month [17, 19, 20]. Little increase of intestinal IgA production usually takes place after 1 year, and a much faster establishment of secretory immunity is often seen in developing countries with a heavy microbial load. The mucosal PC development reflects the progressive microbial stimulation of gut-associated lymphoid tissue (GALT), such as Peyer's patches [23]. Accordingly, only occasional traces of SIgA and SIgM occur in intestinal juice during the first postnatal period, whereas some IgG is often present – reflecting paracellular 'leakage' from the lamina propria that after 34 weeks of gestation contains readily detectable maternal IgG [17, 29]. In addition, some IgG may be actively exported by epithelial FcRn [30].

Uptake of SIgA antibodies from breast milk via the neonatal gut mucosa is negligible and of no importance for systemic human immunity [31, 32], except perhaps in preterm infants [33]. So-called 'gut closure' normally occurs in humans mainly before birth, but the mucosal barrier function may nevertheless be inadequate up to 2 years of age; although the different mechanisms involved remain poorly defined [34], the development of secretory immunity is one decisive variable. Importantly in this context, pIgR-deficient mice that lack SIgA as well as SIgM exhibit aberrant mucosal leakiness [28] and increased uptake of antigens from food and commensal bacteria [35].

Experimental studies have demonstrated a crucial role of microbial colonisation in establishing [36] and regulating [37] the epithelial barrier, including up-regulation of pIgR expression [36]. At least in mice, the beneficial effects of commensal bacteria appear to be mediated significantly via pattern recognition receptors (PRRs) expressed by the gut epithelium, particularly Toll-like receptors (TLRs) [38, 39], which recognise conserved molecular motifs of micro-organisms. Polarised epithe-

lial cells have the ability to dampen the proinflammatory effect of PRR-mediated signals coming from the luminal side [37, 40]. However, after bacterial invasion, PRR signalling from the basolateral side results in NF-κB activation with release of defensins to combat the infection [41].

Altogether, accumulating evidence suggests that barrier-related mucosal homeostasis is largely influenced by 'cross-talk' between epithelial cells and the underlying lamina propria cells (Fig. 3), including macrophages, dendritic cells (DCs) and T cells [42, 43]. In fetal life, murine gut epithelial cells are sensitive to microbial factors such as endotoxin (lipopolysaccharide, LPS) because they express intracellularly the receptor for this conserved microbe-associated molecular pattern (MAMP), namely TLR4 [44]. Therefore, exposure to LPS in the vaginal tract during birth activates temporarily the neonatal gut epithelium so that it subsequently becomes tolerant to MAMPs because of suppressed TLR signalling. In remarkable contrast, such epithelial homeostasis induction does not take place in mice delivered by cesarean section [44]. These experimental observations may be

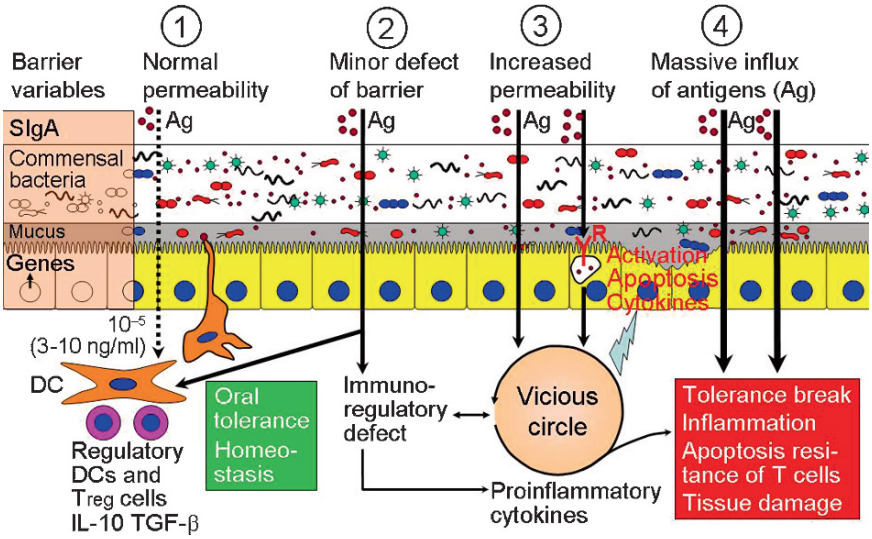

Fig. 3 Role of epithelial barrier function in maintenance of mucosal homeostasis and abrogation of tolerance. Barrier variables in the gut, including secretory IgA (SIgA), are indicated in the orange panel to the left. (1) Normal permeability of gut epithelium allows some uptake of food antigens (Ag), with the amounts detected in circulation after a meal indicated. (2) A minor barrier defect results in increased Ag uptake, but mucosal homeostasis is maintained (green panel) when oral tolerance is adequately induced by quiescent dendritic cells (DC), regulatory T (Treg) cells and suppressive cytokines such as IL-10 and TGF-β. However, if there is a regulatory defect, a vicious circle will develop that reciprocally acts also on the immunoregulatory network. (3) With more skewing towards regulatory dysfunction, the vicious circle will activate the epithelium, and the Ag uptake is enhanced both by increased permeability and aberrant receptor (R) expression apically on epithelial cells. (4) Such adverse development may finally result in epithelial apoptosis, increased secretion of proinflammatory cytokines, chronic inflammation, apoptosis-resistant effector T cells, and tissue damage (red panel)

related to the fact that children delivered by cesarean section are particularly prone to develop food allergy if they have a genetic predisposition for atopy [45].

Mucosal Tolerance Induction

Oral tolerance as defined by experimental feeding in mice, clearly involves more than one mechanism; available data do indeed suggest extensive biological complexity. Variables include genetics, age, dose and timing of postnatal oral antigen administration, antigenic structure and composition, epithelial barrier integrity, and the degree of concurrent local immune activation as reflected by local cytokine profiles and expression of co-stimulatory molecules on antigen-presenting cells (APCs) [12]. In addition, there is an increasing awareness of the suppressive effects of various regulatory T (Treg)-cell subsets induced by conditioned APCs, particularly mucosal DCs as discussed later.

By dampening early immune activation (e.g., expression of the co-stimulatory B7 molecules, designated CD80 and CD86), the shielding effect exerted by maternal SIgA on the breast-fed infant's GALT may contribute to hyporesponsiveness not only against commensal bacteria, but also against dietary antigens such as cow's milk proteins and gluten from wheat [4, 46]. Antibodies to food constituents are present in breast milk, and breastfeeding has been shown to protect significantly against the development of coeliac disease in children, unrelated to the time of solid food introduction [47]. The balance of evidence suggests that exclusive breastfeeding also protects against allergic disorders such as atopic dermatitis and asthma [48–52], particularly in genetically predisposed children [53]. On the basis of such observations, it can tentatively be concluded that mixed feeding, rather than abrupt weaning, appears to promote tolerance to food proteins.

This notion is supported by studies reporting that cow's milk allergy is more likely to develop in infants whose mothers have relatively low levels SIgA antibodies to bovine proteins in their milk [54, 55]. The presence in breast milk of the immunosuppressive cytokine named transforming growth factor (TGF)-β, might further contribute to oral tolerance because of its down-regulatory effect on GALT [46, 56] and enhancing effect on the epithelial barrier [57].

Mucosal Homeostasis and Allergy

Mucosally Induced Tolerance to Exogenous Antigens

Although immunological homeostasis generally prevails in the mucosae, some 5–7% of children up to 3 years of age living in industrialized countries develop food allergy. Recent epidemiological data suggest that nearly 4% of Americans are afflicted with this disorder, and peanut allergy has doubled in American children below 5 years of age in the second half of the past decade [58].

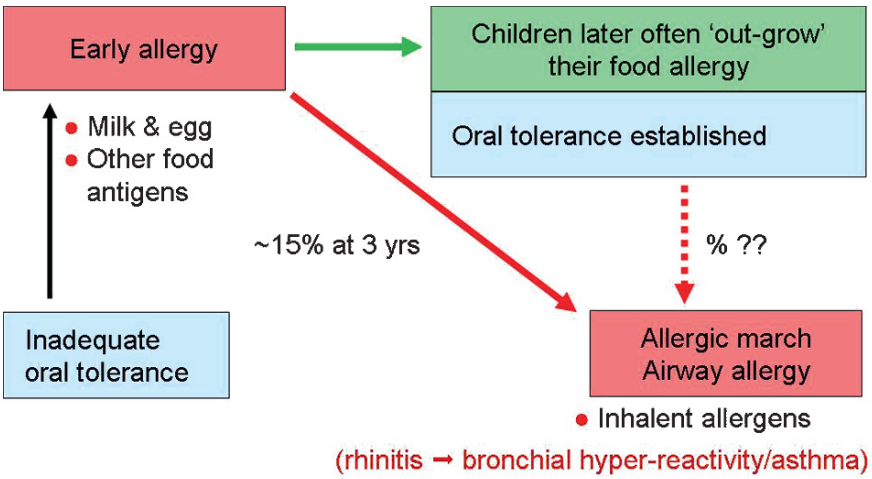

Fig. 4 Schematic depiction of the allergic march from food allergy to airway allergy of increasing severity. At the age of 3 years, approximately 15% of food-allergic children have started the allergic march. The remaining 85% have out-grown their food allergy, but it remains unclear to what extent also these children are predisposed to enter the allergic march (% ??)

Food allergy is associated with serious health consequences and may unfortunately also be the starting point for an 'allergic march' (Fig. 4) – that is, the development of subsequent allergic diseases in the airways, which particularly takes place in atopic individuals with a genetic predisposition to produce IgE antibodies [2]. It is convincingly documented that there has been a striking increase of airway allergy over the last couple of decades, especially in industrialized societies [58–61]. Understanding the immunological mechanisms involved in development of mucosal homeostasis *versus* abrogation of tolerance to dietary antigens is therefore of fundamental interest.

It is believed that oral tolerance may be largely explained by different T cell events such as anergy, clonal deletion and induction of Treg cells (Fig. 5), although other immunosuppressive principles may be involved [46, 62]. For ethical reasons, the existence of mucosally induced tolerance in humans is supported mainly by circumstantial rather than experimental evidence. Thus, the gut mucosa of healthy individuals contains virtually no hyperactivated T cells and hardly any proinflammatory IgG production, and their serum levels of IgG antibodies to food antigens are low [61, 62]. Moreover, the systemic IgG response to dietary antigens tends to decrease with increasing age [63, 64], and a hyporesponsive state to bovine serum albumin has been demonstrated by intradermal testing in adults [65]. Interestingly, nasal antigen application or feeding experiments in healthy individuals induced peripheral down-regulation of T cell responses and to some extent also suppressed systemic humoral immunity [66, 67]. In contrast, oral tolerance could not be induced in patients with inflammatory bowel disease (IBD) where the epithelial barrier function has deteriorated [68].

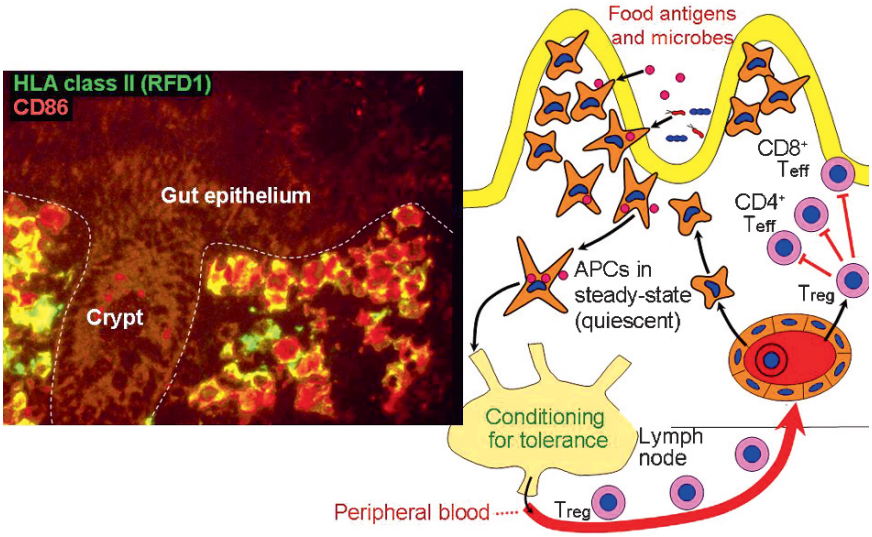

Fig. 5 Uptake of antigens at the intestinal surface and microbial conditioning of antigen-presenting cells (APCs) for tolerance induction. The distribution of APCs below the surface epithelium in normal human colon mucosa is shown with paired immunofluorescence for HLA class II molecules and co-stimulatory molecule B7.2 (CD86) to the left (see colour key). In the schematic depiction to the right, some of the APCs extend their dendrites between epithelial cells to sample luminal antigens. Subepithelial dendritic cells with captured antigens migrate via draining lymph to mesenteric lymph nodes where they either mature to become active APCs, which stimulate T cells for productive immunity, or become conditioned for induction of down-regulatory (suppressive) immunity via generation and expansion of regulatory T (Treg) cells. The Treg cells migrate via efferent lymph to peripheral blood and home to the mucosa to exert their anti-inflammatory control of $CD4^+$ and $CD8^+$ effector T (Teff) cells

Role of Pattern Recognition Receptors

Resident APCs from healthy human gut mucosa are quite inert in terms of immune-productive stimulatory properties [69], and they do not express detectable surface levels of TLR2 or TLR4 [70]. Furthermore, only negligible levels of the LPS receptor CD14 are normally present on these cells, and their proinflammatory cytokine response is usually low after LPS stimulation [70, 71].

These observations support the notion that macrophages and DCs play a central role in mucosal tolerance. Thus, in a quiescent steady state intestinal lamina propria DCs carry penetrating dietary and innocuous microbial antigens away from the mucosa to the mesenteric lymph nodes [72] where the same cells, in a normal maturation process, become conditioned for tolerance induction and drive the expansion of Treg cells [20, 46, 73, 74]. A similar homeostatic mechanism appears to operate in the airways [75]. Hyperactivation of immunological effector cells in the mucosa, with accompanying inflammation, is thereby avoided both initially [76] and also subsequently because of homeostatic control when the generated Treg cells home to the lamina propria (Fig. 5).

Nevertheless, the phagocytic and bacteriocidal activity of mucosal macrophages is maintained [76], which would be important for silent clearance of commensal bacteria normally penetrating into the mucosa in small numbers [77].

Conditioning for oral tolerance induction in mucosa-draining lymph nodes (Fig. 5) appears to depend on appropriate stimulation of the migrating APCs by certain conserved cell wall products of commensal bacteria, the so-called MAMPs (Fig. 6), or even by components of parasites such as helminths [61, 78–80]. Several studies suggest that LPS plays a central role in this early programming of the

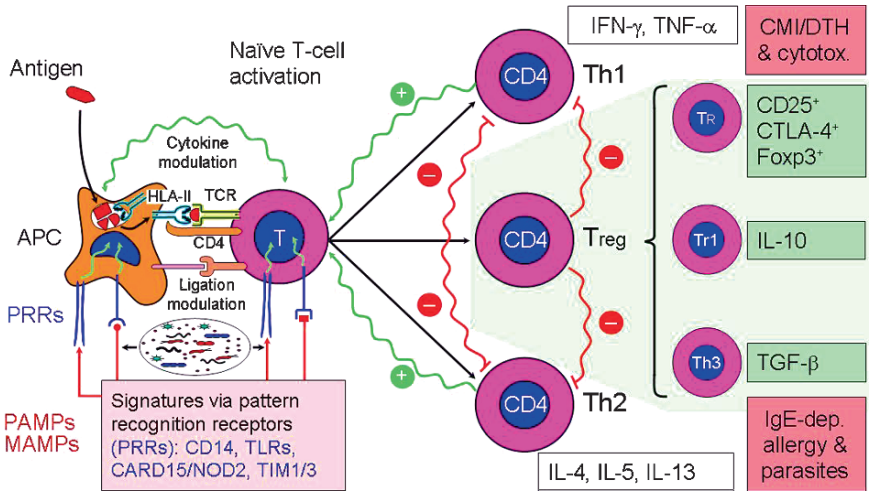

Fig. 6 Decision-making in the immune system is modulated by co-stimulatory signals (ligands and cytokines) from antigen-presenting cells (APCs). Activation of naïve $CD4^+$ T cells takes place after the formation of an immunological synapse with APCs, which take up antigen and process (degrade) it to immunogenic peptides for display to the T-cell receptor (TCR) in the polymorphic grove of HLA class II molecules (HLA-II). The level of co-stimulatory signals in the synapse determines grades of cellular modulation (activation and conditioning). When $CD4^+$ T helper (Th) cells are primed for productive immunity, they differentiate into Th1 or Th2 effector cells with polarized cytokine secretion. Such skewing of the adaptive immune response depends on the presence of microenvironmental factors, including cytokines, as well as signals from microbial components. Bacterial endotoxin (lipopolysaccharide), lipoproteins, unmethylated CpG DNA and other conserved structural motifs are called microbe-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs); they are sensed by cellular pattern-recognition receptors (PRRs) such as CD14, Toll-like receptors (TLRs), etc. Signaling from PRRs stimulates various degrees of activation and functional maturation of APCs and will thereby dictate differential expression of various co-stimulatory signals directing either activation of Th1 cells with predominant production of cytokines such as IFN-γ and TNF-α, or Th2 cells with IL-4, IL-5 and IL-13 secretion. Distinct Th1 and Th2 profiles are further promoted by positive and inhibitory feedback loops as indicated. In addition, under certain as yet fairly unclear conditions, apparently rather immature but yet conditioned APCs may induce various subsets of regulatory T (Treg) cells, which by their cytokines IL-10 and TGF-β, or by direct cellular interactions, can suppress both Th1 and Th2 responses as well as innate immunity and inflammation. IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumour necrosis factor. CMI, cell-mediated immunity; DTH, delayed-type hypersensitivity

immune system [81, 82]; and allergy is associated with functional mutations in PRRs recognising this and other MAMPs, such as CD14, TLR2, TLR4 and NOD [83, 84]. The 'extended hygiene hypothesis' therefore suggests that suboptimal PRR stimulation and delayed maturation of the mucosal immune system contributes significantly to the increasing incidence of not only allergy, but also other immune-mediated inflammatory disorders [61, 85].

This emerging concept has been tested in several clinical studies evaluating the beneficial effect on immune homeostasis exerted by probiotic bacterial preparations derived from the commensal intestinal microbiota [61, 86] and eggs of the porcine helminth (whipworm) *Trichuris suis* [87]. In this context, viable strains of lactobacilli and bifidobacteria have been reported to enhance IgA responses, both in humans and experimental animals, apparently in a T cell-dependent manner [88–93]. In a double-blind study of infants with a family history of atopy, the prevalence of atopic dermatitis was reportedly reduced by 50% at the age of 2 years when the probiotic *Lactobacillus* GG strain was administered to the mothers before birth and to the babies daily for 6 months [94]. The positive effect was subsequently reported to be maintained after 4 years [95].

However, it remains to be shown whether such remarkable results are explained by SIgA-mediated reinforcement of the barrier function, expansion of Treg cells, or the involvement of both these anti-inflammatory mechanism – perhaps combined with direct strengthening of the epithelial integrity (Fig. 3). Notably, atopic dermatitis is associated with loss-of-function mutations in the fillagrin gene which is involved in the epidermal barrier function; similar mutations appear to predispose for the combination of asthma and atopic dermatitis [96]. These striking findings probably reflect that a leaky epithelium is an important variable in allergen penetration leading to induction of allergy.

Innate Decision-Making Directing Adaptive Immunity

The power of the immune system is a result of co-evolution of the host with microorganisms that have shaped its defenses in a state of mutualism [61]. The generally prevailing mucosal homeostasis is remarkable because of the large surface area to be defended – continuously being exposed to perhaps 800 different bacterial species (Figs. 1 and 2). It has been estimated that the human gut microbiota is composed of ~10^{14} bacteria, or approximately ten times the number of body cells, making up a weight of 1–2 kg [61].

According to the original hygiene hypothesis, the increasing incidence of allergy in westernized societies might be explained by reduced or aberrant microbial exposure early in infancy, resulting in too little type 1 helper T (Th1)-cell activity and therefore an insufficient interferon (IFN)-γ level to cross-regulate optimally IgE-inducing Th2-cell responses [97–99]. In this context, an appropriate composition of the commensal microbiota [100] and exposure to foodborne and orofecal microbes [101, 102] probably exert an important homeostatic impact, both by enhancing the SIgA-mediated barrier function and by promoting mucosally

induced tolerance through a shift from a predominant Th2-cell activity in the newborn period [103] to a more balanced cytokine profile later on (Fig. 6).

The extended hygiene hypothesis postulates that induction of Treg cells is an important part of this microbe-driven homeostatic mechanism [61, 85]. Naturally occurring Treg cells with suppressive properties are present in large numbers in fetal mesenteric lymph nodes [104], probably as part of a peripheral tolerance mechanism keeping autoreactive effector T cells in check to avoid inflammation and tissue damage [105]. These Treg cells are apparently induced in the thymus [106, 107]. After birth, the decision between induction of a potentially harmful systemic-type productive immunity against innocuous environmental and dietary proteins, *versus* hyporesponsiveness to such antigens, may be largely instructed in mucosa-draining lymph nodes. As discussed above, the driving force in this homeostatic mechanism appears to be the microbial impact that conditions APCs and T cells for tolerance via PRRs (Figs. 5 and 6).

Thus, MAMPs do not only directly modulate the epithelial barrier function of neonates [44], but also the activation profiles of innate and adaptive immune cells. Appropriate balancing of the immune system therefore appears to depend on a fine-tuned 'cross-talk' between APCs/innate immunity and T cells/adaptive immunity early in the newborn period [20, 61, 85].

A relatively narrow postnatal window apparently exists for such early programming of the immune system – starting when the infants gut mucosa is colonized with commensal vaginal and intestinal bacteria from the mother's birth canal [85, 107]. In healthy individuals, the Th2-skewed cytokine profile of the newborn is then deviated towards a Th1 profile as a sign of immunological maturation. However, in the atopic child the Th2 skewing will continue and thus predispose to allergy. IgE sensitisation may start even in utero [108], although this possibility remains elusive [109, 110] and does not necessarily suggest that the infant is atopic and will go on to become allergic [111]. Instead, the infant may later on be able to develop a balanced immune system. Thus, most children with overt food allergy will become tolerant and clinically outgrow their disorder before the age of 2 years (Fig. 4).

It is important to learn more about the maturation of the immune system in early life to exploit such knowledge for future prevention and treatment of allergy [108]. Fortunately, some plasticity of immunoregulatory pathways persists even after the newborn period so APCs can later on be conditioned to induce Treg cells by environmental factors such as LPS [108] and cell wall lipids from parasites [112]. Transient infestation with porcine helminths has been shown to have a beneficial effect on mucosal homeostasis in adult IBD patients [61, 79, 87].

Homeostatic Role of Commensal Bacteria and Other Biological Variables

Accumulating evidence supports a central role of indigenous gut bacteria in the extended hygiene hypothesis [61]. Thus, the intestinal microbiota of young children in Sweden was found to contain a relatively large number of *Clostridium* spp.,

whereas high levels of *Lactobacillus* spp. and *Eubacterium* spp. were detected in an age-matched population from Estonia [113]; this difference might contribute to the lower incidence of allergy in the Baltic countries compared with Scandinavia [114]. A Finnish study likewise reported that allergic infants had more Clostridia and tended to have fewer bifidobacteria in their stools than non-allergic controls [115]. Absence of early postnatal gut colonisation with a normal commensal microbiota dominated by lactic acid-producing bacteria might likewise contribute to the more than eightfold increased risk for food allergy noted in genetically predisposed children delivered by cesarian section [45, 116].

Such observations encourage studies of possible clinical benefits obtained by introducing probiotic bacterial strains from the indigenous gut microbiota to aid immune regulation, and also prebiobic food constituents favouring the growth of such bacteria, not only in an attempt to prevent allergy but also as biological treatment for autoimmunity and IBD [61, 86, 99, 117, 118]. Balancing the immune system by adjusting the gut microbiota is therefore an intriguing avenue for further research.

The feeding and treatment regimens (e.g., antibiotics) to which the newborn is subjected, and also the nutritional state, have a significant impact on the indigenous microbiota and on gut integrity, and may hence modulate the programming of the mucosal immune system [119, 120]. Intestinal colonisation of lactobacilli and bifidobacteria is promoted by breast milk because it acts as prebiotics through its large amounts of oligosaccharides [46, 120], and it may also contain probiotic bacteria [121]. Cell culture studies have suggested that probiotics may be directly immunomodulatory by enhancing the Th1 profile via induction of IL-12, IL-18 and IFN-γ secretion [122, 123]. Also notably, *Escherichia coli* is a strong inducer of IL-10 secretion, apparently derived both from APCs and Treg cells [124, 125]. IL-10 has been shown to be an important suppressive cytokine in the murine gut [126].

This information implies that the indigenous microbiota could have an impact on mucosal homeostasis beyond that of enchancing the SIgA system and promoting a balanced development of Th1 and Th2 cells [127]. Induction of various Treg subsets may also be influenced by additional factors, for instance, hormones [128]; and especially indoor pollution such as cigarette smoke [129] as well as the nature of certain allergens [130] may skew the cytokine balance in favour of Th2 and allergy.

Immunological homeostasis is, in addition, influenced by nutrition; especially lipid intake such as fish oil enriched in polyunsaturated ω-3 fatty acids may protect against the onset of allergy, but apparently not against established allergic disease [131, 132]. Prevention strategies should therefore be targeted early, perhaps even in utero [133]. Animal experiments have suggested that the ratio of ω-6:ω-3 fatty acids is of special importance for neonatal tolerance induction [134]. This ratio varies in breast milk from different parts of the world, which may contribute to the reportedly variable effects of breastfeeding on allergy prevention [135, 136]. A derivative of ω-3 fatty acids termed Resolvin E1 (RvE1) binds with high affinity to a receptor (ChemR23) on APCs, thereby attenuating NF-κB activation [137]. This may explain the apparent anti-inflammatory effect of ω-3 fatty acids.

In the gut and mesenteric lymph nodes, vitamin A may likewise influence mucosal immune function because it can be metabolised to retinoic acid by DCs and enhance

the gut-homing properties of T and B cells [138, 139]. Such intestinal tropism could aid oral tolerance induction. Thus, vitamin A-deficient mice have dramatically reduced numbers of T cells both in the lamina propria and epithelial compartment [138].

Cow's Milk Allergy as a Model for Mucosal Tolerance Induction

Childhood cow's milk allergy is usually a disease of relatively short duration, which makes this disorder an interesting model to explore oral tolerance induction. Direct evidence for a role of $CD4^+CD25^+$ Treg cells has been obtained from feeding experiments in mice [140–144]. Other identified T-cell subsets possibly involved in mucosal homeostasis are Th3 cells that produce TGF-β, and Tr1 cells that mainly produce IL-10 (Fig. 6).

In a recent study of children that outgrew their allergy after a milk-free period (>2 months), we found a population of $CD4^+CD25^+$ T cells with suppressive function in peripheral blood 1 week after initiating in vivo milk exposure. After a similar challenge, this subset was numerically and functionally at a significantly lower level in children still suffering from cow's milk allergy. Thus, outgrown allergy was associated with a lower in vitro proliferative activity induced by bovine β-lactoglobulin in peripheral blood mononuclear cells (PBMCs) and a higher frequency of circulating $CD4^+CD25^+$ T cells [145].

In the tolerant group, 30% of the $CD4^+CD25^+$ T cells expressed the maturation marker CD45R0, while the corresponding figure was only 5% in the persistently allergic children, both before and after in vivo milk challenge. Depletion of $CD25^+$ cells from PBMCs in the tolerant children caused a fivefold increase in proliferation after in vitro stimulation with β-lactoglobulin, suggesting that the induced T-cell subset contributed to the controlled immune response against cow's milk allergens. This suppressive function appeared to be at least partially cell contact-dependent, while only tentative evidence was obtained for the involvement of suppressive cytokines [145].

Collectively, our study provided the first human data to suggest that induction of oral tolerance to dietary antigens is associated with the development of $CD4^+CD25^+$ Treg cells. Our results could either reflect that these cells were generated in mesenteric lymph nodes (see earlier) or, alternatively, that central Treg cells had become mucosally expanded when children outgrew their food allergy. This novel mechanistic insight might aid the development of new diagnostic tools and perhaps promote a future goal of generating Treg cells for prevention or therapy of allergy.

Cord Blood Cells as a Model for Neonatal Immune Induction

Cord blood mononuclear cells (CBMCs) are of fetal origin and have been commonly used to study neonatal immune responses. Because elevated proliferative responses to

allergens in CBMCs are related to subsequent allergy, this characteristic is considered a suggestive marker for identification of high-risk neonates [146]. CBMC proliferation after in vitro stimulation with allergen has been regarded as evidence of priming of the fetal immune system – reflecting immunological memory induced by intrauterine exposure to dietary or environmental allergens transferred from the mother – most likely via swallowed amniotic fluid and antigen uptake in fetal rudimentary gut-associated lymphoid structures [147]. However, it remains controversial whether the proliferating neonatal T cells indeed have been primed in utero [108–110].

We wanted to examine how exposure to LPS during early food antigen encounter might influence the responsiveness of neonatal T cells, including the generation of Treg cells. We also defined possible differences in this response between neonates with a high risk of allergy due to the family history (FH^+) and controls with no apparent allergy heredity (FH^-). CBMCs from both groups were stimulated both with pure LPS and with bovine β-lactoglobulin in the presence of LPS. When eliminating the non-specific stimulatory effect of LPS, the level of CBMC proliferation distinguished the two groups of neonates, suggesting that the specific response to β-lactoglobulin could represent an early allergy predictor [148]. Moreover, when separating the CBMC proliferation related to various T-cell subsets, clear differences between the FH^+ and FH^- groups were revealed.

The relatively decreased level of functional markers expressed on subsets of neonatal T cells in our study [148], suggested delayed maturation of immunoregulatory capacity in the neonates with hereditary allergy risk (the FH^+ group). Therefore, we also investigated whether the development of putative Treg cells was impaired in this group. After stimulation with LPS both without and in the presence of β-lactoglobulin, phenotypes suggestive of Treg cells ($CD25^+$ or $CD25^{high}$ CTLA-4^+) as well as their proliferative activity were determined by flow cytometry [149]. We furthermore examined the T-cell expression of chemokine receptors CCR4 and CXCR3 and the integrin αE (CD103), because these molecules are involved in the immunological synapse formation (Fig. 6), T-cell homing and immune regulation [150].

Our findings in this in vitro model suggested that when the immune system in early life encounters an exogenous antigen together with LPS, generation of $CD25^+$ Treg cells may be induced. Notably, however, such inductive capacity was found to be impaired in neonates with hereditary allergy risk. This result was supported by a similar difference between the FH^+ and FH^- groups when Treg cells were more convincingly identified by a high CD25 expression combined with CTLA-4 – a phenotype that tended to be associated with suppressed T-cell proliferation [149]. Finally, the putative T-cell potential for compartmentalised immunoregulatory programming, as revealed by elevated CCR4 and CD103 expression after antigen stimulation in the presence of LPS, likewise appeared to be jeopardised in neonates with allergy heredity [150].

Altogether, our in vitro results implied that important immune modulation takes place in early life under the influence of both genes and microbial factors such as LPS on encounter a dietary antigen (β-lactoglobulin). However, the CBMC model can only disclose small fractions of the mechanistic events relevant to tolerance induction. The in vivo situation in the gut is obviously much more complex and

probably involves a multitude of suppressive principles to maintain mucosal homeostasis [20, 46]. In addition to $CD25^+$ Treg cells, also the function of intraepithelial lymphocytes (IELs) may be relevant to tolerance as suggested for human IELs more than a decade ago [12, 46] and strongly supported in a recent mouse study [151].

Immunological Paradigm Shift in Coeliac Disease

Mucosal Immune Activation

It remains elusive which of two tissue modelling mechanisms is most important in causing the flat coeliac lesion in the upper small intestine (Fig. 7): (a) negative effects on the surface epithelium that cause cell damage and loss of enterocytes (villous atrophy) followed by compensatory crypt hyperplasia; or (b) positive effects on the crypt cells directly inducing proliferation, with villous atrophy being only 'apparent' as a result of crypt hyperplasia [4]. There is experimental evidence to support both these possibilities, and the lesion apparently reflects a 'joint venture'.

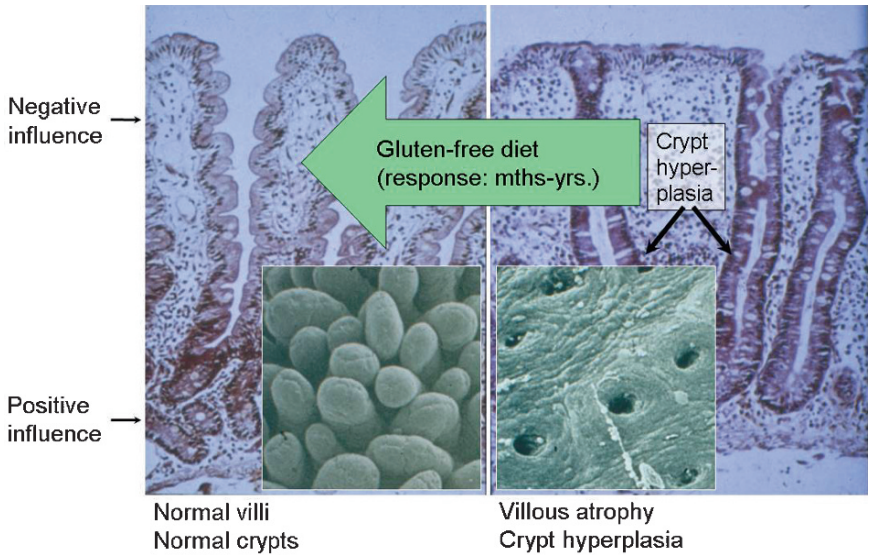

Fig. 7 Histological alterations leading to a flat mucosal lesion (villous atrophy) in coeliac disease (gluten-sensitive enteropathy) may be caused by negative (damaging) effects on the enterocytes, positive (stimulatory) effects on the crypt cells, or most likely a combination of both. Inserts show scanning electron microscopy of normal villi (left) and a flat lesion with crypt hyperplasia where the gland openings reach the luminal surface (right). Large green arrow indicates the healing effect of a gluten-free diet in coeliac patients; such morphological normalization may take from months to years after initiating the treatment

The mucosal lesion of this interesting model disorder most likely involves a complex interplay of many pathophysiological variables on a genetic background [152].

Signs of markedly upregulated cellular and humoral immunity are observed in the untreated coeliac lesion [152]. An important gluten-dependent event is hyperactivation of $CD4^+$ T cells, which can be seen as increased expression of CD25 on T cells both in untreated and partly treated lesions [153]. A reflection of hypersecretion of IFN-γ from activated T cells is the epithelial overexpression of SC/pIgR and HLA-DR (Fig. 8).

The enhanced cellular immune response (Fig. 9, right) can be reproduced ex vivo by exposing duodenal/jejunal biopsy specimens from treated coeliac patients (on a gluten-free diet) to peptides obtained by peptic-tryptic digestion of gluten (or more specifically gliadin, the alcohol-soluble prolamin fraction of gluten). Such in vitro challenge increases strikingly the number of activated ($CD25^+$) gluten-responsive T cells [154], thereby allowing their isolation and cloning [155, 156]. In addition, the active coeliac lesion shows extensive induction of CD25 [152, 153] and HLA-DQ on subepithelial macrophages and DCs [157]. Increased cellular expression of the co-stimulatory molecules intercellular adhesion molecule (ICAM)-1 [158], as well as co-stimulatory CD86 and CD40 [159], is further evidence of

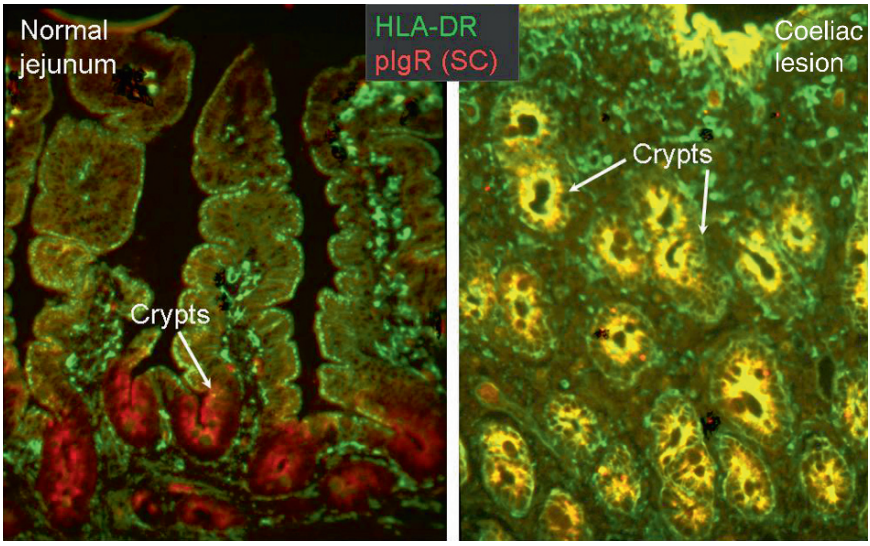

Fig. 8 Paired immunofluorescence staining for HLA-DR and pIgR/SC in tissue section of normal human jejunum (*left*) and jejunal lesion of untreated coeliac disease (*right*). In the normal state, DR is expressed apically by the villous epithelium as well as by numerous macrophages and dendritic cells in the lamina propria, while pIgR/SC expression is typically restricted to the crypt epithelium. In the coeliac lesion the hyperplastic crypts as well as the surface epithelium show coexpression of DR and pIgR/SC (yellow colour). Both components are presumably up-regulated by cytokines, particularly interferon (IFN)-γ, derived from activated T cells and macrophages in the lesion. Immunofluorescence photographs from the author's laboratory

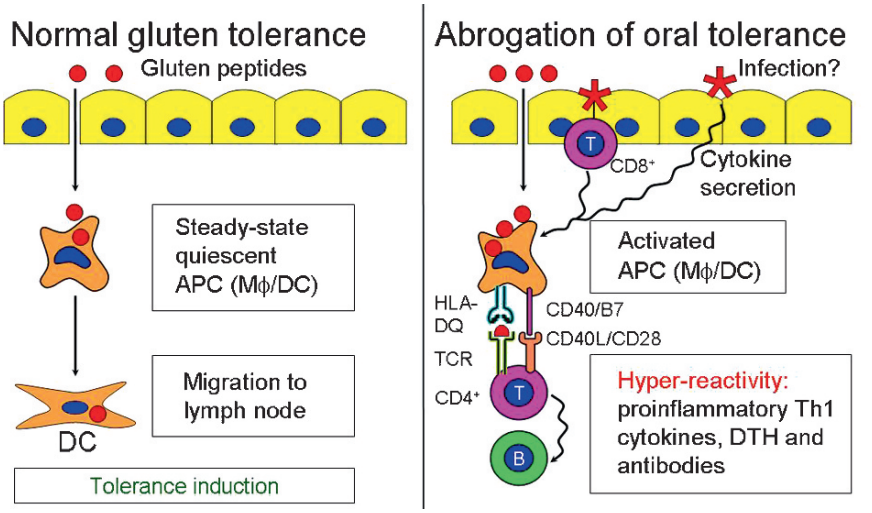

Fig. 9 Depiction of putative mechanisms suggested for intestinal induction of oral tolerance against gluten (*left*) and abrogation of such tolerance in coeliac disease (*right*). In the normal state, when only low-grade activation of subepithelial antigen-presenting cells (APCs) takes place – so that such macrophages (Mφs) and dendritic cells (DCs) are in a steady state (quiescent) – particularly the DCs will migrate quickly to regional (mesenteric) lymph nodes with acquired dietary antigen (e.g., gluten peptides) to become conditioned for tolerance induction. Efficient stimulation of mucosal T cells in the lamina propria is thereby prohibited, which is conducive to mucosal homeostasis. When the epithelium is triggered by gluten peptides or infection, cytokines are released by activated epithelial cells and $CD8^+$ intraepithelial lymphocytes; this causes hyper-activation with HLA-DQ2 expression of APCs. Co-stimulatory molecules such as CD40 and B7 will also be elevated, which allows induction of gluten-reactive proinflammatory $CD4^+$ Th1 effector cells via CD40 ligand (CD40L) and CD28 interaction, respectively. Lamina propria hyper-reactivity may clinically appear as abrogation of tolerance to gluten (coeliac disease), featuring delayed-type hypersensitivity (DTH) and antibody production

mucosal immune activation. It has recently been shown that a putative monocyte-derived $CD11c^+$ mucosal DC subset is most efficient in presenting gliadin peptides to gluten-responsive $CD4^+$ T cells [160].

Induction and Effect of Mucosal Cytokines

The most abundant cytokine secreted by gluten-activated mucosal T cells is IFN-γ, which makes them Th1-like [161, 162]. This cytokine is a major factor in the increased epithelial expression of SC/pIgR and HLA-DR (Fig. 8). Especially together with tumor necrosis factor (TNF)-α [163, 164], IFN-γ may also contribute to increased epithelial permeability [165] and expansion of mucosal PCs [4]. An additional feature of active humoral immunity is constituted by subepithelial complement deposition, which represents an early innate pathogenic insult on the mucosa [166].

The elevated mucosal cytokine production is not sufficient to increase substantially the adhesion molecule repertoire on microvascular endothelium in the coeliac lesion [167]. In IBD lesions, on the other hand, cytokines such as IFN-γ and TNF-α produce intensified endothelial expression of an array of adhesion molecules – rendering leukocyte extravasation rather promiscuous [168]. Thus, coeliac disease is not a classical inflammatory mucosal lesion but rather a reflection of innate and adaptive immune stimulation. While the PC distribution in IBD lesions show a prominent skewing towards proinflammatory IgG production, the IgA-producing mucosal PC phenotype remains remarkably dominating both in treated and untreated disease [4].

Nevertheless, complement activation could be one of several putative initiating innate events in the pathogenesis of coeliac disease, perhaps contributing to a cascade of cytokine release from epithelial and lamina propria cells. Thus, Chowers et al. [169] found that the mucosal mRNA levels for several proinflammatory cytokines increased many thousand-fold following in vivo challenge of rectal mucosa with gluten in coeliac patients, while very little IFN-γ expression was seen initially. Therefore, the characteristic Th1 response to gliadin peptides and other prolamins may develop subsequently only in genetically susceptible individuals [152, 170]; via cell-mediated mechanisms this chronic adaptive response will promote crypt hyperplasia and contribute to the villous atrophy (Fig. 9, right).

Immunogenetics of Coeliac Disease

The T-cell receptor (TCR)α/β of the gluten-reactive mucosal T cells generally shows a molecular restriction that matches the genetic predisposition to coeliac disease and the related gluten-sensitive enteropathy seen in dermatitis herpetiformis. In our part of the world, this susceptibility is mainly associated with a particular HLA-DQ2 heterodimer encoded either *in cis* (DQα1*0,501, β1*0,201) or *in trans* (DQα1*0,505, β1*0,201), and to a minor extent apparently by an HLA-DQ8 (DQα1*03, β1*0302) heterodimer [152, 170]. Other reported associations (e.g., HLA-B8, HLA-DR3, HLA-DR7) reflect linkage disequilibrium on an extended disease-related haplotype. However, the fact that identical twins show some 25% discordance with regard to the clinical presentation of coeliac disease proves that there are pathogenic factors (environmental co-triggers?) in addition to the immunostimulatory prolamins especially represented by gluten-derived gliadin peptides (Fig. 9, right).

Somewhat surprisingly, gluten-reactive $CD4^+$ T-cell clones isolated from individual coeliac patients have been found to become stimulated by prolamins not only from different wheats but also from rye [171]. There are apparently several similar prolamin motifs recognised by the actual TCRα/β, probably comprising 10–15 amino acids that can fit the polymorphic region of the disease-associated HLA-DQ molecules [152, 170, 172] expressed by mucosal APCs (Fig. 9, right). However, a stable 33-mer of α-gliadin (p56–88) is particularly immunogenic after deamidation by tissue transglutaminase [173].

Emerging Two-Signal Model for Coeliac Pathogenesis

Similar to chronic airway allergy [174], coeliac disease is apparently caused by two integrated but principally different mechanisms: signal 1 generated by the innate immune system with as yet undefined genetics, perhaps also involving activated complement as mentioned earlier; and signal 2 representing adaptive immunity mainly driven by HLA-DQ2- or DQ8-restricted $CD4^+$ Th1 cells (Fig. 9, right). Signal 1 is clearly not sufficient to induce chronic disease in the absence of signal 2, but it has an important role in triggering and augmenting signal 2.

Importance of epithelial activation. An increased number of $TCR\alpha/\beta^+$ (mainly $CD8^+$) and $TCR\gamma/\delta^+$ IELs is a hallmark of coeliac disease [175]. These IELs appear to exhibit both cytotoxic and regulatory functions [176–179], but their biological role remains elusive. Notably, proliferation of IELs and activation of lamina propria T cells may occur even in treated patients with no evident ingestion of gluten [180, 181]. Such observations contradict the possibility that epithelial accumulation and activation of IELs is secondary to a $CD4^+$ Th1-cell response in the lamina propria (Fig. 9, right). Importantly, IELs are at least as important for local IFN-γ secretion as $CD4^+$ lamina propria T cells [182, 183], and the intra-epithelial IFN-γ production persists to a variable extent even after introduction of a gluten-free diet [182]. In mice, it has been shown that the IFN-γ-producing capacity of IELs is strain-dependent [184], so there may be related unknown genes in humans involved in the predisposition to coeliac disease.

The antigen specificity of IELs is unknown, although HLA class I-restricted $CD8^+$ T cells reactive with α-gliadin have been identified in blood and intestinal mucosa of treated coeliac patients [185]. These T cells secreted IFN-γ and lysed target cells upon recognition of cognate peptide. Villous atrophy is associated with enterocyte apoptosis [186], perhaps depending on gliadin-specific $CD8^+$ T cells or mediated by the receptor NKG2 on natural killer (NK) cells and $CD8^+$ IELs – interacting with stress-inducible non-classical HLA molecules such as MIC and HLA-E on the enterocytes [187, 188].

Also notably, IELs expressing the NK receptor CD94 specific for HLA-E are increased in coeliac disease [189], and IL-15 can induce the NKG2 signaling pathway [190], while also inhibiting the homeostatic effect of TGF-β [191]. IL-15 is produced both by epithelial and lamina propria cells in active coeliac lesions [192, 193]. Moreover, innate intestinal responses to a selected ‘toxic’ gliadin peptide (p31–49) that does not bind to HLA-DQ2 or HLA-DQ8, resulted in IL-15 production, T-cell activation and enterocyte apoptosis in coeliac patients but not in healthy controls with the disease-predisposition DQ2 genotype [194].

Certain gluten/gliadin peptides have indeed been shown to exert directly innate activation of enterocytes [195], macrophages [196] and DCs [197, 198], but the employed PRRs remain unknown. Enhanced influx of such peptides may be a result of epithelial activation with apical receptor-mediated uptake of antigen-antibody complexes (Fig. 13). Thus, it has been suggested that both toxic and immunostimulatory gliadin peptides, in complex with gluten-specific SIgA, can be subjected to retro-transcytosis which protects them from intracellular epithelial degradation

[199–201]. However, epithelial activation by gliadin may also cause release of zonulin; this endogenous toxin-like molecule acts damaging on the tight junctions, thereby enhancing paracellular uptake of gliadin peptides [202, 203]. Although certain gliadin peptides may directly activate the epithelium, this could also be caused by an (subclinical?) infection (Fig. 9, right).

Pathogenic role of innate signalling. Persistent activation of IELs despite adequate treatment with a gluten-free diet [181], might reflect both a primary and secondary epithelial dysfunction in the coeliac lesion. Because HLA class II-dependent genetic susceptibility alone cannot explain the disease initiation, an increasing focus on innate immunity may provide important clues to the understanding of the complex coeliac pathogenesis. It appears that the primary origin of the disease is activation of the epithelial compartment rather than inflammation which, by definition, depends on activation of the vascular endothelium in the lamina propria (see earlier). Down-regulation of the chemokine receptor CCR9 on both lamina propria T cells and IELs as a sign of activation occurs in coeliac disease (Fig. 10); but after a gluten-free diet this down-regulation is maintained only in the epithelial compartment, reflecting that the IELs are persistently activated even in well-treated patients [204].

In this scenario, signal 1 does not necessarily involve recognition of prolamins as antigens by IELs but, as mentioned above, certain gluten peptides might provide innate triggering of both epithelial and subepithelial cells. Identification of etiological factors and pathogenic mechanisms underlying the epithelial dysregulation in the coeliac lesion could facilitate future curative treatment of this disease.

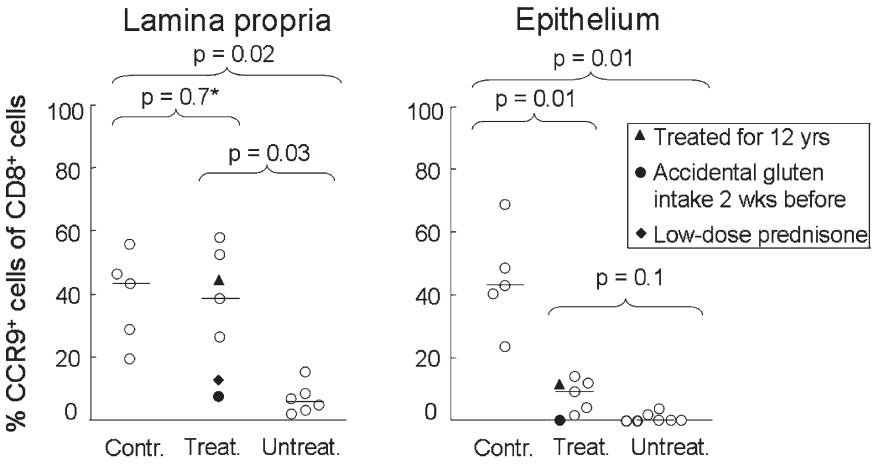

Fig. 10 Persistent activation of the gut epithelium with its intraepithelial $CD8^+$ lymphocytes causes maintained down-regulation of their CCR9 expression in coeliac disease treated with a gluten-free diet. In untreated disease, CCR9 is down-regulated also in the lamina propria (left panel), but the expression level ($CCR9^+$ fraction of $CD8^+$ T cells) increases to that of healthy controls after treatment. A similar increase is not seen in the epithelial compartment (right panel). Adapted from Olaussen et al. [204]

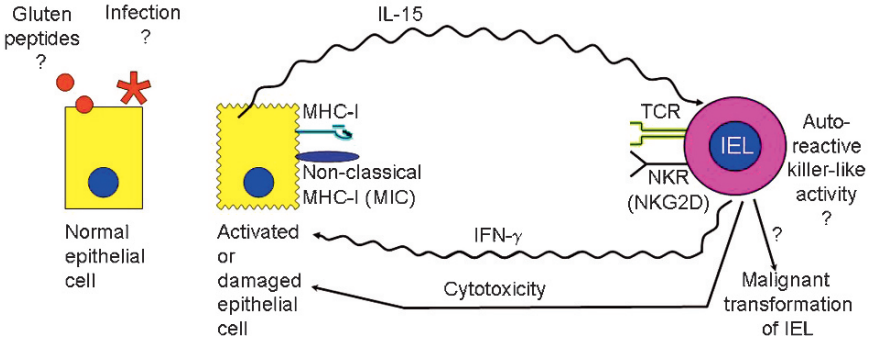

Fig. 11 Model for the putative role of gut epithelial cells and intraepithelial lymphocytes (IELs) in the induction of coeliac disease. In the presence of gluten peptides or infection, subepithelial (not shown) and epithelial coeliac cells become activated and secrete IL-15. Non-classical major histocompatibility complex (MHC) class I molecules (MHC-I) are expressed on the activated epithelium. In turn, IL-15 upregulates natural killer receptors (NKR) variants on IELs, which recognize non-classical MHC-I molecules such as MIC. This could lead to further activation of IELs through two possible mechanisms: (1) reduction of the activation threshold of IELs and their recognition of low-affinity self-antigens; and (2) acquisition by IELs of natural killer-like activity. Hyperactivated IELs can then damage the epithelium by cytotoxicity and interferon (IFN)-γ secretion. In refractory coeliac disease, IELs may also be subjected to malignant transformation. Modified from Green and Jabri [187]

The new paradigm implies a genetically determined primary defect underlying the epithelial sensitivity to gluten peptides and/or other exogenous factors (e.g. microorganisms) inducing mucosal hypersecretion of IL-15 in coeliac patients (Fig. 11). Much work remains to substantiate this new approach to understand the complex pathogenesis of coeliac disease. Emphasis needs to be placed on innate triggering events occurring before activation of gluten-specific $CD4^+$ T cells in the lamina propria. For instance, because IL-15 activates IELs, blocking of this cytokine, which is also expressed in patients with refractory coeliac disease [205], could possibly prevent the intraepithelial development of malignant T-cell lymphoma (Fig. 11).

Conclusions

Many variables influence the induction of peripheral tolerance and productive IgA-dependent local immunity. Some of these variables are reciprocally modulated to achieve mucosal immune homeostasis (Fig. 12). Increased epithelial permeability for exogenous antigens is clearly an important primary or secondary event in the pathogenesis of many diseases, including allergy, coeliac disease and IBD (Fig. 3). The barrier function is determined by the individual's age (e.g. preterm *versus* term infant), genetics, mucus composition, interactions between mast cells, nerves and neuropeptides, concurrent infection and the mucosa-shielding effect of SIgA

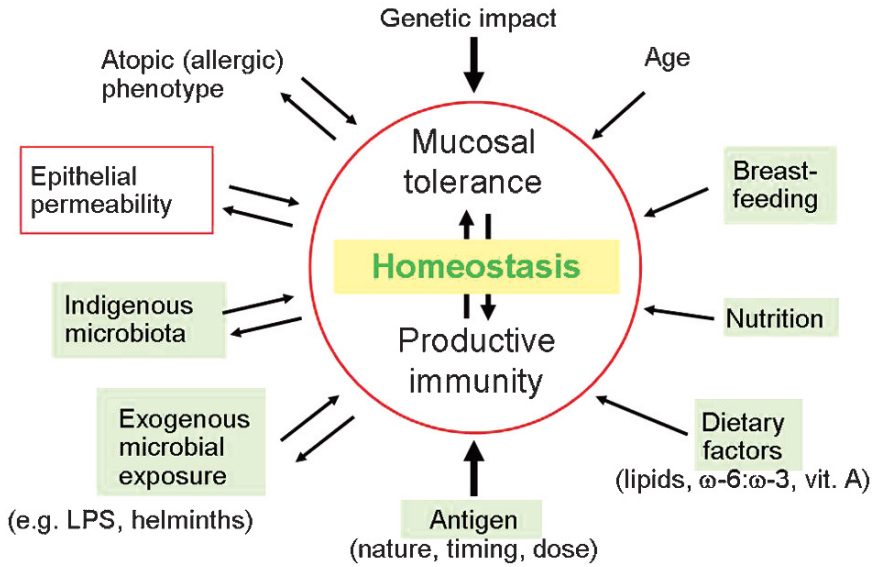

Fig. 12 Summary of variables with an impact on the developing immunophenotype of the infant. Immunological homeostasis in gut mucosa depends on a controlled balance between mucosal tolerance and productive immunity. Several of the components acting on this balance are reciprocally modulated as indicated by bidirectional arrows. Green panels represent components being subject to intervention modalities as discussed in the text. The importance of the epithelial barrier function is highlighted by red frame

provided by breast milk or produced in the infant's gut. The integrity of the epithelium furthermore depends on homeostatic mechanisms such as induction of Treg cells and mucosal tolerance (Fig. 3).

Many studies have suggested that allergy is associated with delayed or impaired development of the IgA system [46]; and an underlying deficiency of antigen-specific SIgA has been documented in a mouse model of food allergy – implying the involvement of secretory antibodies in oral tolerance induction [206]. Indeed, multiple minor dysregulations of both innate and adaptive immunity are reportedly associated with food allergy in children [207]. It is therefore not surprising that several epidemiological reports suggest that breastfeeding protects against allergy [48, 49–52], especially in families with allergic heredity [53]. The same is true for coeliac disease [47]. The remarkable output of SIgA during feeding serves as an optimally targeted passive immunisation of the breast-fed infant's gut [43], and may serve as a positive homeostatic feedback loop [208].

The secretory immune system is clearly of great importance for the epithelial barrier function in the gut because SIgA not only maintains mutualism with the indigenous microbiota but also forms the first line of immune defence against the commensal microbiota and pathogens as well as other harmful agents [23]. In addition, the epithelial barrier function depends on interaction with microbial factors (MAMPs) from the environment and particularly from the indigenous

microbiota, both by direct interaction with PRRs of the epithelium [44] and for induction of mucosal tolerance via mechanisms such as tolerogenic APCs and Treg cells (Fig. 3). In mouse experiments it has indeed been shown that a single immunomodulatory molecule from a commensal gut bacterium can induce crucial modulation and homeostasis of the host's immune system [209].

Despite such expanding knowledge about immune regulation, it remains elusive how the allergic march is driven from food allergy to respiratory allergy on an individual basis [210]. However, clinical problems with certain dietary antigens including gluten are not surprising in view of the relatively short time that has elapsed since the introduction of husbandry and agriculture. The evolutionary adaptation will be slow when the pressure on the genome results from disorders such as allergy and coeliac disease, which generally are not directly deadly on their own. Also, it has to be kept in mind that the current epidemic of allergy is a small price to pay for the remarkable reduction of infant mortality provided by improved hygiene [211].

Hopefully, novel strategies will emerge in the near future to compensate for the missing microbial stimuli apparently needed to induce homeostatic immune regulation. In this respect, molecular refinement of probiotic and prebiotic intervention is an exciting avenue for further research [86].

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Mucosa-Associated Lymphoid Tissue and Dynamics of Lymphoid Cells in the Five Different Compartments in Allergic Diseases

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Introduction

Mucosal surfaces of the respiratory and gastrointestinal tracts are continuously exposed to tremendous amounts of viral and microbial pathogens and allergens. To provide an appropriate protective immune response and maintain a biologically desirable homeostasis at the mucosal epithelium, which forms an important border between the inside and outside of the host, our respiratory and gastrointestinal tracts contain a mucosal immune system [1]. The mucosal immune system can distinguish the nature of antigens encountered via inhalation and ingestion by means of an effective sensing system that includes recently identified antigen pattern-recognition molecules (e.g., toll-like receptor [2]; antigen-specific T-cell receptors; and immunoglobulins, Ig), thereby permitting the induction of protective immune responses when the system recognizes mucosal invasion by microbial pathogens [1]. At the same time, the mucosal immune system is also capable of inducing and creating a quiescent immune condition (unresponsiveness) known as "mucosal tolerance" (e.g., oral tolerance) to environmental antigens, including allergens [3]. Thus, disruption or weakness of mucosal tolerance is a major cause of allergic disease within the surfaces of the body, including the mucosa and the skin.

The mucosal immune system is equipped with a family of mucosa-associated lymphoid tissues (MALTs), which are a component of the host's organized secondary lymphoid tissues and are located in the respiratory and intestinal tracts [1]. Nasopharynx-associated lymphoid tissue (NALT) and bronchus-associated lymphoid tissue (BALT) are MALTs of the upper and lower respiratory tracts, respectively

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[4]. The digestive tract possesses gut-associated lymphoid tissue (GALT), including Peyer's patches (PPs), isolated lymphoid follicles (ILFs), mesenteric lymph nodes (MLNs), cryptopatches, and colonic patches [4]. MALTs have anatomical and histological characteristics that are either unique or common to other systemic secondary lymphoid organs. As is the case for other peripheral lymphoid tissues, MALTs are characterized by their microarchitecture, and particularly the formation of T-cell and B-cell areas (Fig. 1). These lymphocytes migrate into the tissues via high endothelial venues (HEVs) that develop in the MALTs. In comparison with other peripheral lymphoid tissues, MALTs have unique histological characteristics whereby the lymphoid tissue is covered by follicular-associated epithelium (FAE; Fig. 1). FAE secretes several kinds of cytokines and chemokines to induce the recruitment of lymphocytes and dendritic cells (DCs) underneath the epithelium [5]. In addition, the FAE contains professional antigen-sampling cells (microfold cells, M cells) that actively transport mucosally administered luminal antigens to DCs and macrophages in the subepithelial dome (SED) [6]. The presence of antigen-uptaking M cells is necessary to initiate the induction of antigen-specific immune responses in MALTs because the lymphoid tissue lacks afferent lymphatic vessels [7]. Deposition of mucosal antigens thus induces the formation of a germinal center in the B-cell follicles of the MALTs and subsequently induces IgA class switching for the development of antigen-specific IgA-committed B cells. In the

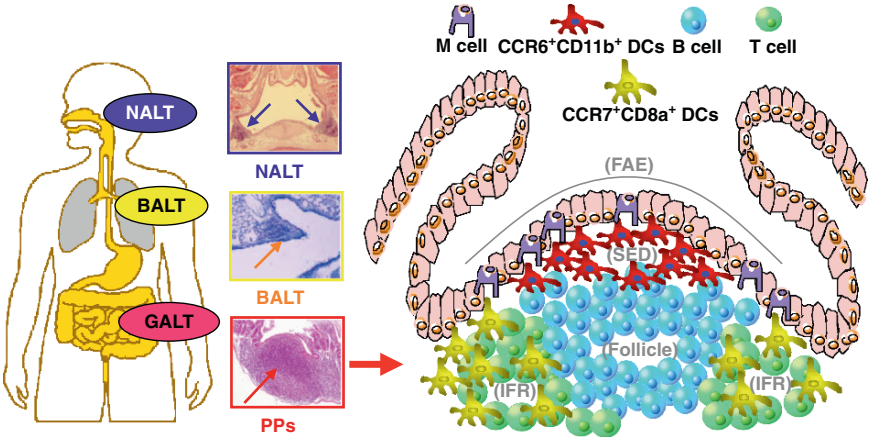

Fig. 1 Anatomy of mucosa-associated lymphoid tissues (MALTs). Nasopharynx-associated lymphoid tissue (*NALT*) is the organized lymphoid tissue located within the nasal cavity. Bronchus-associated lymphoid tissue (*BALT*) develops in the bronchial lower respiratory tract. Gut-associated lymphoid tissue (*GALT*) includes Peyer's patches (*PPs*), isolated lymphoid follicles (ILFs), mesenteric lymph nodes (MLNs), cryptopatches, and colonic patches in the gut. The *right side* of the figure shows the PP component as an example of a MALT. MALTs are generally covered with follicular-associated epithelium (*FAE*) that contains professional antigen-uptaking M cells. The B-cell follicle area is surrounded by a T-cell zone (the interfollicular region, *IFR*). CCL9 and CCL20 produced by PP-FAE and by $CCR6^{+}CD11b^{+}$ dendritic cells (*DCs*) are recruited in the subepithelial dome (*SED*) region. In contrast, $CCR7^{+}CD8\alpha^{+}$ DCs are located in the IFR

T-cell area, naïve $CD4^+$ or $CD8^+$ T cells can differentiate into helper T cells (Th, such as Th1 and Th2) or cytotoxic T lymphocytes (CTLs), respectively [4]. Following antigen priming and stimulation, antigen-primed immunocompetent cells migrate out of the MALTs and enter the blood circulation so they can migrate to distant effector sites, including the lamina propria regions of the aerodigestive tract, glandular tissues, and systemic lymphoid tissues, where they induce protective immunity (e.g., secretory IgA, S-IgA) or quiescent immunity (e.g., oral tolerance). The evidence from studies of MALTs indicates that these are crucial tissues for the induction and regulation of antigen-specific T-cell and B-cell immune responses initiated by the mucosal immune system.

Anatomical and Histological Characteristics of GALT, NALT, and BALT

I GALT

The PP is a well-characterized GALT located antimesenterically in the small intestine. The average number of PPs ranges between 8 and 12 in the murine small intestine. PPs possess efferent lymphatic vessels but no afferent lymphatic vessels, indicating that, in contrast to the other peripheral lymph nodes (pLNs), antigens may not be encountered via the afferent lymphatic vessels [7]. Instead, PPs are covered by FAE that contains professional antigen-sampling M cells responsible for taking up orally deposited luminal antigens. M cells are identified by their unique morphological features, such as the presence of irregular and shortened microvilli at their apical surfaces [6,7], with the formation of a pocket at the basal membrane that contains DCs, macrophages, T cells, or B cells [8]. At least three distinct subsets of conventional DCs have been identified from cell expression of CD11b and CD8α, and their localization is regulated by chemokine receptor expression (e.g., CCR6 and CCR7) in PPs [9]. Indeed, $CCR6^+CD11b^+$ myeloid DCs are localized in the SED region of the PPs, whereas $CCR7^+CD8\alpha^+$ DCs are recruited in the PP interfollicular region (IFR) (Fig. 1) [9]. Among the $CD8\alpha^-CD11b^-$ DCs, CX_3CR1^+ DCs with long dendrites are present within the FAE layer [10]. Infection by *Salmonella* induces the production of CCL20 and CCL9 by the FAE, which consecutively recruit $CCR6^+$ DCs for the initiation of adaptive immune responses against luminal antigens taken up by the M cells [10]. $CD8\alpha^-CD11b^-$ DCs and $CD8\alpha^+CD11b^-$ DCs are capable of inducing a Th1 immune response against viral infection [11]. On the other hand, $CD8\alpha^-CD11b^+$ DCs produce high levels of interleukin 10 (IL-10) [12]. Thus, $CD8\alpha^-CD11b^+$ DCs that produce IL-10 are considered to be among the key regulatory elements for inducing the differentiation of regulatory T (Treg) cells and Th2 cells during the induction of active immunity (e.g., antigen-specific IgA-B cell responses) in the presence of a silent immune system (e.g., oral tolerance). Furthermore, $B220^+Gr1^+CD11c^{int}$ plasmacytoid DCs (pDCs) were found in the IFRs of PPs [13]. PP pDCs are capable of producing large amounts of IL-12, but low levels of type I interferons (IFNs), by

stimulating CpG [14], which might contribute to the generation of appropriate cell-mediated immunity without inducing active inflammation.

PPs possess B-cell follicles with germinal centers under the dome region. In contrast to other systemic secondary lymphoid organs, germinal centers are always present in PPs, except under germ-free conditions [15]. B-cell populations in PPs include IgM^+IgA^- (70%), IgM^+IgA^+ (1%), and IgM^-IgA^+ (4%) cells in naïve mice [16]. Furthermore, Ig^-IgA^+ cells consist of 10% IgA-secreting plasma cell precursors [16]. This evidence suggests that exposure of these tissues to commensal bacteria induces the differentiation of IgA-producing cells in PPs. In addition to the B-cell follicles, some IFRs in PPs contain mainly T cells and DCs (Fig. 1). HEVs are the main entry sites into PPs for circulating lymphocytes. HEVs produce CCL21 and CXCL13, which induce the migration of $CCR7^+$ T cells and $CXCR5^+$ B cells, respectively, into PPs [17,18]. Mucosal addressin cell adhesion molecule-1 (MAdCAM-1) is expressed by the HEVs of PPs. The interaction of MAdCAM-1 and $\alpha4\beta7$ integrin, which is produced by lymphocytes, regulates gut-oriented migration of lymphocytes [19]. FAE also contributes to the recruitment of activated T cells by producing CXCL16 [20]. There is also a lymphatic area on the basal side of the PP. Our recent study demonstrated that IgA^+ plasmablasts in PPs emigrate from the lymph in a manner that depends on the expression of type 1 receptor for sphingosine 1-phosphate (S1P1) [16].

In addition to PPs, the gut mucosal immune system develops ILFs that have been recently characterized as 100 to 200 clusters of miniature versions of the lymphoid structure, each consisting of a B-cell follicle with germinal centers and an FAE that contains M cells [21]. In contrast to the PP, the ILF possesses only a single B-cell follicle that lacks a T-cell area; thus, the ILF has fewer T cells [21]. Interestingly, the B-cell follicle found in the ILF is surrounded by cells that express c-kit and IL-7Rα [21]. Because IL-7Rα^+ cells in ILFs express the retinoic acid receptor-related orphan receptor (ROR) γt, they may be considered as lymphoid tissue inducer (LTi)-like cells [22]. Furthermore, $CD11c^+$ cells are also distributed in the periphery of ILFs [21], although the characteristics of the DCs in ILFs remain to be elucidated. These LTi-like cells and DCs might be involved in inducing formation of the microarchitecture of ILFs or in maintaining the tissue distinction from PPs.

In the large intestine, colonic patches are observed as organized mucosa-associated lymphoid follicles at antimesenteric sites, with an average of 1.4 patches per centimeter of colon length [23]. They have anatomical and histological characteristics similar to those of PPs, including the presence of an FAE that contains M cells as well as distinct T-cell areas and B-cell zones with germinal centers [24]. Interestingly, DCs in colonic patches consist of small populations of $CD11b^+$ DCs and $CD8\alpha^+$ DCs but a high percentage of $B220^+$ DCs [25]. Intrarectal administration of cholera toxin induces the migration of DCs into colonic patches in a CCR7-dependent manner [25]. Inflammatory conditions of the large intestine, such as hapten-induced colitis, enlarge the colonic patches and stimulate the development of germinal centers in the colonic patches [24]. Furthermore, about 50 ILFs have been found in the large intestine [26]. These ILFs are also considered to be sites for the induction of IgA responses [26]. Interestingly, the numbers

of PPs and ILFs in both the small and large intestines of conventional mice are not different from those in germ-free mice, although the architectural maturation differs completely between the two distinctively different microbial conditions [21,26]. These findings indicate that initiation of tissue genesis is not influenced by microbial stimulation signaling.

MLNs, some of the largest lymph nodes in the body, are located in the mesentery and serve as draining lymph nodes for the intestinal mucosal immune system. They possess a central T-cell zone surrounded by a B-cell zone and clusters of follicular DCs (FDCs) [27]. Germinal centers develop in the MLNs of naïve mice [28]. Antigens in the intestinal lumen may enter MLNs and induce the development of germinal centers. Approximately 50% of HEVs in MLNs express both MAdCAM-1 and peripheral node addressin (PNAd) [29]. Because HEVs in MLNs express both mucosa- and systemic-related homing molecules (MAdCAM-1 and PNAdCAM-1, respectively), MLNs may function in interactions between the mucosal and systemic immune systems. Indeed, MLNs induce the expression of CCR9 and $\alpha4\beta7$ integrin on T cells, which enter the intestinal lamina propria [30]. The DCs in MLNs play an important role in generating gut-tropic T cells by producing retinoic acid from vitamin A [31]. In addition, several kinds of DCs migrate into MLNs from the intestinal and systemic compartments (e.g., bone marrow) [32,33]. Thus, MLNs are considered to represent an intersection between the inductive and effector sites via the mucosal circulation system and act as bridges between the mucosal and systemic immune systems.

In 1996, cryptopatches that comprised approximately 1,500 tiny lymphoid clusters were identified in the crypt regions of the villous lamina propria of the murine small and large intestinal mucosa [34]. A large fraction of the lymphocytes in these cryptopatches were progenitors of lymphocytes characterized by the expression of c-kit, IL-7R, and Thy1, but not lineage marker (lin: CD3, B220, Mac-1, Gr-1, and TER-119) [34]. The $c\text{-}kit^{+}lin^{-}$ cells in cryptopatches can differentiate into the T-cell receptors $\alpha\beta$ and $\gamma\delta$ in intestinal intraepithelial T cells [35]. CCR6 is crucial for the recruitment of extrathymic intraepithelial lymphocytes (IELs) from cryptopatches [36]. Therefore, cryptopatches were initially thought to play a role in primary lymphoid organs such as the bone marrow and thymus, and thus to provide a niche for thymus-independent development of IEL T cells [35]. However, this view has been challenged by new evidence that most IELs developed in a thymus-dependent manner [22]. In addition, cryptopatches contain $ROR\gamma t^{+}$ cells, which have recently been considered to be adult LTi-like cells [22]. Thus, cryptopatches may be a major source of LTi-like cells for lymphoid tissue development in the ILFs in some inflammatory conditions.

II NALT

NALT is a MALT located in the upper respiratory tract. In rodents, forehead dissections of the NALT revealed a bell-shaped lymphoid tissue characterized by an accumulation of lymphoid cells on both lateral sides of the nasal cavity (Fig. 1).

Rodent NALT is considered to be the anatomical and functional counterpart of the human Waldeyer's ring [1]. In addition, lymphocyte aggregation accompanied by follicle formation was identified in human nasal mucosa distant from the anatomical location of Waldeyer's ring, especially in the middle conchae of children less than 2 years old [37]. FAE covering the luminal surface of murine NALT contains ciliated columnar epithelial cells, goblet cells, and antigen-sampling M cells [38]. Similar to PPs, NALTs are not equipped with afferent lymphatic vessels. Therefore, NALT M cells are considered to be a principal entry site into the NALT for antigens and pathogens in the nasal cavity. As is the case in PPs, MLNs, and pLNs, HEVs are well developed in NALT [29]. NALT HEVs strongly express PNAd but not MAdCAM-1 [29]. Therefore, the pattern of adhesion molecule expression of HEVs in NALT is similar to that in pLNs rather than those in GALT (PPs and MLNs) [29]. PNAd expression on NALT HEVs is regulated by the LTβR signaling pathway [39]. L-selectin, a ligand of PNAd, is expressed by nearly 90% of NALT lymphocytes [29]. Thus, the interaction of PNAd and L-selectin is important in the extravasation of lymphocytes into NALT through HEVs [29]. Antigen-presenting cells such as DCs are also present in NALT [40]. Following nasal antigen exposure, distinct areas enriched in T cells and B cells have been shown to develop in NALT [40]. In the NALT B-cell region, most B cells belong to the B-2 cell lineage, with few B-1 cells [40]. Although no germinal center is present in the NALTs of naïve mice, nasal administration of antigens (e.g., cholera toxin) induces the formation of a germinal center and an FDC network in the NALT B-cell area [40]. Approximately 20% of mononuclear cells in NALT are $CD3^+$ T cells with a $CD4^+/CD8^+$ T cell ratio of 1.7 [41]. In addition to these effector lymphocytes, which are involved in the induction of active immunity, $Foxp3^+$ Treg cells are also present in NALT [42]. Thus, the tissue is also capable of initiating a mucosal regulatory network for the establishment of mucosal tolerance via the respiratory tract. According to structural and cellular analyses of NALT, the lymphoid tissue is immunologically similar to that of PPs. NALT possesses all the necessary immunocompetent cells for the induction and regulation of both active and quiescent phases of antigen-specific immune responses during nasal exposure to antigens.

III BALT

In 1973, BALT was originally identified as an aggregated lymphoid structure along the bronchus in the lungs of chickens and mammalian species [43,44]. BALT can be observed at the second to fourth branching portions of the bronchial tree in elderly mice (32–40 weeks old), but not in young mice [45]. BALT is not encapsulated, but rather is integrated directly in the lung tissue (Fig. 1). The epithelium that overlies BALT contains *Ulex europaeus* agglutinin-1 $(UEA\text{-}1)^+$ M cells that have morphological and biological characteristics similar to those of the M cells in PPs (e.g., short microvilli, pocket formation, ability to take up antigens) [45]. As in other MALTs (e.g., PPs and NALT), the lymphoid tissue does not possess afferent

lymphatic vessels, but intact efferent lymphatic vessels drain into the regional lymph nodes [46]. Discrete T-cell and B-cell areas that contain DCs can be found in BALT [46]. BALT B cells express mainly IgM and IgA, suggesting the generation of IgA-committed B cells [47]. As in NALT, BALT-associated HEVs express PNAd but not MAdCAM-1 [48]. Interestingly, BALT HEVs differentially express vascular cell adhesion molecule-1 (VCAM-1); this is different from the situation in NALT. Furthermore, the uniqueness of VCAM-1 expression in BALT HEVs contributes to the recruitment of memory T cells [48]. Homing of pulmonary lymphocytes is much more dependent on the interaction between VCAM-1 and β1 integrin than in other lymphoid organs. In the case of pulmonary infection, endothelial VCAM-1 expression mediates the migration of β1 integrin$^+$-activated/memory T cells to the lungs [49]. Therefore, the immune system of the lungs, including BALT, can be distinguished from other systemic and mucosal tissues by the pattern of integrin expression. These anatomical and immunological features of BALT indicate that it qualifies as a member of the MALT family, which is involved in the induction and regulation of antigen-specific immune responses in the respiratory tract and may contribute to protection against respiratory infections. Furthermore, it is notable that BALT contains catecholaminergic nerve fibers and related beta-adrenergic receptors [50]. The nervous system is deeply associated with respiratory physiology, as in the regulation of smooth muscles and vessels in airways [51]. However, the immunological role of nerve fibers in BALT is not yet understood.

Development (Organogenesis) of MALTs

I GALT

The first step of PP genesis is the formation of clusters of VCAM-1$^+$ intercellular adhesion molecule-1 (ICAM-1)$^+$ stromal cells (also called PP organizer cells, PPo cells) at the site of tissue development in the small intestinal wall at embryonic day 15.5 [52]. It was recently demonstrated that PPo cells produce artemin (ARTN), which is known to be a neurotrophic factor derived from the glial cell line (Fig. 2-1) [53]. Its receptor (RET, the receptor tyrosine kinase) is known to critically regulate the development of the enteric nervous system [54]. RET$^+$ hematopoietic cells have been observed in the embryonic intestine and exhibit a unique phenotype (CD45$^+$, CD4$^-$, CD3$^-$, IL-7Rα$^-$, c-kit$^+$, and CD11c$^+$) by means of the expression of cell-surface markers (Fig. 2-1) [53]. RET$^+$ cells produce lymphotoxin (LT) β to induce the activation signal transmitted by LTβR that is expressed on the surfaces of PPo cells in a paracrine manner [53]. Because the LTβR signaling pathway is essential for the development of PPs at the stage of intercellular interactions between PPo cells and IL-7Rα$^+$CD3$^-$CD4$^+$CD45$^+$ PP inducer (PPi) cells [55], the finding further emphasizes the biological importance of the inflammatory cytokine signaling cascade as early as the stage of intercellular interactions between RET$^+$ hematopoietic cells and PPo cells before the genesis contribution from PPi cells. The molecular

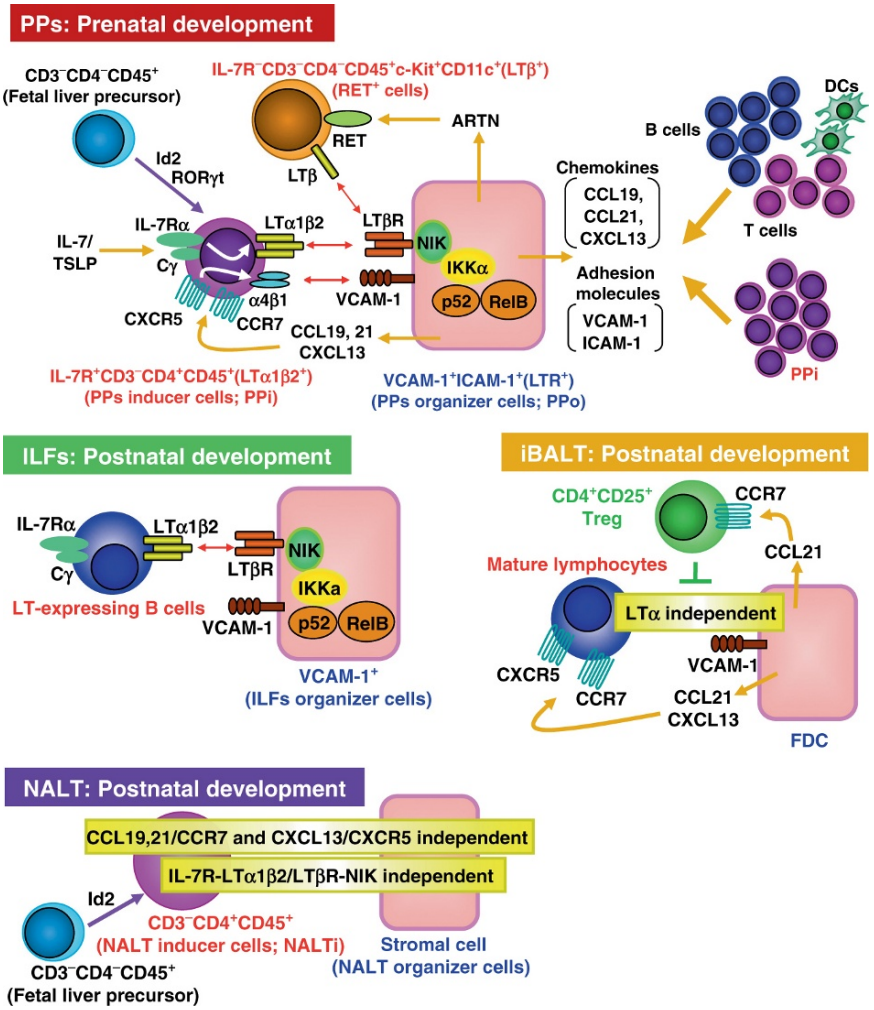

Fig. 2 Molecular and cellular programs involved in MALT organogenesis. Genesis of Peyer's patches (PPs) is initiated during late embryogenesis (**2-1**: prenatal development). *RET* is expressed by LTβ⁺CD11c⁺ cells and migrates to the site of tissue genesis in response to artemin (*ARTN*) produced by VCAM-1⁺ PP organizer (*PPo*) cells. CXCR5 and CCR7 are expressed by interleukin (IL)-7Rα⁺ PP inducer (*PPi*) cells. Migration of PPi cells to PPo cells is regulated by receptors that respond to lymphoid chemokines, including CCL19, CCL21, and CXCL13. Signaling through CXCR5 on PPi cells activates α4β1 integrin, which binds strongly to VCAM-1 and thus interacts with PPo cells. PPi cells are activated by IL-7Rα/Cγ signaling and then express membrane-bound LTα1β2, which stimulates PPo cells through the LTβR/NIK/IKKα/p52/RelB alternative pathway. Activated PPo cells produce chemokines and adhesion molecules that form a positive feedback loop to accumulate PPi cells at the site of tissue genesis, leading to the subsequent migration of leukocytes. The genesis of ILFs occurs after birth (**2-2**: postnatal development). B cells that express lymphotoxin (*LT*) are required for activation of the follicular DCs (FDCs). Expression of IL-7Rα and Cγ is also involved in the genesis of ILF tissue. The NIK-mediated alternative pathway is also essential for the formation of ILFs. NALT organogenesis is initiated postnatally by

interactions between RET and ARTN should now be recognized as the earliest step in the initiation of PP genesis.

PPi cells accumulate in the PP anlagen in the embryonic intestine [56]. PPi cell is a subset of LTi cells that initiates the development of secondary lymphoid organs, including pLNs and NALT, as well as PPs [57–59]. PPi cells are believed to differentiate from hematopoietic stem cells in the fetal liver [58]. Inhibitor of DNA binding/differentiation 2 (Id2) and RORγt are indispensable in the differentiation of PPi cells and pLN inducer (pLNi) cells from the fetal liver [60,61]. Thus, $Id2^{-/-}$ and $ROR\gamma t^{-/-}$ mice lack PPs and pLNs because of their deficiency of PPi cells and pLNi cells [60,61].

RET–ARTN-activated PPo cells interact with PPi cells at E16.5–E17.5 [52,53,58]. PPi cells produce a membrane-type LTβR ligand, LTα1β2, that immediately interacts with neighboring $LT\beta R^+$ PPo cells [56]. IL-7Rα expressed on the inducer cells plays a critical role in the expression of LTα1β2 on PPi cells [56], because the IL-7 produced by epithelial cells appears to stimulate PPi cells. This view is supported by the observation that the formation of PPs is completely impaired in $IL\text{-}7R\alpha^{-/-}$ mice [62] and mice deficient in the common γ (Cγ) chain that forms a complex with the IL-7Rα chain to form a functional receptor [63]. LTα1β2 production induced on PPi cells by the IL-7/IL-7R cascade leads to the activation of $LT\beta R^+$ PPo cells and subsequent production of lymphoid chemokines such as CXCL13 and CCL19 for further recruitment of PPi cells that express the corresponding receptors of CXCR5 and CCR7 in the PP anlagen (Fig. 2-1) [64]. Several studies in gene-manipulated mice have shown that $CXCL13^{-/-}$ or $CXCR5^{-/-}$ mice have few PPs, but $CCR7^{-/-}$ mice have normal numbers of PPs [65–67]. Therefore, the interaction between CXCL13 and CXCR5 is much more important for the initiation of PP genesis by PPi cells than the interaction between CCL19 and CCR7.

Not only chemokines and chemokine receptors but also members of the integrin family mediate interactions between PPi cells and PPo cells. The α4β1 integrin expressed by PPi cells is activated by CXCL13 secreted by PPo cells (Fig. 2-1) [68]. The number of PPs decreases if an activated form of β1 integrin is inhibited in vivo [68]. On the other hand, although PPi cells and PPo cells express α4β7 integrin and its receptor (MAdCAM-1), respectively, blocking the MAdCAM-1–α4β7 integrin interactions does not influence the number of PPs [68]. Therefore, the VCAM-1–α4β1 integrin interaction is also important for the regulation of cell-to-cell contacts between PPi cells and PPo cells, and following the initiation of PP development [68]. This evidence demonstrates that reciprocal interactions between the inducer and organizer cells are regulated by multiple layers of signals mediated

$CD3^-CD4^+CD45^+$ NALT inducer (*NALTi*) cells. NALTi cells do not express either CCR7 or CXCR5, and their migration to the organogenesis site is independent of the lymphoid chemokines. Unlike that of the other tissues in the MALT family, the genesis of NALT is independent of IL-7Rα- and LT-mediated cytokine signaling cascades. The formation of inducible bronchus-associated lymphoid tissue (*iBALT*) is induced postnatally. The lymphoid chemokines are essential for the genesis of iBALT, whereas LT is dispensable. $CCR7^+$ Treg (regulatory T) cells have been shown to inhibit the formation of iBALT

by the chemokine and integrin families, which are essential for the formation of PPs, and that the loss of any part of either of the signaling programs is sufficient to disrupt the development of secondary lymphoid tissue (Fig. 2-1) [57,58].

In the process of PP organogenesis, an LTβR-mediated signaling cascade plays a key role from the beginning of the first steps to late in the process; thus the inflammatory cytokine receptor appears to be a key element in development of the tissue. LTβR induces the activation of NF-κB by the canonical and alternative pathways [69]. In the canonical pathway, the degradation of IκBα induces the nuclear translocation of RelA and p50 dimers [70]. This pathway is rapidly activated and transient [70]. In the alternative pathway, NF-κB-inducing kinase (NIK) processes the inhibitory NF-κB2 p100 precursor protein to form the p52 protein and results in a delayed but sustained activation of primarily RelB-containing NF-κB dimers [71]. The alternative pathway has been implicated in the production of organogenesis-associated chemokines, such as CXCL13, CCL19, and CCL21, and it thereby plays a central role in the development of secondary lymphoid organs (Fig. 2-1) [72]. When the genesis of PPs was examined in gene-manipulated mice with a defect in the alternative pathway, no PPs were developed in the $NIK^{-/-}$, $relB^{-/-}$, and $nfkb2^{-/-}$ mice [73,74]. Therefore, the alternative NF-κB pathway induced by LTβR is essential for PP genesis (Fig. 2-1). In addition, a recent report demonstrated that the canonical pathway mediated by tumor necrosis factor-associated factor 6 (TRAF6) is also involved in PP genesis [75]. Thus, the genesis of PPs operates by both the canonical and the alternative LTβR pathways.

In comparison with the information available on the molecular and cellular mechanisms responsible for PP organogenesis, less information is currently available on the genesis of ILFs and cryptopatches. In general, our present knowledge indicates that the genesis of ILFs generally follows the same rules as in PP organogenesis, but with certain differences. As in the case of PPs, ILFs are absent in $C\gamma^{-/-}$, $LT\alpha^{-/-}$, and *aly/aly* mice (defective NIK mutant) [21]. Signaling mediated by IL-7Rα is also important for ILF genesis as the sizes and numbers of ILFs are reduced in IL-$7R\alpha^{-/-}$ mice [21]. In contrast to the prenatal initiation of PP genesis, ILFs develop postnatally, and they can be first detected 7 and 25 days after birth in BALB/c and C57BL/6 mice, respectively [21]. Since the process of ILF genesis takes place postnatally, environmental antigens may provide the initial signals that trigger the development of ILFs. However, the number of ILFs is not less in germ-free mice than in conventional mice [21]. Interestingly, although the initiation of PP genesis requires PPi cells and is independent of lymphocytes, LT-expressing B cells are required instead of PPi cells for ILF genesis (Fig. 2-2) [76]. It has recently been reported that the expression of RORγt is also essential for the formation of ILFs, suggesting that PPi-like $ROR\gamma t^{+}$ cells might also be involved in ILF genesis [22]. $ROR\gamma t^{+}$ LTi cells are found in ILFs and cryptopatches in the adult murine intestine [22], suggesting that cryptopatches induce the development of ILFs in response to an inflammatory signal [22]. This hypothesis is consistent with the increased number of ILFs under abnormal microfloral conditions in the small intestine [77].

As is the case for ILFs, the development of cryptopatches occurs postnatally, and it can be first observed in 14-day-old C57BL/6 mice [21]. It is difficult to understand the development of cryptopatches, because they are absent in $LT\beta R^{-/-}$ mice

but present in *aly/aly* mice, which lack the alternative LTβR pathway [78]. This evidence indicates that the genesis of cryptopatches may require the canonical NK-κB pathway rather than the alternative pathway. Moreover, it remains controversial whether cryptopatches can develop in LTα$^{-/-}$ mice [21,78]. As indicated earlier, the cellularity of cryptopatches is different from that of the other GALT members, as this tissue is full of premature-stage c-kit^{+}lin^{-} cells instead of mature T cells and B cells. It is still not clear whether so-called LTi cells (e.g., PPi cells or pLNi cells) are essential for the initiation of cryptopatches or whether some c-kit^{+}lin^{-} cells behave as inducer-type cells. In RORγt$^{-/-}$ mice the genesis of cryptopatches is impaired, as is the case for other members of the GALT family, including PPs and ILFs; this finding suggests a role for inducer cells in the organogenesis of cryptopatches [79].

II NALT

Even though two major arms of the mucosal inductive tissues, PPs and NALT, located in the aerodigestive tract share the immunobiological characteristics of microarchitecture, cellularity, and function, distinctive chronological differences are apparent between the two lymphoid tissues. NALT develops postnatally and the anlagen of NALT, with an accumulation of CD3^{-}CD4^{+} inducer cells, are detected 10 days after birth [41]. Although organogenesis of NALT is initiated postnatally, as in the case of ILFs, tissue development is normal in germ-free mice (unpublished data). Therefore, inhaled bacteria and environmental stimulation are not necessary for the initiation of NALT genesis. NALT genesis is initiated by nasal CD3^{-}CD4^{+}CD45^{+} cells (NALT-inducer cells, NALTi cells) that have distinctive biological features that separate them from the well-characterized LTi cells [41]. In contrast to the PPi- and pLNi-mediated tissue genesis cytokine requirements for PPs and pLNs, respectively, the initiation of NALT genesis is totally independent of IL-7Rα- and LTβR-mediated signaling pathways (Fig. 2-2) [41,80]. However, the specific biologically active molecules, including cytokines and chemokines, used by NALTi cells for the induction of NALT genesis remain unidentified.

Lymphoid chemokine CXCL13/CXCR5 signaling is essential for the induction of PP genesis by PPi cells as blocking of its signaling pathway disrupts tissue genesis [58]. However, our previous study demonstrated that lymphoid chemokines (e.g., CXCL13, CCL19, and CCL21) are not involved in the accumulation of NALTi cells in the NALT anlagen [40]. Neither CXCR5 nor CCR7 is expressed by NALT cells, whereas both chemokine receptors are used by PPi cells [40]. Furthermore, the initiation of NALT genesis occurs normally in gene-manipulated mice that lack these chemokines [40]. Two transcriptional regulators (Id2 and RORγt) are essential for tissue genesis in secondary lymphoid organs. Id2$^{-/-}$ mice lack the formation of both PPs and NALT because of the deficiency of the differentiation of LTi [41,60]. Although RORγt is required for the development of LTi cells, and RORγt deficiency prevents the development of secondary lymphoid tissues [61], normal-sized NALT develops in RORγt$^{-/-}$ mice [80]. These distinctive requirements

of organogenesis-associated transcription regulators and the chemokine family indicate that NALTi cells and PPi cells may belong to different subpopulations among the $CD3^{-}CD4^{+}CD45^{+}$ LTi cells. PPi cells that express CXCR5 and CCR7 are regulated by both Id2 and RORγt, whereas NALTi cells that lack CXCR5 and CCR7 expression are controlled by Id2 but not by RORγt (Fig. 2-2).

In contrast to the initiation of NALT genesis, the LTβR signaling pathway and lymphoid chemokines are crucial for the maintenance of the NALT microarchitecture [40,80,81]. Lymphoid chemokines (CXCL13, CCL19, and CCL21) are produced by NALT in adult mice [40,81]. NALT B cells and T cells express lymphoid chemokine receptors (CXCR5 and CCR7, respectively) [40,81]. Histological analysis reveals that, in B-cell areas, a germinal center and an FDC network do not develop in the NALT of $CXCL13^{-/-}$ mice [40,81]. Therefore, CXCL13 is essential for the formation of the B-cell area, including its germinal center and FDC network. Furthermore, the formation of the NALT T-cell area is regulated by interactions between CCL19/CCL21 and CCR7 because *plt/plt* mice, which are deficient in CCL19 and CCL21, lack a T-cell area [40,81]. The NALT of $LT\alpha^{-/-}$ mice has many fewer lymphocytes than that of wild-type (WT) mice, and it has a disorganized structure [41, 80]. Expression of lymphoid chemokines and PNAd is almost absent in the NALT of $LT\alpha^{-/-}$ mice [82]. Furthermore, lack of IκB-kinase-α (IKKα) function results in impaired PNAd expression in the HEVs of NALT and pLNs [83]. IKKα is an essential molecule for the alternative pathway but not the canonical NF-κB pathway in LTβR signaling [70]. Thus, normal development of NALT requires an LTβR-induced alternative NF-κB pathway that regulates the production of lymphoid chemokines and PNAd by the NALT.

III BALT

In general, BALT is rarely detected in young adult mice [84]. However some experimental mouse models show that BALT development is induced by viral infection and by allergic inflammation in the lungs [85]. In humans, BALT was found in 44% of children who were not suffering from pulmonary disease, but the presence of BALT in healthy adult lungs is controversial [84]. However, the development of BALT is induced in some kinds of pulmonary diseases, such as interstitial pneumonia in patients with rheumatoid arthritis [86]. Although the presence of BALT in normal mice and healthy humans is still debated, BALT has been shown to develop 5 days after birth in the lungs of $CCR7^{-/-}$ mice [87]. The formation of BALT is inhibited by the presence of $CCR7^{+}$ Treg cells (Fig. 2-2) [87]. These results suggest that BALT is a tertiary lymphoid tissue induced by exogenous inflammatory stimulation. To support this view, inducible BALT (iBALT) was identified as lymphoid aggregations at the site of the bronchial mucosa in mice with pulmonary influenza virus infection [85]. Allergic asthma induced by ovalbumin also leads to iBALT development in mice [88]. In addition to biological stimulation, exposure to inhaled chemical agents such as diesel

exhaust has been shown to induce iBALT in mice [89]. Interestingly, iBALT development occurs in mice that lack the organogenesis-associated cytokine LTα (Fig. 2-2) [85]. Furthermore, iBALT induced by the influenza virus in $LT\alpha^{-/-}$ mice produces CXCL13 and CCL21 but does not have PNAd-expressed HEVs and FDC [85]. Therefore, even though expression of PNAd and FDC network formation depend on LTα, lymphoid chemokines are produced by iBALT in an LTα-independent manner [85]. As in the case of NALT organogenesis, iBALT develops in $CXCL13^{-/-}$ mice and in *plt/plt* mice [90]. B-cell follicles and FDC do not develop in iBALT in $CXCL13^{-/-}$ mice [90]. The T-cell area in iBALT is disorganized in *plt/plt* mice [90]. These findings indicate that the dependence of iBALT development on lymphoid chemokines is similar to that in NALT. However, despite the presence of NALT in $CXCL13^{-/-}$*plt/plt* mice, iBALT was not observed [90]. Therefore, the cooperation of CXCL13 and CCL21 may be essential for iBALT development, in contrast to NALT development. As mentioned earlier, LTi cells are essential for the organogenesis of secondary lymphoid tissues such as GALT and NALT. In contrast, activated B cells and T cells, rather than LTi cells, appear to be important for the initiation of iBALT formation [90].

Immunological Characteristics of MALTs and the Induction of Protective Immunity Against Pathogens

I GALT

The intestinal epithelium produces enormous amounts of S-IgA in the gut lumen (approximately 40 mg/kg body weight/day in adult humans) [91]. These S-IgAs include both the so-called innate and acquired antibodies, which contribute to the maintenance of intestinal homeostasis and the induction of protective immunity against specific mucosal pathogens, respectively [92]. Innate S-IgA can thus react with intestinal pathogens such as *Salmonella typhimurium* [92]. The potential role of innate S-IgA in prevention of the spread of intestinal pathogens throughout a population, as well as the protection of individuals against infection caused by the bacteria, has also been demonstrated [92]. CX_3CR1^+ DCs are located in the SED of PPs in uninfected mice [10,93] and may help maintain innate immunity and homeostasis in the intestine by means of the induction of innate S-IgA [10,93]. Of course, antigen-specific S-IgA antibodies represent a major part of the adaptive immune response in the intestine [1,3,4]. For induction of the antigen-specific IgA antibody response, GALT (including PPs and MLNs) has been considered to be the key inductive tissue [1,4]. Thus, the delivery of an oral vaccine antigen to PPs has been shown to be an effective immunization regimen for the generation of antigen-specific IgA-mediated protective immunity [94]. M cells located in the FAE of PPs are a gateway cell population responsible for sampling antigens from the gut lumen. Mucosally administered antigens are thought to be taken up and to undergo

transcytosis by M cells without any processing, and they are immediately delivered to antigen-presenting cells, including DCs located in the pockets of M cells, as well as the SED of PPs [8]. For the induction of antigen-specific immune responses, $CCR6^+$ DCs found in the SED region may play a key role, because $CCR6^+$ DCs are recruited into SED regions of PPs after oral infection with *S. typhimurium*, resulting in the induction and activation of pathogen-specific T cells [10]. Our recent study directly showed that oral immunization targeted at M cells effectively induces antigen-specific production of fecal S-IgA and serum IgG [95]. In this study, monoclonal antibody NKM16–2–4, which is specific to M cells, was developed and used as the M cell-targeting vehicle. When a chimeric vaccine formulation that combined NKM16–2–4 and botulinum toxoid was given orally together with a mucosal adjuvant (i.e., cholera toxin), vaccinated mice exhibited botulinum-specific S-IgA and serum IgG antibody responses that provided full protection against botulinum toxin challenge [95]. Taken together, these pieces of evidence provide a theoretical context in which GALT or PPs with M cells are important inductive sites for the initiation of antigen-specific IgA immune responses in the intestine and the creation of a first line of defense against mucosal pathogens [1,3,4].

The other immunologically important aspect of GALT is that it provides a molecular environment for IgA class-switch recombination (CSR) [1]. IgM^+ B cells in PPs differentiate to IgA^+ B cells and produce IgA in response to stimulation by transforming growth factor-β (TGF-β) and mitogenic stimulation (e.g., lipopolysaccharide) [96,97]. Activation-induced cytidine deaminase (AID) is an essential molecule for CSR and somatic hypermutation [98]. Indeed, AID expression is predominantly observed in PPs and ILFs of the intestine [99]. Several reports have shown that unique subsets of DCs in PPs regulate S-IgA production [12,100,101]. $CD11b^+$ DCs in PPs secrete higher levels of one IgA-inducing cytokine (IL-6) than do DCs in the spleen [100]. Therefore, higher amounts of IgA are produced by B cells cocultured with $CD11b^+$ DCs from PPs than with those from the spleen [100]. Although IL-6 is an important cytokine for inducing the differentiation of IgA-committed B cells into IgA-producing cells when B cells are cocultured with $CD11b^+$ DCs from PPs, IL-6 is not the only cytokine capable of supporting IgA production [100]. Thus, other factors might be involved in IgA production regulated by $CD11b^+$ DCs in PPs. Recently it was shown that among the $CD11b^+$ DCs in PPs, DCs that produce tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS) play a crucial role in the induction and regulation of the IgA response [101]. The TNF superfamily, a proliferation-inducing ligand (APRIL), and B-cell-activating factor (BAFF) induce IgA isotype switching in B cells [102,103]. Notably, DCs that produce TNFα/iNOS regulate IgA responses by means of APRIL and BAFF [101].

Naïve T cells in PPs differentiate into Th1 or Th2 cells capable of mediating cytotoxic and humoral immunity, respectively [1,4]. Lymphoid $CD8\alpha^+$ DCs and $CD11b^-CD8\alpha^-$ DCs produce IL-12p70 to generate IFN-γ-producing Th1 cells [12]. Rapidly induced Th1 immune responses are required for protection against primary infection by *Eimeria vermiformis* in the intestine [104]. The failure of rapid Th1 responses in PP-null mice increases the susceptibility of these mice to infection

with *E. vermiformis* [104]. Thus, PPs are important inductive sites for rapid Th1-mediated protective immunity against infection by parasites in the gastrointestinal tract. In contrast, myeloid $CD11b^+$ DCs produce IL-10 to induce the differentiation of Th2 cells that are capable of secreting IL-4 and IL-10 for the induction and regulation of humoral immunity [12]. Since this evidence shows that distinct subsets of the DCs found in PPs play a pivotal role in priming Th1/Th2 differentiation, PPs appear to be major sites for the induction and differentiation of antigen-specific IgA-committed B cells, as well as for the generation of antigen-specific effector T cells involved in both humoral and cell-mediated immunity.

ILFs are also considered to be inductive sites for the generation of immune responses to luminal pathogens, because antigen-specific IgA B cells are generated in ILFs, as is the case in PPs [105]. Polyclonal populations of follicular B-2 B cells in ILFs differentiate to IgA-producing plasma cells [105]. As fecal IgA levels do not decrease in mice that lack PPs but possess ILFs, ILFs appear to be capable of providing a compensatory immune response when there are insufficient PPs [106]. In consistent with this results, PP-null mice, as a result of blockage of LTβR signaling, exhibit an increasing number of mature ILFs that contain germinal centers and large FDC networks [106]. However, antigen-specific S-IgA is not completely supplemented by ILFs in the intestines of PP-null mice [107]. Therefore, ILFs may be compensatory sites for innate and acquired IgA responses in the gastrointestinal tract.

Antigen-specific IgA-committed B cells generated in PPs and ILFs migrate to distant mucosal effector sites by following a unique mucosal migration pathway (Fig. 3) [1,3,4]. Chemokine receptors in the CCR9 and CCR10 family are important gut-imprinting molecules for the migration of IgA-committed B cells to distant intestinal lamina propria regions [108]. Thus, mucosal IgA^+ B cells that originate from PPs preferentially express CCR9 and CCR10 [109]. The CCR10 ligand CCL28 is widely expressed by the mucosal surface of the intestinal and respiratory tracts [110]. Thus, $CCR10^+IgA^+$ B cells migrate to distant mucosal surfaces in response to the chemotactic activity of CCL28 [111]. The CCR9 ligand CCL25 is preferentially expressed by the mucosa in the small intestine but not in the respiratory tract [109]. Molecular interactions between CCR9 and CCL25 permit selective migration of IgA^+ B cells from the GALT into the intestinal lamina propria [111]. These findings indicate that selective chemokines and their receptors dictate both the commonness (e.g., CCL28/CCR10) and compartmentalization (e.g., CCL25/CCR9) of mucosal lymphocyte migration (Fig. 3). Mucosal DCs located in PPs and MLNs play a critical role in the processing of gut imprinting of lymphocytes in the GALT [112]. Thus, DCs from PPs and MLNs, but not those from pLNs, produce retinoic acid, which induces the expression of gut-tropic homing molecules such as CCR9 and $\alpha4\beta7$ integrin on T cells and B cells (Fig. 3) [113,114]. Indeed, mice deficient in vitamin A lack intestinal IgA^+ B cells in their laminae propriae [114]. Thus, the intestinal mucosal immune system is equipped with highly sophisticated machinery for a unique cellular migration system that bridges the inductive and effector sites.

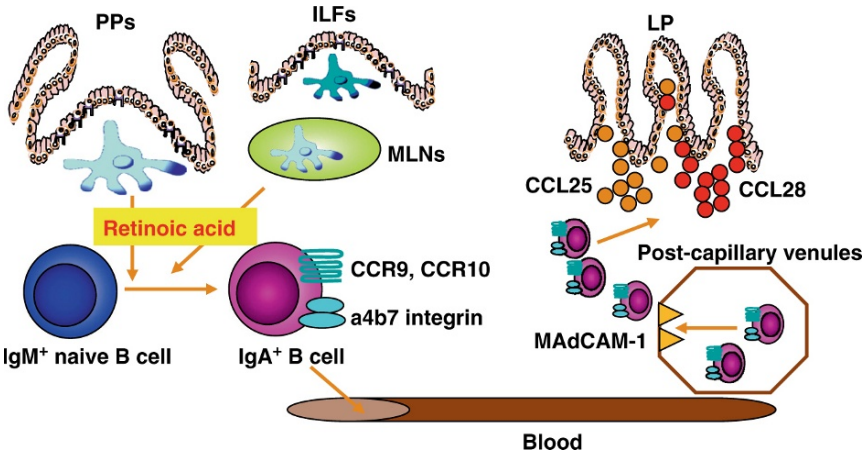

Fig. 3 Induction of an antigen-specific immune response in the intestine. Mucosal B cells and T cells express CCR9 and α4β7 integrin in response to the stimulatory signal provided by DCs in PPs or MLNs. CCR9 and α4β7 integrin are involved in the preferential trafficking of mucosal lymphocytes from PPs to the gut. This gut-imprinting function of mucosal DCs depends on their ability to produce retinoic acid for the activation of mucosal lymphocytes. Mucosal B cells and T cells activated in the PPs, ILFs, and MLNs also express CCR10. These mucosal-activated lymphocytes leave the PPs and first enter the systemic circulation via MLNs and the thoracic duct. Postcapillary venules in the intestinal lamina propria express MAdCAM-1, which recruits circulating α4β7 integrin$^+$ cells that originate in the PPs. CCL25 and CCL28 are produced by lamina propria epithelial cells and preferentially attract the migration of CCR9$^+$CCR10$^+$ lymphocytes to the effective intestinal lamina propria tissue

In addition to the GALT-dependent IgA induction system, recent accumulated evidence has suggested the presence of a GALT-independent IgA induction mechanism in the intestine [4], where antigen-sampling UEA-1$^+$ cells called villous M cells are found outside PPs and ILFs [115]. Villous M cells are capable of taking up intestinal pathogens (e.g., *S. typhimurium*, *Yersinia pseudotuberculosis*, and *Escherichia coli* that express invasin) [115]. Myeloid DCs in the lamina propria also directly sample luminal antigens [93]. Interestingly, the subset of DCs that express CX_3CR1 extends dendrites into the intestinal lumen to capture antigens in response to MyD88-dependent bacterial stimulation [93,116]. Furthermore, *S. typhimurium* is transported by CD18$^+$ phagocytes in the lamina propria that induce a subsequent systemic antigen-specific immune response [117]. Therefore, these unique populations of antigen-sampling cells may cooperatively play a role at the entry sites of luminal antigens and pathogens, independently of PPs and ILFs. Indeed, the antibody response specific to serum tetanus toxoid is not impaired in PP- and ILF-null mice orally immunized with *rSalmonella* capable of producing tetanus toxoid [117]. On the other hand, oral immunization with *rSalmonella* capable of producing tetanus toxoid fails to induce the production of fecal IgA antibodies specific to tetanus toxoid in PP-null mice [118]. Furthermore, an ovalbumin-specific fecal IgA response is induced by cooperation among PPs, ILFs,

and MLNs after oral immunization using ovalbumin combined with cholera toxin [119]. Thus, the GALT and unique antigen-sampling cells in the lamina propria may provide an antigen-specific immune response at intestinal and systemic sites, respectively.

The number of IgA plasma cells is not affected in the small intestinse of $LT\alpha^{-/-}$ mice that lack PPs and ILFs after transfer with bone marrow cells of WT mice [120]. Recent studies have demonstrated that peritoneal B1 cells as well as PP cells are important sources of IgA plasma cells in the lamina propria [121–123]. Given the harsh environment of the gastrointestinal tract, the mucosal immune system is logically furnished with multiple layers of IgA induction pathways that include both GALT-dependent and GALT-independent mechanisms for providing adequate ongoing immunological protection.

II NALT

Nasal immunization is an effective way of activating the mucosal immune system for the induction of antigen-specific IgA immune responses [59]. To this end, NALT is obviously an important inductive site for the generation of immune responses specific to inhaled antigens both in the respiratory tract and systemically [59]. Group A *Streptococcus* is one of the most common pathogens in the respiratory tract. This pathogen infiltrates the respiratory epithelium via NALT M cells. Interestingly, nasal administration of UEA-1 blocks the invasion of group A *Streptococcus* via M cells in the NALT [38]. As streptococcal M protein interacts with fucose-containing glycoproteins on human epithelial cells [124], UEA-1 and M protein may share a binding site on the surfaces of NALT M cells. In addition to the cell-wall component of bacteria (e.g., M protein), sigma-1 originating from a reovirus possesses a high affinity for glycoconjugates that contain α2–3-linked sialic acid and are expressed only on the apical surfaces of M cells [125]. Sigma-1 has been applied to target NALT M cells in the development of a nasal vaccine delivery system [126]. A DNA nasal vaccine coupled with sigma-1 has induced humoral immune responses in both mucosal (nasal and intestinal IgA) and systemic (splenic IgG) organs [126]. In addition, the CTL response was enhanced in the respiratory tract after nasal immunization with sigma-1 [126,127]. The host cell-binding motif of toxins produced by pathogenic bacteria (e.g., the B-subunit of cholera toxin, CT-B) is an effective deliverer of antigen to the mucosal epithelium, including to the FAE of inductive tissues [128]. Nasal immunization using ovalbumin and the B-subunit of the Shiga toxin induces ovalbumin-specific mucosal IgA and systemic IgG antibody responses [129]. The B-subunit of Shiga toxin activates NALT DCs to enhance the expression of costimulatory molecules (CD80, CD86, and CD40), leading to the activation of effector T cells [129]. Therefore, NALT DCs play a pivotal role in initiating and mediating adaptive immunity in the NALT, even though DCs account for less than 5% of NALT mononuclear cells. However, only the basic immunological characteristics of NALT DCs have been

characterized, in contrast to the well-characterized DCs in PPs; thus more detailed analyses are required to clarify the molecular and cellular mechanisms responsible for the common and unique characteristics of NALT DCs.

Single-cell reverse transcriptase-polymerase chain reaction (RT-PCR) analysis has revealed that Th0-type cells are dominant among NALT $CD4^+$ T cells [130]. These T cells are capable of differentiating into Th1 or Th2 cells immediately after antigen presentation by NALT DCs. Nasal administration of an antigen, together with cholera toxin as a mucosal adjuvant, thus induces the production of antigen-specific Th2 capable of producing IL-4 in the NALT [131]. In contrast, nasal vaccination with the antigen expressed by recombinant *Mycobacterium bovis* bacillus Calmette-Guérin leads to a Th1 immune response in the NALT [132].

S-IgA inhibits bacterial adhesion to epithelial cells and prevents nasopharyngeal infection by inhaled pathogens (e.g., *Streptococcus pneumoniae* and *Haemophilus influenzae*) [133]. Nasal vaccination with nontypeable *H. influenzae* and cholera toxin induces the expression of nontypeable *H. influenzae*-specific S-IgA in nasal wash and saliva and inhibits local infection by *H. influenzae* [134]. Phosphorylcholine is a common component of the cell walls of *S. pneumoniae* and *H. influenzae* [135]. The interaction of phosphorylcholine with receptors for platelet-activating factor on epithelial cells is involved in bacterial adhesion to epithelial cells in the respiratory tract [136]. Interestingly, phosphorylcholine-specific S-IgA production induced by nasal immunization using phosphorylcholine combined with cholera toxin prevents nasal infection by both *S. pneumoniae* and *H. influenzae* [137]. Th2 cell production induced in the NALT by nasal exposure to cholera toxin may contribute to the generation of IgA^+ B cells in this inductive tissue. Our study demonstrated that the expression of IgA CSR-associated genes (e.g., AID, the Iα-Cμ circular transcripts, and Iμ-Cα) is restricted to NALT and is not found in the diffuse effector tissues of the nasal passages [99]. This finding indicates that NALT, and not the nasal passage, provides an immunological environment for the generation of IgA^+ B cells from IgM^+B220^+ B cells, as in PPs and ILFs. In contrast to PPs, in which B cells differentiate mainly into IgA^+ B cells, B cells in NALT differentiated into IgG^+ B cells as well as IgA^+ B cells [138]. Furthermore, NALT is an important site for the generation of memory-type B cells that produce high-affinity IgA and IgG antibodies [138]. In addition, NALT is the site for the generation of virus-specific CTL after nasal infection with reovirus [139]. Taken together, these results suggest that the NALT plays a central role in the induction of antigen-specific cellular and humoral immunity mediated by Th1 and Th2, respectively.

The other notable uniqueness of nasal vaccination is the induction of antigen-specific immune responses at mucosal sites in reproductive organs [140]. However, the mechanism responsible for the interaction of the respiratory and reproductive tissue immune systems is still unclear, although nasal immunization has been shown to induce the expression of high levels of CCR10 and $\alpha4\beta1$ integrin on IgA-committed B cells, leading them to efficiently migrate to the respiratory and urogenital tracts, which express the corresponding ligands CCL28 and VCAM-1 [108,109,141]. To develop an effective vaccine that can protect against infectious diseases initiated within the urogenital tract, such as

human immunodeficiency virus (HIV), it will be necessary to further elucidate the molecular mechanism(s) responsible for reproductive imprinting of mucosal lymphocytes in the NALT to permit subsequent cross-communication with urogenital mucosal tissues.

III BALT

BALT can be detected at the division of the bronchus in aged mice [45]. BALT epithelium contains M cells that react to UEA-1 and are covered by microvilli, with pocket formation and infiltration by lymphoid cells [45]. As in the case of M cells located in other MALTs, BALT M cells possess a high ability to take up antigens from the trachea. Interestingly, in young mice cells that resemble UEA-1^+ M cells are found in the bronchial epithelium at some distance from the BALT [45]. Moreover, BALT-independent M cells are present in the bronchus of young mice where *Mycobacterium tuberculosis* has preferentially infiltrated and been transported to draining lymph nodes by macrophages associated with the bronchial M cells [142]. These findings suggest that both BALT-dependent and BALT-independent antigen-sampling pathways mediated by M cells contribute to the recognition of pathogens and the subsequent initiation of pathogen-specific immune responses by immunocompetent cells in the lower respiratory tract.

DCs have been found in the BALTs of some species (mice, pigs, and humans) [85,143,144]. Because of the anatomically and technically difficult nature of the process used to detect and isolate BALT cells from the lungs, the function of these BALT DCs remains speculative. In 2004, iBALT was discovered, and its functional role was investigated in secondary lymphoid tissue-null mice [85]. B-cell follicles containing FDC with IFR rich in T cells appears as iBALT in the lungs after infection or inflammation [85]. In an influenza infection model, iBALT has been shown to be a key inductive site for the generation of influenza-specific $CD8^+$ T cells, which contribute to the induction of protective immunity against the influenza virus [145]. Further, germinal centers develop in iBALT for the generation of influenza-specific IgG and IgA antibody responses. iBALT, developed in the mice that lacked secondary lymphoid tissues, is capable of compensating for the immunological function of the other secondary tissues (e.g., pLNs and spleen) to induce protective immune responses against influenza. On the other hand, induction of an influenza-specific immune response is slower in secondary lymphoid tissue-null mice than in wild-type mice, despite the presence of iBALT contributing to the development of protective immunity. In addition to iBALT-mediated adaptive immunity, innate immunity, such as the production of alveolar macrophages that produce IFN-α, is required for protection of the lungs against infectious respiratory diseases [146]. Because iBALT seems to play a key role in the generation of protective immunity against inhaled pathogens, an obvious and interesting question would be whether iBALT is involved in the induction of allergic responses, including the asthma caused by inhaled allergens.

Role of MALTs in the Induction of Mucosal Tolerance

I Induction of the Regulatory Network by GALT and the Lamina Propria

The mucosal immune system must be capable of distinguishing harmful antigens and pathogens from harmless and self-beneficial antigens and commensal bacteria. To avoid unnecessary responses to such self-beneficial digestive and inhaled antigens and commensal flora, the mucosal immune system can exhibit mucosal tolerance (e.g., oral and nasal tolerance) and thereby create an appropriate homeostasis in the aerodigestive tract [1,3]. Lack or disruption of mucosal tolerance leads to the development of allergic and autoimmune diseases [1,3]. Thus, elucidation and understanding of the cellular and molecular mechanisms of mucosal tolerance are essential for the development of new mucosal immunotherapy capable of controlling allergic and autoimmune diseases.

The PP, a representative organized lymphoid tissue in the GALT, is considered a key site for the induction and maintenance of oral tolerance [1,3]. In the PPs of a murine model of collagen-induced arthritis, oral tolerance induced tolerogenic $CD11b^+$ DCs; this induced the appearance of Treg cells [147]. Tolerogenic $CD11b^+$ DCs in the PPs produced IL-10 and further inhibited the T-cell response to collagen (Fig. 4) [147]. Oral administration of a chimeric protein that combines ovalbumin with CT-B results in the induction of oral tolerance, with increased numbers of $Foxp3^+$ Treg cells, in PPs in a TGF-β-dependent manner [148]. Natural killer T (NKT) cells play a critical role in the regulation of various immune responses, including autoimmune diseases, viral infections, and antitumor responses, as well as in allergic diseases [149,150]. Interestingly, oral tolerance cannot be induced in $CD1d^{-/-}$ mice that lack NKT cells [151]. NKT cells induce tolerogenic DCs for the generation of Treg cells that produce IL-10 and TGF-β in PPs during the induction of oral tolerance (Fig. 4) [151]. These findings indicate that PPs play an important role in the induction of oral tolerance. However, several studies of PP-null mice indicate that oral tolerance can be induced even in the absence of inductive tissue [152–154]. To this end, MLNs have also been shown to contribute to the induction of oral tolerance. $CD8\alpha^+$ pDCs in MLNs are capable of enhancing the suppressive function of T regulatory 1 (Tr1)-like cells (Fig. 4) [155]. A critical role of MLNs in the induction of oral tolerance has further been demonstrated by the lack of tolerance induction in MLN-null mice [156]. This result suggests that MLNs may be more important sites for the induction of oral tolerance than PPs, although it is logical to consider that both PPs and MLNs form a lymphoid tissue network that creates at least a double layer of regulatory machinery which can establish homeostatic conditions despite the harsh environment of the digestive tract.

Recent studies have revealed that antigen-presenting cells localized in the intestinal lamina propria play a central role in the induction of Treg cells or IL-17-producing helper T cells (Th17) that mediate the inhibition and induction, respectively, of inflammatory diseases [157]. Treg cells are induced by IL-10 and TGF-β

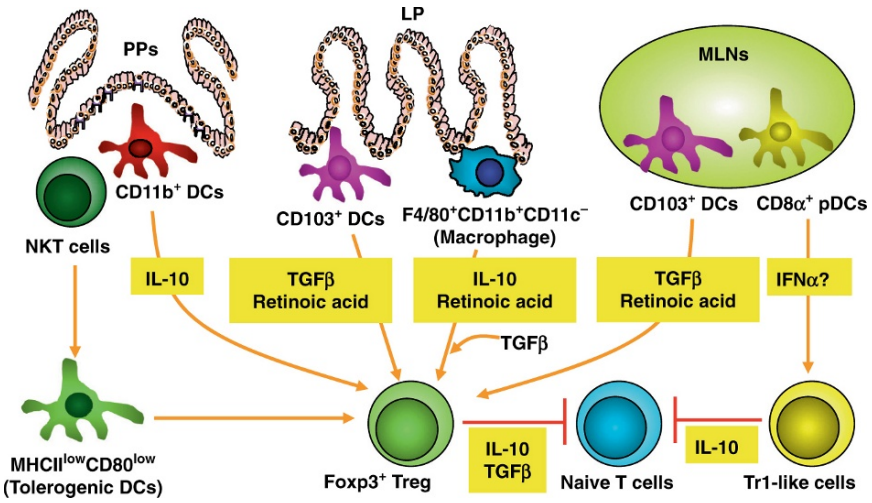

Fig. 4 Induction and regulation of oral tolerance. To create immunological silence in the harsh environment of the digestive tract, multiple layers of molecular and cellular regulatory mechanisms operate to induce and maintain oral tolerance. For example, when CD11b$^+$ DCs in PPs are activated by oral antigens, they produce IL-10 and induce the differentiation of Foxp3$^+$ Treg cells. Natural killer T cells (*NKT* cells) in PPs are also involved in the induction of Foxp3$^+$ Treg cells through the induction of MHCII^{low}CD80^{low} tolerogenic DCs. In the lamina propria (*LP*) compartment, CD103$^+$ DCs produce transforming growth factor-beta (TGF-β) and retinoic acid that create a molecular environment suitable for the generation of Foxp3$^+$ Treg cells. F4/80$^+$CD11b$^+$CD11c$^-$ macrophages are also tolerogenic players in the lamina propria. These macrophages produce IL-10 and retinoic acid, which induce Foxp3$^+$ Treg cells in the presence of TGF-β. In MLNs, CD103$^+$ DCs have been characterized as tolerogenic DCs that produce TGF-β and retinoic acid, which induce Foxp3$^+$ Treg cells. CD8α$^+$ pDCs in MLNs also have an important role in the induction of mucosal tolerance. CD8α$^+$ pDCs produce interferon-alpha (*IFN*-α) and induce the differentiation of IL-10-producing Tr1-like cells

produced by macrophages and stromal cells, respectively, in the lamina propria [157]. Therefore, macrophages in the lamina propria that produce IL-10 create an optimal molecular environment for the differentiation of Treg cells [157]. Although TGF-β induces Treg cells, TGF-β and IL-6 synergistically generate Th17 cells [158]. In contrast to macrophages in the lamina propria, CD11b$^+$ DCs (CD103^{low}) in the lamina propria are capable of inducing Th17 [157]. During inflammation, CD11b$^+$ DCs in the lamina propria may generate Th17 cells in cooperation with IL-6 produced by inflammatory macrophages [159]. CD103$^+$ DCs in the lamina propria and MLNs are also capable of inducing Treg cells via TGF-β and retinoic acid [160,161]. Therefore, the intestinal immune system consists of multiple pathways for the induction of mucosal Treg cells by various types of orchestrated immunocompetent cells, including macrophages, DCs, and NKT cells in both the GALT and the intestinal lamina propria (Fig. 4). These multiple layers of the intestinal regulatory network allow the continuous generation and maintenance of an immunologically quiescent condition (e.g., oral tolerance) that helps the system to deal with numerous environmental and dietary antigens.

II Nasal Tolerance

The respiratory tract is also continuously exposed to inhaled exogenous antigens. The respiratory immune system is thus capable of inducing mucosal tolerance to harmless antigens while also inducing a protective immune response when exposed to harmful antigens such as pathogenic microorganisms. A murine model of surgical removal of the cervical lymph nodes (CLNs), which drain the upper respiratory tract, showed the critical role of these draining lymph nodes for the induction of nasal tolerance [162]. Nasal administration of ovalbumin leads to the induction of functional Treg cells in CLNs in transgenic mice that produce DO11.10 ovalbumin [163]. Recent studies have demonstrated that DCs in CLNs play a critical role in the induction of nasal tolerance [164,165]. In these reports, some key factors (FcγRIIB and indoleamine 2,3-dioxygenase, IDO) expressed by DCs in CLNs have been identified [164,165]. Signaling of FcγRIIB via an immunoreceptor tyrosine-based inhibitory motif downregulates the production of CD40 and the production of IL-12p40 by DCs [164]. Thus, nasal tolerance is not induced in FcγRIIB$^{-/-}$ mice. CLN DCs also produce IDO, an intracellular enzyme that degrades the indole moiety of tryptophan. IDO plays a role in immune tolerance to self- and foreign antigens and tumors [166]. Because inhibition of IDO prevents the induction of nasal tolerance, IDO expressed by CLN DCs is essential for the induction of Treg cells and nasal tolerance [165]. Taken together, these findings suggest that CLNs, the nasal-draining lymph nodes, are required for the induction of tolerance to harmless inhaled antigens in the respiratory tract. In contrast, the involvement of the NALT in the development of nasal tolerance remains to be elucidated. The NALT has a unique population of B220^{+} T cells capable of inhibiting the activation of mature T cells [167]. Therefore, a unique T-cell subset with a suppressor function in the NALT may contribute to the induction of nasal tolerance, with the cooperation of a regulatory network that originates in the CLNs.

Contradictory Roles of MALTs in the Inhibition and Induction of Allergic and Autoimmune Diseases

I Inhibition of Allergic Diseases by MALTs

Allergic diseases, including food allergies, asthma, allergic rhinitis, and atopic dermatitis, are caused by hyperresponsiveness to generally harmless antigens (or allergens such as food and plant antigens). Allergen-specific IgE-mediated hyperresponses are regulated by unnecessary pathological Th2 cells that produce IL-4, IL-5, and IL-13 [168]. We previously found that unnecessary Th2 cells that produce IL-4 were responsible for the development of allergic diarrhea in mice that were exposed to an orally administered allergen and had been presensitized with ovalbumin [169]. Since abundant production of the homodimeric form of IL-12p40 promotes the

development of pathological Th2-mediated allergic diarrhea, nasal administration of plasmid IL-12p70 leads to the inhibition of allergic diarrhea [170]. Nasal administration of plasmid IL-12p70 results in alteration of the p40 homodimer-dominated Th2 environment to a p70/p40 heteromer-induced Th1 condition initiated by NALT DCs that express IL-12p70 and that prevent the pathological Th2 response, leading to the inhibition of allergic diarrhea [170]. In the same mouse model for food allergies, PP-null mice were more susceptible to disease development and had higher levels of antigen-specific IgE antibodies than mice with intact PPs [171]. This study further demonstrated that $Foxp3^+$ Treg cells capable of producing IL-10 were generated in PPs and contributed to inhibition of the allergic response in the intestine [171]. Thus, PPs are essential inductive sites for the control of undesired allergic responses in the intestine. Furthermore, the aforementioned study of nasal plasmid therapy demonstrated that the NALT is capable of modulating allergic inflammatory diseases in distant tissues such the large intestine.

In addition to allergic diseases in the intestine, allergic rhinitis is also controlled by the NALT and its clan regulatory axis [42]. $Foxp3^+CD25^+CD4^+$ Treg cells in the NALT and the CLNs prevent Th2-type allergic responses in the upper respiratory tract of WT mice (Fig. 5) [42]. However, *plt* mice, which lack production of CCR7 ligands, CCL19 and CCL21, exhibit more severe allergic rhinitis than wild-type mice, as recruitment of Treg cells to the NALT and CLN regulatory axis is impaired in the *plt* mice [42]. These findings suggest that the NALT and the CLNs form a regulatory network for the control of allergic responses in the upper respiratory tract by means of CCR7-dependent Treg generation (Fig. 5) [42]. Furthermore, *plt* mice develop increased Th2 responses and severe allergic disease in the lower respiratory tract [172]. In accordance with a similar scenario for the formation of a NALT and CLN regulatory axis in the upper airway, it is possible that asthma could be controlled by the establishment of Treg cells in the BALT (or iBALT) and in regional lymph nodes.

II The GALT as an Initiation Site for Disease Development

It is now well known that *Helicobacter pylori* causes peptic ulcers and gastric adenocarcinomas [173]. $CD4^+$ T cells specific to *H. pylori* that are generated during the infection are implicated as initiators of gastritis [173]. However, gastritis induced by *H. pylori* does not develop in PP-null mice; thus, PPs have been implicated in the initiation of pathological $CD4^+$ T cells specific to *H. pylori* [174]. Subsequently, the coccoid form of *H. pylori* has been shown to be phagocytosed by PP DCs for the presentation of its antigens to Th cells. PPs thus seem to be pathological sites for the induction of $CD4^+$ T cells that are specific to *H. pylori* and somehow preferentially migrate into the stomach to promote the development of gastritis [174]. Furthermore, colonic patches, which are part of the GALT family in the large intestine, promote Th2-type colitis [175]. Thus, LTβR-Ig treatment could inhibit Th2-type colitis induced by trinitrobenzene

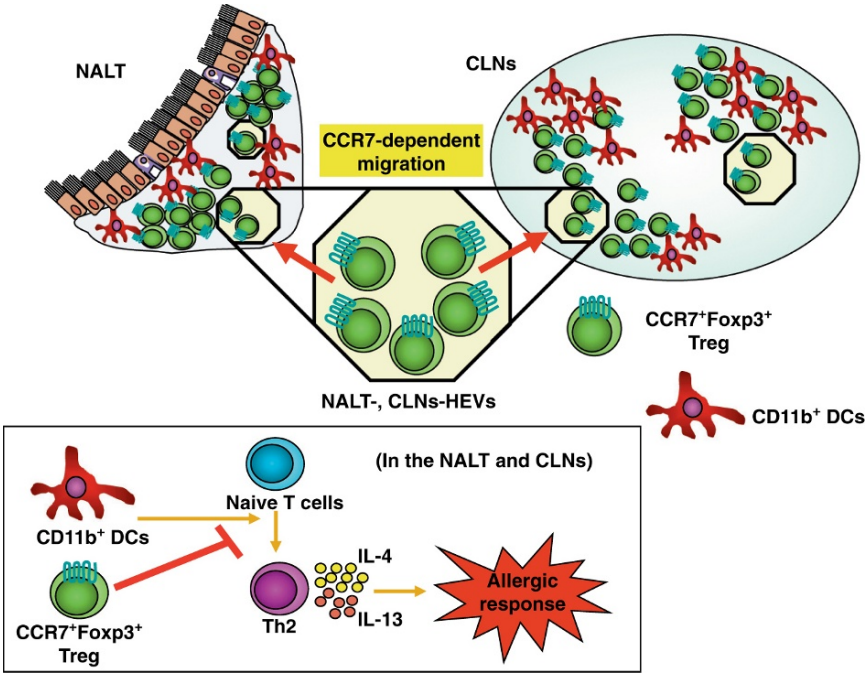

Fig. 5 Role of the NALT and cervical lymph node (*CLN*) regulatory network in the development and control of allergic responses. $CCR7^{+}Foxp3^{+}$ Treg cells migrate to secondary lymphoid organs (including the NALT and the CLNs). This migration depends on CCR7/CCL19 and CCL21. In a nasal allergic reaction, the number of $CD11b^{+}$ DCs increases in the NALT and the CLNs, and these DCs induce the differentiation of naive T cells into Th2-type cells. Because $CCR7^{+}Foxp3^{+}$ Treg cells can inhibit this increase and can reduce the production of allergy-inducing cytokines such as IL-4 and IL-13 by Th2 cells, nasal allergy can be controlled by modulation of the Treg-inducing pathway

sulfonic acid by preventing the development of colonic patches [175]. These findings indicate that the GALT is generally and physiologically a key site for the induction and regulation of desired self-beneficial mucosal immune responses; however, the tissue can also act as an initiation site for inflammatory diseases associated with the gastrointestinal tract.

The development of acute graft-versus-host disease caused by allogenic bone marrow transplantation is inhibited in PP-null mice created by in utero treatment with anti-IL-7Rα monoclonal antibodies [176]. This result demonstrates that PPs provide an opportunity for donor $CD8^{+}$ T cells to obtain specificity to the host's antigens. Therefore, the GALT could play a critical role in inducing severe graft-versus-host disease that affects both systemic and local compartments [176]. On the other hand, a recent study that used $TNF^{\Delta/\Delta}$ mice as a new form of PP-null mice demonstrated that acute graft-versus-host disease developed even in the absence of PPs [177]. Although these contradictory results cannot yet be explained, the different conditions used to eliminate the PPs (i.e., monoclonal antibodies versus gene manipulation) might account for the difference.

III Pathological Contributions by the NALT and the BALT

Several kinds of pulmonary diseases, including viral infections, asthma, and autoimmune diseases, lead to the development of iBALT in both humans and mice [4]. In the lungs of patients with interstitial pneumonia, which is a complication that arises in patients with rheumatoid arthritis and Sjögren's syndrome, iBALT is frequently detected in both the upper and lower lungs as a form of organized lymphoid follicles with germinal centers [86]. Importantly, iBALT has been demonstrated to be the site of the production of autoantibodies capable of reacting with self-antigens such as anticyclic citrullinated peptide antibodies in rheumatoid arthritis patients [86]. Therefore, iBALT may directly influence the development of local inflammation in the lungs of patients with autoimmune diseases.

Our literature review revealed no evidence of a pathological role of the NALT in mouse disease models. On the other hand, the involvement of human tonsils, analogues of the mouse NALT, in the development of immunological diseases has been well investigated, especially in terms of IgA nephropathy [178]. Although human tonsils produce antigen-specific IgA and IgG in response to inhaled pathogens [179,180], harmful antibodies can also be produced by human tonsils [181]. Pathogen-specific IgA antibodies (e.g., against *Streptococcus pyogenes*) produced by the tonsils are considered to be major pathological participants among the IgAs deposited in the glomeruli of patients with IgA nephropathy [178,181]. Abnormal IgA, and especially IgA1, is likely to accumulate and form immune complexes and aggregations in the glomerular mesangium, which induces the production of proinflammatory cytokines in the glomeruli [182]. This evidence may explain why surgical removal of the tonsils can markedly improve the clinical condition of patients with IgA nephropathy [183]. In addition, the tonsils have been implicated in other diseases (e.g., rheumatoid arthritis, pulmoplantar pustulosis, and sternocostoclavicular hyperosteosis) [184,185].

Role of Cross-Communication Between the Mucosal and Skin Compartments in Immunity and Allergy

The skin functions as an immune tissue and a surface barrier that acts as a physical defense (via the keratinized epithelium) and acts in innate and acquired immune responses to external contact antigens [186]. Recent evidence has revealed the presence of immunological cross-communication between the skin and the mucosal immune systems. Transcutaneous immunization can induce antigen-specific systemic and mucosal immune responses in compartments such as the intestines, the salivary glands, and the vaginal mucosa [187–189]. The Langerhans cells, a unique subset of the DC family in the skin, and other dermal DCs migrate to regional lymph nodes after stimulation by antigens, and they subsequently induce antigen-specific immune responses [190]. Furthermore, langerin$^+$ DCs migrate into MLNs after transcutaneous immunization to initiate antigen-specific immune responses

in the mucosal compartment [191]. CCR7 may critically regulate the migration of langerin$^+$ DCs into MLNs [191]. Because MLN-null mice and langerin$^+$ DC-depleted mice show significant decreases in antigen-specific S-IgA production in the intestines after transcutaneous immunization, migration of langerin$^+$ DCs into MLNs from the skin appears to be essential for the induction of antigen-specific S-IgA responses in the gastrointestinal tract [191].

Atopic dermatitis is caused by allergic responses, mainly against food allergens (e.g., eggs, milk, wheat, soy, and peanuts) and aeroallergens (e.g., house dust, weeds, animal dander, and molds) [192]. The intestinal environment is also considered to influence the pathology of atopic dermatitis. Thus, fungal colonization of the intestines increases the severity of atopic dermatitis, and the elimination of intestinal fungi is anticipated to be one of the therapeutic strategies for controlling atopic dermatitis [193,194]. Furthermore, the introduction of probiotics such as lactic acid bacteria has been shown to effectively control atopic dermatitis (Fig. 6) [195]. A recent report showed that production of the regulatory cytokine IL-10 was enhanced in GALT (e.g., PPs and MLNs) as well as in the serum after the consumption of a diet containing heat-treated *Lactobacillus rhamnosus*, although the number of Treg cells did not increase in either the GALT or the spleen [196]. Thus, the study suggested an interesting possibility: that intestinal IL-10 induced by consumption of *L. rhamnosus* may be directly involved in the suppression of atopic symptoms in the skin without influencing the number of Treg cells [196].

Human tonsils are also involved in cross-communication with the skin, and especially in the development of skin diseases [185]. Pustulosis palmaris et plantaris (PPP) is a pustular skin disease associated with focal tonsillar infections. Tonsils from a patient with PPP contained B cells and T cells that reacted to a skin self-antigen (keratin; Fig. 6) [197]. In PPP patients, tonsillar T cells expressed cutaneous lymphocyte-associated antigen (CLA), which mediates the migration of the T cells to the skin (Fig. 6) [198]. As large numbers of CLA$^+$ T cells and their ligand, E-selectin, are observed in the skin lesions of PPP patients, T cells reactive to skin antigens that are generated in the tonsils may preferentially migrate into skin lesions by means of the interaction between CLA and E-selectin, leading to the development of obstinate inflammation [198]. Tonsillectomy has thus been recognized as the most effective and radical treatment for PPP patients [199].

Concluding Remarks

MALTs, including GALT, PPs, NALT, and BALT, are immunologically important elements of the mucosal immune system and are active in the execution of both quiescent and active immune responses against self-beneficial and harmful antigens, respectively, to create appropriate homeostatic conditions in the harsh environment of the aerodigestive tract. Although all MALTs possess a similar microarchitecture, the biological and developmental programs responsible for their respective organogenesis differ greatly. On the basis of recent findings from our laboratories

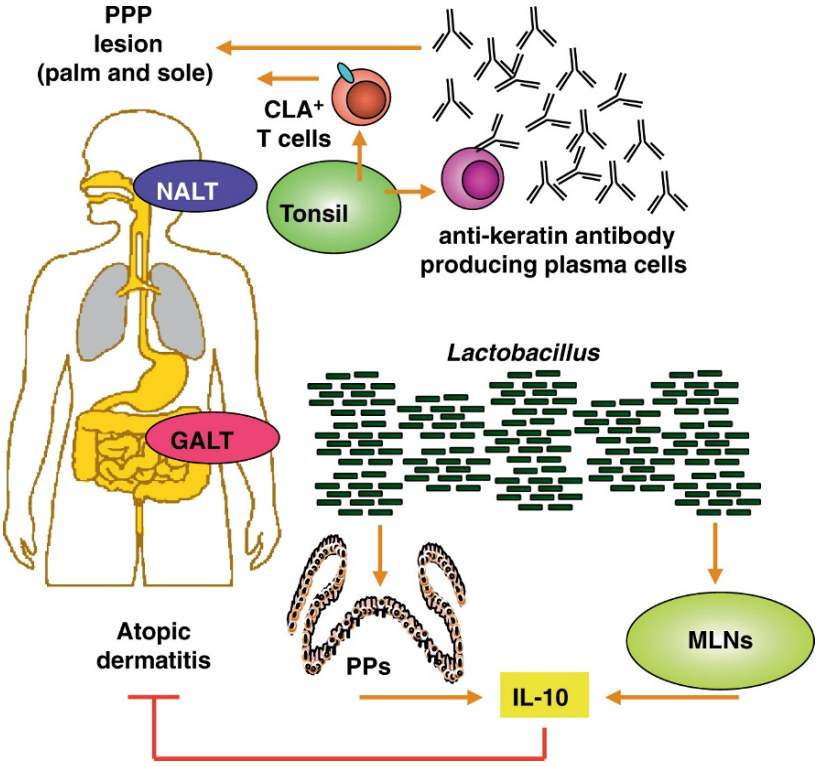

Fig. 6 Involvement of MALTs in atopic dermatitis and the pathogenesis of pustulosis palmaris et plantaris (PPP). In the gut, microfloral bacteria and bacterial components actively regulate the host's immune system. Consumption of probiotics is aimed at influencing and modifying GALT-related immunity by dietary bacteria, including *Lactobacillus* species. For example, a large amount of regulatory cytokine IL-10 is produced in PPs and MLNs after the introduction of dietary *Lactobacillus*, which can lead to inhibition of the onset and development of atopic dermatitis. In the upper respiratory tract, the tonsils may play an important role in the pathogenesis of PPP in the skin. Skin-homing cutaneous lymphocyte-associated antigen (CLA^+) T cells and plasma cells that produce antikeratin antibodies are frequently found in the tonsils of PPP patients. Tonsillectomy has been shown to be an effective treatment for PPP, including the control of clinical skin symptoms

and those of other researchers, we propose that the tissue genesis program of the respiratory-associated lymphoid tissues (NALT and BALT) and of GALT can be classified into at least two separate subsets in accordance with the differences in their dependence on the LTβR signaling pathway. Although development of the GALT is critically regulated by the LTβR signaling pathway, the initiation of NALT and BALT genesis is independent of the LTβR signaling axis. However, the essential molecules that regulate the organogenesis of respiratory-associated lymphoid tissues independently from LTβR-mediated signaling have not yet been identified. In comparison to the organogenesis of PPs and PP-mediated immune responses in

the digestive immune system, relatively little information is available on the respiratory immune system orchestrated by NALT and BALT. Our emphasis should thus be aimed at molecular and cellular characterization of NALT and BALT to improve our understanding of their role in the respiratory immune system as well as their cross-communication with the other mucosal compartments and the skin.

MALTs play a pivotal role in the induction of protective immune responses to luminal pathogens at both mucosal and systemic sites. At the same time, mucosal tolerance is also induced by the mucosal regulatory network orchestrated by the axis between the MALTs and their draining lymph nodes (e.g., MLNs and CLNs). However, little is known about the molecular and cellular mechanisms responsible for the induction, regulation, and maintenance of these opposing immune responses in the mucosal tissues. Recently, the vitamin A metabolite retinoic acid was shown to possess an essential role in controlling the differentiation of Treg cells and Th17 cells, which play a crucial role in the induction of the quiescent and inflammatory immune responses, respectively [200]. Therefore, the luminal environments of the intestinal tract, which include food materials and commensal bacteria, are likely to contain important regulatory molecules for the induction and maintenance of mucosal homeostasis, in addition to regulatory molecules derived from the host immune system.

One of the goals of studying the mucosal immune system is to apply this knowledge in the development of a new generation of prevention and therapy for the control of allergies and infectious and immunological diseases. To accomplish this goal and develop innovative applications, it will be important to combine the strengths of different scientific disciplines. For example, the consolidation of mucosal immunology with plant biology has resulted in the creation of a rice-based antigen production and delivery system that can be used for the development of oral vaccines: CT-B that expresses MucoRice™ [201]. MucoRice, which was generated by manipulated rice genes, is effective in the induction of toxin-specific mucosal and systemic immune responses against challenge with cholera toxin, even when the transgenic rice grains are kept at room temperature for several years [201]. Prolonged oral administration of transgenic rice that expresses T-cell epitopes of pollen antigens is capable of inducing an antigen-specific inhibitory condition for the control of allergic rhinitis in a mouse model [202]. The development of a new generation of effective, nontoxic mucosal modulators and drug delivery systems is another requirement for the development of a human mucosal vaccine system. The development of an antigen delivery system that targets M cells is thought to be an attractive approach for both oral and nasal immunization [95,126,127]. Continuous advances in our understanding of the molecular and cellular bases of the mucosal immune system in humans and in experimental animal models will further facilitate the development of a new generation of innovative mucosal vaccines and immunotherapies for the control of allergic, infectious, and autoimmune diseases.

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