Mia Levite *Editor*

Nerve-Driven Immunity

Neurotransmitters and Neuropeptides in the Immune System

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Preface

Personal Recollection

I still remember these moments very vividly, as if they were today, when at the corner of my eye I saw a lovely young woman waiting patiently for her turn to talk to me. She later turned out to be Dr. Claudia Panuschka – Springer's Editor of Biomedicine/Life Sciences, whom I learned to appreciate professionally, and enjoyed so much working with. Claudia looked as if of she simply wishes to ask me a small question, at the end of my invited lecture on "Glutamate Immunity and Autoimmunity", in an international meeting in Vienna, on Thursday, August 6th 2009. In that talk, I used a novel term – "**Nerve-Driven Immunity**" that I coined a few years before, and like so much using ever since, in papers and lectures, when discussing the relatively new concept of "Neurotransmitters in the Immune System".

When we were finally left alone, Claudia approached me gently, but not regarding a small matter as I thought. Rather, without any prior preparation or hesitation she threw to the air a huge idea that finally gave birth to this book: "As I enjoyed your talk and ideas so much, would you agree to write a book for Springer on 'Nerve-Driven Immunity'?" she asked. This presumably simple question hit me by complete surprise, triggering a burst of fast inner thoughts: "Is she serious? . . . Can I do it? . . . Should I? . . . It could be a wonderful and unique opportunity to raise the awareness of people to this new important topic, which is to a large extent complementally unknown . . . a big challenge . . . a unique scientific and writing adventure . . . but . . . but at the same it would require endless amount of work . . . infinite time . . . huge commitment . . . heavy responsibility . . ."

While debating for a few seconds how should I in fact answer Claudia, I suddenly heard my own voice replying instead of me: "Thank you . . . that's a huge complement . . . I would have to think about it . . . but I already know one thing for certain: if I finally accept your kind and flattering invitation, I wouldn't like to write this book all by myself. It would have to be a team work, and for that I would need the full cooperation of very good people, each contributing a chapter on a different neurotransmitter and its role in the immune system". When Claudia did not reject this idea on the spot I added more obstacles: ". . . It would be very difficult to find such rare people, since only relatively few people work on this topic, and even when I find them, I doubt they would agree to do a very sisyphic work and read, collect,

re-analyze carefully and summarize all the data published thus far in regards to the role of a given neurotransmitter in the immune system (rather than write only about their own work, which is usually the case) . . . and that they would also be willing to write the chapter along my own requested framework and subchapters, which I would probably ask to be similar to all the chapters".

Well . . . since this conversation in 2009 in Vienna, so many things have happened . . . so many discussions between Claudia and me took place, and later so many emails were exchanged between all the great authors of the present book and myself, leading to our present exciting stand point, when we launch this book.

For me, the book exceeds all prior expectations, and I have no words to express how fortunate I feel to have been given this opportunity, and how deeply grateful I am to Springer, to Claudia, and of course to all the very good authors of the book chapters. Without all of you, this book would have never been born. Now, it is hoped that all of you share the enthusiasm and pride seeing this book come to life, and that the future readers of this book will enjoy it and learn from it many new things.

The Book, the Authors, the Vision . . .

Hopefully, our book will become a rich and updated encyclopedia on the novel topic we cover: the major and active role played by many neurotransmitters and neuropeptides in the immune system. Indeed, the book can teach the readers what we know today on the "immune face" of 12 neurotransmitters and neuropeptides: Dopamine, Adrenaline, Noradrenaline, Acetylcholine, Glutamate, GABA, Somatostatin, Neuropeptide Y (NPY), *Vasoactive intestinal polypeptide (VIP)*, Calcitonin gene-related peptide (CGRP), Opioids and Cannabinoids. And it already seems to few of us that this book could easily become a text book for scientists, clinicians and students interested in Immunology, Neurobiology, NeuroImmunology or Pharmacology, and may even serve as the basis for a new Ph.D. or M.D. course on "Neurotransmitters in the Immune System".

The authors of the book chapters are highly qualified scientists and/or clinicians, working in nine different countries: Doina Ganea, USA (VIP); Sabita Roy and Jana Ninković, USA (Opioids); Marco Cosentino and Franca Marino, Italy (Adrenaline and Noradrenaline); Talma Brenner and Eran Nizri, Israel (Acetylcholine); Bernhard Holzmann, Germany (CGRP); Bryndis Birnir, Zhe Jin, Suresh Kumar Mendu and Amol Bhandage, Sweden (GABA); Toomas Talme and Karl-Gösta Sundqvist, Sweden (Somatostatin); Cris Constantinescu and Radu Tanasescu, United Kingdom (Cannabinoids); Yonatan Ganor, France (Glutamate); Mario Delgado, Spain (VIP); Mirjana Dimitrijevic and Stanislava Stanojevic, Serbia and Montenegro (NPY), and myself – Mia Levite, Israel (Dopamine and Glutamate).

Unfortunately, the planned and promised chapters on Serotonin, Substance P and GnRH (I and II) were finally not submitted on time, but may be included in the next updated edition of this book, if and once it will be published.

'NeuroImmunoTransmitters'

As a whole, our 'Nerve-Driven Immunity' book shows beyond any doubt that neurotransmitters and neuropeptides have a huge impact on the immune system, not only on the nervous system. Based' on the impressive wealth of data supporting this notion and covered by this book, I suggest that most if not all of the neurotransmitters deserve now a new title: **'NeuroImmunoTransmitters**', replacing the "old" "classical" and what seems now to be a too narrow title: 'Neurotransmitters', which assigns to these molecules only or primarily effects in the nervous system. The new suggested criteria set herein for being named and considered a 'NeuroImmunoTransmitter' (rather than only a neurotransmitter) are listed below, and fulfilled by most, if not all the neurotransmitters and neuropeptides discussed in our book.

Criteria 1 for being a '*NeuroImmunoTransmitter*': Most if not all types of immune cells ought to express receptors for the respective neurotransmitter/neuropeptide on their cell surface (not only on the mRNA level), and these receptors ought to be functional.

Criteria 2 for being a '*NeuroImmunoTransmitter*': The neurotransmitter/neuropeptide by itself ought to induce direct and potent effects in at least some types of immune cells. The neurotransmitter-induced immune effects can be either an induction/elevation of a given immune function, or rather its suppression, depending on the context (whose parameters are discussed below).

The immune functions discussed throughout the book as those influenced by neurotransmitters/neuropeptides include, but are not limited to, the following: immune proliferation, cytokine secretion, adhesion, spontaneous migration, chemotactic migration (i.e. chemotaxis), cytotoxicity, T-helper polarization and differentiation, suppression of Effector T cells (Teffs) by Regulatory T cells (Tregs), phagocytosis by macrophages, antibody production by B cells, Nitric Oxide (NO) production, expression of various key receptors on the cell surface of the immune cells, inward Ca^2 ion currents, outward K^+ ion currents, expression and activity of NF-kB and various others.

Criteria 3 for being a '*NeuroImmunoTransmitter*': The selective agonists and antagonists of the native neurotransmitter/neuropeptide ought to exert various **direct** effects on the immune cells carrying the specific receptors for the native neurotransmitter/neuropeptide. While the agonists can be usually expected to mimic (at least partially) the effects of the physiological neurotransmitter, the antagonists would usually do the opposite and block the neurotransmitter-induced effects, but often also induce other effects on their own.

Criteria 4 for being a '*NeuroImmunoTransmitter' (optional criteria)*: Immune cells, at least some types, should produce the neurotransmitter, and under certain conditions ought to able to release it to the extracellular milieu. The immune-derived neurotransmitter could then induce autocrine or paracrine effects.

Criteria 5 for being a '*NeuroImmunoTransmitter' (optional criteria)*: The neurotransmitter should be involved directly or indirectly in at least some immune diseases. These diseases can be any autoimmune disease, inflammatory disease,

immunodeficiency, immune malignancy – leukemia and/or lymphoma – or other. The neurotransmitter could also affect the host's immune resistance to infections.

It is suggested that if a currently entitled 'Neurotransmitter' or 'Neuropeptide' fulfills all these five criteria, or at least criteria 1–3, it should be considered as a 'NeuroImmunoTransmitter', or 'NeuroImmunoPeptide'.

'It's a Matter of Context'

A paramount take home message of our book, is that neurotransmitter's effects on immune cells are highly dependent on the context, a phenomenon I call "It's a matter of context". The main factors that determine the context, and dictate the exact effect of a given neurotransmitter on a given immune cell are listed below.

1. *The neurotransmitter concentration*. A different concentration of the very same neurotransmitter often causes a different, and even opposite effect.
   Thus, whenever studying the effect of a given neurotransmitter/neuropeptide on a specific immune function of a given immune cell, it is very important and highly recommended to test several concentrations of that neurotransmitter/neuropeptide and draw a wide range dose–response curve, or at least test three concentration ranges: low ~0.1 nM, medium ~1 μM, or high ~0.1–1 mM. As described in this book, for many neurotransmitters the low ~0.1 nM concentration is the most effective one for the specific induction/elevation or rather suppression of various immune functions, while the very high ~0.1–1 mM concentration is usually non specific and even toxic to the immune cells.
2. *The neurotransmitter receptor subtype/s being activated*. Most if not all neurotransmitters and neuropeptides have more than one receptor, and many of them have a broad family of receptors, which are coded by different genes, and coupled to different downstream elements. Usually, activation of different neurotransmitter receptor subtypes results in very different effects. Also, different immune cells have been shown to express a different composition of neurotransmitter-receptor subtypes. It is thus crucial to identify in each case which specific neurotransmitter receptor subtype/s are expressed on the immune cells being studied, and which one/s mediate the immune effect induced by the physiological neurotransmitter or by its agonist or antagonist.
3. *The activation state of the immune cell being exposed to the neurotransmitter*. One characteristic feature of immune cells is their existence in two very distinct states: a resting/naive state and an activated state, and their frequent shift between these two states. It turns out, as described in the book, that it makes a huge difference if the very same neurotransmitter binds a resting/naive immune cell or rather a cell that has already been activated, by either an antigen, mitogen, CD3/CD28 antibodies, cytokine or any other stimuli, or even an immune cell that is simultaneously activated on the one hand by a neurotransmitter, and on the other hand by any other stimuli. As discussed in this book, at least for some neurotransmitters/neuropeptides, there is also a big difference between the neurotransmitter-receptor subtypes being expressed in a resting/naive immune cell vis-à-vis its activated

counterpart, and activation of an immune cell often causes a downregulation or upregulation of certain neurotransmitter-receptor subtypes. It is thus very important, whenever possible, to study the effects of neurotransmitters on both naive/resting and activated immune cells. And it is also important to remember that what one observes in resting immune cells is not necessarily valid to activated cells, and visa versa.

4. *The specific immune cell subtype being exposed to the neurotransmitter*. There are many different types and subtypes of immune cells, each having its own characteristics and functions. As reported in this book, different immune cell types and subtypes often express a different composition of receptors for the same neurotransmitter/neuropeptides, and therefore respond differently to the very same neurotransmitter/neuropeptide. For example, it has been shown that dopamine at the very same concentration, induces very different effects in $CD4^+$ vis-à-vis $CD8^+$ T cells; in naïve $CD45RA^+$ vis-à-vis $CD45RO^+$ memory T cells, in Teffs vis-à-vis Tregs, and in resting/naive vis-a-vis activated T cells.
   All these parameters determine the exact effect of a given neurotransmitter on a specific immune function of a specific immune cell, and whether it will activate or rather suppress it. Without looking at these details, confusion, misinterpretation, and even contradictory data can be created, and no predication can be made as to how will a given neurotransmitter affect given immune responses of given immune cells.

Few Intriguing Broad Questions Raised by the Book and 'Pleading' to Be Studied, as Their Answers May Be Translated to Improved Understanding and Therapy of Various Human Diseases

Question 1: It is true that immune cells and neuronal cells – which have so many different characteristics, missions and functions – also share much much more in common than we ever realized, especially in terms of the signaling molecules and transmitters they produce, secrete and use for "talking" to cells of other systems, and in the molecules they respond to via cell their surface receptors? The solid evidences covered by our book show we ought to add neurotransmitters and neuropeptides to the list of common molecules shared by neuronal cells and immune cells, and used by both cells for multi-level and multi-system communication. Another type of 'communication molecules' known already to be produced, secreted and used by both immune cells and neuronal cells are various types of cytokines.

Question 2: Can the neurotransmitters that are produced and secreted by immune cells (as shown in this book for most neurotransmitters), i.e. the immune-derived neurotransmitters, bind and affect neuronal cells, and by doing so contribute to the ongoing neuro-immuno cross talks taking place in physiological conditions and needed for maintaining health?

Can in fact the very same neurotransmitter serve for conveying information between four different sets of cellular counterparts: (1) from one neuronal cell to

another (or to the same neuronal cell that secreted it); (2) from a neuronal cell to an immune cell; (3) from an immune cell to a neuronal cell; (4) from one immune cell to another (or to the same immune cell that secreted it)?

The data discussed in the book suggest all these four communication paths might exist for at least some of the neurotransmitters/neuropeptides, but much more evidence is needed, especially for the use of neurotransmitters by certain immune to 'talk' to neuronal cells, as well as to other immune cells.

Question 3: Are there cases in which there is a defect (genetic or acquired) in the ability of immune cells to produce and secrete certain neurotransmitters? If so, is the abnormal secretion of neurotransmitters by immune cells 'felt' by neuronal cells, since they are normally dependent on, and beneficially-affected by, the immune-derived neurotransmitters?

And if this is true, can an abnormal secretion of immune-derived neurotransmitters contribute to impaired neurological function and thereby to various neurological/neuropsychiatric diseases?

Question 4: Can an abnormal secretion of neurotransmitters by neuronal cells lead to an abnormal immune function, since immune cells are normally dependent and beneficially-affected by such nerve-driven neurotransmitters? And if this is true, can an abnormal secretion of neurotransmitters by neuronal cells contribute to impaired immunological function and various immune/autoimmune/inflammatory/immunodeficiency diseases and/or immune malignancies: leukemia and lymphomas?

The Goals of the Book, the Hopes, the Fascinating Journey . . .

The first main goal of this book is to increase the awareness of the scientific and medical communities to the key role played by neurotransmitters, neuropeptides and their receptors in the immune system, both in maintaining health, and in various immunological or neurological diseases. Currently, the receptors, effects and secretion of neurotransmitters in the immune system is to a large extent still unknown to the vast majority of immunologists, neurologists and other scientists and clinicians. It is our aim to stimulate further research and validating studies on this topic (which are so much needed), and to encourage collaborative multidisciplinary work on the various issues discussed in this book.

The second goal and hope is that the novel knowledge covered by the book would lead to the development of new therapies of various human diseases. Some specific pathways and recommendations how to reach such neurotransmitter-based therapies are discussed in several chapters of the book.

The third goal of the book stems from the fact that various neurotransmitters and/or their analogues are in medical use and commonly given to patients with various diseases. It is therefore our aim to draw the attention of clinicians that prescribe such

neurotransmitters analogues to the fact that such drugs most certainly influence immune cells too.

Thus, one may easily envision that once introduced into the body, the neurotransmitters-based drugs could bind immune cells migrating at all times in large numbers in the circulation and present in most body organs (including the brain) and affect them. If so, one can expect immune side effects which could be either positive or negative. It is therefore recommended that whenever a neurotransmitter-based drug is used, its immune effects will be studied and documented as much as possible. Doing so may even lead to modulation of the treatment, either to avoid negative immune side effects or to augment positive ones. And on top of all that, the immune side effects of the neurotransmitters-based drugs, be them as they may, should also be registered in the data sheets of the respective drugs, so that the patients taking such drugs would be fully aware of them.

The forth aim of the book is to hopefully solve what seem to be few inconsistencies and different conclusions reached in different publications regarding the effects of a given neurotransmitter/neuropeptide on a given immune cell population, or on the immune system in general. Also, sometimes, a neurotransmitter or neuropeptide is wrongly called "inhibitory" or "stimulatory", but can in fact induce both opposing effects in different setups and context. The way to solve these discrepancies is once again to pay careful attention in each publication to the small details and to the factors that dictate the context, as specified above under 'It's a matter of context'. Often "the devil is in the details"...

Sailing Towards New Horizons ...

If allowed to end this introduction in a personal viewpoint (like I started it), and by using a metaphor, I would say that reading through our book may be looked at as a fascinating nautical sailing boat journey, in the ocean of science and medicine.

We sail along shores rich in stimulating phenomenon, and between mysterious islands yet to be discovered. On board of the boat are the skilled authors of the book, aside curious readers. The readers may include both scientists that share common interest and knowledge with the authors and contributed to the findings and concepts described in the book, and 'outsiders' in the field that may nevertheless become the followers, collaborators and even navigators of the future cruises.

During our sailing adventure in the sea of life science, more and more secrets are being revealed, new connections and associations are being made, old data is suddenly re-analyzed, some enigmatic issues are being shed with a new light, and many attractive treasures are unveiled from a distance on remote islands, and flagged as ones we wish to return to and explore.

All these trigger our curiosity, imagination and creativity. So Yes ... even after we end the current journey, let's continue sailing towards these new horizons ... and let's devote careful attention and quality time for carrying basic research expeditions

on the one hand, and pharmacological-clinical-therapeutic expeditions on the other hand . . . I foresee that our future cruises will be very interesting and rewarding . . . and I bet there is much more valuable knowledge hidden in these treasure-islands than currently meets our eyes . . .

January 2012 Mia Levite, Israel

Contents

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Neurotransmitter/neuropeptide discussed by the author for its immune effects: Cannabinoids

1 Dopamine in the Immune System: Dopamine Receptors in Immune Cells, Potent Effects, Endogenous Production and Involvement in Immune and Neuropsychiatric Diseases

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1.1 General Introduction: Dopamine and Its Receptors

1.1.1 Nobel Prize for Medicine in 2000 for Discovering Dopamine as an Independent Neurotransmitter

The discovery of dopamine as a neurotransmitter in the brain by Arvid Carlsson at the Laboratory for Chemical Pharmacology of the National Heart Institute of Sweden, approximately 50 years ago, and the subsequent insight provided by Paul Greengard into the cellular signaling mechanisms triggered by dopamine, gained these researchers the Nobel Prize for Medicine in 2000. They showed that dopamine is not just a precursor of norepinephrine (noradrenaline) and epinephrine (adrenaline) (see Fig. 1.1) but also an independent neurotransmitter (Iversen and

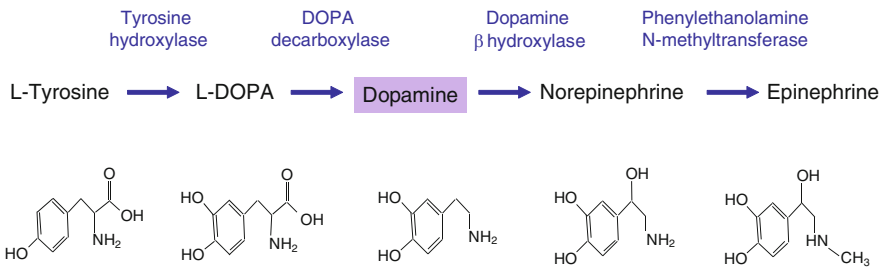

Fig. 1.1 Biosynthesis of dopamine and the other catecholamines, and their chemical structure

Iversen 2007). Since then, dopamine research influenced the development of biological psychiatry and psychopharmacology more than the work on any other neurotransmitter (Iversen and Iversen 2007). Dopamine is known today as one of the principal neurotransmitters in the central nervous system (CNS), involved in several key functions such as behavior, cognition, control of movement, endocrine regulation and cardiovascular function. Dopamine is also an important modulator of peripheral physiologic functions in both humans and animals.

On top of all that, the evidences cited in the chapter show that dopamine exerts very potent effects on a kaleidoscope of key immune functions, and therefore may deserve a new title of a 'Neuro-Immuno-Transmitter', playing a crucial in role in both the nervous system and the immune system. The criteria for this new term, that dopamine undoubtedly fulfills, and the take home messages of the entire chapter are summarized at the very end (part 1.7).

1.1.2 Dopamine Structure and Biosynthesis

Dopamine is classified as a catecholamine. As a member of the catecholamine family, it is a precursor to norepinephrine (noradrenaline) and then epinephrine (adrenaline) in the biosynthetic pathways for these neurotransmitters, drawn in Fig. 1.1.

Dopamine has the chemical formula $C_6H_3(OH)_2–CH_2–CH_2–NH_2$. Its chemical name is "4-(2-aminoethyl) benzene-1,2-diol" and its abbreviation is "DA." Dopamine was first synthesized in 1910 by George Barger and James Ewens at Wellcome Laboratories in London, England. It was named dopamine because it is a monoamine – a compound containing nitrogen formed from ammonia by replacement of one or more of the hydrogen atoms by hydrocarbon radicals – and its synthetic precursor is 3,4-*di*hydr*oxy*p*henyl*a*lanine (L-DOPA) (Fig. 1.1).

Dopamine is not only a member of the catecholamine, but also a member of a group of neurotransmitters called 'Biogenic amines'. This group consists of Dopamine, Norepinephrine, Epinephrine and Serotonin.

1.1.3 Where Is Dopamine Produced, and Where Can Immune Cells 'Meet' Dopamine?

Dopamine is synthesized mainly by nervous tissue. Dopaminergic neurons (i.e. neurons whose primary neurotransmitter is dopamine) are present chiefly in the ventral tegmental area of the midbrain and the substantia nigra pars compacta. Dopamine is also produced by neurons in the arcuate nucleus of the hypothalamus, and secreted into the hypothalamo-hypophysial blood vessels of the median eminence, which supply the pituitary gland. There, in the anterior pituitary, dopamine is the primary neuroendocrine inhibitor of the secretion of prolactin from lactotrope cells (which secrete prolactin in dopamine's absence). In neurons, dopamine is packaged after synthesis into vesicles, which are then released into the synapse in response to a presynaptic action potential. In peripheral tissues, dopamine is released from neuronal cells and is synthesized within specific parenchyma. Dopamine released from sympathetic nerves predominantly contributes to plasma dopamine levels, but recently new functional roles of peripheral dopamine have been found (Rubi and Maechler 2010; Wikipedia 2011). On top of all that, dopamine is undoubtedly synthesized in most if not all immune cells. The evidences for that are summarized herein in part 1.4.

Where can dopamine and immune cells 'meet' one another?

Based on the knowledge accumulated thus far, one may envision that immune cells can 'meet' dopamine under physiological and pathological conditions in all the following organs:

1. **Brain** – Dopamine is produced in the brain. Since activated T cells regularly transmigrate into the CNS across the blood brain barrier (BBB), upon entry of such T cells into the brain they will most probably encounter dopamine.
2. **The lymphoid organs** – The secondary lymphoid tissues are highly innervated by sympathetic nerve fibers that store dopamine at high contents. In addition, as already mentioned above and discussed in length below in part 1.4, lymphocytes and other immune cells are capable of active synthesis and release of dopamine (Bergquist et al. 1994, 1997; Josefsson et al. 1996; Musso et al. 1996; Musso et al. 1997; Bergquist and Silberring 1998; Tsao et al. 1998; Mignini et al. 2003; Ferrari et al. 2004; Cosentino et al. 2007; Flierl et al. 2007, 2009; Nakano et al. 2009a).
3. **Blood** – Under physiological conditions dopamine is present in the plasma in relatively low conc. estimated as ~10^{-10} M–10^{-11} M. Immune cells that are present regularly in the circulation will thus encounter dopamine at this low conc. in the blood yet not necessarily respond to it, since the optimal dopamine conc. for affecting immune cells is higher: ~10^{-8} M and above (see part 1.3). Interestingly, Saha et al. report that in healthy individuals dopamine's plasma conc. is 10.2 ± 0.9 pg/ml, and in cancer (lung carcinoma) patients it is significantly elevated to 48.6 ± 5.1 pg/ml (Saha et al. 2001a).
4. **In other dopamine-containing peripheral organs** – Dopaminergic neurons innervate human peripheral tissues among them the kidney and the hepatic vasculature. Immune cells may enter such tissues and encounter dopamine in them.

1.1.4 Dopamine Receptors: Subtypes, Expression and Function Within and Out of the CNS

In general, dopamine receptors (DRs) have been identified in a number of organs and tissues, which include several regions within the CNS, sympathetic ganglia and postganglionic nerve terminals, various vascular beds, the heart, the gastrointestinal tract, and the kidney. The peripheral DRs influence cardiovascular and renal function by decreasing after load and vascular resistance and promoting sodium excretion. Within the kidney, DRs are present along the nephron, with highest density on proximal tubule epithelial cells (Hussain and Lokhandwala 2003; Wikipedia 2011).

The DRs are a class of metabotropic G protein-coupled receptors that are prominent in the vertebrate CNS. There are at least five subtypes of DRs: D1R, D2R, D3R, D4R, and D5R, which belong to two DR families: The D1 family and the D2 family. There is also some evidence suggesting the existence of possible D6R and D7R, but such receptors have not been conclusively identified. Dopamine is the primary endogenous ligand for all the DR subtypes. At a global level, D1Rs have widespread expression throughout the brain, and the D1R-D2R subtypes are found at 10–100 times the levels of the D3R, D4R and D5R subtypes (Wikipedia 2011).

The dopamine D1-like receptor family: The D1R and D5R are members of the D1-like family of DR's. D1R is encoded by the dopamine receptor D1 gene, while D5R is encoded by the D5 gene. Activation of D1-like family receptors – D1R and D5R, is coupled to the G protein Gαs, which subsequently activates adenylyl cyclase, increasing the intracellular conc. of the secondary messenger cyclic adenosine monophosphate (cAMP) (Wikipedia 2011).

The dopamine D2-like family: the D2R, D3R and D4R are members of the D2-like family of DR's. Activation of these D2-like family receptors is coupled to the G protein Gαi, which directly inhibits the formation of cAMP by inhibiting the enzyme adenylate cyclase. The D2R is encoded by the D2 gene, of which there are two forms: D2Sh (short) and D2Lh (long). The D2Sh form is pre-synaptically situated, having modulatory functions, while the D2Lh form may function as a classical post-synaptic receptor, i.e., transmit information (in either an excitatory or an inhibitory fashion) unless blocked by a receptor antagonist or a synthetic partial agonist. The D3R is encoded by the D3 gene. Maximum expression of D3Rs is noted in the islands of Calleja and nucleus accumbens. The D4R is encoded by the dopamine receptor D4 gene. The D4R gene displays polymorphisms that differ in a variable number of tandem repeats present within the coding sequence of exon 3. Some of these alleles are associated with greater incidence of certain diseases. For example, the D4.7 alleles have an established association with attention-deficit hyperactivity disorder (ADHD) (Wikipedia 2011).

Interestingly, while DR's, alike other G protein-coupled receptors (GPCRs) have been classically thought to work as monomeric entities, the current view of their organization, however, assumes that they are part of highly organized molecular complexes, where different receptors and interacting proteins are clustered. These heteromers have peculiar pharmacological, signaling, and trafficking properties.

GPCR heteromerization, raising different combinatorial possibilities, thus underlies an unexpected level of diversity within this receptor family (Missale et al. 2010). In their recent review, Missale et al. discuss the existence of D1R and glutamate/NMDA receptors heteromers, and D1R-D3R heteromers and their peculiar pharmacological, signaling, and functional properties (Missale et al. 2010).

1.1.5 Abnormalities in Dopamine and/or Its Receptors in the CNS in Neurological and Psychiatric Diseases

Dysfunction of dopaminergic neurotransmission in the CNS has been implicated in a variety of neurological and neuropsychiatric disorders (Kienast and Heinz 2006). These include Parkinson's disease (Fuxe et al. 2006), Schizophrenia (Kienast and Heinz 2006), ADHD (Faraone and Khan 2006; Kienast and Heinz 2006), drug use, abuse and addiction, alcohol dependence (Kienast and Heinz 2006; Di Chiara et al. 2004), social phobia and social anxiety disorder (Schneier et al. 2000, 2008), Obsessive-compulsive disorder (OCD) (Schneier et al. 2008), Tourette's syndrome (Kienast and Heinz 2006), and neuroleptic malignant syndrome (Mihara et al. 2003). Hypersociality, Bipolar disorder, mania as well as hypersexuality are also related to an increase in dopamine. Dopamine disorders in frontal lobes of the brain can cause a decline in neurocognitive functions, especially memory, attention, and problem-solving. D1Rs as well as D4Rs are responsible for the cognitive-enhancing effects of dopamine.

Interestingly, in some of the neuropsychiatric diseases associated with impaired dopamine levels/receptors/signaling, there are reports on abnormalities in DR expression in lymphocytes, and/or in various immune functions, sometimes in significant correlation with the severity of the neuropsychiatric disease. This topic is discussed in part 1.6, for each neuropsychiatric disease separately. On top of all that, there seem to be an association between impaired dopamine levels/receptors/signaling and some immune and autoimmune diseases. This topic is covered in part 1.5, once again for each type of immune/autoimmune disease separately.

1.2 Dopamine Receptors Are Expressed in Most/All Immune Cells

1.2.1 Opening Remarks

It is absolutely clear now that most if not all immune cells express various types of DRs, and that these DRs are functional receptors that play an active role in triggering or suppressing key immune responses. Thus, DRs in immune cells are surely not just 'passive markers' expressed on the RNA level **only**, and not of interest only in the context of their impaired levels and therefore potential diagnosis value in various neurological diseases, as suggested by some studies discussed in part 1.6.

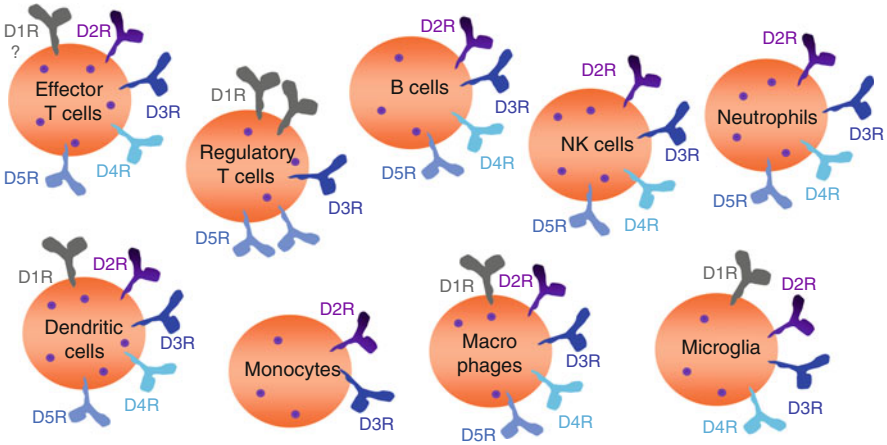

Fig. 1.2 Dopamine receptors are expressed in immune cells. Most, if not all, immune cells express DRs, mainly D2R, D3R, D4R and D5R, yet some also D1R. The expression of the DRs in immune cells was shown both on the mRNA level on the protein levels, and the DR's are present as functional receptors on the cell surface, that bind and respond to dopamine and its analogues. Interestingly, the DR expression in immune cells, especially T cells, often changes dramatically after activation of the cells by strong stimuli such as by antigens, mitogens, cytokines etc. Thus, for example, the levels and types of DRs in T cells is different in resting/naive cells vis-a-vis activated T cells. The DRs expression is also different in various T cell subpopulations such as $CD4^+$ and $CD8^+$ cells; Teffs and Treg cells etc. The figure is based on the findings revealed thus far in various studies (cited in the text of this chapter) and shows the DR subtypes identified so far in effector T cells, regulatory T cells, B cells, Natural Killer (NK) cells, dendritic cells, moncytes, macrophages, microglia and neutrophils

The below paragraphs will review the evidences for the presence of DRs in each type of immune cell. In addition, Fig. 1.2, based on these studies, shows schematically the expression of different DRs in each immune cell type. In most studies only some types of immune cells and some types of DRs were studied. Yet, in at least one comprehensive study by McKenna et al. (McKenna et al. 2002), done on 20 healthy individuals, the protein expression of all DR subtypes was tested by flow cytometry on the cell surface of different leukocyte subpopulations, among them T cells, B cells, monocytes, neutrophils, eosinophils and NK cells (McKenna et al. 2002). As a whole, the three main findings and conclusions of this study were: (1) The D2R, D3R, D4R and D5R, but not D1R, are expressed in most if not all the immune cells tested; (2) Some immune cells have higher levels of DRs than others. In this specific study, B cells showed the highest expression of D2R-D5R; (3) The expression of D3R and D5R is stable and always detected in leukocytes of all individuals, while the expression of D2R and D4R is more variable (McKenna et al. 2002). The detection of DRs in various immune cells reported in this study is in line with earlier and later studies cited below. Yet, in contrast to the findings of this study, T cells were found by several other groups (see part 1.2.3 below) to express high levels of

functional DRs (reviewed by (Levite 2008) and (Sarkar et al. 2010)), and D1R expression, that was not revealed by McKenna et al., was later demonstrated in effector T cells (Teffs) and Regulatory T cells (Tregs) (Cosentino et al. 2007; Nakano et al. 2008), dendritic cells (Nakano et al. 2008), macrophages (Gaskill et al. 2009) and microglia (Farber et al. 2005; Mastroeni et al. 2009).

1.2.2 Dopamine Receptors in Heterogeneous Human Peripheral Blood Lymphocytes (PBL's)

Ricci et al. showed the binding of D3R agonist [3H]7-OH-DPAT to peripheral blood lymphocytes (PBL's), and the reduced [3H]7-OH-DPAT binding upon application of anti-D3R and D4R antibodies, suggesting that human PBL's express D3R and D4R (Ricci et al. 1998). A subsequent study of the same group concluded that D5R is the only D1R-like subtype expressed by human PBL's (Ricci et al. 1999). Kirillova et al. showed by real-time RT-PCR the expression of the DR2-DR5 genes in peripheral blood mononuclear cells (PBMCs), which varied widely among samples, whereas the D1R expression was not detected. The expression levels of the DR3 and DR4 in the PBMCs were comparable with those in the brain for these receptors, and were significantly lower for DR2 and DR5 (Kirillova et al. 2008). On top of all these clear indications for the expression of most if not all DRs in PBLs of healthy people, DRs were also shown in PBLs or in purified T cells of patients with Schizophrenia (Ilani et al. 2001; Kwak et al. 2001) (Boneberg et al. 2006), Parkinson disease (Nagai et al. 1996; Barbanti et al. 1999), Alzheimer's disease (Barbanti et al. 2000a), and migraine (Barbanti et al. 1996). Interestingly, the levels of the DR's in the lymphocytes of patients with these diseases were often significantly different from those in lymphocytes of healthy individuals. The specific findings showing that are cited in part 1.6.

1.2.3 Dopamine Receptors in Human T Cells

Many studies performed by different groups showed that T cells of various subtypes express DRs, mainly D2R, D3R, D4R and D5R (Santambrogio et al. 1993; Levite et al. 2001; Besser et al. 2005; Watanabe et al. 2006; Cosentino et al. 2007; Nakano et al. 2008). For reviews the reader is referred to (Levite 2008) and (Sarkar, Basu et al. 2010). The key studies showing DRs expression in human T cells are cited below in a chronological order.

Study 1: Santambrogio et al. reported on the presence of "recognition sites of the D2R family" in human T cells, based on the binding of [3H] sulpiride – a selective D2R and D3R antagonist (Santambrogio et al. 1993).

Study 2: Levite et al. showed that dopamine activates functional D3R and D2R in purified normal human T cells of healthy volunteers, and triggers β1 integrin function (Levite et al. 2001). Seven D2R/D3R agonists and antagonists were used in that study to identify and confirm that dopamine-induced activation of these T cells indeed occurred via D2R and D3R (Levite, Chowers et al. 2001). These findings are described in further detail in part 1.3.3.1.

Study 3: McKenna et al., in a study already summarized above in the "Opening remarks", used flow cytometry to study DR expression in various types of leukocytes, and reported that $CD3^+$ T cells of healthy volunteers express minimal levels of D2R, D3R, D4R and D5R, and no D1R (McKenna et al. 2002). Once again, the low expression, especially of the D2R, D3R and D4R in T cells, is in contrast to other studies providing solid evidence for the expression of these DRs in human T cells (Levite et al. 2001; Besser et al. 2005; Watanabe et al. 2006; Cosentino et al. 2007; Nakano et al. 2008). Also, D1R expression was later demonstrated in Teff and Tregs, and elegant evidence was provided to support its important role in suppressing Tregs (Cosentino et al. 2007; Nakano et al. 2008). This is discussed in Part 1.3.6.

Study 4: Levite's group showed that normal human T cells of healthy individuals express functional D2R, D3R as well as D1R/D5R, and that dopamine by itself activates triggers the selective secretion of either IL-10, or TNFα or both, by activating either D2R, or D3R or D1R/D5R respectively (Besser et al. 2005). These findings will be described in further detail below, in part 1.3.3.2.

Study 5: Watanabe et al. found that D3R was the predominant DR subtype in the secondary lymphoid tissues, and that it is selectively expressed by naive $CD8^+$ T cells of both humans and mice (Watanabe et al. 2006). The D3R was expressed preferentially on naïve $CD45RA^+$ $CD27^+$ $CD8^+$ T cells. Furthermore, Watanabe et al. showed the following:

(1) Resting $CD4^+$ cells express: (a) low D2R mRNA, (b) low D3R mRNA, (c) hardly any D3R protein; (2) ConA-activated $CD4^+$ cells express: (a) up-regulated D2R mRNA, (b) completely down-regulated D3R mRNA. The protein levels of the DRs were not tested; (3) Resting $CD8^+$ cells express: (a) high D3R mRNA, (b) low D4R mRNA, (c) the D3R protein in 70–80% of the $CD8^+$ T cells; (4) PHA-activated $CD8^+$ cells express: (a) completely down regulated D3R mRNA, (b) completely down regulated D4R mRNA. The protein levels of the DR's were not tested (Watanabe et al. 2006). These findings demonstrate that the mRNA expression of the DRs in $CD4^+$ and $CD8^+$ T cells are different and very sensitive to activation of these cells by other stimuli. It is therefore often modulated dramatically in response to T cell activation by antigens or mitogens.

Study 6: Cosentino et al. showed that human Teffs as well as $CD4^+$ $CD25^+$ Tregs, express both D1-like and D2-like receptors to a similar extent (12–29% of the cells) on their cell surface (Cosentino et al. 2007). The further interesting findings regarding the function of these DRs in Teffs and Tregs are discussed below in part 1.3.6.

Study 7: Nakano et al. detected by flow cytometry the cell surface expression of DRs in naïve and memory T cells purified from the peripheral blood of healthy individuals, and found that $CD4^+$ $CD45RA^+$ naïve T cells and $CD4^+$ $CD45RO^+$ memory T cells express all types of DRs: D1R-D5R, yet to a different extent (Nakano et al. 2008). The authors concluded that while the D1-like-receptors are highly expressed in both naïve and memory T cells, the D2-like-receptors are expressed mainly in memory T cells and only marginally in naive cells (Nakano et al. 2008). The high D1R expression in T cells described in this study

contradicts the findings of McKenna et al. claiming that "D1 was never found" in human T cells (McKenna et al. 2002).

Study 8: Basu et al. showed recently that human T leukemia cell line (Jurkat) express D1R and D2R, alike normal human T cells. However, these DRs seem to function differently in normal and malignant T cells, since the TCR-induced proliferation of activated normal T cells is inhibited by dopamine, but that of Jurkat is not (Basu, Sarkar et al. 2010).

Study 9: Huang et al. found that T cells from the mesenteric lymph nodes of mice express the mRNA of all the five DR subtypes: D1R–D5R (Huang et al. 2010).

1.2.4 Dopamine Receptors in Human B Cells

Four studies showing that B cells express DRs are cited here in a chronological order.

Study 1: Santambrogio et al. detected dopamine D2 binding sites in human B cells using the [3H] sulpiride as a ligand (Santambrogio et al. 1993).

Study 2: Mckenna el al showed that human B cells ($CD19^+$) of seven healthy individuals express the highest level of D2R-D5R, compared to all the other types of leukocytes studied. Out of the seven individuals tested, the D3R was expressed in B cells of all, the D2R and D5R in six, the D4R in two, and D1R in none (McKenna et al. 2002).

Study 3: Watanabe et al., testing for all DRs by RT-PCR, showed that resting human $CD19^+$ B cells faintly express D4R, but none of the other DR subtypes. Upon activation of these cells with PWM, D4R was completely downregulated (Watanabe et al. 2006).

Study 4: Meredith et al. found that with the exception of D4R, D1R-DR5 were variably expressed among normal and neoplastic B cell populations. Transcripts for D1R and D2R were frequently found, whereas D3R and D5R revealed restricted expression (Meredith et al. 2006).

1.2.5 Dopamine Receptors in Human Dendritic Cells

Study 1: Nakano et al. tested by flow cytometry the expression of all DR subtypes in human monocyte-derived dendritic cells (M0-DC) (Nakano et al. 2008). A figure within this study shows that M0-DC cells express all DR subtypes: D1R-D5R, but in unequal levels, the highest being the D1R and D5R (at least in the one representative human volunteer shown). D2R-like antagonist induced DC-mediated Th17 differentiation, while D1R-like antagonist had the reverse effect and inhibited Th17 differentiation (Nakano et al. 2008). These findings will be discussed again in part 1.3.5 below, dealing with dopamine-induced immune effects.

Study 2: Watanabe et al. found by RT-PCT that resting CD14 monocytes faintly expressed D4R, but none of the other DR subtypes (Watanabe et al. 2006).

1.2.6 Dopamine Receptors in Human Macrophages

Gaskill et al. found that macrophages express D1Rs and D2Rs, and that dopamine activated macrophages, by increasing ERK 1 phosphorylation. Dopamine also increased HIV replication in human macrophages via activation of DR2 (Gaskill et al. 2009). In addition, some of the reported effects of either dopamine itself or its agonists and antagonists on macrophage features and effector functions discussed later in part 1.3.7.1 (Ali et al. 1994; Gomez et al. 1999; Sternberg et al. 1987; Hasko et al. 1996), are most probably mediated by DRs expressed in these cells.

1.2.7 Dopamine Receptors in Human Microglia

Microglia, which constitute 20% of the total glial cell population within the brain, are a type of glial cells that are the resident macrophages of the brain and spinal cord, and thus act as the first and main form of active immune defense in the CNS. Microglia are constantly scavenging the CNS for damaged neurons, plaques, and infectious agents (Gehrmann et al. 1995; Kreutzberg 1995; Gehrmann 1996; Aloisi 2001).

Below are two studies reporting on expression of DRs in microglia:

Study 1: Farber et al. used the patch clamp technique as the functional assay and ligands specific to different DRs, and identified a functional expression of D1-like and D2-like DRs in mouse and rat microglia, in culture and brain slices (Farber et al. 2005). The effects triggered by these DRs in microglia will be discussed in part 1.3.7.2.

Study 2: Mastroeni et al. showed that human elderly microglia growing in tissue culture expressed D1R-D4R mRNAs, but not D5R mRNA, and that these cells were also immunoreactive for D1R-D4R, but not for the D5R (Mastroeni et al. 2009).

1.2.8 Dopamine Receptors in Other Human Immune Cells

McKenna et al. reported on DRs expression not only in T cells and B cells as mentioned above, but also in neutrophils, eosinophils and NK cells (McKenna et al. 2002) (see also Fig. 1.2).

1.3 The Direct Effects of Dopamine on Immune Cells: Dopamine Usually Activates Naïve/Resting Immune Cells, but Inhibits Activated Immune Cells

1.3.1 It's a Matter of Context: Dopamine's Concentration, the Dopamine Receptor Subtype/s Being Activated, the Activation State of the Immune Cell and the Specific Immune Cell Subtype, Will All Determine Whether Dopamine Will Activate or Rather Suppress Some of the Immune Responses of This Cell

It is now absolutely clear that the effect of dopamine on an immune cell is highly dependent on the context (illustrated schematically in Figs. 1.3, 1.4, 1.5). In fact, "It's a matter of context" is a term coined by M. Levite (Levite 2008) (see especially Fig. 1.4 in that review), re the effects of neurotransmitters, dopamine

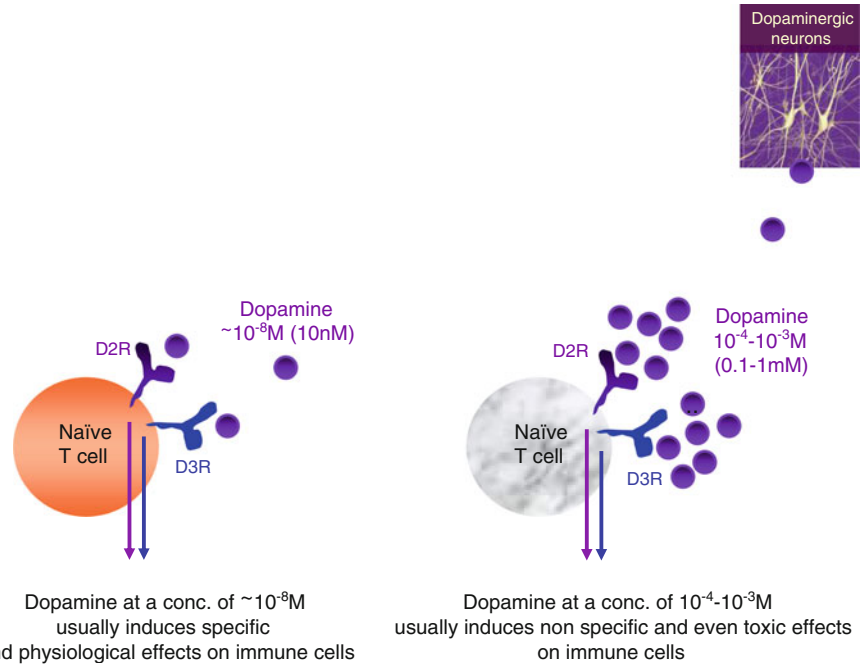

Fig. 1.3 The effects of dopamine on immune cells are highly dependent on the context, and one main factor is dopamine's conc. Thus, different dopamine conc. often induce different and even opposite effects in immune cells. Several examples are discussed in parts 1.3.3–1.3.7. Dopamine's optimal conc. for affecting immune cells in a physiological and specific manner seems to be ~10^{-8} M (0.1nM). While 10–100-fold lower or higher conc. are often still effective, dopamine at very high conc. of ~10^{-4}–10^{-2} M (0.1–10 mM) usually induces non specific and even toxic effects, often resulting in immune cell death

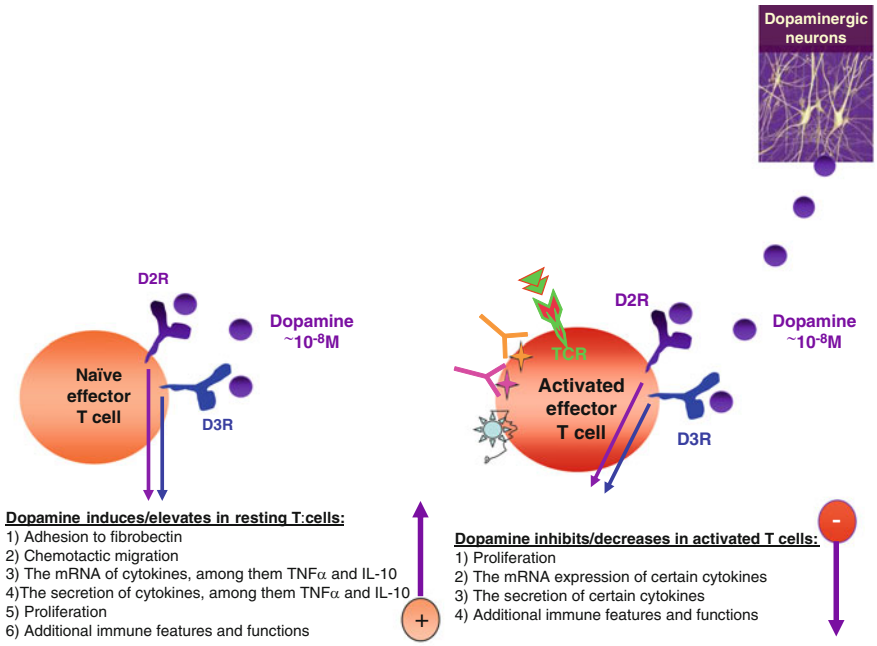

Fig. 1.4 It's a matter of context: Dopamine's effect on immune cells is highly dependent of the activation state of the immune target cell. From the studies published thus far, a certain generalization seems to emerge: dopamine usually activates naïve/resting effector T cells, but suppress already activated T cells, i.e. T cells that before encountering dopamine have been activated by other stimuli such as antigen, CD3/CD38 antibodies, mitogen (i.e. PHA, Con A), cytokines (i.e. IL-2) or even other neurotransmitters. The supporting evidence is discussed in the text of this chapter.

included, on T cell function. Few parameters seem to dictate the context and the final effect of dopamine on a given immune cell, and these are the following:

1. Dopamine's concentration – low: ~10^{-8} M (~0.1nM), medium: ~10^{-6} M (~1 μM), or high: ~10^{-3} M (~1 mM). It turns out that dopamine at these different conc. ranges can induce very different, and even completely opposite effects, in the very same target cell (Fig. 1.3). Several examples are discussed in parts 1.3.3–1.3.7.
2. The activation state of the immune cell being stimulated by dopamine (Fig. 1.4). It makes a huge difference if dopamine binds an immune cell which is in a naïve/resting state (condition 1); rather to a cell that has already been activated by either antigen, mitogen, CD3/CD28 antibodies, cytokine, growth factor or any other stimuli (condition 2), or to an immune cell that is activated simultaneously on the one hand by dopamine, and on the other hand by any other stimuli (condition 3). In most cases, as will be discussed below, when the immune cell is in a naïve/resting state, dopamine will directly trigger or enhance some of its immune functions, but if the cell has already been activated by other stimuli before encountering dopamine, or even simultaneously activated by dopamine and other stimuli, the outcome would be usually suppression.

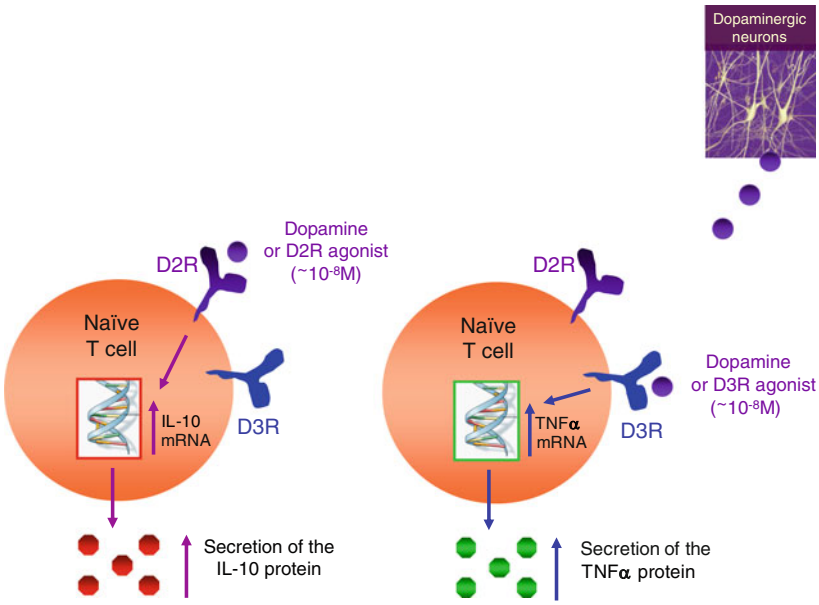

Fig. 1.5 It's a matter of context: Dopamine effects on immune cells are dependent on the specific DR being activated. Thus, activation of different DRs expressed on the same immune cells often induce different and even completely opposite effects. The figure shows schematically the findings revealed by M. Levite and her team (Besser et al. 2005) that support this notion. Additional relevant examples are discussed in the text of this chapter

3. The specific type/subtype of the immune cell that encounters dopamine. For example, dopamine induce different effects in $CD4^+$ vis-à-vis $CD8^+$ T cells (Watanabe et al. 2006); in naïve CD45RA vis-à-vis CD45RO memory T cells (Watanabe et al. 2006), and in Teffs vis-à-vis and Tregs (Cosentino et al. 2007).
4. The specific DR subtype/s being activated by dopamine on the same cell or population of cells. Activation of different DRs in the very same cell or population of cells often leads to different effects (Besser et al. 2005) (Huang et al. 2010). For example, dopamine induces the secretion of different cytokines from the very same naïve peripheral human T cells, via activation of different DR subtypes expressed in these cells. Thus, dopamine on its own in induces a selective secretion of either TNFα only via the D3R, or of IL-10 only via the D2R (Besser et al. 2005).

1.3.2 Suggested Rules for Dopamine-Induced Effects on Immune Cells, and Preliminary Ideas for Possible Therapeutic Use of Dopamine-Induced Effects on T Cells

Together, the fact that "It's a matter of context" and that so many factors determine dopamine's effects on immune cells makes it very hard to predict what would be the exact effect of dopamine on a given immune cell or function, in each specific

Table 1.1 Many types of immune cells contain endogenous dopamine at the range of ~10^{-20}–10^{-17} M per cells The table was reproduced from Table 1 in Bergquist et al. (1998)

Cell type	Average dopamine content (mol cell-1)	Reference
Single cells:		
Human lymphocytes present in the CSF	~2×10^{-18}	[5]
Human $CD4^+$ T cells	~2×10^{-18}	[5]
Human B cells	~3×10^{-19}	[5]
Extracted cell populations:		
Human $CD4^+$ T cells	~2×10^{-18}	[5]
Human B cells	~2×10^{-18}	[9]
Human PBMC cells	~1.6 to 8.6 $\times 10^{-18}$	[9, 10]
Murine spleen cells	~7×10^{-17}	[8]
Murine peritoneal macrophages	~2×10^{-17}	[8]
Murine T cells	~1.9×10^{-18} to $<1.1 \times 10^{-20}$	[8]
Murine B cells	~1.7×10^{-19} to $<6.9 \times 10^{-20}$	[8]

instance. Yet, based on studies done so far and cited below, I would suggest 7 general 'rules' re dopamine's receptors, effects and endogenous production in the immune system, and some preliminary ideas how can the direct effects of dopamine on T cells can be used therapeutically. Hopefully, in the coming years all these suggested rules and ideas will be further studied and validated.

Suggested rule 1: Dopamine plays a very important role in the immune system and thus can be re-named a "Neuro-Immuno-Transmitter" since three major criteria are fulfilled:

1. Most, if not all, immune cells express DRs (Fig. 1.2). The most abundant are D2R, D3R, D4R and D5R, but some immune cells like Tregs, macrophages and microglia also express D1R. The DRs expression in immune cells is very sensitive to the activation state of the cell, and often modified sharply after antigenic or mitogenic stimulation.
2. Dopamine and/or to its selective agonists and antagonists induce direct and potent effects on immune cells, primarily T cells and dendritic cells (DC's), resulting in either augmented or suppressed immune functions and features, depending on the context.
3. Many immune cells produce endogenous dopamine (~10^{-17}–10^{-20} M per cell, see Table 1.1). The immune-derived dopamine may induce autocrine and paracrine immune effects.

Suggested rule 2 (Fig. 1.3): Dopamine's optimal conc. for affecting T cells and other immune cells in a physiological and specific manner is ~10^{-8} M (10nM). While 10–100-fold higher or lower dopamine conc. is often still effective, dopamine at much higher conc. of ~10^{-3}–10^{-4} M (~0.1–1 mM) in most cases induce non specific immune effects, and is often even toxic and kills the immune cells. See for example (Levite et al. 2001; Cosentino et al. 2004; Besser et al. 2005; Watanabe et al. 2006).

Suggested rule 3 (Fig. 1.4): Dopamine usually stimulates naïve T cells (Tsao et al. 1997; Levite et al. 2001; Besser et al. 2005; Watanabe et al. 2006; Strell et al. 2009), but inhibits activated T cells, i.e. T cells that have already been activated

by either antigen, mitogen, CD3 and CD28 antibodies, cytokines etc., or cells that are being activated simultaneously with dopamine and any of these other stimuli (Santambrogio et al. 1993; Bergquist et al. 1994, 1998, 2000; Cook-Mills et al. 1995; Josefsson et al. 1996; Cardoso et al. 1998; ten Bokum et al. 2000; Saha et al. 2001a, b; Carr et al. 2003; Ghosh et al. 2003; Ilani et al. 2004; Sarkar et al. 2006; Schneier et al. 2008; Strell et al. 2009; Huang et al. 2010).

I propose that the potent activation of naïve immune cells, especially T cells, by dopamine or its selective DR agonists (covered in part 1.3.3 below) offers a new attractive strategy in adoptive cell-based therapy, since short ex vivo exposure of the patient's autologous naive T cells to dopamine before they are returned into the body (preferably in a repeated manner, over a prolonged period of time), could safely and potently upregulate their in vivo function, and as such be beneficial in pathologies where these T cells are so critically needed yet often suboptimal such as in cancer, infectious diseases and immunodeficiencies.

In contrast, dopamine-induced inhibition of activated T cells (covered in part 1.3.4) may have therapeutic implications for pathologies requiring down regulation of deleterious activated T cells, like T-cell mediated autoimmune diseases, graft versus host disease (GVHD), graft rejection and others. In such cases, perhaps dopamine (or its agonists) could be infused safely into the body to suppress activated T cells. Of reminder: dopamine infusion is an approved and widely used strategy for the management of cardiovascular disorders and renal dysfunction in intensive care units.

Suggested rule 4 (Fig. 1.5): Activation of different DRs expressed in the same immune cell, or within a given immune cell population, often leads to very different effects. For example see (Besser et al. 2005; Huang et al. 2010).

Suggested rule 5 (Fig. 1.6): Dopamine induce selective adhesion, migration and homing in vivo of naive $CD8^+$ T cells, via the D3R, and as a whole naive $CD8^+$ T cells may be more responsive to dopamine than $CD4^+$ T cells (Watanabe et al. 2006; Saha et al. 2001a, b; Strell et al. 2009).

Dopamine-induced upregulation of $CD8^+$ T cells may also be an attractive cell-based therapeutic approach to upregulate the migration and homing of such cytotoxic cells towards solid tumors, and hopefully also to augment their subsequent attack of the cancer cells.

Suggested rule 6 (Fig. 1.7): Dopamine seems to activate naïve Teffs mainly via the D2R and D3R, but suppress Tregs via the D1R. Dopamine-induced inhibition of Tregs is evident in many features and functions of these cells (Kipnis et al. 2004; Cosentino et al. 2007). Of note, it seems that two different dopamine-induced effects on T cells: first – the direct activation of naive/resting Teffs, and second – the inhibition of Tregs, or in other words the 'suppression of the suppressors', lead to the same result: activation of Teffs by dopamine. I would suggest again that these physiological, safe and potent ways to upregulate T cell function ought to be explored therapeutically.

Suggested rule 7 (Fig. 1.8): Dopamine is released by DCs during antigen presentation to naive $CD4^+$ T cells, and can subsequently play an important role as a T(h) factor in the DC-naive T-cell interface. Moreover, selective DR

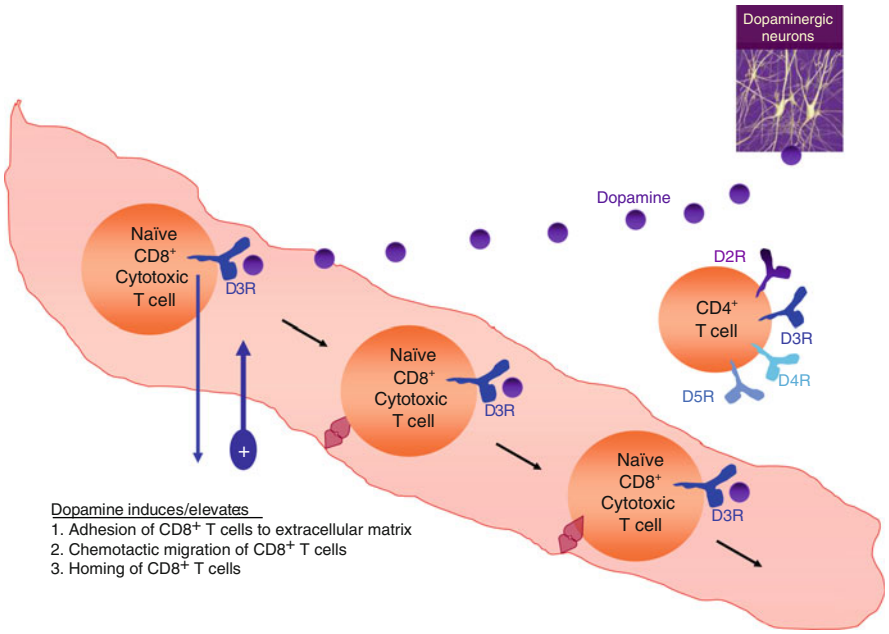

Fig. 1.6 It's a matter of context: dopamine exerts different effects on different immune cell subtypes. In line with this notion, $CD8^+$ T cells seem to be more responsive to dopamine than $CD4^+$ T cells. Indeed, dopamine induces selective adhesion to extracellular matrix, chemotactic migration and homing of $CD8^+$ T cells. The figure illustrates schematically the presumed migration and adhesion of $CD8^+$ T cells in the blood after they have been exposed to dopamine. The figure is based on the findings of (Watanabe et al. 2006) and of (Strell et al. 2009)

antagonists can be used to affect Th17 cell differentiation: antagonizing D1-like receptors inhibits DC-mediated Th17 differentiation, while antagonizing D2-like-receptors induce Th17 differentiation (Nakano and Matsushita 2007; Nakano et al. 2008, 2009a, b, 2011).

1.3.3 Dopamine Activates Naïve/Resting Immune Cells, and Induces Their Adhesion, Migration and Cytokine Secretion

1.3.3.1 Dopamine Induce Adhesion and Migration of Naive Immune Cells

Study 1: *Dopamine induces adhesion of T cells to fibronectin*. Levite et al. showed in 2001 for the first time that dopamine on its own activates naïve normal human T cells, and drives them to function (Levite et al. 2001). Thus, dopamine, at 10^{-8} M (10nM) range, and in the complete absence of any additional molecules, directly interacts with DRs expressed on the cell surface of normal naïve/resting human T cells (purified from the blood of healthy volunteers) and triggers beta1 (β1) integrin-mediated T cell adhesion to fibronectin – a major extracellular matrix

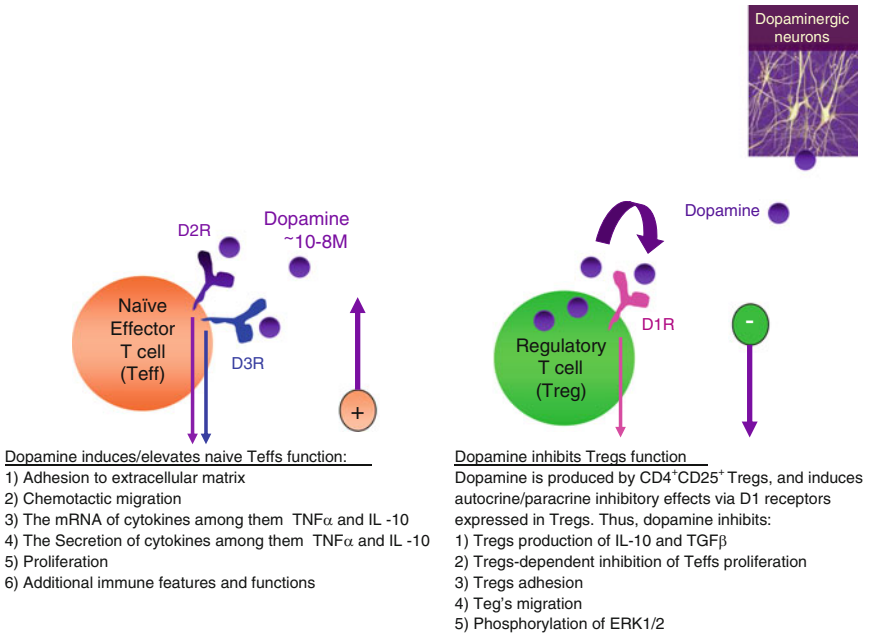

Fig. 1.7 Dopamine usually activates naïve effector T cells, via dopamine D2 and D3 receptors. Yet, dopamine suppress regulatory T cells, via an autocrine effect of endogenously-produced dopamine and the stimulation of dopamine D1 receptors. Thus, when dopamine binds to its receptors in naïve/resting effector T cells, it induces or elevates their: adhesion to extracellular matrix glycoproteins such as Fibronectin, chemotactic migration, the mRNA production and secretion of some very important cytokines including TNFα and IL-10 and proliferation. In contrast, when dopamine binds to its receptors in $CD4^+CD25^+$ regulatory T cells, it suppresses their: production of IL-10 and TGFβ, inhibition of T effector proliferation, adhesion, migration, and phosphorylation of ERK1/2

glycoprotein. Such adhesion to fibronectin is a characteristic feature of activated T cells, and is a very important and critical immune function for trafficking and extravasation of T cells across blood vessels and tissue barriers. Seven D2R/D3R agonists and antagonists were used in this study to identify the DR subtypes with which dopamine specifically interacts to activate T cells and induce adhesion to fibronectin. The D3R agonist, 7-OH-DPAT, mimicked the effects of dopamine, and the effects of both dopamine and 7-OH-DPAT were blocked by a specific D3R antagonist- U-Maleate. Furthermore, the DR agonists bromocriptine and pergolide mimicked the direct effect of dopamine, activated the β1 integrins function, and induced adhesion to fibronectin, while the DR antagonists butaclamol and haloperidol suppressed it, suggesting additional signaling via the D2R subtype. T cell adhesion induced by both dopamine and 7-OH-DPAT was dose dependent, with an optimum reached at 10^{-8} M (10nM). This optimal dopamine conc. range for affecting immune cells is in line with the previous observations on the direct and potent effects of several other neurotransmitters on normal human T cells (Levite 1998, 2008; Levite et al. 1998; Ganor et al. 2003).

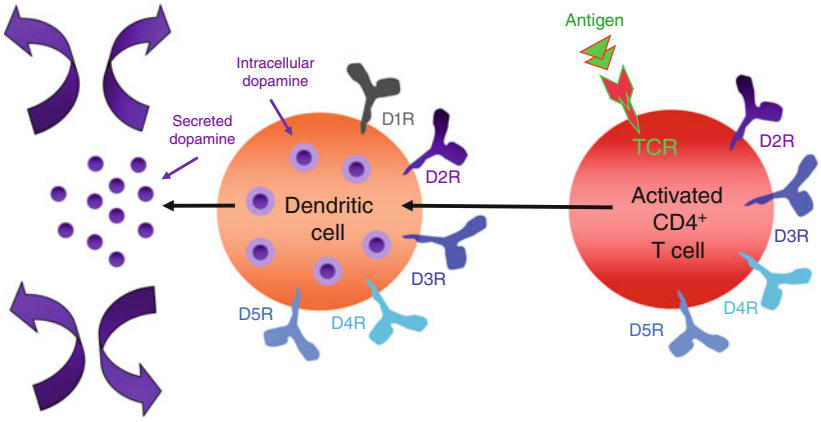

Fig. 1.8 Monocyte-derived dendritic cells (Mo-DCs) store dopamine in the secretary vesicles, and antigen-specific interaction with naive $CD4^+$ T cells induces the release of dopamine-containing vesicles from Mo-DCs. The secreted dopamine may now affect various immune target cells. The figure is illustrates schematically the findings revealed by (Nakano et al. 2009a).

Study 2: *Dopamine induces migration and homing of naive $CD8^+$ T cells via D3R*. Watanabe et al. showed that most naïve resting $CD8^+$ cytotoxic T cells express D3R, and that dopamine at 10^{-7} M (100 nM), and also the selective D3R agonist 7-OH-DPAT, induced adhesion of these cells to fibronectin and ICAM-1(Watanabe et al. 2006). Dopamine at 10^{-8} M (10 nM), 10^{-7} M (100 nM) and 10^{-6} M (1 μM), acting via the D3R, also induced chemotactic migration of human and mouse $CD8^+$ T cells, and of naive $CD45RA^+$ $CD8^+$ T cells, but not of memory/effector $CD45RO^+$ $CD8^+$ T cells (Watanabe et al. 2006). Dopamine was highly synergistic with CCL19, CCL21, and CXCL12 in induction of chemotaxis in naive $CD8^+$ T cells. Furthermore, in their important in vivo studies, Watanabe et al. showed that intraperitoneal injection of mice with 10^{-8} M (0.1 nM) dopamine or 7-OH-DPAT selectively attracted naive $CD8^+$ T cells to the peritoneal cavity. In contrast, treatment of mice with the D3R antagonist U-99194A selectively reduced homing of naive $CD8^+$ T cells into the lymph nodes. The authors concluded that in human and mice naive $CD8^+$ T cells selectively express D3R receptor, and that dopamine plays a significant role in migration and homing of naive $CD8^+$ T cells via D3R (Watanabe et al. 2006). Figure 1.6 attempts to illustrate some of these findings.

Study 3: *Dopamine induces adhesion of $CD8^+$ T cells to endothelium and their spontaneous migration*. Strell et al. showed that dopamine at 10^{-6} M (1 μM) . potently and significantly induced the adhesion of resting/naïve $CD8^+$ cytotoxic T cells to endothelium (Strell et al. 2009). Furthermore, dopamine also induced the spontaneous migration of $CD8^+$ T cells. In contrast, dopamine: (1) had no effect on the migration of CD3/CD28 activated T cells; (2) inhibited the activation of naive $CD8^+$ T cells by CD3/CD28 cross-linking, through downregulation of IL-2 expression via Erk1/2 and NF-kappaB inhibition. These results are in line with 'Suggested rule 2' (part 1.3.2): dopamine often stimulates immune

functions of naïve/resting cells but suppress already activated cells, or cells being co-activated by other stimuli (Strell et al. 2009). These results also support "Suggested rule 5" (part 1.3.2).

Study 4: *Dopamine induces chemotaxis and calcium flux in a mouse pre B cell line stably expressing the human D3R*. Watanabe et al. showed that dopamine at 10^{-8} M (10 nM) and 10^{-9} M (0.1 nM), as well as the selective D3R agonist 7-OH-DPAT at similar conc. induced chemotactic cell migration of murine pre-B cell line (L1.2) transfected with the human D3R, towards the human liver-expressed chemokine (LEC)/CCL16. Dopamine-induced chemotaxis was dose-dependent, and neither higher nor lower conc. were effective (Watanabe et al. 2006). This is in line with the 'Suggested Rule 1' (part 1.3.2). In addition, dopamine at 10^{-7} M (100 nM), 10^{-8} M (10 nM) and 10^{-9} M (0.1 nM), and 7-OH-DPAT at 10^{-7} M (100 nM), induced calcium mobilization in the same L1.2 cells. Dopamine-induced calcium mobilization was suppressed by several antagonists for the D2R, D3R and D4R, but not by antagonists for the D1R and D5R. The authors concluded that dopamine mediates calcium mobilization in these pre-B cells via the D3R (Watanabe et al. 2006).

1.3.3.2 Dopamine Induces Cytokine Secretion by Naïve Human T Cells

Dopamine by itself triggers the mRNA production and the secretion of IL-10 and TNFα by normal naïve human T cells. D3R activation leads to selective TNFα secretion; D2R activation leads to selective IL-10 secretion, while D1/5R activation leads to both. Levite's group (Besser et al. 2005) showed that dopamine on its own increased significantly TNFα and IL-10 secretion by resting normal human T cells, and induced approximately fivefold elevation of the corresponding TNFα and IL-10 mRNA levels, without affecting IFNγ and IL-4. Furthermore, using seven highly selective DR agonists and antagonists, the authors found that dopamine-induced TNFα secretion was evident after 24 h, and was mediated by D3R and/or D1/D5R. In contrast, dopamine-induced IL-10 secretion was evident after 72 h, and was mediated by D2R and/or D1/D5R. These findings showed that dopamine has a very unique ability to trigger a selective secretion of either TNFα only via the D3R, or IL-10 only via D2R. This incredible ability of dopamine to trigger a selective release of either a potent pro-inflammatory cytokine: TNFα, or of a strong anti-inflammatory cytokine: IL-10, differs markedly from the robust and non-selective cytokine-secretion induced by 'classical' TCR-activation (Besser et al. 2005) driven by an antigen or CD3/CD28 antibodies.

1.3.3.3 Dopamine May Augment Proliferation of Immune Cells

Intravenous injection of D1R or D2R agonists enhance splenocyte proliferation, and the same is triggered in vitro *by dopamine or its agonists. Lowering dopamine levels* in vivo *suppress splenocyte proliferation*. Tsao et al. (Tsao et al. 1997) found that intravenous injection into mice of a specific D1R agonist (SKF38393) or D2R agonist (LY171555) enhanced the splenocyte proliferation stimulated by LPS or Con A. Also, in vitro, dopamine, SKF38393 and LY171555 directly promoted cell proliferation to two mitogens: LPS and Con A. Intraperitoneal administration of

MPT, which lowered endogenous dopamine, suppressed splenocyte proliferation in response to LPS and Con A (Tsao et al. 1997).

1.3.3.4 Dopamine at Low-Mid Dose (10^{-7} M) Protects Lymphocytes from Oxidative Stress and Apoptosis

Cosentino et al. found that dopamine at different dose ranges exerted opposite effects on oxidative metabolism and apoptosis in human PBL's (Cosentino et al. 2004). Thus, dopamine at low conc. of $1 \times 10^{-7} - 5 \times 10^{-7}$ M (0.1–5 μM) had a beneficial effect by decreasing ROS levels and apoptosis in human PBL's (Cosentino et al. 2004). Yet, at ~1,000 fold higher conc. of $1 \times 10^{-4} - 5 \times 10^{-4}$ M (100–500 μM), dopamine had a detrimental effect by increasing intracellular reactive oxygen species (ROS) levels and apoptotic cell death through oxidative stress. Dopamine at both 1×10^{-6} and 5×10^{-4} M (1 and 500 μM) partially counteracted the decrease in Cu/Zn superoxide dismutase levels observed in untreated PBLs. However, whereas the effect of the low dose dopamine lasted for the whole incubation period (24 h), the effect of 500 μM dopamine was transient. Dopamine-dependent reduction of ROS levels and apoptosis was prevented by D1R-like (but not D2R-like) receptor antagonism (Cosentino et al. 2004). As a whole, these findings support the "Suggested rule 2" (part 1.3.2) and are drawn schematically in Fig. 1.3.

1.3.4 Dopamine Inhibits Activated T Cells, B Cells and Monocytes, and Suppresses Their Proliferation, Cytokine Secretion and Cytotoxicity

Study 1: *Dopamine inhibits the proliferation and IFNγ secretion by human mitogen-activated T cells*. Bergquist et al. found that dopamine at high conc. of 10^{-4} M (100 μM) or 10^{-5} M (10 μM) suppressed the proliferation and synthesis of IFNγ in Con-A stimulated human T cells (Bergquist et al. 1994).

Study 2: *Dopamine inhibits the activation of splenocytes and thymocytes by Con-A or LPS*. Cook-Mills et al. found that dopamine at 10^{-5} M (10 μM) and even more significantly at 10^{-4} M (100 μM) inhibited the activation of mouse spleen and thymus cells by Con-A or LPS. Other catecholamines exerted similar effects (Cook-Mills et al. 1995). Interestingly, the inhibition of lymphocyte activation by catecholamines could **not** be reversed by antagonists to either DRs or alpha- or beta-adrenergic receptors, suggesting a mechanism independent of these receptors (Cook-Mills et al. 1995).

Study 3: *Dopamine inhibits the proliferation and secretion of IL-2, IL-6 and IFNγ by mouse mitogen-activated T cells*. Josefsson et al. found that dopamine at a high conc. of 10^{-4} M (100 μM) and 5×10^{-4} M (500 μM) suppress the proliferation of ConA-stimulated mouse T cells, and at 500 M (5×10^{-4} M) (but not at lower conc.) also suppressed the secretion of IL-2, IL-6 and IFNγ by such Con-A-stimulated cells (Josefsson et al. 1996).

Study 4: *Dopamine decreases the mitogen-induced proliferation of human T cells and B cells, the synthesis of IFNγ and IL-4, and the production of IgM and IgG*.

Dopamine at high conc. also induces apoptosis of PBMCs. Bergquist et al. found that dopamine at a high conc. of 10^{-4} M (100 μM) and 10^{-5} M (10 μM) decreased significantly the proliferation and the synthesis of IFNγ and IL-4 by Con A-stimulated or PWM-stimulated PBMCs. For reminder: Con-A is a T cell mitogen, while PWM is a mitogen for T cells and B cells. Dopamine at these conc. also caused ~2.8 fold increase in the apoptosis of PBMCs, and increased the synthesis of the apoptotic markers Bcl-2/Bax and Fas/FasL (see the illustration in Fig. 1.3). At much lower conc. of 10^{-8} M (10 nM) dopamine diminished the number of IgM and IgG-producing B cells, suggesting that B cells are more prone to dopamine than T cells (Bergquist et al. 1997).

Study 5: *Dopamine suppress the LPS-induced proliferation and the binding of NF-kB to the TNFα promoter in activated human monocytes*. Dopamine in high conc. of 10^{-5} M (10 μM) and 10^{-4} M (100 μM), but not in lower conc. of 10^{-6} and 10^{-8} M (1 μM and 0.1 nM), suppress the proliferation of LPS-stimulated human peripheral blood monocytes and of transformed monocyte cell lines, and also inhibits the LPS-induced binding of NF-kB to the TNFα promoter (Bergquist et al. 2000). This supports "Suggested rules 2 and 3".

Study 6: *Dopamine inhibits the proliferation, cytotoxicity and cytokine secretion of CD3-activated T cells*. Saha et al. found that dopamine at a conc. of ~48.6 pg/mL, similar to the increased dopamine plasma levels they measured in cancer patients, caused the following effects: (1) inhibited significantly the in vitro IL-2-induced proliferation of CD3-activated $CD4^+$ and $CD8^+$ human T cells; (2) inhibited the cytotoxic ability of Lymphokine Activated Killer T cells (LAK-T); (3) increased the intracellular cAMP levels in these cells (Saha et al. 2001a, b). In contrast, dopamine at a lower conc. of ~10.2 pg/mL, similar to that measured in the plasma of healthy individuals, failed to induce such inhibitory effects (Saha et al. 2001a, b). Interestingly, $CD8^+$ T cells were more vulnerable to dopamine-mediated inhibition than $CD4^+$ T cells. The dopamine-induced inhibition of the proliferation and cytotoxicity of the activated T cells was abrogated by 90% using a D1R/D5R antagonist, but not by antagonists to D2R, D3R or D4R. Together, these findings support "Suggested rules 2, 3, 4 and 5" (part 1.3.2). Saha et al. concluded that dopamine elevated levels such as in the plasma of cancer patients, inhibits activated T cells via D1R-mediated stimulation of intracellular cAMP (Saha et al. 2001a, b). Unfortunately, the authors of this study did NOT test: (a) the effects of dopamine at a conc. of ~10^{-8} M, reported by previous studies to be optimal for affecting T cells; (b) the effects of dopamine on naive/resting T cells.

Study 7: *Dopamine inhibits the IL-2, IFNγ and the IL-4 secretion and the expression of the non-receptor tyrosine kinases Lck and Fyn in CD3-activated T cells*. Gosh et al. reported that dopamine inhibited IL2, IFNγ and IL-4 release by anti-CD3 antibody-activated T cells (Ghosh et al. 2003). Dopamine also suppressed the expression of the non-receptor tyrosine kinases, Lck and Fyn, which are the initial and pivotal signaling steps in T cell receptor (TCR)-mediated downstream signaling cascades, leading to cytokine release and to the subsequent clonal expansion of these immune effector cells (Ghosh et al. 2003).

Study 8: (In vivo/In vitro): *Infusion of dopamine into mice inhibits the number of IFNγ-producing Con-A stimulated splenocytes*. Carr et al. found that daily infusions for 5 days of L-dopa into mice, resulted in 2.2-fold increase in the in vitro Con-A-stimulated proliferative response of splenocytes removed of these mice (Carr et al. 2003). In contrast to this upregulation, the L-dopa infusions, as well as chronically infused dopamine (via subcutaneously implanted osmotic pumps), reduced the number of IFNγ-producing cells within the in vitro cultures of the anti-CD3-stimulated spelenocytes. No statistically significant effect was observed on the supernatant levels of IFNγ, and the number of IL-4 producing cells was not affected too. All the effects were mediated by D2-like receptors. (Carr et al. 2003).

Study 9: *A dopamine D2R/DR3 ligand increases the IFNγ mRNA levels but decreases the IL-4 and IL-10 mRNA levels in mitogen-activated T cells*. Ilani et al. found that treatment of mitogen (PHA) + IL-2-activated human $CD4^+$ T cells with quinpirole, a D2R/D3R ligand, increased their IFNγ mRNA levels, but reduced their pronounced IL-4 and IL-10 mRNA levels. The same treatment of $CD8^+$ T cells with quinpirole resulted in increased IFNγ mRNA. These changes were medicated by the D3R. Based on their results, the authors suggested that stimulation of D2R in activated $CD4^+$ T cells cause a Th2 to Th1 shift, and trigger IFNγ production in activated $CD8^+$ T cells (Ilani et al. 2004). Unfortunately, the authors did **not** study: (1) The effects of dopamine itself (not only of a D2R/D3R ligand) on $CD4^+$ and $CD8^+$ cells; (2) The effects of dopamine also on naive/resting (not only on activated) T cells; (3) The protein (not only mRNA) levels of the studied cytokines. It should be kept in mind that the cytokine proteins released by the cells (not their respective mRNAs) are the most important molecules for binding to their specific cytokine receptors in target cells (paracrine or autocrine) and for inducing meaningful effects.

Study 10: *Dopamine D4 agonists down regulate the proliferation and IL-2 secretion of CD3/CD28 activated T cells by up-regulating Krupple-like Factor-2 expression and inhibiting Erk1/2 and NF-kB*. Sarkar et al. found that the addition of D4R specific agonists (PD 168, 077 or ABT 724 trihydrochloride) at 10^{-6} M (1 μM) to CD3/CD28-activated human T cells inhibited their proliferation, IL-2 release and iERK1/ERK2 phosphorylation (Sarkar et al. 2006). In contrast to their inhibition of the activated T cells, the D4R agonists did not affect naive/resting T cells (in line with "Suggested rule 3"). The inhibition of the activated T cells was associated with the expression of Kruppel-like factor-2 (KLF2) – a transcription factor that regulates quiescence in T cells. Lower or higher conc. of the D4 agonists failed to exert these effects, showing again how critical is the exact dopamine conc, being used (in line with "Suggested Rule 2"). The findings of Sarkar et al. suggest that stimulation of D4R in activated T cells induce T cell quiescence by up-regulating KLF2 expression through inhibition of ERK1/ERK2 phosphorylation (Sarkar et al. 2006).

Study 11: *Dopamine inhibits IL-2 mRNA and the secreted IL-2 protein levels, Erk1/2 phosphorylation and NF-kB of CD3/CD28-activated T cells*. Strell et al. found that dopamine at 10^{-6} M (1 μM) induced the following effects: (1) Inhibited the

activation of $CD8^+$ cells by CD3 and CD28 antibodies; (2) Decreased IL-2 mRNA levels in these cells 24 h after activation; (3) Decreased the levels of IL-2 secretion; (4) Reduced the phosphorylation of Erk1/2 and NF-kB in these CD3/CD28-activated T cells (Strell et al. 2009).

Study 12: *Dopamine D1R-like agonist inhibits IFNγ production in mitogen-activated mouse T cells, without affecting IL-4; A D2R-like agonist inhibits IFNγ, proliferation, cAMP and phosphorylated CREB, but elevates IL-4 in such cells*. Huang et al. showed that purified T cells from the mesenteric lymph nodes of mice express the mRNA of all five D1R–D5R subtypes (Huang et al. 2010). Agonist of D1-like receptors (SKF38393), reduced the IFNγ production, and an antagonist (SCH23390) blocked this effect, without affecting the proliferative response, IL-4 production, cAMP content or CREB activation of the lymphocytes. In contrast, agonist of D2-like (D2, D3 and D4) receptors (Quinpirole), decreased the IFNγ but increased the IL-4 production, attenuated the lymphocyte proliferation to Con A, and diminished the cAMP content and the phosphorylated CREB level in these lymphocytes. All the quinpirole-induced changes were reversed by dopamine D2R-like antagonist haloperidol. The main conclusions of this study were: (1) Mouse T cells express all DR subtypes; (2) The D2-like receptors are more important in modulating T cell function than the D1-like receptors; (3) In activated mouse T cells, the D2-like receptors are involved in suppression of T helper 1 (Th1) cell function and enhancement of Th2 cell function through negative link to cAMP-CREB pathway (Huang et al. 2010).

1.3.5 Dopamine as an Immune Modulator Between Dendritic Cells and T Cells

Study 1: *Antagonizing D1-like receptors in dendritic cells later cultured with T cells, suppresses Th17, augments IFNγ and suppresses experimental autoimmune encephalomyelitis. In contrast, antagonizing D2-like-receptors augments Th17 production*. Nakano et al. used a DC-T cell differentiation assay composed of human monocyte-derived dendritic cells (MO-DCs) and allogeneic naïve $CD4^+$ T cells, and found that a D1R-like antagonist (SCH23390) at 10^{-6} M (1 μM), inhibited Th17 production, augmented IFNγ, and prevented experimental autoimmune encephalomyelitis (EAE) in vivo (Nakano et al. 2008). In contrast, a D2R-like antagonist L750667 at 10^{-6} M (1 μM) led to high IL-17 production. The further findings of this study re EAE are described below in part 1.5.5.1 (Nakano et al. 2008). While the results of this study support the notion that activation of different DRs within the same immune cell population can lead to opposite effects, and while they may have therapeutic implications for inhibiting Th17-mediated diseases, they do not teach us what dopamine itself is doing at the DC-T cell interface under physiological and/or pathological conditions.

Study 2: *Dopamine released by dendritic cells polarizes Th2 differentiation*. In a later study Nakano et al. demonstrated that human Mo-DCs stored dopamine in the secretary vesicles, and that antigen-specific interaction with naive $CD4^+$

T cells induced the release of dopamine-containing vesicles from Mo-DCs (Nakano et al. 2009a). This interesting observation is illustrated schematically in Fig. 1.8. Furthermore, when naïve $CD4^+CD45RA^+$ T cells were simultaneously activated by dopamine at 10^{-8} M or 10^{-7} M (10 nM or 100 nM) and CD3/CD28 antibodies, their secretion of IL-4 and IL-5 was up regulated. Of note, this original and interesting observation is the basis of "Suggested rule 7". Furthermore, Nakano et al. found that when naïve $CD4^+CD45RA^+$ T cells were simultaneously activated by dopamine at 10^{-8} or 10^{-7} M and CD3/CD28 antibodies, their secretion of IL-4 and IL-5 was up regulated (Nakano et al. 2009a). (Of note, these findings are somewhat surprising and contradict several others showing that simultaneous exposure of immune cells to dopamine and other stimuli like CD3/CD28 antibodies prevents their activation, or in fact leads to their inhibition). Nakano et al. also co-cultured adjuvant-activated MO-DCs and CD3/CD28-activated HLA-DR non shared allogeneic $CD4^+$ T cells (in what they call "DC-mediated T-cell differentiation assay") and found that the pre-treatment of the MO-DCs with selective D2R antagonists before their interaction with the CD3/CD28-activated $CD4^+$ T cells, led to: (1) High IL-5 and low IFNγ secretion (i.e. high IL-5:IFNγ ratio); (2) Growth of cells expressing CCR4 (a Th2-type chemokine receptor); (3) A decrease in CXCR3. These findings suggested that antagonizing D2R's cause a shift towards Th1 response. In addition, when dopamine release from Mo-DCs was inhibited, T cell differentiation shifted toward T(h)1. This suggested that once dopamine is present, the opposite occurs. Together, all these findings suggest that dopamine released by DCs functions as a T(h)2-polarizing factor in the DC-naive T-cell interface (Nakano et al. 2009a).

Review: *Role of dopamine in the physiology of T cells and dendritic cells:* Pacheco et al. review the emerging role of dopamine as a regulator of DCs and T cells physiology and, in turn, immune responses. Moreover, the authors discuss how alterations in the dopamine-mediated immune regulatory mechanisms could contribute to the onset of immune-related disorders (Pacheco et al. 2009).

1.3.6 Dopamine Inhibits Regulatory T Cells, Thereby 'Suppressing the Suppressors'

Study 1: *Dopamine reduces the suppressive activity, adhesion and migration of naïve and activated $CD4^{\pm}CD25^{\pm}$ regulatory T cells, via dopamine D1 receptors expressed in these cells.*

Kipnis et al. found that while naïve Tregs, or Tregs that have been activated by anti-CD3 antibodies, IL-2 and irradiated APC's, significantly inhibit the proliferation of Teffs, the incubation (2 h) of Tregs with dopamine significantly reduced the suppressive activity of naïve and activated Tregs (Kipnis et al. 2004). Thus, the proliferation of Teffs co-cultured with either activated or naïve Tregs that had been incubated with dopamine at 10^{-5} M (10 μm) and 10^{-7} M (0.1 μm), but not at lower conc. of 10^{-9} M (1 nM) was more than twofold higher than the proliferation of Teffs co-cultured with Tregs that were

not incubated with dopamine. A D1/5R agonist (10^{-5} M) reproduced dopamine's effect, and a D1R/D5R antagonist (10^{-5} M) prevented it. Kipnis et al. further found that: (1) Tregs express significantly more D1R than Teffs, (2) Dopamine at 10^{-5} M caused a slight but consistent decrease in CTLA-expression in Tregs; (3) Dopamine decreased the levels of IL-10 produced by Tregs (but did not affect FoxP3 expression), (4) Dopamine and a D1/5R agonist (both at 10^{-5} M) down regulated: (a) the phosphorylation of ERK1/2 in Tregs; (b) The adhesion of Tregs to CSPG (extracellular matrix proteins often associated with injured tissues), but not their adhesion to fibronectin; (c) The migration of Tregs towards MDC (a chemokine for CCR-4) but not towards SDF-1; (d) The expression of CCR-4 mRNA (but not of CXCR-4 and CCR-8); (5) A systemic injection of dopamine or a D1R/5 agonist significantly enhanced, via a T-cell-dependent mechanism, the protection against neuronal death after CNS mechanical and biochemical injury (Kipnis et al. 2004) Together, the results of this study show that dopamine reduces the suppressive activity and trafficking of Tregs, through D1-like receptors, found in this study to be abundantly expressed by Tregs. Some of the findings of this study are illustrated in Fig. 1.7.

Study 2: *Dopamine is produced by Tregs and subserves as an autocrine/paracrine stimuli that downregulates Treg's production of IL-10 and TGFβ, and Treg-dependent inhibition of Teffs proliferation*. Cosentino et al. found that $CD4^+CD25^+$ Tregs constitutively express TH, the rate-limiting enzyme in the synthesis of catecholamines, and contain substantial amounts of dopamine, as well as norepinephrine, and epinephrine, which are released upon treatment with reserpine (known to deplete monoamine neurotransmitters in synapses) (Cosentino et al. 2007). Catecholamine release resulted in reduced production of IL-10 and TGFβ by Tregs, and in down-regulation of Treg- dependent inhibition of Teffs proliferation, that occurred without affecting the production of TNFα and IFNγ. Cosentino et al. further found that Tregs and Teffs express both D1-like and D2-like dopaminergic receptors to a similar extent (12–29% of the cells) on the cell membrane. Yet, catecholamine-dependent down-regulation of Tregs was selectively reversed by pharmacological blockade of D1-like receptors, which in Tregs only (and not in Teffs) are also expressed at the level of mRNA, and are functionally coupled to intracellular production of cAMP. These findings indicated that in human Tregs endogenous catecholamines subserve an autocrine/paracrine loop involving dopaminergic pathways leading to the down-regulation of Treg function (Cosentino et al. 2007). This conclusion is illustrated schematically in Fig. 1.7.

1.3.7 Dopamine, as well as Dopaminergic Analogues and Drugs, Exert Several Effects on Macrophage and Microglia Activity

Several studies, cited here in a chronological order, show that dopamine affect the phagocytosis by macrophages, the cell surface expression of their macrophage Fc-gamma receptors, their LPS-induced TNFα and NO production, and their

cytokine secretion. Dopamine also affects the migration, potassium currents and NO release of microglia cells.

1.3.7.1 Macrophages

Study 1: *Dopamine modulates IFNγ-induced phagocytosis by murine macrophages*. Sternberg et al. found that dopamine, as well as 5-HT and histamine, modulate IFNγ-induced phagocytosis in murine bone marrow macrophages, through their respective receptors, (Sternberg et al. 1987). The dopamine-induced effect was blocked by spiperone and pyrilamine, both of which have been shown to block dopaminergic effects in other systems.

Study 2: *Dopamine increases phagocytosis of E. coli and sheep red blood cells (SRBC) by chicken macrophages, and the level of Fc-receptor positive macrophages*. Ali et al. found that dopamine, as well as norepinephrine and epinephrine, all at 0.1 and 0.25 μg/ml, increased the phagocytosis of *E. coli* and sheep red blood cells (SRBC) by macrophages. The percentage of Fc-receptor positive macrophages increased after exposure to dopamine, or to norepinephrine or epinephrine. Dopamine, norepinephrine or epinephrine were toxic for macrophages at 1–5 μg dose range, resulting in 25–50% cell death (Ali et al. 1994).

Study 3: *Dopamine receptor agonists and antagonists injected to mice modulate the LPS-induced TNFα and nitric oxide production by peritoneal macrophages*. Haskó et al. found that pretreatment of mice with D2R agonists: Bromocryptine or Quinpirole, caused a blunting of both the TNFα and NO responses to LPS injected intraperitoneally. An antagonist of D2R (Sulpiride) decreased the LPS-induced TNFα plasma levels in a dose-dependent manner, and inhibited the LPS-induced NO production by peritoneal macrophages. Bromocryptine or Quinpirole blunted both the TNFα and NO response to LPS. A D1R antagonist did not alter LPS-induced TNFα production, but inhibited LPS-induced NO production. These results indicated that: (1) The D2Rs are involved in the modulation of both LPS-induced TNFα and NO production; (2) The D1Rs regulate the production of NO (Hasko et al. 1996).

Study 4: *Dopaminergic drugs alter macrophage Fcgamma receptors expression*. Macrophage Fc-gamma receptors have an important role in host defense and in the pathophysiology of immune-mediated disorders. Alteration of splenic macrophage Fc-gamma receptors expression predisposes to severe infection. Gomez et al. used an experimental model in the guinea pig to assess the effect of commonly used dopaminergic drugs on the expression of macrophage Fc-gamma receptors (Gomez et al. 1999). Three dopa-agonists: Bromocryptine, Leuprolide, and Pergolide, and seven dopa-antagonists: Chlorpromazine, SCH 23390, Metochlopramide, Sulpiride, Veralipride, Alizapride, and Cisapride, were studied. Treatment with the dopa-agonists enhanced the clearance of IgG-sensitized RBCs, the in vitro binding of IgG-sensitized RBCs by isolated splenic macrophages, and the cell surface expression of the macrophage Fc-gamma receptors. The Dopa-antagonists had the opposite effect and impaired macrophage Fc-gamma receptors expression. These alterations of macrophage

Fc-gamma receptors expression were mediated by both D1Rs and D2Rs, with a major participation of D2Rs. The authors concluded that dopaminergic drugs alter the clearance of IgG-coated cells by affecting the expression of splenic macrophage Fc-gamma receptors (Gomez et al. 1999).

Study 5: *Dopamine suppresses IL-12 p40 and increases IL-10 production by LPS-stimulated macrophages, via a beta-adrenoceptor-mediated mechanism (rather than via dopamine receptors)*. Hasko et al. found that treatment of LPS-stimulated J774.1 macrophages with dopamine at 10^{-8}–10^{-4} M (0.01–100 μM) decreased the release and the mRNA accumulation of IL-12 p40, in a conc. dependent manner (Hasko et al. 2002). Yet, this inhibitory effect of dopamine was NOT mediated by DRs, but rather by beta-adrenoceptors. Dopamine also stimulated the production of the anti-inflammatory cytokine IL-10 in both J774.1 cells and peritoneal macrophages via both beta-adrenoceptor-dependent and independent mechanisms. These results suggested that dopamine has multiple anti-inflammatory effects which are not mediated by DR's (Hasko et al. 2002).

Study 6: *Dopamine antagonist decreased oxidative burst and PMA-induced burst in rat macrophages*. Carvalho-Freitas evaluated the effects of the dopamine antagonist Domperidone (DOMP) and Prolactin (PRL) on macrophage activity of rats (Carvalho-Freitas et al. 2008). Oxidative burst and phagocytosis of peritoneal macrophages were evaluated by flow cytometry. In vitro incubation of rat macrophages with 10^{-8} M (10 nM) dopamine antagonist DOMP decreased oxidative burst (after 30 min) and PMA-induced burst (after 2 and 4 h). Based on all the findings of this study the authors suggested that macrophage functions are regulated by an endogenous dopaminergic tone, and that both PRL and dopamine exert their action by acting directly on the peritoneal macrophage (Carvalho-Freitas et al. 2008).

1.3.7.2 Microglia

Study 1: *Dopamine inhibits nitric oxide production by microglia*. Chang et al. found that dopamine, as well as norepinephrine and epinephrine, potently inhibited NO production by N9 microglial cells (Chang and Liu 2000). In contrast, Dopa, the immediate precursor of the catecholamine biosynthesis pathway (see Fig. 1.1), was a weak inhibitor, except at very high conc. The inhibitory effect of the catecholamines was mimicked by an alpha-adrenergic receptor agonist and by a beta-adrenergic receptor agonist, but not by forskolin or analogs of cAMP. Western blot analysis indicated that the catecholamines caused a slight decrease in the formation of inducible NO synthase. These results suggest that catecholamines have the ability to block NO production by microglia (Chang and Liu 2000).

Study 2: *Dopamine inhibits potassium currents in microglia. Chronic dopamine receptor stimulation enhances migratory activity and attenuates LPS-induced NO release*.

Faber et al. studied mouse and rat microglia in culture and brain slices, and found that they express functional DRs (Farber et al. 2005). Using the patch clamp

technique and specific ligands to different DRs, D1 and D2-like receptors were identified. They inhibited the constitutive potassium inward rectifier and activated potassium outward currents in a subpopulation of microglia. Chronic DR stimulation enhanced migratory activity and attenuated the LPS-induced NO release (Farber et al. 2005).

1.4 Dopamine Is Produced Endogenously in Most Immune Cells, and Under Certain Conditions Released to the Extra Cellular Milieu. The Immune-Derived Dopamine Can Induce Autocrine and Paracrine Effects

Dopamine is undoubtedly produced in many, if not all, types of immune cells, and under certain conditions can be released to the extracellular milieu by these cells, resulting in autocrine and paracrine effects. The multiple evidences supporting these conclusions were discovered and published by several groups (Bergquist et al. 1994, 1997; Josefsson et al. 1996; Musso et al. 1996; Musso et al. 1997; Bergquist and Silberring 1998; Tsao et al. 1998; Ferrari et al. 2004; Cosentino et al. 2007; Flierl et al. 2007, 2009; Nakano et al. 2009a). The key reports on these studies are cited below in a chronological order. Among these publications, the sixth paper cited below (Bergquist et al. 1998) is the most relevant and comprehensive study done so far on this topic, and contains a very informative data, reconstituted herein in Table 1.1.

Study 1: *Discovery of endogenous catecholamines in lymphocytes and evidence for catecholamine regulation of lymphocyte function via an autocrine loop.* Bergquist et al. revealed, by capillary electrophoresis with electrochemical detection, that catecholamines and their metabolites are present in single lymphocytes and extracts of T cell and B cell clones (Bergquist et al. 1994). Pharmacological inhibition of TH reduced catecholamine levels, suggesting catecholamine synthesis by lymphocytes. Interestingly, the intracellular dopamine levels were increased by extracellular dopamine, suggesting a cellular-uptake mechanism. Furthermore, incubation of lymphocyte with dopamine at 10^{-5} M (10 μM) and 10^{-4} M (100 μM), or with its precursor L-dopa, resulted in a dose-dependent inhibition of Con A-stimulated proliferation and their synthesis of IFNγ. The authors also reported (yet the data was not shown) that incubation with dopamine at conc. of 10^{-5} to 5×10^{-4} M (10–500 μM) completely abolished the production of antibodies by B cells (Bergquist et al. 1994).

Study 2: *Peripheral human T lymphocytes contain catecholamines, and are able to synthesize them from normal precursors in physiologic concentrations.* Musso et al. studied the catecholamine content in PBLs and their ability to synthesize catecholamines in vitro (Musso et al. 1996). The catecholamines were separated by high performance liquid chromatography (HPLC) and determined in the supernatant by electrochemical detection, as well as being determined after

ultrasonic cell disruption in mononuclear leukocytes, adherent cells (monocytes/macrophages), total lymphocytes, and B cell and T cell-enriched fractions. T cells contained L-Dopa and Norepinephrine (NE), whereas B cells contained only L-Dopa. After the addition of [3H]-L-Dopa (10^{-8} and 10^{-7} M) to the incubation medium, [3H]-dopamine and [3H]-NE appeared. By increasing the conc. of L-Dopa in the medium ($<10^{-6}$ M), catecholamines were detected in the supernatant as well. These findings showed that peripheral human T cells contain catecholamines and are able to synthesize them from normal precursors in physiologic conc. (Musso et al. 1996). In a subsequent study, Musso et al. found that L-tyrosine and nicotine induce synthesis of L-Dopa and norepinephrine in human lymphocytes (Musso et al. 1997). The conclusion of this study was that Acetylcholine might regulate catecholamine synthesis in lymphocytes through an activation of the rate limiting enzyme TH.

Study 3: *Mouse spleen cells, macrophages, as well as B cell and T cell hybridomas contain endogenously-produced dopamine. Dopamine at high conc. inhibits the secretion of IL-2, IL-6 and IFNγ by Con A-stimulated T cells.* Josefsson, E., Bergquist, J. and their colleges found that mouse spleen cells and macrophages contained on average 7×10^{-17} and 2×10^{-17} mole dopamine per cell, respectively (Josefsson et al. 1996). Several mouse B cell and T cell hybridomas also contained endogenously-produced dopamine in levels ranging from 7×10^{-20} to 2×10^{-18} mole dopamine per cell. The dopamine production of lymphocytes was blocked by the TH inhibitor alpha-methyl-p-tyrosine, whereas incubation with the precursor L-DOPA increased the dopamine content. Dopamine at a high conc. of 10^{-4} M (0.1 mM) and 5×10^{-4} M (0.5 mM) suppressed the proliferation of Con A-stimulated mouse T cells, and dopamine at 5×10^{-4} (but not at lower conc.) also suppressed also the secretion of IL-2, IL-6 and IFN of such Con A-stimulated cells (Josefsson et al. 1996).

Study 4: *Lymphocytes are capable of active synthesis of dopamine*. In a later study Bergquist et al. used a single cell analysis with capillary electrophoresis – a technique capable of detecting zeptomole quantities (10^{-21} mole) of neurochemical species – to demonstrate that lymphocytes are capable of active synthesis of dopamine and norepinephrine. In addition, both inhibition of dopamine uptake and inhibition of packing of catecholamines into vesicles, resulted in significantly lower levels of dopamine and norepinephrine. Exposure of lymphocytes to catecholamines at low conc. of 10^{-8} M (10 nM) led to decreased proliferation, differentiation, IFNγ, IL-4 and immunoglobulins (Bergquist et al. 1997).

Study 5: *Immune cells express TH, and its expression level is increased during cell growth*. Tsao et al. found, by flow cytometric analysis, that T cell hybridoma cells express TH that catalyzes the initial rate-limiting step of catecholamine biosynthesis (Tsao et al. 1998). Furthermore, temporal studies indicated that the expression of TH increased during the T hybridoma cell growth. As a whole, the findings of this study suggested that T cells express TH which is correlated to cell growth, and that dopamine released from these cells may

bind to the receptors to act in an autocrine or paracrine manner (Tsao et al. 1998).

Study 6: Review: *Lymphocytes present in human cerebrospinal fluid contain endogenous dopamine and other catecholamines. Dopamine is present in CD4$^+$ T cells, B cells, PBMCs, mouse spleen cells, peritoneal macrophages, as well as in T cells and B cell hybridomas*. Bergquist et al. used capillary electrophoresis of single lymphocytes obtained directly from human cerebrospinal fluid (CSF), and demonstrated the presence of endogenous dopamine in an average concentartion of 2×10^{-18} mol in these cells (Bergquist et al. 1994, 1998). Other catecholamines were also detected. In addition, single cloned CD4$^+$ T cells and B cells contained 3×10^{-18} and 3×10^{-19} mol catecholamines per cell respectively (Bergquist et al. 1994, 1998). Furthermore, freshly isolated PBMCs have been found to hold dopamine at a basal level of 1.6×10^{-18} mol cell. Spleen cells contain ~7×10^{-17} mol dopamine per cell, whereas peritoneal macrophages contain 2×10^{-17} mol dopamine per cell. Dopamine was also found in different T cell and B cell hybridomas, at a concentration varying from ~7×10^{-18} mol to below the detection limit (~1×10^{-19}) (Bergquist et al. 1998). Furthermore, the levels of catecholamines in isolated and extracted human lymphocyte nuclei were determined with capillary electrophoresis and electrochemical detection. Dopamine was found in the nuclei at levels of ~$5.3 \pm 2.6 \times 10^{-21}$ mol, and in PBMCs whole-cell extracts at a conc. of $2.6 \pm 0.6 \times 10^{-18}$ mol cell. These findings suggested that 0.1–0.2% of the total amount of catecholamine (dopamine included) is situated inside the nuclear membrane, and indicated that catecholamines exert their regulatory mechanism on lymphocytes through interaction with nuclear components (Bergquist et al. 1994, 1998).

Study 7: *Human lymphocytes produce endogenous catecholamines through PKC activation of TH. Activation of D1Rs inhibit TH mRNA expression and intracellular production. Inhibition of intracellular catecholamine production in PBMCs promote cell survival*. Ferrari et al. report that activation of human PBMCs triggered endogenous production of catecholamines, through protein kinase (PK) C-dependent induction of TH (Ferrari, Cosentino et al. 2004). Activation of human T cells and B cells with a PKC activator induced TH mRNA expression, followed by an increase in the amount of intracellular catecholamines. Furthermore, co-incubation of human PBMCs with dopamine or dopaminergic D1R-like agonist SKF-38393 inhibited the TPA-induced TH mRNA expression and increased intracellular catecholamines, and these effects were antagonized by the D1R-like antagonist SCH-23390. It was thus suggested that in human T cells and B cells PKC activation leads to TH mRNA expression and subsequent increase of intracellular catecholamines, which can be inhibited by D1R-like activation. Inhibition of intracellular catecholamine production in human PBMCs promoted cell survival through reduction of activation-induced apoptosis (Ferrari et al. 2004).

Study 8: *Human CD4$^{\pm}$CD25$^{\pm}$ regulatory T cells contain endogenous dopamine and other catecholamines, which subserve an autocrine/paracrine inhibitory*

loop, resulting in down-regulation of Treg function. Cosentino et al. showed that $CD4^+CD25^+$ Tregs constitutively express TH, and contain substantial amounts of dopamine, as well as norepinephrine and epinephrine. These catecholamines were released upon treatment with reserpine (Cosentino et al. 2007). The further interesting findings of this study were already discussed above (part 1.3.6, study 2).

Study 9: *Phagocyte-derived catecholamines enhance acute inflammatory injury*. Flierl et al. investigated in their study published in Nature (Flierl et al. 2007) whether phagocytes are capable of de novo production of catecholamines, suggesting an autocrine/paracrine self-regulatory mechanism by catecholamines during inflammation, as has been described for lymphocytes. They found that exposure of phagocytes to LPS led to a release of catecholamines and to an induction of catecholamine-generating and degrading enzymes, indicating the presence of the complete intracellular machinery for the generation, release and inactivation of catecholamines. To assess the importance of these findings in vivo, the authors used two models of acute lung injury. Blockade of alpha2-adrenoreceptors or catecholamine-generating enzymes greatly suppressed lung inflammation, whereas the opposite was the case either for an alpha2-adrenoreceptor agonist or for inhibition of catecholamine-degrading enzymes. The results of this study identified phagocytes as a new source of catecholamines, which enhance the inflammatory response (Flierl et al. 2009). In a subsequent study, Flierl et al. found that upregulation of phagocyte-derived catecholamines augments the acute inflammatory response (Flierl 2009 #198).

Study 10: *Human monocyte-derived dendritic cells store and release dopamine, which polarizes Th2 differentiation*. Nakano et al. demonstrated that human Mo-DCs stored dopamine in secretary vesicles, and that dopamine storage in these cells was enhanced by Forskolin and D2R-like antagonists, via increasing cAMP formation. Antigen-specific interaction with naive $CD4^+$ T cells induced the release of dopamine-containing vesicles from Mo-DCs (illustrated schematically in Fig. 1.8). In naive $CD4^+$ T cells, dopamine dose dependently increased cAMP levels via D1-like receptors and shifted T cell differentiation to T(h)2, in response to anti-CD3 and anti-CD28 antibodies. Furthermore, dopamine D2R-like antagonists, such as Sulpiride and Nemonapride, induced a significant DC-mediated T(h)2 differentiation. When dopamine release from Mo-DCs was inhibited the T cell differentiation shifted toward T(h)1. These findings identified DCs as a new source of dopamine, which functions as a T(h)2-polarizing factor in DC-naive T cell interface (Nakano et al. 2009a).

1.5 Dopamine and/or Its Receptors Expressed in Immune Cells May Be Involved in Several Immune and Autoimmune Diseases

1.5.1 Dopamine's Involvement in Autoimmune Diseases, Among Them: Multiple Sclerosis, Systemic Lupus Erythematosus, Rheumatoid Arthritis and Diabetes Mellitus

1.5.1.1 Multiple Sclerosis and Experimental Allergic Encephalomyelitis

Study 1: Giorelli et al. found diminished mRNA and protein levels of D5R (but not of D3R) in PBMCs of untreated multiple sclerosis (MS) patients (Giorelli et al. 2005). Dopamine reduced T cell proliferation, secretion of IFN-γ, and production of matrix metalloproteinase-9 (MMP-9) mRNA in PBMCs from controls, but not from MS patients. By contrast, reduced levels of D3R and renewed dopamine-associated regulatory functions were found in PBMCs from IFN-beta treated MS patients. Based on all these findings, the authors suggested that failure of the dopaminergic system of lymphocytes may lessen the threshold of T cell activation and sustain the pathogenic cascade of MS (Giorelli et al. 2005).

Study 2: Dijkstra et al. found that a therapeutic effect of Bromocriptine (BCR) – a potent D2R agonist – on experimental allergic encephalomyelitis (EAE) – an animal model for demyelinating diseases, particularly MS. Injection of BCR at daily intervals after the onset of clinical signs of chronic relapsing form of EAE reduced both the severity and the duration of the clinical signs. The BCR treatment did not affect the severity and duration of the first attack, but reduced the duration of the subsequent second attack. Thus, BCR treatment seem to improve the clinical course of EAE in animals with ongoing disease (Dijkstra et al. 1994).

Study 3: Nakano et al. found that a D1R-like antagonist (SCH23390) inhibited DC-mediated Th17 differentiation, and had the ability to prevent EAE in mice. Spleen cells from EAE mice showed decreased IL-17 production when SCH23390 was administered. Adoptive transfer of DCs treated with SCH23390 successfully prevented EAE. These findings indicated that antagonizing D1-like-receptors on DCs inhibits Th17 differentiation, thereby leading to an amelioration of EAE (Nakano et al. 2008).

1.5.1.2 Systemic Lupus Erythematosus (SLE)

Study 1: Depressive-like behavior is the most profound manifestation of autoimmunity-associated behavioral syndrome in lupus-prone MRL-lpr mice. Sakic et al. found that depressed MRL-lpr mice have decreased dopamine in the brain in the paraventricular nucleus (PVN) and median eminence (ME), decreased serotonin levels in the PVN, and enhanced levels in the hippocampus, as well as decreased norepinephrine (NE) levels in the prefrontal cortex (Sakic et al. 2002). Behavioral deficits correlated with the changes in PVN and median eminence. These results are consistent with the hypothesis that imbalanced neurotransmitter regulation of the hypothalamus-pituitary axis plays an important

role in the etiology of behavioral dysfunction induced by systemic autoimmune disease (Sakic et al. 2002).

Study 2: Ballok et al. found that autoimmunity-induced destruction of mesonigral and mesolimbic dopaminergic pathways may contribute to the etiology of aberrant behavior in an animal model of neuropsychiatric lupus (Ballok et al. 2004a, b).

Study 3: Chun et al. found evidences supporting the notion that development of systemic autoimmunity alters the sensitivity of the dopaminergic system and renders MRL-lpr mice prone to 'self-injurious behavior' (Chun et al. 2008).

1.5.1.3 Rheumatoid Arthritis (RA)

Study 1: Nakashioya et al. found that a selective D1R antagonist suppressed the severity of collagen-induced arthritis in mice, without affecting neither the serum levels of antibodies to type II collagen, nor the splenic Th1/Th17 differentiation (Nakashioya et al. 2010).

Study 2: Nakano et al. found that dopamine is present in DCs in the synovial tissue of RA patients, and significantly increased in RA synovial fluid. A D1R-like antagonist inhibited cartilage destruction in a human RA/SCID mouse chimera model. Dopamine, via D1-like receptors, elevated IL-6-dependent IL-17 production by $CD4^+$ T cells activated by CD3/CD28 antibodies (Nakano et al. 2011). Taken together, these findings suggested that elevated dopamine levels in RA synovial fluid play a role in RA, that dopamine released by DCs induce IL-6-Th17 axis and cause aggravation of synovial inflammation of RA, and that blocking D1-like receptors can be of benefit in RA. These findings seem to be the first evidences for pathological dopaminergic signaling in RA (Nakano et al. 2011).

1.5.1.4 Diabetes Mellitus

Non-obese diabetic (NOD) mice exhibit susceptability to spontaneous development of autoimmune dependent diabetes mellitus (IDDM). Hashimoto et al. demonstrated that in the pancreas of NOD mice, islet infiltrates appear to be composed of mononuclear cells positive for IL-23R, one of the specific markers for Th17. Thereafter, NOD mice were orally administered with the dopamine D1-like receptor antagonist SCH23390, from week 6 to 26. At week 26, 67% and 25% of mice developed Diabetes in the control and the SCH23390 groups, respectively ($p < 0.05$). A histological examination of SCH23390-treated mice exhibited a typical normal islet structure with no signs of periductal and perivascular infiltrates, whereas the islets from vehicle controls showed insulitis. In week 26, spleen cells were re-stimulated with anti-CD3 and anti-CD28 antibodies in vitro and exhibited an augmentation of IFNγ induction and the suppression of IL-17 induction in the SCH23390-treated mice. These findings indicated that antagonizing D1-like-R suppresses IL-17 expression, thereby leading to a decreased occurrence of NOD (Hashimoto et al. 2009).

1.5.2 Dopamine Receptors in Neutrophilic Airway Inflammation/Asthma

Nakagome et al. found recently that a D1R-like antagonist significantly suppressed OVA-induced neutrophilic airway inflammation in mice, and also inhibited the production of IL-17 and infiltration of Th17 cells in the lung. Further, a D1R-like antagonist suppressed the production of IL-23 by lung $CD11c^+$ APCs (Nakagome et al. 2011). Based on their findings the authors proposed that antagonizing D1-like receptors could be a new strategy for treating neutrophil-dominant severe asthma (Nakagome et al. 2011).Yet, the study can not answer the question whether dopamine itself promotes neutrophilic airway inflammation, and this question awaits further investigation.

1.5.3 Dopamine May Increase HIV Replication in Macrophages and the Neurological and Cognitive Impairments Associated with AIDS

Study 1: Berger et al. found that the CSF dopamine mean values were significantly lower in the HIV-1-seropositive patients with ($P < 0.0001$) or without ($P < 0.0001$) neurological disease than in HIV-seronegative patients. Interestingly, there was a very strong correlation between the $CD4^+$ lymphocyte counts and the CSF dopamine levels in the neurologically symptomatic patients. The authors concluded that HIV-1 infection appears to have an effect on the CNS dopaminergic systems, as reflected in levels of CSF dopamine (Berger et al. 1994).

Study 2: Gaskill et al. (2009) found that: (1) Macrophages express D1R and D2R; (2) Dopamine activates macrophages by increasing ERK 1 phosphorylation; (3) Dopamine increases HIV replication in human macrophages; the mechanism by which dopamine mediates this change is by increasing the total number of HIV-infected macrophages; (4) The dopamine-induced increase in HIV replication is mediated by activation of D2R. These findings show that dopamine can aggravate HIV infection, and also suggest that elevation of dopamine, which in turn activates macrophages infected with viruses, can be a common mechanism by which drugs of abuse enhance HIV replication in macrophages. Thus, the drug abuse-heightened levels of CNS dopamine could increase viral replication, thereby accelerating the development of HIV-associated neurocognitive disorders (Gaskill et al. 2009).

Study 3: Kumar et al. found that HIV-1-infected individuals have 45% decrease in dopamine levels in the substantia nigra region of the brain, which is significantly correlated with the low level of neurological and cognitive performance (speed of information processing, learning, memory, verbal fluency, and average T scores across domains) (Kumar et al. 2011).

1.5.4 Dopamine and Hematologic Cancers: Leukemia and Lymphoma

Study 1: Wick et al. showed that Levodopa and dopamine analogs have anti-tumor effects in experimental leukemia in vivo (Wick 1981).

Study 2: Meredith et al. found that dopamine targets cycling B cells for oxidative attack, in a mechanism **independent** of DRs and dopamine transporters, and discuss the implications of these findings for non-Hodgkin's lymphoma (Meredith et al. 2006).

1.6 In Some Neurological and Psychiatric Diseases Associated with Dopamine Abnormalities in the Brain, There Is Also Abnormal Expression of Dopamine Receptors in Lymphocytes, and/or Abnormal Immune Features and Functions

As already discussed in part 1.1.5, impairments in the dopaminergic neurotransmission in the CNS play a role in a variety of neurological and neuropsychiatric disorders.

The below cited findings show that altered levels of DRs expressed in immune cells, and/or altered response of immune cells to dopamine, and/or various other immune abnormalities, may play a role in some of these neurological and psychiatric diseases.

1.6.1 Schizophrenia

Three independent and interesting studies performed by three different groups show increased D3R mRNA in lymphocytes of Schizophrenia patients, and some other abnormalities. Yet continuation studies are highly needed for unveiling the meaning and reason for this modification, and whether DR3 and maybe other DR's in some types of lymphocytes are playing in fact an active role in Schizophrenia.

Meanwhile, here is the essence of the evidences accumulated thus far:

Study 1: Lymphocytes of Schizophrenia patients have twofold increase in their D3R receptor mRNA levels, and this elevation is not affected by treatment (Ilani et al. 2001).

Study 2: Lymphocytes of drug free Schizophrenia patients have elevated D3R mRNA levels compared to controls and drug-medicated Schizophrenics, and elevated D5R mRNA levels compared to drug-medicated Schizophrenics. The increased DR expression in lymphocytes correlated with more severe psychiatric symptoms (Kwak et al. 2001).

Study 3: T cells of schizophrenic patients have significantly higher expression of D3R and lower D4R mRNA levels (Boneberg et al. 2006).

1.6.2 Parkinson's Disease

Parkinson's disease (PD) is a degenerative disorder of the central nervous system resulting from the death of dopamine-containing cells in the substantia nigra, a region of the midbrain.

Study 1: Nagai et al. found that lymphocytes of PD patients express lower levels of D3R mRNA and binding sites compared to age-matched controls (Nagai et al. 1996).

Study 2: Barbanti et al. found that lymphocytes of patients with PD have higher density of dopamine D1-like and D2-like receptors, and that these levels are back to normal after 3 months therapy with Levodopa or Bromocriptine (Barbanti et al. 1999).

Study 3: Patients with de novo idiopathic PD have lower levels of IL-2 and IFNγ in their blood, and these abnormal cytokine levels are corrected after Amantadine treatment (Wandinger et al. 1999).

Study 4: Fiszer et al. showed that patients with PD have in their CSF monocytes that express high HLA-DR, and in their blood a decreased percentage of $CD45RA^+$ "naive" and an increased percentage of $CD45RO^+$ "memory" T cells (Fiszer 2001).

Study 5/Review: U. Fiszer asked if PD has an immunological basis, and discuss the evidence and its therapeutic implications (Fiszer et al. 1994; Fiszer 2001, 2004). The evidence include the occurrence of autoantibodies against neuronal structures, and high numbers of microglia cells expressing the histocompatibility glycoprotein human leukocyte antigen-DR in the substantia nigra (Fiszer et al. 1994; Fiszer 2001, 2004). An infectious cause for PD is discussed, as well as the data on disturbed cellular and humoral immune functions in peripheral blood of PD patients. An elevated gamma $delta^+$ T cell population and increased immunoglobulin G immunity in CSF to heat shock proteins have been found in PD. Cytokines and apoptosis-related proteins are elevated in the striatum in patients with PD. Furthermore, activated glial cells may participate in the neuronal cell death in PD by providing toxic substances. Fiszer concluded that the immune system is involved in the pathogenesis of PD, but admitted it is still impossible to determine whether the disturbances described above constitute a primary or secondary phenomenon (Fiszer et al. 1994; Fiszer 2001, 2004).

Study 6: Hisanaga et al. found that patients with PD displayed a significantly greater population of circulating $CD3^+$ CD4 $bright^+$ CD8 $dull^+$ lymphocytes than age-matched control subjects ($P = .005$) and patients with cerebrovascular disease ($P = .002$) (Hisanaga et al. 2001). The increase in these cells appeared to continue for at least 17 months. These T cells also expressed CD45RO and Fas, markers for activated T cells, while CD1a, a marker for thymic T cells, was negative, suggesting that these cells are mature T cells with immune activities. As $CD4^+$ $CD8^+$ T cells increase after some specific viral infections, the continuous increase in CD4 $bright^+$ CD8 $dull^+$ T cells shown in this study may indicate post infectious immune abnormalities that are possibly associated with the pathogenesis of this slowly progressive, multifactorial neurodegenerative disease (Hisanaga et al. 2001).

Study 7: Bas et al. studied 30 untreated and 34 treated patients with PD and found a numeric decrease in helper T cells (higher in $CD4^+CD45RA^+$ than in $CD4^+CD29^+$) and B cells, and a rise in activated $CD4^+CD25^+$ lymphocytes that was correlated with lymphocyte depletion (Bas et al. 2001). All these alterations were independent of Levodopa treatment. In addition, when striatal dopamine depletion was performed in rats with either MPP(+) or 6-OHDA, it was found that MPP(+) but not 6-OHDA can increase $CD4^+CD25^+$ lymphocytes. Based on their findings the authors speculated that mechanisms other than dopamine deficit may explain the immune activation in PD (Bas et al. 2001).

Study 8: Or et al. concluded that humoral immunity may play a role in the pathogenesis of PD, based of their findings that all the patients with PD included in their study had: (1) A significant dopamine neuron loss negatively correlating with disease duration; (2) Increased inflammatory HLA immunopositive microglia throughout the disease; (3) IgG (but not IgM) binding on dopamine neurons. Lewy bodies were strongly immunolabelled with IgG. A mean of 30% ± 12% of the dopamine nigral neurons were immunoreactive for IgG in PD, with the proportion of IgG immunopositive neurons negatively correlating with the degree of cell loss in the substantia nigra, and positively correlating with the number of HLA immunopositive microglia. The high affinity activating IgG receptor, FcgammaRI, was expressed on nearby activated microglia. These findings and additional ones revealed in this study are consistent with an immune activation of microglia leading to the targeting of dopamine nigral neurons for destruction in both idiopathic and genetic cases of PD (Orr et al. 2005).

Study 9: Rajda et al. found higher intracellular dopamine content in lymphocytes of PD patients receiving a high dose of L-Dopa as compared to lymphocytes from healthy controls and from PD patients treated with a low dose of L-Dopa. The dihydroxyphenylacetic acid to dopamine ratio was significantly lower in the high-dose L-Dopa-treated PD patients than in the controls. These findings suggested that the dopamine content and metabolism in the peripheral lymphocytes of patients with PD are influenced by L-Dopa (Rajda et al. 2005).

1.6.3 Alzheimer's Disease

Clinical and pathological evidence points to an involvement of dopamine in Alzheimer's disease (AD). Barbanti et al. tested dopamine D1-like and D2-like receptors on PBLs of 20 patients with AD and in 25 healthy controls by radioligand binding assay techniques using SCH 23390 and 7OH-DPAT as radioligands. The density of dopamine D1-like receptors and the affinity of [3H]SCH 23390 and [3H] 7OH-DPAT binding to PBL were similar in both groups investigated. AD patients revealed a lower density of dopamine D2-like receptors on PBL than controls ($P = 0.\ 0016$). The pharmacological profile of [3H]SCH 23390 and [3H] 7OH-DPAT binding to PBL was consistent with the labeling of D5R and D3R subtypes, respectively. The reduced density of dopamine D2-like receptors on

PBLs is consistent with the observation of changes in the expression of D2-like receptors in dopaminergic brain areas in AD. These findings supported the hypothesis of an involvement of dopamine in AD, even in those patients with no evidence of Parkinsonism, behavioral abnormalities or psychosis (Barbanti et al. 2000).

1.6.4 Migrane

Study 1: Barbanti et al. investigated the D5R expression in PBL's of 11 migraine patients and 10 healthy control subjects. A radioligand binding technique showed that an antagonist of D1-like (D1/D5) receptors (SCH 23390) bound specifically to PBL's of migraineurs and control subjects in a manner consistent with the labeling of a D5R. In migraineurs a statistically significant higher density of lymphocyte D5R compared with controls was evident, whereas the affinity of the radioligand was unchanged. The increased density of D5R in PBLs may reflect the dopaminergic hypersensitivity displayed by migraineurs (Barbanti et al. 1996).

Study 2: In a later study, Barbanti et al. found that migraine patients show an increased density of D3R and D4R on lymphocytes compared with controls (Barbanti et al. 2000). The authors proposed that this up-regulation might reflect central and/or peripheral DR hypersensitivity due to hypofunction of the dopaminergic system (Barbanti et al. 2000).

1.6.5 Depression

Rocc et al. found that the D4R mRNA levels were significantly decreased in the PBMCs of untreated depressed patients as compared to controls. The abnormally low D4R mRNA expression returned to control levels after paroxetine treatment, when patients achieved a significant improvement of depressive symptoms (Rocc et al. 2002).

1.7 Summary and Concluding Remarks

Based on all the data discussed in this Chap. 1 we believe that dopamine deserves now a new title – a 'Neuro-Immuno-Transmitter' – a novel term coined herein, since it is deeply involved in the activities of the immune system, not only in those of the nervous system. My six criteria for this new title 'Neuro-Immuno-Transmitter', that dopamine undoubtedly fulfills, are:

Criteria 1: Most of not all immune cells express DRs. Different immune cells have different levels of dopamine D1R-D5R subtypes. The expression of the DRs in immune cells was shown both on the mRNA level on the protein levels, and the DR's are present as functional receptors on the cell surface that bind and respond to dopamine and to its analogues. Interestingly, the DR expression in immune

cells, especially in T cells, often changes dramatically after activation of the cells by strong stimuli such as by antigens, mitogens, cytokines etc. Thus, for example, the levels and types of DRs in T cells is different in resting/naive cells vis-a-vis activated T cells. DR expression also in different in various T cell subpopulations such as $CD4^+$ and $CD8^+$ cells; Teff and Treg cells etc.

Criteria 2: Dopamine by itself induces direct and potent effects in most immune cells. Dopamine either triggers or suppress many vital immune functions, depending on the context, its conc., the activation state of the target immune cell, the cell subtype, and the DRs expressed on it's cell surface. Dopamine usually stimulates naïve/resting T cells, but inhibits already activated T cells, or T cells that are exposed simultaneously to dopamine and other stimuli. Dopamine's optimal conc. for affecting T cells and other immune cells in a specific and physiological manner is ~10 nM (10^{-8} M). While 10–100-fold higher or lower dopamine conc. are often still effective, dopamine at much higher conc. of ~0.1–1 mM (10^{-3}–10^{-4} M) induces non specific immune effects, and is often even toxic and kills the immune cells.

Criteria 3: Dopamine receptor agonists and antagonists have potent effects on immune cells.

Criteria 4: Most immune cells produce dopamine, although at very low conc. of ~ 10^{-18} mol dopamine per cell. In some conditions the immune-derived dopamine can be released to the extracellular milieu, and able to induce autocrine or paracrine effects.

Criteria 5: Dopamine and/or its receptors seem to be involved in various immune and autoimmune diseases, among them Multiple Sclerosis, Systemic Lupus Erythematosus, Rheumatoid Arthritis, Diabetes Mellitus, HIV infection and the resulting immunodoficiency, hematologic cancers: T cell leukemia and lymphoma, and others.

Finally, according to few publications, there are some abnormalities in the expression of DRs in lymphocytes, and/or in several immune functions in various neurological/neuropsychiatric diseases associated with abnormal dopamine in the brain. Among these are: Schizophrenia, Parkinson's disease, Alzheimer's disease, migraine, stress & depression. Based on these relatively small no of publications one may hypothesize that when the dopamine levels in the brain are abnormal, the immune system 'feels' it and influenced by it too, since the dopamine-induced immune effects that may be induced under normal conditions would be impaired or lacking now in the neuropsychiatric disease. One may further speculate that the abnormal immunity due to the abnormal dopamine levels could even be directly or indirectly responsible for some of the symptoms of the neurological/neuropstchiatric disease.

Hopefully, many more new studies would be performed in the coming years on dopamine and its receptors in the immune system in health and disease, which would reveal a handful of exciting findings, which would be of interest and importance to a broad spectrum of scientists and clinicians, mainly those dealing with Neurology and Neuropathology, Immunology, Immunopathology and Psychiatry. Currently, my 'suspicion' is that there is much much more than meets the eye...

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Nerve Driven Immunity: Noradrenaline and Adrenaline

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Marco Cosentino and Franca Marino

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2.1 Noradrenaline and Adrenaline as Classical Neurotransmitters and Neurohormones

Noradrenaline and adrenaline belong to catecholamines, a family of chemical compounds containing a catechol or 3,4-dihydroxyphenyl group and an amine function. Together with dopamine, they are the most abundant and important catecholamines in the human body and are all produced from tyrosine, a non-essential amino acid which is both obtained from dietary proteins or in turn synthesized from the essential amino acid phenylalanine by the enzyme phenylalanine hydroxylase. Noradrenaline is synthesized from dopamine by dopamine β-hydroxylase and is converted to adrenaline by phenylethanolamine *N*-methyltransferase (Fig. 2.1). The natural stereoisomers are L-(-)-(R)-noradrenaline and adrenaline.

Adrenaline was the first hormone to be isolated in a pure state. After Georg Oliver's empirical observation in 1893 of the effect of an extract of sheep's adrenal gland on human blood vessels, John Abel obtained the impure form of the active principle in 1897 and named it as "epinephrine" (from the Greek roots *epi* and *nephros*, i.e. "on the kidney"), but it was Jokichi Takamine in 1900 the first to isolate adrenaline as pure crystalline base. He immediately patented the process for isolating the hormone, which was then marketed under the proprietary name of Adrenalin® ("near the kidney", from Latin roots *ad* and *renes*). Noradrenaline was synthesized a few years later (the prefix "nor" is the acronym of *nitrogen öhne radikal*, indicating the absence of a methyl group), but it was only in 1949 that this molecule was proved by Ulf von Euler in Stockholm to be the main sympathomimetic neurotransmitter in humans (Sneader 2005).

Adrenaline (and, as a consequence, noradrenaline) was introduced as the British Approved Names (BAN) in the United Kingdom and British Commonwealth, while in the US epinephrine (and norepinephrine) was adopted as the United States Approved Name (USAN). Epinephrine and norepinephrine later became also the World Health Organization Recommended International Nonproprietary Names (rINN) for these compounds. Although the use of rINN is now mandatory in the European Union, adrenaline and noradrenaline represent the sole exception to the rule and retain their BAN on grounds of safety (Sneader 2005).

Noradrenaline and, to a lesser extent, adrenaline act as neurotransmitters in the central and peripheral nervous systems. Chromaffin cells in medulla of adrenal glands also produce adrenaline (~80% in humans) and noradrenaline (~20%), which are directly released into the blood upon stimulation by the sympathetic nervous system through preganglionic fibers originating in the thoracic spinal cord. In the central nervous system, the *locus coeruleus* (LC) is the most important noradrenergic nucleus, its axons projecting rostrally to hippocampus, septum, hypothalamus and thalamus, cortex and amygdala, dorsally to cerebellum, and caudally to spinal cord. Noradrenaline in LC neurons plays an important role in attention, arousal and vigilance to salient and relevant external stimuli, and regulates hunger and feeding behavior exerting a stimulatory effect on feeding possibly acting directly on the hypothalamus. Adrenaline-containing neurons in the central nervous system are mainly localized in the medullary reticular formation and their axons project both rostrally and caudally, possibly participating in the

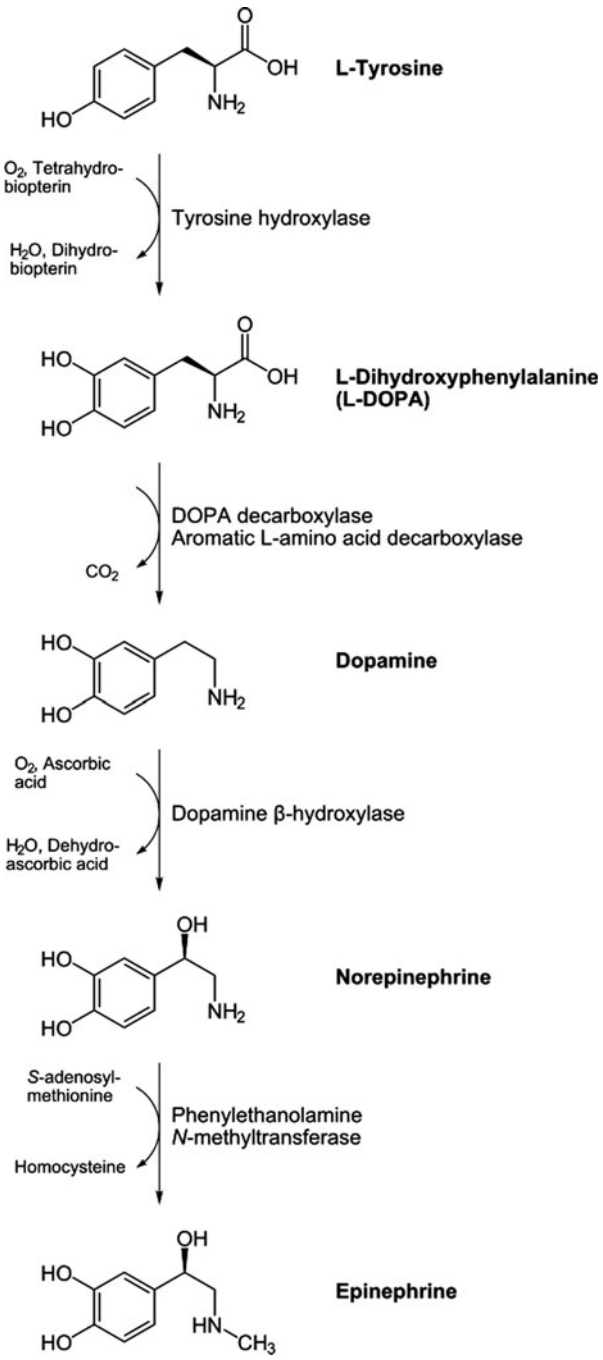

Fig. 2.1 Biosynthesis of the catecholamines adrenaline (epinephrine) and noradrenaline (norepinephrine). The synthesizing enzymes are shown to the *right* of each *arrow*, while enzyme cofactors are shown to the *left* (reproduced from the Wikimedia Commons – http://commons.wikimedia.org)

coordination of eating and in visceral activities such as blood pressure regulation. In the peripheral nervous system, noradrenaline is the principal transmitter of most autonomic sympathetic postganglionic fibers. Main peripheral actions of noradrenaline and adrenaline include: contraction of certain types of smooth muscle (blood vessels supplying skin, kidney, and mucous membranes), stimulation of exocrine glands (e.g. salivary and sweat glands), relaxation of certain other types of smooth muscle (gut wall, bronchi, blood vessels supplying skeletal muscle), increases of heart rate and force of contraction, metabolic actions (increased glycogenolysis in liver and muscle, lipolysis in adipose tissue), endocrine actions (e.g. modulation of the secretion of insulin and renin). An extensive discussion of noradrenaline and adrenaline neurochemistry, anatomy and physiology can be found in Feldman et al. (1997).

2.1.1 Adrenoceptors

Noradrenaline and adrenaline exert their effects by acting on 7-transmembrane, G-protein coupled receptors called "adrenergic receptors" or "adrenoceptors" (ARs). ARs are expressed in virtually all peripheral tissues and within the central nervous system and are involved in the control of blood pressure, myocardial contractile rate and force, airway reactivity, as well as a variety of metabolic and central nervous system functions. AR agonists and antagonists are currently used to treat a variety of diseases, including hypertension, angina pectoris, congestive heart failure, asthma, depression, benign prostatic hypertrophy, and glaucoma. Additional conditions where these agents proved useful include shock, premature labor and opioid withdrawal, and as adjunct medications in general anesthesia.

ARs were first divided into α and β, based on the rank order of potency of selected agonists, and subsequently, both the α and β types were further divided into α_1, α_2, β_1 and β_2 subtypes (Bylund et al. 1994). Current classification is based on both pharmacological and molecular evidence and includes three major types – α_1, α_2 and β – each further divided into three subtypes. ARs can be either pre- or postsynaptic. Presynaptic ARs are mainly of the α_2 type and mediate inhibition of neurotransmitter release, while postsynaptic receptors include all AR types. In peripheral tissues, α-ARs are expressed in smooth muscle cells, in particular in the vasculature, and usually mediate contraction, while β-ARs can be found in heart (β_1 and to a minor extent β_2, which mediate contraction), in smooth muscle cells (mainly β_2, inducing relaxation), and in skeletal muscle (β_2, possibly inducing hypertrophy). Indeed, β_2-ARs are expressed in virtually all normal human cell types. Usually, α_1- and β_1-ARs are located in the immediate proximity of sympathoadrenergic terminals and are therefore the main target of noradrenaline released upon nerve stimulation. On the contrary, α_2- and β_2-ARs may be located far from nerve terminals (extrajunctional receptors) and may represent the preferential target of circulating noradrenaline and adrenaline. ARs are also widely distributed in the brain. AR physiology and pharmacology is summarized in Table 2.1, while detailed and continuously updated information is provided in Bylund et al. (2011).

Table 2.1 Classification, nomenclature, molecular and cellular physiopharmacology of adrenoceptors (based on Bylund et'al. 2011)

Name	Previous and unofficial names	Agonists[a] (affinity)[b]	Antagonists[a] (affinity)[b]	Transduction mechanisms (effectors)	Tissue distribution	Physiological functions
α_{1A}	α_{1a}	(-)-Adrenaline (6.3)	Tamsulosin (10.0–10.4)	G_q/G_{11} (phospholipase C stimulation, calcium channel)	Human heart, liver, cerebellum, cerebral cortex, predominant subtype in human prostate and urethra	Contraction of urethral smooth muscle, contraction of skeletal muscle resistance arteries
	α_{1c}	Noradrenaline (5.8–6.0)	Silodosin (10.4)			
	Adrenergic receptor	Oxymetazoline (8.0–8.2)	Prazosin[c] (9.5)	Phospholipase D stimulation		
		Phenylephrine (5.2–5.4)		Protein kinase C		
		Methoxamine (5.0–5.2)				
α_{1B}	α_{1b}	(-)-Adrenaline (6.5)	Prazosin[c] (9.6–9.9)	Mitogen activated protein kinases	Human spleen and kidney, human somatic arteries and veins	Contraction of arteries and veins
		(-)-Noradrenaline (6.2)	Tamsulosin (9.5)			
		Oxymetazoline (6.5)				
		Phenylephrine (pIC_{50}: 6.3–7.5)				
		Methoxamine (4.0)				
α_{1D}	$\alpha_{1a/d}$	(-)-Noradrenaline (7.4)	Tamsulosin (9.8–10.1)		Human aorta, blood vessels of human prostate, human bladder	Constriction of arteries, urethral contraction, contraction of corpus cavernosum
	α_{1A}		Prazosin[c] (9.5–10.2)			
	Adrenergic receptor	(-)-Adrenaline (7.2)				
		Oxymetazoline (6.1–6.4)				
		Xylometazoline (6.0)				
		Phenylephrine (5.9)				

(continued)

Table 2.1 (continued)

Name	Previous and unofficial names	Agonists[a] (affinity)[b]	Antagonists[a] (affinity)[b]	Transduction mechanisms (effectors)	Tissue distribution	Physiological functions
α_{2A}	α_2-C10	(±)-Adrenaline (5.6–8.3)	Lisuride (10.3)	G_i/G_o (adenylate cyclase inhibition, potassium channel, calcium channel, phospholipase A2 stimulation)	Human brain > spleen > kidney > aorta = lung = skeletal muscle > heart = liver	Presynaptic inhibition of noradrenaline release, hypotension, sedation, analgesia, hypothermia
	RG20		Yohimbine (8.4–9.2)			
		Noradrenaline (5.6–8.4)	Phentolamine (8.4)			
		Oxymetazoline[d] (8.0)				
		Guanfacine[d] (7.3)				
		Clonidine[d] (7.2–9.2)				
α_{2B}	α_2-C2	Noradrenaline[d] (5.6–9.1)	Lisuride (9.9)		Human kidney >> liver > brain = lung = heart = skeletal muscle (also reported in aorta and spleen)	Vasoconstriction
	RNG		Phentolamine (8.2)	G_s (adenylate cyclase stimulation)		
		(±)-Adrenaline[d] (5.2–6.2)	Yohimbine (7.9–8.9)			
		Clonidine[d] (6.7–9.5)				
		Guanfacine (5.8–6.5)				
		Oxymetazoline (5.5–6.2)				
α_{2C}	α_2-C4	Noradrenaline (5.9–8.7)	Lisuride (9.9)		Human brain ≥ kidney (also reported in spleen, aorta, heart, liver, lung, skeletal muscle)	Presynaptic inhibition of noradrenaline release
		(±)-Adrenaline (5.8–6.2)	Rauwolscine (9.1)			
		Oxymetazoline[d] (6.7)	Yohimbine (8.5–9.5)			
		Clonidine[d] (6.0–7.8)				
		Brimonidine[d] (5.7–7.6)				

β_1		(±)-Adrenaline (6.0)	Carvedilol (9.5)	G_s (adenylate cyclase stimulation)	Pineal gland, skeletal muscle, liver, superior cervical ganglion, heart, lung, adrenal cortex, cardiac myocytes, brain	Increase of cardiac output (heart rate, contractility, automaticity, conduction), renin release from juxtaglomerular cells, lipolysis in adipose tissue
			Betaxolol (8.8)			
		Noradrenaline (6.0)	(-)-Propranolol (8.2–8.9)			
		Isoprenaline (6.6–7.0)				
		Denopamine[d] (5.8)		G_i/G_o (guanylate cyclase stimulation)		
		Dobutamine[d] (5.5)				
β_2		(±)-Adrenaline (6.2)	Timolol (9.7)		Lung, lymphocytes, skin, liver, heart	Smooth muscle relaxation, striated muscle tremor and glycogenolysis, increase of cardiac output, increase of acqueous humor production in eye, glycogenolysis and gluconeogenesis in liver, insulin secretion
		Noradrenaline (5.4)	Carvedilol (9.4–9.9)			
		Salmeterol (8.8)	Propranolol (9.1-9.5)			
		Isoprenaline (6.4)				
		Dobutamine[d] (6.2)				
β_3	Atypical β adrenoceptor	Noradrenaline (4.7–6.3)	Carvedilol (9.4)		Adipose, gall bladder > small intestine > stomach, prostate > left atrium > bladder	Lipolysis, thermogenesis, relaxation of miometrium and colonic smooth muscle cells, vasodilatation of coronary arteries, negative cardiac inotropic effect
		(±)-Adrenaline (3.9-4.7)	Propranolol (6.3–7.2)			
		BRL 37344 (6.4–7.0)	Nadolol (6.3)			
		Isoprenaline (5.1–6.2)				
		SR58611A (5.2)				

[a] Besides adrenaline and noradrenaline, representative agonists and antagonists were selected on the basis of higher affinity, with preference for drugs approved for therapeutic use. For investigational agents as well as for comparative affinity profiles see Bylund et'al. (2011).
[b] Unless otherwise specified, affinity (i.e., a measure of how strongly the ligand binds to the receptor) is expressed in terms of pK, that is the negative logarithm to base 10 of the equilibrium dissociation constant, K in molar concentration units (Neubig et'al. 2003).
[c] Inverse agonist.
[d] Partial agonist.

2.1.2 Sympathoadrenergic Innervation of Lymphoid Organs

Both primary (bone marrow and thymus) and secondary (spleen and lymph nodes) lymphoid organs are innervated by autonomic efferent nerve fibers which are mainly sympathoadrenergic. Indeed, the sympathetic nervous system represents, together with the hypothalamic-pituitary-adrenal axis, the major pathway involved in the cross-talk between the brain and the immune system (reviewed in Elenkov et al. 2000). ARs expressed on immune cells are usually located far from noradrenergic varicosities of sympathetic nerves, thus representing a prominent example of extrajunctional receptors and possibly also of volume transmission mode of intercellular communication (Agnati et al. 2010). Several excellent reviews addressed over the years the origin, pattern of distribution and targets of sympathetic nerves in lymphoid organs, their neurochemical signaling (Felten et al. 1985; Felten and Felten 1988; Felten 1991; Straub 2004), as well as the functional and clinical significance of age-induced changes in sympathetic-immune interactions (Bellinger et al. 1992; Madden et al. 1995, 1997, 1998; Friedman and Irwin 1997) and the consequences of dysregulated sympathetic nervous system in stress responses (Irwin 1994; Nagatomi et al. 2000; Marshall and Agarwal 2000; Sloan et al. 2008) and in the development and progression of immune-mediated diseases (Bellinger et al. 1992, 2008; Madden et al. 1995; Friedman and Irwin 1997; Marshall and Agarwal 2000; Frohman et al. 2001; Wrona 2006; Straub et al. 2006; del Rey and Besedovsky 2008; Benarroch 2009).

2.2 Expression of ARs and Effects of Noradrenaline and Adrenaline on Immune Cells

In the study of nervous system-immune system interactions, sympathoadrenergic pathways received extensive attention and, as a result, a huge amount of literature is available regarding the occurrence and the functional relevance of ARs on immune cells, which have been the subject of several excellent reviews (e.g. Kohm and Sanders 2000, 2001; Elenkov et al. 2000; Kin and Sanders 2006; Nance and Sanders 2007; Flierl et al. 2008). Among ARs, β_2-ARs are usually the most expressed on immune cells and they have been consequently regarded as the main receptors mediating the immune effects of noradrenaline and adrenaline. It was only over the last two decades that evidence began to increase regarding the occurrence of other ARs, in particular the α_1-AR subtype (reviewed by Kavelaars 2002). Also thanks to novel molecular techniques allowing the sorting of highly purified cell subsets, it is becoming increasingly evident that different immune cell populations express distinct patterns of ARs, which in turn undergo up/downregulation upon cell maturation, activation, etc. In summarizing current knowledge regarding ARs on immune cells, attention will be devoted mainly to studies addressing specific cell types (and not, e.g. peripheral blood mononuclear cell preparations). In addition, also in view of the large amount of available literature, results obtained in human

samples will be preferentially considered. Finally, here it will be only mentioned the issue of sympathoadrenergic control of thymic function, which has been recently reviewed (Leposavić et al. 2008).

2.2.1 T and B Lymphocytes

2.2.1.1 AR Expression

Although in initial studies using radioligand binding techniques the density of β-ARs was reported as apparently similar on human T and B lymphocytes (Pochet et al. 1979; Bishopric et al. 1980), subsequently it was shown that T cells exhibited a lower number of binding sites than B cells (Bidart et al. 1983; Paietta and Schwarzmeier 1983), with T helper and T suppressor cells showing similar binding capacities (Landmann et al. 1984). Another study however showed the following rank order of β-AR (likely β_2-AR) density: T suppressor > T cytolytic > T helper (Khan et al. 1986). These findings were confirmed by another study showing that B lymphocytes had the greatest number of β-ARs with 12.1 ± 1.8 fmol/10^6 cells, followed by CD8+ T lymphocytes with 3.4 ± 0.4 fmol/10^6 and CD4+ T lymphocytes with 1.2 ± 0.1 fmol/10^6 cells (Karaszewski et al. 1990). In another study, the same authors reported 2.8 ± 0.3 fmol/10^6 cells in CD8+CD28- (suppressor) T cells versus 1.4 ± 0.4 fmol/10^6 cells in CD8+CD28+ (cytotoxic) T cells (Karaszewski et al. 1991).

The density of β-ARs on human lymphocytes is possibly subject to rapid changes. In a longitudinal study in ten healthy subjects, the absolute values of circulating CD4+ and CD8+ T lymphocyte frequency showed little variation, β-AR density variance was greater on both cell subsets (Anstead et al. 1998). Interestingly, it was reported that the expression of β_2-ARs is increased in both T and B cells after physical stress and returns to normal after 30 min rest (Ratge et al. 1988). Another factor affecting β-AR density is the level of cell activation. Cultivation of human peripheral blood T-lymphocytes in the presence of interleukin-2 (IL-2) and phytohemagglutinin (PHA) increases β_2-AR expression (Korichneva and Tkachuk 1990), as well as isoprenaline-induced cAMP production, possibly by a calcium-independent mechanism (Carlson et al. 1994). PHA stimulation has been reported to prevent both sequestration of β-ARs and their dissociation from G_s proteins in response to isoprenaline stimulation (but not the functional uncoupling from adenylyl cyclase) (Carlson et al. 1994). In human CD4+ and CD8+ T lymphocytes, exposure to IL-1β has no effect on β_2-AR expression, however incubation with IL-2 up-regulated β_2-ARs on CD8+ cells (Wahle et al. 2001).

No evidence is currently available regarding the expression of β_1- or β_3-ARs in human lymphocytes with the only notable exception of the occurrence of β_1-AR mRNA (together with β_2- but not β_3-AR mRNA) in human CD4+CD25+ T regulatory cells (Cosentino et al. 2007), however their membrane expression as well as their possible functional relevance has never been investigated so far. Expression of β_1-ARs in CD4+CD25+ T cells was recently confirmed and it was

tentatively proposed that they may mediate the effects of stress on this specific cell subset (Freier et al. 2010). Expression of β_3-AR mRNA was reported in only one study in concanavalin A (Con A)-stimulated human T lymphocytes, but no correlation found with specific functional responses (Borger et al. 1998).

As regards α-ARs, only a few evidence exists regarding human T and B lymphocytes. Ligand binding studies suggest the occurrence of α_2-ARs in human CD4+ and CD8+ T lymphocytes, as well as their upregulation after in vivo administration of adrenaline (Jetschmann et al. 1997). On the contrary, no direct evidence exists regarding the expression of α_1-ARs in T and B lymphocytes in humans. However, it was shown that α_1-ARs can be induced in peripheral blood mononuclear cells stimulated with the T lymphocyte-preferring mitogen PHA, suggesting that their expression may occur at least on activated T cells (Rouppe van der Voort et al. 2000).

2.2.1.2 Functional Responses to AR Activation

Stimulation of β-ARs has been reported to down-regulate IL-2 receptors in both mitogen-stimulated lymphocytes and IL-2-dependent T lymphocyte cell lines (Feldman et al. 1987). In agreement with these observations, it was shown that treatment of human subjects with the β-AR antagonist propranolol increased spontaneous and PHA-induced IL-2R expression as well as IL-2 generation in circulating lymphocytes (Malec et al. 1990), although the actual involvement of β-ARs in this effect of propranolol has been subsequently questioned (Mangge et al. 1993). The β-AR isoprenaline inhibits the anti-CD3 mAb-induced proliferation of T cells, without synergistic effects with dexamethasone. Isoprenaline-induced inhibition is however nearly completely overcome by the addition of anti-CD28 mAb to anti-CD3 mAb-stimulated T cells (Elliott et al. 1992). The inhibitory effect of isoprenaline on anti-CD3 mAb-induced proliferative response of T cells affects also their CD4+, CD8+, or CD45RO+ subsets, as well as anti-CD3 mAb-induced IL-.2 production, although to a lower extent than PGE2 (Bartik et al. 1993). Such difference may be explained on the basis of differential activation of cAMP-induced PKA I and II isozymes (Bauman et al. 1994). In contrast to freshly isolated T cells however, β_2-AR-inhibition of Th1 (IFN-γ) and Th2 (IL-4, IL-5) cytokine production and induction of CREB phosphorylation is reduced in polarized T helper cells, possibly as the result of a generalized loss of the negative feedback by receptors coupled to the AC/cAMP system (Heijink et al. 2003). In CD8+ human T lymphocytes activation of β-ARs has been shown to decrease the peak current amplitude and to increase the rate of inactivation of the delayed rectifier K^+ current. Since inhibition of the delayed rectifier K^+ current has been found to decrease the proliferative response in T lymphocytes, β-AR-induced modulation of K^+ current may well serve as a feedback control mechanism limiting the extent of cellular proliferation (Soliven and Nelson 1990). Concanavalin A (Con A)-induced production of IFN-γ, GM-CSF, and IL-3 (but not IL-4) by human T lymphocytes is inhibited by activation of β_2-ARs (but not of β_1- or β_3-ARs, although at least β_3-AR mRNA was detectable in Con A-activated cells) (Borger et al. 1998).

Although activation of β-ARs on human lymphocytes is usually believed to result in antiinflammatory effects, evidence exists that immunostimulatory effects may occur depending on the time of exposure and degree of cell differentiation and activation. Indeed, expression of β_2-ARs occurs on resting and activated B cells, naive CD4+ T cells, T helper 1 (Th1)-cell clones and newly generated Th1 cells, but not in Th2-cell clones and newly generated Th2 cells (Sanders et al. 1997; Kohm and Sanders 1999). In agreement with such observations, noradrenaline may promote IL-12-mediated differentiation of naive CD4+ T cells into Th1 effector cells, and increase the amount of IFN-γ produced by Th1 cells (Swanson et al. 2001), but has no apparent effect on IL-4-mediated differentiation of Th2 cells (Sanders et al. 1997). The effect of noradrenaline seems to depend on the state of cell activation even in B lymphocytes, which produce more IgG1 and IgE when noradrenaline is added either during antigen processing or within the first 12 h of culture with Th2 cells (Kasprowicz et al. 2000), while in plasma cells noradrenaline decreases antibody production (Melmon et al. 1974).

2.2.2 Natural Killer Cells

Several lines of evidence indicate that Natural Killer (NK) cells express high levels of β-AR. Maisel et al. (1990), studying β-AR expression and cAMP production in lymphocyte subsets sorted by positive selection with specific monoclonal antibodies, found the highest number of receptors (1,934 ± 122 sites/cell) in CD16+CD56+ NK cells, which after physical exercise increased (to 2,617 ± 289 sites/cell) together with isoprenaline-stimulated cAMP accumulation.

Both noradrenaline and adrenaline decrease NK cell cytotoxicity, pharmacological evidence suggesting the involvement of β-AR (likely β_2-AR) pathways (Whalen and Bankhurst 1990; Takamoto et al. 1991), however adrenaline resulted in stimulation of NK cell cytotoxicity at lower (submicromolar-picomolar) concentrations (Hellstrand et al. 1985). In vitro, β_2-AR activation on NK cells also results in reduction of cell adhesion to endothelial cells (Benschop et al. 1994, 1997). The in vivo relevance of this effect is confirmed by the observation that in human subjects both adrenaline and noradrenaline modulate the migratory capacity of human NK cells via spleen-independent β_2-AR mechanism (Schedlowski et al. 1996; Benschop et al. 1997).

Human NK cells express also α-ARs. In CD16+ lymphocytes, ligand binding studies indeed showed the presence of β_2-, α_1-, α_2- but not β_1-AR, and infusion of adrenaline (but not noradrenaline) significantly decreased β_2- and α_1-AR numbers on NK cells. Expression of α_2-ARs as well was decreased by adrenaline infusion on NK cells, but increased in CD4+ and CD8+ T lymphocytes (Jetschmann et al. 1997). No evidence exists so far however regarding the functional relevance of α-AR pathways in human NK cells.

2.2.3 Monocytes/Macrophages

Extensive evidence support the occurrence of β-AR on human monocytes/macrophages: β_2-ARs are usually regarded as mainly antiinflammatory, while recent evidence suggest under certain circumstances the occurrence of β-AR (possibly β_1-AR)-mediated proinflammatory responses (Grisanti et al. 2010). Alpha-ARs can also occur upon appropriate stimulation and their functional role is presently a matter of active investigation.

The expression of β-ARs on human monocytes was initially documented by means of classical binding experiments, which showed that β-AR density on human circulating monocytes was about 2,400 binding sites/cell and increased to 3,220 binding sites/cell after physical exercise (Ratge et al. 1988). Recently however, by means of flow cytometry, it was shown that β_2-AR expression on monocytes was elevated in anticipation of an acute bout of resistance exercise and decreased during the exercise (Fragala et al. 2011). Expression of β-ARs on human macrophages is regulated upon activation in a stimulus-dependent manner, suggesting that changes in receptor number can be regulated with different states of cell maturation and function (Radojcic et al. 1991). Indeed, during the maturation of human monocytes to macrophages in vitro, despite a functional adenylyl cyclase system, β-AR responsiveness is lost (Baker and Fuller 1995). In particular, it was recently shown that as human monocytes adhere to surfaces to begin differentiation into macrophages, they selectively lose their surface β_2-ARs and hence become insensitive to the inhibitory effects of β_2-AR agonists on LPS-induced TNF-α production, and this has been as a possible explanation to the lack of significant anti-inflammatory effect of β_2-AR agonists on alveolar macrophages or in clinical asthma (Ezeamuzie et al. 2011). On the other side, β_2-AR agonists like terbutaline, salmeterol and formoterol have been shown to inhibit to a variable extent the release of inflammatory mediators such as LTB4, PGE2 and IL-1β from human monocytes possibly through β-AR-independent mechanisms (Linden 1992). Prolonged β_2-AR stimulation up-regulates cAMP phosphodiesterase activity in human monocytes (Manning et al. 1996), thus leading to receptor desensitization. In U937 cells, which are commonly used as a model of human monocytes, β_2-ARs are the main subtype of β-ARs expressed and their expression is lower on undifferentiated (monocytes) than in PMA-differentiated U937 (macrophages), and care should be therefore exerted when using these cells to study the physiopharmacology of β-ARs in human monocytes (Izeboud et al. 1999).

Noradrenaline and adrenaline acting on human monocytes through β-ARs (possibly, β_2-ARs) have been usually regarded as anti-inflammatory. Pharmacological evidence suggests that β-ARs activation may inhibit the production of oxygen radicals (Schopf and Lemmel 1983), upregulate TNF receptors and inhibit TNF (Guirao et al. 1997), reduce the phagocytosis of *C. albicans* (Borda et al. 1998), inhibit LPS-induced macrophage inflammatory protein-1 α (MIP-1 α), which has an important role in the development of inflammatory responses during infection by regulating leukocyte trafficking and function (Li et al. 2003). In particular, selective activation of β_2-ARs may inhibit the production of TNF-α and of IL-6 and increase

the production of IL-10 in PMA-differentiated U937 human macrophages (Izeboud et al. 1999), and down-regulate IL-18-induced intercellular adhesion molecule (ICAM)-1 expression and IL-12, TNF-α and IFN-γ production (Takahashi et al. 2003) as well as LPS-induced IL-18 and IL-12 production in human monocytes, suggesting that the stimulation of β_2-ARs might be beneficial in the treatment of sepsis through inhibiting LPS-elicited IL-18 (Mizuno et al. 2005). On the other side, reversal of the inhibitory effects of noradrenaline and adrenaline on human monocytes may result in immunostimulation. In agreement with this hypothesis, recently the β_2-AR antagonist propranolol has been shown to reduce circulating immunosuppressive M2b monocytes in severely burned children, suggesting that the increased susceptibility of severely burned patients to opportunistic pathogens might be controlled by propranolol (Kobayashi et al. 2011).

Evidence exists however that, under certain conditions, β-AR stimulation may lead to proinflammatory responses. Indeed, β_2-AR activation in unstimulated monocytes may increase the production of IL-18, although it had no effect on IL-12, TNF-α, IFN-γ and IL-10, possibly due to β_2-AR-induced inhibition of IL-18-elicited upregulation of both CD40 and CD40 ligand (CD40L/CD154) expressions (Takahashi et al. 2004). Moreover, in human monocytes stimulated with either LPS of IL-1, treatment with β_2-AR agonists was shown to increase the production not only of the antiinflammatory cytokine IL-10 but also of the pro-inflammatory chemokine IL-8 (Kavelaars et al. 1997). In vitro, adrenaline may upregulate the surface expression of L-selectin on human monocytes possibly through a partial contribution of β-ARs (Rainer et al. 1999), and noradrenaline and adrenaline, possibly through the activation of β_2-ARs, up-regulated MMP-1 and potentiated LPS-induced expression of MMP-1 in peripheral blood monocytes and monocyte-derived macrophages (Speidl et al. 2004). Activation of β_2-ARs may also result in upregulated IL-4-induced CD23 (low affinity IgE receptor/Fc epsilon RII) expression in human monocytes (Mencia-Huerta et al. 1991), resulting in potentiated IgE/anti-IgE-induced production of IL-6 (Paul-Eugene et al. 1992, 1994), IgE (Paul-Eugene et al. 1993) as well as generation of superoxide anion and of nitric oxide, and of TxB2 (Paul-Eugène et al. 1994). Isoprenaline treatment was reported to increase phorbol ester-induced production of TNF-α, IL-12, and nitric oxide, while it decreased inflammatory mediator production in combination with LPS stimulation (Szelenyi et al. 2006). Isoprenaline also increased LPS-induced production of IL-1β in human monocytes, however this effect – according to pharmacological evidence – was due to the activation of β_1-ARs, which were directly observed in the monocytic cell line THP-1 by immunoblot techniques as well as by radioligand binding studies (Grisanti et al. 2010).

Although pharmacological evidence suggesting the possible occurrence of functional α-ARs in human monocytes enhancing the synthesis of complement components (Lappin and Whaley 1982), direct evidence for their existence was provided only recently. In particular, in human monocytes expression of α_{1B}- and α_{1D}-AR mRNA could be obtained by culturing human circulating monocytes with dexamethasone or the β_2-AR agonist terbutaline (Rouppe van der Voort et al. 1999) or with LPS, resulting in the activation of ERK-2 (Rouppe van der Voort et al.

2000b). Alpha-ARs are also expressed in the human THP-1 monocytic cell line, where α_{1B}- and α_{1D}-AR mRNA are respectively upregulated and reduced by the proinflammatory cytokines TNF-α and IL-1β (but not by IL-6 or IL-8) (Heijnen et al. 2002).

Fragmentary evidence is available regarding the functional relevance of α-AR in human monocytes. In a recent study, by means of radioligand binding techniques a homogenous α_{1B}-AR subtype population was characterized on monocytes, which changed to a heterogeneous receptor subtype expression pattern when differentiated to macrophages. The selective α_1-AR agonist phenylephrine synergistically increased LPS-induced IL-1β production and this effect was blocked in the presence of a selective α_1-AR antagonist as well as of inhibitors of protein kinase C (PKC) (Grisanti et al. 2011). It should be also mentioned that in human whole blood incubated with PHA and LPS, noradrenaline and the α_2-AR agonist clonidine reduced the production of TNF-α, while the α_2-AR antagonist yohimbine inhibited the production of IL-1RA (Maes et al. 2000). Pharmacological evidence however is not convincing and no data were provided regarding the actual expression of functional α_2-ARs on these cells.

2.2.4 Dendritic Cells

Extensive evidence exists for the presence and the functional relevance on murine dendritic cells (DCs) of ARs, which mediate sympathetic nervous system influence on DCs-T cells interactions thus contributing to the shaping of the appropriate adaptive immune response (reviewed by Maestroni (2005, 2006)). Murine DCs express both α_1- and β_2-ARs, which play opposite roles in the modulation of cell migration, the former being stimulatory while the latter being inhibitory. Exposure of both skin and bone marrow-derived DCs to noradrenaline after stimulation with bacterial toll-like receptor (TLR) agonists results in decreased IL-12 and increased IL-10 production and, as a consequence, impaired T helper-1 (Th1) priming. On the other side, reduced noradrenaline activity in the skin may contribute to contact sensitizers-induced Th1 responses (Maestroni 2004). Noradrenaline has also been shown to activate β_2-AR-mediated cAMP-PKA pathways to enhance DC production of IL-33, resulting in direct Th2 differentiation and possibly contributing to the stress-related progression of Th2-associated disorders (Yanagawa et al. 2011). Interestingly, however, it has been shown that sympathoadrenergic modulation of the skin innate and adaptive immune response occurring after stimulation with TLR-2 but not TLR-4 agonists may promote a Th1 adaptive response possibly relevant to Th1-sustained autoimmune inflammatory skin diseases (Manni and Maestroni 2008). In agreement with these findings, it has been recently reported that β_2-AR agonists like salbutamol bias DCs preexposed to TLR-2 and nucleotide-binding oligomerization domain (NOD) 2 agonists towards increasing the Th17/Th1 cell ratio finally resulting in an IL-17 immune response, which may be relevant to the defense against extracellular bacteria, the pathogenesis of inflammatory diseases as well as the antitumor response (Manni et al. 2011). Recently,

pharmacological evidence was also provided for the occurrence on murine DCs of α_2-ARs, which may mediate enhancement of antigen capture, possibly contributing to explain immune enhancement following acute stress (Yanagawa et al. 2010).

The extensive knowledge about sympathoadrenergic regulation of murine DCs and its potential relevance to an array of disease conditions is in sharp contrast with the very few information available regarding human DCs. It has been reported that in human dendritic cells stimulated via CD40 activation of β_2-ARs increases intracellular cAMP and inhibits IL-12 production, resulting in inhibition of Th1 and promotion of Th2 differentiation (Panina-Bordignon et al. 1997). More recently, it was shown that in human dendritic cells obtained by differentiating human cord blood CD34+ precursor cells, noradrenaline acting through β_2-ARs and increased cAMP inhibited LPS-stimulated production of IL-23, IL-12 p40, TNF-α and IL-6 without affecting IL-10 (Goyarts et al. 2008). This response pattern is similar to that obtained in mouse skin DCs (Maestroni 2005, 2006), thus suggesting that noradrenaline may regulate human skin DC function resulting in decreased Th1 differentiation of CD4+ T cells. These findings provide new insight into the immunological consequences of the clinical use of β_2-AR agonists and may suggest new approaches for the treatment of Th1-mediated diseases.

2.2.5 Granulocytes

Limited data exist regarding AR expression and function in human granulocytes. Available evidence concern nearly only polymorphonuclear cells (PMN) and β-AR. Expression of β-AR on human PMN has been investigated only by means of ligand binding assays which indicated the existence on average of 1,700–2,200 binding sites per cell (Pohl et al. 1991; Schwab et al. 1993) or 39–61 fmol/mg of protein (Boreus et al. 1986; Gurguis et al. 1999b). In one study, the possible existence of α_2-AR was excluded based on both ligand binding and functional experiments in either PMN and differentiated HL-60 cells, a promyelocytic cell line frequently used as a model for neutrophils (Musgrave and Seifert 1994).

The functional relevance of β-AR on human PMN is also debated. Low concentration of the β-AR agonist isoprenaline inhibited the respiratory burst induced by the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) or by calcium ionophores (A23187, ionomycin), as well as leukotriene B4 generation (Nielson 1987). In another study, however, production of IL-8 and expression of the adhesion molecules CD15, CD44, and CD54 was only slightly reduced by adrenaline and only at very high concentrations (1 mM), suggesting that, although functionally coupled to signaling cascades, the functional relevance of β-AR in PMN is limited. It should be however considered that β-AR desensitization has been reported after activation of PMN respiratory burst (Vago et al. 1990). Decreased β-AR responsiveness in PMN has been reported in the elderly (Cotter and O'Malley 1983).

Expression of β-AR on circulating PMN was found decreased in essential hypertension (Corradi et al. 1981), juvenile type I diabetes mellitus (Schwab et al.

1993), and increased in post-traumatic stress disorder (Gurguis et al. 1999a). Physical exercise may decrease PMN β-AR, however only after acute heavy resistance exercises (Ratge et al. 1988; Fragala et al. 2011).

2.2.6 Microglia

In the central nervous system (CNS), microglial cells are involved in phagocytosis and neuroinflammatory responses, triggering or amplifying both innate and acquired immune responses and in particular contributing to T-cell activation within the CNS. Microglia are therefore usually considered the CNS mononuclear phagocytes (Ghorpade et al. 2008). No data exist regarding the existence and functional relevance of adrenergic mechanisms in human microglia, therefore main evidence obtained in the murine model will be reviewed hereafter.

Direct evidence for the expression of AR on microglial cells has been recently provided by Hertz et al. (2010), who showed, by means of microarray and immunohistochemistry, the occurrence in murine microglia of β_2-AR and possibly of β_1-AR and α_{2A}-AR. β-AR activation in these cells results in increased IL-1 β, TNF-α and IL-6 expression through signal transduction mechanisms involving cAMP and cAMP-dependent protein kinase (Tomozawa et al. 1995) as well as ERK1/2 and P38 MAPK (Wang et al. 2010). Interestingly, however, NA acting on β-AR has been shown to induce also IL-1ra and IL-1 type II receptor expression in murine microglia enriched cultures and to protect cortical neurons against IL-1 β-induced neurotoxicity. In this study, IL-1 β expression was not affected by NA (McNamee et al. 2010). In agreement with these observations, exposure to both β1- and β2-AR agonists decreased the levels of secreted TNF-α, IL-6 and monocyte chemoattractant protein-1, prevented microglia activation and was antiinflammatory and neuroprotective in LPS-treated murine hippocampal slices (Markus et al. 2010). Notably, it has been recently shown that, in a rat model of monoarthritis, spinal glia, as well as dorsal root ganglion primary afferent neurons, express α2-AR and the α2-AR agonist dexmedetomidine exerted analgesic effects involving the blockade of spinal glial activation (Xu et al. 2010).

2.2.7 Astrocytes

Astrocytes provide mechanical and functional support for neurons, there is however also evidence that they contribute to neuroinflammation upon severe challenges by releasing pro-inflammatory molecules (e.g. TNF-α, IL-1, IL-6) and possibly by contributing to antigen presentation under autoimmune response, although this latter function needs further investigation (Jana et al. 2008).

Human astrocytes express mainly β_2-AR, which play a key role in glycogen metabolism, regulation of immune responses, release of neurotrophic factors, as well as in the astrogliosis that occurs in response to neuronal injury. Accordingly, downregulation of the astrocytic β_2-AR-pathway might be involved into a number

of neurological conditions such as multiple sclerosis, Alzheimer's disease, human immunodeficiency virus encephalitis, stroke and hepatic encephalopathy (reviewed in Hertz et al. (2004) and Laureys et al. (2010)). It has been recently reported however that β-AR stimulation together with TNF-receptor triggering may also induce synergistic IL-6 expression in astrocytes, an effect which warrants further investigation also in view of the role of uncontrolled expression of IL-6 in the CNS in neurodegeneration and glioma development (Spooren et al. 2011).

Circumstantial evidence also exists regarding the occurrence of α_1-AR, e.g. in astrocytes from human optic nerves (Mantyh et al. 1995). The human U373 MG astrocytoma cell line, widely used as a model system for the investigation of astrocyte function, express a single class of α_1-AR, the α_{1B}-AR subtype, which are coupled to phosphoinositide hydrolysis and calcium mobilization, and which mediate a mitogenic response to α_1-AR-agonists (Arias-Montaño et al. 1999).

2.3 Endogenous Noradrenaline and Adrenaline in Immune Cells

Traditional criteria to assess the role of a substance as neurotransmitter in the nervous system usually include (see e.g. Purves et al. 2001):

- Presence of the substance within the cell (either synthesized by the cell or taken up from other cells that release it).
- Stimulus-dependent release.
- Mechanisms for removal (i.e. by degradation or reuptake).
- Action on target cells through specific receptors (effects mimicked by exogenous application of the substance in appropriate amounts).

As regards immune cells, evidence for the presence and the functional relevance of ARs has been reviewed in the previous section. Hereafter, data regarding endogenous noradrenaline and adrenaline, their synthesis, storage, release and removal will be discussed and experimental evidence about their possible functional role in immune cells will be summarized.

2.3.1 Presence and Synthesis

Noradrenaline and adrenaline (as well as dopamine and their major metabolites) have been identified in several types of immune cells, such as: murine lymphocytes (Josefsson et al. 1996), peritoneal macrophages (Spengler et al. 1994), bone marrow derived mast cells (Freeman et al. 2001), human peripheral blood mononuclear cells (Musso et al. 1996; Bergquist and Silberring 1998; Marino et al. 1999; Cosentino et al. 2002a), various lymphocyte subsets (Bergquist et al. 1994; Cosentino et al. 2000), including CD4+CD25+ regulatory T lymphocytes (Cosentino et al. 2007), granulocytes (Cosentino et al. 1999), and hematopoietic cell lines (Cosentino et al. 2000). Table 2.2 summarizes available information obtained in human immune cells.

Table 2.2 Endogenous noradrenaline and adrenaline in human immune cells

Cells	Treatments	Analytical assay	Noradrenaline[a]	Adrenaline[a]	Ref.
Peripheral blood mononuclear cells		HPLC-ED	0.206 ± 0.030[b]		Musso et al. (1996)
Peripheral blood mononuclear cells		HPLC-ED	0.129 ± 0.005[b]		Musso et al. (1997)
Peripheral blood mononuclear cells		Mass spectrometry	170.0817[c]		Bergquist and Silberring (1998)
Peripheral blood mononuclear cells		HPLC-ED	0.125 ± 0.015	0.856 ± 0.469	Marino et al. (1999)
Peripheral blood mononuclear cells	48 h-culture	HPLC-ED	1.153 ± 1.121	0.450 ± 0.539	Cosentino et al. (2002a)
Peripheral blood mononuclear cells	ACh 60-120 μM, 1 h	HPLC-ED	0.173 ± 0.003[b]		Musso et al. (1997)
Peripheral blood mononuclear cells	nicotine 250 μM, 1 h	HPLC-ED	0.216 ± 0.026[b]		Musso et al. (1997)
Peripheral blood mononuclear cells	tyrosine 50 μM, 1 h	HPLC-ED	0.176 ± 0.014[b]		Musso et al. (1997)
Peripheral blood mononuclear cells	PHA 10 μg/mL, 48 h	HPLC-ED	38.533 ± 9.629	35.717± 6.011	Cosentino et al. (2002a)
Freshly isolated lymphocytes		HPLC-ED	0.227 ± 0.039[b]		Musso et al. (1996)
Total lymphocytes		Flow cytometry		402 ± 70[d]	Pallinger and Csaba (2008)
CD3+ T lymphocytes		HPLC-ED	1.26 ± 0.69	0.45 ± 0.27	Cosentino et al. (2000)
CD3+ T lymphocytes		Flow cytometry		421 ± 78[d]	Pallinger and Csaba (2008)
CD3+CD4+ T lymphocytes		Flow cytometry		437 ± 84[d]	Pallinger and Csaba (2008)
CD3+CD8+ T lymphocytes		Flow cytometry		420 ± 106[d]	Pallinger and Csaba (2008)
CD4+CD25- effector T lymphocytes		HPLC-ED	1.32 ± 1.18	0.30 ± 0.24	Cosentino et al. (2007)
CD4+CD25+ regulatory T lymphocytes		HPLC-ED	25.61 ± 15.23	25.16 ± 15.86	Cosentino et al. (2007)
CD19+ B lymphocytes		HPLC-ED	1.18 ± 0.57	0.69 ± 0.31	Cosentino et al. (2000)
Granulocytes		HPLC-ED	84.61 ± 1.58	11.2 ± 2.0	Cosentino et al. (1999)
Granulocytes		HPLC-ED	0.21 ± 0.04	0.05 ± 0.01	Cosentino et al. (2000)

(continued)

Table 2.2 (continued)

Cells	Treatments	Analytical assay	Noradrenaline[a]	Adrenaline[a]	Ref.
Granulocytes		Flow cytometry		not detected	Pallinger and Csaba (2008)
CD14+ monocytes		HPLC-ED	0.82 ± 0.70	0.35 ± 0.25	Cosentino et al. (2000)
CD45+CD14+ monocytes		Flow cytometry		549 ± 80[d]	Pallinger and Csaba (2008)
Jurkat (T lymphoblastoid)		HPLC-ED	0.67 ± 0.48	0.03 ± 0.00	Cosentino et al. (2000)
NALM-6 (pre-B)		HPLC-ED	1.83 ± 0.72	0.87 ± 0.58	Cosentino et al. (2000)
U937 (promonocytic)		HPLC-ED	0.55 ± 0.13	0.07 ± 0.01	Cosentino et al. (2000)

[a] Means ± SD, expressed as 10^{-12} mol/10^6 cells, unless otherwise indicated.
[b] pmol/10^7 cells.
[c] *m/z* values in 4–10 × 10^9 PBMC/L.
[d] Detected with affinity isolated highly antigen-specific antibodies to epinephrine [Ab 35020, Lot 293141].

Intracellular levels of noradrenaline and adrenaline (as well as of dopamine, the direct precursor of noradrenaline) sharply increase in human peripheral blood mononuclear cells after 48–72 h stimulation with phytohemagglutinin (Cosentino et al. 2002a) as well as with anti-CD3/anti-CD28 monoclonal antibodies (Cosentino M., unpublished observations). Similar results were subsequently published in rodent lymphocytes stimulated with concanavalin A, and in the same study by use of immunohistochemistry it was shown that TH-positive cells in rodent lymphoid organs had highest density in lymph nodes and lowest density in thymus (Qiu et al. 2004). Mitogen-stimulated increase of intracellular catecholamines is in line with the reported upregulation of ARs occurring in lymphocytes following mitogen, glucocorticoid or proinflammatory cytokine treatment (see e.g. Zoukos et al. 1994; Rouppe van der Voort et al. 2000) and suggests a preferential involvement of intracellular catecholamines-operated pathways in activated immune cells. According to pharmacological evidence, endogenous synthesis of catecholamines occurs through protein kinase C (PKC) activation and the contribution of intracellular Ca^{++}-dependent mechanisms (Cosentino et al. 2002a).

The existence of a classical pathway (Fig. 2.1) for the synthesis of noradrenaline and adrenaline in immune cells is suggested by the expression of the enzyme tyrosine hydroxylase (TH, EC 1.14.16.2), the first and rate-limiting enzyme in the synthesis of catecholamines, which undergoes upregulation following cell stimulation, as well as by the ability of the TH inhibitor α-methyl-*p*-tyrosine, the RNA-polymerase inhibitor actinomycin D and the protein synthesis inhibitor cycloheximide to prevent intracellular enhancement of catecholamine levels (Cosentino

et al. 2002a, b; Reguzzoni et al. 2002). Only fragmentary evidence however exists regarding the expression and activity in immune cells of other key enzymes such as phenylethanolamine *N*-methyltransferase (Andreassi et al. 1998; Ziegler et al. 2002) or dopamine β-hydroxylase (Giubilei et al. 2004).

In human peripheral blood mononuclear cells stimulated in vitro with phytohemagglutinin, TH mRNA expression and catecholamine production occurs only in T and B lymphocytes and is reduced by dopamine (but not by noradrenaline or adrenaline) through dopaminergic D1-like receptor-dependent mechanisms which include inhibition of TH gene transcription (Ferrari et al. 2004). The proinflammatory cytokine interferon-γ exerts similar effects and its action is counteracted by interferon-β (Cosentino et al. 2005). TH expression and catecholamine production are on the contrary enhanced by agents which induce catecholamine release (see next section).

2.3.2 Storage and Release

Intracellular storage of catecholamines in human lymphocytes occurs in reserpine-sensitive compartments (Marino et al. 1999; Cosentino et al. 2000, 2007), which suggests the involvement of vesicular monoamine transporters (VMAT) similar to those expressed in neural and neuroendocrine cells (Henry et al. 1994), in agreement with preliminary evidence suggesting the occurrence of VMAT-1 and 2 in rat thymus and spleen (Mignini et al. 2009) and possibly also in human peripheral blood lymphocytes (Amenta et al. 2001)

In particular, in human activated lymphocytes release can be effectively induced by biological agents such as interferon-β (Cosentino et al. 2005) or by physiological stimuli such as elevation of extracellular K^+ concentrations ($[K^+]_e$) (Cosentino et al. 2003). High $[K^+]_e$ is characteristic of various pathological conditions and is per se a sufficient stimulus to activate integrin-mediated adhesion and migration of T cells (reviewed in Levite 2001). An excess $[K^+]_e$ may thus both assist the recruitment of lymphocytes to an injured tissue and lead to local increase of catecholamines, which in turn may act upon lymphocytes themselves and/or upon neighboring cells. It should be noted that, at least in vitro, catecholamine release from activated lymphocytes increases extracellular catecholamines from pico-nanomolar up to submicromolar concentrations (and possibly to even higher values in the vicinity of the cells), which may be well within the effective concentration range to exert immunomodulating effects.

Release of catecholamines is usually associated with induction of TH mRNA expression and increased catecholamine production (Cosentino et al. 2000, 2005), a response which closely resembles the increased activity of catecholamine-synthesizing pathways observed in neurons following depletion with reserpine, and which has been ascribed to increased TH activity (see e.g. Mallet 1996).

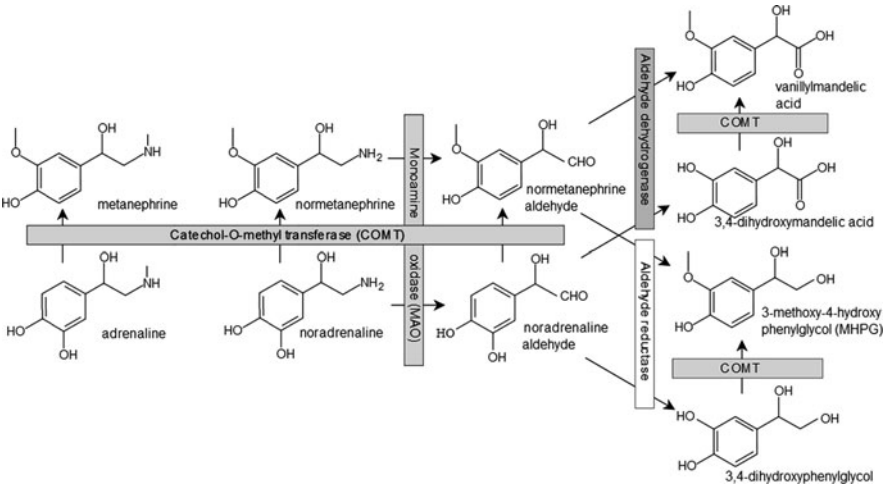

Fig. 2.2 Degradation of adrenaline and noradrenaline (modified from the Wikimedia Commons – http://commons.wikimedia.org)

2.3.3 Mechanisms for Removal

Signal termination of noradrenaline and adrenaline as neurotransmitters and hormones is the result of reuptake through specific membrane transporters and/or of degradation, mainly through monoamine oxidase (MAO)- and catechol-O-methyl transferase (COMT)-mediated pathways (Fig. 2.2).

In the synapse of noradrenergic neurons, termination of the action of noradrenaline is brought about by NET (NorEpinephrine Transporter) (see e.g. Mandela and Ordway 2006). In immune cells however the only indirect evidence for the presence of NET was provided nearly three decades ago, when Audus and Gordon (1982) described in murine lymphocytes a single population of desipramine-binding sites with an apparent dissociation constant (Kd) of about 0.4 nM. More convincing evidence however exists in immune cells for the expression and the functional relevance of DAT (DopAmine Transporter) (reviewed in Marazziti et al. 2010). Interestingly, the affinity of noradrenaline for NET and for DAT is about the same (see e.g. the PDSP K_i database – http://pdsp.med.unc.edu/pdsp.php). Evidence exists that incubation of human peripheral blood mononuclear cells with the NET inhibitor desipramine or with the DAT inhibitor GBR 12909 increased the extracellular levels of both dopamine and noradrenaline (Marino et al. 1999), an observation which is compatible with the occurrence of both transporters on the human lymphocyte membrane. No data however exist about the possible differential expression of catecholamine transporters on different immune cell populations, on the effects of cell activation and/or on their functional role, and it remains to be established whether the immunomodulating effects of monoamine uptake inhibitors (see e.g. Berkeley et al. 1994) can be attributed to a direct effect on NET and/or DAT.

Indirect evidence for the existence of classical enzymatic pathways for the degradation of noradrenaline and adrenaline in immune cells is provided by the identification of all the main metabolites of these catecholamines in phagocytes as well as in lymphocytes in both rodents and humans (Bergquist et al. 1994, Bergquist and Silberring 1998; Spengler et al. 1994; Musso et al. 1996; Marino et al. 1999; Cosentino et al. 1999, 2000, 2002a, 2007; Freeman et al. 2001) (Fig. 2.2). Occurrence in human immune cells of both MAO and COMT is also supported by functional assays and pharmacological experiments. Investigations on COMT activity were so far very limited (Bidart et al. 1981) and its eventual connections with modulation of immune response was never examined. On the contrary MAO expression and activity in immune cells has received significantly more attention, not only as a marker of neurodegenerative and neuropsychiatric disease (Tsavaris et al. 1995; Jiang et al. 2006). Various groups indeed provided experimental evidence indicating that MAO activity occurs in both human granulocytes and lymphocytes and it is predominantly of the B type (Pintar and Breakefield 1982; Thorpe et al. 1987; Balsa et al. 1989). Support to its functional relevance has been provided mainly by use of pargyline, which leads to increased catecholamine levels in concanavalin A-stimulated rodent lymphocytes (Qiu et al. 2005) as well as in human peripheral blood mononuclear cells (Marino et al. 1999) and granulocytes (Cosentino et al. 1999).

Recently, evidence has been provided that MAO type A is expressed in human monocytes in particular after incubation with interleukin-4, and it has been suggested that upregulation of MAO-A expression may contribute in switching naive monocytes into a resolving phenotype (Chaitidis et al. 2004, 2005).

2.3.4 Functional Relevance

Possible strategies to study the role and the functional relevance of endogenous noradrenaline and adrenaline production in immune cells include:

- Effect of AR antagonists
- Interference with synthesis/degradation
- Interference with intracellular storage/release/uptake

Modulation of synthesis and release could be obtained through both pharmacological and non-pharmacological approaches (e.g. suppression of expression of key proteins – TH, VMAT, etc. – by means of gene silencing techniques). Available evidence however has been provided so far mainly through pharmacological experiments (Table 2.3). The first evidence that endogenous noradrenaline and adrenaline may subserve autocrine/paracrine regulatory loops in immune cells was obtained by Spengler et al. (1994), who showed that in mouse peritoneal macrophages stimulated with LPS the β-AR selective antagonist propranolol increased and the α_2-AR selective antagonist idazoxan decreased tumor necrosis factor-α production, which – together with the presence of intracellular noradrenaline in these cells – was taken as an evidence of the existence of an adrenergic

Table 2.3 Functional role of endogenous noradrenaline and adrenaline in immune cells

Experimental model	Treatments	Experimental approach	Functional role	Notes	Reference
In vitro, mouse peritoneal macrophages	LPS	α-AR antagonism with idazoxan, β-AR antagonism with propranolol	β-AR-mediated decrease and α_2-AR-mediated increase of tumor necrosis factor-α production		Spengler et al. (1994)
In vitro, mouse peritoneal macrophages	LPS	α-AR antagonism with idazoxan, β-AR antagonism with propranolol	β-AR-mediated decrease and α_2-AR-mediated increase of tumor necrosis factor-α production	Effects increased in animals with streptococcal-cell-wall-induced arthritis	Chou et al. (1998)
In vitro, murine phagocytes	LPS		LPS induced release of catecholamines and induction of catecholamine-generating and degrading enzymes		Flierl et al. (2007)
In vivo, rats with acute lung injury		α_2-AR agonists/ antagonists, inhibitors of catecholamine synthesizing/ degrading enzymes	α_2-AR-mediated increase of lung inflammation, suppressed by α_2-AR antagonists or inhibitors of catecholamine synthesizing enzymes		Flierl et al. (2007)
In vitro, mouse peritoneal macrophages		adrenaline, noradrenaline	NFκB activation, enhanced release of tumor necrosis factor-α, IL-1β, IL-6 and MIP-2		Flierl et al. (2009)
In vivo, rats with immune complex-induced acute lung injury		α_2-AR antagonism with RX821002	α_2-AR-mediated increase of the severity of acute lung injury	Effect increased in adrenalectomized rats	Flierl et al. (2009)
In vitro, rodent lymphocytes	Con A	TH inhibition with α-methyl-*p*-tyrosine, MAO inhibition with pargyline, α-AR blockade with phentolamine,	β-AR-mediated inhibition of IL-2 production		Qiu et al. (2004, 2005)

(continued)

Table 2.3 (continued)

Experimental model	Treatments	Experimental approach	Functional role	Notes	Reference
		β-AR blockade with propranolol			
In vitro, rodent lymphocytes	Con A	TH inhibition with α-methyl-*p*-tyrosine, MAO inhibition with pargyline, α_1/α_2- and β_1/β_2-AR blockade	α_1- and β_2-AR-mediated increase of apoptosis	Involvement of cAMP-PKA- and PLC-PKC-linked CREB-Smac/DIABLO pathways	Jiang et al. (2007, 2009)
In vitro, human peripheral blood mononuclear cells	PHA	TH inhibition with α-methyl-*p*-tyrosine	Catecholamine-mediated increase of apoptosis		Cosentino et al. (2002b)
In vivo, human lymphocytes			Intracellular levels of adrenaline and noradrenaline correlate with basal and isoprenaline-stimulated cAMP		Knudsen et al. (1996)

autocrine loop, which is even more pronounced in macrophages obtained from rats with streptococcal-cell-wall-induced arthritis (Chou et al. 1998).

Involvement of endogenous catecholamines and α_2-ARs in the regulation of innate immunity was further demonstrated showing that exposure of rodent phagocytes to lipopolysaccharide catecholamine release together with induction of catecholamine-generating and degrading enzymes, and blockade of α_2-ARs or pharmacological inhibition of catecholamine synthesis suppressed (while α_2-AR agonism or inhibition of catecholamine-degrading enzymes enhanced) lung inflammation in two rodent models of acute lung injury (Flierl et al. 2007). These results were confirmed in adrenalectomized rodents, which show greatly enhanced catecholamine release from phagocytes as well as enhanced expression of TH and DBH in these cells (Flierl et al. 2009). It is thus suggested that noradrenaline and adrenaline directly activate NFκB at least in rodent phagocytes, causing enhanced release of proinflammatory cytokines such as tumor necrosis factor-α, IL-1β and IL-6, resulting in amplification of the acute inflammatory response via α_2-ARs (Flierl et al. 2009).

Experimental evidence for the functional relevance of endogenous noradrenaline and adrenaline also exists in rodent lymphocytes. Qiu et al. (2004) reported that stimulation with concanavalin A markedly increased both TH expression and

catecholamine content in lymphocytes and that inhibition of TH with α-methyl-*p*-tyrosine significantly facilitated concanavalin A-induced IL-2 production, suggesting that endogenous catecholamines may exert a tonic inhibition on the production of this cytokine. In further studies (Qiu et al. 2005), the same group showed that rodent lymphocyte proliferation is increased by α-methyl-*p*-tyrosine but decreased by the monoamine oxydase inhibitor pargyline, which at the same time increased intracellular cAMP, the second messenger acted upon by β-ARs. By use of AR antagonists, evidence indeed was provided that the effects of pargyline required activation of β-ARs, possibly through increased levels of noradrenaline and/or adrenaline.

In human immune cells, the first evidence for a functional role of endogenous noradrenaline and adrenaline was provided by Knudsen et al. (1996), who showed that intracellular levels of both monoamines in circulating lymphocytes from healthy subjects were strongly correlated with both basal and isoprenaline-stimulated cAMP in these cells. In a subgroup of subjects, variability in endogenous lymphocyte concentration of adrenaline also correlated with concomitant changes in number of NK (CD3-CD56+) cells and cAMP. More direct evidence came a few years later from studies in human peripheral blood mononuclear cells, where stimulation with phytohemagglutinin induces the synthesis of catecholamines through induction of TH and its pharmacological inhibition with α-methyl-*p*-tyrosine results in decreased activation-induced apoptosis (Cosentino et al. 2002b). Similar findings were subsequently obtained in rodent lymphocytes activated with concanavalin A, where the proportion of apoptotic cells as well as the expression of apoptosis-related genes and proteins, Bax, Fas, Fas-Ligand and caspase-3 were decreased by α-methyl-*p*-tyrosine but increased by the monoamine oxydase inhibitor pargyline, which on the contrary decreased the expression of the antiapoptotic protein Bcl-2 (Jiang et al. 2007). In separate experiments, by use of a pharmacological approach, it was shown that the effects of pargyline-increased endogenous catecholamine levels on rodent lymphocyte apoptosis were mediated by cAMP-PKA- and PLC-PKC-linked CREB-Smac/DIABLO pathways coupled to α_1- and β_2-ARs (Jiang et al. 2009).

In summary, at least circumstantial evidence exists for each of the criteria needed to establish the role of noradrenaline and adrenaline as transmitters in immune cells. Nonetheless, systematic application of such criteria to specific immune cell types and functional conditions is still lacking, and for these reasons noradrenaline and adrenaline should be still referred to as "putative" transmitters in immune cells.

2.4 Noradrenaline, Adrenaline and Immune-Mediated Disease

A huge amount of evidence exists regarding the involvement of sympathoadrenergic neuroimmune mechanisms in several disease conditions. Hereafter, specific attention will be dedicated to *multiple sclerosis*, *rheumatoid arthritis* and

cancer with emphasis on human data. Other diseases in which catecholamine modulation of the immune response has been suggested as pathogenetic and/or therapeutic mechanism will be also summarized.

2.4.1 Multiple Sclerosis

Multiple sclerosis (MS) is an organ-specific autoimmune disorder characterized by inflammation, demyelination and axonal loss in the CNS (Noseworthy et al. 2000; Frohman et al. 2006). Classical observations in rats with experimental allergic encephalomyelitis (EAE) support the relevance of sympathoadrenergic mechanisms in MS pathogenesis (Bellinger et al. 1992; Chelmicka-Schorr and Arnason 1999). In particular, EAE is worsened by chemical sympathectomy (Chelmicka-Schorr et al. 1988) and suppressed by β-adrenergic agonists (Wiegmann et al. 1995).

Several lines of evidence indicate that adrenergic pathways contribute to MS in immune system cells as well as in glial cells. Indeed, membrane expression of β_2-ARs in PBMC is upregulated in MS (Arnason et al. 1988; Karaszewski et al. 1990, 1993; Zoukos et al. 1992) possibly in relation to disease activity (Zoukos et al. 1994). Increased expression of β_2-ARs seems specific for CD8+CD28- T lymphocytes (Karaszewski et al. 1990, 1993). In circulating PBMC, gene expression of β_2-ARs (and of other G protein-coupled receptors like dopaminergic receptors D5) and responsiveness to the β-AR agonist isoprenaline are on the contrary downregulated in untreated patients (suggesting the occurrence of receptor uncoupling) but restored in IFN-β-treated subjects (Giorelli et al. 2004). Intracellular levels of catecholamines are also affected in lymphocytes of MS patients. Peripheral blood lymphocyte levels of adrenaline have been reported to be significantly higher in the first-attack of MS whilst noradrenaline levels were significantly lower during remissions (Rajda et al. 2002). In stimulated lymphocytes from MS patients, no difference was observed in noradrenaline or adrenaline levels in comparison to cells from healthy controls, however cells from patients with chronic-progressive MS or relapsing-remitting MS in relapse produced less dopamine (Cosentino et al. 2002a). In view of the role of stimulation-induced increase of endogenous catecholamines in activation-induced apoptosis of lymphocytes (Cosentino et al. 2002a), this finding was tentatively linked to the occurrence of impaired apoptotic mechanisms, which in MS can contribute to survival of autoreactive cells (Pender 1998; Macchi et al. 1999; Comi et al. 2000; Sharief et al. 2002).

Additional evidence for the relevance of adrenergic (and dopaminergic) lymphocyte-related mechanisms come from the observation that in vitro interferons (IFNs) profoundly affect endogenous catecholamines in human lymphocytes: while IFN-β increases their production following stimulation with PHA and induces their extracellular release, IFN-γ profoundly decreases catecholamines production as well as the mRNA expression of the catecholamines-synthesizing enzyme tyrosine hydroxylase, and coincubation with both IFNs prevents the inhibitory effect of IFN-γ,

as well as the enhancing/releasing effect of IFN-β (Cosentino et al. 2005). The relevance of these findings for MS is confirmed by the observation that in lymphocytes from patients treated with IFN-β for 12 months the production of catecholamines hugely increased and was less sensitive to IFN-γ-induced inhibition. Expression of mRNA for TH and β_2-AR (and also dopaminergic receptors D5) was already enhanced after 1 month and further increased up to 6–12 months of treatment (Zaffaroni et al. 2008). Thus, in MS patients IFN-β treatment enhances the ability of lymphocytes to produce CA, and restores the efficiency of β_2-AR- (and dopaminergic receptor-) operated pathways, which could result in reduced T cell proliferation and IFN-γ secretion (Giorelli et al. 2004). Indeed, β_2-ARs have been already regarded as a promising target in the pharmacotherapy of MS and in particular salbutamol has been proposed as add-on therapy in patients with MS (Makhlouf et al. 2002). In-depth understanding of the complex (dys)regulation of β_2-AR pathways in lymphocytes of MS patients also in relation to treatment with immunomodulating agents could allow better exploitation of the potential benefits of drugs acting on β_2-ARs.

As regards glial cells, consistent evidence indicates that astrocytes of MS patients are deficient in β_2-AR, both in normal-appearing white matter as well as in chronic active and inactive plaques (De Keyser et al. 1999; Zeinstra et al. 2000). These observations led to the speculation that in MS astrocytes may serve as primary (facultative) antigen-presenting cells due a failure to on one side of β_2-AR-mediated suppression of class II major histocompatibility complex molecules (De Keyser et al. 2003). Astrocyte β_2-AR dysregulation however may contribute to MS pathogenesis and progression through several other mechanisms, including on one side deficient suppression of nitric oxide and proinflammatory cytokine production and glutamate uptake, and on the other side deficient glycogenolysis and production of trophic factors (De Keyser et al. 2004), as well as also contributing to reduced perfusion of normal-appearing white matter (De Keyser et al. 2008).

Astrocytes as therapeutic targets in MS were recently challenged in a proof of concept study in MS subjects by use of fluoxetine, which activates protein kinase (PK) A in astrocytes. PKA is physiologically activated by β_2-AR-mediated cAMP increase and in turn suppresses coactivator class II transactivator (CIITA), which regulates major histocompatibility (MHC) class II molecule transcription (De Keyser et al. 2010). Direct activation of PKA could in principle bypass the functional deficiency of astrocytes, however preliminary results need to be confirmed and extended in larger, randomized studies.

2.4.2 Rheumatoid Arthritis

Extensive experimental and clinical evidence support the occurrence of dysregulated immune system and response to the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system in rheumatoid arthritis (RA), and targeting the neuroimmune network is increasingly regarded as an attractive therapeutic strategy (reviewed in: Baerwald et al. (2000), Wahle et al. (2002a), Lorton et al. (2003), Straub et al. (2005)).

Several studies over the last two decades documented modification of AR signaling in immune cells of patients with RA, such as decreased density and affinity of β_2-ARs on PBMC and in particular on CD8+ lymphocytes, showing negative correlation with disease activity (Baerwald et al. 1992b, 1997) and serum IL-2R levels (Krause et al. 1992). Reduction of β_2-ARs may be even more pronounced in synovial fluid lymphocytes, with impairment of the suppressive effect of catecholamines on anti-CD3-induced lymphocyte proliferation (Baerwald et al. 1997, 1999). In RA patients with high disease activity, a shift to α_1-AR-mediated catecholamine effects upon PBMC reactivity could also be observed (Wahle et al. 1999). Indeed, noradrenaline and adrenaline fail to shift anti-CD3/anti-CD28-induced T-cell cytokine responses toward a Th2 profile and in particular CD8+ T cells are responsible for the impaired adrenergic control of IFN-γ production (Wahle et al. 2006).

In patients with RA the density of β_2-ARs is decreased also on peripheral CD19+ B lymphocytes (a finding which occurs also in patients with systemic lupus erythematosus and with systemic sclerosis), and negatively correlates with systemic disease activity. Furthermore, although basal intracellular cAMP levels in these cells are raised, the increase of cAMP upon stimulation of β_2-ARs is low (Wahle et al. 2001). Cell death induced by exposure to β_2-AR agonists is also diminished in RA CD19+ B lymphocytes exhibiting decreased β_2-AR density (Wahle et al. 2002b).

It should be mentioned however that at least one study found no changes in β_2-ARs on RA lymphocytes, while showing a significant decrease in G-protein-coupled receptor kinase (GRK) activity, with reduced protein expression of GRK-2 and GRK-6, possibly as a result of increased cell exposure to proinflammatory cytokines. RA lymphocytes showed a significantly increased cAMP production and inhibition of TNF-α production after β_2-AR stimulation (Lombardi et al. 1999).

Evidence for dysregulated sympathoadrenergic modulation of the immune response is available also in juvenile RA (JRA), a subset of arthritis occurring in children, which may be transient or chronic. Indeed, patients with JRA have an altered function of the autonomic nervous system associated with increased central noradrenergic outflow, which is associated with changes in the response of leukocytes via β_2-ARs: leukocytes of patients with active JRA have a lower cAMP response to a β_2-AR agonist, presumably due to increased cAMP-phosphodiesterase activity in these cells (Kuis et al. 1996). In particular, exposure of JRA patients (but not healthy controls) to a noradrenergic stressor results in enhanced LPS-induced IL-6 production by peripheral blood cells. In addition, PBMC of patients with JRA express mRNA encoding α_1-ARs, predominantly of the α_{1D}-AR subtype, which on the contrary are undetectable in cells from healthy subjects (Rouppe van der Voort et al. 2000a). In subjects with polyarticular JRA (but not in healthy controls or subjects with the oligoarticular form of the disease), α_1-ARs expressed on circulating lymphocytes mediate noradrenaline-induced production of the proinflammatory cytokine IL-6 (Heijnen et al. 1996).

Factors contributing to dysregulated sympathoadrenergic tuning of the immune response may include β_2-AR gene variants. Responsivity of β_2-ARs are affected by

polymorphisms at positions 16 and 27, which determine the propensity for agonist-induced downregulation and associated subsensitivity (Green et al. 1993, 1995). According to genetic studies performed in northern Sweden (Xu et al. 2005) and in Germany (Malysheva et al. 2008), carriage of Arg16 and of Gln27 was associated with RA, carriage of Gln27 was associated with activity of the disease and in combination with non-carriage of Arg16 with higher levels of rheumatoid factor, and homozygosity for Arg16 exhibited the greatest risk for RA in combination with HLA-DRB1*04. Association of Arg16 and of Gln27 was not found in a population of children with JRA (Pont-Kingdon et al. 2009). According to current models of β_2-AR kinetics, homozygous Arg16 would be relatively resistant to downregulation by endogenous catecholamines, while homozygous Gln27 would be relatively sensitive to downregulation (Liggett 2000).

In RA the sympathoadrenergic tuning of the immune response is extensively dysregulated also at the local level, in synovial tissue, where sympathetic innervation is reduced while sensory innervation is increased, and the differential patterns of innervation are dependent on the severity of the inflammation (Miller et al. 2000). Local noradrenaline production is maintained by TH+ cells, mainly synovial macrophages, and its levels correlate with the degree of inflammation and with spontaneous IL-8 secretion, while density of TH+ cells correlates positively with spontaneous secretion of IL-6, IL-8, and MMP-3 (Miller et al. 2002). In RA patients treated with corticosteroids, synovial tissue shows decreased spontaneous cytokine secretion, less T cells, CD163+ macrophages and TH+ cells, reduced inflammation and reduced noradrenaline secretion (Miller et al. 2002). In vitro, in human synoviocytes noradrenaline inhibits IL-8 and TNF production (Miller et al. 2002), as well as the production of the proinflammatory bactericidal alpha-defensins human neutrophil peptides 1–3 (HNP1-3) (Riepl et al. 2010), suggesting that the loss of sympathetic nerve fibers with low resting noradrenaline levels is crucial for the development of the inflammatory process, possibly through a shift from β-to-α adrenergic signaling in the progressing course of the inflammatory disease (β-to-α adrenergic shift) (reviewed by Straub and Härle 2005). Noradrenaline secreted by TH+ cells occurring in synovial tissue during RA would thus represents an antiinflammatory mechanism to counteract local inflammation. Indeed, in RA patients clear evidence has been provided that systemic secretion of cortisol together with local production of noradrenaline are required to lower synovial inflammation (Straub et al. 2002a), while on the contrary systemic infusion of adrenaline (e.g. during a typical stress reaction) may result in lowered endogenous cortisol production and consequently increased inflammation (Straub et al. 2002b). Further evidence for a critical role of local production of catecholamines by TH+ cells in RA synovium has been recently provided by showing that increased catecholamine release induced after blockade of vesicular monoamine transporter 2 (VMAT2) results in strong reduction of TNF (occurring through cAMP increase but possibly without involvement of classical β-ARs) and amelioration of inflammation in an animal model of RA (Capellino et al. 2010). Local catecholamine-producing cells may thus represent a novel target for the pharmacotherapy of RA,

possibly in the context of neuroimmunopharmacological strategies aimed at restoring the global autonomic balance (Koopman et al. 2011).

Finally, it has been reported that, at least in vitro, α_2-AR stimulation of type A (macrophage-like) and B (fibroblast-like) synoviocytes produced an increase and a decrease in the respective cell number, probably through Gi-coupled PLC activation and the resulting stimulation of the PKC betaII/MAP kinase (Mishima et al. 2001), providing preliminary evidence for a role of α_2-ARs in RA.

2.4.3 Cancer

Evidence showing a direct connection between sympathoadrenergic function and tumor development has been obtained mainly in animal models, where activation of the sympathoadrenergic system through either stressful events or direct stimulation of β-ARs usually leads to compromised resistance to tumor development and metastasis (Stefanski and Ben-Eliyahu 1996; Shakhar and Ben-Eliyahu 1998), although it was shown that also chemical denervation may lead to tumor growth, thus suggesting a complex role of the sympathetic nervous system in the regulation of antitumor immunity (Brenner et al. 1992). Enhancement of tumor progression is usually ascribed to β-AR-mediated decrease of NK activity (Shakhar and Ben-Eliyahu 1998; Ben-Eliyahu et al. 2000; Ben-Eliyahu et al. 2000a), although noradrenaline has also been shown to inhibit the generation of specific antitumor cytotoxic T lymphocytes (Kalinichenko et al. 1999). Interestingly, impairment of NK activity and reduced antitumor resistance following stress and β-AR stimulation seem to be affected by age (Page and Ben-Eliyahu 2000) as well as by gender (Page et al. 2008). Recently, the prophylactic use of type-C CpG oligodeoxynucleotides (CpG-C ODN) was shown to improve NK activity and immunocompetence, potentially reducing metastatic dissemination after enhanced sympathetic stress responses (Goldfarb et al. 2009) and in association with pharmacological blockade of β-ARs and COX inhibition it was proposed as a potential approach to limit postoperative immunosuppression and metastatic progression (Goldfarb et al. 2011).

AR modulation of immune response may be relevant also to cancer vaccine strategies. Indeed, Botta and Maestroni (2008) found that β_2-AR antagonism along with TLR2 activation at the site of intradermal cancer vaccination may either enhance the resulting antitumor response or be tolerogenic in dependence of the maturation state of the transferred DCs, suggesting that manipulation of β_2-ARs expressed in the site of DCs inoculation may influence the efficacy of the antitumor response.

Activation of β-ARs may also result in direct stimulation of tumor proliferation, e.g. in human colon adenocarcinoma where adrenaline stimulates cell growth via both β_1- and β_2-AR- and COX-2-dependent pathways (Wong et al. 2011), while antagonism at β-ARs may have direct antitumor effects, e.g. in pancreatic cancer cells where β_2-AR blockade synergizes with gemcitabine to induce apoptosis (Shan et al. 2011).

Epidemiological studies support the hypothesis that exposure to β_2-AR antagonists (beta blockers) may indeed reduce cancer progression and mortality, e.g. in melanoma (De Giorgi et al. 2011) and in breast cancer (Powe et al. 2010; Barron et al. 2011), although conflicting results have also been reported (Shah et al. 2011). Larger epidemiological studies as well as well-designed randomized clinical trials are needed for several cancer types to establish the potential of AR manipulation as antitumor therapy.

A role for ARs has been proposed long time ago also in the proliferation of hematological malignancies. Very low β-AR density and loss of adenylate cyclase activity is a well characterized feature of pathologic cells from chronic and acute lymphocytic leukemia (Sheppard et al. 1977; Paietta and Schwarzmeier 1983), although at least one study in intact B lymphocytes from patients with chronic lymphocytic leukemia found normal β-AR binding and a single overexpressed population of α_2-ARs (Goin et al. 1991). Loss of β-AR function and class I major histocompatibility complex antigen surface expression was also reported in the murine S49 lymphoma cell line in association with a higher rate of proliferation (Cremaschi et al. 1994), and β-AR activation induces apoptosis in these cells via G (s)α and PKA, possibly providing a means to control proliferation of immature T cells (Yan et al. 2000). A decreased number of β-ARs involved in cell proliferation was described also on the T cell lymphoma BW5147 (Cremaschi et al. 2000). Impaired AR expression in circulating cells from patients with chronic lymphocytic leukemia was shown to be specific for β_2-ARs and to be associated with disease progression (Kamp et al. 1997). Whether activation of β-ARs may represent a therapeutic strategy in leukemias remains however to be established. Indeed, although accumulation of cAMP has been shown to increase the chemosensitivity of chronic lymphocytic leukemia (CLL) cells, the proapoptotic effect of the long acting β_2-AR agonists salmeterol and formoterol in these cells have been shown to be independent from β_2-AR activation (Mamani-Matsuda et al. 2004). On the other side, endogenous adrenaline together with prostaglandins has been recently shown to mediate the promoting effects of stress on leukemia progression at least in animal models through suppression of NK activity, thus providing the rationale to explore the therapeutic potential of β-AR blockers and COHX inhibitors even in patients with hematological malignancies (Inbar et al. 2011).

It should also be mentioned that noradrenaline and adrenaline may act through specific ARs to increase the synthesis of proangiogenic factors, thus promoting tumor growth (while dopamine through dopaminergic receptors may have opposite effects by suppressing the actions of vascular permeability factor/vascular endothelial growth factor-A). These issues have been recently reviewed by Chakroborty et al. (2009).

2.4.4 Other Diseases

The density of β_2-ARs on circulating lymphocytes is decreased in several other chronic inflammatory diseases, including *Crohn's disease* (Krause et al. 1992) and

systemic lupus erythematosus (Baerwald et al. 1992a; Wahle et al. 2001). Evidence regarding the role of the sympathetic nervous system in systemic lupus erythematosus has been recently reviewed (del Rey and Besedovsky 2008).

Patients with *myasthenia gravis* (MG) have decreased β_2-AR density on peripheral blood mononuclear cells (Xu et al. 1997) and circulating antibodies and T cells that react with β_1- and β_2-ARs (Yi et al. 1996), which might be implicated in the few patients with myasthenia gravis who have heart disease (Xu et al. 1998).

In *Alzheimer's disease* (AD), β-ARs on circulating lymphocytes were usually studied as peripheral markers of central disruption of adrenergic transmission, as described in AD post-mortem brains. Although initial studies provided no clear evidence of disrupted β-AR responsiveness in lymphocytes of AD subjects (Oppenheim et al. 1984; Gietzen et al. 1989), later it was shown that isoprenaline-induced cAMP increase may be reduced (Garlind et al. 1997) GRK2 expression may be increased (Leosco et al. 2007). Interestingly, using a cDNA microarray representing 3,200 distinct human genes it was shown that in AD lymphocytes the α_{2C}-AR gene is among 20 candidate genes whose expression is altered, although its eventual physiopathological and clinical meaning remains a matter of speculation (Kálmán et al. 2005). Recently, in murine microglial cells it was shown that both noradrenaline and isoprenaline promote amyloid β peptide uptake and degradation through activation of β_2-ARs, thus providing a potential link between central noradrenergic neurotransmission and neuroinflammatory mechanisms in AD (Kong et al. 2010).

Although extensive evidence exists regarding the role of neuroimmune mechanisms in *allergy and asthma* (Marshall 2004), sympathoadrenergic modulation of immunity in this field received attention mainly regarding the ability of β_2-AR agonists to exert anti-inflammatory effects (Hanania and Moore 2004). Nonetheless, peripheral blood lymphocytes of patients with asthma have reduced β-AR binding capacity (Hataoka et al. 1993), and in long-term smokers with mild to moderate chronic obstructive pulmonary disease (COPD) smoking cessation is associated with a significant increase in T lymphocyte β_2-AR density (Leader et al. 1994), which is probably not only a good marker of change in pulmonary response to β_2-AR agonists but, first of all, an indicator of reduced chronic low-grade systemic inflammation.

Lymphocyte β-ARs received considerable attention in *cardiac disease*, however usually only as a as a surrogate tissue for myocardium to assess β-AR function, despite the apparent absence of any direct relationship between lymphocyte and myocardial β-AR density (Dzimiri and Moorji 1996). For instance, it was shown that plasma noradrenaline and adrenaline were increased and lymphocyte β-AR density was reduced in patients with congestive ischemic disease and coronary artery bypass grafting was associated with clinical and hemodynamic improvement as well as with improvement of lymphocyte β-AR density and function (Chello et al. 1995). In another study in patients with chronic severe heart failure it was reported that decreased lymphocyte β-ARs were increased after treatment with angiotensin converting enzyme inhibitors, which also induced significant improvement in cardiac function (Townend et al. 1993). Density of lymphocyte β-ARs was shown to be reduced also in infants and children with heart failure secondary to

congenital heart disease (Wu et al. 1996) and in patients with rheumatic heart valvular disease (Dzimiri et al. 1995). Only in the last decade however increased sympathetic activity and altered immune function occurring in chronic heart failure began to be more carefully considered, for instance showing that in subjects with chronic heart failure reduced β-AR on lymphocytes results in impaired β-adrenergic control of lymphocyte activation, which in turn contributes to the chronic low-intensity inflammation occurring in heart disease (Werner et al. 2001).

Noradrenaline and adrenaline exert extensive effects on innate immunity, as discussed in previous sections. Monocytes/macrophages as well as granulocytes are affected by catecholamines and can themselves produce and utilize these transmitters (reviewed by Flierl et al. 2008), which may have significant relevance for *bacterial infections and sepsis*. The therapeutic effects of α_2-AR antagonism or pharmacological inhibition of catecholamine synthesis in rodent models of acute lung injury has been discussed previously (Flierl et al. 2007, 2009). Recently, it has been shown that the β-AR antagonist propranolol may control the susceptibility of severely burned patients to opportunistic pathogens by reducing the occurrence of immunosuppressive M2 monocytes (Kobayashi et al. 2011).

Evidence exists that highly stressful events may promote *viral infections* (e.g. herpes simplex virus type-1 and varicella zoster virus) through activation of the sympathetic nervous system. For instance, catecholamines directly stimulate the human cytomegalovirus immediate-early (IE) enhancer/promoter in monocytic cells via beta-2 adrenergic receptors, possibly leading to the development of an active human cytomegalovirus infection in latently infected patients (Prösch et al. 2000). Noradrenaline has also been shown to accelerate human immunodeficiency virus (HIV) replication in quiescently infected PBMC via β-AR and PKA activation (Cole et al. 1998). In the central nervous system, HIV coat protein gp120 may interfere with the β-AR-mediated regulation of astrocytes and microglia and may alter astroglial "reactivity" thus promoting neuroinflammation and impairing defense against viral and opportunistic infections (Levi et al. 1993).

Decreased β-ARs have been consistently reported in subjects with *depression* (reviewed by Werstiuk et al. 1990), and electroconvulsive therapy has been reported to increase lymphocyte β-AR responsivity (Mann et al. 1990). Decreased lymphocyte β-ARs may occur also in *panic disorder* (Brown et al. 1988), and even in normal subjects with *increased tension and anxiety* traits (Yu et al. 1999). These findings however need to be evaluated in the context of dysregulated immunity occurring in depression (reviewed by Blume et al. 2011).

2.5 Concluding Remarks

Sympathoadrenergic mechanisms represent the main channel of communication between the nervous system and the immune system, and the origins of neuroimmunology itself can be traced back to the understanding of the role of noradrenaline and adrenaline in the modulation of the immune response. It may therefore sound paradoxical that so much remains to elucidate before AR-mediated

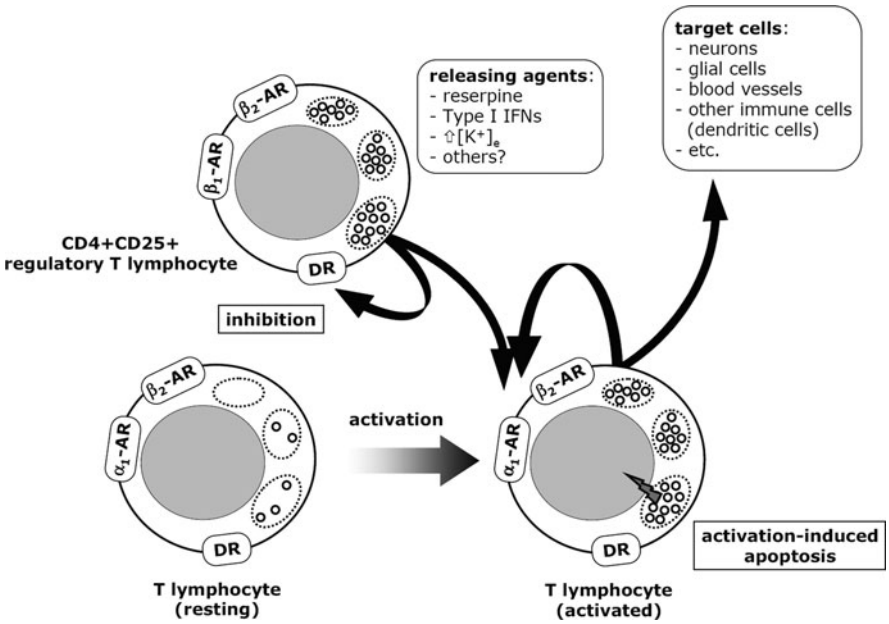

Fig. 2.3 Speculative scheme depicting the cellular network sustained by endogenous catecholamines in human lymphocytes. CD4+CD25+ regulatory T lymphocytes constitutively express TH, the key enzyme in the synthesis of catecholamines, dopaminergic receptors, and α- and β-ARs, and contain high amounts of catecholamines stored in reserpine-sensitive compartments. Upon release, endogenous catecholamines may subserve autocrine/paracrine modulatory loops, leading to e.g. impaired suppressive activity of CD4+CD25+ regulatory T lymphocytes toward mitogen-induced T lymphocyte proliferation (Cosentino et al. 2007). In addition to the chromaffin granule depletant reserpine, candidate agents that may induce the release of catecholamines from lymphocytes include type I IFNs (Cosentino et al. 2005), tetrabenazine, as well as even high $[K^+]_e$ (Cosentino et al. 2003). The picture also shows that, in the absence of stimulation, effector T lymphocytes express dopaminergic receptors and α- and β-ARs and contain trace amounts of catecholamines. Upon stimulation, intracellular catecholamines increase by several 10-fold, and expression and function of both dopaminergic receptors and ARs may also undergo significant changes. Under these conditions, endogenous catecholamines either may directly affect cell survival and apoptosis from within the cell (lightning bolt) (Cosentino et al. 2002a), or they can be released (*arrows*) to act upon lymphocytes themselves and/or upon neighboring cells. The picture does not include the potential role of catecholamines that are normally present in the extracellular space or that can be released from sympathoadrenergic terminals innervating lymphoid organs and tissues, nor does it include catecholamines, which lymphocytes can encounter when they enter the brain in physiological (or pathological) situations

pathways can be fully exploited as pharmacotherapeutic targets. Indeed, according to experimental and clinical evidence, several key points awaiting clarification are critical for the therapeutic efficacy (or failure) of agents acting on catecholaminergic mechanisms:

- Adrenergic and dopaminergic receptors exist in multiple subtypes which are expressed on immune cells with specific patterns in each cell subset, where each of them is involved in the control of well defined functions;

- Receptor dysregulations occurring in disease states is not only specific for the receptor type but even for the cell subset(s).
- Receptors may be acted upon not only by exogenous but also by endogenous catecholamines directly produced by immune cells (Fig. 2.3).
- Dynamic changes occur to receptor expression and responsiveness (and to endogenous catecholamine production) during treatment with immunomodulatory drugs (e.g. the case of IFN-β in MS).

Exploitation of AR-operated mechanisms in human immune cells as targets for therapeutic interventions can be approached by several pharmacological strategies, not limited to direct ligation of membrane receptors by agonists/antagonists. The catecholamine systems can be indeed modulated at several levels:

- Intracellular uptake and storage (e.g. using selective inhibitors of NET or VMAT).
- Receptor activation/blockade by selective agonists (e.g. β-AR agonists used in asthma) or antagonists (α-AR antagonists, e.g. antihypertensives; β-AR antagonists, e.g. beta-blockers).
- Inhibition of catecholamine synthesis (e.g. by using the anti-hypertensive agent alpha-methyl-p-tyrosine, a TH selective inhibitor).
- Enhancement of catecholamine synthesis (e.g. by using the anti-Parkinson drug L-DOPA).
- Inhibition of catecholamine metabolism (e.g. by use of MAO or COMT inhibitors).

Reserpine is currently used as an anti-hypertensive agent, although its use is limited by a burden of adverse effects. Other therapeutic agents however have been shown at least in vitro to interfere with the storage of catecholamines in lymphocytes (Fig. 2.3).

A wide array of sympathoadrenergic agents is currently used for various indications with a usually favorable therapeutic index, and represent therefore an attractive source of potentially novel immunomodulating agents with significant therapeutic potential.

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Acetylcholine and Cholinergic Modulation of Immune Responses

3

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Contents

3.1 Introduction

Acetylcholine (ACh) is a phylogenetically ancient molecule involved in cell-cell signaling in almost all life forms. ACh was first proposed as a mediator of cellular function by Hunt in 1907, and in 1914 Dale pointed that its action mimicked the response of parasympathetic nerve stimulation (Dale 1914). Loewi, in 1921,

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provided evidence for ACh release by nerve stimulation (Siegel et al. 1998). Furthermore, Dale's experiments showed that separate receptors can explain the variety of actions of ACh. Subtyping of the receptors was based on the pharmacological activity of two alkaloids: nicotine and muscarine.

Studies of the chemical structure of ACh pointed that its *trans* conformation is the active conformation at muscrinic receptors (Portoghese 1970), while NMR studies show that the acetoxy and quaternary nitrogens are too close together for this conformation to exist when ACh is bound to the nicotinic receptor (Behling et al. 1988). Thus, the bound conformations of ACh are flexible and differ substantially with the receptor subtype. It is well known that the structural modifications that enhance or diminish activity on muscarinic receptors are very different from those modifications that influence activity on nicotinic receptors (Siegel et al. 1998). Subsequently, it was found that the nicotinic receptors are not identical and muscarinic receptors also exhibit distinct subtypes. Molecular cloning techniques enabled further classification of the muscrinic and nicotinic receptors based on their primary structure.

ACh synthesis occurs as a single step reaction by the enzyme cholineacetyltransferase (ChAT) from choline and acetylcoenzyme A and the neurotransmitter is packed into vesicles in the neuronal terminus. It is released to the synaptic space upon neuronal activation, and then interacts with its receptors.

ACh as a neurotransmitter can be found in brain areas associated with cognitive function such as the hippocampus, amygdala and cerebral cortex. It has a central role in the autonomic system: it is the main neurotransmitter in the preganglionic neurons in both the sympathetic and parasympathetic systems and the postganglionic neurotransmitter in the parasympathetic system. ACh is also responsible for motor function, as it is present in the neuro-muscular junction (NMJ).

As mentioned above cholinergic receptors can be divided to muscarinic and nicotinic (mAChRs and nAChRs, respectively). mAChRs are seven transmembrane (7TM) G-protein coupled receptors. They comprise of five subtypes and are usually divided to two distinct categories: M_1, M_3 and M_5 are coupled to G_q receptors, mediating their effects through activation of phospholipase C and intracellular calcium mobilization; M_2 and M_4 mAChRs are coupled to G_i receptors and mediate their effects by reducing cAMP intracellular levels (Langmead et al. 2008).

nAChRs are pentameric receptors comprised of different α and β subunits. There are nine α subunits and four β subunits, allowing for various combinations with different physiological function and ligand affinity. The α7 nAChR is a homopentamer with each α7 subunit containing four trans-membrane domains that are encoded by exons 7–10. Mice in which these exons were deleted showed no expression of α7 protein or mRNA (Orr-Urtreger et al. 1997). It has a relatively low affinity to nicotine in comparison to the heteropentameric receptors, but high affinity to α-bungarotoxin (α-btx) (de Jonge and Ulloa 2007).

The nAChRs at the NMJ are very well characterized; they are comprised of two types, the fetal α1β1γδ and the adult α1β1εδ. These receptors co-assemble as 2:1:1:1 and require two ACh molecules for activation. There is a high and low affinity binding site at the α-δ and the α-γ/ε interface, respectively. After birth there

is a dramatic increase in transcription of the ε subunit which is assembled into the receptor. The α1β1εδ nAChRs aggregate at the NMJ, are more stable to degradation and have a more rapid response to agonist compared to the α1β1γδ receptor (Fagerlund and Eriksson 2009). In addition, animal studies have demonstrated that the neuronal α7 nAChR is also found in the muscle membrane during development and denervation, indicating that this receptor might be associated also with endplate stabilization and synaptogenesis (Corriveau et al. 1995; Fischer et al. 1999).

The termination of signaling in the cholinergic system is achieved by degradation of ACh by acetylcholinesterase (AChE), a serine hydrolase. AChE has several isoforms, all of which are a product of alternative splicing of a single gene. Exon 2 encodes for the catalytic activity of the enzyme and is common to all isoforms (Grisaru et al. 1999). The termination of signaling by degradation of the neurotransmitter is unique to the cholinergic system, as other neurotransmitters dissociate from their cognate receptor and are then cleared from the synaptic space by re-uptake mechanisms. Thus cholinergic signaling and synaptic transmission may be regulated by modulation of AChE activity.

AChE inhibitors (AChEIs) inhibit the AChE catalytic activity leading to increased ACh levels. Edrophonium, pyridostigmine, neostigmine, tacrine and rivastigmine are some of the clinically useful AChEIs. They differ in their pharmacokinetic properties: edrophonium has a very short half life; edrophonium and pyridostigmine do not cross the blood–brain barrier (BBB), whereas rivastigmine and tacrine do. These variations in pharmacokinetics allow adaptation of a specific agent for a specific need avoiding unwanted side-effects.

3.1.1 Cholinergic Agents in Human Use

Various cholinergic agents are in human use. In many instances their use is short term such as atropine, a muscarinic antagonist, for symptomatic bradycardia or muscle relaxants used during general anesthesia. We will focus here on cholinergic agents used in the chronic set-up.

3.1.1.1 Acetylcholinesterase Inhibitors

The main clinical indications for AChEIs treatment are myasthenia gravis (MG) and Alzheimer's disease (AD).

In MG there is an antibody mediated auto-immune attack on the nAChR in the postsynaptic membrane of the NMJ. Some antibodies may block the ACh site and thus prevent receptor activation. However, the majority of the auto-antibodies are non blocking-antibodies which mediate their effect by increasing the rate of receptor degradation and complement activation. Both processes lead ultimately to reduction of the number of nAChR in the NMJ. Clinically, disease manifestation is muscular weakness (Drachman 1994). AChEIs are used both for the diagnosis and treatment of MG. AChE inhibition increases ACh concentration and compensates for the reduced number of muscular nAChR. Short acting AChEIs, like edrophonium, serve for diagnostic testing-patients with MG show improved

symptoms after inoculation. Pyridostigmine is an orally available, non-competitive AChEI that despite its short half-life serves as the main therapeutic agent in MG for many years.

However, recent evidence gained from experimental autoimmune myasthenia gravis (EAMG), the animal model for MG, indicate that AChEIs may posses other mechanism of action. It was shown that AChEIs not only improved survival and stamina, but also improved various immunological parameters, such as T-cell proliferation, antibody production and chemokine levels in the muscles (Brenner et al. 2003). These alterations in immunological parameters are not expected if the only role of AChEIs was cholinergic up-regulation and subsequent activation of the muscular nAChR.

AD is the most common dementia comprising about two-thirds of all diagnosed dementias. The cognitive deterioration is manifested as a decline in memory, judgment, language, decision-making and orientation to surroundings (Nussbaum and Ellis 2003). Pathologically, the disease is characterized by neuronal and synaptic loss in the cortex and hippocampus, both areas associated with cognitive function. Another area which typically degenerates early in the disease is the basal nucleus of Meinert, which is a major source of cortical cholinergic input. Therefore AD is almost invariably associated with a disruption of cholinergic balance. Extracellular plaques containing β-amyloid and intracellular neurofibrillary tangles containing hyperphosphorylated tau protein accompany, and may actually induce, neuronal loss (Desai and Grossberg 2005). There is also prominent activation of astrocytes and microglia near the plaques, attesting to an innate immune response (Monsonego and Weiner 2003). In addition, a T-cell dependent component in AD was identified (Monsonego et al. 2003). Treatment of AD may be directed against each of the components of the pathological process: (1) neuroprotective strategies (against neuronal loss); (2) antioxidant and anti-inflammatory treatment (against accompanying inflammatory-induced neurotoxicity); (3) external compensation for specific neurotransmitter loss (like AChEIs); (4) specific therapy against β-amyloid (halting aggregation or deposition, enhancing clearance) and tau hyperphosphorylation.

Actually, treatment is dependent on disease stage: In early stages, centrally acting AChEIs are used in an attempt to restore cholinergic input and partially ameliorate the memory loss (Cummings 2004). As no anti-amyloid therapy is currently available, AChEIs constitute the only specific treatment for mild to moderate AD. Several AChEIs have been approved for the treatment of AD. These drugs penetrate the blood–brain barrier and act by enhancing cholinergic transmission in brain areas associated with cognitive functions and suffering from neuronal loss. The reduction in ACh breakdown exerted by these drugs allows compensation for the decrease in viable neurons. This treatment is, therefore, a symptomatic treatment with debatable efficacy (Birks 2006; Kaduszkiewicz et al. 2005). In any event, two clinical observations suggest that enhancement of cholinergic transmission is not the only mechanism of action of AChEIs. The first is that in 20% of AD patients treated with AChEIs, cognitive function is stabilized for 24 months. This effect is surprising in light of the symptomatic influence of AChEIs

treatment. The second is that upon discontinuation of drug treatment, the deterioration in cognitive function returns to the predicted course more slowly than expected. And again, if AChEIs were only enhancing cholinergic transmission, deterioration should quickly return to the untreated patients course, since the half-life of AChEIs is relatively short (Giacobini 2003). Several alternative mechanisms were proposed based on experimental data: (1) AChEIs increase the secretion of soluble fragments of amyloid precursor protein (sAPP). This effect is dependent on AChE inhibition and involves activation of M_1 and M_3 mAChRs (Nitsch et al. 1992). The observation that treatment of AD patients with a selective M_1 muscarinic agonist decreased cerebral spinal fluid levels of β-amyloid supports this notion (Hock et al. 2003). (2) It was shown that AChE is associated with amyloid plaques: the presence of AChE accelerated amyloid fibril formation, and increased the neurotoxicity of the complexes (Inestrosa et al. 2000). A hydrophobic region close to the peripheral anionic site of the enzyme is responsible for this interaction. Hence, AChEIs that bind to this site may prevent the transformation of β-amyloid to amyloid fibril aggregates (Inestrosa et al. 2005).

Overall, it seems that in two settings in which AChEIs are used, i.e. MG and AD, evidence exists that these agents do more than enhancement of cholinergic signaling and symptomatic improvement.

3.1.1.2 Nicotine

Cigarette smoking is the most common preventable cause of death and disease. It is implicated in various diseases, the predominant of which are lung cancer, chronic obstructive pulmonary disease and atherosclerosis. However, despite the harmful effects of smoking, epidemiological data indicate that smokers have relatively less incidence of inflammatory diseases such as ulcerative colitis (UC), sarcoidosis, and maybe even AD (Sopori 2002). Tobacco is a complex chemical mixture, containing more than 4,500 compounds in particulate and vapor phase. However, animal studies defined nicotine as the active substance in cigarette smoke responsible for this anti-inflammatory effect. This makes nicotine the most common cholinergic agent in human use. Could this anti-inflammatory effect of nicotine be explained in terms of the cholinergic neuronal system? One has to recall that there exists a major pathway connecting the nervous and the immune system, namely the hypophysis-pituitary-adrenal axis (HPA). The final outcome of this axis activation, the release of corticosteroids, has the ability to modulate the immune system in almost every aspect: immune cell activation, cytokine secretion, inflammatory mediators' secretion and apoptosis of immune cells. Indeed, it was shown that nicotine administration could enhance the release of corticosteroids in vivo, and that at least part of the immunosuppressive effects of nicotine were abolished in the absence of intact HPA axis (Sopori et al. 1998). However, as we will show subsequently, the immunosuppressive effects of nicotine could be also demonstrated directly on immune cells due to the presence of the immune, non neuronal, cholinergic system.

3.2 Non-neuronal Immune Cholinergic System

ACh is present in the vast majority of human cells, including epithelial, mesothelial endothelial cells and immune cells. Cholinergic components including ACh, ChAT, AChE and mAChR and nAChrs have been identified in numerous non-neuronal cells and tissues including keratinocytes, urinary bladder, airway epithelial cells, vascular endothelial cells, reproductive organs, cancer cells and immune cells. Here, we will focus on the immune cholinergic system.

It has been shown that lymphocytes and macrophages posses a complete cholinergic system, which includes the ability to synthesize and degrade ACh (by ChAT and AChE, respectively) (Kawashima and Fujii 2003) as well as various subtypes of muscarinic and nicotinic cholinergic receptors (Sato et al. 1999): all subtypes of mAChR were shown to be expressed on blood bank donors, although not consistently. nAChR muscle type subunits, i.e. α1, β1 and ε, were not expressed by the same cells. We could not detect them even after immune activation (Nizri et al. 2006). Among the neuronal type, α2, α5 and α7 were the most consistently expressed. Whether this expression variability among individuals has relation to nicotine exposures or has any clinical implication, is currently unknown. Activation of immune cells increased their ability to synthesize and secrete ACh by increased ChAT expression. This was verified by measurement of extracellular levels of ACh (Fujii et al. 1996). Murine macrophages and microglia were also shown to express α7 RNA and protein (Shytle et al. 2004; Wang et al. 2003). Mouse B cells were shown to express α4, α5, α7, β2 and β4 nAChR subunits. The number of α7 subunits increased with B-cell maturation, while α4 and α5 were expressed in immature B-cells (Skok et al. 2007).

3.3 Effects of Cholinergic Signaling on Immune Cells

3.3.1 Macrophages

ACh was shown to decrease pro-inflammatory cytokine production by activated macrophages. Murine macrophages were activated by lipopolysaccharide (LPS), an endotoxin produced by all gram-negative bacteria and implicated in the pathogenesis of septic shock, to secrete TNF-α, IL-1β and IL-6, all pro-inflammatory cytokines. ACh at the μM range significantly reduced this cytokine secretion. ACh had no effect on the secretion of IL-10 (Borovikova et al. 2000). The effects of ACh could be recapitulated by nicotine, but not by muscarine. Subsequent work done by the same group (Wang et al. 2003) indicated that the α7 nAChR was involved in this anti-inflammatory effect of nicotine, since it was abolished in $\alpha7^{-/-}$ derived macrophages or wild-type (WT) macrophages treated with anti-sense to the α7 mRNA. The mechanism implicated in this effect was reduced NF-κB-mediated transcription, and indeed all the pro-inflammatory cytokines whose production was reduced by nicotine contain an NF-κB binding site in their promoter (Barnes and Karin 1997).

As expected, same effects of nicotine could be demonstrated on microglia, the brain-residents macrophages (Shytle et al. 2004). Microglia express both α7 nAChR mRNA and protein. Its activation with nicotine or ACh, again in the μM range, on LPS-stimulated microglia, resulted in about 50% TNF-α reduction. This was mediated through MAP-kinase inhibition.

3.3.2 T-Cells

T-cell proliferation is a key step according to the clonal immune theory, in which the T-cell expressing the receptor that identifies the pathogen or immune activator, proliferate to create the active immunological clone.

It was found that human mononuclear cells and cell lines express mRNA for muscarinic and nicotinic AChRs (Sato et al. 1999). This group of investigators showed that all the cell lines tested expressed m4 and m5 subtype mRNAs, whereas the expression of m1, m2 and m3 varied among the cell lines.

The expression of the neuronal subunits α2, α3, α5, α6, α7, β2 and β4 in the same cell lines also varied and was present in 2–7 of them. Whereas, α4 and β3 was not found in any of these cells (Table 3.1).

It was shown that human T-cells from healthy donors stimulated by various mitogens increase their cholinergic synthesizing machinery and expression of cholinergic receptors (Kawashima and Fujii 2003; Nizri et al. 2006). Immune activation did not result in de-novo expression of cholinergic receptors, but rather increased the level of receptor expression pre-existing in the unactivated immunological state. Nicotine and ACh in the μM range could inhibit T-cell proliferation. Interestingly, muscarinic activation could increase T-cell proliferation, and when muscarinic blockers such as atropine were used in conjuction with cholinergic agents, they increased the inhibitory effect of the later (Nizri et al. 2006). Accordingly, it was reported that mAChR activation increased the production of pro-inflammatory mediators by enhancement of c-fos and iNOS transcription

Table 3.1 Expression of muscarinic receptor subtypes and nicotinic receptor subunits mRNA by human T and B cell lines

	Muscarinic subtype			Nicotinic receptor subunits										
Cell line	m1	m2	m3	m4	m5	α2	α3	α4	α5	α6	α7	β2	β3	β4
CEM (T)	+	−	+	+	+	−	+	−	+	+	+	−	−	+
HPB-ALL (T)	−	+	+	+	+	+	−	−	+	+	+	−	−	+
HUT-78 (T)	+	+	+	+	+	+	−	−	+	+	−	−	−	−
Jorkat (T)	−	−	−	+	+	−	−	−	−	−	−	−	−	+
MOLT-3 (T)	−	−	+	+	+	−	+	−	+	+	−	−	−	+
BALL-1 (B)	+	−	−	+	+	−	−	−	+	−	−	+	−	+
Daudi (B)	−	+	+	+	+	+	−	−	+	+	+	−	−	−
NALM-6 (B)	−	−	−	+	+	−	−	−	+	−	−	+	−	+

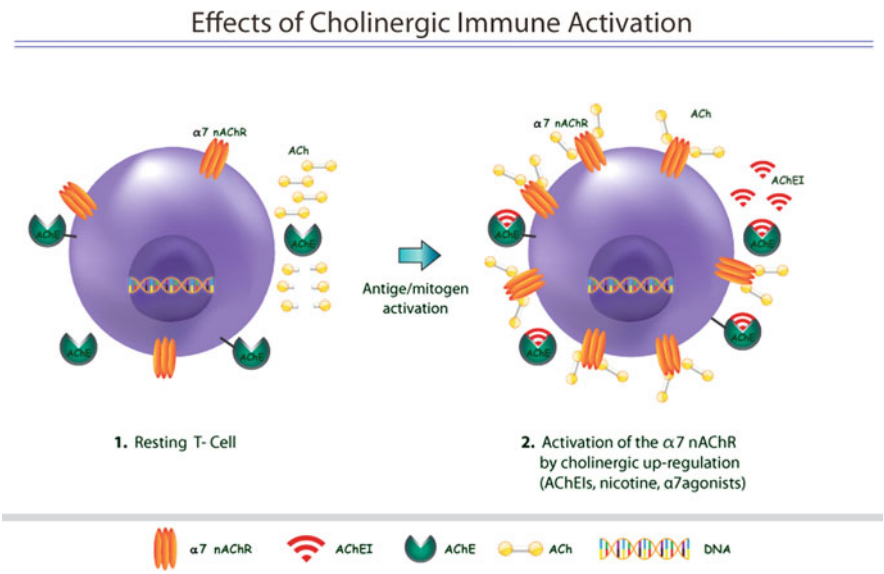

Fig. 3.1 The α7nAChR is expressed on T-cells and its' stimulation exerts anti-inflammatory effects. (**a**) Under basal conditions extracellular level of ACh is low due to its degradation by AChE. (**b**) Cholinergic up-regulation by AChEIs (or nicotinic agonists) increases the ACh concentration and activation of the α7nAChR occurs as well as an increase in the number of these receptors (Nizri et al. 2009)

(Fujii and Kawashima 2000). Thus cholinergic stimulation could mediate pro or anti-inflammatory effects based on muscarinic or nicotinic receptor activation, respectively.

These findings could be reproduced also by AChEIs. Rivastigmine or edrophonium could also decrease T-cell proliferation, an inhibition that was α7 nAChR-dependent (Nizri et al. 2006; Nizri et al. 2008). We have shown that inhibition of AChE caused extracellular increase in ACh concentration which could increase cholinergic receptor activation (Fig. 3.1). This mechanism is similar to the mechanism by which AChE function in activation of the NMJ in MG or compensate for loss of cholinergic transmission in AD. This forms the base for the immunological activity of AChEIs described below.

As described above a nAChR receptor is responsible for the cholinergic anti inflammatory effects. We have shown that like in macrophages, the α7 nAChR is expressed by T-cells and responds to cholinergic and immunological stimuli. This receptor is expressed on naïve T-cells, its expression is augmented following immune activation (antigen or mitogen induced) in both mRNA and protein level (Nizri et al. 2006; Nizri et al. 2007b; Nizri et al. 2009). Cloning of this receptor demonstrated 99.7% identity to the neuronal α7 (Razani-Boroujerdi et al. 2007). This apparent identity should not deceive: it was demonstrated that for Ca^{2+} channels even a single amino acid substitution may change permeability and

function of the channel (Matza and Flavell 2009). The specific electrophysiological activity of the α7 nAChR in T-cells was not investigated. This receptor-coupled Ca^{2+} channel generates rapid Ca^{2+} influxes; however, it is possible that immunological function of this receptor depends on release of intracellular Ca^{2+} stores, rather than Ca^{2+} extracellular influx (Tracey 2009).

One key aspect of CD4-T-cell function is their lineage identity. In 1986 Mosmann et al. initially proposed a model whereby $CD4^+$ T cells are subdivided into two independent subsets with distinct effector functions (Mosmann et al. 1986). Th_1 and Th_2 subsets are divided on the basis of cytokine expression and bioactivities as well as helper function. Th_1 cells secrete predominantly IFN-γ, IL-2, IL-3 and TNF-α, and control cell-mediated functions such as the activation of macrophages, while Th_2 cells secrete IL-4, IL-5, and IL-13 and lead to the stimulation of humoral immunity. Central to this model is the ability of the cytokines of a particular Th subtype to further promote the expansion of that subtype population while simultaneously inhibiting the development of the other subset. Recently it was discovered that Th linage differentiation is associated with specific transcription factors (TFs). These are crucial to the development of Th lineage, as determined by genetic targeting models. T-bet is the TF responsible for Th_1 differentiation and acts by remodeling of chromatin at the IFN-γ promoter. GATA-3 has the same function in Th_2 lineage, affecting the IL-4 promoter (Murphy and Reiner 2002). Recently, a novel Th subset was defined, the Th_{17}. This newly defined Th subset was implicated in various autoimmune diseases, such as inflammatory bowel disease, collagen-induced arthritis and experimental autoimmune encephalomyelitis (EAE, see below) (Langrish et al. 2005). IL-17 is a pro-inflammatory cytokine which drives the secretion of other pro-inflammatory cytokines (such as IL-1, IL-6 and G-CSF) and chemokines from endothelial cells, stromal cells and fibroblasts (Gutcher and Becher 2007). IL-17 is secreted by a specific CD4 subset, along with IL-22, TNF-α, IL-6 and IL-23 (Liang et al. 2006). The Th_{17} subset has its own TFs responsible for effector cells differentiation: the orphan nuclear receptors ROR-γt (Borovikova et al. 2000) and ROR-α (Yang et al. 2008).

Various investigators (Nizri et al. 2008; Shi et al. 2009) have shown that activation of the α7 nAChR by nicotine decreases Th_1 cytokine production (such as TNF-α, IFN-γ, IL-2) while the production of IL-4, a prototype Th_2 cytokine, is increased (Nizri et al. 2009). This phenotype was demonstrated also in the mRNA transcription of lineage specific TFs: nicotine decreased T-bet, but increased GATA-3 transcription. α7 nAChR activation also suppressed Th17 activity: IL-17, IL-21 and IL-22 expression was reduced. However, levels of ROR-γT and ROR-α were not changed, indicating that treatment affected Th17 activity, but not differentiation (Fig. 3.2).

$CD8^+$ T-cells are implicated in various biological processes among them immunity against viral pathogens and graft rejection. A common used model for T-cell alloreactivity, which essentially measure $CD8^+$ reactivity, is the mixed lymphocyte reaction model. In this model, recognition of alloantigen T-cells generates IL-18 production which further augments various co-stimulatory and adhesion molecules

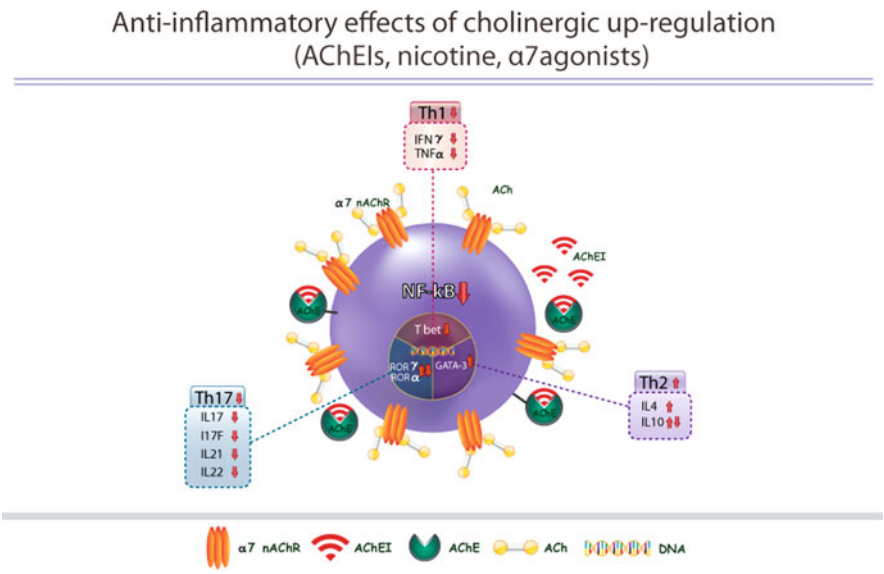

Fig. 3.2 The anti-inflammatory effects of activation of the T-cell cholinergic system. α7nAChR activation by cholinergic agonists (nicotine, ACh, AChEIs) has immunomodulatory effects; suppressing Th1 and Th17 pro-inflammatory pathways, and enhancing Th2 lineage (It should be noted that α7nAChR$^{-/-}$ T-cells were refractory to such cholinergic stimulations)

expression along with IFN production. It was shown that nicotine inhibits this over expression of co-stimulatory and adhesion molecules, and also suppresses inflammatory cytokine production. These effects on T-cells were abolished in the presence of specific α7 nAChR antagonists and hence were attributed to the α7 nAChR (Takahashi et al. 2007).

Over all, α7 nAChR stimulation was shown to modulate T-cell activity extensively including T-cell proliferation, cytokine production and specific T-cell proliferation and function. The effects of cholinergic stimulation were demonstrated on both $CD4^+$ and $CD8^+$ T-cells. Another important T-cell subset affected by nicotine is regulatory T-cell ($FOXP3^+CD4^+CD25^+$). Preliminary data show that treatment with nicotine increase their number (Shi et al. 2009), however, delineation of specific function of these cells under cholinergic modulation is lacking.

3.3.3 B-Cells

Nicotine was shown to affect the size of the B-cell niche in bone-marrow (BM) and this effect was β2-dependent, as the number of B-cells in BM was affected in β2$^{-/-}$. However, this receptor did not affect the number of B-cells in the spleen, where the α7 nAChR was the dominant receptor.

Mice deficient in one of the nAChR subunits had less serum IgG and less IgG producing cells. However cells from deficient animals had larger antibody production in response to activation, due to increased CD40 expression. Interestingly, the inhibitory effects of nicotine on antibody production could only be demonstrated in the absence of the β2 subunit. Nevertheless, again α7 nAChR had inhibitory immunological function (Skok et al. 2007).

3.3.4 Dendritic Cells

Until now, there is no direct evidence for nAChR expression on dendritic cells (DCs). We and others have shown decreased antigen presentation following cholinergic activation (Nizri et al. 2008; Nizri et al. 2009; Shi et al. 2009). It was also shown that co-stimulatory molecules such as CD80, CD86 and MHCII expression on APCs is reduced upon nicotine treatment (Shi et al. 2009). However, these results were obtained following in vivo treatment with nicotine, which could attest to an indirect effect of nicotine on DCs either through other cell types such as T-cells, or through corticosteroids. However, in vitro treatment of human dendritic cells with nicotine decreased their phagocytic activity and pro-inflammatory cytokine secretion. DCs treated with nicotine failed to mount an effective Th_1 response in T-cells. This points to a direct effect of nicotine on DCs, but still does not point to a specific molecular target. In light of the aforementioned effects of α7 nAChR on immune cells, it seems plausible that these effects are mediated also by the same receptor.

3.4 Cholinergic Signaling in Immune Responses: Paracrine or Nerve-Driven Regulation?

The existence of the immune cholinergic system paves the way for nerve-immune interactions. Indeed initial work done on this system showed that increased vagal activation by vagus nerve stimulation decreased macrophages TNF-α and IL-1β production in an α7 dependent mechanism. This work implicated vagal innervation of the liver as the location of a nerve-immune "synapse". It was shown that liver TNF-α production differed between control and vagotomized animals. This production was suppressed following vagal stimulation (Borovikova et al. 2000). Subsequent work by the same group identified the spleen as the major site of nerve-immune interaction. Specific (dorsal branch) vagal transection, abolished the effects of vagal nerve stimulation on pro-inflammatory cytokine production (Huston et al. 2006). This vagal branch innervates the celiac plexus and indirectly, the spleen. These results were further confirmed in splenctomized animals: vagal innervation of the spleen mediated its anti-inflammatory activity (Huston et al. 2006; Rosas-Ballina et al. 2008).

These and other findings led to the definition of the "cholinergic anti-inflammatory reflex", in which the nervous system takes a part in the control of the magnitude and quality of immune response. The afferent arm of the reflex consists of vagal afferents which are activated due to inflammation initiated by pathogen-associated molecular patterns (activators of Toll-like receptors), pro-inflammatory cytokines (such as IL-1β) or endogenous markers of damage (intracellular molecules present in plasma, like high-mobility group B protein 1- HMGB-1) (Tracey 2009). Such a pathway was demonstrated in the initiation of sickness behavior in rats. When IL-1β is injected intraperitonealy, it initiates sickness behavior in animals, manifested as fever, anorexia, acute phase reactions and decreased arousal. This effect is abolished in vagotomized rats, pointing to the vagus nerve as the afferent route for initiation of sickness behavior. Subsequent work showed that the glomus cells present in the vicinity of vagal afferents sense IL-1 and release dopamine which stimulates vagal action potentials (Niijima 1996). This neural network provides the CNS with the ability to monitor inflammatory reactions occurring in epithelial compartments, and also to initiate a neural reaction to this process. The efferent arm of this reflex is efferent vagal neurons which release ACh on sites of the reticulo-endothelial system and modulate both macrophages and T-cell activity. It is important to note that although ACh levels can be easily measured in the spleen, cholinergic nerve endings were never identified in the spleen, despite intensive efforts. Cathecholaminergic innervation of the spleen is well described, including close contact between T-cell and macrophages and cathecholaminergic neuron endings. These sympathetic nerve fibers can be activated in the celiac ganglion by either sympathetic nerves originating in the spinal cord, as part of the classical sympathetic system, or by vagal neurons originating from the brain as part of the parasympathetic system. It is possible that vagal activation of the splenic nerve induces immune cells residing in the spleen to produce ACh (Rosas-Ballina et al. 2008; Tracey 2009).

It should be noted that immune cells posses the ability to synthesize ACh by themselves and that this production is augmented following immune activation. This may point for a role of ACh as internal mediator of immune function, similar to cytokines and other immune active molecules. Of note, ACh existence in evolution preceded the appearance of the CNS. There is a possibility that it arose first as an inflammatory mediator in immune response (Kawashima and Fujii 2003).

However, even if the presence of the immune cholinergic system attests to neuro-immune interactions, and to the ability of the CNS to modulate even this aspect of physiological function, the existence of the immune cholinergic system highlights novel targets for pharmacological intervention. Research done by our group utilized AChEIs and nicotinic agonists for the modulation of neuroinflammatory conditions with success in pre-clinical models (Nizri et al. 2005, 2006, 2007b, 2008, 2009). Others have shown the efficacy of nicotinic agonists or cholinergic stimulation of immune cells in various diseases including septic endotoxemia model (Huston et al. 2007), collagen induced arthritis (van Maanen et al. 2009), pancreatitis (van Westerloo et al. 2006) and colitis (Ghia et al. 2007).

3.5 Involvement of Cholinergic Transmission in Clinical and Experimental Disease States

As outlined above, cholinergic transmission stimulators and agonists were recently exploited in various inflammatory conditions, induced by the innate or the adaptive immune system (reviewed in Tracey 2009). Here we will focus on the most extensively studied models.

3.5.1 Experimental Endotoxemia and Septic Shock

Sepsis is the most common cause of death in intensive care units, and despite decades of clinical and pre-clinical research, little progress has been made in the treatment of this phenomenon. Sepsis is defined as systemic inflammatory reaction syndrome (SIRS) in the presence of suspected or proven infection (Russell 2006). Key to this definition is the interaction between pathogens, usually gram negative, and a systemic response manifested as fever, tachycardia, leukocytosis or tachypnea. In its severe and devastating form, septic shock, sepsis is manifested as decreased blood pressure necessitating aggressive fluid resuscitation and inotropics to maintain adequate tissue perfusion. The pathogenesis of sepsis is usually attributed to the innate immunological system: activation of this system by pathogen-associated molecular patterns and their cognate receptors on innate immune cells induces secretion of pro inflammatory cytokines and mediators. These further induce activation of neutrophils, macrophages and platelets. The septic state is defined as a cytokine storm and a state in which immune system over-activation can cause damage to the organism more than the inciting pathogen (Russell 2006). Drawbacks of this hyper inflammatory theory of sepsis have been presented (Hotchkiss and Karl 2003), the delineation of which is not in the scope of this chapter.

The first work describing the cholinergic anti-inflammatory pathway used the experimental endotoxemia model, in which a septic state is induced in experimental animals using endotoxin (LPS). It was demonstrated that vagus nerve stimulation increased animals' survival after induction of sepsis. Vagotomized animals were more susceptible to death than sham operated animals. Similarly, vagus nerve stimulation maintained mean arterial blood pressure in comparison to control animals (Borovikova et al. 2000). Subsequent work by the same group utilized vagus nerve stimulator, a device in clinical use for the treatment of intractable epilepsy, to treat sepsis in experimental animals. Again, vagus nerve stimulation with transcutaneous device reduced both serum pro-inflammatory levels and animals' mortality (Huston et al. 2007).

The use of the vagus nerve may not be limited only for anti-inflammatory interventions but also for the assessment of the inflammatory set point in individuals. It was shown that heart rate variability is a measure of vagal activity. A correlation could be demonstrated between heart rate variability and the tendency to develop inflammatory diseases and their severity. For example, it was shown that

vagal activity as measured from heart rate variability in septic patients admitted to intensive care unit was correlated with survival, length of hospital stay and complications (Pontet et al. 2003). Similar findings were observed for several auto-immune and inflammatory conditions (Tracey 2009).

3.5.2 Experimental Autoimmune Encephalomyelitis

First established in 1933 by Rivers, experimental autoimmune encephalomyelitis (EAE) is a widely used model for the study of multiple sclerosis (MS). MS is an inflammatory disease of the CNS. It is the most common cause for neurological disability in the young (Sospedra and Martin 2005). The clinical manifestations usually include fatigue, muscle weakness, spasticity, gait and bladder dysfunction, vision abnormalities, cognitive and affective disorders (Kesselring and Beer 2005). The cognitive dysfunction in MS consists of memory and attention impairment, reduced speed of information processing and a decrease in verbal fluency (Gilchrist and Creed 1994). Immunomodulatory treatment can slow the cognitive decline in MS patients, but there is still a need for symptomatic treatment (Henze et al. 2006).

EAE is an inflammatory disease of the CNS in which myelin components are the focus of the autoimmune attack. Since the disease is induced by a known antigen, study of the pathogenesis of EAE has led to many immunological insights, allowing lessons from EAE to be generalized and applied to other autoimmune diseases (Steinman and Zamvil 2006). $CD4^+$ T-cells are sensitized in the periphery against protein-components of the myelin sheath, such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP). These encephalitogenic T-cells then migrate to the CNS, and upon additional activation by resident antigen-presenting-cells (APC) such as microglia, astrocytes and a subpopulation of dendritic cells (Greter et al. 2005), initiate plaque formation. Damage mechanisms in the CNS include secretion of cytokines by pathogenic T-cells, mainly of the Th_1 and Th_{17} lineages (see below), but also activation of glial cells to secrete both pro-inflammatory cytokines and inflammatory mediators such as nitric oxide (NO). Overall, this process leads to destruction of the myelin sheath, axonal damage and even loss (Hemmer et al. 2002). The clinical manifestation of this process is neurological motor dysfunction that can be quantified using a specific score. The $MOG_{35–55}$-induced model of EAE is characterized by an acute inflammatory phase, followed by a chronic phase of neurological deficit. This pattern is more compatible with the common course of MS.

The clinical neurologic deficit is accompanied by specific alterations in histological sections from spinal cord and brain tissue. These include inflammatory infiltrates containing macrophages and T-cells in neural tissue, together with axonal damage and loss. Moreover, based on the encephalitogenic T-cell migration pattern and the well known disease kinetics, the model affords analysis of various immunological parameters at different phases of the disease (Hjelmstrom et al. 1998; Wolf et al. 1996).

Until recently, EAE was considered a Th_1 mediated disease. Several lines of evidence supported this notion: Th_1 cytokines (like TNF-α and IFN-γ) were up-regulated in inflammatory plaque (Traugott and Lebon 1988), Th_1 polarized encephalitogenic T-cells could adoptively transfer EAE (Ben-Nun et al. 1981) and more recently, it was shown that mice deficient in T-bet are resistant to EAE (Bettelli et al. 2004). Furthermore, data regarding IL-12 also reinforced the Th_1 hypothesis. IL-12 is secreted by APCs upon antigen presentation and plays a major role in the development of Th_1 lineage (Gutcher and Becher 2007). Structurally, it is a heterodimeric cytokine comprised of two subunits p40 and p35. Animals deficient in p40 were resistant to EAE (Segal et al. 1998), and antibodies to IL-12 ameliorated disease symptoms (Leonard et al. 1995).

However, several experimental flaws appeared in this theory. IFN-γ knock-out (KO) mice developed more severe EAE than wild-type mice (Ferber et al. 1996). The same is true for IFN-γ receptor KO animals (Willenborg et al. 1996). In fact, there were reports that IFN-γ administration ameliorated clinical signs of EAE (Voorthuis et al. 1990), whereas treatment with antibodies to IFN-γ exacerbated EAE (Billiau et al. 1988). Moreover, deletion of p35, the light chain of IL-12, did not confer resistance to EAE, but actually increased disease severity (Becher et al. 2002; Gran et al. 2002). So while p40 is essential for EAE induction, p35 is not. Indeed, it was discovered that p40 can dimerize with another subunit, p19, to form IL-23, a novel cytokine which belongs to the IL-6 super-family. Thus, the resistance of $p40^{-/-}$ mice to EAE actually reflects IL-23 deficiency phenotype. This was later confirmed in $p19^{-/-}$ mice. While these mice were resistant to EAE, they had no defect in Th_1 lineage development (Cua et al. 2003). IL-23 is secreted by APC and acts on the IL-23 receptor (IL-23R) which is composed of the p40 binding protein IL-12Rβ1 subunit and the signaling IL-23R subunit. IL-23R is present on the surface of various cells of the immune system, including activated/memory T cells, NK cells, DCs, monocytes, and macrophages. IL-23R is highly expressed on murine memory CD4+ T cells and is expressed at low levels on naive T cells, permitting unique effects of IL-23 on this cell type (Gutcher and Becher 2007). The importance of IL-23 in the development of autoimmunity stems from its role in the differentiation of IL-17 secreting T-cells (Th_{17}). This newly defined Th subset was implicated in various autoimmune diseases, such as inflammatory bowel disease, collagen-induced arthritis and EAE (Langrish et al. 2005).

As could be expected from in vitro studies showing decreased Th_1 and Th_{17} reactivity under cholinergic stimulation, decreased T-cell proliferation and reduced antigen presentation by APCs, activators of the α7 nAChR suppressed effectively EAE clinical severity. We used both AChEIs as α7 activators and direct α7 agonists such as nicotine.

Treatment with various AChEIs decreased EAE severity by about 40% (Nizri et al. 2006; Nizri et al. 2008). This effect was abolished in the presence of nicotinic blockers. Moreover, sustained release preparation increased treatment efficacy and yielded 70% reduction in EAE severity. The clinical amelioration was accompanied by improvement in histopathological parameters of damage, such as axonal loss, demyelination and microglial activation (Nizri et al. 2008).

As rivastigmine is used in clinical practice for the treatment of cognitive dysfunction in AD, we hypothesized that it could also ameliorate memory impairment associated with EAE. We measured cognitive impairment in the Morris water maze (MWM) assay after EAE induction before appearance of clinical signs. Indeed, EAE was associated with cognitive impairment, which was absent in mice injected with adjuvant without induction of CNS inflammation. This fact can be explained by inflammatory infiltration of immune cells, culminating in axonal perturbation in brain areas associated with this function, like the hippocampus, an area known to be associated with MWM performance. Importantly, treatment with rivastigmine ameliorated performance in the MWM to naïve mice level. Analysis of hippocampal brain sections demonstrated decreased inflammatory infiltrates in rivastigmine-treated animals. The presence of cognitive impairment in mice with EAE further validates it as a model of MS, and paves the way for testing potential drugs for MS-induced cognitive dysfunction in this model. A similar beneficial effect of rivastigmine on spatial memory function was reported in acute EAE with cholinergic up-regulation and increased neuronal growth factor (NGF) production (D'Intino et al. 2005). The use of rivastigmine, an approved drug for cognitive dysfunction in MS seems plausible and recently AChEI use in MS was tested successfully in a clinical trial with donepezil (Krupp et al. 2004).

Treatment with nicotine in continuous release preparation suppressed EAE clinical score by 70%. Under this protocol, CNS infiltration by $CD4^+$ and $CD11b^+$ cells was also reduced (Nizri et al. 2009). The dose used in our experiments (2 mg/ks, s.c.) was significantly less than that used in human clinical trials in ulcerative colitis (Thomas et al. 2005). This was done in order to minimize side effects, which were not reported in our experiments. However, this also affords using higher doses in resistant cases. Indeed, another group reported similar effects of nicotine with 13 mg/kg in similar preparation (Shi et al. 2009). In the same report, nicotine was also shown to inhibit the adoptive transfer form of EAE, a fact that points to the effects of the treatment on T-cells.

Nicotine is known to increase corticosteroids release by activation of the hypothalamic-pituitary-adrenal axis (Seyler et al. 1984). To exclude the possibility that the effects of nicotine on EAE depend on corticosteroids, EAE in adrenalectomized mice was treated with nicotine. The effects of nicotine were not dependent on intact adrenal function, because EAE was inhibited to the same extent in the adrenalectomized mice (Nizri et al. 2009). These results are in accordance with previous published results regarding chronic nicotine treatment (Singh et al. 2000). Treatment with nicotine of $\alpha7^{-/-}$ EAE-induced mice did not alter disease severity, indicating that the effects of nicotine depend on this receptor (Nizri et al. 2009).

To summarize, there is a firm evidence that cholinergic agents can down-regulate CNS inflammation in EAE. However, the inflammatory phase in MS gives way to neurodegenerative phase, in which axonal loss in the absence of inflammation predominates. In fact, most patients convert to this phase in the late course of the disease. Could cholinergic modulation affect this stage of disease? Preliminary results from our laboratory indicate a possible up-regulation of neurotrophic agents induced by cholinergic stimulation. If so, the use of cholinergic modulation for CNS inflammation would gain novel aspects.

3.5.3 Alzheimer's Disease

As outlined above, AChEIs are used in AD to compensate for the loss in cholinergic transmission caused by degeneration of cholinergic neurons. Does the existence of the cholinergic immune system change our view on the effects of AChEIs treatment? Is there a possibility that at least part of the effects of AChEIs are due to an anti-inflammatory effect?

As noted above, there is an inflammatory component in AD. Inflammation in AD seems to be a double-edged sword. On one hand, activation of the adaptive immune system against β-amyloid, either by active or passive immunization, constitutes a strategy for AD therapy (Bard et al. 2000; Schenk et al. 1999). Further along this line, it was also found that mice deficient in complement activity were affected to a greater extent by amyloid deposits, implicating a beneficial activity of the innate immune system (Wyss-Coray et al. 2002). On the other hand, it seems that although the primary activation of the immune system is intended to clear the amyloid plaques, when clearance fails, the chronic over-activation of the inflammatory process becomes detrimental (Akiyama et al. 2000; Wyss-Coray and Mucke 2002). Indeed, various epidemiological studies revealed an inverse relationship between the use of anti-inflammatory agents and AD (Akiyama et al. 2000; Vlad et al. 2008). Despite these findings, clinical trials with non-steroidal anti-inflammatory drugs (both cyclooxygenase-1 and cyclooxygenase-2 inhibitors) (Aisen et al. 2003; Scharf et al. 1999) or with prednisone (Aisen et al. 2000), reported negative outcomes. A randomized-controlled trial of primary prevention of AD using celecoxib and naproxen in the treatment arms, reported negative results (Martin et al. 2008). There was even a tendency of treatment with naproxen to become detrimental. Thus, anti-inflammatory treatment of AD as a sole modality is questionable.

Nevertheless, in view of the inhibitory effect of ACh on pro-inflammatory cytokine production by microglia described above (Shytle et al. 2004), the loss of cholinergic transmission described in both aging and in AD may favor an activated state of microglia.

Astrocytes also express α7 nAChR, and this expression is up-regulated in the brains of AD patients (Teaktong et al. 2003). Therefore, it is possible that AChEIs affect astrocytes and microglia in the same way as they affect T cells: increasing the interaction of ACh with α7 nAChR, and so harness the anti-inflammatory effects of this receptor. Indeed, evidence from human subjects using AChEIs points to an immunomodulating effect of these drugs. Long term AChEIs treatment induced a Th1 to Th2 shift expressed by prototype cytokine production (Reale et al. 2004, 2006). Thus, AChEIs may affect the inflammatory activity of various cell types participating in AD associated inflammation. This, in turn, could affect neuronal loss and cognitive function.

Figure 3.3 summarizes the dual effects of acetylcholinesterase inhibitors in human diseases AD, MS and MG. In AD, a local inflammatory response is present near brain amyloid plaques. Cholinergic up-regulation by AChEIs improves cognitive function and may also down-regulate inflammation by activating α7nAChR on immunocompetent cells. In EAE the animal model for MS, prominent

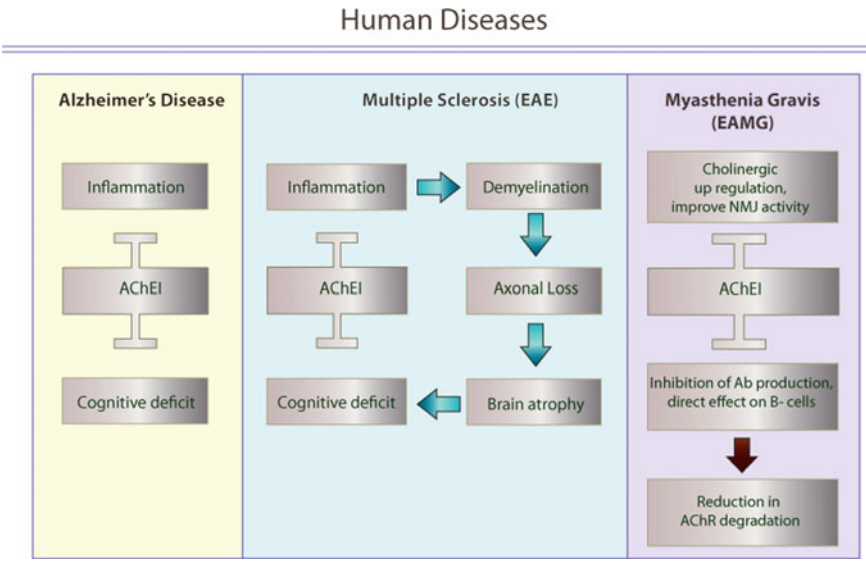

Fig. 3.3 The dual effects of acetylcholinesterase inhibitors in human diseases and in the related experimental models. In Alzheimer's disease, a local inflammatory response is present near brain amyloid plaques. Cholinergic up-regulation by AChEIs improves cognitive function and may also down-regulate inflammation by activating α7nAChR on immunocompetent cells. In the animal model for multiple sclerosis, prominent inflammatory lesions lead to demyelination and axonal loss. The cholinergic up-regulation exerted by AChEIs could be beneficial due to both inflammatory suppressing properties that may limit the axonal damage and induce an improvement of cognitive dysfunction (Nizri et al. 2006, 2008). In MG and experimental autoimmune MG (EAMG) the cholinergic balance is impaired at the NMJ. Treatments with AChEIs induce cholinergic up-regulation and improvement of muscle weakness. In addition, AChEI may affect T-cell responses as well as antibody production by B-cells which may lead to normalization of neuromuscular transmission and immunomodulation of the immune cells involved in disease pathogenesis (Brenner et al. 2003; Nizri et al. 2007c)

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Glutamate in the Immune System: Glutamate Receptors in Immune Cells, Potent Effects, Endogenous Production and Involvement in Disease

4

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4.1 Extended Summary

Glutamate, an amino acid, is the principal excitatory neurotransmitter within the vertebrate nervous system. Glutamate is involved in most aspects of normal brain function including cognition, memory and learning, and also plays major roles in the development of the central nervous system, including synapse induction and elimination, and cell migration, differentiation and death. Glutamate further plays a signaling role in peripheral organs and tissues, such as the heart, kidney, intestine, lungs, muscles, liver, ovary, testis, bone, pancreas and the adrenal, pituitary and pineal glands.

In this review, we discuss the involvement of glutamate and its receptors in the immune system, and argue that glutamate is in fact not only a **neurotransmitter**, but a **'Neuro-Immuno-Transmitter' –** a novel term coined herein, since four major criteria are undoubtedly met.

First: Glutamate receptors (GluRs), both ionotropic and metabotropic, are highly expressed in various immune cells, among them T cells, B cells, macrophages, and dendritic cells. Interestingly, different GluRs, or different levels of certain GluRs, are expressed in resting and activated T cells.

Second: Glutamate by itself, as well as glutamate agonists and antagonists, bind GluRs expressed in many types of immune cells, and can either trigger or suppress key immune functions. The exact glutamate-induced effect is determined by the context, and especially by glutamate's concentration and whether the cells are naïve/resting or rather activated. There is a marked difference between the response of naïve/resting and activated T cells to glutamate.

Third: Glutamate is produced by immune cells of several types, among them neutrophils, monocytes/macrophages and activated microglia. Furthermore, immune-derived glutamate has functional consequences on various target cells, and can for example bind GluRs expressed in human brain and dermal microvascular endothelial cells, resulting in decreased cell permeability.

Fourth: Glutamate seems to contribute to certain immune diseases, among them hematological cancers: T-leukemia and T-lymphoma, autoimmune diseases like Multiple Sclerosis, and human immunodeficiency virus (HIV) type 1 infection. On top of all that, GluR antibodies (Abs), i.e. anti-AMPA GluR3, anti-NMDA NR1 and anti-NMDA NR2A/B Abs, seem to play a role in 'Autoimmune Epilepsy', Encephalitis and Sytemic Lupus Eerythematosus. GluR Abs are found in serum and/or cerebrospinal fluid of patients, and induce detrimental effects on glutamate signaling, and on the viability of neuronal and glial cells in-vitro and in-vivo. On top of all that, we speculate that in the coming years new evidence would be revealed, showing that glutamate and/or its receptors are in fact 'guilty' in a kaleidoscope of additional immune diseases. This, of course, may open new avenues for medical interventions.

To conclude, glutamate, GluRs and GluR Abs seems to play an active, broad, potent and important role in both the nervous system and the immune system under physiological and pathological conditions. All the above mentioned topics are discussed in this book chapter.

4.2 Glutamate

Glutamate (Fig. 4.1) is an amino acid that functions as an excitatory neurotransmitter. The excitatory action of glutamate in the mammalian brain and spinal cord has been known since the 1950s, but only during the late 1970s it became widely recognized that glutamate is the principal excitatory neurotransmitter within the vertebrate nervous system (Meldrum 2000). Glutamate is in fact involved in most aspects of normal brain function including cognition, memory and learning, and also plays major roles in the development of the central nervous system (CNS), including synapse induction and elimination, as well as cell migration, differentiation and death (Danbolt 2001; Foster and Fagg 1984; Komuro and Rakic 1993; Mayer and Westbrook 1987). Glutamate also plays a signaling role in peripheral organs and tissues, among them the heart, kidney, intestine, lungs, muscles, liver, ovary, testis, bone and pancreas, and the adrenal, pituitary and pineal glands. In these tissues, glutamate may be important in mediating cardio-respiratory, endocrine and reproductive functions, which include hormone regulation, heart rhythm, blood pressure, circulation and reproduction (for detailed reviews see Nedergaard et al. 2002; Hinoi et al. 2004; Gill and Pulido 2001). Importantly, although highly regulated, glutamate levels may be extremely different in the CNS and in the periphery under physiological and pathological conditions (see Sect. 4.4 below). Furthermore, excess glutamate causes massive cell death in the nervous system by a mechanism called excitotoxicity, which plays a cardinal role in numerous neurological diseases and injuries.

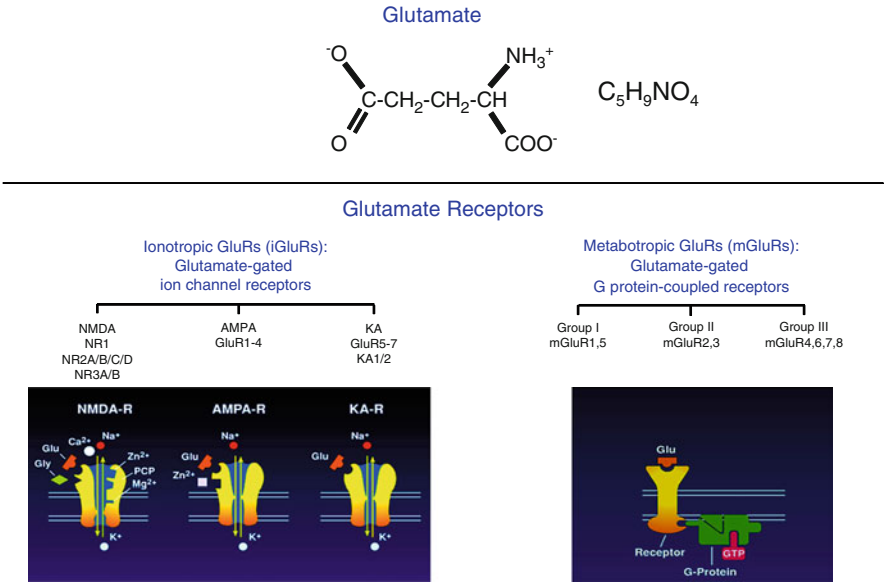

Fig. 4.1 Glutamate and glutamate receptors. The amino acid glutamate is the major excitatory neurotransmitter in the mammalian CNS and is involved in most aspects of physiological brain function and development. Glutamate has a further signaling role in peripheral organs and tissues and in endocrine cells. In contrast, excessive exposure to elevated levels of glutamate that leads to neuronal death (a process termed excitotoxicity) plays a key role in numerous pathological conditions. Glutamate has two major classes of receptors: ionotropic glutamate receptors (iGluRs) that are ion-channels gated by glutamate, and metabotropic glutamate receptors (mGluRs) that are coupled to G-proteins. Each of these two receptor classes is further subdivided to specific receptor subtypes or groups, which contain multiple subunits

4.3 Glutamate Receptors

The vast majority of neuronal and glial cells express glutamate receptors (GluRs) of several types on their plasma membranes (Danbolt 2001). The GluRs (shown schematically in Fig. 4.1) are divided into two main groups: (1) The ionotropic glutamate receptors (iGluRs), which are ion channels opened/gated by glutamate; (2) The metabotropic glutamate receptors (mGluRs), which are G protein-coupled receptors (GPCRs) activated/gated by glutamate. The mGluRs belong to the large superfamily of GPCRs that activate intracellular signal transduction pathways (Tanabe et al. 1992; Masu et al. 1991; Monaghan et al. 1989; Hollmann and Heinemann 1994; Kew and Kemp 2005). Glutamate can activate all types of iGluRs and mGluRs. Yet, there are many glutamate agonists and antagonists that bind selectively to particular types of GluRs and activate or block their activity, respectively.

4.3.1 The Ionotropic Glutamate Receptors

The iGluRs are subdivided into three groups according to their pharmacology, structural similarities, and the type of synthetic agonist that activates them: (1) The N-methyl-D-aspartate (**NMDA**) receptors; (2) The alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (**AMPA**) receptors; and (3) The 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (Kainate; **KA**) receptors (Hollmann et al. 1989). iGluRs are found mainly in the CNS, where they have extremely important roles in many brain functions. On top of that, numerous iGluR subunits are expressed also in peripheral tissues outside the CNS. Their role in these tissues is out of the scope of the current review, and the reader may refer to Nedergaard et al. (2002), Hinoi et al. (2004), Gill and Pulido (2001) for further details on this important topic. Finally, as cited and discussed below, various types of immune cells, primarily T cells, clearly express high levels of iGluRs on their cell surface. The iGluRs in immune cells are functional receptors that upon direct binding of glutamate or its agonists or antagonists exert potent immune effects.

4.3.1.1 The AMPA Glutamate Receptors

The AMPA iGluRs (Fig. 4.1) are found in many parts of the brain, and are the most commonly expressed receptors in the nervous system. AMPA receptors are also expressed outside the CNS, for example in human T cells, and in other immune cells, as discussed later herein.

The AMPA iGluRs are both glutamate receptors and cation channels that are integral to the plasticity and synaptic transmission at many postsynaptic membranes. As to their structure, the AMPA iGluRs are homo- or hetero-oligomers composed of the GluR1-GluR4 subunits (Keinanen et al. 1990), and assemble as functional tetramers (Rosenmund et al. 1998). Thus, each AMPA receptor has four sites to which glutamate or AMPA iGluR agonists can bind, one for each subunit (Mayer 2005b). The binding site is believed to be formed by the N-tail and the extracellular loop between transmembrane domains three and four (Armstrong et al. 1998). When glutamate or AMPA agonists bind, these two loops move towards each other, opening the channel pore. Thus, the channel opens when two sites are occupied (Platt 2007), and increases its current as more binding sites are occupied (Rosenmund et al. 1998). The AMPA iGluR permeability to Ca^{2+} and other cations, such as Na^{+} and K^{+}, is governed by the GluR2 subunit. If an AMPA iGluR lacks a GluR2 subunit, it will be permeable to Na^{+}, K^{+} and Ca^{2+}. The presence of a GluR2 subunit will almost certainly render the channel impermeable to Ca^{2+}. This is determined by post-transcriptional modification – RNA editing – of the Q/R editing site of the GluR2 mRNA. Native AMPA iGluRs contain GluR2 and are therefore impermeable to Ca^{2+} ions (Kew and Kemp 2005).

4.3.1.2 The NMDA Glutamate Receptors

The NMDA iGluRs (Fig. 4.1) are the predominant molecular devices for controlling synaptic plasticity and memory function (Li and Tsien 2009). Alike

the AMPA receptors, the NMDA iGluRs are expressed broadly in the CNS, but also outside it, for example in T cells, as discussed later in this review.

The NMDA iGluRs are hetero-oligomers composed of two obligatory NR1 subunits and two or three of the four regulatory subunits NR2A-D, which co-assemble to form a tetramer (Rosenmund et al. 1998) or pentamer (Hawkins et al. 1999). The NR1 subunit is necessary for Ca^{2+} conductivity of the receptor ion channel, while the NR2 subunits determine the electrophysiological and pharmacological properties of the receptor. A third subunit NR3A/B is a regulatory subunit that decreases the receptor's channel activity (Das et al. 1998), and assembly of NR3 with NR1 subunits creates a functional glycine receptor that is not activated by glutamate (Chatterton et al. 2002). Activation of NMDA iGluRs requires binding of two agonists, glutamate and glycine, at their bindings sites on NR2 and NR1 subunits, respectively (Kew and Kemp 2005). In addition, membrane depolarization is needed to abrogate the blockade of these receptors by Mg^{2+}, to render the receptors highly permeable to Ca^{2+} and Na^{+} ions (Moriyoshi et al. 1991; Collingridge and Singer 1990). Thus, a unique property of the NMDA iGluRs is their voltage-dependent activation, a result of the ion channel block by extracellular Mg^{2+} ions. This allows the flow of Na^{+} ions and small amounts of Ca^{2+} ions into the cell, and K^{+} ions out of the cell to be voltage-dependent. The Ca^{2+} flux through NMDA iGluRs is thought to play a critical role in synaptic plasticity, a cellular mechanism for learning and memory. The NMDA iGluRs are hence distinct in two ways: first, they are both ligand-gated and voltage-dependent; second, they require co-activation by two ligands – glutamate and glycine. Antagonists of the NMDA iGluRs are used as anesthetics for animals and sometimes humans, and are often used as recreational drugs due to their hallucinogenic properties, in addition to their unique effects at elevated dosages, such as dissociation. Common NMDA iGluRs antagonists include: Amantadine, Ketamine, Phencyclidine (PCP), Nitrous oxide, Dextromethorphan and Dextrorphan, Memantine, Ethanol, Riluzole, Xenon, HU-211 (also a cannabinoid), Lead and others.

4.3.1.3 The KA Glutamate Receptors

The KA iGluRs (Fig. 4.1) have a somewhat more limited distribution in the brain compared to the AMPA and NMDA iGluRs, and their function is still not well defined. The KA receptors play a role in both pre and postsynaptic neurons (Huettner 2003). The KA postsynaptic receptors are involved in excitatory neurotransmission, while the presynaptic KA receptors have been implicated in inhibitory neurotransmission by modulating release of the inhibitory neurotransmitter gamma-amino-butyric acid (GABA). Unlike AMPA iGluRs, KA iGluRs play only a minor role in signaling at synapses (Song and Huganir 2002). Rather, KA iGluRs may have a restrained role in synaptic plasticity, affecting the likelihood that the postsynaptic cell will fire in response to future stimulation (Mayer 2005a). Activating KA iGluRs in the presynaptic cell can affect the amount of neurotransmitters that are released (Mayer 2005a; Schmitz et al. 2001). This effect may occur quickly and last for a long time, and the effects of repetitive stimulation of KA iGluRs can be additive over time (Mayer 2005a; Schmitz et al. 2001).

The ion channel formed by KA iGluRs is permeable to Na^+ and K^+ ions, but impermeable to Ca^{2+}. The KA iGluRs are composed of tetrameric assemblies of GluR5-7 and KA1/2 subunits. GluR5-7 subunits can form homomeric functional receptors, as well as combine with KA1 and KA2 to form heteromeric receptors with distinct pharmacological properties. KA1 and KA2 subunits do not form homomeric functional receptors (Keinanen et al. 1990; Lerma 2006).

4.3.2 The Metabotropic Glutamate Receptors

The mGluRs (Fig. 4.1) perform a variety of functions in the central and peripheral nervous systems. For example, they are involved in learning, memory, anxiety, and the perception of pain (Ohashi et al. 2002). The mGluRs are found in pre and postsynaptic neurons in synapses of the hippocampus, cerebellum and the cerebral cortex (Hinoi et al. 2001), as well as in other parts of the brain and in peripheral tissues (Chu and Hablitz 2000). Again, as discussed in this chapter, immune cells, primarily T cells, express mGluRs which are functional receptors in these cells, and upon their activation by glutamate or its mGluR agonists induce immune effects.

Like other types of metabotropic receptors, mGluRs have seven transmembrane domains that span the cell membrane (Platt 2007). Unlike iGluRs, mGluRs are not ion channels. They activate biochemical cascades, leading to the modification of other proteins. This can lead to changes in the synapse's excitability, for example by presynaptic inhibition of neurotransmission (Sladeczek et al. 1993) or modulation and even induction of postsynaptic responses (Bonsi et al. 2005; Endoh 2004; Platt 2007). The mGluRs are also subdivided into three groups, termed group I, II and III (See Fig. 4.1), based on sequence similarity, pharmacology and intracellular signaling mechanisms. Group I mGluRs (mGluR1 and 5) are associated with G_q protein and coupled to phospholipase C (PLC), while group II (mGluR2 and 3) and III (mGluR4, 6, 7 and 8) are associated with G_i and G_0 proteins and negatively coupled to adenylate cyclase. These eight mGluRs are products of different genes (Pin and Duvoisin 1995; Masu et al. 1991; Tanabe et al. 1992). mGluRs function as homodimers, with two glutamate molecules being required for full receptor activation (Kew and Kemp 2005).

4.4 Glutamate Receptors Expressed in Immune Cells

The vast majority of studies that investigated expression of GluRs in immune cells focused on lymphocytes of the T lineage, i.e. T cells. Hence, we will first describe GluR expression in T cells, and then describe the few studies that documented the expression of GluRs in other immune cells.

4.4.1 T Cells Express Ionotropic and Metabotropic Glutamate Receptors

4.4.1.1 Glutamate Binds to Normal Human T Cells with High Affinity

The first indirect evidence for the possible expression of GluRs in T cells was provided by a study testing the interaction of radiolabeled glutamate with naïve/resting normal human T cells (Kostanyan et al. 1997). [^{3}H]glutamate was found to bind specifically to such T cells with a very high affinity (Kd $= 2.36 \times 10^{-7}$ M), and the binding was inhibited by glutamate-containing dipeptides. Moreover, binding of [^{3}H]glutamate conjugated to dextran demonstrated that GluRs are expressed on the outer membrane of the T cells (Kostanyan et al. 1997). Yet, the exact identification of the specific GluR subtypes expressed in these T cells was not reported. Following this pioneering binding study, subsequent reports provided direct evidence for the expression of a plethora of iGluRs and mGluRs in human and rodent T cells (Table 4.1).

4.4.1.2 Naïve/Resting Normal Human T Cells Express on Their Cell Surface High Levels of AMPA Ionotropic Glutamate Receptors That Contain the GluR3 Subunit. Upon T Cell Receptor Activation, GluR3 Is Transiently Eliminated from the T Cell Surface by Granzyme B That Is Released by the Activated T Cells Themselves

The first demonstration of high expression and function of iGluR, of the AMPA subtype containing the GluR3 subunit, on the cell surface of normal human T cells (purified from blood of healthy individuals) was made in fact in our own studies (Ganor et al. 2003). By using several different methodologies, among them GluR3 specific RT-PCR, Western blotting, flow cytometry and immunofluorescent microscopy, we demonstrated for the first time that normal resting human T cells express high levels of iGluR AMPA GluR3 mRNA, as well as the GluR3 protein on their cell surface (Ganor et al. 2003). Furthermore, sequencing showed that the T cell-expressed GluR3 is identical with the brain GluR3. Interestingly, in a subsequent study we revealed that upon activation of the normal human peripheral T cells via their T cell receptor (TCR) with anti-CD3/CD28 antibodies (an in-vitro experimental approach that mimics T cell activation by an antigen presented to T cells by antigen presenting cells), GluR3 was eliminated from the surface of these T cells (Ganor et al. 2007). This process was mediated by the proteolytic enzyme granzyme B that was secreted by the activated T cells themselves, and that cleaved GluR3 from the T cell surface in an autocrine/paracrine manner (Ganor et al. 2007). Expression of GluR3 on the T cell surface was restored few days after TCR activation, when the cells reverted to their naïve/resting phenotype. This process of eliminating GluR3 transiently from the T cell surface by granzyme B may have an important regulatory role, and seems to operate also in neurons, as the neuronal GluR3 was also reported to serve as a substrate for granzyme B-mediated cleavage (Gahring et al. 2001). On top of all the above, we postulate that this proteolytic process may also have pathological consequences, since it may be relevant to the

Table 4.1 Expression of a plethora of GluRs in immune cells

Cells	GluRs identified	Methods used	References
iGluRs			
Normal T cells			
Human T cells, resting	AMPA GluR3	RT-PCR, sequencing, Western blotting, flow cytometry, microscopy	Ganor et al. (2003, 2007)
Human PBLs, resting	NMDA NR1, NR2B	RT-PCR, sequencing, flow cytometry	Miglio et al. (2005b)
Human PBMCs, resting	NMDA NR1	Flow cytometry	Mashkina et al. (2007)
Human PBLs, PHA-activated	NMDA NR1, NR2A/B/D	RT-PCR, sequencing, flow cytometry	Miglio et al. (2005b)
Rat T cells, resting	NMDA NR1	RT-PCR	Boldyrev et al. (2004)
Mouse thymocytes, resting	NMDA NR1, NR2A/B	RT-PCR, flow cytometry, microscopy	Affaticati et al. (2011)
Cancer T cells			
Human T cell leukemia (Jurkat)	AMPA GluR3	RT-PCR, sequencing, flow cytometry	Ganor et al. (2003, 2009)
	AMPA GluR2/4	RT-PCR	Stepulak et al. (2009)
	KA GluR6/7, KA1/2	RT-PCR	Stepulak et al. (2009)
	NMDA NR1, NR2B	RT-PCR, sequencing, flow cytometry, microscopy	Miglio et al. (2005b, 2007)
	NMDA NR2A-D, NR3A/B	RT-PCR	Stepulak et al. (2009)
Human T cell lymphoma (HuT-78)	AMPA GluR3	RT-PCR, sequencing, flow cytometry	Ganor et al. (2003, 2009)
Autoimmune T cells			
Human lymphocytes from MS patients	AMPA GluR3	RT-PCR, Western blotting	Sarchielli et al. (2007)
Mouse anti-myelin basic protein T cells	AMPA GluR3	RT-PCR, sequencing, Western blotting, flow cytometry	Ganor et al. (2003)
Other immune cells			
Human B cells (tonsilar, peripheral, 8866 line)	KA GluR6/7, KA1/2	RT-PCR, Western blotting, flow cytometry	Sturgill et al.
Rat alveolar macrophages (NR8383)	NMDA NR1, NR2B	RT-PCR, Western blotting, microscopy	Dickman et al. (2004)

(continued)

Table 4.1 (continued)

Cells	GluRs identified	Methods used	References
mGluRs			
Normal T-cells			
Human PBMCs, resting	Group I: mGluR5	RT-PCR, flow cytometry, microscopy	Pacheco et al. (2004, 2006)
Human PBMCs, resting	Group I: mGluR5, mGluR1	RT-PCR	Chiocchetti et al. (2006)
Human PBMCs, PHA/Anti-CD3/antigen-pulsed DCs-activated	Group I: mGluR5, mGluR1	RT-PCR, flow cytometry, microscopy	Pacheco et al. (2004, 2006)
Human PBMCs from healthy individuals and ALS patients	Group I: mGluR1b	RT-PCR	Poulopoulou et al. (2005a)
	Group II: mGluR2, mGluR3		
	Group III: mGluR8		
Rat T cells, resting	Group III mGluRs	RT-PCR	Boldyrev et al. (2004)
Rat thymocytes (medullary > cortical)	Group I: mGluR5	Immunohistochemistry, Western blotting	Rezzani et al. (2003)
	Group II: mGluR2/3		
	Group III: mGluR4		
Mouse thymocytes, resting	Group I: mGluR5 (mature thymocytes), mGluR1 (immature thymocytes)	RT-PCR, Western blotting, flow cytometry	Storto et al. (2000)
	Group II: mGluR2/3		
Cancer T cells			
Human T cell leukemia (Jurkat)	Group I: mGluR5, mGluR1	RT-PCR, flow cytometry	Pacheco et al. (2004), Chiocchetti et al. (2006)
	Group I: mGluR5, mGluR1	RT-PCR	Stepulak et al. (2009)
	Group II: mGluR2, mGluR3		
	Group III: mGluR4, mGluR6, mGluR7		
Human T cell leukemia (FRO, SUP-T1)	Group I: mGluR1 (FRO, SUP-T1), mGluR5 (FRO)	RT-PCR	Chiocchetti et al. (2006)
Human T cell lymphoma (HuT-78, H9)	Group I: mGluR1 (HuT-78), mGluR5 (HuT-78, H9)	RT-PCR	Chiocchetti et al. (2006)
Other immune cells			
Human monocytes-derived macrophages	Group I: mGluR5, mGluR1	RT-PCR	Chiocchetti et al. (2006)
Human B lymphoblasts (SKW6.4)	mGluR5	RT-PCR, flow cytometry	Pacheco et al. (2004)
Mouse DCs (medullary > cortical)	Group I: mGluR5	Immunohistochemistry, Western blotting	Rezzani et al. (2003)
	Group II: mGluR2/3		
	Group III: mGluR4		

generation of a GluR3-derived autoantigenic peptide and later of GluR3 Abs found in 'Autoimmune Epilepsy' (Levite 2002). This topic is discussed in further details in Sect. 4.7 below.

Other studies further demonstrated the expression of iGluR of the NMDA family in human T cells. By using specific RT-PCR, sequencing and flow cytometry, normal resting human T cells were found to express the NR1 (Mashkina et al. 2007; Miglio et al. 2005b) and the NR2B subunits (Miglio et al. 2005b). Upon activation of the T cells with the mitogen phytohaemagglutinin (PHA), NR1 and NR2B levels increased and the NR2A and NR2D subunits were also expressed (Miglio et al. 2005b). These results show that the subunit composition of NMDA iGluRs in normal human T cells is altered following activation: while resting T cells express NR1 and NR2B-containing NMDA receptors, activated T cells express NR1 and NR2A/B/D-containing NMDA receptors. Interestingly, as discussed above, the AMPA GluRs are also altered following T cell activation, as shown by the disappearance of AMPA GluR3 from the cell surface of activated T cells (Ganor et al. 2007).

4.4.1.3 Normal Human T Cells Express Metabotropic Glutamate Receptors, and the Expression of Some mGluRs Is Modified by Activation of the Cells

The first mGluR subtypes identified in normal human T cells were mGluR5 and mGluR1 (Pacheco et al. 2004), which belong to the mGluRs group I. Several methodologies, including RT-PCR, flow cytometry and immunofluorescent microscopy, demonstrated the expression of these mGluRs at the RNA and protein levels. Interestingly, mGluR5 was expressed constitutively in naïve/resting human T cells, and in T cells activated by either PHA, the anti-CD3/TCR antibody OKT3, or antigen-pulsed dendritic cells (Pacheco et al. 2004, 2006). In contrast, mGluR1 was expressed only in activated, but not in naïve/resting normal human T cells (Pacheco et al. 2004, 2006). This is another example for the modification of GluRs composition after TCR activation, and hence for the differences in GluRs expression in resting vs. activated T cells. Another study confirmed at the RT-PCR level the constitutive expression of mGluR5 in both resting and activated human T cells, but showed the presence of mGluR1 transcripts also in resting T cells (Chiocchetti et al. 2006). Finally, RT-PCR was used to show that peripheral T cells from healthy individuals expressed transcripts for mGluR1, 2, 3 and 8, while T cells from patients with Amyotrophic Lateral Sclerosis (ALS) – a progressive and fatal neurodegenerative disease – showed reduced expression of mGluR2, but not the other mGluRs tested (Poulopoulou et al. 2005a). The possible relevance of this modified mGluR2 expression in T cells to the pathology of ALS is still unknown.

4.4.1.4 Mouse and Rat T Cells Express Ionotropic and Metabotropic Glutamate Receptors

Various GluR subtypes are expressed in T cells of non-human origin. In rat lymphocytes, RT-PCR was used to demonstrate the expression of the iGluR NMDA NR1 subunit, as well as group III mGluRs. Other GluRs were not expressed

in these lymphocytes (Boldyrev et al. 2004). In rat thymus, a wider variety of mGluRs was expressed: immunohistochemical analysis and Western blotting revealed that T cells residing in the medulla express high levels of mGluR5 (group I) and moderate levels of mGluR2/3 (group II) and mGluR4 (group III), all of which showed decreased expression in T cells residing in the cortex (Rezzani et al. 2003). mGluRs were also shown to be differentially expressed in mouse thymocytes: RT-PCR, Western blotting and flow cytometry revealed the expression of group I mGluR5 in mature $CD4^+CD8^+$ and $CD4^+CD8^-$, but not in immature $CD4^-CD8^-$ thymocytes; Group I mGluR1 showed an opposite pattern of expression; and group II mGluR2/3 were similarly expressed in all subsets (Storto et al. 2000). Finally, a recent study provided evidence that mouse thymocytes might also express iGluRs: PCR, confocal microscopy and flow cytometry were used to show the expression of NMDA iGluR subunits NR1, NR2A and NR2B. The NR1 subunit was expressed at higher levels on $CD4^+$ or $CD8^+$ single-positive thymocytes, compared to $CD4^+CD8^+$ double-positive cells (Affaticati et al. 2011).

Taken together, the above studies discussed in parts 4.4.1.1–4.4.1.4**,** show that normal human T cells, as well as mouse and rat T cells, definitely express iGluRs and mGluRs, and that T cell activation and/or maturation is an important mechanism able to either down- or up-regulate expression of specific iGluRs and mGluRs in normal T cells. As a result, resting and activated T cells express different GluRs, or different levels of these receptors.

4.4.1.5 Cancerous T Cells and Autoimmune T Cells, of Human and Mouse Origin, Express Ionotropic and Metabotropic Glutamate Receptors

Not only normal T cells express various types of GluRs. High levels of iGluRs and/or mGluRs are also expressed in cancer T cells: T-leukemia and T-lymphoma, as well as in autoimmune T cells (Table 4.1), such as encephalitogenic anti-myelin basic protein T cells that induce Experimental Autoimmune Encephalitis (EAE) - an animal model for Multiple Sclerosis (MS). A detailed description of GluRs expression in such autoimmune cells, and their relevance to cancer and MS, are discussed in Sect. 4.7 below.

4.4.2 Glutamate Receptors in Immune Cells Other Than T Cells: B Cells, Macrophages and Dendritic Cells

Few studies provided evidence for the expression of different GluRs in immune cells that do not belong to the T cell lineage. The key ones are cited below.

Study (1) A recent study demonstrated for the first time the expression of KA iGluRs in human B cells, and showed that human tonsilar B cells, $CD19^+$ peripheral B cells, and the B cell line 8866 express transcripts and proteins for the GluR6, GluR7, KA1 and KA2 subunits (Sturgill et al. 2011). Interestingly, the authors further demonstrated that activation of such KA iGluRs by glutamate and KA increased IgE and IgG synthesis and cell proliferation (Sturgill et al.).

Study (2) Human monocytes-derived macrophages express both mGluR5 and mGluR1 (Chiocchetti et al. 2006), and rat alveolar macrophages (NR8383 cell line) express the NMDA subunits NR1 and NR2B (Dickman et al. 2004).

Study (3) In rat thymus, medullary dendritic cells (DCs) express high levels of mGluR5 and moderate levels of mGluR2/3 and 4, all of which are absent in cortical DCs (Rezzani et al. 2003).

Together, the studies cited here in part 4.4 show that GluRs are expressed in T cells and B cells, the two major types of lymphocytes that constitute the adoptive immune system, as well as in DCs and macrophages, two extremely important types of immune cells that belong to the innate immune system, but also bridge between the innate and the adoptive immune systems. Yet, while a great deal has been learned already about the effects of glutamate on T cells, the outcome of glutamate binding to other types of immune cells is to a large extent unknown. Accordingly, further studies are now needed to unveil the biological relevance of the interaction between glutamate and its receptors in such immune cells.

4.5 The Direct Effects of Glutamate on T Cell Function

4.5.1 Glutamate Binds Directly to Its Receptors in T Cells and Induces Potent Effects on T Cell Function. Glutamate's Concentration and the Activation State of T Cells Are Major Factors in Dictating the Response to Glutamate. Thus, Resting and Activated T Cells Respond Differently to Glutamate

Collectively, several studies provide now clear evidence that glutamate on its own, directly and potently affects different T cell features and functions. This is in line with the direct effects of other neurotransmitters/neuropeptides on T cells (Levite 2008).

Glutamate-induced effects on T cells can be either activation or inhibition of a given T cell feature or function, depending on the context, and mainly on glutamate's concentration and the activation state of the T cell. Thus, one can expect a different response to glutamate in the following three conditions:

Condition 1: The T cells being exposed to glutamate are in naïve/resting state, and not stimulated by any other stimuli;

Condition 2: Before encountering glutamate, the T cells have already been activated by other stimuli, such as antigen, mitogen, CD3 and CD28 antibodies, cytokines, growth factors or others;

Condition 3: The T cells are being simultaneously activated by glutamate and any other stimuli.

Concerning glutamate's dose, this is indeed a very important factor which dictates the functional outcome of the interaction between glutamate and T cells, since glutamate induces different immune effects at low nanomolar (~10^{-9}–10^{-8} M), mid micromolar (~10^{5} M), or high millimolar (~10^{-3} M) concentrations (see Fig. 4.2).

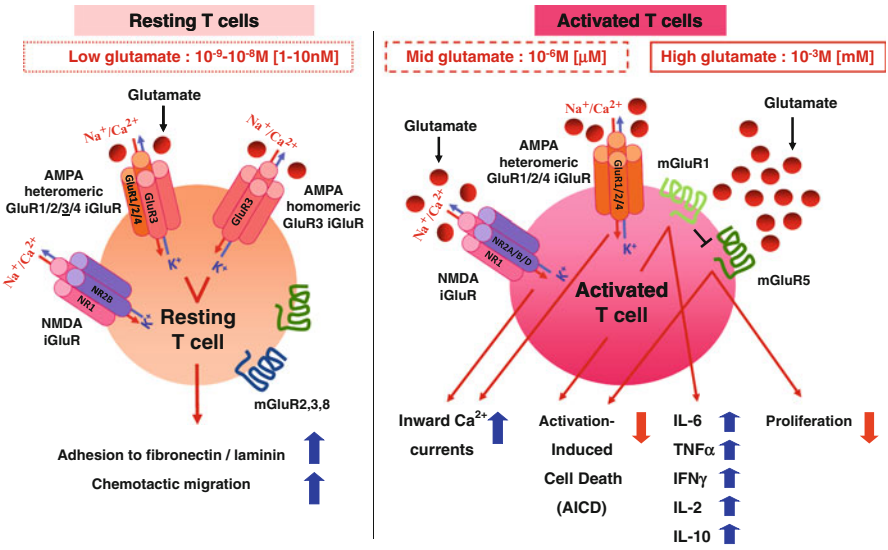

Fig. 4.2 The direct effects of glutamate on human T cell function. (*Left*) Naïve/resting T cells express the GluR3 subunit of AMPA iGluRs. Whether AMPA receptors in resting T cells are homomeric tetramers containing only GluR3, or heteromeric tetramers containing also other AMPA iGluR subunits is still unknown. Low levels of glutamate at the nanomolar range act via GluR3 to increase T cell adhesion and chemotactic migration (Ganor et al. 2003). Such interactions may take place in the normal CNS and brain fluids, and may serve to assist T cells in their survey or exit of the CNS. Resting T cells express also the NMDA iGluR subunits NR1 and NR2B, as well as mGluR2, 3, 5 and 8, but their role in mediating resting T cell function is still elusive. (*Right*) Activated T cells lose GluR3 expression, due to potent enzymatic cleavage mediated by granzyme B, which is released by activated T cells and cleaves GluR3 of the cell surface (Ganor et al. 2007). GluR3 appears again once the cells return to the resting phase (Ganor et al. 2003, 2007). In parallel to losing GluR3, the activated T cells are no longer responsive to glutamate's pro-adhesive and pro-migratory effect, evident at low nanomolar glutamate levels, and when the T cells are naïve/resting. Yet, such cells may express other AMPA iGluR subunits (that still needs to be formally demonstrated), as well as NMDA iGluRs composed of NR1 and NR2A/B/D and mGluR1 and 5. Mid levels of glutamate at the micromolar range act via both iGluRs and mGluRs to promote cell proliferation, by increasing iCa^{2+} via iGluRs and decreasing apoptosis via mGluRs. Such interactions may take place in blood under normal physiological conditions, and may be part of the normal process of T cell proliferation following antigen binding. In addition, excess levels of glutamate at the millimolar range, present in blood and/or CNS in a variety of pathological conditions, may activate mGluR5 to decrease proliferation and mGluR1 to increase cytokine secretion. Such interactions may control the expansion of the activated cells and help combating the disease

These different concentrations actually reflect the in-vivo levels of glutamate under normal and healthy physiological conditions on the one hand, and excess glutamate levels in numerous pathological conditions, on the other.

In the healthy cerebrospinal fluid (CSF) and brain extracellular fluid, glutamate is present at $\leq 10^{-6}$ M (Meldrum 2000). In the plasma of healthy individuals glutamate is present at a concentration of 10^{-5}–10^{-4} M (Divino Filho et al. 1998;

Graham et al. 2000; Meldrum 2000; Reynolds et al. 2002). Yet, glutamate levels are highest within the synaptic cleft and may reach a concentration as high as 10^{-3} M (Meldrum 2000). While these are the values for a normal healthy body, glutamate's concentrations increase substantially, both in the plasma and in the brain, in numerous pathological conditions. Thus, glutamate's plasma levels may increase substantially well above 10^{-4} M, either in a kaleidoscope of pathologies conditions, such as immune deficiency (Droge et al. 1993; Eck et al. 1989b; Ferrarese et al. 2001) and cancer (Droge et al. 1988; Eck et al. 1989a; Ollenschlager et al. 1989) (see Sect. 4.7 below), and glutamate's brain levels increase in a variety of disorders that display a neuroinflammatory component, such as MS (see Sect. 4.7 below), traumatic brain injury, acute brain anoxia/ischemia, epilepsy, glaucoma, meningitis, ALS, and Alzheimer's, Huntington's disease and Parkinson's disease (see Meldrum 2000; Sattler and Tymianski 2001), and (Pacheco et al. 2007) for specific numerical examples of elevation in glutamate levels). Importantly, excess glutamate is highly detrimental to neuronal cells, as it mediates over-activation of GluRs leading to neuronal death by a mechanism termed excitotoxicity (Sattler and Tymianski 2001).

As already mentioned above, another factor that crucially affects the responsiveness of T cell to glutamate is their activation state, i.e. whether they are naïve/resting or rather already activated, primarily since different GluRs are expressed in naïve/resting T cells and in activated T cells (see Fig. 4.2).

In the paragraphs that follow, we summarize the reported glutamate-mediated effects on various T cell functions. Each functional outcome is described while taking into account the concentrations of glutamate inducing the effect, the activation state of the cells, and the GluRs involved (Table 4.2). Finally, we suggest a model that incorporates all these findings into a unifying presentation of the effects of glutamate on T cells in health and disease (Fig. 4.2).

4.5.2 The Effects of Glutamate on Proliferation, Intracellular Ca^{2+} (iCa^{2+}) and Apoptosis

4.5.2.1 Glutamate at a Very High Millimolar Concentration Range (10^{-3}–10^{-2} M) Suppresses the Proliferation of Activated T Cells, Most Probably via Metabotropic Glutamate Receptors

An early observation suggested that elevated plasma glutamate levels correlate with a reduction in the mitogenic response of blood lymphocytes against pokeweed mitogen (Droge et al. 1988). Such an inverse correlation was reported for plasma glutamate ranging from 5×10^{-5} to 2×10^{-4} M (a range we refer to as mid-high), corresponding to the levels of glutamate measured in the plasma of either healthy individuals or patients with carcinoma, respectively (Droge et al. 1988). Later studies confirmed this initial observation, and provided direct evidence that glutamate hinders T cell proliferation. Glutamate at a high millimolar concentration range (10^{-3}–10^{-2} M) inhibited the proliferation of T cells induced by PHA or anti-CD3 ± CD28 antibodies. Importantly, even at these high concentrations,

Table 4.2 The effects of glutamate and GluR agonists/antagonists on human T cell function

Activation state	Concentration tested	Effect	References
Proliferation			
PHA-activated	10^{-4}–10^{-3} M Glu	Inhibition of proliferation (at 10^{-3} M)	Lombardi et al. (2001)
PHA-activated Anti-CD3-activated	10^{-4}–10^{-2} M Glu	Inhibition of proliferation by Glu (IC_{50} = 2.14, 0.54, 0.87 mM for cells activated with PHA, anti-CD3, anti-CD3/CD28, respectively)	Lombardi et al. (2004)
Anti-CD3/CD28-activated	iGluR agonists: NMDA, (S)-AMPA, KA	No effect of iGluRs agonists	
PHA-activated	10^{-6}–5 × 10^{-3} M	Inhibition of PHA-induced proliferation (maximal effect at 2 × 10^{-4} M (+)-MK801/2 × 10^{-3} M D-AP5)	Miglio et al. (2005b)
	NMDA iGluR antagonists: (+)-MK801, D-AP5	No effect on IL-2-induced proliferation of T-cell blasts	
Anti-CD3-activated	1–5 × 10^{-4} M mGluR agonists: Group I agonist (S)-3,5-DHPG mGluR5 agonist CHPG	Inhibition of proliferation by CHPG Abrogation of inhibition by DHPG	Pacheco et al. (2004)
DC-activated T cells	~10^{-6}–5 × 10^{-5} M Glu (released from antigen-pulsed DCs to medium)	Anti-proliferative effect following Glu depletion Increase in proliferation by NPEP	Pacheco et al. (2006)
	mGluR5 antagonist MPEP 10^{-6} M mGluR1 antagonist CPCCOEt 10^{-4} M	Inhibition of proliferation by CPCCOEt	
Intracellular Ca^{2+} (iCa^{2+})			
PHA-activated Anti-CD3-activated	10^{-9}–10^{-3} M Glu iGluR agonists: NMDA, (S)-AMPA, KA mGluR agonist: prototype agonist (1S,3R)-ACPD	Increase in iCa^{2+} by Glu and iGluR agonists (effective range 10^{-7}–10^{-5} M, maximal effect at 10^{-6} M) No effect of mGluR agonist	Lombardi et al. (2001)
PHA-activated Anti-CD3-activated	10^{-4} M mGluR group I agonist (S)-3,5-DHPG	No effect of mGluR agonist on iCa^{2+}	Pacheco et al. (2004)

(continued)

Table 4.2 (continued)

Activation state	Concentration tested	Effect	References
Apoptosis			
PHA-activated	10^{-8}–10^{-4} M Glu iGluR agonists: NMDA, (S)-AMPA, KA mGluR agonists: Prototype agonist (1S,3R)-ACPD Group I agonists quisqualate and (S)-3,5-DHPG mGluR5 agonist CHPG Group II agonist L-CCG-I Group III agonist L-AP4	Inhibition of AICD and FasL expression by Glu (maximal effect at 10^{-6} M) and prototype and group I mGluR agonists No effect of groups II and III mGluR or iGluR agonists	Chiocchetti et al. (2006)
Adhesion and migration			
Resting	10^{-14}–10^{-4} M Glu iGluR agonists: AMPA, KA	Increase in adhesion to laminin and fibronectin (effective range 10^{-12}–10^{-6} M, maximal effect at 10^{-9} M) Increase in chemotactic migration towars CXCL12/SDF1 at 10^{-8} M	Ganor et al. (2003)
Cytokine secretion			
Anti-CD3-activated	5×10^{-4}–5×10^{-3} M Glu	Increased IFNγ and IL-10 secretion at 10^{-3} M Decreased IFNγ, IL-10 and IL-5 secretion at 5×10^{-3} M	Lombardi et al. (2004)
DC-activated T cells	~10^{-6}–5×10^{-5} M Glu (released from antigen-pulsed DCs) mGluR5 antagonist MPEP 10^{-6} M mGluR1 antagonist CPCCOEt 10^{-4} M	Inhibition of early IL-6 secretion via mGluR5, abrogation of effect via mGluR1 Increase in late IL-6, TNFα, IFNγ, IL-2, IL-10 secretion via mGluR1	Pacheco et al. (2006)

glutamate did not affect the proliferation of normal naïve/resting human T cells (Lombardi et al. 2001, 2004; Pacheco et al. 2004), showing the marked differences between glutamate-induced effects on resting vs. activated T cells. The iGluRs agonists (S)-AMPA and KA, tested at the same concentration range, did not affect the proliferation of PHA-activated T-cells (Lombardi et al. 2004). Interestingly, while the iGluR agonist NMDA was similarly ineffective as AMPA and KA (Lombardi et al. 2004), the NMDA GluR antagonists D-AP5 and (+)-MK801 both inhibited PHA-induced (but not IL-2-induced) T cell proliferation (Miglio et al. 2005b). Together, these results suggest that AMPA and KA iGluRs do not mediate the anti-proliferative effect of glutamate on T cells. The exact contribution

of the NMDA iGluRs to this effect requires further studies, which should better test the effects of several types of NMDA agonists on their own, as well as the effect of glutamate at mid-high and low concentrations, in the absence or presence of NMDA antagonists, on the proliferation (and hopefully also other responses) of resting vs. activated T cells.

In fact, the inhibitory effect of glutamate on the proliferation of activated T cells might be mediated by mGluRs, as the selective mGluR5 agonist CHPG (5×10^{-4} M) inhibited proliferation of anti-CD3-activated T cells (Pacheco et al. 2004). As such inhibitory effect was abrogated by the non-selective mGluR1/5 agonist (S)-3,5-DHPG (10^{-4} M), and coupled to the demonstration that both mGluR5 and mGluR1 are expressed in activated T cells (Table 4.1), these results suggest that glutamate might play a dual role in T cell function: on the one hand inhibiting T cell proliferation via mGluR5, and on the other hand reverting this effect via mGluR1 (Pacheco et al. 2004). Similar observations were indeed reported later, showing that glutamate released by antigen-pulsed DCs (see Sect. 4.6 below) acted initially via mGluR5 to impair T cell proliferation, but later stimulation of mGluR1 abrogated the anti-proliferative effect mediated by mGluR5 to allow robust T cell proliferation (Pacheco et al. 2006).

4.5.2.2 Glutamate at Low-Mid Nanomolar to Micromolar Concentrations Increases iCa^{2+} in Activated T Cells via Ionotropic Receptors. The Effect Is Not Seen in Resting T Cells or at Higher Glutamate Concentrations

Ca^{2+} signaling in response to antigenic stimulation is essential for proliferation of T cells (Guse 1998). Several studies reported that glutamate can clearly affect Ca^{2+} signaling in T cells, and that the effect is once again dependent first on glutamate's concentration, and second on the activation state of the T cells. Thus, at low-mid nanomolar to micromolar concentrations, glutamate increased iCa^{2+} in activated (but not in resting) T cells (Lombardi et al. 2001). This iCa^{2+} potentiating effect induced by glutamate showed a bell-shape concentration-dependent relationship and was effective at 10^{-7}–10^{-5} M with a maximal effect observed at 10^{-6} M. In contrast, glutamate at higher concentrations of 10^{-4}–10^{-3} M was ineffective (Lombardi et al. 2001). The reported iCa^{2+} increase by glutamate in activated T cells was mediated by iGluRs, as the effect was mimicked by the iGluRs agonists NMDA, (S)-AMPA and KA at a similar concentration range, and several iGluRs antagonist and blockers (i.e. D-AP5, (+)-MK801, NBQX and KYNA) inhibited glutamate- and iGluR agonists-mediated iCa^{2+} rise (Lombardi et al. 2001). Interestingly, the prototype mGluR agonist (1S,3R)-ACPD was ineffective at the above concentration range (Lombardi et al. 2001), as well as the non-selective mGluR1/5 agonist (S)-3,5-DHPG, tested at concentrations up to 10^{-4} M (Pacheco et al. 2004).

Taken together with the observations discussed above in part 4.5.2.1, it seems that glutamate induces two different effects on activated T cells, at different concentration ranges and via different GluRs: (1) glutamate at low-mid nanomolar to micromolar concentrations increases iCa^{2+} in activated T cells via iGluRs;

(2) glutamate at a very high millimolar concentration range inhibits the proliferation of activated T cells via mGluR5.

Interestingly, neither of these effects is induced by glutamate in naïve/resting human T cells.

4.5.2.3 Glutamate Affects Kv1.3 Voltage-Gated Potassium Currents in Resting T Cells, and Exerts Opposite Effects at Different Concentrations: Micromolar Glutamate Potentiates, While Higher, up to Millimolar Glutamate, Suppresses T Cell Kv1.3 Currents

How does glutamate increase Ca^{2+} signaling (the effect described above in part 4.5.2.2)? Ca^{2+} influx requires a negative membrane potential as its driving force, which in T cells is provided by voltage-gated K^+ (Kv) channels (Lin et al. 1993), and especially the Kv1.3 channel (Cahalan et al. 2001). Interestingly, micromolar concentrations of glutamate (10^{-6}–10^{-5} M) potentiated Kv1.3 currents in normal resting human T cells, while higher concentrations (up to 10^{-3} M), suppressed such currents (Poulopoulou et al. 2005b). These observations provide a plausible mechanism for the distinct effects of glutamate on Ca^{2+} signaling and proliferation. Unfortunately, the ability of glutamate to modulate Kv1.3 currents was tested only in resting human T cells, and therefore additional studies are necessary to test the effects of glutamate at different concentrations on voltage-gated K^+ currents in activated T cells, and to find out if these correlate with the glutamate-induced effects on iCa^{2+} on the one hand, and on proliferation on the other, in such activated T cells.

4.5.2.4 Glutamate Protects T Cells from Activation-Induced Cell Death (AICD) via Metabotropic Glutamate Receptors, and Also Inhibits FasL Expression Involved in AICD

It is very well known that antigen stimulation and TCR triggering of T cells induce robust proliferation, cytokine secretion, and the upregulation of many other T cell features and functions. In contrast, chronic TCR stimulation, or re-stimulation of T cells within a short period after the first antigenic stimulation, induces apoptosis of the cells via a mechanism termed Activation-Induced Cell Death (AICD), which is crucial for maintenance of peripheral tolerance and for limiting an ongoing immune response (Green et al. 2003). As glutamate at high concentrations clearly suppresses the proliferation of activated T cells (discussed above in part 4.5.2.1), one may speculate that glutamate may also induce T cell apoptosis and promote AICD. Yet, the opposite was found to be true: glutamate at a broad concentration range of 10^{-8}–10^{-4} M suppressed AICD (Chiocchetti et al. 2006). Thus, glutamate in fact seems to contribute to improved/prolonged T cell survival by protecting the cells from apoptosis. Glutamate exerted its maximal effect at 10^{-6} M, and this effect was mimicked by the prototype mGluR agonist (1S,3R)-ACPD, as well as by the non-selective group I mGluR agonists quisqualate and (S)-3,5-DHPG and the selective mGluR5 agonist CHPG (Chiocchetti et al. 2006). In line with that, several group I mGluRs antagonists and blockers (i.e. AIDA, LY367385 and MPEP) antagonized

glutamate- and mGluR agonists-mediated inhibition of AICD in activated T cells (Chiocchetti et al. 2006). The protection of activated T cells from AICD by glutamate was further shown to result from inhibition of FasL expression (Chiocchetti et al. 2006), which is known to be involved in AICD (Green et al. 2003).

Together, the above-discussed data show that:

1. At **low-mid concentration**, glutamate induces the following effects in activated T cells via different GluRs expressed in these cells: (a) Increase in iCa^{2+} via iGluRs; (b) Improved/prolonged survival in conditions of AICD via mGluRs.
2. In contrast, **high concentrations** of glutamate have an anti-proliferative effect on mitogen- or CD3/CD28 Ab-activated T cells, probably via mGluRs.

Such unique ability of glutamate to induce somewhat opposing effects on activated T cells at different dose ranges may be important for either limiting or maintaining ongoing immune responses, respectively. Yet, additional studies are needed for exploring whether glutamate indeed exerts similar effects in-vivo in physiological and/or pathological conditions.

4.5.3 Glutamate at Low Nanomolar Concentrations Induces Adhesion to Fibronectin and Laminin, as well as Chemotactic Migration to CXCL12/SDF-1 of Naïve/Resting Human T Cells, via AMPA Ionotropic Glutamate Receptors

Following our discovery that normal resting human T cells express high levels of iGluR AMPA GluR3 on their cell surface (Ganor et al. 2003), we investigated whether glutamate, operating via such AMPA receptors, can on its own trigger T cell function in naïve/resting T cells. We found that glutamate at low-mid concentrations of 10^{-9}–10^{-6} M induces adhesion of normal resting human T cells to two major glycoproteins of the extracellular matrix (ECM): fibronectin and laminin (Ganor et al. 2003). T-cell adhesion to ECM is a very important and required T cell function, needed for migration, extravasation and homing of T cells into tissues under physiological and pathological conditions. This pro-adhesive effect of glutamate showed a bell-shape concentration-dependent relationship, effective at a very broad low-mid concentration range of 10^{-12}–10^{-6} M, with a maximal effect observed at low 10^{-9} M. Glutamate-induced T cell adhesion to fibronectin and laminin was mediated via AMPA iGluRs, since it was mimicked by the AMPA iGluRs agonists AMPA and KA, and blocked by several AMPA iGluRs antagonist such as CNQX and NBQX. Glutamate induced the adhesion of normal human T cells to the ECM glycoproteins via activating specific adhesion receptors expressed on the T cell surface, namely the $\alpha5\beta1$ and $\alpha6\beta1$ integrins. We proved this by demonstrating that anti-VLA-5 monoclonal antibody, which blocks the $\alpha5$ integrin chain that binds fibronectin, blocked glutamate-induced T cells adhesion to fibronectin, and likewise the anti-VLA-6 monoclonal antibody, which blocks the $\alpha6$ integrin chain that binds laminin, blocked glutamate-induced adhesion to laminin (Ganor et al. 2003). Interestingly, upon TCR activation and granzyme B cleavage of GluR3 from the cell surface, human T cells 'lost' their glutamate-induced adhesion

to laminin (Ganor et al. 2007), suggesting a possible mechanism by which activated T cells shut off the pro-adhesive effect of glutamate via shedding their surface GluR3.

Glutamate on its own (10^{-8} M again) induced an additional very important function: T cell chemotactic migration. Thus, glutamate increased the CXCR4-mediated chemotactic migration of naïve/resting normal human T cells towards the key chemokine CXCL12/SDF-1 (Ganor et al. 2003), which is a key player in cell migration in health and disease. Glutamate-induced T cell chemotaxis was mediated by activation of CXCR4, the specific receptor for CXCL12/SDF-1, expressed on the cell surface of the T cells since anti-CXCR4 monoclonal antibody blocked the effect. These results demonstrate that glutamate, at very low nanomolar concentrations and via acting on AMPA iGluRs highly expressed in normal naïve/resting human T cells, has both a pro-adhesive and a pro-migratory effect on such cells.

How does glutamate affect T-cell integrin function? The answer is still unknown but we speculate that this process may be linked to the depolarization and opening of voltage-gated Kv1.3 channels expressed in naïve/resting T cells, since we previously reported that β1 integrins are physically associated with such channels, and that T cell adhesion can be induced by depolarization (Levite et al. 2000). Hence, a possible scenario might be that glutamate at low concentrations induces depolarization of resting T cells, thereby opening Kv1.3 channels and inducing outward K^+ currents in such cells (Poulopoulou et al. 2005b), and this in turn leads also to integrin activation and to the subsequent T cell adhesion to ECM glycoproteins.

4.5.4 Glutamate Affects the Secretion of Several Cytokines by T Cells

All immune responses absolutely require, and are completely dependent on, autocrine and paracrine cytokine signaling, i.e. the secretion of specific cytokines at the right time and place by immune cells, and the binding of these cytokines to their cognate receptors expressed by other immune cells, as well as on those that secreted them. T cells secrete and respond to a variety of cytokines, and different T cell subpopulations secrete and respond preferentially to different cytokines. $CD4^+$ T helper (Th) cells are crucial for orchestrating the adoptive immune response by recruiting and activating other cells of the immune system, and these activities are also dependent on specific cytokines.

Traditionally, Th cells were classified as either Th1 or Th2 cells based on cytokine secretion, signaling pathways and lineage-specific transcription factors. Th1 cells mainly secrete IFNγ and IL-2 and promote immunity against intracellular pathogens, while Th2 cells secrete IL-4, IL-5, IL-10 and IL-13 and promote humoral responses and the defense against extracellular parasites. More recently, several additional T cell subsets have been identified, among them Th9 and Th17

cells (Nakayama and Yamashita 2010; Zhou et al. 2009). As T cell effector function critically depends on cytokine secretion, any stimuli able to stimulate, modulate or suppress the cytokines secreted by T cells may have important outcomes on the type, efficiency and control of the T cell-mediated immune reactions in response to viruses, bacteria, cancer or any other stimuli.

Can glutamate affect cytokine secretion by T cells? Several studies have provided evidence that glutamate can indeed do so. At high concentrations of 10^{-3} M glutamate increased IFNγ and IL-10 secretion by anti-CD3 activated T cells, but at five times higher concentration glutamate decreased IFNγ, IL-10 and IL-5 secretion by these T cells. Once again, glutamate had different effects on naïve/resting vs. activated T cells, since glutamate had no effect on cytokine secretion by naïve/resting cells (Lombardi et al. 2004). Glutamate-induced suppression of IFNγ secretion by activated T cells may involve iGluRs, as the effect on IL-2-stimulated T cells was mimicked by NMDA at 5×10^{-4} M (Mashkina et al. 2007).

In contrast to these glutamate-induced effects exerted at very high concentrations of $1 - 5 \times 10^{-3}$ M, glutamate at ~1,000-fold lower concentration of 10^{-6} M may operate via mGluRs to modulate IL-6 production and enhance the secretion of TNFα, IFNγ, IL-2 and IL-10 (Pacheco et al. 2006). Glutamate released by antigen-pulsed DCs (see part 4.6.3 below) acted on mGluR5 expressed in T cells, in the context of a DC-T cell co-culture (i.e. the immunological synapse), to impair early IL-6 production. At later time points, when antigen-pulsed DCs induced T cell activation and expression of mGluR1, glutamate operated via mGluR1 to counteract the suppressive effect on IL-6 production and also enhanced the secretion of TNFα, IFNγ, IL-2 and IL-10 (Pacheco et al. 2006). Of note, although the levels of glutamate secreted by DCs to the co-culture media were estimated at the micromolar range, the actual concentration of glutamate within the immunological synapse might in fact be higher. Taken together, the studies cited above show clearly that glutamate has the ability to increase or suppress the secretion of several key T cells cytokines. Yet, further studies are needed to elucidate the exact effects exerted by glutamate at different concentrations on the secretion of specific cytokines by various T cell subpopulations, among them Th1, Th2, Teffs, Tregs, Th17, $CD4^+$, $CD8^+$ and others. Exploring these effects can be very rewarding scientifically and even clinically.

4.5.5 Proposed Summary for the Effects of Glutamate on T Cell Function

Based on the multitude of studies discussed above, we propose the following model to describe the functional dialogues between glutamate, its receptors – the different types of GluRs – and human T cells (Fig. 4.2), taking place in three different conditions:

Condition 1: Activated T cells encountering physiological mid micromolar levels of glutamate

Activated T cells express on their cell surface: (1) NMDA iGluR subunits NR1 and NR2A/B/D; (2) AMPA iGluRs that do not contain the GluR3 subunit; (3) mGluR5 and mGluR1. The expression of these GluRs allows the activated T cells to respond to the physiological levels of glutamate at a mid concentration range (10^{-6}–10^{-4} M) present in blood. Such interactions lead to increased iCa^{2+} (via iGluRs) and decreased AICD (via mGluRs) of the activated T cells.

All these glutamate-induced effects may contribute to an improved T cell survival and function following the encounter of T cells with various antigens derived either from invading microbial and/or viral threats, or from tumor antigens, when the cancer is attacked by T cells. One may envision that glutamate-induced protection of activated T cells from AICD may be especially important in conditions of chronic/repeated T cell exposure to such antigens.

Condition 2: Activated T cells encountering pathological excess millimolar levels of glutamate

Activated T cells express mGluRs that allow the cells to respond to elevated levels of glutamate (10^{-3} M concentration range), present in the plasma and/or in the CNS in various pathologies. Activated T cells are expected to encounter such excess glutamate in these two very different body locations, i.e. blood and CNS, since they are constantly migrating in the circulation, and routinely cross the blood–brain barrier (BBB) and enter the CNS for immunosurveillance and neuroprotection in physiological and pathological conditions. The interaction of mGluRs expressed by activated T cells with excess glutamate present in many pathological conditions may have functional outcomes, leading to decreased T cell proliferation and increased cytokine production. These could be important for preventing or controlling the expansion of the activated cells on the one hand, and for improved combat of diseases on the other.

Condition 3: Naïve/resting T cells encountering physiological low levels of glutamate

At normal conditions, most of the T cells in the body are in a naïve/resting a state. Furthermore, few days after TCR activation, T cells revert to a naïve/resting phenotype. We found that in parallel to that reversion, T cells regain their expression of the AMPA GluR3 that was cleaved of their cell surface by granzyme B after their TCR activation. Yet, the cells lose now their expression of mGluR1. So some GluRs are regained, while others are lost upon entering the resting phase. The exposure of naïve/resting T cells to the low glutamate levels present in the healthy brain fluids triggers their adhesion to fibronectin and laminin, and induces their chemotactic migration towards key chemokines present in the CNS. This could assist T cells in their patrol and survey of the CNS, and maybe also in their exit back to the periphery. As the levels of glutamate in blood are higher under normal physiological conditions (at the micromolar range), the interactions between such high glutamate and resting T cells in the periphery may not necessarily be productive.

4.6 Production of Glutamate by Immune Cells

Neuronal cells produce glutamate, and this is most probably the major source for glutamate under physiological conditions. Upon release, glutamate exerts various effects on neuronal cells, as well as on other target cells among them immune cells, as discussed above. In addition, published evidences point now to the ability of various immune cells to release glutamate from their own sources. The main evidences supporting this notion are summarized below.

4.6.1 Neutrophils Secrete Glutamate, Which in Turn Decreases Endothelial Cell Permeability

Neutrophils are the most common type of white blood cells, comprising about 50–70% of all white blood cells. Neutrophils are phagocytic cells that can ingest other cells, though they do not survive the act. Neutrophils are the first immune cells to arrive at a site of infection through chemotaxis. Activated neutrophils were shown to secret glutamate, which subsequently acted on mGluR1, 4 and 5 expressed by human brain and dermal microvascular endothelial cells, resulting in decreased cell permeability (Collard et al. 2002). These findings suggest that neutrophil-derived glutamate may be an important factor that alters endothelial cell permeability during injury and/or inflammation. Whether this process contributes, for instance, to the in vivo transmigration and entry of activated T cells across blood vessels into the brain, is an important yet still open question that surely deserves more investigation.

4.6.2 Monocytes/Macrophages and Activated Microglia Cells Secrete Glutamate, Which in Turn Leads to Neuronal Death

Early observations suggested that activated microglia (featuring a phenotype similar to brain macrophages) could secrete glutamate, which acts on NMDA iGluRs to induce neuronal cell death (Piani et al. 1991, 1992). These results were later confirmed by many studies, demonstrating that the activation of both monocytes/macrophages and microglia may lead to the secretion of NMDA iGluR-activating substances, including glutamate itself, which in turn are able to cause excitotoxic neuronal death. Importantly, this process, by which macrophages/microglia-derived glutamate leads to neuronal death, is accepted as an important process contributing to neuronal damage in human immunodeficiency virus type 1 infection (for review see Kaul et al. 2005).

4.6.3 Immature Dendritic Cells Release Low Levels of Glutamate, While Activated Mature Dendritic Cells Release Much Higher Levels of Glutamate

A recent study reported that immature DCs were able to release low levels of glutamate. Interestingly, DC maturation (induced by either lipopolysaccharide [LPS], TNFα, or the superantigen staphylococcal enterotoxin A [SEA]) markedly enhanced the capacity of DCs to release glutamate (Pacheco et al. 2006). Moreover, glutamate was also released by antigen-pulsed DCs that were co-cultured with T cells, suggesting that glutamate release from DCs is a general feature of antigen presentation across the immunological synapse formed between DCs and T cells. More recently, these results were confirmed by showing that mouse DCs also secret glutamate, which in turn acted on mouse thymocytes in the context of the immunological synapse (Affaticati et al. 2011).

4.7 Involvement of Glutamate and Its Receptors in Cancer, Autoimmune Diseases and Human Immunodeficiency Virus Type 1 Infection

4.7.1 Cancer

4.7.1.1 Glutamate Antagonists Block the Growth of Solid (Non Immune) Tumors: Glioma, Breast and Lung Carcinoma, Colon Adenocarcinoma and Neuroblastoma

Clear evidence has accumulated to support a role of glutamate and its receptors, both iGluRs and mGluRs, in promoting tumor growth, while glutamate antagonists block it. Few examples include the following: (1) Blocking Ca^{2+}-permeable AMPA iGluRs in human glioblastoma cells inhibited cell locomotion and induced apoptosis (Ishiuchi et al. 2002); (2) Treatment of gliomas with the NMDA iGluR antagonists MK801 or memantine slowed tumor growth (Takano et al. 2001); (3) The NMDA iGluR antagonist dizocilpine exerted an anti-proliferative effect preferably on colon adenocarcinoma, astrocytoma, and breast and lung carcinoma cells. In addition, breast and lung carcinoma, colon adenocarcinoma and neuroblastoma cells responded most favorably to the AMPA iGluR antagonist GYKI52466. Such anti-proliferative effects of iGluR antagonists were Ca^{2+}-dependent and resulted from decreased cell division and increased cell death (Rzeski et al. 2001; Stepulak et al. 2005); (4) Blocking group II mGluRs reduced the growth of glioma cells in-vivo (Arcella et al. 2005), and mGluR3 and mGluR4 controlled the proliferation of brain tumor cells (Nicoletti et al. 2007). Importantly, many studies have also shown that GluRs are expressed in a variety of neuronal and non-neuronal cancer cell lines and tumors (Kalariti et al. 2005; Stepulak et al. 2009).

4.7.1.2 T-leukemia and T-lymphoma Express Ionotropic and Metabotropic Glutamate Receptors, and Glutamate, via AMPA Receptors, Promotes These Cancerous T Cells by Augmenting Their in vivo Extravasation and Their Expression of Matrix Metalloproteinase 9 (MMP-9) and CD147. Glutamate, via Metabotropic Receptors, Also Augments iCa^{2+} and the Expression of the Early Ca^{2+}-Inducible Genes c-fos and c-jun in T-leukemia

Various cancerous immune cells express GluRs, as shown in Table 4.1. Moreover, we showed in our own studies that human T-leukemia (Jurkat) and T-lymphoma (HuT-78) cell lines express on their cell surface high levels of the specific AMPA iGluR GluR3 subunit (Ganor et al. 2003, 2009), the very same iGluR subunit we found in high levels in resting normal human T cells (Ganor et al. 2003, 2009).

Human T-leukemia cells (Jurkat) express also the AMPA iGluR GluR2 and GluR4 subunits (Stepulak et al. 2009); the KA iGluR GluR6, GluR7, KA1 and KA2 subunits (Stepulak et al. 2009); and the NMDA iGluR NR1, NR2A-D and NR3A, B subunits (Miglio et al. 2007, 2005b; Stepulak et al. 2009). Group I mGluRs are also expressed in several human T-cancerous cell lines: mGluR5 in T-leukemia (Jurkat, FRO) and T-lymphoma (H9, HuT-78), and mGluR1 in T-leukemia (Jurkat, FRO, SUP-T1) and T-lymphoma (HuT-78) cell lines (Pacheco et al. 2004; Chiocchetti et al. 2006). Another study confirmed the expression of group I mGluRs in T-leukemia (Jurkat) cells, and further demonstrated that these cells also express group I and II mGluRs (Stepulak et al. 2009). In addition, mGluRs are expressed by other types of cancerous immune cells, which are not of T cell origin: both group I mGluR5 and mGluR1 were shown to be expressed in human monocytic leukemia cells (THP-1) (Chiocchetti et al. 2006), and mGluR5, but not mGluR1, was reported in B lymphoblast SKW6.4 cells (Pacheco et al. 2004).

Coupled with the early studies reporting on elevated plasma glutamate levels in patients with malignancies (Droge et al. 1988; Eck et al. 1989a; Ollenschlager et al. 1989), the above observations suggest an important role for glutamate/GluRs in tumor biology, including T cell cancers. Indeed, several studies have clearly demonstrated that glutamate affects directly the behavior of T-leukemia and T-lymphoma cells (illustrated schematically in Fig. 4.3).

Investigating the effect of glutamate on iCa^{2+} rise in human T-leukemia Jurkat cells, Miglio et al. showed that group I mGluRs, i.e. mGluR5 and mGluR1 are expressed in Jurkat cells (see above), and evoke iCa^{2+} increase. Thus, at a broad concentration range of 10^{-7}–10^{-3} M, the prototype mGluR agonist (1S,3R)-ACPD, the non-selective mGluR1/5 agonist (S)-3,5-DHPG, and the selective mGluR5 agonist CHPG produced such effect (Miglio et al. 2005a). Moreover, several selective group I mGluR antagonists (i.e. AIDA, LY367385, MPEP) antagonized the effect of DHPG (Miglio et al. 2005a). The reported rise in iCa^{2+} resulted also in up-regulation of the early Ca^{2+}-inducible genes *c-fos* and *c-jun* (Miglio et al. 2005a). As the protein products of these genes play important roles in the regulation of the cell cycle (Tay et al. 1996), these findings suggest that glutamate might

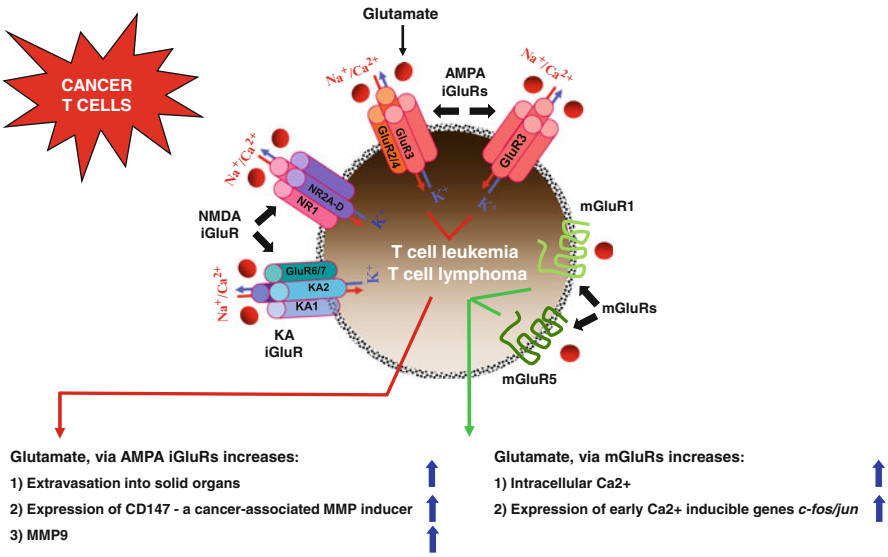

Fig. 4.3 Glutamate promotes T leukemia and T lymphoma via iGluRs and mGluRs expressed in these cells. Glutamate, in nanomolar range, such as that found primarily in the CSF and brain extracellular fluids (Meldrum 2000), activates its AMPA iGluRs expressed in T leukemia and T lymphoma and induces the following: (1) Elevated extravasation in-vivo into solid organs; (2) Elevated expression of CD147, a cancer-associated MMP inducer and MMP9, also associated with cancer metastasis (Ganor et al. 2009). Also, glutamate, via mGluRs expressed in T leukemia and T lymphoma induces the following: (1) Elevated intracellular Ca^{2+}; (2) Elevated expression of the early Ca^{2+} inducible genes c-fos and c-jun (Miglio et al. 2005a)

modulate Jurkat cell function by activating multiple downstream signaling events that regulate cell proliferation and cytokine mRNA transcription.

Miglio et al. further showed that the NMDA iGluR antagonists (+)-MK801 and D-AP5 at concentrations of $1 - 5 \times 10^{-4}$ M, inhibited the growth of Jurkat T-leukemia cells by promoting their apoptosis (Miglio et al. 2007). Interestingly, direct treatment with either glutamate or NMDA induced adhesion of the T-leukemia cells to fibronectin. This pro-adhesive effect showed a bell-shape concentration-dependent relationship, was effective at 10^{-8}–10^{-5} M with a maximal effect observed at 10^{-6} M glutamate and 10^{-5} M NMDA, and was blocked by the NMDA iGluRs antagonists (+)-MK801 and D-AP5 (Miglio et al. 2007). As we previously reported that glutamate induces such pro-adhesive effect also in normal resting non-cancer human T cells (Ganor et al. 2003), these observations suggest that activation by glutamate of iGluRs in T cells, and possibly other integrin-expressing cells, is a common mechanism by which glutamate controls T cell adhesion.

Finally, our own studies revealed several other cancer-promoting functional outcomes of the interaction between glutamate and its AMPA iGluRs expressed in human T cell cancers. Thus, ex vivo treatment of Jurkat human T-leukemia cells with glutamate at low concentration of 10^{-8} M enhanced their subsequent in-vivo

engraftment into chick embryo liver and chorioallantoic membrane (Ganor et al. 2009). In correlation with this pro-metastatic in-vivo effect, glutamate also induced in-vitro a significant elevation of the cancer-associated matrix metalloproteinase (MMP) inducer CD147, as well as increased the secretion of the cancer-associated MMP-9 in the human T-cancerous cells (Ganor et al. 2009). These multiple and potent glutamate-induced effects were mediated by iGluR AMPA receptors expressed in these cancerous T cells, since the effect was mimicked by AMPA, and blocked by the AMPA antagonist CNQX (Ganor et al. 2009). These findings suggest that glutamate may facilitate the spread of T-leukemia and T-lymphoma in-vivo and their penetration from the circulation into solid organs.

Importantly, the reported pro-adhesive and pro-metastaic effects of glutamate on T cell cancers seem to be operational at low concentrations of 10^{-9} M, such as those found primarily in the CSF and brain extracellular fluids (Meldrum 2000). This argues that increased engraftment of T cell cancers would reach its optimum within the CNS, after such immune cancer cells cross the BBB, enter the nervous system, and encounter glutamate that will operate via iGluRs. Importantly, in the periphery, the interaction of T cell cancers with glutamate would rather increase their proliferation, in line with all the above-mentioned findings demonstrating that GluR antagonists limit tumor growth. Such increased glutamate-mediated cancer cell proliferation would probably be operational at higher glutamate levels, since a multitude of early studies clearly indicated that glutamate levels might increase in the plasma of patients with malignancies (Droge et al. 1988; Eck et al. 1989a; Ollenschlager et al. 1989).

4.7.2 Multiple Sclerosis

Multiple Sclerosis (MS) and its animal model Experimental Autoimmune Encephalomyelitis (EAE) are demyelinating diseases caused primarily by autoreactive T cells, which enter the CNS and attack the nerve enwrapping myelin sheath (Hemmer et al. 2002; Merrill and Benveniste 1996). In addition, myelin-producing cells of the CNS (i.e. oligodendrocytes) and some axons are lost as a result of the inflammatory attack on the CNS.

4.7.2.1 AMPA GluR3 Is Highly Expressed in Mouse EAE-Inducing Anti-myelin Basic Protein T Cell Mouse Clones; Treatment of EAE-Afflicted Mice and Rats with Antagonists of AMPA Ionotropic Glutamate Receptors Results in Substantial Amelioration of the Disease

Interestingly, two previous studies reported that treatment of mice (Pitt et al. 2000) or rats (Smith et al. 2000) sensitized for EAE, with NBQX, the AMPA iGluR antagonist, resulted in substantial amelioration of disease. The authors concluded that NBQX was beneficial for EAE since it blocked AMPA iGluRs expressed in neuronal or glia cells.

In addition to this interpretation and conclusion, we proposed that NBQX suppressed EAE in these studies because on top of inhibiting GluRs in neurons and glia, it blocked AMPA iGluRs expressed in the autoagressive encephalitogenic T cells that cause the disease. Hence, we believe that by blocking the T cell expressed AMPA iGluRs, NBQX could have prevented the activation of the autoagressive T-cells by glutamate released from nerve endings at the sites of inflammation/damage in the CNS, thereby reducing their pathogenic potential and conferring EAE suppression (Ganor et al. 2003). Our proposal is based on: (1) our demonstration that iGluR AMPA GluR3 is highly expressed not only in normal human T cells, but also in mouse encephalitogenic EAE-inducing anti-myelin basic protein T cell clones (Ganor et al. 2003); (2) the evidences discussed above in parts 4.5.2.2, 4.5.2.4, and 4.5.4, showing the multiple beneficial effects of glutamate at low-mid concentration on activated T cells. For reminder, glutamate-induced effects on activated T cells, mediated by various types of GluRs, include increase in iCa^{2+} currents and the improvement/prolongation of T cell survival in conditions of AICD by protection from apoptosis and inhibition of FasL expression. In view of all these, one can envision that a glutamate antagonists able to prevent the beneficial effects of glutamate on activated T cells would also be able to inhibit activated autoimmune cells in EAE/MS.

4.7.2.2 Lymphocytes of MS Patients Express iGluR AMPA GluR3, Which Is Upregulated During Relapse and in Patients with Neurological Evidence of Disease Activity. Furthermore, Glutamate Augments the Proliferation and Chemotactic Migration of the Autoreactive T Cells

In line and confirmation with the observations described above in part 4.7.2.1, a later important study by Sarchielli et al. demonstrated that iGluR AMPA GluR3 is expressed in lymphocytes of MS patients, and that its expression is upregulated during relapse and in patients with neurological evidence of disease activity (Sarchielli et al. 2007). An equally exciting additional finding made in this study was that either glutamate or AMPA (10^{-8}–10^{-5} M) enhanced the proliferation of the autoreactive T cells in response to myelin-derived proteins, and also augmented the chemotactic migration of these autoimmune T cells towards CXCL12/SDF-1 (Sarchielli et al. 2007). Another study also reported that the inhibition of PHA-induced cell proliferation caused by 10^{-3} M glutamate (see above) was lower in T cells of MS patients (Lombardi et al. 2003).

Interestingly, adhesion to laminin in the CNS plays a crucial role in the recruitment, transmigration and penetration of myelin autoreactive T cells: the parenchymal basement membranes containing certain laminin isoforms were found to be permissive for encephalitogenic T cell transmigration, while those containing others were restrictive (Sixt et al. 2001). Based on our previous findings, showing that glutamate on its own induces adhesion of naïve/resting human T cells to laminin, as well as to fibronectin (Ganor et al. 2003), we speculate that encounter of resting T cells with glutamate could cause their adhesion to laminin-containing

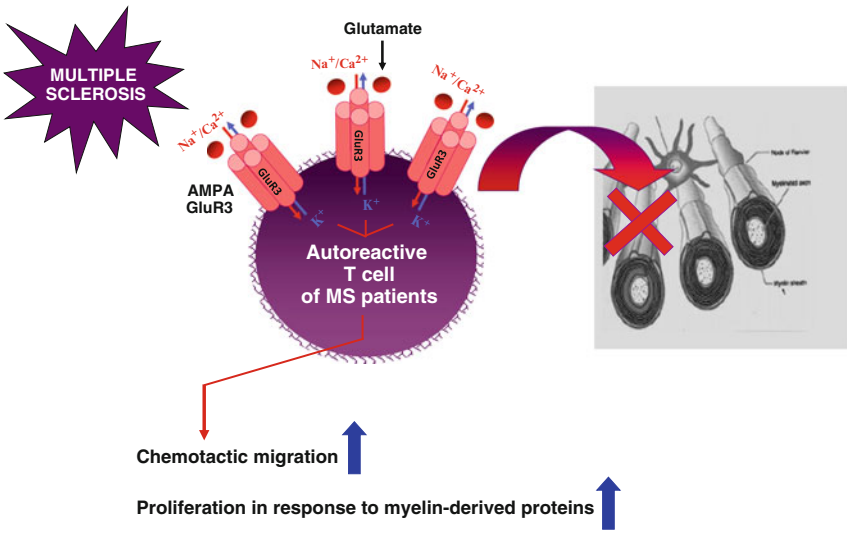

Fig. 4.4 Glutamate and its receptors may play an important role in Multiple Sclerosis (MS) and Experimental Autoimmune Encephalomyelitis (EAE). The main evidences supporting an important role played by glutamate and its AMPA iGluRs in MS and EAE are the following: (1) The iGluR AMPA GluR3 subunit is expressed in lymphocytes of MS patients, and its expression is upregulated during relapse and in patients with neurological evidence of disease activity (Sarchielli et al. 2007); (2) Glutamate or AMPA (10^{-8}–10^{-5} M) enhances the proliferation of the autoreactive T cells in response to myelin-derived proteins, and also augments the chemotactic migration of these autoreactive Tcells towards CXCL12/SDF-1 (Sarchielli et al. 2007); (3) AMPA GluR3 is expressed in mouse encephalitogenic anti-myelin basic protein T cell clones (Ganor et al. 2003); (4) There is an abnormal response to glutamate of T lymphocytes from MS patients (Lombardi et al. 2003); (5) Glutamate/AMPA receptor antagonists block EAE in mice and rats (Pitt et al. 2000; Smith et al. 2000)

brain parenchyma, and could further promote their directional migration towards chemokines secreted in specific sites within the CNS in MS.

Together, these findings support the idea that glutamate-induced activation of MS-associated T cells may indeed play a vital role in MS (illustrated schematically in Fig. 4.4), and if this is indeed true, blocking glutamate signaling in MS may turn to be beneficial.

4.7.3 Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is a chronic systemic inflammatory disorder that may affect many tissues and organs, but principally attacks synovial joints. The process produces an inflammatory response of the synovium (synovitis) secondary to

hyperplasia of synovial cells, excess synovial fluid, and the development of pannus in the synovium. Although the cause of RA is unknown, autoimmunity plays a pivotal role in both its chronicity and progression, and RA is considered a systemic autoimmune disease. A recent review discusses a possible involvement of glutamatergic signaling machineries in the pathophysiology of RA (Hinoi and Yoneda 2011). The authors cover recent molecular/biological analyses, including their own, which propose a novel function for glutamate as an extracellular signal mediator operating in an autocrine and/or paracrine manner in several peripheral and non-neuronal tissues, including the bone and cartilage. In particular, a drastic increase was demonstrated in the endogenous levels of both glutamate and aspartate in the synovial fluid with intimate relevance to increased edema and sensitization to thermal hyperalgesia in experimental arthritis models. In their review, the authors outline the role of glutamate in synovial fibroblasts in addition to the possible involvement of glutamatergic signaling machineries in the pathogenesis of joint diseases such as RA.

4.7.4 Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is a systemic autoimmune disease (or autoimmune connective tissue disease) that can affect any part of the body. SLE most often harms the heart, joints, skin, lungs, blood vessels, liver, kidneys, and nervous system. As occurs in other autoimmune diseases, the immune system attacks the body's cells and tissues, resulting in inflammation and tissue damage. It is a type III hypersensitivity reaction caused by antibody-immune complex formation. In a recent study, high-performance liquid chromatography was used for glutamate measurements in freshly isolated, non-cultured peripheral T cells of patients with SLE and healthy controls. The authors found that the mean ± SD serum concentrations of glutamate were lower in patients with either clinically quiescent SLE (77 ± 27 μM [n = 18]) or active SLE (61 ± 36 μM [n = 16]) than in healthy controls (166 ± 64 μM [n = 24]) (Poulopoulou et al. 2008).

4.7.5 'Autoimmune Epilepsy' and 'Autoimmune Encephalitis' Mediated by Glutamate Receptor Antibodies

It is known and widely accepted that excess glutamate contributes to epilepsy. In fact, epilepsy is conceived as a disease caused by either too much glutamate-mediated excitation or too low gamma-amino-butyric acid (GABA)-mediated inhibition. In line with this notion, excess glutamate or its agonists are highly neurotoxic and causes seizures in animal models. For decades, epilepsy was considered a brain disorder caused solely by various neurological factors, and not by any immunological factors like antibodies (Abs). Breaking this dogma, a series of studies and publications by several groups including ours over the last ~15 years provided plentiful evidence in support of 'Autoimmune Epilepsy', a term coined in 2002 by M. Levite (Levite 2002) and used in subsequent studies (Ganor et al. 2004,

2005a), referring to human epilepsies in which deleterious Abs, primarily iGluR Abs, are found in serum and/or CSF of the epilepsy patients, and where such Abs are suspected to contribute to the initiation and/or worsening of the seizures themselves, and/or to any of the neuropsychobehavioral impairments that often accompany the seizures (Levite and Ganor 2008). For review of the vast majority of relevant publications on the topic of 'Autoimmune Epilepsy' and GluR Abs in human diseases the reader is refereed to (Levite and Ganor 2008; Vincent et al. 2011) and for some original papers to (Andrews et al. 1996; Andrews and McNamara 1996; Antozzi et al. 1998; Baranzini et al. 2002; Ganor et al. 2004, 2005a; Levite et al. 1999; Levite and Hermelin 1999; Mantegazza et al. 2002; Rogers et al. 1994; Twyman et al. 1995; Tziperman et al. 2007; Wiendl et al. 2001).

In summary, three types of GluR Abs were found thus far in patients with epilepsy, encephalitis and/or SLE, and most if not all of them can cause brain damage. The main features of these three types of GluR Abs are summarized below, as well as shown in schematically Fig. 4.5 and its detailed legend.

1. **Anti-AMPA-GluR3 (GluR3) Abs**. These Abs are directed primarily against amino acids (aa) 372–395 of GluR3, termed GluR3B peptide, or against aa 245–274 of GluR3 termed GluR3A peptide. The GluR3 Abs, mainly GluR3B Abs, were found thus far in ~30% of patients with different types of epilepsy. Furthermore, studies of our own group, as well as of others, showed that these GluR3 or GluR3B Abs can often activate GluRs and damage neurons and glia in-vitro and in-vivo. Thus, GluR3B Abs bind to neurons, possess a unique ability to activate their glutamate-receptor antigen, kill neurons and glia in-vitro and in-vivo (either by excitotoxicity or by complement fixation independent of receptor activation), and cause multiple brain damage. In animal models (mice, rats or rabbits), GluR3 Abs may cause seizures, augment their severity or modulate their threshold (for review of all these effects see (Levite and Ganor 2008) and the studies cited therein).

   Interestingly, in one of our studies we showed by electrophysiological recordings that affinity purified GluR3B Abs on their own activate GluR3-containing homomeric and heteromeric AMPA receptor complexes and induce the characteristic ion currents, without the requirement of neuronal, glial or blood ancillary molecules. Furthermore, the ion currents induced by GluR3B Abs were completely blocked by CNQX, a selective AMPA agonist (Cohen-Kashi Malina et al. 2006). Thus, GluR3B Abs can partially mimic glutamate.

   In other studies, we showed that GluR3B-immunized mice and rats exhibited significant brain pathology in-vivo (Ganor et al. 2005b; Levite and Hermelin 1999). The neuropathological findings included, for example, thickening of cerebral meninges with lymphocyte infiltrates, cerebellar cortical abiotrophy with loss of Purkinje cells, occasional reactive gliosis, and loss of mature neurons compensated by increased neurogenesis. In a recent still unpublished study, we also observed that GluR3B-immunized mice exhibit lower threshold to chemoconvulsant-induced seizures, and suffer from several neuropsychiatric and behavioral impairments compared to several control groups (Goldberg H, Ganor Y and Levite M, paper in preparation). Finally, in this study we also found

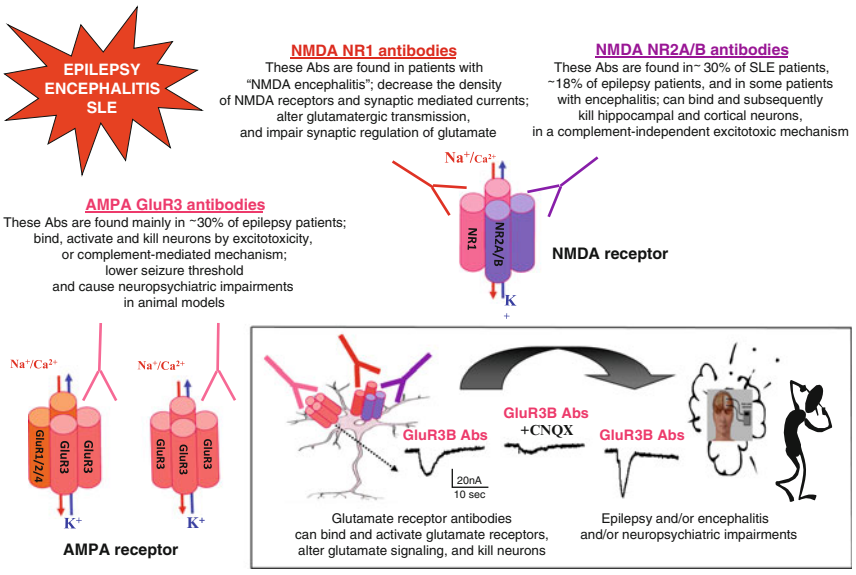

Fig. 4.5 Glutamate receptor autoantibodies (Abs) of several types are found in patients with Epilepsy, Encephalitis and Systemic Lupus Erythematosus (SLE), and can cause brain damage. Three types of glutamate receptor Abs were found thus far in patients with epilepsy, encephalitis and/or (SLE), and they are all detrimental to the CNS. The three glutamate receptor Abs types are: (1) **AMPA GluR3 Abs**. GluR3 Abs, especially those directed against the B peptide of GluR3, termed GluR3B Abs, are found mainly in ~30% of epilepsy patients. These AMPA GluR3 Abs bind, activate and kill neurons, and cause brain pathology in animal models (for review see Levite and Ganor 2008). In addition, we recently found in GluR3B-immunized mice that GluR3B Abs lower seizure threshold and cause neuropsychiatric and behavioral impairments, and are also frequent among epilepsy patients with neuro/psycho/cognitive impairments (Goldberg et al., paper in preparation). In addition, by electrophysiological recordings we showed that affinity purified GluR3B Abs activate GluR3-containing homomeric and heteromeric AMPA receptor complexes and induce the characteristic ion currents, without the requirement of neuronal, glial or blood ancillary molecules. Furthermore, the ion currents induced by the GluR3B Abs were completely blocked by CNQX, a selective AMPA agonist (Cohen-Kashi Malina et al. 2006) (*insert*). (2) **NMDA R1 (NR1) Abs**. These Abs are directed against the NR1 subunit of the NMDA iGluRs. According to several recent studies, the NMDA NR1 Abs cause 'Anti-NMDA receptor encephalitis' – a severe, treatable and potentially reversible disorder presenting with memory deficits, psychiatric symptoms, autonomic instability including hypoventilation and seizures (Dalmau et al. 2011; Florance et al. 2009; Irani and Vincent 2011). (3) **NMDA NR2A/B Abs**. These Abs are directed against the NR2 subunit of the NMDA iGluRs and were found thus far in ~30% of SLE patients (with our without neuropsychiatric problems), ~18% of epilepsy patients, and in some patients with encephalitis. Once they reach the CNS, the NMDA NR2A/B Abs can bind and subsequently kill neurons in the hippocamous or amygdala via induction of excitotoxicity and apoptosis (Huerta et al. 2006; Kowal et al. 2004; Levite and Ganor 2008)

a striking, surprising and significant correlation in epilepsy patients between the presence of GluR3B Abs in their serum and the presence of their neuro/psycho/cognitive impairments. For remider: various neuropsychiatric impairment sometimes accompany the seizures.

2. **Anti-NMDA-R1 (NR1) Abs**. These Abs are directed against the NR1 subunit of NMDA iGluRs. According to several recent studies, NMDA NR1 Abs cause 'Anti-NMDA receptor encephalitis' - a severe, treatable and potentially reversible disorder presenting with memory deficits, psychiatric symptoms, autonomic instability including hypoventilation and seizures (Dalmau et al. 2011; Florance et al. 2009; Irani and Vincent 2011). Importantly, Hughes et al. demonstrated recently that patient's NMDA receptor Abs cause a selective and reversible decrease in NMDA surface receptors and synaptic localization that correlate with patient's Abs titers (Hughes et al. 2011). The authors further found that the mechanism of this decrease is selective Ab-mediated capping and internalization of surface NMDA receptors (since Fab fragments from patient's Abs did not decrease NMDA receptor density). This is in line with another study of this group (Moscato et al. 2011). Furthermore, in another study, Manto et al. showed that CSF of patient with NMDA Abs and encephalitis increased the concentrations of glutamate in the extracellular space. The increase was dose-dependent and was dramatic with purified IgG. Patient's CSF also impaired both the NMDA- and the AMPA-mediated synaptic regulation of glutamate (Manto et al. 2011). Taken together, all these findings show that NR1 Abs can clearly impair glutamate signaling and cause neurological abnormalities.
3. **Anti-NMDA-NR2A/B (NR2) Abs**. These Abs are directed against the NR2 subunit of the NMDA iGluRs. They were found in 18% of epilepsy patients tested thus far, ~30% of SLE patients (with our without neuropsychiatric problems), and in some patients with encephalitis. Once the NR2A/B Abs reach the CNS, they can bind and subsequently kill neurons in the hippocamous or amygdala via induction of excitotoxicity and apoptosis (Huerta et al. 2006; Kowal et al. 2004; Levite and Ganor 2008).

In summary, the three types of GluR Abs, directed against AMPA GluR3, NMDA NR1 or NMDA NR2A/B, are found in human patients, mainly suffering from epilepsy and encephalitis, and are detrimental to the nervous system.

What triggers the autoimmune production of all these GluR Abs is still unknown. Yet, at least for the AMPA GluR3 Abs, we proposed that the AMPA GluR3 expressed in peripheral T cells may be the primary source of the GluR3-derived antigens for these Abs, which later damage neurons. We also proposed that the cleavage of the AMPA GluR3 from the surface of activated T cells by granzyme B that is produced and released by such activated cells, and the subsequent shedding of GluR3B-containing fragments into the extracellular milieu is a key immunogenic event, which at certain inflammatory conditions leads to the production of the detrimental GluR3B Abs (Ganor et al. 2007). We further speculate that the genetic background of the individual may play a role too in dictating whether autoimmunity to GluR3 Abs will develop or not. Our suggested models/scenarios for the production of GluR3 Abs are drawn schematically and discussed in detail in our recent review (Levite and Ganor 2008). All the above suggestions call for further studies.

4.7.6 Human Immunodeficiency Virus Type 1 (HIV-1) Infection

Patients who are infected with the human immunodeficiency virus type 1 (HIV-1) were found to have, on average, markedly elevated plasma glutamate levels (Eck et al. 1989b). Moreover, increased glutamate levels were reported in CSF of patients with HIV-1 dementia. Importantly, the levels of glutamate in the CSF, but not in the plasma, were related to the degree of dementia and brain atrophy. Of remider: HIV-1 reaches the brain following entry of HIV-1-infected monocytes/macrophages across the BBB. Although neurons are not infected with HIV-1, the predominant pathways to neuronal injury in HIV-1 dementia include indirect effects through release of macrophage, microglial and astrocyte toxins, as well as direct injury by viral proteins. Among the released toxins is glutamate, which is produced by macrophages themselves (see Sect. 4.6 above) and overstimulates neuronal GluRs, resulting in neuronal death by excitotoxicity, similar to the events taking place in other neurodegenerative diseases (for review see Kaul et al. 2005). These observations suggest that increased CSF glutamate, most probably produced locally by infected macrophages (and other cell types) plays a pathogenic role in HIV-1 dementia, thus supporting the treatment of these patients with GluR antagonists.

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GABA Is an Effective Immunomodulatory Molecule in the Brain and in the Periphery

5

Zhe Jin, Suresh Kumar Mendu, Amol Bhandage, and Bryndis Birnir

Contents

5.1 Introduction

For a long time it was thought that the brain was an immune privilege organ. In recent years it has become increasingly clear that there is an extensive cross-talk between the nervous and the immune system and somewhat surprisingly, the immune cells themselves do appear to express components of neuronal neurotransmitters systems that may be regulated by the neurotransmitters themselves. What function the neurotransmitters, their ion channels, receptors and transporters have in immune function and regulation is an emerging field of study. Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian central nervous system (CNS). GABA is made and released by a variety of interneurons and some projection neurons in the brain and GABA activates GABA-A and GABA-B receptors. The GABA-A receptors are pentameric chloride channels that can be made from 19 different subunits that are grouped into eight different families (α1-6, β1-3, γ1-3, δ, ε, π, θ, ρ1-3) (Olsen and Sieghart 2009). Normally the channels are heteropentameres formed by two alphas, two betas and a third subunit-type with the exception of channels formed by the

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ρ subunit, they can be homomeric. All neurons have GABA-A ion channels but the subtypes expressed by the different regions in the brain and by the different types of neurons varies. The channels are either located at synapses where they generate phasic currents or outside of synapses where they are termed extrasynaptic channels and generate tonic currents (Birnir and Korpi 2007). The GABA-B receptor is a G-protein coupled receptor and has a modulatory effect on neuronal excitability (Marshall et al. 1999). GABA is produced by decarboxylation of the amino acid glutamate by the enzyme glutamic acid decarbolylase (GAD) that exists in two isoforms GAD65 and GAD67. The two GAD isoforms have different subcellular location with GAD67 distributed evenly throughout the neuronal cytoplasm whereas the GAD65 is often associated with synaptic vesicles (Buddhala et al. 2009). GABA is metabolized into succinic semialdehyde by the action of the enzyme GABA transaminase (GABA-T).

In the past, activation of GABA-A channels was thought to be solely inhibitory but it is now clear that the intracellular chloride concentration can vary during development and even within individual neurons can intracellular chloride gradients exist (Zilberter et al. 2010). As a consequence of this, activation by GABA of the GABA-A channels can lead to either depolarization or hyperpolarization of the membrane potential leading to excitation or inhibition, respectively, of the neuron (Fig. 5.1).

GABA is not only present within the CNS but has also been identified in many organs such as the pancreas, pituitary, testes, gastrointestinal tract, ovaries, placenta, uterus and adrenal medulla (Gladkevich et al. 2006) and at least cells in the pancreatic islets, adrenal gland and testes express GAD. In recent years it has become evident that cells of the immune system may also produce GABA and express the GABA-A ion channels, GABA transporters and GABA-B receptors (Table 5.1). A misconception that has contributed to the ignorance of the role of

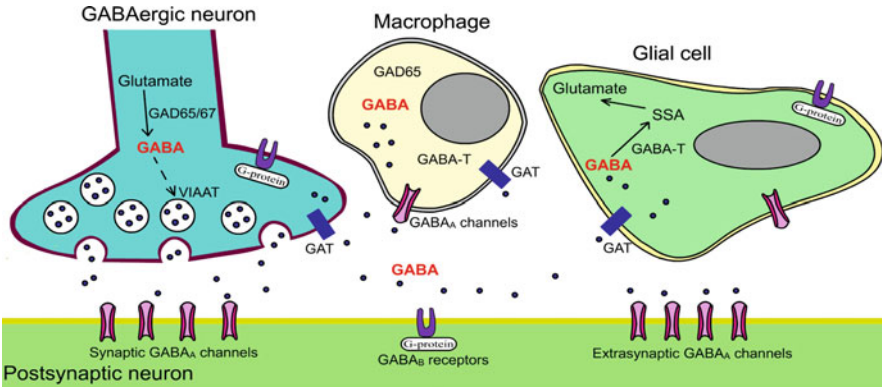

Fig. 5.1 Schematic figure showing the various components of the GABA signalling system. *GABA* gamma-aminobutyric acid, *VIAAT* vesicular inhibitory amino acid transporter, *GAT* GABA transporter, *GABA-T* GABA transaminase, *GAD* glutamic acid decarboxylase, *SSA* succinic semialdehyde

Table 5.1 Components of the GABA signaling system in immune cells

Species	Origin/cell type	GABA signalling component	Function	References
Human	Monocytes	GABA-A α1 mRNA		Alam et al. (2006)
	$CD4^+$ T cells	GABA-A α1, α3, β2 mRNA, α1 protein		
	$CD8^+$ T cells	GABA-A β2 mRNA, α1 protein		
	Irradiated B cells	GABA-A α1, α3, β2 mRNA, α1 protein		
	PBMC	GABA-A α1, α3, α4, β2, β3, γ2, δ, ε mRNA, α1 protein	GABA decreases $[Ca^{2+}]$ inside	
	PBMC	Functional GABA-A channels	Blocking of GABA-A channels prevents pressure-induced macrophage phagocytosis	Shiratsuchi et al. (2009)
	PBMC, macrophages	GABA present		Stuckey et al. (2005)
	Psoriatic skin/ macrophages, lymphocytes, neutrophils	GABA present (macrophage and lymphocytes), GABA-A α protein (macrophages lymphocytes and neutrophils)		Nigam et al. (2010)
	Peripheral T lymphocytes	GAD67, VIAAT, GABA-T mRNA, GAT-1 mRNA (50% of resting cell), GAT-2 mRNA(activated cell) GABA-A α1, α6, δ, ρ2 (resting cells), GABA-A α1, α3, α6, β3, γ2, δ, ρ2 mRNA (activated cells), functional GABA-A channels	GABA decreases T cell proliferation	Dionisio et al. (2011)
	Monocytes	GABA-A β2 mRNA	Blocking of GABA-A channels reverses the inhibition of monocyte migration and phagocytosis by anaesthetics	Wheeler et al. (2011)
	PBMC	Functional GABA-B receptors	GABA-B receptor agonist baclofen decreases PBMC chemotaxis, decreases TNF- α production, decreases immune cell infiltration in a mouse model for allergic contact dermatitis	Duthey et al. (2010)

(continued)

Table 5.1 (continued)

Species	Origin/cell type	GABA signalling component	Function	References
	Neutrophils	GABA-B receptor protein, GAD65/67 protein	GABA-B receptor agonist baclofen increases neutrophil-chemotaxis	Rane et al. (2005)
Rat	$CD4^+$ or $CD8^+$ T cells	GABA-A α1, α2, α3, α4, α6, β3, γ1, δ, ρ1, ρ2 mRNA, functional GABA-A channels	100 nM GABA decreases T cell proliferation	Mendu et al. (2011)
Mouse	T cells	Functional GABA-A channels	GABA decreases proliferation and IL-2 production in stimulated T cell	Tian et al. (1999)
	$CD4^+$ T cells from NOD mice	GABA-A α1, α2, β1, β2, δ mRNA (naïve T cells), GABA-A α1, α2, β1, β2, γ3, δ mRNA (activated T cells)	GABA decreases inflammatory T cell response and cell cycle progression	Tian et al. (2004)
	Peritoneal macrophages (non-stimulated and stimulated)	GABA-A α1, α2, β3, δ mRNA, α1 protein	GABA decreases IL-6 and IL-12 production in macrophages	Reyes-Garcia et al. (2007)
	Spleen cells from GAT-$1^{+/+}$ and$^{-/-}$ mice	GABA-A α1, α2, α5, β1, β2, δ, γ1, γ3 mRNA, GAT-1 mRNA, GAT-1 protein	GAT-1 deficiency increases T cell proliferation and cytokine production	Wang et al. (2008)
	$CD4^+$ T cell	GAT-$1^{+/+}$ compared to GAT-1 $^{-/-}$ mice	GAT-1 deficiency increases CD^+ T cell proliferation and cell cycle entry but decreases apoptosis	Wang et al. (2009)
	Macrophages, DCs and $CD4^+$ T cells	GAD65 protein (DC, macrophage) increased in stimulated cells, GABA pressent (DC, macrophage, $CD4^+$ T cell), GABA-A β1 and ε mRNA (resting and stimulated macrophage), GABA-T mRNA (macrophages, $CD4^+$ T cells), GAT-2 mRNA (macrophage, $CD4^+$ T cells), functional GABA-A channels (macrophages)	GABAergic agents decrease cytokine production from APCs and ameliorate EAE	Bhat et al. (2010)
	APCs and T cells		GABA decreases both T-cells autoimmunity and APC activity and ameliorate EAE	Tian et al. (2011)

(continued)

Table 5.1 (continued)

Species	Origin/cell type	GABA signalling component	Function	References
Cell lines	Human $CD4^+$ H9 T cell line	GABA-A α1, α4, β1 mRNA GABA-A α and β protein	GABA decreases T cell-dependent cytotoxicity	Bergeret et al. (1998)
	Human $CD4^+$ Jurkat J6 cell line	GABA-A α1, α3, α4, α6, β1, β2, β3, γ2, ε, θ mRNA, α1 protein		Alam et al. (2006)
	Human HL60 cell line	α1 protein		Alam et al. (2006)
	Human monocytic cell line THP-1	Functional GABA-A channels	Blocking of GABA-A channels prevents pressure-induced macrophage phagocytosis	Shiratsuchi et al. (2009)
	Human monocytic cell line THP-1	GABA-A α4, β2, γ1, δ mRNA, functional GABA-A channel	Blocking of GABA-A channels reverses the inhibition of monocyte migration by anesthetics	Wheeler et al. (2011)
	Mouse EAE $CD4^+$ T cell line	GABA-A α1, α4, β2, β3, γ1, δ mRNA (resting cells), GABA-A α1, α4, β3, δ mRNA (activated cells), functional GABA-A channels	100 nM GABA decreases T cell proliferation	Bjurstom et al. (2008)
	Mouse RAW 264.7 macrophage cell line	GABA present resting cells > activated cells		Stuckey et al. (2005)

GABA gamma aminobutyric acid, *PBMC* peripheral blood monocytes, *VIAAT* vesicular inhibitory amino acid transporter, *GAT* GABA transporter, *GABA-T* (GABA transaminase), *GAD* glutamic acid decarboxylase, *DC* dendritic cells, *EAE* experimental autoimmune encephalomyelitis, *APC* antigen presenting cells, *IL* – interleukin

GABA outside of the nervous system is that until recently it was thought that GABA-A channels were only fully activated by millimolar (mM) concentrations of GABA. Those high mM concentrations of GABA exist only in the synaptic cleft and only for a very short period (ms) during synaptic transmission whereas outside of the synapse, the GABA concentration is in the submicromolar range. It is now clear that extrasynaptic GABA-A channels can be activated by more than million times lower GABA concentrations than their synaptic counterparts and may be saturated by nano to micromolar GABA concentrations (Lindquist and Birnir 2006; Jin et al. 2011). Importantly, submicromolar GABA concentrations may be present e.g. around neurons in the brain, within the pancreatic islets and in blood (Petty et al. 1999; Wendt et al. 2004; de Groote and Linthorst 2007; Oresic et al. 2008).

5.2 The GABA Signaling Machinery in Immune Cells

The neuronal GABAergic system is composed of four primary parts: the GABA-A channels, the GABA-B receptor, the GABA transporters and the enzymes that make GABA and others that break it down. So far there are relatively few studies that have looked at the GABA system in immune cells (Table 5.1). The GABA-A transcripts are present in immune cells but it varies which subunits have been detected. Tian et al. (2004) identified α1, α2, β1, β2, γ3 and δ and in $CD4^+$ T cells from the type-1 diabetic NOD mice whereas Bhat et al. (Bhat and Steinman 2009) did not identify any of the subunits examined in $CD4^+$ T cells from an Encephalomyelitis (EAE) mouse model. In an EAE cell line α1, α4, β2, β3, γ1 and δ were detected (Bjurstom et al. 2008) and in $CD4^+$ and $CD8^+$ T cells from rats α1, α2, α3, α4, α6, β3, γ1, δ, ρ1 and ρ2 were identified out of the 19 subunits examined (Mendu et al. 2011). As only in two of the studies was the expression of all the different subunits was examined, it is possible that more subunits types can be detected in the T cells from the mouse models or alternatively, the GABA-A subunits expression may be regulated according to e.g. the state of activation of the cells. Clearly the T cells express GABA-A subunits but what specifies the subtypes in the cells remains to be determined. Similarly, α1, α2, β3 and δ have been detected in cultured peritoneal macrophages and β1 and ε in macrophages isolated from the EAE mouse model (Bhat and Steinman 2009). Human peripheral monocytes express α1, α3, β2 and the δ subunits (Alam et al. 2006). So far, other immune cells like e.g. dentritic cells, NK or B cells have not been reported to express the GABA-A channels subunits. These results demonstrate that immune cells from rats, mice and humans do have the necessary building blocks to form GABA-A ion channels.

There have been four plasma membrane GABA transporters identified (GAT1-4). All of these transporters mediate ion-coupled secondary active transport of GABA across the plasma membrane but only two of them have, so far, been detected in immune cells. Transcripts for GAT-1 or GAT-2 have been identified in human peripheral lymphocytes (Dionisio et al. 2011) and also in EAE mice T cells but differ in whether the transcript is detected in resting (Bhat et al. 2010) or activated cells (Wang et al. 2008). Interestingly both Wang et al. (2008) and Bath et al. (2010) isolated macrophages form EAE mice but only Bath et al. (2010) detected GABA transporters in these cells and then only in stimulated macrophages.

Only one study so far has examined the expression of the GABA-T and the GABA transporter that transports GABA into synaptic vesicles (VIAAT) in immune cells. In peripheral human monocytes transcripts were detected for the two genes and the VIAAT was additionally identified by immunohistochemistry (Dionisio et al. 2011). Whether the cells have synaptic like vesicles containing GABA is not known. The enzymes responsible for the GABA production have been detected in T cells, macrophages and dendritic cells (Bhat et al. 2010; Dionisio et al. 2011). GAD65 has been detected in dendritic cells and macrophages (Bhat et al. 2010) from EAE mice whereas GAD67 has been detected in human peripheral

monocytes (Dionisio et al. 2011). GABA is secreted by stimulated mice macrophages and T cells (Bhat et al. 2010; Soltani et al. 2011) and GABA was detected in extracts from human peripheral blood macrophages (Stuckey et al. 2005). The G-protein coupled GABA-B receptor has been implicated in chemotaxis of immune cells (Rane et al. 2005; Duthey et al. 2010).

The results from the different laboratories vary somewhat. The reasons for this are not clear but may reflect variations between species (humans, rats and mice), strains of animals or animal models being used, the state of activation of the cells or classes of cells or even different subtypes of GABA-A ion channels, GABA transporters expressed or concentrations of GABA or drugs applied.

5.3 Effects of GABA on Immune Cells

The GABA signaling system is active in the immune cells and can affect a variety of functional properties of the cells like cytokine secretion, cell proliferation, phagocytic activity or chemotaxis. Nevertheless, much remains to be discovered, as we know relatively little about how these processes are linked to GABA and the GABAsystem in the cells. Bergeret et al. (1998) reported that GABA modulates cytotoxicity of immunocompetent cells expressing GABA-A ion channel subunits. GABA opens the plasma membrane GABA-A ion channels in the immune cell generating a chloride current across the membrane (Bjurstom et al. 2008; Bhat et al. 2010; Mendu et al. 2011). GABA application can result in decreased cytokine secretion and proliferation of T cells (Tian et al. 2004; Bjurstom et al. 2008; Mendu et al. 2011) or have no effects on these properties of the cells (Bhat and Steinman 2009). In lymphocytes exposure to GABA reduced but did not abolish the transient increase in intracellular calcium concentration that is associated with activation of the cells (Alam et al. 2006). Macrophages can also express functional GABA-A channels (Bhat et al. 2010). In these cells agents that mimic GABA e.g. muscimol or drugs that increase the ambient GABA concentration e.g. vigabatin and gabaculine decrease cytokine production (Reyes-Garcia et al. 2007; Bhat et al. 2010). GABA has been protective in mouse models of type 1 diabetes where GABA application was associated with decreased level of inflammatory cytokines including IL-1β, TNF-α, IFN-γ and IL-12 (Tian et al. 2004; Soltani et al. 2011). GABA also decreased secretion of IL-6 and IL-12 from stimulated mouse macrophages *in vitro* (Reyes-Garcia and Garcia-Tamayo 2009). The GABA transporters have been reported to modulate cytokine production and T cell proliferation. In GAT-1 knock-out mice both proliferation and IFN-γ secretion is increased in the T cells relative to cells from wild-type mice (Wang et al. 2008). Furthermore, pharmacological modulation of human peripheral monocytes with drugs acting at the GABA-A channels has been shown to impair the function of the cells in classical immunological chemotaxis and phagocytotic assays (Wheeler et al. 2011).

5.4 GABA in Immune and Autoimmune Diseases

The cross-talk between the immune cells and the affected tissue is complex but nevertheless the relatively few studies published to-date imply that the GABA signaling system is an important part of the disease environment. GABA is decreased in serum of MS patients (Demakova et al. 2003), oral GABA treatment down-regulates inflammatory responses in a mouse model of rheumatoid arthritis (Tian et al. 2011), disregulation of GABA metabolism precedes pancreatic islets autoimmunity in children who later progress to type 1 diabetes (Oresic et al. 2008) and GAD is a major autoantibody in type 1 diabetes (Lernmark et al. 1978). What role the GABA system has in these diseases remains nevertheless to be clarified. The realization that GABA-A ion channels can be activated by very low, sub-micromolar to as low as picomolar, GABA concentrations (Lindquist and Birnir 2006; Jin et al. 2011), and not only the high millimolar concentrations that are present at the neurological synapse, suddenly makes GABA a potential effector molecule in many tissues of the body, e.g. in blood, pancreatic islets, cerebrospinal fluid and, of course, in the brain, where the ambient GABA concentration is in the submicromolar range (Tossman et al. 1986; de Groote and Linthorst 2007; Soltani et al. 2011). In 2004 Tian et al. (2004) showed that increasing the systemic GABA concentration delayed the onset and incidence of type 1 diabetes in NOD/scid mice. Similarly, in 2010 Bath et al. (2010) showed that increasing the GABAergic activity ameliorated ongoing paralysis in EAE, the mouse model for multiple sclerosis, via inhibition of inflammation. GABA has also been proposed to have a role in rheumatoid arthritis (Kelley et al. 2008; Tian et al. 2011) and psoriasis (Nigam et al. 2010).

Conclusion

Several recent studies show that the immune system is capable of synthesizing and releasing the classical neurotransmitter GABA (γ-aminobutyric acid). GABA influence on the function of immune cells is apparent in many processes such as activation or suppression of cytokine secretion, proliferation and it can modulate migration. The immune cells may not only encounter this neurotransmitter when it is secreted by the cells themselves or when the immune cells enter the brain but also in parts of the body like in the lymph nodes, the islets of Langerhans and in blood. It is now clear that GABA-A channels located outside of synapses can be activated by more than million times lower GABA concentrations than their synaptic counterparts (Lindquist and Birnir 2006; Bjurstom et al. 2008; Jin et al. 2011; Mendu et al. 2011) and importantly, submicromolar GABA concentrations are present around the neurons in the brain, within the pancreatic islets and in blood. Immune cells from rodents and humans do have the necessary building blocks to form GABA-A ion channels that can be activated by these low, submicromolar GABA concentrations (Bjurstom et al. 2008; Mendu et al. 2011). The absence of a presynaptic terminal defines these channels in the immune cells as extrasynaptic-like channels. Physiologically this seems reasonable as the cells will not encounter the high, synaptic-like mM concentrations of GABA but rather, will be exposed to

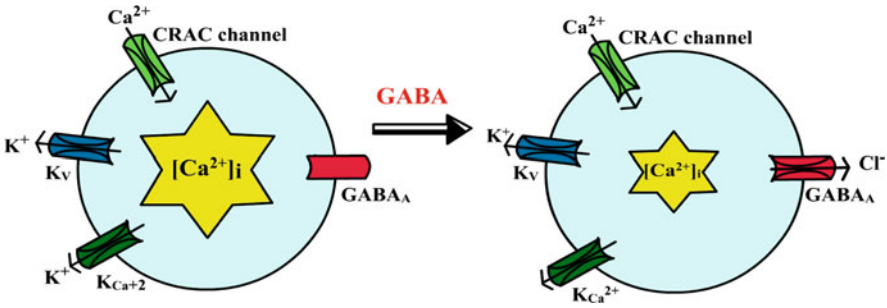

Fig. 5.2 A cartoon depicting an immune cell and ion channels that may affect the intracellular calcium concentration. Opening potassium channels increases whereas opening GABA-A channels decreases Ca^{2+} entry from the external milieu into the cell. Calcium, in turn, regulates the cell's proliferation and cytokine production. GABA-A Cl^-channel, CRAC (Ca^{2+} release-activated Ca^{2+}) channel, Kv (voltage-gated K^+) channel, $K_{Ca}{}^{2+}$ (calcium-activated K^+) channel. An *arrow* through the channel indicates an open channel (Tian et al. 2004; Bjurstom et al. 2008)

submicromolar GABA concentrations as they travel in the blood or enter the brain or the pancreatic islets. When GABA opens the GABA-A channels in the immune cells' plasma membrane, the membrane potential will change and can thereby affect a number of cellular processes including the entry of calcium into the cell by decreasing the driving force on the calcium ion (Fig. 5.2). Despite the relative limited number of studies, so far, there are significant discrepancies between the results e.g. in terms of what GABA-A channel subunits and therefore what channel subtypes are expressed in the immune cells. This matters as the different subtypes vary in their properties such as their affinity for GABA, the efficiency of conducting ions and changing the membrane potential plus their pharmacological profile. The results may reflect difference between species (humans, rats and mice) or some experimental condition such as different strains of animals or animal models, different state of activation of the immune cells or classes of cells and in some cases, limited number of subunits being examined. GABA appears to have a role in autoimmune diseases like MS, type 1diabetes and rheumatoid arthritis and modulate the immune response to infections (Bhat and Steinman 2009; Mendu et al. 2011; Soltani et al. 2011; Tian et al. 2011; Wheeler et al. 2011). Neuroinflammation is also involved in epileptogenic processes in the brain (for review see Vezzani et al. 2008) where infiltration of leukocytes into the brain (Fabene et al. 2008; Zattoni et al. 2011) and increased production of pro-inflammatory cytokines (Li et al. 2011) have been shown to be associated with the induction and progression of epilepsy. In a variety of epilepsy models several cytokines can modulate the neuronal GABA system (Vezzani et al. 2008) highlighting the cross-talk that takes place between the immune and the nervous systems.

Despite the few studies of the GABA signaling system in the immune cells to-date, it is clear that it is an integral part of the mammalian immune system. In the

near future it will be important to unfold the effects and the underlying mechanisms of GABA modulation of the immune cells. Modulation of the functional properties of the GABA system in immune cells provides several promising targets for the treatment of inflammatory diseases.

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The authors declare that they have no conflict of interest.

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The Effects of Opioids on Immune Cells, Functions and Diseases

6

Jana Ninković and Sabita Roy

Contents

6.1 Opioids

Opioid analgesics have been the foundation of human pain management for centuries. In spite of their many debilitating side effects, prescription opioids remains a gold standard for treatment of chronic pain. The human body naturally produces its own, endogenous opiate-like substances in order to modulate pain mechanisms. Endogenous opioids mediate our reactions to painful stimuli by

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binding to opioid receptors which are widely distributed throughout most tissues and organ systems.

6.1.1 Structure and Site of Production

6.1.1.1 Endogenous Opioids

During the decade spanning the mid-1970s to the late 1980s, more than 20 different endogenous opioid peptides were identified and shown to possess differential affinity for the different opioid receptor types (Evans 2004). The endogenous opioids are derived from three opioid protein precursors by a process of selective proteolytic cleavages. Although there is a wide variety of endogenous peptides, they consist of three major classes: enkephalins, endorphins, and dynorphins. All endogenous opioids have an N-terminal enkephalin sequence (Tyr-Gly-Gly-Phe-Met/Leu), with many peptides containing a C-terminal extension which modulates receptor selectivity and susceptibility to degradation by extracellular proteases (reviewed by Weber et al. 1983).

Pro-enkephalin contains multiple repeats of the enkephalin sequence, seven in the human pro-enkephain precursor. The enkephalins are small, five amino acid peptides that exist in two forms, leucine enkephalin and methionine enkephalin. Pro-opiomelanocortin (POMC) contains beta-endorphin, 31 amino acid peptides that contain the met-enkephalin sequence, and shares the precursor protein with adrenocorticoptrophic hormone (ACTH), a critical pituitary hormone for coordination of stress responses. The endorphins and enkephalins act primarily on mu and delta opioid receptors. Finally, pro-dynorphin contains three leu–enkephalin core opioid sequences analogous to pro-enkephalin, differential processing of the core sequences leads to generation of multiple opioid peptides. The dynorphins exist in several forms that range from 10 to 17 amino acids in length, and they exert their effects primarily on kappa receptors. The biological significance of the multiplicity of endogenous opioids is still unclear.

Early studies in 1970s, and 1980s concluded that production of endogenous opioids was limited to the cells of the nervous system. However, each opioid peptide precursor has a unique pattern of expression, with POMC transcripts restricted to the pituitary, the arcuate nucleus of the hypothalamus and cells in the nucleus of the solitary tract, whereas both pro-enkephalin and pro-dynorphin have a considerably more expansive distribution (Akil et al. 1984). β-endorphin production is localized mostly in the pituitary and hypothalamus, while enkephalins are more widely distributed through the neuraxis found mainly in the cortex and spinal cord as well as the adrenal medulla, while dynorphin production is limited to the hypothalamus and the brainstem. Table 6.1 summarizes the sites which have been known to generate endogenous opioids. Recent findings have shown that all opioid peptides are also found in leukocytes. Endorphins processing from POMC have been studied most extensively (Rittner et al. 2005). POMC processing occurs in the endoplasmic reticulum and the trans-Golgi network. The enzymatic machinery required for this process includes

Table 6.1 Site of production of endogenous opioids

Opioid	Site of production	Reference
β-endorphin	*Pituitary	Bloom et al. (1977)
	*Hypothalamus	Moon et al. (1973)
		Pelletier et al. (1977), Pelletier (1980)
	Nucleus of the solitary tract	Bloch et al. (1978)
		Jacobowitz and O'Donohue (1978)
	T cell, B cell, monocyte, macrophage	Krieger et al. (1977)
		Pelletier (1980)
		Stein (1995)
		Rittner et al. (2005)
Enkephalin	Cortex spinal chord	Elde et al. (1976)
	Adrenal medulla	Hokfelt et al. (1977)
		Khachaturian et al. (1982a)
	Gi tract	Uhl et al. (l978)
		Watson et al. (1981)
	T cell, B cell, monocyte, macrophage	Rittner et al. (2005)
Dynorphin	*Hypothalamus	Khachaturian et al. (1982b)
	Brainstem	Vincent et al. (1982)
		Watson et al. (1981)
	T cell, B cell, monocyte, macrophage	Weber et al. (1982)
		Rittner et al. (2005)

*Classical loci for production of peptides derived from POMC

carboxypeptidase E, the pro-hormone convertases 1 (PC1) and PC2, and the binding protein 7B2 (Mousa et al. 2004). β-endorphin, POMC, and all processing enzymes have been located in leukocytes in the blood and within inflamed tissue in rats (Mousa et al. 2004). Thus, leukocytes can process POMC into functionally active β-endorphin. Furthermore, met-enkephalin, dynorphin, and endorphins are also detectable in leukocytes of inflamed tissues. Some opioid-containing immune cells identified to date are T and B-lymphocytes, granulocytes, and monocytes/macrophages (Cabot et al. 1997; Mousa et al. 2001a; Przewlocki et al. 1992; Rittner et al. 2001). Thus, opioid peptides are processed and present in the circulation and in the immune cells infiltrating injured tissue.

In macrophages, monocytes, granulocytes, and lymphocytes, β-endorphin is present in secretory granules arranged at the cell periphery, ready for exocytosis. During the early inflammation, as the leukocytes migrate to the site of infection, they (along with the resident cells) secrete various chemokines such as CXCL8, CXCL1, IL-6, IL-1 etc. which lead to hyperalgesia. In the late inflammation mocrophages and lymphocytes secrete IL-4, IL-10 and IL-13 inhibiting the hyperalgesic pathways at several different stages lading to analgesia (see Fig. 6.1 for details). In addition leukocytes can also release opioid peptides following stimulation from corticotropin-releasing factor (CRF) and IL-1β. The effects are

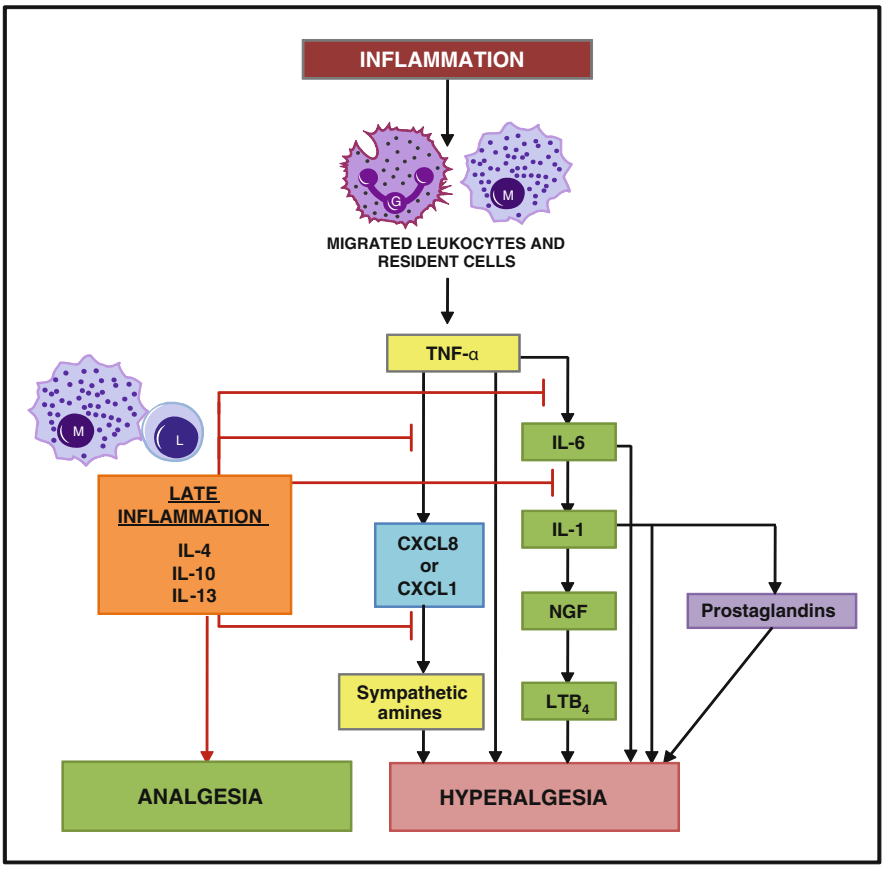

Fig. 6.1 Hyperalgesic and analgesic mechanisms in inflammation. In early inflammation, leukocytes, e.g., granulocytes (G) and monocytes (M), migrate into the inflamed tissue. Here, these leukocytes as well as resident cells release cytokines including TNF-α, interleukins (ILs), chemokines [CXC chemokine ligand 8 (CXCL8), CXCL1], NGF, and secondary mediators, such as sympathetic amines, leukotriene B_4 (LTB_4), and prostaglandins, culminating in hyperalgesia. TNF-α, IL-6, and IL-1 can also have direct hyperalgesic effects on nociceptors. During ongoining, late inflammation, lymphocytes (L) and monocytes/macrophages (M) start to produce anti-inflammatory cytokines, such as IL-4, IL-10 and IL-13. These cytokines inhibit the proinflammatory cytokines, such as TNF-α, IL-1, and IL-6, and block the cascade, resulting in analgesia

mediated by CRF and IL-1β receptors (coexpressed by opioid-containing leukocytes) via a calcium-dependent mechanism and are mimicked by elevated extracellular concentrations of potassium (Cabot et al. 1997). This is consistent with a regulated pathway of neuronal or endocrine release from secretory vesicles. Furthermore, noradrenalin (NA) can release β-endorphin from leukocytes *in vitro* following activation of adrenergic receptors (Schafer et al. 1996). Adrenergic α1, β2, and to a lesser degree, α2 receptors are expressed on β-endorphin-containing inflammatory cells located in close proximity to sympathetic nerve fibers in inflamed

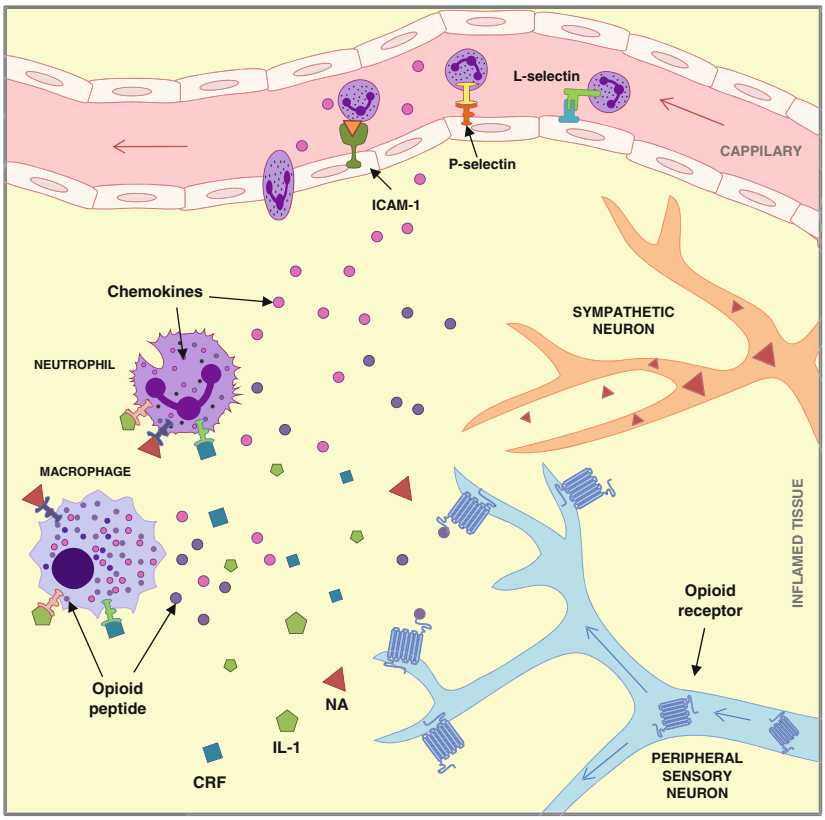

Fig. 6.2 Inflammation induced migration of opioid-producing leukocytes and opioid secretion. Resident macrophages of the inflammed tissue release chemokine gradient to recruit neutrophils form the blood stream. Chemokine secretion leads to upregulation of adhesion molecules (P-selectin, ICAM-1, etc.) on the capillary endothelium which facilitates neutrophil rolling, adhesion and extravasation. Once extravasated, leukocytes can be stimulated by releasing agents such as corticotropin-releasing factor (CRF), interleukin-1β (IL-1) and/or noradrenaline (NA). CRF, IL-1, and NA (derived from sympathetic neurons) elicit opioid release by activating their respective receptors on leukocytes. Opioids bind to peripheral opioid receptors (produced in dorsal root ganglia and transported to peripheral endings of sensory neurons) and produce analgesia by inhibiting the excitability of these neurons. Opioid agonists have easier access to neuronal opioid receptors during inflammation because inflammation disrupts the perineurium (normally a rather impermeable sheath encasing peripheral-nerve fibers). *Arrow* in the blood vessel and sensory neuron indicates the direction of the events

paws. Chemical ablation of these fibers has been shown to abolish intrinsic opioid analgesia (Rittner et al. 2005). In summary, CRF, IL-1β, as well as sympathetic neuron-derived NA can act on their respective receptors on leukocytes to release opioid peptides (Fig. 6.2). Leukocytes are able to exert analgesic effects by releasing opioid peptides which bind to opioid receptors of the nociceptors in the periphery.

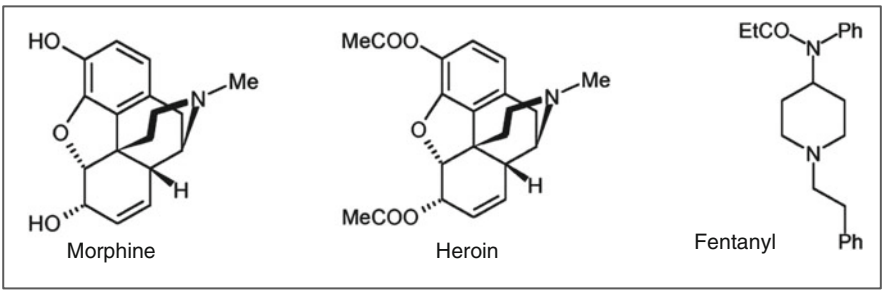

Fig. 6.3 Chemical structures of μ receptor agonists and antagonists

6.1.1.2 Exogenous Opioids

Of the exogenous opioids, morphine the principal alkaloid of opium, has been most extensively studied. Gates and Tschudi were the first to successfully synthesize the complete molecule of morphine in 1952 (Gylbert 1973). The overall structure of morphine consists of two planes. The first plane containing a benzene ring, an oxide ring, and a carboxylic ring while the second plane contains a carbocyclic ring and an ethenamine ring, O2 and N (Fig. 6.3) (Gylbert 1973). Morphine is metabolized in the liver via N-dealkylation and glucoronidation at the third position morphine-3-glucuronide (M3G) or the sixth position morphine-6-glucuronide (M6G). Although M3G is the most common metabolite (accounts for 50% of the metabolites produced), they exert no biological activity when bound to μ-opioid receptor. In contrast, M6G, although less prevalent (accounts for 10% of the metabolites produced) can induce an analgesic effect upon binding to the μ-opioid receptor (Dahan et al. 2008).

The synthetic morphine derivatives fentanyl and heroin have similar efficacy and addictive properties as morphine, yet these two drugs differ in their onset and duration of action (see Fig. 6.3 for chemical structures). Rapid onset of fentanyl and heroin are attributed to their highly lipophilic profiles making these drugs readily available to cross the blood–brain barrier.

6.1.2 Opioid Receptors

Opioids and opioid peptides selectively bind to the opioid receptors. Classical opioid receptors are seven transmembrane G protein-coupled receptors (GPCRs) and have three major receptor subtypes μ (mu for morphine), δ (delta for deferens because it was first identified in mouse vas deferens) and kappa κ (kappa for ketocyclazocine – an agonist that is a benzomorphan derivative)(Lord et al. 1977). As a class, GPCRs are of fundamental physiological importance, mediating the actions of the majority of known neurotransmitters and hormones. Opioid receptors are particularly intriguing since they are activated both by endogenously produced opioid peptides and by exogenously administered opioid drugs, such as morphine (Waldhoer et al. 2004).

Analgesia induced by opioids is predominately mediated through the μ opioid receptor. Endogenous opioids have been implicated in activating all three receptor types. β-endorphins and enkephalins bind to μ and δ, while dynorphin binds predominately to the κ receptor. μ opioid agonists (endogenous and exogenous) induce analgesic effects by regulating both the pre- and post-synaptic sensory neurons. At the pre-synaptic neurons, opioids bound to μ opioid receptors (MOR) block voltage-gated calcium (Ca^{2+}) channels and hence, block Ca^{2+} influx. Lower intracellular Ca^{2+} leads to an inhibition of excitatory neurotransmitter release from presynaptic vesicles. Activation of MOR on postsynaptic terminals promotes the efflux of potassium (K^+) via K^+ channels. The net effect of active MOR receptor results in hyperpolarization of the post-synapse causing inhibition of neuronal firing. Studies have shown that MOR effects the pre- and post-synaptic neuron synergistically, thereby decreasing the perception of pain (Glaum et al. 1994; Kohno et al. 1999; Yoshimura and North 1983).

Opioid receptors are G protein coupled receptors that are classified in two distinct classes: classical (μ, δ, κ) and non-classical opioid receptors (Goodsell 2005). Three decades of extensive pharmacological studies have uncovered a variety of opioid receptor types, however only four have been cloned to date: μ, δ, κ and the n-opioid receptor [initially called LC132 (Bunzow et al. 1994), ORL-1 (Mollereau et al. 1994), or nociceptin/orphanin FQ receptor (Meunier et al. 1995). Although only four receptor genes have been discovered, there is substantial pharmacological evidence to suggest the existence of additional opioid receptor phenotypes.

The sigma receptor (σ = sigma for SKF10047) was initially classified as an opioid receptor (Martin et al. 1976). However, since the time it was cloned in 1996 (Hanner et al. 1996), it has become evident that the sigma receptor is a single transmembrane-spanning protein targeted by other drugs of abuse, for example phencyclidine and its analogues (for review see (Monassier and Bousquet 2002)). The sigma receptor is no longer regarded as a member of the opioid receptor family. Moreover, a variety of other opioid receptors have been described on the basis of pharmacological profiles that did not match any of the classical receptors. These include a ζ (zeta) receptor, which has recently been cloned and classified as an opioid growth factor receptor (OGFr) with no homology to the classical opioid receptors (Zagon et al. 1991, 2000). In addition, a λ (lambda) receptor and a β-endorphin-sensitive ε (epsilon) opioid-binding site have been described (Wuster et al. 1979). However, these receptors are poorly characterized, and proof of their existence by identifying their respective genes is still lacking (Waldhoer et al. 2004).

6.1.3 Opioid Receptor Functions

Opioid receptors belong to the class A (Rhodopsin) family of Gi/Go protein-coupled receptors with an extracellular N-terminal domain, seven transmembrane helical domains connected by three extracellular and three intracellular domains, as

well as an intracellular C-terminal tail. Seven-transmembrane helices of opioid receptors are arranged sequentially in a counterclockwise fashion, to form a tight helical bundle. Together with the extracellular domains of the receptor, this provides a dynamic interface for the binding of various opioid ligands. The opioid receptors are about 60% identical to each other with greatest homology in the transmembrane helices, and the greatest diversity in their N and C termini as well as their extracellular loops.

Opioids initiate a signal through a G protein cascade. When morphine binds to an opiate receptor, the receptor changes shape and interacts with a G protein inside the cell. The activated receptor causes the G protein to replace its GDP molecule with a GTP, causing the G protein to break into Gα and Gβγ subunits. The half with the GTP molecule then diffuses along the membrane until it finds its target. Opioid receptor activation leads to upregulation of the cAMP pathway, which is believed to be a mechanism of opiate tolerance and dependence (reviewed by Nestler (2001)). Opioids acutely inhibit the functional activity of the cAMP pathway (indicated by cellular levels of cAMP and cAMP-dependent protein phosphorylation). With continued opiate exposure, functional activity of the cAMP pathway gradually recovers, and increases far above control levels following removal of the opiate (e.g. by administration of the opioid receptor antagonist naloxone). These changes in the functional state of the cAMP pathway are mediated via the induction of adenylyl cyclase and protein kinase A (PKA) in response to chronic administration of opioids. Induction of these enzymes accounts for the gradual recovery in the functional activity of the cAMP pathway that occurs during chronic opiate exposure (tolerance and dependence) and activation of the cAMP pathway observed on opiate withdrawal (Nestler 2001).

6.1.4 Classical Target Cells

Originally, before the discovery of opioid receptors on leukocytes, it was thought that opioids exert their effects solely through an indirect pathway by the binding of opioid receptors expressed in the central nervous system (CNS). One school of thought postulates that opioid receptors are expressed only in the CNS, and therefore the classical target cells of the opioid mediated analgesia were neuronal cells of the CNS. Morphine's modulation of the pain mechanisms and immune system occurs through the activation of the hypothalamo-pituitary-adrenal axis (HPA) and the stress-responsive neuroendocrine pathway (Peterson et al. 1993).

HPA axis is a complex set of direct influences and feedback interactions among the hypothalamus, the pituitary gland, and the adrenal (or supradrenal) glands (Fig. 6.4). The interactions among these organs constitutes the HPA axis, a major part of the neuroendocrine system that controls reactions to stress, and through release of stress hormones (corticosteroids (CORT)), exerts immunosuppressive effects.

However, more recently, studies supporting a direct role of opioids on immune system are gaining more acceptance primarily with the discovery of opioid

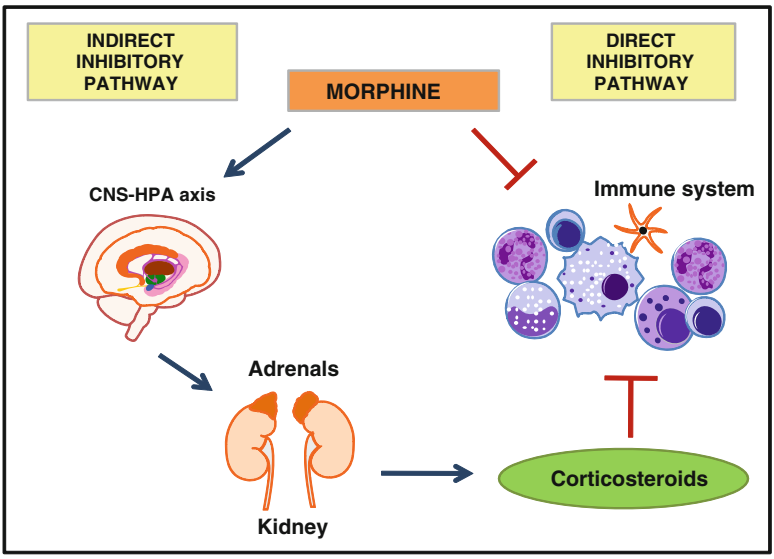

Fig. 6.4 Pathways of opiate induced immune suppression. Morphine can modulate immune system via direct and indirect pathway. Indirectly morphine acts on CNS and the hypothalamic-pituitary-adrenal (HPA) axis which leads to release of corticosteroids, immunosuppressive hormones which lead to suppression of the immune system. Direct inhibitory pathway requires direct interaction of opioids on cells of the immune system

receptors on the immune cells (Table 6.2). Opioid alkaloids and peptides such as morphine and the endogenous peptides, including β-endorphin and the dynorphin peptides, directly modulate the functions of lymphocytes and other cells involved in host defense and immunity (Bidlack et al. 2006). The concept of direct and indirect morphine action was first introduced through work with morphine dependant rodents. Findings by our group and others indicate that morphine-induced immunosuppression is mediated by the MOR and that although some functions are amplified in the presence of CORT or sympathetic activation, the inhibition of IFN-gamma synthesis, and modulation of macrophage-cytokine synthesis is CORT-independent and only partially dependent on sympathetic activation (Bryant et al. 1991; Casellas et al. 1991; Perez-Castrillon et al. 1992; Peterson et al. 1987). Although the current research focus has shifted to direct effects of opioids on immune cells, when looking at *in vivo* models of drug abuse and immunomodulation, it is important to consider the role of stress mechanisms mediated by the HPA axis.

6.1.5 Clinical Use

Opioids remain the gold standard for chronic pain management, in spite of the adverse side-effects resulting from their use. A number of opioids are available for

Table 6.2 Opioid receptor expression in immune cells

Cell type	μ	δ	κ	Reference
T cell	CEM cell line, human CD4+ T cells	MOLT cell line, human cell line, murnie splenic T-cells,	MOLT cell line, human immature thymic T-cells, murine thymocytes and splenocytes	Chuang et al. (1995)
				Wick et al. (1996)
				Sharp et al. (1998)
				Ignatowski and Bidlack (1998)
B cell	CEM cell line, Raji line	Human cell line	CEM cell line	Chuang et al. (1995)
				Gaveriaux et al. (1995)
				Suzuki et al. (2001)
Dendritic cell	Human and murine primary DC	Human and murine primary DC	Murine DC	Makarenkova et al. (2001)
				Kirst et al. (2002)
Macrophage/ monocyte	Primary rat peritoneal mac., human and simean mono	Human cell line	Macrophage-like murine cell line $P388D_1$	Chuang et al. (1995)
				Sedqi et al. (1995)
				Lopker et al. (1980)
				Carr et al. (1991)
Neutrophil	Human granulocytes	Human granulocytes	Murine bone marrow neutrophils	Lopker et al. (1980)
				Falke et al. (1985)
				Stefano et al. (1993)
				Kulkarni-Narla et al. (2001)
Microglia	Human fetal microglia Murine microglia	Murine microglia	Human fetal microglia Murine microglia	Chao et al. (1996)
				Stiene-Martin et al. (2001)

clinical use, including morphine, hydromorphone, levorphanol, oxymorphone, methadone, meperidine, oxycodone, and fentanyl (Inturrisi 2002). Opioids are often prescribed for management of cancer pain, post-operative pain, as well as chronic pain in individuals with late stage HIV. Morphine and fentanyl are often used to alleviate severe pain, while codeine is used for milder pain. Other examples of opioids prescribed to relieve pain include propoxyphene (Darvon); hydromorphone (Dilaudid); and meperidine (Demerol), which are used less often because of their side effects. In addition to their effective pain-relieving properties, some of these

medications can be used to relieve severe diarrhea (for example, Lomotil, also known as diphenoxylate) or severe coughs (codeine). Methadone and buprenorphine, are synthetic opioids that are used for treatment of addiction. They eliminate withdrawal symptoms and relieve craving. Methadone has been used successfully for more than 30 years for treatment of opioid addiction, while buprenorphine, has been approved more recently for treating addiction to heroin and other opiates.

Naltrexone and naloxone are opioid receptor blockers which are clinically used to prevent relapse and treat overdose (respectively). Naltrexone is a long-acting opioid receptor blocker that can be employed to help prevent relapse. It is not widely used, however, because of poor compliance, except by highly motivated individuals (e.g. physicians at risk of losing their medical license). This medication is only used in patients who have already been detoxified, since it can produce severe withdrawal symptoms in a person continuing to abuse opioids.

Studies examining morphine's effect vary greatly in terms of doses and concentrations used. Chronic morphine treatment has been observed to lead to morphine plasma levels of 11–1,440 ng/ml in cancer patients using morphine for pain management at a dose of 2.5–90 mg every 4 h (Aherne et al. 1979). Therefore concentrations used in most studies, in order to mimic physiological doses, range from 10 nM to 1 μM.

6.2 Opioid Receptor Expression in Immune Cells

Opioid receptors are expressed throughout the body, in various tissues and cell types. They are found in the periphery, at pre-synaptic and postsynaptic sites in the spinal cord dorsal horn, and in the brain stem, thalamus, and cortex, in what constitutes the ascending pain transmission system. Receptors are also found in the structures that comprise the descending inhibitory system that modulates pain at the level of the spinal cord (Inturrisi 2002). Until recently it was thought that opioid receptors were only expressed in the central nervous system. However, recent findings have proven that opioid receptors are expressed by the cells of the immune system such as T cells, B-cells, and macrophages (for details see Table 6.2) (Chuang et al. 1995).

The earliest examination of opioid receptor expression used pharmacological and ligand binding studies to provide support for the existence of opioid receptors on the cells of the immune system. The advent of genetic cloning and polymerase chain reaction (PCR) techniques provided an essential tool for addressing the existence of opioid receptors on cells of the immune system. These techniques enabled an alternative way to demonstrate expression of all three opioid receptors on several immune cells, including CD4+ T helper cells (reviewed in (Sharp et al. 1998)). Specifically, using reverse transcriptase-polymerase chain reaction (RT-PCR) cDNA clones of the μ (Chuang et al. 1995), δ, and κ (Wick et al. 1996) opioid receptors were obtained from several immune cells. Chuang et al. demonstrated the expression of the MOR gene in various cell types including, the human hybrid B and T cell CEM line, the Raji line (human B cells), human CD4+ cells, human monocytes

and macrophages, and various others (Chuang et al. 1995). In addition, studies have demonstrated the existence of delta and kappa opioid cDNA transcripts in MOLT-4 and CEM T cell lines as well as in human peripheral blood lymphocytes using similar techniques (Wick et al. 1996). Furthermore, existence of MOR transcripts has been demonstrated to be expressed in rat peritoneal macrophages, while the delta opioid receptor has been found in inactivated mouse thymocytes (Sedqi et al. 1995a; Sedqi et al. 1996). In all cases, the transcripts obtained from the immune cells were nearly identical to opioid receptor cDNAs isolated from neuronal cells.

These observations suggest that opioids may be directly mediating their diverse array of effects by binding to receptors expressed on cells of the immune system. It is also important to note that the cDNA clone, AT7–5EU, was isolated after a screen of an activated human lymphocyte cDNA library in search of homology to brain opioid receptors. This clone was demonstrated to encode for the opioid 'orphan' receptor, and the protein coding region shared complete homology with a reported opioid 'orphan' receptor cloned from human brain. There have also been reports that implicate the existence of novel, non-classical opioid receptors and binding sites on immune cells that are selective for morphine (Sharp et al. 1998). The importance and relevance of all of these findings (summarized in Table 6.2) are centered on the idea that opioids, both endogenous and exogenous, may be exerting their myriad of effects on the immune system in a direct and indirect manner.

6.3 Effects of Opioids on Immune Cells

Several research studies provide strong support for the role of chronic morphine in indirect modulation of both adaptive and innate immune systems. Although, support for an indirect effect of morphine modulation is strong (Vallejo et al. 2004; Wang et al. 2005), recently focus has shifted to exploring how morphine may directly exert its suppressive effects on innate immune cells by binding to their opioid receptors. The multifaceted immunosuppressive actions of morphine add to the complexity of identifying targets of its inhibition.

6.3.1 Indirect Modulation of Immune Functions

Morphine and the endogenous opioids can lead to immunosuppression indirectly by activating hypothalamic-pituitary-adrenal (HPA) axis and stress pathways. Psychological stressors are known to stimulate the HPA axis and the sympathetic nervous system resulting in the release of corticosterone and catecholamines respectively. These molecules affect various immune parameters and can alter overall immune competence of the individual (Li et al. 2000). The immunoregulation of corticosteroids is mediated by specific binding of glucocorticoids to glucocorticoid receptors which are expressed in all leukocytes. Corticosteroids have been shown to promote the immune response during acute stress and to inhibit the immune response during chronic stress (Dhabhar and McEwen 1997b). Endogenous opioid

peptides, in contrast, are known to be elevated by both acute and chronic stress, and play a critical role in regulating stress-induced changes of the immune system (Sharp et al. 1998). Some studies have shown that blockade of endogenous opioids with naloxone results in attenuation or reversal of stress-induced immune alterations (Yin et al. 2000; Dhabhar and McEwen 1997a, b).

Indirect modulation of the immune system by opioids was first demonstrated by Bryant et al. who administered morphine to both adrenalectomized and sham animals and found that morphine implantation significantly increased corticosterone levels in sham animals. In addition, morphine implanted animals displayed spleen and thymic atrophy as well as impaired lymphocyte proliferative responses. Interestingly, these morphine induced effects were absent in adrenalectomized mice, indicating that the rise in corticosterone can be a mediator of imunosuppressive effects (Bryant et al. 1991). Our group has also addressed the question of morphine's indirect signaling, by examining the contribution of the HPA axis. Morphine mediated elevations in corticosterone levels were demonstrated to be mediated by the μ-opioid receptor, proved by a loss in corticosterone increases in mu opioid receptor knockout (MORKO) mice (Roy et al. 2001b). Further studies utilizing MORKO mice demonstrated that morphine-induced immune suppression is regulated by the μ-opioid receptor, and that only a few deficits are amplified in the presence of corticosterone, such as inhibition of IFN-γ synthesis and activation of macrophage cytokine production (Wang et al. 2002a). When examining the effects of stress on immune processes using a restraint stress model our group demonstrated that stressed WT mice had decreased splenocyte numbers accompanied by increased apoptosis. This effect was lost in MORKO mice, despite the finding that corticosterone concentrations were similar for both WT and MORKO animals. Together these results indicate that the μ-opioid receptor is involved in stress-induced immunosuppression, and that this effect is not entirely mediated by a glucocorticoid pathway (Wang et al. 2002b).

Endogenous opioids are peptides that are a known component of the stress response. The role of the endogenous opioids in the CNS is fairly well established, but their presence in the cells of the immune system has only recently been acknowledged. β-endorphin (BE) is constitutively synthesized, its concentrations are independent of opioid plasma concentration and it can be elevated in response to certain stimuli, such as stress. Pharmacological studies examining regulation of BE have revealed that dopamine and γ-aminobutyric acid (GABA) inhibit, while serotonin stimulates BE production (Panerai and Sacerdote 1997). The existence of endogenous opioids in the cells of the immune system suggests that they may serve a relevant function and towards that end many groups have examined possible roles of the endogenous opioids in immune functioning.

It was demonstrated by Sacerdote, et al. that treatment of splenocytes with the antagonist naloxone resulted in an increase in IL-2 and IFN-γ production accompanied by a decrease in IL-4 and IgG antibody titers. These results suggested that naloxone treatment polarizes T helper cells towards a Th1 effector population, and it was implicated that naloxone may be removing the regulatory effects of endogenous opioids that may shift the balance towards Th2 (Sacerdote et al. 2000). Interestingly, it was demonstrated that administration of met-enkephalin before

restraint stress abolished stress-induced immune alterations including elevations of glucocorticoids. However, met-enkephalin administration alone had effects on the immune system similar to those seen following stress, including decreased NK activity and a deficit in the PFC response (Marotti et al. 1996). In summary, the work described in this section clearly points to a role for endogenous opioids in the modulation of the immune system, and like stress and exogenous opioids, it appears that these compounds have an inhibitory mode of action.

The question of whether morphine has a direct effect on immune functioning by acting on opiate receptors on immune cells, or whether morphine is acting indirectly by activating receptors in the CNS to release catecholamines and/or steroids, which then indirectly effect immune parameters, is an area which requires further examination.

6.3.2 Direct Modulation of Immune Functions by Opioids

6.3.2.1 Modulation of Adaptive Immune Response

Modulation of adaptive immune system has first been observed in morphine treated rodents where morphine treatment led to decreased splenic and thymic weight resulting in reduced function of T cells and their precursors (Bryant et al. 1988a, 1988b, 1991). Such morphologic changes indicate the magnitude of morphine's imunosuppressive capabilities.

Morphine and T-Cells

Morphine has been observed to modulate various aspects of T cell functions (see Table 6.3 for details). Chronic morphine treatment leads to a reduction in cell viability, proliferative response, T-helper cell function, as well as reduced CD4/CD8 population *in vivo*. Additionally, chronic morphine treatment *in vitro* has been shown to significantly decrease the production of IL-1β, IL-2, TNF-α, and IFN-γ from mouse splenocyte cultures, as well as stimulate the production of anti-inflammatory cytokines TGF-β1 and IL-10 (Pacifici et al. 2000). Furthermore, our group has demonstrated that *in vitro* morphine treatment of PBMCs or splenocytes results in T helper cell differentiation towards the Th2 lineage (Roy et al. 2001a). Mechanistically morphine treatment impairs mitogen stimulated lymphocyte proliferation by interfering with transcriptional activation of the IL-2 gene (Roy et al. 1997), as well as interfering with IFN-γ promoter activity through two distinct cAMP dependent pathways, specifically the NF-κβ and AP-1/NFAT pathways (Wang et al. 2003). Low dose morphine treatment of lymph node derived T lymphocytes results in impaired Con A induced proliferation and IL-2 and IFN-γ production, accompanied by an increase in apoptosis. These effects were abolished in the absence of μ-opioid receptor, in MORKO mice (Wang et al. 2001). Other investigators have also investigated the role of the MOR in morphine induced immunosuppression, and have noted that morphine induced lymphoid organ atrophy and diminished NK cell activity is lost in MORKO mice demonstrating the essential role of the mu-opioid receptor (MOR) in morphine mediated immune deficits (Gaveriaux-Ruff et al. 1998).

Table 6.3 Morphine suppresses immune cell function

Cell type	Morphine effect	Dose	Reference
PBMC	Suppressed activity	3.2 mg/kg/day sub q. for 2 years	Carr and France (1993)
	Th1 → Th2 shift	100 ng/ml for 4 days	Roy et al. (2001a)
	↓ superoxide production	10 nM, 1 pM	Peterson et al. (1987)
NK cells	Suppressed activity	3.2 mg/kg/day sub q. for 2 years	Carr and France (1993)
T cells	↓ number	3.2 mg/kg/day sub q. for 2 years	Carr and France (1993)
	↓ IFN-γ promoter activity via ↑ cAMP	1 μM, 10 nM	Wang et al. (2003)
B cells	↓ mitogenic responses of splenic B cells to LPS	s.c. implant of 75 mg morphine pellet for 3 days	Bhargava et al. (1994)
			Bryant et al. (1988a)
	↓ numbers in mouse spleens	s.c. implant of 75 mg morphine pellet	Bussiere et al. (1992)
Murine macrophages	↓phagocytosis	1 μM for 17 h	Tomei and Renaud (1997)
	↓ respiratory burst activity	10 nM, 1 pM	Peterson et al. (1987)
	↓NO release	10 and 30 mg/kg/day, 15 days	Singh and Singal (2007)
	↓ chemotaxis	1 μM, 10 μM, 100 nM	Martin et al. (2010)
			Perez-Castrillon et al. (1992)
	↑ intracellular growth of *Leishmania donovani*	10 and 30 mg/kg/day, 15 days	Singh and Singal (2007)
Neutrophil	↓ chemotaxis ↓ respiratory burst activity ↑ intracellular bacterial growth	s.c. implant of 75 mg morphine pellet for 3 days	Martin et al. (2010)
			Wang et al. (2005)
Murine splenocytes	↓ IL-1β, IL-2, TNF-α, IFN-γ, ↑ TGF-β1, IL-10 production	s.c. at doses of 20 mg/kg in a 0.1 ml volume for 24 h	Pacifici et al. (2000)
	Th1 → Th2 shift	100 ng/ml for 4 days	Roy et al. (2001a)
Murine thymocytes	↓ activation of IL-2 gene	1 μM for 17 h	Roy et al. (1997)

Morphine and B Cells

In contrast to T-cell research, work on morphine's effect on function of B cells is limited. First findings reported by Lefkowitz et al. (2000), indicated that that morphine injection reduced the number of antibody-forming cells in the mouse spleen following immunization with sheep red blood cells. These findings were

further supported by several other groups that found *in vivo* morphine treatment (pellet implantation) led to reduction of the mitogenic responses of splenic B cells to bacterial lipopolysaccharide (LPS) (Bhargava et al. 1994; Bryant et al. 1988a; Bussiere et al. 1992). Since formation of antibody response requires interaction of macrophages, T cells, and B cells, modulation of antibody producing capacity does not necessarily mean that the effect of the drug is on B cells and that morphine could be affecting any of the three cell types (Eisenstein and Hilburger 1998). Bussiere et al. (1993), found that morphine's inhibition of antibody responses could be restored with addition of untreated macrophages or with addition of macrophage cytokines (IL-1, IL-6 or Interferon-γ (IFN-γ), suggesting that the morphine-induced suppression is due in part to a deficit of macrophage activity. Furthermore, Weber et al. (1987), demonstrated that morphine's modulation of antibody responses was T-cell dependent, but not to a T cell independent antigen, suggesting that morphine did not directly affect B cell function (Eisenstein and Hilburger 1998).

6.3.3 Modulation of Innate Immune Response

The modulation of innate immune system has been observed on several levels. Morphine treatment modulates leukocyte recruitment, cytokine secretion and bacterial clearance. By decreasing the proliferative capacity of macrophage progenitor cells morphine treatment inhibits numbers of macrophages that are available to respond to an infection (Roy et al. 2006). In addition, morphine delays leukocyte migration, which affects the numbers of phagocytes recruited to the site of infection and ultimately suppresses the capacity of macrophages to ingest opsonized pathogens (Casellas et al. 1991; Szabo et al. 1993; Tomei and Renaud 1997). Collectively, these findings suggest that the macrophage is a key cellular target for the suppressive effects of morphine on the antibody response (Bussiere et al. 1993). Although morphine modulates both innate and adaptive immune systems, defects in innate immunity seem to have broader consequences, with modulation of macrophage functions playing an essential role. Therefore examining morphine mediated modulation of macrophage processes will be the main focus of our discussion

6.3.3.1 Morphine and Macrophages

Our lab first demonstrated that morphine modulation of several immune functions is attributable to the MOR, including macrophage phagocytosis and secretion of TNF-α, since these effects were abolished in morphine treated MORKO mice (Roy et al. 1998a).

Macrophages form the first line of defense against pathogens, and play an essential role in innate immunity through their phagocytic and bactericidal roles as well as through their ability to recruit other cells to the site of infection. Therefore any defects in macrophage function can be detrimental for the host. Macrophages have been at the center of several studies due to significant role they play in morphine mediated immune suppression.

Morphine Modulation of Macrophage Phagocytosis

Morphine treatment leads to suppression of peritoneal macrophage phagocytosis as well as inhibition of respiratory burst activity and chemotaxis (Perez-Castrillon et al. 1992). Due to inhibition of phagocytosis, bacteria are inadequately removed and since respiratory burst is inhibited, morphine attenuates bacterial killing which together with inhibited phagocytosis leads to increased bacteremia and bacterial escape from latency as shown by our group and others (Bhaskaran et al. 2001; Lugo-Chinchilla et al. 2006; Wang et al. 2005). Human studies and rodent models of drug abuse indicate that morphine impairs the ability to eradicate infection by inhibiting phagocytosis. *In vivo* models of morphine abuse have shown that morphine inhibits phagocytosis by non-elicited and elicited macrophages in a naltrexone reversible manner indicating involvement of classical opioid receptors (Rojavin et al. 1993). Subsequent *in vitro* studies indicate that morphine inhibits Fcγ receptor (FcgR) mediated phagocytosis essential for internalization of extracellular pathogens, and that inhibition of phagocytosis occurs through μ and δ opioid receptors (Szabo et al. 1993; Tomassini et al. 2004). Studies by our group confirmed that morphine mediated inhibition of phagocytosis was abolished in μ opioid receptor knockout mice (MORKO) mice, adding further evidence for the role of MOR in these functions (Roy et al. 1998a). Additionally, it was observed that *in vitro* administration of endogenous opioid peptides such as leu- and met-enkephalin (delta receptor agonists) are able to inhibit phagocytosis of opsonized sheep red blood cells (Casellas et al. 1991).

In addition to inhibiting macrophage phagocytosis, several studies support that morphine attenuates bacterial killing as evident by increased bacterial loads or sepsis (Wang et al. 2005; Hilburger et al. 1997). In mice, chronic morphine has been shown to modulate bacterial killing by inhibition of NO release (Bhaskaran et al. 2007; Menzebach et al. 2004). Our laboratory's previous data and several other studies indicate that chronic morphine, by inhibition of NO release, increases susceptibility to bacterial infection, resulting in bacteremia and bacterial invasion of the CNS (Asakura et al. 2006; Bhaskaran et al. 2007; Wang et al. 2005). A recent study by Singh and Singal (2007), notes that morphine administration has a dose-dependent biphasic modulation in *Leishmania donovani* infected mice and peritoneal macrophages *in vitro*, via a NO-dependent mechanism. They show that morphine administration in the nanomolar range was protective against *L. donovani* infection, while morphine concentrations in the micromolar range led to augmented parasite growth in macrophages.

Furthermore, morphine has been implicated in the inhibition of superoxide production. Several groups studying morphine's effect on infection examined superoxide release as a mechanism of bacterial killing, noticed that morphine inhibits superoxide production in neutrophils (Sharp et al. 1985; Simpkins et al. 1986; Welters et al. 2000). In addition to exogenous opioids, endogenous opioids had similar inhibitory effects where pretreatment with endogenous opioid peptides leucine or methionine enkephalins reduced neutrophil's ability to generate superoxide production in response to the *Escherichia coli* product, N-formyl methionyl leucyl phenylalanine (FMLP) (Sharp et al. 1985; Simpkins et al. 1986). Morphine

mediated suppression of superoxide production was reproduced in human peripheral mononuclear cells in studies done by Peterson et al., which examined respiratory burst activity in response to phorbol myristate acetate (PMA) (Peterson et al. 1987, 1989).

In addition to inhibition of bacterial clearance, morphine treatment leads to inhibition of macrophage recruitment and function during an innate immune response. A study carried out by Grimm et al. showed a significant decrease in macrophage chemotaxis when cells were preincubated with morphine, or met-enkephalin (Grimm et al. 1998b). They concluded that morphine's inhibition of subsequent macrophage chemotaxis occurs upon direct binding to the macrophage MOR, and that this activation of MOR leads to the phosphorylation and desensitization of chemokine receptors CCR1, CCR2, CXCR1 and CXCR2. Desensitized chemokine receptors are therefore unable to elicit a response when their ligands are present.

In the presence of the endotoxin LPS, suppression of cytokines IL-6 and TNF-α was seen following morphine treatment (Roy et al. 1998b). The transcription factor NFκB, responsible for up-regulation of several cytokines including IL-6, TNF-α, NO and IL-10, was also suppressed following morphine treatment.

Morphine and Neutrophils

Although it has been observed for some time that chronically administered morphine modulates neutrophil chemotaxis and function, controversy still exists in determining which mechanisms are at play. A growing body of literature supports morphine's suppressive effects on recruitment and immune functions of neutrophils during an innate immune response. Exogenous opioid treatment of peripheral human blood neutrophils leads to inhibition of IL-8-induced chemotaxis (Grimm et al. 1998a). Conversely, Simpkins et al. reported an increase in neutrophil chemotaxis following endogenous opioid (β-endorphin) treatment (Simpkins et al. 1984). The discrepancy of the latter finding may in part be explained by the differences in affinity of morphine and β-endorphins to the MOR on immune cells. Furthermore, acute morphine treatment leads to inhibition of neutrophil cytokines involved in regulation of wound healing (Martin et al. 2010). We recently showed in a wound healing model that morphine treatment resulted in a significant delay and reduction in both neutrophil and macrophage recruitment to the wound site. The delay and reduction in neutrophil reduction was attributed to altered early expression of keratinocyte derived cytokine and was independent of macrophage inflammatory protein-2 expression, whereas suppression of macrophage infiltration was attributed to suppressed levels of the potent macrophage chemoattractant, called monocyte chemotactic protein-1.

Taken together, the complexity by which morphine acts as an immunosuppressor on migration and functional activity of innate immune responders, particularly neutrophils and macrophages, poses a compromising environment that proves detrimental to the host's ability to eradicate pathogens.

6.4 Autocrine and Paracrine Opioid Signaling

Endogenous opioids are capable of paracrine and autocrine signaling. Cells of the CNS and cells of the immune system are capable of generating endogenous opioids. Interestingly, exogenous opioids are capable of acting directly on the immune cells as well as indirectly by activating the HPA axis.

It is accepted that inflammatory mediators released from leukocytes contribute to the generation of pain. However, it is less well known that immune cells also produce mediators that can effectively counteract pain. These include anti-inflammatory cytokines and opioid peptides (Machelska 2007). Physiological pain, triggers a warning mechanism which functions to minimize tissue damage. During the inflammatory response various pro-inflammatory and pro-analgesic mediators are released in order to activate specialized peripheral pain signaling sensory neurons ("nociceptors"). Trigeminal and dorsal root ganglia (DRG) contain nociceptor cell bodies, which give rise to myelinated Aδ and unmyelinated C fibers. Peripheral terminals of Aδ and C fibers transduce and propagate noxious stimuli from peripheral tissues (such as skin, muscles, joints, and viscera) to the dorsal horn of the spinal cord and thereafter to the brain. At spinal and supraspinal sites the integration of signals from pro-analgesic neurotransmitters, environmental and cognitive factors eventually results in the sensation of pain (Woolf and Salter 2000). Inflammation in the periphery leads to increased synthesis and axonal transport of opioid receptors in DRG neurons, resulting in upregulation of their surface expression and enhanced G-protein coupling at peripheral nerve terminals (Ji et al. 1995; Mousa et al. 2001b). Followed by disruption in perineurial barrier allowing access of opioids to access their respective receptors and modulate the pain signals emanating from the site of inflammation (Antonijevic et al. 1995).

Another way by which leukocytes are able to control inflammatory pain is by recruiting other opioid-containing leukocytes to the site of inflammation. During the inflammatory response leukocytes are recruited to the site of infection through chemokines, cytokines and upregulation of adhesion molecules. Studies by Machelska et al., have shown that pretreatment of rats with selectin blocker (fucoidin), or selective antibodies against ICAM-1, integrins (α_4 and $\beta_{2)}$, or against the chemokines (CXCL1 and CXCL2/3) lead to a substantial decrease in the number of opioid-containing immune cells accumulating in inflamed tissue, and consequently abolished endogenous peripheral opioid analgesia (Machelska et al. 1998, 2002). In addition, the migration of opioid-containing leukocytes into injured tissues appears to be modulated by mechanisms involving signaling from the CNS. Schmitt et al. (2003), have shown that intrathecally injected morphine, in a dose dependant manner, significantly decreases the number of β-endorphin-containing leukocytes in inflamed rat paws, and attenuates peripheral endogenous analgesia. These findings indicate that effective central inhibition of pain signals inhibit the recruitment of opioid-containing leukocytes to injured tissues (Machelska 2007).

These studies support a paracrine role of endogenous opioids on the regulation of pain through either leukocyte mediated opioid release signaling via the nociceptors, or though the central opioid mechanisms utilized to limit opioid secretion at

the inflammatory site. Paracrine and autocrine signaling of opioid-containing leukocytes is important in immune suppression. Leukocyte chemotaxis and key immune functions are significantly impaired in the presence of opioids. By secreting opioids, leukocytes can inhibit their own immune functions as well as those of other leukocytes present at the inflammatory site. Opioid mediated inhibition of cytokine and chemokine release inhibits further recruitment to the site of inflammation leading to reduced inflammatory signals and potential pain reduction. Therefore, opioids released from leukocytes can modulate pain by acting through nociceptors and DRG, as well as by inhibiting inflammation.

6.5 Opioids and Disease

Morphine's immunosuppressive effects have been observed for centuries. Recently, as the prescription of opioid-based pain relievers began to rise, opportunistic infections have followed the same trend (Compton and Volkow 2006; Wang et al. 2008). Prevalence of opioid use and abuse is undisputed, and has impacted a wide range of individuals in both the drug abuse population as well as the patients in clinical settings.

Immunosuppression in opioid abusers has been observed clinically and anecdotally. Although clinical studies examining opioid mediated immune suppression are limited, animal studies indicate morphine's immunosuppressive abilities through increased incidence of many bacterial and viral infections. Several groups show that intravenous drug abusers have a greater incidence of infection than non-abusers (Hussey and Katz 1950; Louria et al. 1967). The documentation that opioids, such as morphine, have the potential to modulate immunity is consistent with their ability to alter immune responsiveness to microbial agents (Cabral 2006). Extensive research in the area of morphine induced immune suppression noted that opioid addicts present with high prevalence of tuberculosis, bacterial pneumonias, abscesses, CNS infections as well as viral hepatitis A, B, and C, and high rate of HIV infections (Haverkos and Lange 1990; Louria et al. 1967; Reichman et al. 1979).

Several groups indicate a linkage between intravenous opioid use and increased incidence of infections in humans. McCoy et al. (2004) examined the prevalence of HIV-1 and HCV among injection drug users in Miami, Florida. Results of multivariate analyses indicated a direct correlation between years of heroin use and HCV infection. Furthermore, retrospective studies as well as seroepidemiological analyses indicate that injection users of opioids, such as heroin, have an increased incidence of disease including that attributable to HIV infection (Horsburgh et al. 1989; Joe et al. 1991; Nemoto et al. 1990; Spittal et al. 2003).

A seminal study by Tubaro et al. (1983), observed that following single daily injections of morphine given 24–72 h prior to iv injection of fungus *Candida albicans*, resulted in increased lethality in mice from the organism. This study demonstrated that morphine was able to increase the number of viable *Candida albicans* in the kidney in a dose-dependent manner. More recent studies indicate

similar results, where mice implanted with slow release morphine pellet presented with sepsis, which was manifested by increased bacterial loads in liver, spleen and peritoneal cavities (Hilburger et al. 1997). Additionally, our laboratory has shown that *in vivo* chronic morphine treatment followed by intranasal inoculation with *Streptococcus pneumoniae* markedly delayed neutrophil recruitment, increased bacterial burden in the lung, spleen and blood, with a subsequent increase in mortality (Wang et al. 2005). Morphine's immunosuppressive effects were first noted in its ability to increase susceptibility to infection, as well as accelerate the rate of their progression. Interestingly, *S. pneumoniae* is one of the most common diagnoses among opiate abusers; it is responsible for more than 25% of all cases of pneumonia, and is still associated with an overall mortality rate of 23% among hospitalized patients. Drug abuse has been determined to be a significant risk factor for the development of community-acquired pneumonia since *Pneumococcal* clearance requires the cooperation of both innate and adaptive immunity. Epidemiological data suggests that HIV-positive drug abusers progress to symptomatic AIDS more rapidly than those who do not use drugs, therefore, additional longitudinal studies addressing the enhancement of disease in immunocompromised individuals are warranted (Cabral 2006).

Clinical studies examining effects of opioids in clinical setting are scarce. The difficulty in clinically determining the extent or longevity of opioid immune modulatory effects, is primarily because drug abusers may use multiple drugs. The studies that have been reviewed suggest that illicit drugs act, at least, as cofactors that can increase the severity of infection by microbial agents by altering host resistance. This decrease in host resistance may be a consequence of immunosuppressive action on the activities of macrophages, T lymphocytes, and NK cells. The mechanisms by which these drugs increase susceptibility to infection have not been fully delineated. Considering previously discussed studies, a convergent mode of action by which drugs of abuse affect immunity and increase susceptibility to infection appears to be that they affect cytokine and chemokine expression and, in so doing, alter the homeostatic balance of proinflammatory versus anti-inflammatory mediators. The documented evidence that illicit drugs alter antimicrobial activity *in vivo* and *in vitro*, indicates that their use presents a potential risk of decreased resistance to infections in humans.

Conclusion

This chapter summarizes the current understanding of the roles opioids play in neuro-immunity. We delineate opioid receptor functions and distributions as well as the role of endogenous and exogenous opioids on the immune system and pain mechanisms. Signaling and acting directly through immune cells or acting via the HPA axis, immunosuppressive effects of opioids have been observed in several different models. Ability of the immune cells to produce opioid peptides, as well as their expression of opioid receptors has led to an interesting paradigm shift. Original thoughts of opioids being secreted by and acting solely on the nervous system, has recently been diverted to investigation of opioid secreting leukocytes and the role they play in modulation of traditional pain mechanisms.

In spite of a multitude of research conducted in this field, a gap in understanding of mechanisms underlying these processes still exists. Although many advances have been made in understanding the effects of endogenous and exogenous opioids on immune responses, the real clinical relevance of these effects is not completely clear. Enhancing our knowledge and understanding of opioid mediated immune suppression and mechanisms involved in these processes is essential to development of new and improved therapies for chronic pain management.

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The Effects of Somatostatin on Immune Cells, Functions and Diseases

7

Toomas Talme and Karl-Gösta Sundqvist

Contents

7.1 Introduction

Somatostatin (SST) is a ubiquitous neuropeptide hormone that was first extracted from bovine hypothalamus as an inhibitor of growth hormone secretion (Brazeau et al. 1973). The SST gene is a very ancient gene present in all vertebrate classes (Tostivint et al. 2004). Since its discovery in 1973, SST has stimulated a plethora of studies investigating its multiple physiological actions in a great variety of tissues. The continued scientific interest has been evident over the years including the cloning of a family of five somatostatin receptors (SSTRs) in 1992 (Yamada et al. 1992; O'Carroll et al. 1992; Panetta et al. 1994), the characterization of the related neuropeptide corticostatin in 1996 (de Lecea et al. 1996; Tostivint et al.

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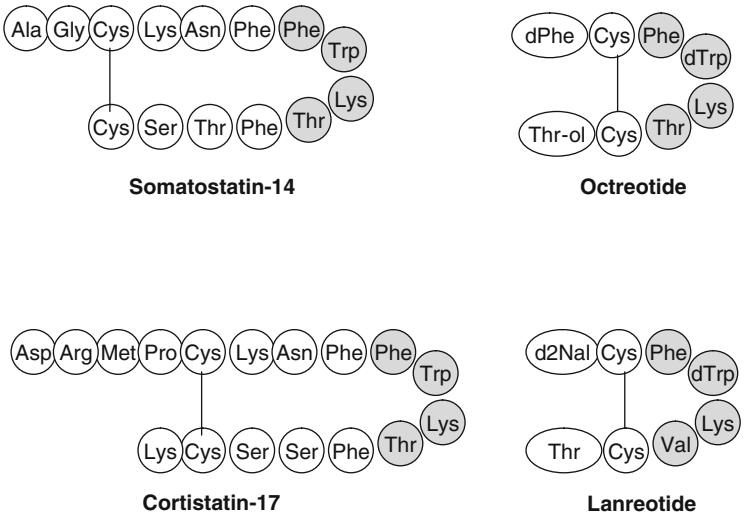

Fig. 7.1 Amino acid sequences of somtostatin-14, cortistatin-17, and of the synthetic octapeptide analogues octreotide and lanreotide. The *circles in grey* denote the amino acids essential for binding with the somatostatin receptors

1996), and the development of hundreds of synthetic SST analogues, some of them with clinical applications. SST exists in two bioactive forms, as a 14 amino acid peptide (Brazeau et al. 1973) and as a cogener of somatostatin-14 extended at the N-terminus called somatostatin-28 (Pradayrol et al. 1980) (Fig. 7.1). SST is widely distributed in the central and peripheral nervous system but also present in endocrine pancreas, gut thyroid, prostate, placenta, adrenals, kidneys and skin (Luft et al. 1974; Arimura et al. 1975; Dubois 1975; Hokfelt et al. 1975; Orci et al. 1975; Pelletier et al. 1975; Polak et al. 1975; Patel and Reichlin 1978; Johansson and Nordlind 1984). SST is present in the peripheral nervous system in sympathetic and sensory neurons innervating lymphoid organs and may thus influence functional responses of lymphocytes and antigen-presenting cells (Aguila et al. 1991; Felten et al. 1985). SST is expressed in both cortical and medullary thymic epithelial cells (Solomou et al. 2002). SST suppresses the synthesis and secretion of growth factors such as growth hormone and insulin-like growth factor 1. SST inhibits gastrointestinal hormones that include gastrin, cholecystokinin, serotonin, glucagon, vasoactive intestinal peptide, and others (Alberti et al. 1973; Koerker et al. 1974; Zhang et al. 1991; Philippe 1993; Nelson-Piercy et al. 1994; Kleinman et al. 1995; Ballian et al. 2006; Corleto 2010). There is evidence from several test systems that SST can modulate the responses of lymphocytes to mitogens and T cell antigen receptor (TCR)/CD3 stimulation and even that SST influences adhesion and motility of lymphocytes. SST stimulates adhesion of lymphocytes to extracellular matrix components (ECM) (Levite et al. 1998). SST inhibits proliferation directly by regulating tyrosine kinase, tyrosine phosphatase, nitric oxide synthase, cyclic

guanosine 3′, 5′-cyclic monophosphate–dependent protein kinase, and RAS/extracellular signal–regulated kinase signalling pathways (Pyronnet et al. 2008). SST also has antiangiogenic properties and can induce apoptosis (Sharma and Srikant 1998; Woltering 2003; Pyronnet et al. 2008). Glucagons, growth-releasing hormone, neurotensin, corticotrophin-releasing hormone, calcitonin gene-related peptide and bombesin are potent stimulators of SST secretion, while opiate and GABA are inhibitors (Patel et al. 1991; Epelbaum et al. 1994). Inflammatory cytokines have also shown regulatotory effects on SST secretion: IL-1, IL-6, IL-10; INF-γ, and TNF-α thus stimulate whereas TGF-β inhibits SST release (Scarborough et al. 1989; Quintela et al. 1997; Elliott 2004).

7.2 Somatostatin Receptors

SST mediates its functions through five receptor subtypes, SSTR1-5, belonging to the family of seven transmembrane domain G protein coupled receptors coded by genes localized on different chromosomes (Schonbrunn and Tashjian 1978; Patel et al. 1995; Reisine and Bell 1995; Patel et al. 1996; Olias et al. 2004). All SSTR subtypes exist in a single protein isoform, except for SSTR2, which is alternatively spliced into SSTR2A and SSTR2B isoforms (Patel et al. 1995, 1996). The six known SSTR proteins are well characterized and widely expressed in neuronal and nonneuronal tissues (Weckbecker et al. 2002; Olias et al. 2004). SSTR ligand binding inhibits their adenylate-cyclase activity and regulates calcium and potassium channels (Weckbecker et al. 2002; Schonbrunn 2008). G-protein-coupled receptors (GPCRs) exist both as monomers and dimers or higher-order oligomers, representing assemblies either with their peers or with other classes of GPCR (“heterodimers”). Activation by ligand induces SSTR dimerization, both homo- and heterodimerization which alter functional properties, such as ligand binding affinity, and agonist-induced receptor internalization and up-regulation (Rocheville et al. 2000b). For example heterodimerization of SSTR2A and SSTR3 receptors inactivated SSTR3 function (Pfeiffer et al. 2001), and SSTR2 and SSTR5 heterodimers markedly extended cell growth inhibition in coexpressing cells (Grant et al. 2008). SSTR have not only been shown to form dimers within their own family but also to other related families, such as the dopamine, opioid and epidermal growth factor receptors (Rochville et al. 2000a; Pfeiffer et al. 2002; Watt et al. 2009). Although many such heterodimers of G protein-coupled receptors have been reported only a few have shown functional relevance so far. Molecules that target receptor-receptor interactions may also provide opportunities for novel drug discovery (Filizola 2010).

In the immune system SSTRs are localized in the germinal centers of lymphoid follicles (Reubi et al. 1992, 1998). MRNA for SSTRs as well as SST binding sites are expressed by T and B lymphocytes and monocytes (Dalm et al. 2003b; Talme et al. 2001). Normal non-activated T cells express SSTR1-4, activated T cells

selectively express SSTR5, leukaemia cells express SSTR2-4 and thymocytes express SSTR2 (Talme et al. 2001). Isolated thymocytes generally express SSTR2A and SSTR3 whereas thymic epithelial cells selectively express SSTR1 and SSTR2A (Ferone et al. 2002). SST ligation of SSTR receptors mediate inhibition of adenylate cyclase and cAMP/protein kinase A via pertussis sensitive GTP binding proteins that may account for the inhibitory actions of SST reported in multiple in vitro and in vivo systems as further discussed below.

7.3 Somatostatin and Lymphocytes

Numerous effects of somatostatin on immune cell functions have been described *in vitro* as well as *in vivo*. It is difficult to compare these studies, as experiments were carried out with cells from different species and under different experimental conditions. Lower concentrations (10^{-12}–10^{-10} M) are characteristic of levels of neuropeptides found in blood, whereas higher concentrations (10^{-7}–10^{-6} M) may be found at nerve endings (Stanisz et al. 1986). SST (10^{-12}–10^{-10} M) inhibits proliferation of human T lymphocytes by alloantigens, PHA and CONA as well as the expression of the activation markers CD25 and CD69 (Payan et al. 1983; Casnici et al. 1997). SST further inhibits T cell proliferation induced via CD28 and CD2 but has been reported to reduce proliferation by anti-CD3 antibodies only marginally (Casnici et al. 1997). The inhibitory effects of SST are more pronounced when the lymphocytes are stimulated by alloantigen than with a polyclonal activator. SST (10^{-14}–10^{-10} M) has been reported to reduce the production of IFN-γ by mononuclear cells and T cells in murine schistosomiasis mansoni infection, probably through SSTR2 since the effect was blocked by an antiserum against this receptor (Blum et al. 1992; Weinstock and Elliott 1998). Schistosoma parasites induce granulomas and granuloma macrophages produce SST mRNA in response to IFN-γ and IL-10 (Weinstock and Elliott 1998). These findings are interesting since they demonstrate that the parasite's production of SST may suppress a Th1 response and promote a Th2 response which has obvious implications to the many situations when the immune system avoids strong reactions to prevent organ damage or preferentially develops Th1 or Th2 responses. Thus, the hygiene hypothesis of allergy and autoimmunity predicts that exposure to non-pathogenic bacteria protects individuals from developing autoimmune and atopic disorders perhaps by down-regulating potentially harmful Th1 and Th2 responses and giving preference to Treg cell responses (Kamradt 2005) . It is an attractive hypothesis that an adaptive immune system with minimal risk to undergo abnormal proliferative responses to self antigens and allergens is endowed with effective inhibitory pathways that promote tolerance as reported for interactions between PD1, CTLA4 and their ligands (Fife et al. 2009; Schneider et al. 2006). SST may provide immunosuppressive activity that contributes to tolerance and thus prevent adverse proliferative T cell responses leading to autoimmunity or allergy.

SST (10^{-8} M) has also been found to trigger secretion of IL-2 and IFN-γ in T cell lines without a concomitant stimulation of T cell proliferation showing that cytokine production and proliferation are dissociable events and that neuropeptides have specific effects that probably reflects properties of the target cell and the context of the stimulation (Levite 1998). The SST analogue octreotide (SMS 201–995) (10^{-12}–10^{-11} M), which shows an affinity for SSTR2, 3 and 5, also inhibits lymphocyte proliferation (Casnici et al. 2004).The SST analogues octreotide (10^{-11}–10^{-10} M) and pasireotide (10^{-12} M) also have an antiproliferative effect on human lymphocyte proliferation, that was suggested to be an apoptotic phenomenon mediated through SSTR2a, in the case of octreotide, and through SSTR3 by pasireotide (Lattuada et al. 2007). In contrast to octreotide, which enhances IL-10 and inhibits IFN-γ, pasireotide inhibits both IL-2 and IFN-γ. In both sets of experiment the different behaviour of the two analogues could be due to their different affinity to the SSTR subtypes. Interestingly, octreotide (10^{-12}–10^{-11} M) enhances IL-10 secretion in both alloantigen and PHA-activated human blood lymphocytes and the results obtained suggest that IL-10 produced by T cells is responsible for the antiproliferative effect of octreotide (Casnici et al. 2004). Octreotide has been reported to inhibit the secretion of IL-12 from blood mononuclear cells (Komorowski et al. 2001). One interesting example of a regulatory effect of SST in lymphocytes is that the neuropeptide is present in aqueous humor in the eye and has been reported to induce production of α-melanocyte stimulating hormone (α-MSH) in anti-CD3-stimulated primed T cells (Taylor and Yee 2003). α-MSH was interpreted to be responsible for the suppressive effect of SST on IFN-γ production. In epithelial cell- and neural cell-derived tumors TGF-ß serves as a growth inhibitor at the beginning of tumor development but later becomes a growth accelerator for transformed tumors. The growth-inhibitory effect seems to reflect that TGF-ß induces the production of SST and potentially activates the negative growth autocrine loop of SST, which leads to the downstream induction of multiple growth inhibitory effectors (Leu et al. 2008).

The chemokine CXCL12 is a potent stimulator of T lymphocyte migration within three-dimensional type 1 collagen gels and SST (10^{-12}–10^{-8} M) inhibits this stimulatory effect whereas a number of other neuropeptides and opioids do not affect CXCL12-induced lymphocyte migration (Talme et al. 2004).T cell migration within collagen as well as the inhibitory effect of SST is independent of adhesive interactions between cells and collagen gels. SST does not influence the cell surface expression of the CXCL12 receptor CXCR4 and therefore probably affects the motile process or its regulation. It is interesting in this context that CXCL12 has been shown to trigger cell surface expression of endogenous thrombospondin-1 in human T cells (Liu et al. 2009). In the light of the fact that SST induces inhibitory effects on secretion this points to the possibility that SST may inhibit T cell migration through inhibition of exocytosis of thrombospondin-1.

In contrast to its inhibitory effect on migration SST (10^{-11}–10^{-5} M) enhances adhesion of human T cells to fibronectin and to a certain extent also to collagen type IV and laminin (Levite et al. 1998). Calcitonin-gene related peptide (CGRP) and

Table 7.1 Table summarizing the effects of somatostatin and somatostatin analogues on cells of the immune system

Immune cells	Effects	References
T cells	Inhibits production of IFN-γ	Blum et al. (1992)
	Inhibits T cell proliferation	Casnici et al. (1997)
	Inhibit expression of CD25 and CD69	Casnici et al. (1997)
	Stimulates production of IL-2 in T cell lines	Levite (1998)
	Enhances adhesion to ECM	Levite (1998)
	Suppresses Th1 response and promotes a Th2 response	Weinstock and Elliott (1998)
	Inhibits IL-12 secretion (octreotide)	Komorowski et al. (2001)
	Increases production of ά-MSH	Taylor and Yee (2003)
	Increases IL-10 production (octreotide)	Casnici et al. (2004)
	Inhibits CXCL 12-induced migration	Talme et al. (2004)
	Inhibit production of IL-2 and IFN-γ (pasireotide)	Lattuada et al. (2007)
B cells	Reduce the secretion of IgA in B lymphocytes from spleen, Peyer's patches and mesenteric lymph nodes	Stanisz et al. (1986)
	Inhibits immunoglobulin production in peripheral blood lymphocytes and lamina propria mononuclear cells	Fais et al. (1991)
	Stimulates proliferation and immunoglobulin formation in B lymphoblasts	Rosskopf et al. (2003)
Macrophages/ dendritic cells	Inhibits production of IFN-γ	Blum et al. (1992)
	Produces SST	Weinstock and Elliott (1998)
	Inhibits IL-12 secretion	Komorowski et al. (2001)
	Inhibits responsiveness to Helicobacter	Kao et al. (2006)
	pylori by suppressing IL-12 release	
	Inhibit TNF-α secretion in Crohn's disease	Taquet et al. (2009)

neuropeptide Y also promote lymphocyte adhesion to fibronectin whereas substance P, which coexists with CGRP in peripheral sensory nerve endings including those innervating lymphoid organs blocks T cell adhesion to fibronectin induced by CGRP, neuropeptide Y, SST, macrophage inflammatory protein-1β and PMA (Levite et al. 1998). A comparison of the SSTR expression in various leukemic T cell lines and the responsiveness of the same cells to SST and its analogues in adhesion tests suggest that SSTR2 and/or 3 are responsible for the enhancement of adhesion to extracellular matrix components (Talme et al. 2001). As it comes to B cells, SST (10^{-8} M) has been shown to reduce the secretion of IgA from murine CONA-stimulated lymphocytes from spleen, Peyer's patches and mesenteric lymph nodes (Stanisz et al. 1986). B cells exclusively express the SSTR isoform 2A which seems to be involved in SST-induced (10^{-7} M) signal transduction to proliferation and immunoglobulin formation (Rosskopf et al. 2003). The effects of SST on the immune system are summarized in Table 7.1.

7.4 Somatostatin and Antigen Presenting Cells

Activation of T cells during adaptive immune responses is initiated by the capture and processing of antigen by antigen presenting cells. These then present peptide fragments of the antigens in recognizable form to T cells combined with MHC class II antigens together with the appropriate concomitant signals. Macrophages, dendritic cells and B lymphocytes are the major professional antigen presenting cells although also other cell types may present antigen to T cells. Monocytes, macrophages and dendritic cells do not seem to produce SST as demonstrated at the mRNA level (Dalm et al. 2003a). All these cell populations express cortistatin but in contrast to lymphocytes that express mRNA for SSTR1-5 (Talme et al. 2001), monocytes, macrophages and dendritic only seem to express SSTR2 (Dalm et al. 2003a). Dendritic cells produce IL-12 in response to bacterial infections and IL-12 promotes the Th1 response needed for bacterial clearance (Méndez-Samperio 2009). There is evidence that SST (10^{-10}–10^{-8} M) inhibits the responsiveness of dendritic cells to Helicobacter pylori by suppressing IL-12 release and consequently inhibits the capacity of activated dendritic cells to prime T cells (Kao et al. 2006). Interestingly, IL-4 mediates resolution of Helicobacter-induced gastritis and this IL-4-induced resolution is not observed in SST null mice suggesting that IL-4 mediates resolution of gastritis through SST. Further support for this concept comes from the finding that treatment with the somatostatin analogue octreotide leads to resolution of chronic gastritis (Kao et al. 2006).

7.5 Cortistatin Binds SSTR's and Shares Pharmacological and Functional Properties with SST

Cortistatin (CST) is a neuropeptide with strong structural similarity to somatostatin (Fig. 7.1). CST was discovered in 1996 by L. de Lecea and comprises cortistatin-14 (present in rat and mouse), cortistatin-17 (human), and cortistatin-29 (human) (de Lecea et al. 1996). CST binds with high affinity to all five SSTR subtypes (Criado et al. 1999). CST is a widely distributed neuropeptide present in neural and non-neural tissues such as endocrine organs and pancreas. In the immune system CST, but not SST, is expressed by T and B lymphocytes, T and B cell lines, thymocytes, monocytes/macrophages and dendritic cells (Dalm et al. 2003a, b; de Lecea and Castano 2006; van Hagen et al. 2008). CST and SST exhibit the same endocrine activities. CST is also a ligand for the human growth hormone (GH)-secretagogue receptor (GHSR), also known as the ghrelin receptor, that does not bind SST, suggesting that CST represents a link between the ghrelin and the SST systems (Broglio et al. 2007). A group of synthetic small molecular weight compounds, growth hormone secretagogue (GHS), binds to GHSR and induces production of GH by the pituitary gland. GH enhances development of the thymus and promotes the engraftment of human T cells in SCID mice (Koo et al. 2001). Ghrelin, a 28 amino acid peptide in humans, is a natural ligand for GHSR (Kojima et al. 1999). Ghrelin as well as GHS(-)R mRNA expression has been detected in

Table 7.2 Table summarizing the effects of cortistatin on cells of the immune system

Immune cells	Effects	References
T cells	Increases IL-10 production	Gonzales-Rey (2006)
	Increases TGF-ß production	
	Reduces development of self-reactive Th1 cells	
	Induces peripheral expansion of Treg cells	
Macrophages	Inhibits the production of TNF-α, IL-6, IL-1β, IL-12	Gonzales-Rey (2006)
	Inhibits production macrophage-inflammatory protein	
	(MIP)-2 and Rantes	
	Inhibits realease of nitric oxide (NO) and free radikals	

human T and B cells and neutrophils (Dixit et al. 2004; Hattori et al. 2001). However, the wide distribution of GHSR suggests that ghrelin, have multiple roles and subsequent studies have demonstrated that ghrelin is a potent inhibitor of proinflammatory cytokines including IL-6, TNF-α, IL-1β, GMCSF, IL-12 and IL-17 (Dixit et al. 2009). Ghrelin has also been shown to down-regulate the serum levels of the proinflammatory cytokines TNF-α and IL-6 in a rat model of sepsis through activation of the vagus nerve (Wu et al. 2007). The classical SST analogues octreotide, lanreotide and vapreotide bind GHSR with an affinity lower than that of ghrelin (Gauna and van der Lely 2005). CST-14 as well as CST-17 binds to GHSR with an affinity comparable to ghrelin (Broglio et al. 2007).

The fact that various human immune cells, including lymphocytes and antigen-presenting cells, produce CST and its levels correlate with cell differentiation and activation state suggests that CST may be a major endogenous regulatory factor in the immune system (Dalm et al. 2003a, b) (Table 7.2). Administration of CST (25–250 μg/kg mouse) delays the onset, decreases the frequency and reduces the severity of various experimental models of sepsis (Gonzalez-Rey et al. 2006a), rheumatoid arthritis (Gonzalez-Rey et al. 2007), and Crohn's disease (Gonzalez-Rey et al. 2006b). CST treatment impairs early events that are associated with the initiation and establishment of autoimmunity to self-components, as well as later phases that are associated with the evolving immune and destructive inflammatory responses. The anti-inflammatory action of CST is exerted at different levels of the innate immunity. CST reduces the development of self-reactive Th1 cells, their entry into target organs, the release of pro-inflammatory cytokines and chemokines as well as the recruitment and activation of macrophages and neutrophils (Gonzalez-Rey and Delgado 2008). This is accompanied by a decreased production of inflammatory cytokines (TNF-α, IFN-γ, IL-12, IL-6, IL-18 and IL-1β) and chemokines (Rantes and MIP-2) by activated macrophages (Gonzalez-Rey et al. 2006a, b). In addition, CST down-regulates the release of free radicals and nitric oxide in macrophages (Gonzalez-Rey et al. 2006a, b). At the same, CST stimulates the production of anti-inflammatory cytokines such as IL-10 (Gonzalez-Rey et al. 2006a). Many of these immunomodulatory effects are shared by SST (Krantic 2000; Pinter et al. 2006). Interestingly, CST induces the peripheral expansion of new antigen-specificCD4 + CD25+ forkhead box P3 (FoxP3) + Treg cells, with

suppressive activity on self-reactive T cells (Gonzalez-Rey et al. 2006b), by a mechanism dependent on the production of the immunosuppressive cytokines IL-10 and/or TGF-β. This indicates that CST participates in maintaining immune tolerance by regulating the balance between pro-inflammatory and anti-inflammatory factors, and by inducing the emergence of regulatory T cells with suppressive activity against autoreactive T cell effectors (Gonzalez-Rey and Delgado 2008). The effects of CST on the immune system are summarized in Table 7.2.

7.6 Somatostatin in Inflammatory Diseases

SST has been reported to suppress inflammation in animal model systems (Lembeck et al. 1982; Karalis et al. 1995; Fioravanti et al. 1995). In a rat model of carrageenan-induced inflammation glucocorticoids seem to reduce the volume and cellularity of the inflammatory exudate through stimulation of local SST expression (Karalis et al. 1995). SSTR4 receptor knockout mice exhibit markedly enhanced inflammation in response to carageenan-induced edema, adjuvant-induced arthritis, oxazolone-induced delayed hypersensitivity of the skin and airway inflammation elicited by intranasal lipopolysaccharide administration (Helyes et al. 2009). These results suggest that SST through SSTR4 has a potent protective effect against immunologic changes leading to edema formation, inflammatory pain and hypersensitivity responses. SSTR4 receptors are present on lymphocytes, vascular endothelial cells, smooth muscle cells and synoviocytes that all play key roles as effectors and targets of inflammatory diseases implicating this particular receptor as a promising target for the development of anti-inflammatory drugs. The SSTR4 agonist J-2156 inhibits neurogenic and vascular inflammatory reactions in rats and mice further supporting a key role of this receptor for development of anti-inflammatory therapy (Helyes et al. 2006).

Somatostatin and rheumatoid arthritis. Rheumatoid Arthritis (RA) is a chronic, multisystem, autoimmune disease characterized by persistent inflammatory synovitis. The chronic inflammation leads to development of pannus, an aggressive inflammatory tissue where activated T lymphocytes, macrophages, B cells, and their products and active angiogenesis play major roles leading to progressive destruction of joints (Harris 1990). SST has multiple modulatory effects on the immune system and the function of synovial cells, as well as anti-angiogenic, antiproliferative and analgesic properties, which makes SST an attractive candidate for use as a therapeutic agent in immune-mediated diseases such as RA (Paran et al. 2001). SST (10^{-9} M) inhibits the proliferation of human lymphocytes, the production of immunoglobulins by B lymphocytes, and neutrophil chemotaxis (Fais et al. 1991; Kolasinski et al. 1992). As an antagonist of substance P, SST modulates neurogenic inflammation and pain perception (Kolasinski et al. 1992). Receptors for SST have been demonstrated on lymphocytes and macrophages and on the synovial membrane in patients with active RA (van Hagen et al. 1994). In vitro SST (10^{-10} M) has been shown to inhibit proliferation, mRNA expression and synthesis of IL-6 and IL-8, as well as production of matrix metalloproteinases in synovial

cells (Takeba et al. 1997). Other effects of SST relevant to the treatment of RA are its analgesic properties and its ability to inhibit angiogenesis, thus potentially suppressing pannus formation (Chrubasik et al. 1984; Barrie et al. 1993). Somatostatin analogue treatment with Sandostatin LAR (Novartis, Basel, Switzerland) 2 mg attenuates histological findings of inflammation and increases mRNA expression of IL-1β in the articular tissues of rats with ongoing adjuvant-induced arthritis (Paran et al. 2005) Intra-articular knee injections in with SST 14 (50 μg at 15-day intervals) improved pain, morning stiffness and knee function in patients with RA (Ciocci et al. 1994). Treatment with a long acting SST analogue containing octreotide have led to significant clinical improvement in a subset of patients with active, refractory RA in a couple of small open studies, but further large, placebo controlled studies are required to evaluate this drug as a potential DMARD for patients with RA. (Paran et al. 2001; Koseoglu and Koseoglu 2002)

Somatostatin and psoriasis. Psoriasis is a common inflammatory skin disease which affects about 2% of the population. The commonest variant is the plaque form psoriasis, with well demarcated erythematous infiltrated lesions covered with silvery scales. It has been hypothesized that stressful events and local trauma cause the release of neuropeptides, such as substance P from sensory nerves in the skin which, in turn, may initiate the development of psoriasis lesions in predisposed individuals (Farber et al. 1986; Mallbris et al. 2005). This theory is supported by case reports of patients in whom cutaneous nerve damage resulted in clearance of their psoriasis at that site, but with reappearance of the skin lesions after recovery of cutaneous sensation (Farber et al. 1990). SST immunoreactive nerve fibers and cells with dendritic morphology are found in human skin (Johansson and Nordlind 1984; Johansson and Vaalasti 1987; Talme et al. 1997). SST immunoreactivity has also been reported in Merkel cells (Wollina and Mahrle 1992; Fantini and Johansson 1995). Serial biopsies taken from psoriasis lesions during topical treatment with clobetasol (a potent corticosteroid) and calcipotriol (a vitamin D_3 analog) showed a significant reduction in the number of SST-positive cells in the dermis during healing induced by both therapies (Talme et al. 1999). All five SSTRs are expressed both in normal human skin as well as in lesional skin of psoriasis and atopic dermatitis (Hagstromer et al. 2006). Psoriasis patients have significantly higher levels of SST than controls in the serum (Geisner et al. 2007). SST has been used in several open-label trials as treatment for psoriasis and psoriatic arthritis (Weber et al. 1982; Venier et al. 1988; Matucci-Cerinic et al. 1988; Matt et al. 1989). Although the test protocols in the different trials are not comparable, the compiled data suggest that SST probably improves psoriasis and psoriasis arthritis.

Somatostatin and inflammatory bowel disease (IBD). Intestinal inflammation is controlled by various immune cells interacting via molecular mediators including neuropeptides. SST has significant effects on digestive system physiology and modulates gastrointestinal functional activities, such as motility, secretion, and absorption (Corleto 2010). In the gastrointestinal tract most of the SST is contained in mucosal [delta] cells distributed with different densities in neurons intrinsic to the submucosal and myenteric plexuses, and in pancreatic islet [delta] cells (Costa et al. 1977). SST release in the gastrointestinal tract can be stimulated by luminal

factors (mechanical, HCL, fat, glucose) or by circulating factors (peptides, amines, free fatty acids, prostaglandins) and various drugs. Conversely, SST can be luminally inhibited by peptones, $NaHCO_3$, or circulating agents such as peptides, hormones, neurotransmitters, and amines (Corleto 2010).

There is both morphological and experimental evidence for crucial involvement of extrinsic sensory neurons and neuropeptides in the pathogenesis (Engel et al. 2010). Activation of sensory neurons is accompanied by a release of the neuropeptides CGRP and substance P, which induce neurogenic inflammation characterized by vasodilatation, plasma extravasation, and leukocyte migration (O'Connor et al. 2004). Chemical desensitization or surgical denervation of sensory nerves attenuated experimental colitis (Takami et al. 2009). The genetic deletion or pharmacological blockade of receptor channels on nociceptive sensory neurons was also demonstrated to be effective in treating experimental colitis, supposedly by inhibiting neuropeptide release (Engel et al. 2010). Neurogenic inflammation can be suppressed by SST released from sensory nerve terminals upon stimulation (Green et al. 1992; Pinter et al. 2006). SST exerts a short-lasting systemic anti-inflammatory by distribution through the systemic circulation effect (Szolcsanyi et al. 1998). This neuronally-dependent anti-inflammatory effect of SST is supposed to be largely mediated by SSTR4 and – to a lesser extent – SSTR1 (Helyes et al. 2001; Pinter et al. 2002). The course of intestinal inflammatory responses is tightly coordinated by the extensive communication between the immune system and the enteric nervous system, among which the bidirectional mast cell-neuron interaction within the intestinal wall plays a role. Recent research suggests that SST is able to inhibit this self-reinforcing network by simultaneously suppressing the inflammatory activities of both neurons and mast cells (Van Op den Bosch et al. 2009). In murine experimental colitis, administration of octreotide (10 μg/rat) and CST (25–250 μg/kg mouse) were found to effectively reduce mucosal damage and intestinal inflammatory responses, mainly through modulation of several cell types residing in the widely scattered gutassociated lymphoid tissue (Eliakim et al. 1993; Gonzales-Rey et al. 2006b). In Crohn's disease (CD) and ulcerative colitis (UC) the total numbers of SST-containing endocrine cells were decreased in number compared with the controls. This decrease was related to the degree of inflammation in CD; the higher the grade of inflammation, the lower the number of SST-containing cells suggesting that absence of such cells may signify a loss of immunosuppressive activity in the gastrointestinal tract (Watanabe et al. 1992). SSTR were present in high density in most intramural veins, but not in arteries, of intestines in florid CD or UC but were undetectable in the veins of noninflamed control intestine (Reubi et al. 1994a). A significant increase (more than 400 times) in SSTR5 mRNA level was observed in CD patients peripheral blood mononuclear cells (PBMCs) and four tested SST analogues were found to significantly inhibit TNF-α secretion of CD patients PBMCs (Taquet et al. 2009). The compiled results of these studies suggest that SST/SSTR interactions may be important for the pathogenesis of IBD and are disturbed in CD disease.

7.7 Somatostatin and Somatostatin Analogues in Clinical Applications

Owing to its antiangiogenic, antiproliferative and analgesic properties SST has been used in clinical trials as treatment of various inflammatory diseases and neuroendocrine tumors. Although SSTRs are widely distributed in the human body as good therapeutic targets, somatostatin is rapidly degraded and clinical usefulness is limited due to a half-life of 2–3 min making it difficult to use as a therapeutic. However, synthetic analogues have been developed that are more stable and can be used in clinical applications. The long acting analogues octreotide and lanreotide have been approved for the treatment of gastroenteropancreatic neuroendocrine tumors, acromegaly, complications after pancreas surgery and bleeding oesophagus varices. Octreotide and lanreotide are more stable than natural SST but have restricted receptor affinity profiles with high affinity for SSTR2 and lower affinities for SSTR3 and SSTR5 (Weckbecker et al. 2002) (Fig. 7.1). Compared with SST, octreotide contains three substituted amino acids (D-Phe, LThr[ol] D-Trp) that make it resistant to metabolic degradation and increases its in vivo half-life (Fig. 7.1) (Bauer et al. 1982). It was tested in clinical trials for patients with carcinoid syndrome and approved by the Food and Drug Administration for patients with hormone-producing neuroendocrine tumors such as carcinoid tumors as well as pancreatic tumors (glucagonoma, VIPoma) in 1987. Octreotide was originally formulated for subcutaneous injection, but the long-acting formulation Sandostatin LAR (Novartis, Basel, Switzerland) can be given as a monthly injection. Other analogues have been developed, such as lanreotide (BIM23014) and a long-acting form of lanreotide is Somatuline Autogel (IPSEN, Paris, France) (Cai et al. 1986; Caron et al. 1997). Other subtype-selective and multi-somatostatin nonpeptide SST analogues are under development (Rohrer et al. 1998; Lohof et al. 2000; Hofland and Lamberts 2003; Zatelli et al. 2007; Schonbrunn 2008). The multi-somatostatin analogue SOM230 (pasireotide) has high affinity for SSTR1, SSTR2, SSTR3 and SSTR5 (Weckbecker et al. 2002), and is in clinical trials for treatment of pituitary adenomas and octreotide-resistant carcinoids (Zatelli et al. 2007). The multi-somatostatin analogue KE108 has high affinity for all five known SSTRs (Reubi et al. 2002). Although multi-somatostatin analogues were designed to mimic the natural actions of SST, SOM230 and KE108 have cell signalling properties distinct from those of SST; they mobilize calcium and induce phosphorylation of extracellular signal–regulated kinase, whereas SST is an agonist in these pathways (Cescato et al. 2010). The most frequent adverse effects during treatment with SST analogues include abdominal pain with cramps, diarrhoea, nausea, and pain at the injection site. Less frequent side effects include cholelithiasis, bradycardia, and diabetic glucose tolerance (Oberg et al. 2004).

Gastroenteropancreatic neuroendocrine tumors (GEP-NETs), such as carcinoid tumors, glucagononoma and VIPoma, are heterogeneous tumors, in terms of clinical and biological features, that originate from the pancreas or the intestinal tract. GEP-NETs may cause hormone hypersecretion and associated symptoms. Most GEP-NETs overexpress the SSTR2 (Reubi 2007). SST analogues are the best

therapeutic option for functional neuroendocrine tumors because they reduce hormone-related symptoms and also have antitumor effects. Numerous studies have shown that SST analogues are effective in patients with hormone-producing GEP-NETs, and pooled data of octreotide and lanreotide trials from the past 20 years show a mean symptomatic response rate of 73% (range 50–100%) (Oberg et al. 2004, 2010). Long-acting formulations of SST analogues stabilize tumor growth over long periods. The development of radioactive analogues for imaging and peptide receptor radiotherapy has also improved the management of GEP-NETs. Carcinoid crisis is a severe condition that occurs in a subgroup of patients with carcinoid tumors, characterized by flushing, fluctuations in blood pressure, and bronchoconstriction. Long-acting SST analogues prevent carcinoid crisis. Carcinoid heart disease secondary to serotonin production of liver metastases was previously a common cause of death (30%) that has been significantly reduced (4%) by the introduction of SST analogue therapy (Oberg et al. 2010). A new treatment modality for patients with inoperable or metastasized endocrine GEP-NETs is the use of radiolabeled SST analogues and the results obtained with radiolabeled octreotide are very encouraging in terms of tumor regression and symptomatic improvement (Kwekkeboom et al. 2010).

Pancreatic resections are associated with high morbidity (30–60%) and mortality (5%). Synthetic analogues of SST are advocated by some surgeons to reduce complications following pancreatic surgery. Somatostatin analogues reduce perioperative complications but do not reduce perioperative mortality. In those undergoing pancreatic surgery for malignancy, they shorten hospital stay. Based on the current available evidence, SST and its analogues are recommended for routine use in patients undergoing pancreatic resection for malignancy (Koti et al. 2010).

Acromegaly, a chronic disabling disease with a prevalence of 50–60 cases per million population, is almost invariably caused by a GH-secreting pituitary adenoma, and rarely by eutopic or ectopic GHRH production. Elevated GH and IGF-1 are the hallmarks of this endocrine disturbance, resulting in soft tissue and skeletal growth and deformations, with cardiovascular, respiratory, neuromuscular and metabolic complications, as well as impairment of other pituitary functions. About 80% of the GH-secreting tumors are macroadenomas, many of them leading to mass-effect manifestations as visual impairment and headaches (Holdaway and Rajasoorya 1999; Melmed 2006). The mortality rate is three times higher than of the normal population, mainly due to cardiovascular diseases. Many efficacious therapeutic approaches are currently available to acromegaly control, namely pituitary surgery, radiotherapy and medical therapy with dopamine agonists and SST analogues (Kumar et al. 2009; Melmed 2006). About two thirds of acromegalic patients can be controlled by treatment with octreotide or lanreotide. (Freda et al. 2005; Murray and Melmed 2008; Bronstein 2010).

Variceal bleeding is still a life-threatening complication of liver cirrhosis with portal hypertension responsible for an appreciable rate of morbidity and mortality. In patients with actively bleeding varices, octreotide can be used in conjunction with endoscopic band ligation of the varices (Moitinho et al. 2001).

SSTRs have been identified in vitro in a large number of human neoplasias. A high incidence and density of SSTRs are found, in particular, in neuroendocrine tumors, such as pituitary adenoma, pancreatic islet cell tumor, carcinoid, pheochromocytoma, paraganglioma, medullary thyroid cancer, and small cell lung carcinoma (Reubi 1997). Tumors of the nervous system including meningioma, neuroblastoma, and medulloblastoma also often express a high density of SSTRs (Kwekkeboom et al. 2010). But also tumors not known to be classically originating from endocrine or neural cells, such as lymphoma, breast cancer, renal cell cancer, hepatocellular cancer, prostate cancer, sarcoma, and gastric cancer, can express SSTRs (Kwekkeboom et al. 2010). In the majority of these tumors, the SSTR2 is predominantly expressed, although low amounts of other SSTR subtypes may be concomitantly present (Reubi et al. 2001). The expression of SSTRs is not specific for tumoral pathologies. For instance, active granulomas in sarcoidosis express SSTRs on the epithelioid cells, and inflamed joints in active rheumatoid arthritis express SSTRs, preferentially located in the proliferating synovial vessels (Reubi et al. 1994).

Since the SSTR-ligand complex is internalized by the cell radiolabeled SST analogues have been developed for imaging analyses and targeted radiotherapy of SSTR-expressing tumors (Reubi 2003; Cescato et al. 2006; Bodei et al. 2006; van Essen et al. 2009). Peptide receptor scintigraphy in man started with the demonstration of SSTR-positive tumors in patients using a radioiodinated SST analogue (Krenning et al. 1989). Indium-labeled [^{111}In-DTPA^{0}] octreotide is the most commonly used agent for SSTR scintigraphy today. Also, over the past decade, positron emission tomography (PET) tracers for SSTR imaging (SRI) were developed, and the superiority of the image quality as well as the increased sensitivity in tumor site detection using these newer tracers and PET cameras has been reported by several research groups. Starting in the 1990s, attempts at treatment with radiolabeled SST analogues were undertaken in patients with inoperable and/or metastasized neuroendocrine tumors. Improvements in particularly the peptides used (with higher receptor affinity) and the radionuclides that were applied together with precautions to limit the radiation dose to the kidneys and the bone marrow, led to better results with a virtually negligible percentage of serious adverse events (Kwekkeboom et al. 2010).

Conclusions

SST is a neuropeptide hormone widely distributed in 14 amino acid and 28 amino acid containing forms in the central and peripheral nervous system but also present in endocrine pancreas, gut thyroid, prostate, placenta, adrenals, kidneys and skin. SST mediates its functions through five receptor subtypes belonging to the family of seven transmembrane domain G protein coupled receptors. SST is generally regarded as an inhibitory peptide and can function either locally on neighbouring cells or distantly through the circulation. SST suppresses the synthesis and secretion of growth factors such as growth hormone and insulin-like growth factor 1 and inhibits gastrointestinal hormones. SST is also present in the peripheral nervous system in sympathetic and sensory

neurons innervating lymphoid organs and thus may influence functional responses of lymphocytes and antigen-presenting cells. SST has been shown to exert functional effects on the immune system in vitro, including cytokine- and antibody production, lymphocyte migration and proliferation, and to inhibit inflammation in animal models. Cortistatin shares homology with SST, binds with high affinity to all five SSTR subtypes and also shares many pharmacological and functional properties with SST. Lymphocytes express SSTR subtypes and also produce cortistatin pointing to the possibility that cortistatin rather than SST is an endogenous ligand for SSTR in the immune system. The increased knowledge of tissue-specific expression of the five SSTRs, their capacities for internalization and downregulation, their subtype-specific intracellular messengers, the possibility of forming functionally distinct homodimers or heterodimers as well as the possible interactions of SST, CST and other neuropetides makes the actual in-vivo mechanism of action of SST complex and there are still many interesting questions to be answered. Developments of animal models such as SSTR knockout mice may result to better understand the direct and indirect effects of SST functions in vivo. SST and SST analogues have been applied in several pilot studies for the treatment of refractory immune mediated diseases, such as RA and psoriasis. Long-acting SST analogues have been developed for the treatment of neuroendocrine tumors and acromegaly, and radiolabeled SST analogues are in use for diagnostic and therapeutic applications of human neoplasias expressing SSTR's. New SST analogues are also in clinical trials as treatment of various diseases.

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Neuropeptide Y: The Story, the Players, the Outcomes

8

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Contents

8.1 Introduction to the NPY Story

Neuropeptide Y (NPY) is a peptide consisted of 36 amino acids. It is designated by a capital letter Y due to the presence of many tyrosine residues which are abbreviated by the letter Y in the single letter amino acid code. NPY is one of the most evolutionary conserved peptides, originally isolated from pig brain (Tatemoto et al. 1982; Larhammar 1996). Due to a very wide tissue distribution of NPY and its

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significance for the human and animal physiology, peptides with high structural homology to NPY, e.g., peptide YY (PYY), pancreatic polypeptide (PP) and their truncated forms NPY2-36, NPY3-36 and PYY3-36, sharing amino acid backbone that forms a hair-pin turn called the PP-fold (Fuhlendorff et al. 1990), are now specified as members of NPY family. NPY was previously considered a companion and amplifier of norepinephrine activity. It is now known that NPY could be stored alone in small vesicles of sympathetic nerves, and in combination with catecholamines in large vesicles (Fried et al. 1985). Although NPY is preferentially released under conditions of high frequency nerve stimulation, the endogenous NPY modulates the effects of norepinephrine at both high and low levels of sympathetic nerve activity (Han et al. 1998).

In healthy humans, average NPY concentrations in plasma range from less than 0.5 pM up to 30 pM, and its plasma half-life is around 12 min (Grouzmann et al. 1989, 2001; Reich et al. 2007). In the rat, NPY concentration in plasma and in central nervous system was found to be also in picomolar range (Thompson et al. 1995). It should be noted, however, that in rats platelets serve as an extra neuronal source of NPY, suggesting that platelet release could be a major source of circulating peptides in this species (Myers et al. 1988).

NPY is a peptide with highest distribution in central nervous system where it induces feeding behavior and regulates energy balance (Sindelar et al. 2004), decreases synchronized neuronal discharges (Kopp et al. 1999), nociception (Broqua et al. 1996), anxiety (Heilig 1995), and sexual behavior (Schneider 2004). In the periphery, NPY has a potent mitogenic activity and is chemotactic for vascular smooth muscle cells and the endothelial cells, and also stimulates angiogenesis (Zukowska-Grojec et al. 1998). Independently from its other functions, NPY is a strong vasoconstrictor and cardiodepressant (Zukowska-Grojec and Vaz 1988). NPY gene polymorphism leads to more stress-releasable form of the NPY, which is associated with hyperlipidemia, increased carotid atherosclerosis, and higher tendency for coronary disease in Northern European population (Niskanen et al. 2000). NPY-related peptides have been found within the colon, liver and gallbladder. Moreover, two molecular forms (PYY and PYY3-36) have been purified from the human intestine (Elitsur et al. 1994). Both NPY and PYY are potent inhibitors of intestinal fluid secretion and motility, exerting antidiarrheal actions (Sawa et al. 1995; Souli et al. 1997). Involvement in so many diverse physiological processes labeled the NPY the “universal soldier” (Pedrazzini et al. 2003).

8.1.1 The Way It Works: Y Receptors

The members of the NPY peptide family bind to five different Y-receptor subtypes (Y1, Y2, Y4, Y5 and y6) in mammals (Michel et al. 1998; Berglund et al. 2003). All Y receptor subtypes except for the Y3, have been cloned. A high diversity of Y receptor subtypes and the extremely conserved structure of NPY, PYY and PP make this multireceptor/multiligand system unique. The Y1, Y2 and Y5 receptor subtypes show a very low sequence homology to each other (about 30%), but all

exhibit a high affinity for both NPY and PYY (Larhammar et al. 2001). The Y4 receptor preferentially binds PP, whereas the y6 (written with a lower case y as it has no physiological correlate) seems to be a pseudogene in primates and exhibits quite different pharmacological properties in mice versus rabbits. Regardless of the structural heterogeneity of different receptor subtypes, it is evident that all of them share a single common Y receptor ancestor (Larhammar et al. 2001). All four receptor subtypes belong to the class I rhodopsin-like family of G-protein – coupled receptors. They modulate a variety of pathways through coupling with inhibitory GTP-binding protein (Gi/o), resulting in the inhibition of the enzyme adenylyl cyclase. An additional mechanism of cell signaling involves the inositol phosphate dependent /independent mobilization of Ca++ from the intracellular storage (Herzog et al. 1992; Mullins et al. 2002). Similarly to the other G protein-coupled receptors, the phosphorylation of Y1 receptor following ligand binding results in its rapid internalization and reduction in the number of receptors at the cell surface (Gicquiaux et al. 2002). While the Y1 receptor undergoes fast desensitization, this may not be the case with the Y2 and Y4 receptor subtypes. Nevertheless, the resultant action of endogenous NPY and the related peptides depends on complex intracellular pathways as well as the interactions between the Y receptors themselves, like receptor internalization, recycling, degradation and receptor oligomerization.

The Y receptors are widely distributed within the central nervous system, where they co-localize with the NPY-producing nerve cells. They are also expressed at the periphery by many cell types, including the immune cells. Central effects of NPY, such as the reduction of blood pressure and heart rate, and decrease of anxiety and depression are mediated via the Y1 receptors. Y1 receptor is involved in the feeding in response to NPY (McLaughlin et al. 1991) and the regulation of ethanol consumption (Schroeder et al. 2003). In addition, the ability of NPY to regulate arousal is mediated by the Y1 receptors, while the Y2 receptors are also involved in an opposing fashion. At the periphery, the Y1 receptor mediates most of the vascular (Malmstrom 1997; Zukowska-Grojec et al. 1998) and antinociceptive effects of NPY. The Y2 receptor is involved in suppression of transmitter release (Wahlestedt et al. 1985) and the feeding behavior (Sainsbury et al. 2002), while the Y2 receptor in the gut mediates antisecretory activity (Goumain et al. 2001). The Y4 receptor has been related to the regulation of the secretion and motility of the gut (Gehlert 1998), while the brain Y5 receptors are involved in the regulation of the feeding (Cabrele and Beck-Sickinger 2000), limbic seizures (Marsh et al. 1999) and anxiety behavior (Sajdyk et al. 2002).

Besides the considerable differences in the structure of Y receptor subtypes, there is also a difference in the affinity of these receptors for the NPY and the NPY related peptides (Berglund et al. 2003). The interactions between the Y receptors and their respective ligands have been mostly focused on Y1 receptor. The Y1 and Y2 receptor subtypes show high affinity for the NPY and PYY, but bind the PP with lower affinity. Conversely, due to a remarkable selectivity of Y4 receptor subtype for PP over the NPY and PYY, it is designated as the PP-preferring receptor. The most potent endogenous agonists of the Y5 receptor are the NPY and PYY; however, PP is also able to bind to this receptor with a rather good affinity. Y1,

Y2 and Y5 receptor subtypes bind to the C terminal portion of the NPY and PYY molecules. While the Y1 receptor requires a complete N-terminal part, the Y2 and Y5 receptors bind to N-terminally truncated NPY and PYY peptides with only minor reduction in affinity.

8.1.2 The Way It Concludes: The NPY-Degrading Enzymes

Dipeptidyl peptidase 4 (DP4, CD26), belonging to a serine protease enzymes, is the primary peptidase involved in the N terminal truncation of the NPY and PYY leading to formation of NPY3–36 and PYY3–36 (Mentlein 1999; Gorrell 2005). These N-terminally truncated derivatives of the NPY and PYY lose their efficacy at the Y1 receptor but remaining active especially toward the Y2 receptor. It is well known that most DP4/CD26 activity is membrane expressed, mainly located on the vasculature/endothelial cells and on hepatocytes. However, there is also a strong circulating DP4-like activity. DP4-like peptidases, DP8 and DP9, are also capable of cleaving the NPY with lower efficiency compared to DP4, whereas no NPY cleavage could be demonstrated by action of DP2 (Frerker et al. 2007). Aminopeptidase P from smooth muscle cells removes the terminal amino acid from the NPY, therefore generating an additional Y2 receptor specific agonist, the NPY 2-36 (Mentlein and Roos 1996). DP4 is also responsible for the inactivation of incretin hormones, glucagon-like peptide 1 and glucose-dependent insulinotropic polypeptide that regulate post-prandial glucose excursion by increasing insulin secretion and decreasing glucagon release. Thus, the inhibition of DP4 activity prolongs the life of incretins leading to better blood sugar control in type 2 diabetes mellitus patients (Peters 2010). Indeed, three DP4 inhibitors are now approved for the treatment of type 2 diabetes mellitus: Januvia® (sitagliptin, Merck & Co.) and Onglyza® (saxagliptin, Bristol-Myers Squibb Co. and AstraZeneca Pharmaceuticals) were approved by the FDA in 2006 and 2009, respectively, while vildagliptin (Galvus®, Jalra® and Xiliarx®, Novartis) was approved by European Medicines Agency during 2007 and 2008 for use within the EU. DP4 is implicated in various immune responses via its interaction with several immunologically active molecules such as extracellular adenosine deaminase and tyrosine phosphatase CD45. In addition, DP4 was shown to degrade a number of chemokines (Morimoto and Schlossman 1998). DP4/CD26 is an important cofactor required for the activation and proliferation of macrophages, T and B lymphocytes. Due to significant roles of DP4 in immune system functions, a possible deleterious or disturbing influence of systemic inhibition of DP4 enzyme activity should be taken into consideration, aside from a desired improvement in glucose tolerance. For instance, neutralizing enzymatic activity of DP4 completely abolishes the proangiogenic effect of the NPY and might impair closure of the endothelial wounds (Ghersi et al. 2001). In addition, it has been suggested that DP4 functions as a tumor suppressor, so downregulation of DP4 enzymatic activity might be an important event in the progression of many cancers (Gorrell 2005).

8.2 The NPY and the Immune Cells: Are There Doors to Knock on?

The existence of Y receptors in different types of immune cells has been verified at molecular level (at both transcription and translation) via identifying receptor mRNA by PCR, and by labeling with receptor specific antibodies, as well as at the functional level as a result of pharmacological manipulations with receptor specific agents.

The Y1 receptor subtype is the first Y receptor cloned from rat spleen lymphocytes and it has been found to have identical nucleotide sequence as the Y1 receptor in the brain (Petitto et al. 1994). However, the expression levels of both Y1 receptor mRNA and membrane receptor protein in spleen lymphocytes were lower when compared to their expression levels in the brain. Y1 receptor mRNA has been detected in spleen T cells from naïve mice and in lymph node cells from immunized mice (Bedoui et al. 2003). Immune cells show differences in the levels of Y1 receptor mRNA detected. High levels of Y1 receptor expression have been found in dendritic cells, NK cells, and mast cells, and lower levels in T cells, B cells, and macrophages in mice (Wheway et al. 2005). Resident immune cells express low level of Y1 receptor, and Y1 receptors were also detected on recruited CD4+ T lymphocytes during inflammation (Rethnam et al. 2010).

The presence of Y1, but not Y2 and Y5 receptors mRNA was detected on rat peripheral blood mononuclear cells (Bedoui et al. 2002; Nave et al. 2004). Nevertheless, Y2 receptor mRNA transcription in rat peripheral blood mononuclear cells can be induced by in vitro stimulation with lipopolysaccharide (LPS) (Nave et al. 2004). While the Y1 receptor is prevalent in lymphocytes and monocytes, it has been shown that human neutrophils contain mRNA encoding all cloned receptor subtypes, i.e. Y1, Y2, Y4 and Y5, with Y4 receptor being dominant in comparison with other three subtypes (Bedoui et al. 2008). Stimulation of neutrophils with N-formyl-methionine-leucine-phenylalanine (fMLP) significantly increased the levels of Y2 receptor mRNA but did not affect Y1, Y4 and Y5 receptor mRNA. Approximately the same levels of Y1, Y2 and Y5 membrane receptors expression have been detected on carrageenan-elicited rat air-pouch granulocytes (Dimitrijević et al. 2010). Recent study discovered the presence of Y1R+ CD43+ granulocytes among recruited immune cells during pulpal inflammation in rats, but not in immune cells in normal pulp (Rethnam et al. 2010).

8.3 Are the Immune Cells "Immune" to the NPY Effects?

8.3.1 Migration

Migration of lymphocytes to specific sites or tissues is a prerequisite for their local protective effect against infectious agents, contributing to successful immune defense. However, if uncontrolled, it might take part in the development of autoimmune and allergic reactions. The presence of NPY immunoreactive nerve fibers

in lymphoid organs (Muller and Weihe 1991; Romano et al. 1991) and mucosal-associated lymphoid tissues (Nohr and Weihe 1991; Sipos et al. 2006) suggested the role of NPY in immune cells recruitment. Several studies in mice showed enhanced migration of leukocytes stimulated in vitro with NPY (10^{-12} to 10^{-8} M) and chemotactic agent fMLP (De la Fuente et al. 1993; Medina et al. 1998). However, the effects of NPY on lymphocyte chemotaxis vary with respect to the cell type and the age. Although NPY augmented the fMLP-stimulated chemotaxis of lymphocytes at a wide range of concentration, the most effective dose of NPY (10^{-10} M) was higher than normal values of NPY concentration in plasma (10^{-11} to 10^{-12} M) (Reich et al. 2007). Furthermore, it appeared that NPY is itself a chemoattractant for human monocytes at physiological concentration (10^{-11} M) (Straub et al. 2000). Despite the significant chemoatractive feature, NPY (10^{-8} M) did not stimulate the migration of human T lymphocytes into a collagen matrix (Talme et al. 2008). In contrast, the NPY suppressed the migration capacity of cell line Raw 264.7 towards living *L. major* promastigotes, at various concentrations (10^{-10} to 10^{-5} M) (Ahmed et al. 2001).

Extravasation of leukocytes into inflamed tissues is dependent on cells adherence to endothelium and the extracellular matrix. In resting human T cells NPY (10^{-12} to 10^{-8} M) stimulates β1 integrin-mediated adhesion to fibronectin, a major glycoprotein component of the extracellular matrix (Levite et al. 1998). Since NPY did not influence expression of α4β1 and α5β1 integrins on T cells, the proadhesive effect of the NPY most likely was achieved by increasing the affinity of T cells for fibronectin. The proadhesive action of NPY is mediated by the Y2 receptor, and it involves diverse intracellular signal transduction pathways, including characteristic G protein signaling protein tyrosine kinase, protein kinase C, and phosphatidylinositol -3 kinase.

The increase in rat monocyte adherence capacity by the NPY in vitro was also mediated via Y2 receptors (Nave et al. 2004). NPY (10^{-12} to 10^{-8} M) in vitro mainly elevated the adherence capacity of leukocytes in mice, but the effect was dependant on the cell type and the age (De la Fuente et al. 2000; Medina et al. 2000b). Interestingly, NPY was ineffective in aged mice that already exhibited the increased adherence capacity. In line with that, the pharmacological concentration of PYY of 10^{-6} M stimulated the macrophage adherence capacity in adult, but had no influence in aged rats (Stanojević et al. 2006). While the proadhesive effect of NPY might be beneficial under endotoxemic conditions by reducing tissue immigration of monocytes (Nave et al. 2004), the resistance to NPY modulation could be beneficial in old age. Namely, as aging is associated with the increased susceptibility to infection and decreased function of epithelial barriers of the skin and mucosal tissues facilitating the invasion of pathogens, the absence of NPY proadhesive effect in advanced age could limit the inflammatory reaction.

It has been reported that intravenous NPY dose-dependently affected blood leukocyte mobilization and composition in the rat (Bedoui et al. 2001). High-dose NPY increased leukocyte numbers via mobilization of CD161+ NK-cells, CD4+ T-lymphocytes and CD9+ monocytes, while low-dose NPY significantly decreased NK-cells and IgM+ B-cell numbers. While Y1 receptors on peripheral

blood mononuclear cells mediated the inhibition of leukocyte mobilization, the stimulating effects of NPY on leukocytes mobilization are mediated via the Y5 receptor on non-immune cells (Bedoui et al. 2002). These findings indicated that NPY exerts opposite direct and indirect effect on leukocyte mobilization through the action on different Y receptor subtypes and different cells.

8.3.2 Proliferation

Several studies showed that NPY (10^{-12} to 10^{-8} M) in vitro suppressed mitogen-stimulated proliferation of lymphocytes in rodents but did not affect their basal level of proliferation (Soder and Hellstrom 1987; Medina et al. 1999, 2000a; Puerto et al. 2005). The NPY-induced suppression of Concanavalin A (Con A)-stimulated proliferation of lymphocytes was accompanied by the decreased IL-2 production (Puerto et al. 2005). However, the modulatory capacity of NPY appeared to be particularly dependent on the age of mice, as it increased the Con A-stimulated proliferation of lymphocytes originated from senescent mice, indicating that NPY may have beneficial effect on immune response in advanced age (Puerto et al. 2005).

NPY (10^{-12} to 10^{-9} M) also suppressed proliferation of mouse lymphocytes in response to plate-bound anti-CD3 antibody (Wheway et al. 2007). In a murine model of cutaneous leishmaniasis, splenocyte proliferation upon in vitro challenge with *Leishmania major* parasites was inhibited by NPY at 10^{-5} M in the resistant strain, but not in a susceptible strain of mice (Ahmed et al. 1999). On the other hand, NPY at 10^{-7} M enhanced splenocyte IFN-γ secretion in susceptible mouse strain, indicating the NPY involvement in the pathophysiology of cutaneous leishmaniasis. NPY in vitro (10^{-12} to 10^{-6} M) enhanced mitogen-stimulated proliferation of human colonic lamina propria lymphocyte indirectly, by increasing the production of IL-1β in monocytes (Elitsur et al. 1994; Hernanz et al. 1996).

8.3.3 Th1/Th2 Balance

Despite the ambiguous effects of NPY on lymphocyte mitogen-stimulated proliferation, the modulation of Th cytokines secretion appeared to be the most important action of NPY in the regulation of immune response. Following the antigenic stimulation, the Th1 lymphocytes normally secrete IL-2 and IFN-γ, the Th2 cells secrete primarily IL-4 and IL-10, while the Th0 cells are capable of secreting both Th1 and Th2 cytokines. Presumably, it appears that NPY drives Th2 cytokines production over Th1 cytokines production and shifts the Th1/Th2 balance towards the Th2 immune response. NPY greatly enhanced IL-4 production and inhibited IFN-γ in mouse splenocytes upon stimulation with a plate-bound anti-CD3 antibody and mouse helper T cell clones stimulated with antigen in vitro (Kawamura et al. 1998).

Interestingly, NPY (10^{-8} M) directly (in the absence of any additional factors) induced the secretion of cytokines from antigen-specific Th1 and Th2 cell lines derived from mice (Levite 1998; Levite 2000). The NPY-induced T cell cytokine secretion was not correlated with the increased cell proliferation, as was the case with antigen-driven cytokine secretion. The ability of NPY to induce the secretion of a given cytokine varies between the T cell subsets. Particularly, NPY stimulated the secretion of Th1 cytokines, IL-2 and IFN-γ from Th1 clone, but also stimulated the secretion of Th2 cytokine IL-4 from the same T cell clone. However, NPY directly stimulates Th2 T cell clone to secrete IFN-γ, without affecting IL-4 and IL-10 secretion. On the other hand, NPY was ineffective in stimulating cytokine secretion from Th0 clone. Therefore, NPY may induce simultaneously the secretion of both Th1 and Th2 cytokines from either Th2 or Th1 cells, but cannot initiate Th0 cell differentiation to either Th1 or Th2 cell type. Even more significantly, the capacity of the NPY to modulate antigen-driven cytokine secretion in vitro has been acknowledged. The NPY-induced (10^{-8} M) increase of IL-4 production in antigen-stimulated Th1 clone along with elevation of IL-2 and IFN-γ in antigen-stimulated Th2 clone revealed that NPY in vitro was capable to break the commitment of single Th1 and Th2 cells already engaged in a distinct pattern of cytokine secretion (Levite 1998, 2000). Overall, NPY could play a significant role in the regulation of the local immune response by modulating T cells migration and cytokine secretion.

8.3.4 NK Activity

It has been reported that NPY (10^{-12} to 10^{-9} M) in vitro significantly suppressed NK activity of human peripheral blood lymphocytes (Nair et al. 1993). This finding at least partly explained the stress induced suppression of NK activity coupled with the increased plasma levels of NPY following stress (Irwin et al. 1992). Besides, stress induced elevation of plasma levels of catecholamines and NPY has been related with reduced splenic NK activity in aged rats (Irwin et al. 1992), and intravenous NPY administration produced a dose-dependent inhibition of splenic NK activity (Saurer et al. 2006). As NK cells play a major role in the MHC unrestricted recognition of virally infected cells and in the rejection of tumors, the NPY-induced suppression of NK activity connects stress with cancer and viral infection. Quite the reverse, NPY in vitro mainly stimulated NK activity in leukocytes derived from lymph nodes and thymus (De la Fuente et al. 2001b), as well as from peritoneal exudates of adult and mature mice (Puerto et al. 2005). An inhibition of NK activity was observed only in splenocytes from young mice. Generally, the effects of NPY on NK cell activity differ between human and rodents. Although aging has been related to the increased absolute number of NK cells and decreased NK cell cytotoxicity and IFN-γ production, on a "per cell" basis, in both humans and rodents, the question remains whether NPY can increase NK cell activity of human peripheral blood leukocytes in aged individuals.

8.3.5 Production of Cytokines

Extensive investigations of the effect of NPY on macrophages discern that all of their activities are modulated by NPY. This is noteworthy because of macrophages involvement in both innate and adaptive immune responses, and especially because of macrophages ability to secrete a number of potent biologically active molecules. For instance, NPY (10^{-10} M) increased the Con A-induced secretion of IL-1β in human peripheral blood monocytes (Hernanz et al. 1996) and peritoneal macrophages in mice (De la Fuente et al. 2001a; Puerto et al. 2005). The modulatory capacity of NPY regarding the IL-1β secretion varied in mice of different age. The stimulation of IL-1β release by Con A was significantly increased in the presence of NPY (10^{-11} M) in adult mice, while it was decreased for the remaining ages studied. However, NPY decreased macrophages production of TNF-α following stimulation with LPS (Puerto et al. 2005) and increased the production of TGF-β1 in macrophage cell line Raw 264.7 (Zhou et al. 2008), signifying the potential of NPY to constrain inflammatory reaction.

8.3.6 Phagocytosis

Phagocyting bacteria and foreign particles is critical for the successful elimination of pathogen by macrophages and for the presentation of antigen to lymphocytes and initiation of specific immune response. NPY at various concentrations (10^{-10} to 10^{-5} M) suppressed the phagocytic and leishmanicidal capacities of cell line Raw 264.7 (Ahmed et al. 2001). Similarly, both NPY and PYY decreased the phagocytosis of zymosan, which binds to the complement receptor CR3 (also known as Mac-1, or CD11b/CD18), in both resident and elicited peritoneal macrophages in the rat (Dimitrijević et al. 2005; Stanojević et al. 2006, 2007). The NPY-induced suppression of phagocytic function in zymosan-stimulated macrophages was mediated through Y2 and Y5 receptors. Quite the opposite, NPY and PYY (10^{-12} to 10^{-8} M) stimulated the phagocytosis of latex beads in resident peritoneal macrophages derived from mice (De la Fuente et al. 1993, 2000, 2001a). Since the macrophage phagocytic activity in mice significantly increased due to aging, the NPY-induced suppression of latex beads phagocytosis in aged mice maintained macrophage phagocytic functions at physiologically adequate level. Furthermore, NPY in vitro decreased phagocytic capacity of human and rat granulocytes, via Y1/Y2 and Y1 receptors, respectively (Dimitrijević et al. 2006; Bedoui et al. 2008).

8.3.7 Production of Reactive Oxygen Species (ROS) and Nitric Oxide (NO)

The majority of studies on macrophage H_2O_2 and superoxide anion production demonstrated stimulatory effect of NPY in rats (Dimitrijević et al. 2005; Stanojević et al. 2007) and mice (De la Fuente et al. 1993, 2000, 2001a). NPY and PYY at a wide range of concentration (10^{-12} to 10^{-6} M) increased oxidative burst in rat

macrophages stimulated with phorbol myristate acetate (PMA) that involves activation of protein kinase C, through Y1 and Y2 receptors (Dimitrijević et al. 2005). Quite the opposite, a specific agonist of Y5 receptor significantly suppressed oxidative burst in PMA-stimulated macrophages, showing the Y1/Y2 and Y5 receptor interplay in the modulation of macrophage ROS production. Similar receptor interaction in the regulation of H_2O_2 production has been previously suggested for macrophage δ1, δ2, μ and κ opioid receptors (Stanojević et al. 2008).

Pharmacological doses of NPY in vitro induced a considerable decrease in rat granulocyte peroxide production, mediated by the Y2 and Y5 receptors activation (Dimitrijević et al. 2006), and significantly intensified the respiratory burst in human granulocytes, mediated by the Y5 receptor (Bedoui et al. 2008). The opposing effects of NPY on ROS production in rat and human granulocytes may result from different activation status of these cells prior to the NPY treatment in vitro. Specifically, rat granulocytes have been isolated from sterile inflammation site, i.e. carrageenan-elicited cells from air-pouch, while human granulocytes were isolated from peripheral blood of healthy donors.

Regarding the NO production by the LPS-activated resident peritoneal macrophages, the stimulatory effect of NPY (10^{-12} to 10^{-6} M) was observed only in cells derived from young rats (Dimitrijević et al. 2008). Studies investigating the effects of specific agonists of Y receptors on macrophage NO production indicated the role for Y1 and Y2, and excluded the mediation via Y5 receptors. Likewise, NPY increases the LPS-stimulated NO production in rat granulocytes via the Y1 receptor (Dimitrijević et al. 2006).

The modulatory capacity of NPY depends on interaction of different Y receptor subtypes expressed on the membrane of inflammatory cells. The effects of NPY on phagocytosis and ROS and NO production in inflammatory cells involve Y1 and Y2/Y5 receptors' interaction. Furthermore, a subcutaneous injection of NPY in hind paw modulates the local inflammatory response in the rat. This modulation occurs via Y1/Y5 receptor interaction (Dimitrijević et al. 2002). Table 8.1 summarizes the effect of NPY on the activity of lymphocytes, monocytes/macrophages and granulocytes. An overview of the effects of Y receptor specific agonists and antagonists on inflammatory cells functions is given in Table 8.2.

In conclusion, numerous investigations provided evidence for a significant role for NPY in the regulation of key features of innate immune functions. Particularly, the potential of NPY to modulate phagocytosis of macrophages and accordingly affect their ability to present antigen to T lymphocytes, led to the conclusion that NPY also plays a role at the crossroads between the innate and the adaptive immunity (Bedoui et al. 2007).

8.4 The NPY Production by the Immune Cells: A Blast from the Past?

NPY is synthesized in postganglionic nerves innervating lymphoid organs (Romano et al. 1991). Close proximity between the NPY positive nerve fibers and the immunocompetent cells has been demonstrated in spleen (Meltzer et al. 1997) and

Table 8.1 The effects of NPY/PYY in vitro on immune and inflammatory cells functions

	Cell origin (Species)	References
Lymphocytes		
Chemotaxis		
↑ fMLP-stimulated	Lymph node	De la Fuente et al. (1993)
↓ fMLP-stimulated	Thymus (m)	
Adherence		
↑	Peripheral blood T cells (h)	Levite et al. (1998)
↑↓	Lymph node, thymus, spleen, peritoneal exudate (m)	Medina et al. (2000b)
Proliferation		
↓ Con A-stimulated	Lymph node, thymus, spleen, peritoneal exudate (m)	Medina et al. (1999, 2000a), Puerto et al. (2005)
↑ Con A-stimulated	Lamina propria (h)	Elitsur et al. (1994)
↓ PHA-stimulated	Lymph node (gp)	Soder and Hellstrom (1987)
↓ Anti-CD3-stimulated	Lymph node (m)	Wheway et al. (2007)
↓ Mixed lymphocyte reaction	Lymph node (m)	Wheway et al. (2005)
Th1/Th2 cytokines production		
↑ *L. major*-stimulated IFN-γ	Spleen (m)	Ahmed et al. (1999)
↓ Con A-stimulated IL-2	Peritoneal exudate, spleen, lymph node (m)	Medina et al. (2000a), Puerto et al. (2005)
↑ Anti-CD3-stimulated IL-4	Spleen (m)	Kawamura et al. (1998)
↓ Anti-CD3-stimulated IFN-γ		
↓ Antigen-stimulated IFN-γ	Th1 clone (m)	Kawamura et al. (1998)
↑ Antigen-stimulated IL-4	Th2 clone (m)	
↑ Antigen-stimulated IL-4	Th1 clone (m)	Levite (1998)
↑ Antigen-stimulated IL-2 and IFN-γ	Th2 clone (m)	
NK cells activity		
↑	Lymph node,thymus	De la Fuente et al. (2001b)
↓	Spleen (m)	
↓	Peripheral blood (h)	Nair et al. (1993)
Monocytes/macrophages		
Chemotaxis		
↑fMLP-stimulated	Peritoneal exudate (m)	De la Fuente et al. (1993, 2000), Medina et al. (1998)
↑ unstimulated	Peripheral blood (h)	Straub et al. (2000)
↓ *L. major* – stimulated	Raw 264.7 cells	Ahmed et al. (2001)

(continued)

Table 8.1 (continued)

	Cell origin (Species)	References
Adherence		
↑	Peritoneal exudate (m)	De la Fuente et al. (1993, 2000)
↑ LPS-stimulated	Peritoneal exudate (r)	Nave et al. (2004)
↓	Peritoneal exudate (r)	Stanojević et al. (2006)
Cytokine production		
↑ LPS-stimulated IL-1β	Peripheral blood (h)	Hernanz et al. (1996)
↑ LPS-stimulated TGF-β1	Raw 264.7 cells	Zhou et al. (2008)
↓ LPS-stimulated TNF-α	Peritoneal exudate (m)	De la Fuente et al. (2001a)
↑ Con A-stimulated IL-1β		
Phagocytosis		
↓ *L. major*	Raw 264.7 cells	Ahmed et al. (2001)
↓ Zymosan	Peritoneal exudate (r)	Dimitrijević et al. (2005), Stanojević et al. (2006, 2007)
↑ Latex beads	Peritoneal exudate (m)	De la Fuente et al. (1993, 2000)
ROS and NO production		
↑ PMA-stimulated H_2O_2	Peritoneal exudate (r)	Dimitrijević et al. (2005), Stanojević et al. (2007)
↑ Latex beads-stimulated O_2^-	Peritoneal exudate (m)	De la Fuente et al. (1993, 2000)
↑ LPS-stimulated NO	Peritoneal exudate (r)	Dimitrijević et al. (2008)
Granulocytes		
Adherence		
↑	Air pouch (r)	Dimitrijević et al. (2010)
Phagocytosis		
↓ Zymosan	Air pouch (r)	Dimitrijević et al. (2006, 2010)
↓ *E. coli*	Peripheral blood (h)	Bedoui et al. (2008)
ROS and NO production		
↓↑ PMA-stimulated H_2O_2	Air pouch (r)	Dimitrijević et al. (2006, 2010)
↑ fMLP-stimulated ROS	Peripheral blood (h)	Bedoui et al. (2008)
↑ LPS-stimulated NO	Air pouch (r)	Dimitrijević et al. (2006)

Con A concanavalin A, *E. coli Escherichia coli*, *fMLP* N-formyl-methionine-leucine-phenylalanine, *h* human, *gp* guinea pig, H_2O_2 hydrogen peroxide, *L. major Leishmania major*; *LPS* lipopolysaccharide, *m* mice, *NO* nitric oxide, *PHA* phytohemagglutinin, *PMA* phorbol myristate acetate, *r* rat, *ROS* reactive oxygen species

in inflamed mucosa. The distance between the nerve fibers and the cells is found to be anywhere from 200 nm to 1 μm (Sipos et al. 2006), therefore allowing for a direct effect of nerve-derived NPY on the immune cells. In turn, the immune stimulation was found to affect the NPY content in central nervous system (Kim et al. 2007;

Table 8.2 The effects of NPY and NPY-related peptides in vitro on inflammatory cells activity: Contribution of Y1, Y2 and Y5 receptor subtypes

	Cells (Species)	Effect	Agonist	YR	Antagonized by (YR)	References
Adherence to						
Fibronectin	T cells (h)	↑	NPY			Levite et al. (1998)
		↑	NPY_{18-36}	Y2		
Plastic (LPS-stimulated)	Elicited macrophages (r)	↑	NPY		BIIE0246 (Y2)	Nave et al. (2004)
		↑	NPY_{13-36}	Y2,5		
Plastic		↓	PYY		BIBO3304 (Y1)	Stanojević et al. (2006)
	Air-pouch granulocytes (r)	↑	PYY		BIBO3304 (Y1)	Dimitrijević et al. (2010)
Cytokine production						
LPS-stimulated TGF-β1	Raw 264.7 cells	↑	NPY		PD160170 (Y1)	Zhou et al. (2008)
Phagocytosis of						
Zymosan	Elicited macrophages (r)	↓	NPY		BIBO3304 (Y1)	Dimitrijević et al. (2005)
					BIIE0246 (Y2)	
		↑↓	LP-NPY	Y1,5		
		↓	NPY_{3-36}	Y2,5		
		↓	NPY_{13-36}	Y2,5		
		↓	hAAib-NPY	Y5		
	Air-pouch granulocytes (r)	↓	NPY			Dimitrijević et al. (2006)
		↓	LP-NPY	Y1,5		
		Ø	NPY_{13-36}	Y2,5		
		Ø	hAAib-NPY	Y5		
		↓	PYY		BIBO3304 (Y1)	Dimitrijević et al. (2010)
		↓	LP-NPY	Y1,5		
		Ø	NPY_{3-36}	Y2,5		
E. coli	Peripheral blood granulocytes (h)	↓	NPY		BIBO3304 (Y1)	Bedoui et al. (2008)
					BIIE0246 (Y2)	
		↓	LP-NPY	Y1,5		
		↓	NPY_{13-36}	Y2,5		
		Ø	PP	Y4		
		Ø	D-Trp-NPY	Y5		

(continued)

Table 8.2 (continued)

	Cells (Species)	Effect	Agonist	YR	Antagonized by (YR)	References
ROS and NO production						
PMA-stimulated H_2O_2	Elicited macrophages (r)	↑	NPY		BIBO3304 (Y1)	Dimitrijević et al. (2005)
					BIIE0246 (Y2)	
		Ø	LP-NPY	Y1,5		
		↑	NPY_{3-36}	Y2,5		
		↑	NPY_{13-36}	Y2,5		
		↓	hAAib-NPY	Y5		
	Air-pouch granulocytes (r)	↓	NPY		BIIE0246 (Y2)	Dimitrijević et al. (2006)
					L152804 (Y5)	
		↓	LP-NPY	Y1,5		
		↓	NPY_{13-36}	Y2,5		
		↓	hAAib-NPY	Y5		
		↓	PYY			Dimitrijević et al. (2010)
		↓	LP-NPY	Y1,5		
		↓	NPY_{13-36}	Y2,5		
fMLP-stimulated ROS	Peripheral blood granulocytes (h)	↑	NPY		BIBO3304 (Y1)	Bedoui et al. (2008)
		↑	LP-NPY	Y1,5		
		Ø	NPY_{13-36}	Y2,5		
		Ø	PP	Y4		
		↑	D-Trp-NPY	Y5		
LPS-stimulated NO	Resident macrophages (r)	↑	NPY			Dimitrijević et al. (2008)
		↑	LP-NPY	Y1,5		
		↑	NPY_{3-36}	Y2,5		
		Ø	hAAib-NPY	Y5		
	Air-pouch granulocytes (r)	↑	NPY			Dimitrijević et al. (2006)
		↑	LP-NPY	Y1,5		
		Ø	NPY_{13-36}	Y2,5		
		Ø	hAAib-NPY	Y5		

D-Trp-NPY D-[Trp^{32}]-NPY, *E. coli Escherichia coli, fMLP* N-formyl-methionine-leucine-phenyl-alanine, *hAAib-NPY* [hPP1-17, Ala^{31}, Aib^{32}]-NPY, *h* human, *H_2O_2* hydrogen peroxide, *LP-NPY* [Leu^{31},Pro^{34}]-NPY, LPS, lipopolysaccharide, *NO* nitric oxide, *PMA* phorbol myristate acetate, *r* rat, *ROS* reactive oxygen species

Du et al. 2010). In periphery, the stimulation by LPS induces the NPY production by the immunoreactive neurons in the vicinity of IgA – producing lymphocytes in the mouse ileum lamina propria. This finding suggests the involvement of NPY in the modulation of IgA secretion following exposure to microorganisms or toxins (Shibata et al. 2008).

A possibility of NPY production by some immune cells independently from neurons was hypothesized based on the fact that chemical sympathectomy did not change the spleen NPY content (Lundberg et al. 1985). Likewise, high levels of NPY expression were seen in leukocytes isolated from renal grafts that were denervated during surgery (Holler et al. 2008). In line with that is a study by Lambert and colleagues who showed that Langerhans cells, the predominant dendritic cell subset present in the skin, synthesize both the NPY and PYY (Lambert et al. 2002). The presence of both NPY mRNA and NPY peptide was detected in rat and mouse spleen, bone marrow, and peripheral blood cells. In situ hybridization confirmed that the NPY-like immunoreactivity in megakaryocytes originated from de novo synthesis of NPY from mRNA present in these cells (Ericsson et al. 1987). What is more important, the amount of NPY mRNA and the NPY detected in monocytes are in the range of those found in human and rat nervous tissue (Lundberg et al. 1983; Beck et al. 1993).

However, it has been suggested that genes controlling the production of NPY and related peptides are inducible in the immune system (Schwarz et al. 1994). In human peripheral blood mononuclear cells low level of NPY mRNA was observed under resting conditions. Following the stimulation with PMA or mitogens the NPY mRNA transcription was strongly induced in monocytes and B lymphocytes, but not in T lymphocytes (Schwarz et al. 1994). Although previous study also disclosed that NPY mRNA is expressed in vivo at sites where persistent low-level of activation of lymphocytes and monocytes occurs, like in human tonsils (Schwarz et al. 1994), the other study showed that tonsilar lymphocytes produce NPY only after additional in vitro cell activation (Bracci-Laudiero et al. 1996a). In addition, the NPY positive lymphocytes were found in patients with chronic gastritis and not in normal stomach mucosa (Sipos et al. 2006). In contrast to all of the previous studies, strong NPY expression was seen in the resting rat blood leukocytes (Holler et al. 2008).

That NPY could be not only produced and secreted by the cells of the immune system, but can also modulate the functions of cell(s) in which it was produced and the functions of the neighboring immune cells, was documented by study in which the addition of Y1 receptor antagonist to cultured activated macrophages reduced their production of cytokines IL-12 and TNF-α (Wheway et al. 2005). This indicates that NPY produced by the activated macrophages is required for their normal proinflammatory cytokines production. In addition, both Y1 receptor - deficient macrophages and dendritic cells produced less IL-12 and TNF-α (Wheway et al. 2005). Taking into account that the observed immune changes are due to the deficiency of Y1 receptor in immune cells and not due to secondary effects resulting from the lack of the Y1 receptor in other tissues (as confirmed by use of chimeric animals), it is reasonable to assume that the NPY may function in an

autocrine fashion to modulate the function of antigen presenting cells via Y1 receptor. Several pharmacological studies also suggested the autocrine/paracrine action of NPY in the stimulated immune cells. Considering that specific Y1 and Y2 receptor antagonists suppressed oxidative burst in rat granulocytes and macrophages, the involvement of endogenous NPY and/or NPY-related peptides in the tonic regulation of cellular oxidative metabolism were suggested (Nave et al. 2004; Dimitrijević et al. 2005, 2010). Similarly, other endogenous neuropeptides were implicated in the regulation of macrophage activity (Taherzadeh et al. 1999; Vujić et al. 2004).

The question arises as what would be the physiological meaning of that extraneuronal neuropeptide production? If NPY is already produced in neurons and secreted from nerve endings that directly contact immune cells, why would immune cells additionally create this peptide? It has been documented that immune cells can produce other neuropeptides (Weinstock and Elliott 1998), that locally regulate immune reactions in an autocrine/paracrine way. These ultra-short loops serve to fine tune the immune events without any necessity for higher order rank of regulation. While the immune cells pool might be a reservoir of neuropeptides for the situations in which nerves get injured or become inactive, one could not expect that the immune cell machinery for neuropeptide production was created only for that unique purpose. It is reasonable to assume that the presence of neuropeptides in both the immune and the nervous system is a reminiscence to lower organisms in which neuropeptides were stored in cell less committed to the specialized systems. High efficiency of neuropeptides to fulfill different demands probably led to a neuropeptide function promiscuity in evolutionary more complex organisms, as well as to the development of their more sophisticated way of action while at the same time keeping the old modes in pace. Likewise, the amphibian skin peptide termed SPYY showing pharmacological and structural properties closely resembling those of NPY, exhibits a non-receptor mediated antibiotic activity (Vouldoukis et al. 1996). Mouse skin Langerhans cells synthesize the NPY and PYY, especially upon bacterial LPS stimulation (Lambert et al. 2002). However, due to the evolution of immune cells, Langerhans cells -derived NPY protects skin directly by non-receptor lytic action, and by modulating antigen presenting cell activity through the autocrine/paracrine Y receptor-specific mechanism. The modulation of antigen presenting cells in this way was previously suggested for NPY (Wheway et al. 2005).

NPY has a great importance for the immune system function during stressful conditions. It has been shown that exposure to stress greatly enhances NPY release from peripheral nerves and subsequently augments its plasma level (Castagne et al. 1987). Stress, by increasing plasma levels of proinflammatory cytokines, induces nerve growth factor (Hristova and Aloe 2006), that as well stimulates the NPY release from immune cells (Bracci-Laudiero et al. 1996a). Flooding the immune cells and the Y receptors with supraphysiological doses of NPY from different sources result in the inhibition of T cell activity via Y1 receptors (Wheway et al. 2005). Considering that NPY induces strong activation of antigen presenting cells, the blunted T cell response is most likely a mechanisms that counterbalances the effects from NPY-overstimulated antigen presenting cells, which might otherwise

lead to autoimmunity (Wheway et al. 2005). Interestingly, previous exposure of rats to stress diminished the effect of NPY in vitro on macrophage functions, indicating reduced responsiveness of these cells to stress mediator such as NPY (Stanojević et al. 2007). This suggests that NPY might be a good candidate for one of the stress mediators that are, beside corticosteroids (Barnum et al. 2007), responsible for the phenomenon of the stress-induced sensitization or habituation of a particular response, including the immune cell functions.

8.5 The Strange Case of NPY in Immune-Mediated Diseases

Considering the wide distribution of NPY family of peptides and Y receptors and their involvement in the array of different physiological functions, it is not surprising that these peptides are associated with human pathology in many diverse conditions.

8.5.1 Cancer

Overexpression and/or perturbation of NPY and its receptor system at different levels have been reported in various types of cancers. The NPY gene was recently identified as hypermethylated at its promoter CpG island loci in a cancer-specific manner in hepatocellular carcinomas (Shin et al. 2010) and neuroblastomas (Abe et al. 2008). Having in mind that promoter CpG island hypermethylation has been recognized as an important mechanism for inactivating tumor suppressor genes or tumor-related genes, the NPY gene hypermethylation might be responsible for the loss of tumor growth control. Higher NPY immunostaining in prostate cancer compared with benign epithelium may represent autocrine/paracrine stimulus for cell growth (Rasiah et al. 2006) and angiogenesis (Kitlinska et al. 2005). Indeed, mitogen-activated protein kinase pathway was associated with proliferation of prostate, breast, melanoma and fibrosarcoma cancer cells (Aguirre-Ghiso et al. 2003), and this pathway mediates the proliferative actions of NPY (Hansel et al. 2001). Tumor-promoting effect of NPY was described in neuroblastomas and blocking of Y2 receptor was sufficient to inhibit neuroblastoma growth in vivo by inhibiting cell proliferation and angiogenesis and by increasing apoptosis (Kitlinska et al. 2005; Lu et al. 2010). The Y1 and/or Y2 receptors are also found in pheochromocytomas and paragangliomas, (Korner et al. 2004) and in renal cell carcinomas and nephroblastomas (Korner et al. 2005). Y1 and Y5 receptors are expressed in primary human breast carcinomas and up-regulated by estrogen in a receptor-specific manner (Amlal et al. 2006). Y5 receptor-selective antagonist inhibited cell growth and induced cell death in Y5 receptors-expressing breast cancer cells (Sheriff et al. 2010). High level of NPY mRNA was found in bone marrow, peripheral lymphoblasts and in malignant B-cell precursor lymphoblasts, together with the elevated plasma level of NPY in children with B-cell precursor acute lymphoblastic leukemia (Kogner et al. 1992). What is more important, the

elevated plasma NPY level at diagnosis appears to correlate with a favorable outcome in pediatric leukemia, while chemotherapy rapidly normalizes it, implying tumoral origin of increased systemic NPY (Kogner et al. 1992). In adults, genotype analysis revealed that single nucleotide polymorphism in NPY gene was associated with the increased risk of non-Hodgkin lymphoma or follicular lymphoma (Skibola et al. 2005). Increased NPY secretion, predisposed by NPY gene polymorphism, probably led to the loss of the appropriate immune surveillance, leading to enhanced growth of transformed B-cells and progression to lymphoma (Skibola et al. 2005).

In contrast, PYY was shown to inhibit the proliferation of breast and prostate cancer cells, probably via Y4 receptors (Grise et al. 1999; Yu et al. 2002) and pancreatic cancer cells via Y2 receptors (Liu et al. 1995). In addition, Y1 signaling inhibits the growth of specific sarcomas (Korner et al. 2008). These direct effects of the NPY peptide family on tumor cells are thought to be potent enough to overcome their angiogenesis-promoting effect and point to diverse effects of NPY in cancer pathology (Tilan and Kitlinska 2010). Figure 8.1 shows harmful and beneficial effects of NPY/PYY in cancer.

Clinically, tumoral Y receptors may be targeted with NPY analogs coupled with adequate radionuclides or cytotoxic agents for a scintigraphic tumor imaging and/or tumor therapy. Via binding to the corresponding receptors expressed on tumor cells, radioisotopes or cytotoxic molecules coupled to peptide analogs are delivered directly into the tumor cells, resulting in high intratumoral drug concentrations, while systemic side effects and drug resistance may be significantly reduced (Schally and Nagy 1999). The corresponding radionuclide-coupled NPY analogs suitable for this purpose have been synthesized (Langer et al. 2001; Zwanziger et al. 2008). Y receptors are also suggested as promising new targets in cancer therapy, such as Y2 receptor in therapy of neuroblastoma (Lu et al. 2010), or Y5 receptor in therapy of breast cancers (Sheriff et al. 2010).

8.5.2 Autoimmunity

Genetic analysis of the BB rat, one of the best models of insulin-dependent diabetes mellitus, revealed that development of type 1 diabetes, involves (among other genes) and lymphopenia (Lyp) gene that is tightly linked to the neuropeptide Y (Npy) gene on chromosome 4 (Jacob et al. 1992). Besides, it has been recently found that NPY might be a possible new minor autoantigen in diabetes mellitus type 1, as one of the secretory vesicle-associated proteins that are targets of the autoimmune response (Hirai et al. 2008).

In autoimmune-mediated chronic inflammatory diseases such as systemic lupus erythematosus and arthritis, plasma and/or synovial NPY levels are increased, mostly reflecting the increased sympathetic tonus over the course of the diseases (Harle et al. 2006), while the densities of NPY- containing nerve fibres are decreased, as in rheumatoid synovium (Pereira da Silva and Carmo-Fonseca

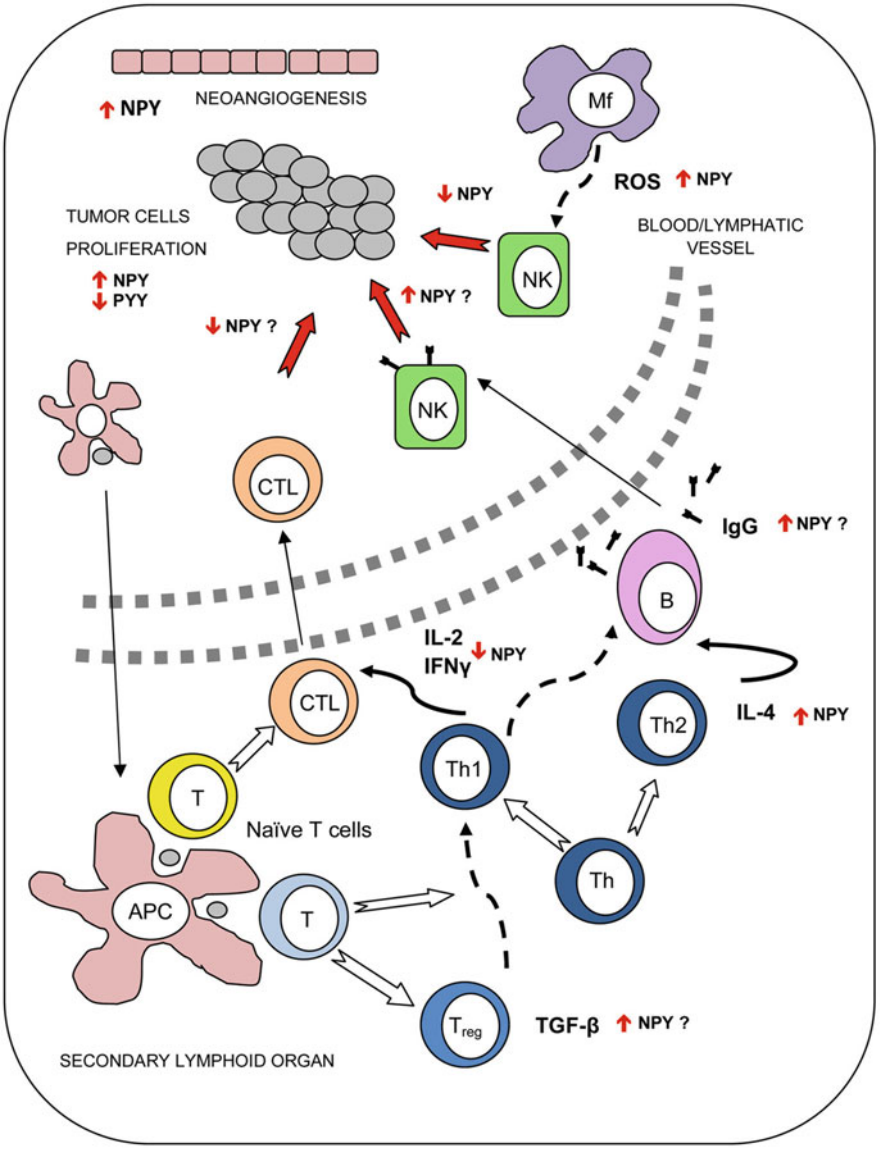

Fig. 8.1 Multifaceted role of NPY in cancer. The mechanisms underlining the tumor promoting action of NPY presumably comprise: (1) stimulation of tumor cells proliferation; (2) angiogenesis within tumor tissue; (3) direct suppression of the NK cell activity; and (4) indirect suppression of the NK cell activity through the elevation of macrophage ROS production. The effects of NPY on lymphocyte proliferation and cytokine production in vitro suggest an additional mechanism i.e. the reduced generation of cytotoxic T lymphocytes. Moreover, the effect of NPY on Treg cell mediated suppression of anti-tumor immunity has not been established yet. Alternatively, the anti-cancer action of NPY and related peptides involves suppression of cell growth in certain types of tumors and a predictable potentiation of antibody-dependent cell-mediated cytotoxicity

1990). In the hybrid mouse strain that spontaneously develop an autoimmune disease similar to human systemic lupus, NPY is significantly increased in the spleen and in the inflamed kidneys in parallel with the progression of the disease (Bracci-Laudiero et al. 1998). Thus, an enhanced local level of NPY may be correlated with the lymphocyte activation and the extensive lymphoproliferation induced by overexpression of nerve growth factor in spleen during the development of systemic lupus (Bracci-Laudiero et al. 1996b), indicating a role of NPY in the maintenance of the inflammation. Plasma NPY levels were increased in several other immune-mediated conditions, such as polymyositis and dermatomyositis (Liu et al. 2004), during exacerbations of asthma (Groneberg et al. 2004), or in atopic dermatitis (Salomon and Baran 2008). In addition, the NPY has been proposed for therapeutic applications in allergic rhinitis, as local pretreatment with NPY reduced nasal obstruction and mucus secretion evoked by allergen challenge in allergic patients with no systemic absorption (Lacroix and Mosimann 1996; Lacroix et al. 1996).

The concentration of NPY is reduced in cerebrospinal fluid of patients with multiple sclerosis (Maeda et al. 1994). Considering that NPY can induce the shift of the Th1/Th2 balance towards the Th2 phenotype (Levite 1998), it was speculated whether the NPY could alter the course of rat experimental autoimmune encephalomyelitis (EAE), an accepted rodent model of human multiple sclerosis mediated by CD4+ Th1 lymphocytes (Bedoui et al. 2003). Indeed, Bedoui and coworkers (2003) found that the repetitive subcutaneous administration of NPY or Y1 receptor specific agonists ameliorated the symptoms and disease severity in a dose-dependent fashion. Besides, the autoreactive T lymphocytes from the NPY-treated EAE mice secreted significantly lower amounts of IFN-γ when stimulated with the specific autoantigen. The authors reported that the NPY directly affected T cells and their Y1 receptors, and not the antigen-presenting cells. NPY also elevated the IgG1-IgG2a ratio (by decreasing IgG2a titers, without altering IgG1 titers) of autoantigen-specific antibodies, which is indicative of favoring the Th2 response. Figure 8.2 illustrates potential targets of modulatory action of NPY in EAE. In addition, an increased expression of CD26 molecules on T lymphocytes observed in multiple sclerosis patients (Reinhold et al. 2002) results in a decreased amount of intact NPY available for Y1 receptor signaling and disease control. In line with that, pharmacological inhibition of CD26 activity successfully suppresses the clinical course of EAE including an active TGF-β1-mediated antiinflammatory effect at the site of pathology (Steinbrecher et al. 2001). In contrast, the Y1 receptor deficient mice or the normal mice treated with specific Y1 receptor antagonist displayed reduced clinical and histological signs of experimentally induced acute colitis (Hassani et al. 2005). Having in mind that this model of colitis is highly dependent on the activation of macrophages (Cooper et al. 1993), and that NPY stimulates macrophages proinflammatory activity, hindering NPY by antagonists or the NPY antisense oligodeoxynucleotides (Pang et al. 2010) may be the useful therapeutic approach to the treatment of ulcerative colitis.

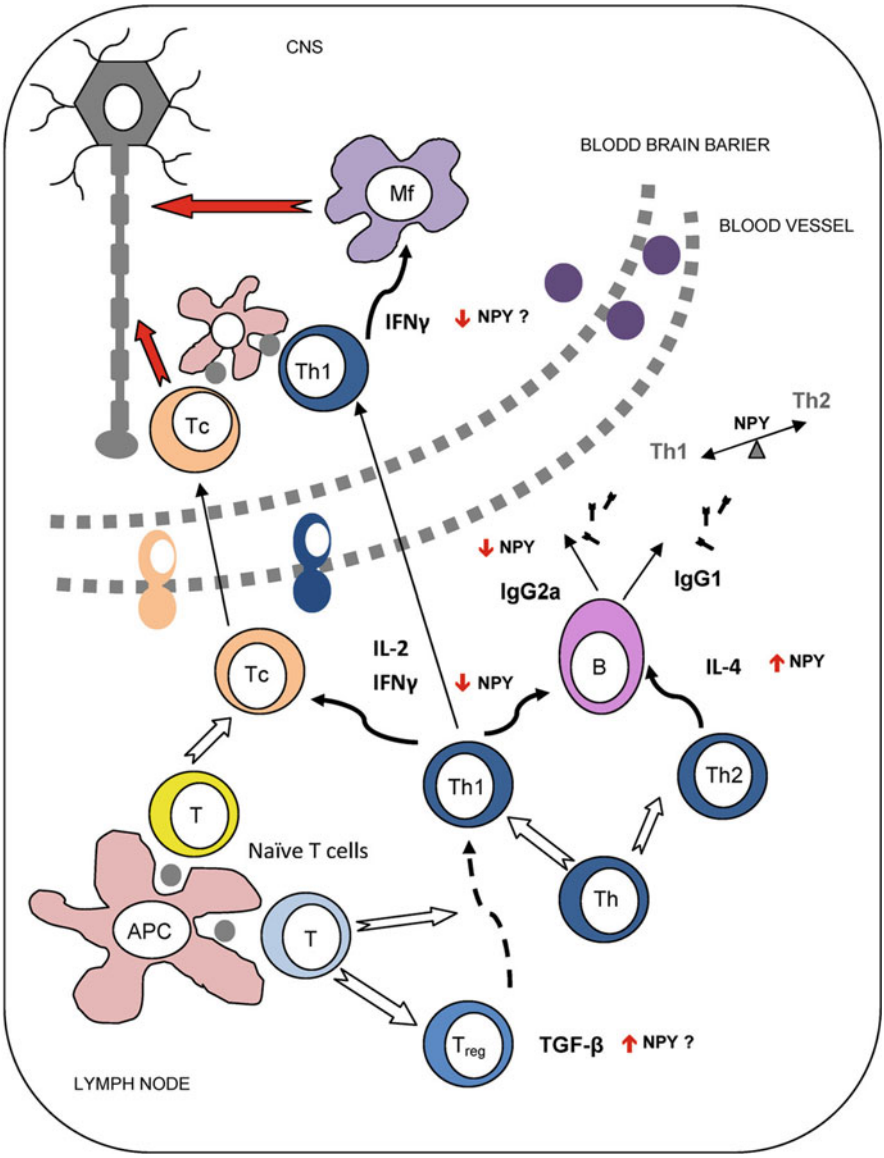

Fig. 8.2 Beneficial effect of NPY in EAE. The capability of NPY to skew the Th immune response toward the Th2 arm provides the plausible explanation for the suppression of EAE in mice treated with NPY in vivo. Precisely, NPY induces: (1) decrease of autoantigen-stimulated proliferation of lymphocytes; (2) diminution of IFN-γ production by autoantigen-stimulated lymphocytes; and (3) suppression of IgG2a autoantibody production. Two key questions are still without answer: (1) Does NPY affect the function of Treg cells in EAE? and (2) Is NPY involved in the modulation of local inflammatory reaction in the CNS, and what are the possible targets for NPY action?

8.6 NPY: The Take Home Message

What at first seemed to be a small group consisting of the NPY and the related peptides isolated from the brain and the intestine where they mediated the basic energy regulating and fluid resorptive functions, respectively, at the present time grew to be a very interesting contemporary, although actually ancient, cohort of peptides mediating almost every physiological process examined. The ability of the immune system to respond to NPY by expressing the Y receptors, as well as to produce the NPY upon stimulation, combined with the role of NPY in the aethiology/pathogenesis/cure of immune-mediated diseases, paves the way for the future growth in the field of NPY research by opening an avenue for potential new pharmacological treatments.

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Vasoactive Intestinal Peptide: Immune Mediator and Potential Therapeutic Agent

9

Mario Delgado and Doina Ganea

Contents

9.1 Introduction

9.1.1 Discovery, Structure, Synthesis and General Functions of VIP

As its name indicates, the vasoactive intestinal peptide (VIP) is a 28-aa peptide that was originally isolated from the porcine small intestine by Said and Mutt (1970) based on its vasodilatatory actions. After its isolation, VIP was rapidly identified in the central and peripheral nervous system (Said and Rosenberg 1976) and was since

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recognized as a widely distributed neuropeptide. Beside its expression by neurons in various brain areas, VIP can be found as a neuropeptide in many organs and tissues, including heart, lung, thyroid, kidney, urinary and gastrointestinal tracts, genital organs and immune system (Henning and Sawmiller 2001). In addition, many endocrine and immune cells produce VIP under different physiological and pathological situations. The widespread distribution of VIP is correlated with its involvement in a large variety of biological activities, which include the stimulation of vasorelaxation in several tissues in addition to the gut. VIP also acts as a neuromodulator in brain and was proposed to be associated with the "visceral forebrain system" that influences and temporarily overrides brainstem control of cardiovascular, respiratory, and gastrointestinal functions. Thus, VIP increases cardiac output, bronchodilatation, smooth muscle relaxation and regulates secretory processes in the gastrointestinal tract and gastric motility. In the endocrine system, VIP releases prolactin, luteinizing hormone, and growth hormone from the pituitary, and regulates the release of insulin and glucagon in the pancreas, depending on the level of glucose. It also acts on the exocrine pancreas to increase the bicarbonate output. VIP also promotes analgesia, hyperthermia, learning and behavior, has neurotrophic effects and regulates bone metabolism, circadian rhythms and embryonic development.

Based on its sequence and structure, VIP belongs to a family of structurally related peptide hormones that include secretin, glucagon, growth hormone releasing factor, glucagon-like peptide-1 and -2, helodermin, gastric inhibitory peptide and pituitary adenylate cyclase-activating polypeptide (PACAP, which exists in two amidated forms, PACAP-27 and PACAP-38, and shows approximately 70% identity with VIP). The amino acid sequences of the various members of the VIP family are depicted in Fig. 9.1a. It is assumed that this superfamily of peptides resulted from the duplication of a common ancestral gene, which then diverged extensively.

VIP, like other members of this family, has a secondary structure, characterized by long α-helical structure at the C terminus. VIP is proposed to have an initial N terminus sequence of eight amino acid residues, probably with two β-turns, followed by two helical segments at residues 7–15 and 19–27 connected by a region of undefined structure that confers mobility to the molecule (Fry et al. 1989).

VIP is synthesized from a precursor molecule (preproVIP), which also contains a VIP-related peptide called PHM (peptide with N-terminal histidine and C-terminal methionine amide) in humans or PHI (peptide with N-terminal histidine and C-terminal isoleucine amide), its counterpart in other mammalian species (Fig. 9.1b). The discovery of VIP and PHM/PHI sequences on the same gene and mRNA suggests that both peptides are co-synthesized in the same tissues. However, VIP and PHI/PHM are not always found in the same cell, suggesting that alternative processing of the nuclear precursor RNA or differential protein processing may occur, resulting in cells expressing either VIP or PHI. The fact that all brain cells synthesizing VIP mRNA also contain PHM mRNA points out that the differential regulation could be at the protein processing level.

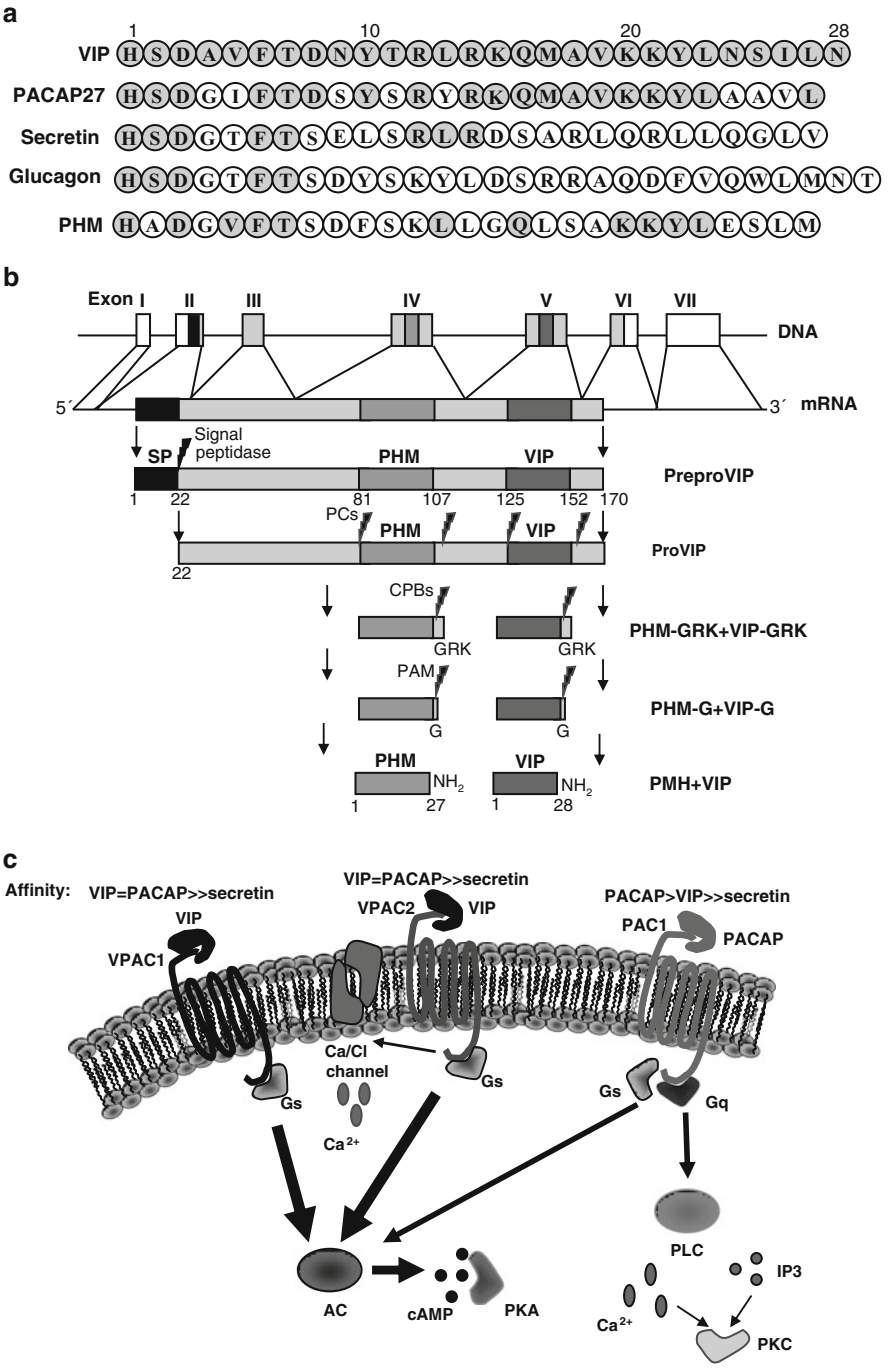

Fig. 9.1 VIP sequence, gene and receptors. (**a**) Sequence of VIP and related peptides of the family. VIP shows 68% identity with PACAP27. (**b**) VIP gene structure (DNA and RNA): the

9.1.2 VIP in the Clinic

Although VIP has shown a plethora of physiological functions, its translation to the clinic has been scarce and occurred only recently. To date, VIP (known as Aviptadil in clinic) has been successfully applied in three different clinical conditions: erectile dysfunction, pulmonary hypertension and sarcoidosis.

Erectile dysfunction is becoming an increasingly common problem and although oral therapies offer first-line treatment for many men, they are contraindicated or ineffective in a substantial group of patients. VIP (25 μg) has been combined with phentolamine mesylate (1–2 mg) in a product called Invicorp (Plethora Solutions, UK) for intracavernosal injection for the management of moderate to severe erectile dysfunction. The two active components have complementary modes of action; VIP has a potent effect on the veno-occlusive mechanism, but little effect on arterial inflow, whereas phentolamine increases arterial blood flow with no effect on the veno-occlusive mechanism. Sufficient arterial blood supply and a functional veno-occlusive mechanism are prerequisites for the attainment and maintenance of a functional erection. Clinical studies showed that Invicorp is effective in more than 80% of men with erectile dysfunction, including those who have failed to respond to other therapies and is associated with a very low incidence of penile pain and negligible risk of priapism (Dinsmore et al. 1999).

Based on its bronchodilatory properties and on the fact that low levels of VIP were detected in serum and lungs of patients with pulmonary hypertension, VIP have been proposed for the treatment of idiopathic pulmonary arterial hypertension. Daily inhalation of 200 μg VIP over a 3 month period resulted in a reduction of pulmonary arterial pressure (Petkov et al. 2003), and a single inhalation of 100 μg VIP caused a temporary but significant selective pulmonary vasodilation, an improved stroke volume and mixed venous oxygen saturation, and patients experienced a pulmonary vascular resistance reduction of >20% (Leuchte et al. 2008). More recently, Prasse et al. (2010) reported in a phase II clinical study that use of

Fig. 9.1 (Continued) human VIP gene (8,837 bp), located on chromosomal region 6p24, contains seven exons, each encoding a distinct functional domain on the protein precursor or its mRNA. VIP is synthesized from a precursor molecule (preproVIP), which also contains PHM. The 170 amino acid prepro-VIP is metabolized by a signal peptidase in the endoplasmic reticulum to yield the 148 amino acid pro-VIP. Pro-VIP is cleaved by prohormone convertases (PCs) to VIP-GKR (prepro-$VIP_{125–155}$) and PHM-GKR (prepro-$VIP_{81–110}$). VIP-GKR and PHM-GKR are then cleaved by carboxypeptidase-B like enzymes (CPB) to VIP-G and PHM-G, which can then be metabolized and amidated in C-terminus by peptidyl glycine α-amidating monooxygenase (PAM) enzymes to VIP and PHM. (**c**) Three receptors for VIP, belonging to family B1 of the G-protein-coupled receptors, have been described: $VPAC_1$ and $VPAC_2$, which show high affinity for both VIP and PACAP, and PAC_1 that shows selective affinity for PACAP. The three receptors are coupled to Gαs proteins, increase the levels of cAMP through the activation of adenylate cyclase (AC) and activate the PKA pathway. In addition, PAC1 is coupled to activation of phospholypase C (PLC), elevation of intracellular calcium and phosphoinositides (In3P) and subsequent activation of PKC. Finally, VPAC2 is also coupled to a membrane calcium/chloride channel

nebulized VIP (50 μg, four inhalations per day) for 4 weeks in patients with active chronic sarcoidosis resulted in a reduction of inflammatory markers in the bronchoalveolar lavage fluid.

All these clinical studies, beside its good efficacy, demonstrated the safety of VIP administration.

9.2 VIP Receptors and Signaling in Cells of the Immune System

9.2.1 VIP Receptors in General

VIP and the two alternatively processed forms of PACAP elicit their biological action through binding to a subset of specific membrane receptors belonging to the large family B of G protein-coupled receptors (GPCRs). These receptors share a common molecular architecture, consisting of seven transmembrane-spanning domains, which are linked to one another by three extracellular (EC1, EC2, and EC3) and three intracellular (IC1, IC2, and IC3) loops, a long amino-terminal extracellular domain, and an intracellular carboxyl-terminus. To date, three types of receptors that can interact with VIP and PACAP have been cloned (Fig. 9.1c). According to the International Union of Pharmacology nomenclature (Harmar et al. 1998) these VIP/PACAP-receptors have been classified as follows: VPAC1 (also known as VIP1, VIP/PACAP type II, or PVR 2) and VPAC2 (also known as VIP2, VIP/PACAP type III, or PVR 3). Both receptors bind VIP and PACAP with equal affinity (Kd $\approx$ 1 nM) and activate primarily the adenylate cyclase pathway. The PAC1 (also known as VIP/PACAP type I receptor, or PVR 1) shows a high affinity for PACAP-27 and PACAP-38 (Kd $\approx$ 0.5 nM), but a much lower affinity for VIP (Kd $>$ 500 nM). This PACAP preferring receptor activates both adenylate cyclase and phospholipase C and exists in at least eight different variants that result from alternative splicing of a single transcript and inclusion or exclusion of one or two cassettes, the hip and hop cassettes.

VPAC1 is expressed in brain (especially in the cerebral cortex and hippocampus) and in peripheral tissues such as liver, lung, and intestine, and immune cells (see below). VPAC2 has been localized in brain (especially the thalamus and suprachiasmic nucleus, and lower levels in the hippocampus, brainstem, spinal cord, and dorsal root ganglia) and in a number of peripheral tissues, including the pancreas, skeletal muscle, lung, heart, kidney, adipose tissue, testis, stomach, and in smooth muscle of blood vessels and of the gastrointestinal and reproductive tracts. PAC1 has been detected predominantly in brain (olfactory bulb, thalamus, hypothalamus, dentate gyrus of the hippocampus and cerebellum) and in the adrenal medulla. The wide distribution of these receptors provides clear evidence that VIP has many target sites and functions.

9.2.2 VIP Receptors on Immune Cells

In general, VIP receptors in the immune system share the same molecular basis of ligand-receptor interaction as in other cells and tissues. The expression of fully functional VIP receptors in the immune system was first claimed in human peripheral blood lymphocytes in the early 1980s (Guerrero et al. 1981) through binding techniques (using ^{125}I-VIP as a ligand) and adenylyl cyclase measurements. Later on, VIP binding sites were identified in human peripheral lymphocytes and monocytes and in murine lymphocytes and macrophages (Delgado et al. 2004b). Depending on the cell type and source, the analysis of the binding data indicate a single class of high affinity binding sites or two classes of binding sites (a low number of high affinity and a high number of low affinity binding sites), with the high affinity sites having a Kd between 0.2 and 1 nM.

The cloning and molecular characterization of the three VIP receptor genes allowed the study of the pattern of expression of VIP receptors in immune cells. Table 9.1 summarizes the expression of genes encoding VIP receptors in different cells or tissues of the human and rodent immune systems. In general, it is accepted that VPAC1 is constitutively expressed in lymphocytes (including thymocytes, CD4 and CD8 T cells in peripheral lymphoid organs), macrophages, monocytes, dendritic cells (DCs), microglia and mast cells, and that VPAC2 is scarcely expressed in these cells when they are in a naïve or resting state, but its expression is induced following stimulation in lymphocytes, monocytes and macrophages (see Table 9.1 for references). PAC1 is only expressed by cells of the macrophage/monocyte lineage, including microglia and osteoclasts. Interestingly, the expression of PAC1 is lost during differentiation of monocytes or bone marrow precursors to DCs. In addition, different functional and biochemical studies indicate that the PAC1 isoform expressed in macrophages, monocytes and microglia binds VIP and PACAP with similar affinities, clearly differing from the PAC1 isoforms expressed in the central nervous system.

Despite the immunoregulatory properties of VIP in isolated neutrophils (Palermo et al. 1996), the presence of VIP receptors is controversial. VIP increases on cAMP levels in human neutrophils (Palermo et al. 1996) might be mediated through non-receptor mechanisms (Pedrera et al. 1994). Further research is needed to evaluate the mechanisms of action of VIP in granulocytes.

Most of the studies using specific agonists and antagonists for the different VIP receptors have established that VPAC1 is the major mediator in the immunomodulatory effects of VIP, both in vitro and in vivo, with a moderate involvement of VPAC2, and minimal or none for PAC1 (Delgado et al. 2004b; Gonzalez-Rey and Delgado 2007). However, the development of mice deficient for VPAC2 or PAC1 revealed that both receptors must participate in the effects of VIP on the immune system, since increased susceptibility for inflammatory disorders have been described in both PAC1-KO and VPAC2-KO mice (Goetzl et al. 2001 Martinez et al. 2002, 2005; Lauenstein et al. 2010; Samarasinghe et al. 2011). Although VPAC1-deficient mice are not presently available due to the crucial role played by this receptor during the embryonic development, a critical role for VPAC1 in the

Table 9.1 VIP receptors on immune cells

Immune cells	Receptors VPAC1	VPAC2	PAC1	Comments	References
Lymphocytes					
Thymocytes:					
Murine	+	−(+)	−	VPAC1 expressed on DP and SP $CD4^+$ and $CD8^+$. VPAC2 inducible upon stimulation	Delgado et al. (1996), Johnson et al. (1996)
Murine	+	+	−	VPAC2 involved in SP $CD4^+$ differentiation	Pankhaniya et al. (1998)
Human	+	+	−	VPAC2 mainly expressed	Lara-Marquez et al. (2001)
CD4 T cells CD8 T cells	+	−(+)	−	Spleen and lymph node cells. VPAC2 induced upon TCR/antigen stimulation and inflammation	Gomariz et al. (1994), Delgado et al. (1996), Johnson et al. (1996), Metwali et al. (2000)
Human PBLs	+	−(+)	−	VPAC1 decreases after stimulation. VPAC2 low levels	Lara-Marquez et al. (2001)
B cells	+	−	ND	Controversial. VPAC1 mRNA detected on rat, but not on mouse. Few VIP-biding sites described on human B cells	Gomariz et al. (1994), Johnson et al. (1996), Washek et al. (1995)
Mast cells	+	+	−	VPAC2 decreases after stimulation and in atopic dermatitis	Washek et al. (1995); Groneberg et al. (2003)
Granulocytes					
Neutrophils	ND	ND	ND	Suggested VIP-binding sites by functional studies, although with pharmacological VIP doses (>> Kd of VPACs)	
Macrophages					
Monocytes	+	−(+)	+	VPAC2 induced after inflammatory stimulation	Delgado and Ganea (2001b), Lara-Marquez et al. (2001), Dewit et al. (1998)
Alveolar MΦ	+	+	ND	VPAC1 increases after lung inflammation	Kaltreider et al. (1997), Groneberg et al. (2001)
Peritoneal MΦ	+	−(+)	+	VPAC2 induced after stimulation in cell lines	Delgado et al. (1996), Pozo et al. (1997), Delgado et al. (1998)
Microglia	+	−	+	VPAC2 unresponsive to inflammatory stimulation	Delgado et al. (2002a) Kim et al. (2000)
Osteoclasts	+	−	+		Ransjo et al. (2000)
Dendritic cells					
Langerhans cell	+	+	−		Torii et al. (1997)
BM-DCs	+	+	−		Delgado et al. (2004c)

DP double positive, *SP* single positive, *BM-DCs* bone marrow-derived DCs, *MΦ* macrophages, *PBLs* peripheral blood lymphocytes, *ND* not determined

regulation of immune disorders has been reported in humans. Reduced expression of VPAC1 has been associated with reduced responses to VIP in immune cells of patients with autoimmune and chronic inflammatory disorders such as ankylosing spondylitis, rheumatoid arthritis and osteoarthritis (Delgado et al. 2008a; Juarranz

et al. 2008; Paladini et al. 2008)Interestingly, the reduced expression of VPAC1 in arthritic subjects was genetically associated with a polymorphism found the 3′UTR region of the VPAC1 gene (Delgado et al. 2008a; Paladini et al. 2008). Moreover, the MicroRNA 525–5p, which specifically targets the 3′UTR region of the VPAC1 gene, decreased the expression of VPAC1 mRNA in human activated monocytes (Cocco et al. 2010). A decrease in VPAC2 expression in Th1 cells of patients with multiple sclerosis has been also described, although no associations with genetic polymorphisms were found in this case (Sun et al. 2006) However, specific polymorphisms in the VPAC2 gene appear to be associated with autism and altered VIP levels were detected in the blood of newborns later diagnosed with autism spectrum disorders (Asano et al. 2001; Nelson et al. 2001). These data suggest that defects in the VIP receptor/signaling system predisposes to a higher frequency of autoimmune disorders.

Similar to other tissues and cells, VPAC1, VPAC2 and PAC1 are coupled to adenylate cyclase activation and subsequent activation of the protein kinase A (PKA) pathway in immune cells (Delgado et al. 2004b). Moreover, VIP binding to PAC1 activates phospholipase C and protein kinase C (PKC) in macrophages and monocytes. By using specific stimulators or inhibitors of both signaling pathways, numerous studies have demonstrated that the cAMP/PKA pathway is the main signaling pathway involved in the anti-inflammatory action of VIP in macrophages, monocytes, DCs and microglia (Fig. 9.2a), and that is uniquely involved in the regulation of the T lymphocyte response (Fig. 9.2b). In addition, a PKA-independent pathway also participates in the VIP deactivation of macrophages and monocytes by inhibiting the nuclear translocation of the transcription factor NFκB (Fig. 9.2a), although it does not seem to be involved in the effect of VIP on microglia and DCs. Finally, the participation of PAC1 and PKC pathway has been demonstrated only in the stimulatory effect of VIP on IL-6 production in resting macrophages (Martinez et al. 1998).

9.3 Direct Effects of VIP on Immune Cells: In Vitro and In Vivo Studies

VIP, one of the best studied immunoregulatory neuropeptides, affects both innate and adaptive immune responses, and acts as a major anti-inflammatory factor in animal models of inflammatory and autoimmune diseases (Fig. 9.3). Recent observations in patients and experimental models of inflammatory/autoimmune diseases support the role of VIP as an immune modulator very attractive for the design of new therapeutic strategies in immune disorders.

Some general considerations should be taken in account about the data reviewed in this section. All the in vitro studies were performed with naïve VIP at an effective dose around 1–10 nM, which corresponds to the Kd of the VIP receptors expressed on immune cells. In general, VIP administration, in the absence of any immune stimulus, did not show significant effects, and VIP showed higher efficiencies when administered together or immediately after stimuli. Most of the in vivo experiments

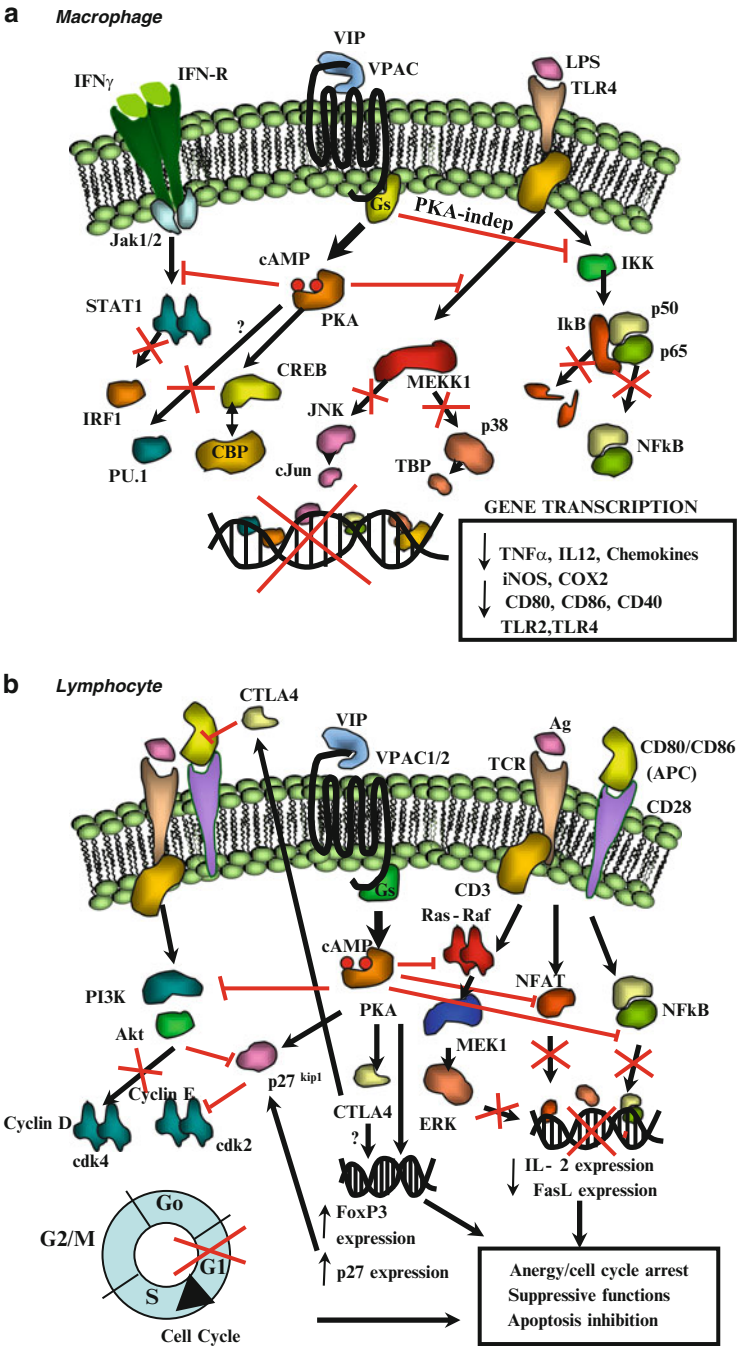

Fig. 9.2 Molecular mechanisms and transcription factors involved in the VIP signalling in immune cells. (**a**) VIP signalling during inflammatory response (macrophage, microglia and

were performed in mice with a VIP dose of 1–5 nmol (2–15 μg/mouse), in general through systemic i.p. administration. Although they showed improved stability, when specific VIP receptors agonists were used, none of them resulted in higher efficiencies than VIP, suggesting that VIP must act through multiple receptors.

9.3.1 Effects of VIP on the Innate Immune Response

To eliminate invading pathogens, the immune system mounts two different but interconnected responses, i.e. innate and adaptive immunity. The innate immune response occurs following ligation of pattern-recognition receptors (PRRs) by foreign molecular patterns shared by groups of pathogens, and is characterized by phagocytosis/endocytosis, release of oxygen and nitrogen radicals, and production of proinflammatory cytokines and chemokines. The major cell types involved in innate immunity are neutrophils, macrophages and DCs in the periphery, and

Fig. 9.2 (Continued) DCs). Binding of VIP to VPAC1 induces cAMP and activates PKA, and exerts several effects: (1) inhibition of IFNγ-induced Jak1/Jak2 phosphorylation, STAT1 activation and binding to promoters of inflammatory genes (CD40, CXCL10, iNOS, COX2) which are dependent of the regulation by IRF-1; (2) inhibition of various MAPK cascades, initiated with the suppression of MEKK1/MEK4 and of MEKK1/MEK6 activities, and subsequent inhibition of Jun-kinase (JNK) and p38 MAPK activities, respectively. Consequently there is a change in the composition of transcription factor AP-1 with c-Jun being replaced by JunB, and inhibition of TATA-box binding protein (TBP) phosphorylation and nuclear translocation. Moreover, through a PKA-independent mechanism, VIP-VPAC1 interaction inhibits IκB-kinase activity and suppresses nuclear translocation and activation of the transcription factor NFκB. AP1, TATA-box protein and NFκB act in concert to activate gene transcription of most of inflammatory cytokines and chemokines as well as co stimulatory molecules. In parallel, VIP-induced PKA activation stimulates cAMP-responsive element binding (CREB) factor to compete with NFκB for co activators, such as p300-CBP, required for transcription of inflammatory genes. LPS, bacterial lipopolysaccharide. (**b**) VIP signalling on T lymphocytes. VIP binding to VPAC (mainly VPAC1) increases cAMP and activates PKA, which could regulate T cell cycle and activation at multiple levels. First, VIP down regulates the PI3K-Akt pathway, and consequently, the activity of cdk4-cyclin D complexes, which induce genes involved in DNA replication and progression through S phase of cell cycle. VIP also increases the levels of the cdk-inhibitor $p27^{kip1}$ by inhibiting the Akt-mediated phosphorylation/degradation of $p27^{kip1}$ and by inducing $p27^{kip1}$ gene expression. Thus, VIP promotes $p27^{kip1}$ binding and inactivation of the cyclin E-cdk2 complexes that result in arrest of the cell in G1 phase. Second, VIP inhibits signaling through the Ras-Raf1-MEK1-ERK1 cascade by decreasing Ras activity and also by impairing Raf1-Ras interaction. This deactivates AP-1 and reduces its binding to the IL-2 promoter. Moreover, VIP decreases the nuclear translocation of NFκB and NFAT, also required for IL-2 transcription. Since IL-2 is a mitogenic factor for T cells, its inhibition by VIP contributes to an anergic state. Third, VIP increases the expression of both soluble and membrane forms of CTLA4, which are critically involved on the induction on the expression of transcription factor FoxP3 and on the regulatory/suppressive activity of the VIP-treated T cells. Finally, by decreasing NFAT and NFκB signalling, VIP inhibits FasL expression in activated T cells and subsequent apoptosis induced by FasL-Fas interaction. *Red back crossed arrows* represent inhibitory signals

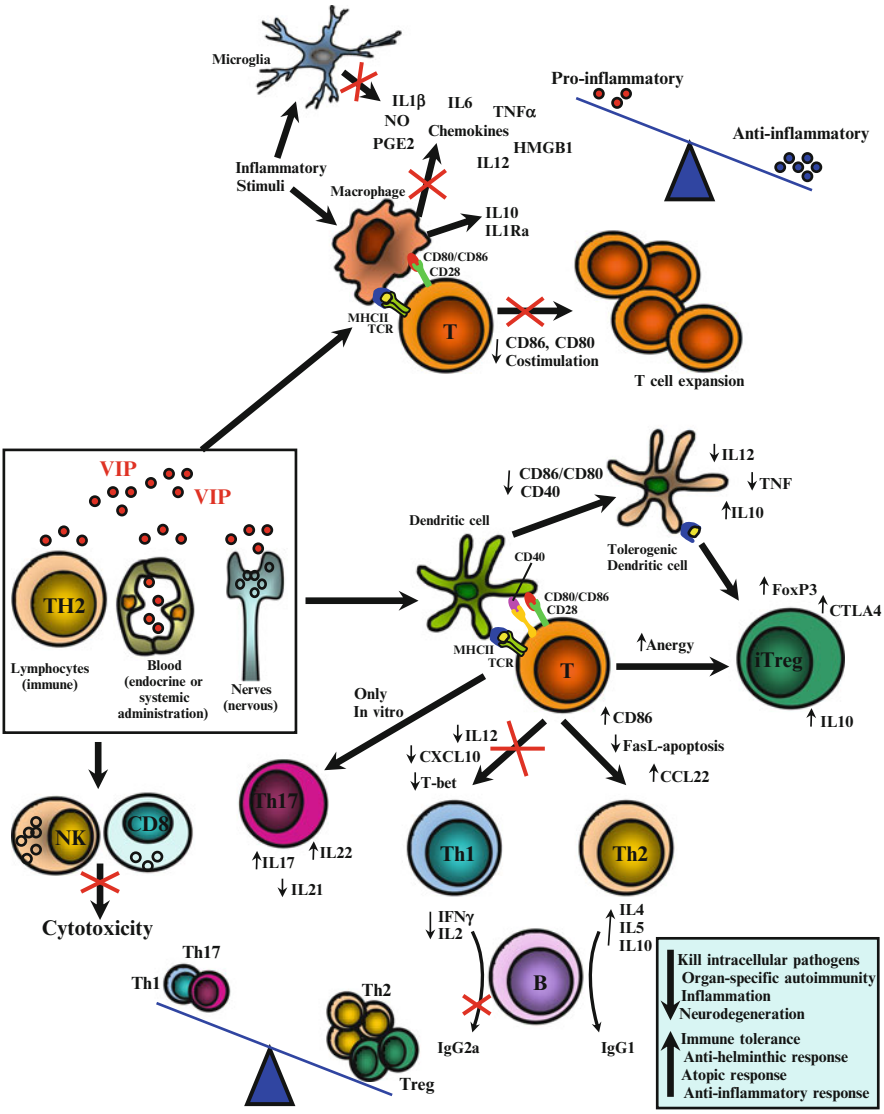

Fig. 9.3 VIP regulates key events of the innate and adaptive immune responses. VIP is released in the context of an immune response through different sources: nerves release VIP as a neuropeptide in lymphoid organs, lymphocytes (mainly Th2 CD4 and T2 CD8 cells) secrete VIP under stimulation, and blood contains variable amounts of VIP as a consequence of endocrine secretion or a therapeutic systemic administration of the peptide. VIP can impair the inflammatory response directly acting on macrophages and microglia by inhibiting the production of inflammatory mediators (cytokines, chemokines, lipids and free radicals) and by inducing the production of anti-inflammatory cytokines (IL-10 and IL-1Ra). In addition, VIP deactivates the costimulatory response of macrophages and DCs on T cells, limiting the clonal expansion of Th1 cells under inflammatory conditions. On the other hand, VIP regulates the adaptive response in different ways. First, VIP inhibits the differentiation of Th1 cells and favors the expansion of Th2 cells through

microglia in the central nervous system. In addition to their immediate function in killing pathogens, macrophages, microglia, and especially DCs have the capacity to process antigen and initiate an adaptive immune response, through stimulatory and costimulatory contacts with naïve T cells bearing the appropriate TCR. Although the immune response is required for the successful elimination of pathogens, an uncontrolled inflammatory response can lead to tissue damage, organ failure, and death. Therefore, endogenous anti-inflammatory factors such as glucocorticoids, lipid mediators, anti-inflammatory cytokines, and neuropeptides such as VIP play an important role in the successful resolution of the inflammatory response (Fig. 9.3).

9.3.1.1 VIP Inhibits the Expression and Release of Proinflammatory Mediators from Activated Macrophages and Microglia

Following signaling through PRRs, macrophages, DCs, and microglia secrete a plethora of proinflammatory cytokines and chemokines. Exogenous VIP inhibits the production of TNF, IL-6, IL-12, the induction of iNOS, and stimulates the production of the anti-inflammatory cytokine IL-10 in LPS-stimulated macrophages and microglia primarily through VPAC1 [reviewed in (Delgado et al. 2004b)]. In addition, recent reports indicate that VIP inhibits COX2 expression in LPS/IFNγ-activated macrophages, DCs and microglia, downregulates LPS-activated macrophage-derived high mobility group box-1 (HMGB1), an essential late-occurring cytokine involved in lethal endotoxemia and sepsis, and suppresses the inflammatory response of microglia exposed to beta-amyloid fibrils or to the neurotoxin MPTP in a model of Parkinson's disease (Chorny and Delgado 2008; Delgado and Ganea 2003a; Delgado et al. 2008c). In agreement with the in vitro studies, local i.c.v. VIP administration prevents LPS-induced neurodegeneration, microglia activation, and TNF, IL-1β, and iNOS expression in an in vivo model of neuroinflammation (Delgado and Ganea 2003a, c; Kim et al. 2000).

Accumulation of immune cells at the site of pathogen invasion is mediated through inflammatory chemokines released primarily by innate immune cells. VIP inhibits the expression of several chemokines, i.e. CXCL1/KC, CXCL2, CCL2, CCL3, CCL4, and CCL5 in mouse macrophages and microglia, and IL-8 in human peripheral blood monocytes stimulated with bacterial endotoxin (Delgado

Fig. 9.3 (Continued) various non-excluding mechanisms that involve regulation of DC functions, Th1-differentiating factors, chemokines and apoptosis. B cell responses and switching IgG isotype are indirectly affected by this VIP effect vias Th2-responses. Second, VIP induces the emergence of Treg cells with suppressive effects on activated T cells through two mechanisms that imply direct effects on naïve T cells and indirect actions on the generation of tolerogenic DCs. The effect on Th17 differentiation is more controversial, and contradictory results have been obtained in vitro (showing VIP-mediated Th17 responses) and in vivo (showing an apparent impairment on Th17 responses). Finally, VIP inhibits cytotoxicity exerted by natural killer (NK) cells and CD8 T cells. As a consequence, the VIP effects on the innate and adaptive immune responses results in diminished inflammation and organ-specific autoimmunity and in restoration of immune tolerance, that could be therapeutically exploited on autoimmune and inflammatory diseases and transplantation

and Ganea 2001a, 2003b; Delgado et al. 2002a). In agreement with the effect on chemokines, VIP administration led to a significant reduction in neutrophil, macrophage, and lymphocyte recruitment to the peritoneal cavity in a model of acute peritonitis (Delgado and Ganea 2001a).

9.3.1.2 Signaling Pathways Involved in the Inhibitory Effect of VIP

VIP affects the expression of pro- and anti-inflammatory factors in LPS − and LPS + IFNγ-stimulated macrophages and microglia by regulating the expression and/or transactivating activity of a plethora of transcription factors such as AP-1, NFκB, CREB, and IRF-1 (Delgado 2002; Delgado and Ganea 2000a, b, 2001b; Delgado et al. 1998) (Fig. 9.2a).

In addition to the inhibition all these downstream signaling pathways, VIP also reduces the capacity of monocyte/macrophages to detect toll like receptors (TLR)-mediated signaling by preventing the upregulation of various TLRs. TLRs represent the major membrane-bound family of PRRs. Gomariz and colleagues showed that daily administration of 1 nmol VIP in the experimental colitis inhibited TLR-2 and TLR-4 expression in colonic extracts and on macrophages, DCs and lymphocytes from mesenteric lymph nodes (Arranz et al. 2008; Gomariz et al. 2005). VIP was also shown to inhibit LPS-induced upregulation of TRL-4 in human rheumatoid synovial fibroblasts (Gutierrez-Canas et al. 2006) This effect was shown to be mediated via inhibition of PU.1 (Foster et al. 2007).

9.3.2 Effects of VIP on the Adaptive Immune T Cell Response

Following recognition of antigenic peptides complexed to MHC class II, CD4 T lymphocytes proliferate and differentiate into Th1, Th2, Th17, and inducible peripheral Treg (iTreg) effectors. CD4 T cells are major targets for VIP regulation, with VIP affecting both activation of naïve T cells and their differentiation into effector cells (Fig. 9.3).

9.3.2.1 VIP Inhibits the Capacity of Antigen-Presenting Cells (APCs) to Initiate Adaptive Immunity

The link between innate and adaptive immunity is built on the capacity of APCs, primarily DCs to present processed antigen to naïve CD4 T cells and induce T cell proliferation and differentiation. In addition to TCR signaling, optimal T cell stimulation requires costimulatory signals, provided by CD40, CD80 and CD86 expressed and upregulated following APC stimulation. VIP prevents the upregulation of CD80 and CD86 in LPS-activated macrophages and DCs, resulting in a significantly reduced capacity to stimulate allogeneic or antigen-specific syngeneic CD4 T cells in vivo and in vitro (Delgado et al. 1999b, 2004c). VIP was also reported to directly affect IL-2 gene expression in activated CD4 T cells and to inhibit T cell proliferation induced by mitogenic factors and stimulation through TCR, primarily through cAMP induction and subsequent effects on the transcription factors NFAT and AP-1 (Wang et al. 2000) (Fig. 9.2).

9.3.2.2 Effects of VIP on CD4+ T Cell Differentiation

VIP Inhibits Th1 and Promotes Th2 Differentiation, Survival, and Migration

Activated CD4 T cells differentiate into several types of effector cells which differ primarily in their cytokine profile. The best characterized effectors are Th1, Th2, and the more recently described Th17 cells. Recent studies indicate that neuropeptides, particularly VIP, inhibit Th1 and favor Th2 differentiation. In vitro, VIP-treated macrophages and DC induce IL-4 and IL-5 (Th2 cytokines) and inhibit IFNγ and IL-2 (Th1 cytokines) in primed CD4 T cells. In vivo, administration of VIP to immunized mice results in a reduction in the number of IFNγ-secreting and an increase in the number of IL-4-secreting cells [reviewed in (Ganea et al. 2003)]. A similar Th2 preference was established in vivo for endogenous VIP in transgenic mice overexpressing the human VPAC2 receptor in CD4 T cells, whereas the Th1 response prevailed in VPAC2-deficient mice (Goetzl et al. 2001; Voice et al. 2003). These studies confirm the concept that VIP affects the Th1/Th2 balance in vivo and indicate the prevalent role of VPAC2 in this process.

A number of nonexcluding mechanisms contribute to the VIP-induced Th2 bias (Fig. 9.3). VIP affects Th1/Th2 generation indirectly by inhibiting IL-12 production in activated APCs, and directly by blocking IL-12 signaling through the inhibition of JAK2/STAT4 phosphorylation and by inducing c-Maf and JunB, master Th2 transcription factors (Liu et al. 2007; Voice et al. 2004) Finally, VIP also preferentially supports the survival of Th2, but not Th1 effectors. In hosts that received transgenic CD4 T cells, followed by immunization and VIP administration, transgenic T cells recovered 62 days later exhibited a phenotype typical of memory Th2 cells ($CD44^{hi}$, L-$selectin^{lo}$, $CD45RB^{lo}$, and IL-4 and IL-5 but not IFNγ or IL-2 producers) (Delgado et al. 2002b). Mechanistic studies established that the VIP-induced preferential survival of Th2 effectors is due to the inhibition of FasL and granzyme B expression, and correlates with higher levels of VPAC1/2 expression on Th2 cells (Sharma et al. 2006).

In addition to promoting Th2 differentiation and survival, VIP also affects Th1/Th2 migration in a differential manner. VIP promotes Th2 and inhibits Th1 migration, by promoting antigen-stimulated DC expression and release of CCL22, a Th2-attracting chemokine and inhibiting the release of the Th1-attracting chemokine CXCL10 stimulated with LPS and antigen (Jiang et al. 2002). In agreement with the in vitro studies, *i.p.* administration of VIP-treated DC pulsed with antigen led to the preferential accumulation of Th2 effectors in the peritoneal cavity (Delgado et al. 2004a).

Effects of VIP on Th17 Differentiation

A recently discovered new lineage of CD4 effector T cells, the Th17 cells, was shown to play a major role in autoimmunity (Harrington et al. 2005; Park et al. 2005) Th17 cells differentiate in the presence of TGFβ and IL-6, and IL-23 is required for expansion and maintenance of their functional phenotype. Th17 dominate the inflammatory response in several autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, psoriasis, and Crohn's disease (Fujino et al. 2003; Kebir et al. 2007) There are only a few studies related to the effects of VIP on Th17

differentiation and function. In a model of type I diabetes, VIP administration to NOD mice (every other day from 4 to 30 weeks of age; 2.5 nmol VIP; *i.p.*) resulted in delayed disease onset and reduced pancreatic expression of the major Th17 cytokines, i.e. IL-17 and IL-22, suggesting an inhibitory effect of Th17 differentiation or function (Jimeno et al. 2010). Similar results were reported in a rat model of collagen-induced arthritis following VIP treatment (every other day from day 0 to 35, 5 nmol VIP) with VIP suppressing clinical symptoms, reducing the percentage of splenic IL-17$^+$ T cells and the expression of STAT3 and RORγt (Deng et al. 2010)

The in vivo VIP inhibition of Th17 observed in models of autoimmune diseases is in agreement with the anti-inflammatory effect of VIP reported previously. However, a rather surprising observation was made recently regarding the in vitro effects of VIP on Th17 differentiation. In contrast to the reduction in Th17 cells observed following in vivo VIP administration, increasing numbers of IL-17$^+$ T cells were observed in vitro upon culture of murine resting CD4 T cells with TGFβ and VIP. Similar to classical Th17 cells differentiated in the presence of TGFβ and IL-6, the TGFβ/VIP-induced Th17 produce IL-17A and IL-22; however, they differ from classical Th17 cells by not producing IL-21 (Yadav et al. 2008). Whether VIP induction of Th17 also occurs in vivo remains to be determined. However, the fact that VIP appears to replace IL-6, a major pathogen-induced cytokine, in the induction of Th17, raises the possibility that VIP contributes to the maintenance of homeostatic levels of Th17.

9.3.3 VIP and Tolerance

Although DCs are essential for the stimulation of antigen-specific T cells, they also function to establish and maintain tolerance. DCs regulate the response of effector T cells by causing anergy or deletion of stimulated T cells, and by inducing regulatory T cells (Treg). Deficiencies in Treg were reported in several human autoimmune diseases, and have been documented in experimental autoimmune models (Buckner 2010; Sakaguchi et al. 2010). DC inducing tolerance, i.e. tolerogenic DC (tDC), exhibit a rather heterogenous phenotype, including immature, semi-mature and in some cases even mature DC (Maldonado and von Andrian 2010) Similar to Treg, tDC can be divided into natural (ntDC) and inducible (itDC) tolerogenic DCs. ItDC are of major interest since they can be induced with biological and pharmacological agents and manipulated to present specific autoantigens. Together with galectin 1, vitamin D3, IL-10 and TNF, VIP belongs to the group of biological agents that induce itDC (Maldonado and von Andrian 2010).

9.3.3.1 VIP-Mediated itDC Induction

Differentiation of murine bone marrow-derived DC (BMDC) in the presence of VIP (10^{-8} M) led to the development of DC with a tolerogenic phenotype, i.e. low expression of CD40/80/86, reduced production of TNF and IL-12, and increased secretion of IL-10 following LPS stimulation. In vitro and in vivo generated DC_{VIP}

induced IL-10-producing $CD4^+Foxp3^+$ Treg which inhibited the proliferation of allogeneic or syngeneic Ag-specific T cells and were able to transfer tolerance to naïve recipients (Chorny et al. 2005; Delgado et al. 2005b). More recently, BMDC were transduced during differentiation with a lentiviral vector expressing VIP and shown to develop a tolerogenic phenotype which was dependent on the endogenous VIP secreted by the transduced DC (Toscano et al. 2010). Similar results were obtained with human blood monocyte-derived DC differentiated with IL-4 and GM-CSF in the presence of VIP (10^{-8} M). VIP led to the generation of human tDC which in turn induced IL-10 producing human CD4 and CD8 Treg. Both types of human Treg suppressed the proliferation/activation of antigen-specific Th1 cells (Gonzalez-Rey et al. 2006a)

9.3.3.2 VIP Induces Treg

In addition to its effect on inducing Treg through the generation of human itDC, VIP also induced human Treg directly in cultures of CD3/CD28-stimulated human CD4 T cells (Anderson and Gonzalez-Rey 2010; Pozo et al. 2009). Interestingly, VIP-treated human trophoblast cells co-cultured with maternal peripheral blood mononuclear cells also induced high numbers of $Foxp3^+CD4^+CD25^+$ T cells which expressed TGFβ and secreted IL-10, which might represent a direct effect of autocrine VIP on fetal survival (Fraccaroli et al. 2009). The only report on VIP treatment in patients supports the induction of Treg. Prasse and colleagues reported recently that nebulized VIP administered to patients with sarcoidosis resulted in increased numbers of $CD4^+FoxP3^+CD127^-CD25^+$ Treg in the bronchoalveolar lavage (Prasse et al. 2010).

VIP administration has been also reported to induce Treg in experimental models. Inoculation of 5 nmol of VIP together with a low dose of antigen (Ag) to TCR-transgenic mice resulted in the expansion of the $CD4^+CD25^+Foxp3^+$ Treg which inhibited proliferation of Ag-specific T cells, transferred suppression and inhibited Th1 responses in vivo (Delgado et al. 2005a). VIP administration in collagen-induced arthritis (CIA), murine type I diabetes and experimental autoimmune encephalomyelitis (EAE) also resulted in the generation of Treg usually associated with a decrease in Th17 (Chen et al. 2008; Delgado et al. 2005a; Deng et al. 2010; Fernandez-Martin et al. 2006; Jimeno et al. 2010). Of obvious clinical significance is the observation that administration of VIP to arthritic mice resulted in less severe clinical symptoms associated with the generation of $CD4^+$-$CD25^+Foxp3^+$ Treg in the draining lymph nodes and joints. Upon transfer into mice with established disease, the Treg from VIP-treated arthritic mice suppressed and ameliorated disease progression in the recipients (Gonzalez-Rey et al. 2006b). Along the same lines, in vivo delivery of a VIP-expressing lentiviral vector (10^8 copies/mouse) to arthritic mice at different phases of the disease resulted in significant disease amelioration concomitant with a reduction of the inflammatory and autoimmune process and the induction of $CD4^+CD25^+Foxp3^+$ Treg in the draining lymph nodes (Delgado et al. 2008b).

An interesting dichotomy became apparent recently regarding the effects of VIP in EAE. Initial studies using exogenous VIP administration established quite clearly

that VIP has a beneficial effect in EAE, and that this is associated, at least partially, with the generation both in the periphery and the central nervous system of $CD4^+CD25^+Foxp3^+$ Treg which inhibit the proliferation of encephalitogenic Th1/Th17 cells, the major players in EAE (Fernandez-Martin et al. 2006; Gonzalez-Rey et al. 2006c). Similar effects were reported with the structurally related neuropeptide PACAP. The protective effect of PACAP in EAE was confirmed in PACAP-deficient mice, which developed a more severe disease associated with increased expression of proinflammatory cytokines and chemokines and decreased expression of Foxp3 in spinal cord, and lower numbers of $CD4^+CD25^+Foxp3^+$ Treg in draining lymph nodes (Tan et al. 2009). In contrast VIP-deficient mice are almost completely resistant to EAE. In agreement with the previously reported anti-inflammatory effect of exogenous VIP, the VIP-deficient mice developed a robust Th1/Th17 response. However, although CD4 T cells entered the meningeal and perivascular areas, their infiltration in spinal cord parenchyma was severely impaired (Abad et al. 2010), suggesting that VIP plays a dual role, affecting T cell differentiation as previously reported but also promoting immune cell infiltration in the central nervous system.

9.3.3.3 Use of DC_{VIP} in Cellular Therapy

The potential use of DC_{VIP} in cellular therapy is aimed at the in vivo generation of Ag-specific Treg and was described for the first time in 2005 (Delgado et al. 2005b). Administration of Ag-pulsed DC_{VIP} (3×10^5 cells, *i.v.*) to mice inoculated with Ag-specific TCR transgenic T cells resulted in the generation of Treg characterized by reduced in vitro proliferation, reduced levels of IL-2 and IFNγ, and increased Foxp3 and IL-10 expression. The Treg were specific for the Ag carried by the DC_{VIP} and were able to transfer Ag-specific tolerance to naïve recipients (Delgado et al. 2005b).

Cellular therapy with DC_{VIP} was then tested in models of inflammatory/autoimmune diseases. In experimental arthritis, DC_{VIP} pulsed with collagen II were administered to mice with established disease and shown to stop disease progression, reduce T cell proliferation and IFNγ production. This was an Ag-specific event, since DC_{VIP} pulsed with OVA did not affect CIA although they did inhibit OVA-induced DTH (Chorny et al. 2005). Inoculation of DC_{VIP} also significantly ameliorated clinical TNBS-induced colitis, inhibited a variety of macrophage-derived proinflammatory mediators, and generated IL-10-secreting Treg which suppressed autoreactive T cells (Gonzalez-Rey and Delgado 2006). In a bone marrow transplantation model, DC_{VIP} were shown to prevent graft-versus-host disease while maintaining the graft-versus tumor response through the generation of Treg (Chorny et al. 2006). More recently, lentiVIP-transduced DCs were shown to be have a therapeutic effect in EAE and sepsis models (Toscano et al. 2010).

A promising future development of these findings is the possibility to generate tolerogenic VIP-expressing human monocyte-derived DCs that could be loaded with relevant autoantigens and used in the treatment of chronic autoimmune diseases.

9.4 Effect of VIP in Inflammatory and Autoimmune Diseases

The evidence reviewed in the previous section indicates that, through its potent and diverse anti-inflammatory and/or immunosuppressive actions, VIP is part of a feedback circuit that limits ongoing inflammatory and immune responses. Recent studies proved the VIP relevance to human health. Treatment with VIP decreases the frequency and severity of various experimental models of sepsis, pancreatitis, hepatitis, respiratory inflammatory disorders, neurodegenerative disorders, rheumatoid arthritis, inflammatory bowel disease, type I diabetes, multiple sclerosis, Sjogren's syndrome, and autoimmune uveoretinitis (see Table 9.2 for references and details). In disorders characterized by an exacerbated inflammatory response, such as endotoxemia, sepsis, chronic obstructive pulmonary disease, pancreatitis and hepatitis, the beneficial effect of VIP is exerted through the downregulation of a wide spectrum of inflammatory cytokines (mainly TNF, IL-6, IL-12 and IFNγ), chemokines and mediators of oxidative stress, at both systemic and local levels. VIP have been proven effective in neurodegenerative diseases characterized by inflammation, such as spinal cord injury (Dickinson et al. 1999; Kim et al. 2000) brain trauma (Delgado and Ganea 2003c; Favrais et al. 2007), and Parkinson's disease (Delgado and Ganea 2003a; Korkmaz et al. 2010), by diminishing neuroinflammation and neurodegeneration through its effect on microglia.

In the case of autoimmune disorders, the therapeutic effects of VIP are associated with the reduction of both early events that are associated with the initiation and establishment of autoimmunity, and of later phases are associated with an evolving immune and destructive inflammatory response (Fig. 9.4). VIP impairs the development of self-reactive Th1 and Th17 cells, their entry into the target organs, the release of pro-inflammatory cytokines and chemokines, and the subsequent recruitment and activation of macrophages and neutrophils. Although VIP can directly regulate the activation of macrophages and T cells, VIP also regulates the immune response through the modulation at multiple levels of the differentiation and activation of DCs and through the induction of Treg (Fig. 9.4).

Despite the effectiveness of VIP in experimental models of inflammation and autoimmunity, only two clinical trials have been performed to date using VIP on patients with immune disorders. In a phase I clinical trial sponsored by the National Institutes of Health (NCT00004494 clinical trial, http://www.ClinicalTrials.gov), Dr. Said has demonstrated the effectiveness of systemic VIP administration (i.v., 1 mg/kg) in patients with sepsis and pulmonary distress syndrome. In a recent open label phase II clinical trial, VIP (administered by inhalation for 28 days) has been proven clinically effective in patients with sarcoidosis, a systemic disease of unknown etiology characterized by the formation of granulomas especially in the lung. Sarcoidosis courses with both inflammatory and autoimmune components, with an increased production of TNF, IL-17 and IFNγ in the lung, exacerbated activation of pulmonary macrophages and Th1 cells and decreased numbers of Treg. VIP treatment diminished all the inflammatory markers in the lung, deactivated macrophages, and led to an increase in Treg numbers (Prasse et al. 2010).

Table 9.2 Therapeutic effects of VIP on inflammatory and autoimmune models

Immune disorder Experimental models	VIP dose/via	Mechanisms of action	References
Sepsis/endotoxemia			
LPS injection	1–5 nmol/i.p., once, within 4 h after sepsis	Inhibition of inflammatory cytokines (TNF, IL6, IL12, IFN), NO and chemokines	Delgado et al. (1999a), Chorny and Delgado (2008)
E. coli injection		Main targets: macrophages/ monocytes	
CLP	5 nmol/i.p., 4 times, initiated 12 h sepsis	Inhibition of late mediators: HMGB1	Chorny and Delgado (2008)
		Cell targets: macrophages	
CLP	LentiVIP-DCs, i.p., once within 1 h sepsis	Inhibition of inflammatory cytokines and chemokines	Toscano et al (2010)
Rheumatoid arthritis			
CIA	1–5 nmol/i.p., 5 times, at onset and with established signs	Inhibition of inflammatory and autoreactive Th1/Th17 responses, induction of Th2 responses and antigen-specific Treg in DLNs and joints	Delgado et al. (2001), Fernandez-Martin et al. (2006), Chen et al. (2008), Williams (2002), Deng et al. (2010)
CIA	VIP-tolDCs/i.p., once, at onset	Inhibition of autoreactive Th1 responses, and induction of antigen-specific Treg in DLNs and joints	Chorny et al. (2005)
CIA	Lenti-VIP/i.p., once, at onset and with established signs	Inhibition of inflammatory and autoreactive Th1 responses in DLNs and joints	Delgado et al. (2008b)
Inflammatory bowel disease			
TNBS-colitis	1–5 nmol/i.p., once, 12 h after TNBS	Inhibition of inflammatory and autoreactive Th1 responses and induction of Th2 responses in DLNs and colon	Abad et al. (2003, 2005)
TNBS-colitis	>5 nmol/i.p., once	Caution: Increase of colitis and mortality	
TNBS-colitis DSS-colitis	VIP-tolDCs/i.p., once, 12 h after TNBS	Inhibition of inflammatory and autoreactive Th1 responses and induction of Treg in DLNs and colon	Gonzalez-Rey and Delgado (2006)
Multiple sclerosis			
MOG-induced EAE PLP-induced EAE	1–5 nmol/i.p., 5 times, at onset and with established signs	Inhibition of inflammatory and autoreactive Th1 responses, induction of Th2 responses and antigen-specific Treg in DLNs and CNS	Gonzalez-Rey et al. (2006c), Li et al. (2006)
MOG-induced EAE	VIP-tolDCs/i.p., once at onset	Inhibition of inflammatory and autoreactive Th1 responses and induction of Treg in DLNs and CNS	Chorny et al. (2005)
PLP-induced EAE MOG-induced EAE	LentiVIP-DCs/i.v., once at onset	Inhibition of inflammatory responses on CNS	Toscano et al. (2010)

(continued)

Table 9.2 (continued)

Immune disorder Experimental models	VIP dose/via	Mechanisms of action	References
Type 1 diabetes			
STZ-diabetes	1 nmol/i.p., for 28 days	Inhibition of oxidative stress and inflammation in pancreas	Yu et al. (2011)
CAD-NOD mice	DNA-VIP/i.m., once at 2 day before induction	Not determined	Herrera et al. (2006)
NOD mice	5 nmol/i.p., for 3 months, initiated 4 weeks before onset	Inhibition of inflammatory and autoreactive Th1/Th17 responses and induction of Th2 and Treg in pancreas and spleen	Rossignoli et al. (2006), Jimeno et al. (2010)
Uveoretinitis			
hIRBP-EAU	2 nmol/i.p., 10 times at onset and on established disease, also with VIP-treated macrophages	Inhibition of inflammatory and Th1 responses on eye and induction of Treg responses in spleen	Keino et al. (2004), Camelo et al. (2009)
Sjogren's disease			
NOD mice	AAV-VIP, in salivary glands, once before onset	Inhibition of inflammatory and Th1 responses in salivary glands	Lodde et al. (2006)
Lung inflammation			
Experimental COPD	5 mol/intratracheal, once, 1 h after smoke	Decrease on neutrophil infiltrarion in lung, inhibition of inflammatory cytokines	Onoue et al. (2010)
Human sarcoidosis	Inhalation 15 nmol/day, 28 days on established chronic disease	Decrease on inflammation cytokines on lung, inhibition of macrophage activation and induction of Treg	Prasse et al. (2010)
Hepatitis/pancreatitis			
Cerulein/LPS-pancreatitis	5 nmol/i.p., twice before and after LPS	Decrease of inflammatory cytokines in pancreas	Kojima et al. (2005)
ConA-hepatitis	5 nmol/i.p., twice before and after ConA	Decrease of inflammatory cytokines and induction of IL10 in liver	Luo et al. (2009)

CIA collagen-induced arthritis, *CLP* cecal ligation and puncture, *CAD* cyclophosphamide accelerated diabetes, *ConA* concanavalin A, *NO* nitric oxide, *HMGB1* high mobility group box-1, *i.m.* intramuscular injection, *i.p.* intraperitoneal injection, *i.v.* intravenous injection, *VIP-tolDCs* *VIP*-induced tolerogenic DCs, *lenti-VIP* lentivirus vectors expressing VIP, *LentiVIP-DCs* DCs transduced with lentivirus expressing VIP, *DLN* draining lymph nodes, *CNS* central nervous system, *EAE* experimental autoimmune encephalomyelitis, *NOD* nonobese diabetic mice, *STZ* streptozotocin-induced diabetes, *hIRBP-EAU* human interphotoreceptor retinoid-binding protein-induced experimental autoimmune uveoretinitis, *AAV-VIP* adeno-associated virus expressing VIP, *COPD* chronic obstructive pulmonary disease

One of the major obstacles for the translation of VIP-based treatments into viable clinic therapies is related to its sensitivity to degradation by peptidases. Table 9.3 shows the general strategies proposed to increase the VIP half-life and to improve its targeted tissue delivery. We must also keep in mind the possibility of

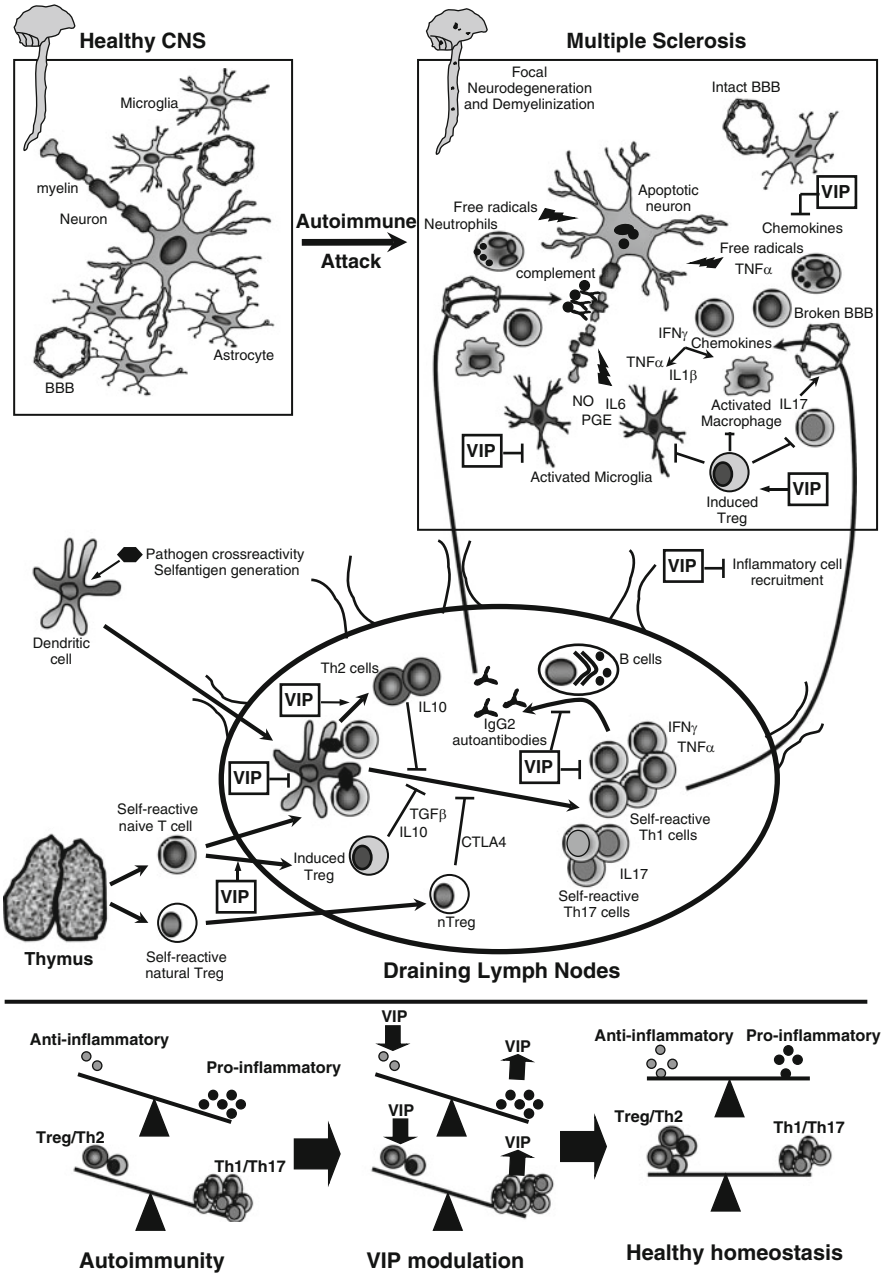

Fig. 9.4 VIP restores tolerance in autoimmune disorders acting at multiple levels. Loss of immune tolerance compromises immune homeostasis and results in the onset of autoimmune disorders (multiple sclerosis is depicted as an example). Most of the immune-mediated destruction of self tissues occurs via specific recognition of self-antigens, followed by a typical effector

using VIP to generate ex vivo Ag-specific itDCs and Treg for treatments related to autoimmunity and transplantation.

9.5 VIP Expression in Immune Cells and Its Endogenous Immune Role

Two different sources of VIP have been described in lymphoid organs. The first and most evident are the nerve endings. Immunohistochemical studies demonstrated the presence of VIPergic nerve fibers in both central (thymus) and peripheral (spleen, lymph nodes and mucosal-associated lymphoid tissue) lymphoid organs, reinforcing the idea of an established anatomical link between the central nervous system and the immune system (Bellinger et al. 1996). The second and probably more relevant source of VIP are the immune cells themselves. Lymphoid cells seem to be the major producers of VIP in lymphoid organs, since the autonomic denervation of thymus and spleen did not change the content of VIP in these organs (Bellinger et al. 1997). Table 9.4 summarizes various works describing the immune cells that express and secrete VIP. Numerous studies indicate that lymphocytes are the main source of VIP in the immune environment. In central and peripheral lymphoid organs both CD4 and CD8 T cells express VIP mRNA, express and process the preproVIP precursor, and secrete naïve VIP, especially under

Fig. 9.4 (Continued) immune response, and/or occasionally as a byproduct of unchecked non-specific inflammation or overly vigorous responses to chronic infections. The initial stages of the disease take place in peripheral lymphoid organs, are associated with initiation and establishment of autoimmunity to self components, and involve the development of self-reactive Th1 and Th17 cells by DCs presenting self-antigens. In addition, Th1 cells promote the secretion of IgG2 autoantibodies by self-reactive B cells and the deposition of immune-complexes in the target tissue. Progression of the autoimmune response involves the entry of self-reactive Th1/Th17 cells into the affected organ, release of proinflammatory cytokines and chemokines and subsequent recruitment and activation of inflammatory cells (macrophages and neutrophils). In addition, autoantibodies activate complement, neutrophils and mast cells. Later events are associated with the evolving immune and destructive inflammatory responses, where inflammatory mediators (cytokines, oxygen and nitrogen reactive species and free radicals) produced by infiltrating cells and resident cells (microglia in this case) definitively participate in tissue destruction. Naturally occurring $CD4^+CD25^+$ Treg (nTreg) or induced Treg cells are key players in maintaining tolerance by their suppression of self-reactive Th1 and Th17 cells. Unbalance of Treg versus Th1/Th17 cells, or of anti-inflammatory cytokines versus proinflammatory factors, are the cause of autoimmune disorders. In order to reinstall tolerance on autoimmune disorders, VIP reinforces various mechanisms. (**a**) VIP decreases Th1 cell functions through direct actions on differentiating T cells, or indirectly by regulating DC functions. Consequently, the inflammatory and autoimmune responses are inhibited because the infiltration/activation of neutrophils and macrophages and deposition of immune complexes are avoided. (**b**) VIP inhibits the production of inflammatory cytokines, chemokines and free radicals by macrophages and resident cells, avoiding the inflammatory response and its cytotoxic effect against self-tissue components. (**c**) VIP induces the new generation of peripheral Treg that suppress activation of self-reactive T cells, and indirectly generates Treg through the differentiation of tolerogenic DCs. *Arrows* indicate a stimulatory effect. *Back-crossed lines* indicate an inhibitory effect. BBB, blood–brain barrier

Table 9.3 Strategies for improving VIP therapy on immune disorders

Aim	Strategy	Reference
Increasing VIP stability	Aminoacid substitutions to increase stability of naïve VIP	Onoue et al. (2010)
	Cycling the structure	Tang et al. (1995)
Decreasing degradation by peptidases	Combination with inhibitors of peptidases	Jungraithmayr et al. (2010)
	Combination with neuropeptide-binding proteins (as other neuropeptides)	
Increasing VIP potency	Combination with phosphodiesterase-inhibitors	Foey et al. (2003)
Improving VIP delivery	Gene therapy: VIP-expressing adenovirus or lentivirus vectors	Herrera et al. (2006), Delgado et al. (2008), Lodde et al. (2006)
	Trojan horses: DCs expressing VIP	Toscano et al. (2010)
	Insertion on micelles or nanoparticles	Fernandez-Montesinos et al. (2009), Onyüksel et al. (1999)
Cell-based therapy	Treg or tolDCs generated ex vivo with VIP	Chorny et al. (2005), Gonzalez-Rey and Delgado (2006)

Table 9.4 VIP production by immune cells

Immune cells	Comments	References
Lymphocytes		
Thymocytes	Expressed on DP and SP $CD4^+$ and $CD8^+$	Gomariz et al. (1993, 1994)
CD4 T cells	Spleen and lymph node cells. Induced upon TCR/antigen stimulation and inflammation	Gomariz et al. (1993), Leceta et al. (1994)
CD8 T cells		
Th2 cells	Main immune cell producing VIP, especially upon antigenic stimulation	Delgado and Ganea (2001c)
Th1 cells	Basal production of VIP, not increased by antigen stimulation	Delgado and Ganea (2001c)
B cells	Detected only by RT-PCR	Gomariz et al. (1993)
Mast cells	First evidence of VIP production by immune cells. Beside naïve VIP, they produce VIP fragments ($VIP_{10–28}$)	Cutz et al. (1978), Wershill et al. (1993), Goetzl et al. (1988)
Granulocytes		
Neutrophils	Also produce VIP fragments: $VIP_{6–28}$	O'Dorisio et al. (1980), Goetzl et al. (1989)
Basophils	Mainly of leukemic origin	Wershill et al. (1993)
Eosinophils	Mainly on granulomatous lesions on Schistosomiasis infection	Aliakbari et al. (1987), Weinstock and Blum (1990)
Macrophages	Controversial. Negative expression on most studies, but VIP expressed on mononuclear fraction on human PBLs and on macrophages in Schistosomiasis infection	Lygren et al. (1984), Metwali et al. (2002), Delgado et al. (1996)
Dendritic cells	Not determined	

DP double positive, *SP* single positive, *PBLs* peripheral blood leukocytes

inflammatory and antigenic stimulation. Importantly, Th2 CD4 and T2 CD8 T cells specifically respond to antigen stimulation by producing considerable amounts of VIP. Therefore, VIP has been lately considered as a type 2 cytokine. Interestingly, beside the production of naïve VIP, neutrophils and mast cells secrete VIP fragments ($VIP_{6–28}$ and $VIP_{10–28}$) that fail to signal through the VIP receptors, but have been found recently to exert antimicrobial activities (Delgado et al. 2009; El Karim et al. 2008) probably as part of the natural immune response against pathogens.

Endogenous VIP produced by immune cells plays a critical role in the control of the immune response. Early studies showed increased levels of VIP in inflammatory and autoimmune conditions such as sepsis and rheumatoid arthritis, presumably in an attempt to keep the immune response under control (Arnalich et al. 1994; Brandtzaeg et al. 1989). In fact, VIP-deficient mice are more likely to die from LPS-induced septic shock and more prone to develop bronchial asthma and pulmonary hypertension (Hamidi et al. 2006). More important, Voice and coworkers (2003) demonstrated the involvement of T cell-derived VIP on the regulation of Th differentiation in a study in which elimination of VIP from activated T cells with VIPase IgG resulted in the readjustment of the Th1/Th2 balance. In humans, there is also a correlation between high levels of VIPase autoantibodies and low levels of VIP in patients with lupus and autoimmune thyroiditis (Bangale et al. 2003). Finally, the studies of susceptibility to inflammation and Th1-driven responses in VIP receptors-deficient mice or of association of decreased VIP receptors in patients susceptible to rheumatoid arthritis and multiple sclerosis also indirectly support the involvement of endogenous VIP in the maintenance of immune homeostasis.

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Nerve-Driven Immunity: The Effects of Neurotransmitters on Immune Cells, Functions and Disease

10

CGRP

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Contents

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Abbreviations

CGRP	Calcitonin gene-related peptide
CLR	Calcitonin receptor-like receptor
CTR	Calcitonin receptor
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
NGF	Nerve growth factor
NOD mouse	Non-obese diabetic mouse
PBMC	Peripheral blood mononuclear cells
PKA	Protein kinase A
RAMP	Receptor activity-modifying protein
RCP	Receptor component protein
T_H cell	T helper cell
TLR	Toll-like receptor

10.1 Introduction

The neuropeptides α- and β-CGRP are structurally related to the hormones calcitonin, adrenomedullin and amylin (Wimalawansa 1997; Muff et al. 2004). α-CGRP is predominantly produced in the central and peripheral nervous systems and is generated by tissue-specific alternative splicing of the primary RNA transcript of the calcitonin gene. β-CGRP is produced by a structurally related, but distinct, gene that is a pseudogene for calcitonin. In contrast to α-CGRP, β-CGRP is primarily found in the gut, the pituitary gland and in the immune system. Both α- and β-CGRP are produced as precursors that are cleaved at the amino- and carboxyterminal ends to produce mature peptides. The sequences of α- and β-CGRP differ by only one amino acid in rats and three amino acids in humans. Consequently, the biological activities of α- and β-CGRP are largely identical with the possible exception of pentagastrin-stimulated gastric acid secretion in humans (Beglinger et al. 1988).

Nerve fibers with immunoreactivity for CGRP are detected in bone marrow, thymus and lymph nodes, but are sparse in spleen and non-inflamed gut-associated lymphoid tissues (Weihe et al. 1991). Nerve fibers containing CGRP were found to branch off into non-vascular regions of lymphoid organs such as the paracortical T cell areas of lymph nodes and, in addition, were frequently detected in close proximity to tissue mast cells and macrophages (Stead et al. 1987; Weihe et al. 1989, 1991; Metcalfe et al. 1997). Anatomical contacts were also demonstrated between CGRP-containing nerves and Langerhans cells in the epidermis of the skin (Hosoi et al. 1993).

Nerve fibers containing CGRP also associate with mast cells in inflamed tissues (Stead et al. 1987; Naukkarinen et al. 1996; Metcalfe et al. 1997). During inflammation, activation of mast cells triggers the exocytosis of tryptase, which may cleave protease-activated receptor-2 at the membrane of peripheral projections of

spinal afferent neurons thereby stimulating the local release of CGRP (Steinhoff et al. 2000). Other substances considered to be involved in the release of CGRP from neurons include bradykinin and prostaglandins (Jenkins et al. 2001; Andreeva and Rang 1993).

Thus, there is an extensive network of hard-wired connections between lymphoid tissues and CGRP-containing nerves, which may provide the anatomical basis for reciprocal interactions between the immune and nervous systems both during physiological conditions and inflammation. This chapter will review the influence of CGRP on the regulation of innate and adaptive immune responses and will outline current concepts for the molecular basis of these effects.

10.2 Structure, Signaling and Distribution of the CGRP Receptor in the Immune System

10.2.1 Structure and Cellular Trafficking of the CGRP Receptor

Studies using CGRP receptor antagonists such as $CGRP_{8\text{-}37}$, $CGRP_{12\text{-}37}$, or Cys $(ACM)^{2,7}$-hαCGRP have suggested the existence of two CGRP receptor subtypes, which were termed $CGRP_1$ and $CGRP_2$ receptors (Poyner 1995). Molecular characterization of receptors engaged by CGRP has revealed that the $CGRP_1$ receptor is composed of the seven-transmembrane domain protein CLR and the type-I transmembrane protein RAMP1 (Hay et al. 2006; Walker et al. 2010; Parameswaran and Spielman 2006). In contrast, the $CGRP_2$ receptor phenotype results from CGRP acting at the structurally related amylin and adrenomedullin receptors. Adrenomedullin subtype 1 and 2 receptors are generated by dimerization of CLR with RAMP2 and RAMP3, while complexes of CTR with RAMP1 or RAMP3 function as amylin subtype 1 and 3 receptors. Based on these structural studies it was proposed that the term $CGRP_2$ receptor should no longer be applied and that the term CGRP receptor should be used instead of $CGRP_1$ receptor (Hay et al. 2008).

CLR belongs to the secretin receptor family of G protein-coupled receptors (Schiöth and Fredriksson 2005). Heterodimerization with RAMP members is required for translocation of CLR to the cell membrane (McLatchie et al. 1998; Hilairet et al. 2001a). Both RAMP1 and CLR participate in CGRP binding and are therefore essential for CGRP receptor signaling (Hilairet et al. 2001b). Interaction of different RAMP members with CLR also results in the modification of the terminal glycosylation of the receptors, but these changes do not appear to be important for ligand binding (Fraser et al. 1999; Hilairet et al. 2001b). The cytosolic surface of the CLR/RAMP1 heterodimer associates with the peripheral membrane protein RCP, which contributes to CGRP receptor signal transduction, most likely by stabilizing the interaction of CLR with $G\alpha_S$ (Evans et al. 2000; Prado et al. 2002). Binding of CGRP leads to internalization and desensitization of the CLR/RAMP1 complex by clathrin-coated pit-mediated endocytosis (Bomberger et al. 2005; Hilairet et al. 2001a). Transient stimulation of cells with CGRP induces recycling of the CLR/RAMP1 complex, which is promoted by

endothelin-converting enzyme-1-mediated degradation of CGRP in early endosomes (Cottrell et al. 2007; Padilla et al. 2007). In contrast, sustained stimulation with CGRP targets the CLR/RAMP1 complex to lysosomal degradation thereby preventing resensitization of cells (Cottrell et al. 2007).

10.2.2 CGRP Receptor Signaling

Activation of the CGRP receptor is generally accepted to result in $G\alpha_S$-mediated activation of adenylate cyclase with a subsequent increase in cellular cAMP levels and activation of PKA (Walker et al. 2010). PKA has been implicated in numerous biological effects of CGRP including vasodilation, pain transmission and negative regulation of inflammatory responses (Brain and Grant 2004; Benemei et al. 2009; Sternberg 2006; Harzenetter et al. 2007). CGRP, acting through the cAMP and PKA pathway, was shown to upregulate and activate the transcription factor CREB thereby regulating gene expression in neuronal and glial cells (Anderson and Seybold 2004; Vause and Durham 2010). In addition, CGRP may induce Erk-1/2 and p38 mitogen-activated protein kinase activities in a PKA-dependent manner (Parameswaran et al. 2000).

CGRP was also reported to activate signaling through $G\alpha_{q/11}$ proteins leading to the activation of PLC-β1 and intracellular calcium release in osteoblasts, astrocytes and epithelial cells (Drissi et al. 1998; Aiyar et al. 1999; Morara et al. 2011). Moreover, CGRP induced the membrane translocation of PKCε in alveolar epithelial cells (Wang et al. 2005). It should be considered, however, that these studies did not investigate whether CGRP signaling mediated by $G\alpha_{q/11}$ proteins is driven by the canonical CGRP receptor composed of RAMP1 and CLR or by complexes of RAMP members with CTR. For example, in cells that express both CLR and CTR, CGRP may also activate CTR/RAMP1 dimers, which are known to couple to $G\alpha_{q/11}$ proteins (Morfis et al. 2008). In addition, it is unclear whether this signaling pathway is engaged in immune cells, because studies using dendritic cells failed to show that CGRP triggers Ca^{2+} transients, although dendritic cells express RAMP1, CLR and RCP and respond to CGRP exposure by elevation of cellular cAMP levels (Harzenetter et al. 2007).

10.2.3 Expression of CGRP Receptor Components in Immune Cells

Expression of CLR, RAMP1 and RCP has been demonstrated for epidermal Langerhans cells (Ding et al. 2008) and dendritic cells (Carucci et al. 2000; Harzenetter et al. 2007), while CLR and RAMP1 have been found in macrophages (Fernandez et al. 2001). CLR, RAMP1 and RCP have also been detected in human $CD34^+$ hematopoietic progenitor cells, but, notably, are absent in mature peripheral blood neutrophils (Harzenetter et al. 2002). Although expression of CGRP receptor components on B and T lymphocytes has not been reported at the molecular level, these cells readily respond to treatment with CGRP indicating expression of functional receptor complexes.

10.3 Functional Effects of CGRP on Immune Cells

Modulation of cellular functions by CGRP has been reported for T and B lymphocytes, antigen-presenting cells and hematopoietic progenitors. The effects of CGRP are mostly consistent with a function in the negative regulation of inflammatory responses and a shift of T cell-mediated responses towards a T_H2 phenotype (Table 10.1).

Table. 10.1 Functions of CGRP in the immune system

Targets	Activities	Concentrations
Antigen-presenting cells	MCH class II, CD86 expression ⇩	0.1–100 nM[a]
	IL-12, IL-1, TNFα, CCL4 release ⇩	
	Activation of T_H1 cells ⇩	
	Activation of T_H2 cells ⇧	
	Migration of immature dendritic cells ⇧	
	Migration of mature dendritic cells ⇩	
T cells	Cytokine release of T_H1 cells ⇩	1–100 nM
	T_H1 polarization of $CD4^+$ T cells ⇩	
	T_H2 polarization of $CD4^+$ T cells ⇧	
	Ahesion to fibronectin ⇧	
	Migration into collagen matrices ⇧	
B cells	IL-7-induced pre-B cell proliferation ⇩	0.1–10 nM
Hematopoietic progenitors	Formation of granulomonocytic colonies	0.1 pM–100 nM[b]
	from $CD34^+$ progenitors ⇧	
Disease models	LPS shock (mouse)	1–10 μg/mouse[c]
	TNFα, mortality, liver injury ⇩	
	Diabetes (mouse)	n.a.[d]
	Incidence, hyperglycemia, insulitis ⇩	
	Colitis (rat)	n.a.[e]
	Ulceration, local inflammation ⇩	
	Skin inflammation (mouse)	2 μg/mouse[f]; 10–100 nM[g]
	T cell-mediated hypersensitivity ⇩	

[a] Maximal effects on antigen-presenting cells except for migration of immature dendritic cells were observed at 100 nM CGRP. Inhibition of immature dendritic cell migration was found to be most effective at 0.1–10 nM CGRP

[b] Effective concentrations varied strongly between human and mouse hematopoietic progenitor cells

[c] Single doses of CGRP were administered intraperitoneally or intravenously

[d] Not applicable. Results are derived from transgenic mice or gene transfer experiments

[e] Not applicable. Results are derived from CGRP neutralization experiments

[f] A single dose of CGRP was administered intradermally

[g] Epidermal cells or Langerhans cell-enriched epidermal cells were pretreated *in vitro* with CGRP and used as an immunogen

10.3.1 Effects on Antigen-Presenting Cells

CGRP induces cAMP elevation in murine Langerhans cells (Asahina et al. 1995b) and bone marrow-derived dendritic cells (Harzenetter et al. 2007), whereas mature human dendritic cells may also respond by transient mobilization of intracellular Ca^{2+} (Carucci et al. 2000). Functional studies revealed that the capacity of Langerhans cells to stimulate the proliferation of murine T cells by presenting alloantigens, ovalbumin or pigeon cytochrome C is inhibited by CGRP (Hosoi et al. 1993; Asahina et al. 1995b). Similarly, when human monocytes or dendritic cells were used as antigen-presenting cells and treated with CGRP, the proliferative response of allogeneic T cells was greatly reduced (Fox et al. 1997; Carucci et al. 2000). Moreover, the recall response to tetanus toxoid of human PBMC was impaired in the presence of CGRP (Fox et al. 1997). CGRP was reported to down-regulate the expression of MHC class II antigens and the costimulatory receptor CD86 and to inhibit the release of IL-12p40, IL-1β, TNFα and CCL4 by antigen-presenting cells thereby providing possible explanations for the inhibitory effects of CGRP (Fox et al. 1997; Torii et al. 1997; Asahina et al. 1995b; Carucci et al. 2000; Harzenetter et al. 2007).

Recent studies have extended these findings and indicate more complex effects of CGRP on the function of antigen-presenting cells. While treatment of Langerhans cells by CGRP impaired antigen-stimulated production of IFN-γ, CXCL9 and CXCL10 by T_H1 cells, production of IL-4, CCL17 and CCL22 by T_H2 cells was augmented. These results are consistent with the inhibitory effect of CGRP on IL-12 production of macrophages and dendritic cells (Fox et al. 1997; Torii et al. 1997). It therefore appears that CGRP may not serve as a general suppressor of antigen-presenting cell function, but may rather modulate the function of these cells to promote T_H2 polarization (Ding et al. 2008).

10.3.2 Effects on T Lymphocytes

In addition to its effects on antigen-presenting cells, CGRP may also influence adaptive immune responses by directly acting on T lymphocytes. CGRP increases cellular cAMP levels in established T_H1 cells, but had no such effect on T_H2 cells (Wang et al. 1992). Importantly, treatment of T_H1 cells with CGRP inhibits the mitogen-induced elevation of IL-2, TNFα and IFN-γ at the mRNA level (Wang et al. 1992). These findings were extended by a study showing that CGRP inhibits IFN-γ production of antigen-stimulated murine T_H1 clones, but does not influence IL-4 production by T_H2 clones (Kawamura et al. 1998). Analysis of non-polarized $CD4^+$ T cells revealed that CGRP inhibits production of both IFN-γ and IL-4 when cells are stimulated through CD3 (Tokoyoda et al. 2004). In the presence of CD28-mediated costimulatory signals, CGRP markedly enhanced IL-4 production, but impaired IFN-γ release. CGRP was found to activate the cAMP/PKA signaling pathway in naive $CD4^+$ T cells and the effects of CGRP on T_H cell polarization were mimicked by a synthetic cAMP analogue. Considered together, the available

data suggest that CGRP acts both on T cells and antigen-presenting cells to promote the differentiation of T_H2 cells from naive $CD4^+$ T cells and to impair both T_H1 polarization and the function of established T_H1 cells.

10.3.3 Effects on B Lymphocytes

Several reports have indicated that CGRP influences the differentiation of B lymphocytes. In the pre-B cell line 70Z/3, CGRP causes a prolonged elevation of cAMP and inhibits the LPS-stimulated up-regulation of surface immunoglobulin and μ and κ mRNA levels (McGillis et al. 1993). In addition, CGRP appears to exert direct effects on primary B cells. CGRP was found to inhibit IL-7-induced colony formation, when added to cultures of $B220^+IgM^-$ B cell precursors isolated from murine bone marrow (Fernandez et al. 2000). In addition to direct effects on B lymphoid cells, CGRP was found to stimulate bone marrow stroma cells, but not bone marrow macrophages, to release a soluble factor that inhibits pre-B cell colony formation (Fernandez et al. 2000). The results of these *in vitro* studies were confirmed under *in vivo* conditions by experiments showing that administration of CGRP to mice reduces the number of IL-7-responsive B cell progenitors in bone marrow (Schlomer et al. 2007). Complementary studies have shown that CGRP induces a transient elevation of IL-6 and TNFα mRNA levels in bone marrow stromal cell cultures and that addition of exogenous IL-6 and TNFα inhibits pre-B cell colony formation driven by IL-7 (Fernandez et al. 2003). However, because CGRP was reported to also augment LPS-induced IL-6 production of bone marrow-derived macrophages (Tang et al. 1998; Fernandez et al. 2001), which do not release an inhibitory factor (Fernandez et al. 2000), and because experiments investigating potential effects of IL-6 and TNFα neutralization have not been reported, it appears unclear whether the inhibitory effect of CGRP on pre-B cell colony formation is mediated by IL-6 and/or TNFα.

10.3.4 Effects on Myelopoiesis

The CGRP receptor components RAMP1, CLR and RCP are expressed in human $CD34^+$ hematopoietic progenitor cells (Harzenetter et al. 2002). Both RAMP1 and CLR are down-regulated during granulocytic differentiation *in vitro* and are not detected on mature peripheral blood neutrophils (Harzenetter et al. 2002). Formation of granulomonocytic, but not erythroid or mixed, colonies by purified human $CD34^+$ cells is enhanced in the presence of CGRP (Harzenetter et al. 2002). Consistent with these findings, CGRP was found to stimulate colony formation of murine granulomonocytic progenitor cells (Broome et al. 2000). These results suggest that CGRP may have a function in promoting the generation of myeloid cells.

10.3.5 Effects on Immune Cell Migration and Adhesion

The ability of immune cells to interact with components of the extracellular matrix is essential for their homing and migration to inflamed tissues. CGRP was reported to stimulate adhesion of human T cells to fibronectin under static conditions (Levite et al. 1998). T cell adhesion to fibronectin is mediated by VLA-4 and VLA-5 suggesting that CGRP may be able to activate the ligand binding capacity of integrins. Interestingly, the effects of CGRP on T cell adhesion were antagonized by substance P (Levite et al. 1998). In addition, CGRP induced the migration of naive and CD3-stimulated human T cells into collagen-containing matrices (Talme et al. 2008). CGRP increased the migration of $CD4^+$ and $CD8^+$ T cells to the same extent, but had no effect on the migration of B cells. The influence of CGRP on dendritic cell migration was found to be maturation-dependent (Dunzendorfer et al. 2001). Whereas CGRP has potent chemotactic activity for immature dendritic cells, it inhibits the chemokine-induced migration of mature dendritic cells. These studies therefore suggest that the local release of CGRP promotes the accumulation and arrest of T cells and antigen-presenting cells at sites of infection and inflammation.

10.3.6 Mechanisms of CGRP Action on Immune Cells

Several mechanisms have been proposed to explain the effects of CGRP on immune cell functions. In human PBMC stimulated with inactivated *Staphylococcus aureus* or in a murine dendritic cell line stimulated with LPS and GM-CSF, CGRP weakly increased the production of IL-10 (Fox et al. 1997; Torii et al. 1997). Addition of antibodies directed against IL-10 prevented CGRP-induced down-regulation of dendritic cell CD86 expression and partially reverted the inhibitory effect of CGRP on IL-12p40 production by PBMC (Fox et al. 1997; Torii et al. 1997). In contrast to these studies, CGRP did not significantly alter the production of IL-10 by murine bone marrow-derived dendritic cells stimulated with an agonist of TLR2 (Harzenetter et al. 2007). Moreover, analysis of IL-10-deficient dendritic cells directly demonstrated that the inhibitory effect of CGRP on TNFα production was independent of IL-10 (Harzenetter et al. 2007). Experiments showing that IL-10 antibodies did not reconstitute IFN-γ production by human PBMC treated with CGRP are consistent with these findings (Fox et al. 1997). Considered together, these studies indicate that some anti-inflammatory activities of CGRP may be mediated by IL-10, but, in addition, clearly establish the existence of an IL-10-independent pathway of CGRP function.

It has also been proposed that exposure of immune cells to CGRP may impair activation of NF-κB. Using thymocytes from NF-κB reporter mice, it was shown that CGRP inhibits constitutive NF-κB-driven gene expression (Millet et al. 2000). Interestingly, inhibition of NF-κB activity was observed in CD4/CD8 double positive, but not CD4 single positive, thymocytes. It should be noted, however, that this study also found CGRP to induce thymocyte apoptosis raising the question

as to whether the effects of CGRP on NF-κB activation may be indirect and result from an increased rate of cell death. Additional studies have shown that treatment of Langerhans cells with CGRP prior to stimulation with LPS partially inhibits IKKβ phosphorylation, IκBα degradation and NF-κB DNA-binding activity (Ding et al. 2007). However, the mechanism by which CGRP may influence the induction of NF-κB remains unclear. Because studies with bone marrow-derived dendritic cells have shown that CGRP does not influence canonical TLR signaling including NF-κB activation (Harzenetter et al. 2007) it appears conceivable that, under certain experimental conditions, CGRP may activate NF-κB by indirect pathways such as inhibition of an autocrine stimulatory loop mediated by TNFα or the induction of IL-10, which is well known to inhibit NF-κB in dendritic cells (Bhattacharyya et al. 2004).

Treatment of murine dendritic cells with CGRP inhibits the production of inflammatory cytokines such as TNFα and CCL4 (Harzenetter et al. 2007). This effect was found to be mediated by the cAMP/PKA signaling pathway leading to the rapid up-regulation of the transcriptional repressor, ICER (Harzenetter et al. 2007). Gene knock-down experiments directly demonstrated that inhibition of dendritic cell TNFα production by CGRP is dependent on ICER (Altmayr et al. 2010). Treatment of dendritic cells with CGRP caused a premature repression of TLR2-induced *Tnfa* gene expression, but did not interfere with the initiation of transcriptional activity. This effect was explained by the finding that CGRP prevented binding of ATF-2, but not NF-κB, to the *Tnfa* promoter with a concomitant increase in the recruitment of ICER (Altmayr et al. 2010). Previous studies showing that, in T cells, treatment with forskolin or PGE_2 leads to the induction of ICER, interaction of ICER with the composite NF-AT/AP-1 site of the *Il2* promoter and inhibition of IL-2 production (Bodor and Habener 1998; Bodor et al. 1996) and that ICER may bind to a CRE site in the *Ccl4* promoter thereby attenuating production of CCL4 in activated T cells (Barabitskaja et al. 2006) are in accordance with these results. Together, these studies therefore suggest a model whereby CGRP triggers the rapid expression of ICER, which competes with ATF-2 or other activating transcription factors for binding to CRE sites in promoters of inflammatory genes, leading to a robust repression of gene transcription (Fig. 10.1).

10.4 Involvement of CGRP in Immune-Mediated Diseases

The local and systemic concentrations of CGRP increase during inflammation. Although little is known about the role of endogenous CGRP in immune-mediated diseases, treatment of experimental animals with CGRP has been reported to markedly attenuate pathogenesis in various models of acute and chronic inflammation (Table 10.1).

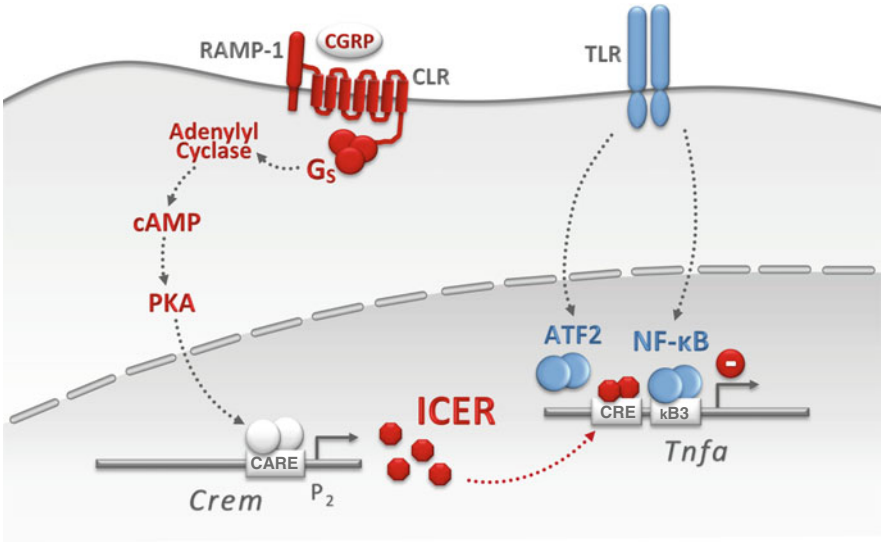

Fig. 10.1 *Model for the control of gene expression by CGRP*. Treatment of immune cells with CGRP induces elevation of cellular cAMP levels and activation of PKA leading to the upregulation of the transcriptional repressor ICER. Transcription of ICER is driven by binding of transactivating transcription factors to cAMP-responsive sites (CARE) present in the intronic promoter (P_2) of the *Crem* gene. ICER binds to CRE sites in target genes thereby preventing recruitment of stimulatory transcription factors such as ATF2. Due to a lag phase caused by the upregulation of ICER, CGRP treatment of cells does not interfer with the initiation of gene transcription, but causes premature repression of established gene expression. This mechanism has been demonstrated to explain the inhibitory effect of CGRP on TLR-stimulated TNFα production by dendritic cells. Additional data supporting this model have been described for the control of IL-2 and CCL4 production by activated T cells

10.4.1 Sepsis

Several studies have shown that systemic levels of CGRP are increased in human patients with sepsis as compared with control subjects (Joyce et al. 1990; Arnalich et al. 1995; Beer et al. 2002). Moreover, CGRP levels directly correlated with disease severity as indicated by comparing CGRP levels of septic shock and sepsis patients (Arnalich et al. 1995) or of sepsis survivors and non-survivors (Beer et al. 2002). High levels of CGRP in sepsis patients may contribute to sepsis-associated macrophage deactivation and suppression of T_H1 responses or may be the result of an exacerbated mixed antagonist response. In addition, it was shown that CGRP acts as a potent vasodilator (Brain et al. 1985) and that CGRP levels correlate with hemodynamic variables (Arnalich et al. 1995) and increase in the late hyperdynamic phase of sepsis just before the onset of the hypodynamic phase (Zhou et al. 2001). It is therefore conceivable that CGRP also contributes to the hypotension and cardiovascular collapse during septic shock.

In a mouse model of endotoxemia, administration of CGRP was shown to attenuate mortality caused by injection of high-dose LPS (Gomes et al. 2005). The protective effect of CGRP correlated with the reduced production of TNFα and an increased release of IL-10 (Gomes et al. 2005). Treatment of mice with CGRP also prevented septic liver injury induced by the combined administration of LPS and D-galactosamine (Kroeger et al. 2009). CGRP decreased serum TNFα levels, while IL-10 levels were increased. However, the protective effect of CGRP was independent of IL-10, because it was also observed in IL-10-deficient mice (Kroeger et al. 2009). Interestingly, hepatic ICER mRNA levels were elevated in CGRP-treated mice suggesting that ICER mediates the inhibitory effects of CGRP on TNFα production also under *in vivo* conditions (Harzenetter et al. 2007; Kroeger et al. 2009).

Murine endotoxemia causes a transient increase in serum CGRP levels (Tsujikawa et al. 2007). Consistent with potent anti-inflammatory activities of endogenous CGRP and the function of RAMP1 as an essential component of the CGRP receptor, serum levels of various inflammatory cytokines including TNFα, IL-12, IFN-γ, IL-6 and CCL2 were elevated in *Ramp1*$^{-/-}$ as compared with *Ramp1*$^{+/+}$ mice following administration of LPS (Tsujikawa et al. 2007).

10.4.2 Diabetes

The development of insulin-dependent diabetes mellitus in the NOD mouse is dependent on both CD4 and CD8 T cells. Transgenic NOD mice expressing human CGRP in pancreatic β cells showed a markedly reduced incidence of diabetes (Khachatryan et al. 1997). The protective effect of transgenic CGRP expression was associated with a reduced mononuclear cell infiltrate of islets, but not with a systemic immunosuppressive effect of CGRP. These findings were confirmed by CGRP gene transfer experiments in mice (Sun et al. 2003). When autoimmune diabetes was induced by multiple low-dose streptozotocin administration, CGRP expression reduced the incidence of diabetes, ameliorated hyperglycemia, increased serum insulin levels and inhibited lymphocyte infiltration into islets. In contrast to the local expression of CGRP in β cells (Khachatryan et al. 1997), elevated serum levels of CGRP following gene transfer were also associated with an inhibition of T_H1, but not T_H2, responses (Sun et al. 2003). Thus, both local and systemic expression of CGRP may protect from T cell-driven autoimmune diabetes.

10.4.3 Inflammation of the Gut

The role of CGRP in experimental inflammation of the gut was investigated in rats following rectal administration of trinitrobenzene sulfonic acid. Macroscopic damage and ulceration as well as neutrophil accumulation in the distal colon were enhanced by neutralization of CGRP indicating that endogenous CGRP may protect rats from acute inflammatory damage of the gut (Reinshagen et al. 1998).

Subsequent studies revealed that expression of CGRP in normal and inflamed colon tissue is dependent on NGF and that NGF neutralization aggravates experimental colitis (Reinshagen et al. 2000). Consistent with these findings, expression of CGRP was reported to be under control of NGF in peripheral sensory neurons, B cells and monocytes (Lindsay and Harmar 1989; Bracci-Laudiero et al. 2002, 2005). It therefore appears that NGF promotes the expression of endogenous CGRP in the inflamed gut, which, in turn, ameliorates tissue damage.

10.4.4 Inflammation of the Skin

In a model using antigen-pulsed epidermal cells as an immunogen, *in vitro* treatment of cells with CGRP prior to antigen pulsing ablated their ability to sensitize mice and resulted in an impaired delayed-type hypersensitivity response (Hosoi et al. 1993; Asahina et al. 1995b; Asahina et al. 1995a). The antigenicity of epidermal cells in this model is dependent on the presence of Langerhans cells suggesting that the effect of CGRP is due to suppression of the antigen-presenting capacity of these cells (Hosoi et al. 1993). When treated with CGRP in the presence of IL-10 neutralizing antibodies, epidermal cells elicited a normal delayed-type hypersensitivity response (Torii et al. 1997). In addition, intradermal injection of CGRP before epicutaneous sensitization of mice with haptens attenuated contact hypersensitivity responses, whereas administration of CGRP after sensitization or at a distant site had no effect (Asahina et al. 1995a). Considered together, these studies indicate that treatment of antigen-presenting cells with CGRP or the presence of CGRP at the local site of antigen exposure potently inhibits T cell-mediated hypersensitivity reactions by a mechanism that may involve IL-10.

10.5 Production of CGRP by Immune Cells

Production of β-CGRP, but not α-CGRP was observed in human and rat T lymphocytes stimulated with mitogens (Wang et al. 2002; Xing et al. 2000). Addition of the CGRP receptor antagonist, $CGRP_{8–37}$, to block the activity of endogenous CGRP enhanced proliferation and IL-2 release of T cells. Resting human B cells were found to express low amounts of CGRP, but expression was markedly upregulated in *ex vivo* isolated B lymphoblast and B cells activated *in vitro* with anti-IgM and IL-4 or NGF (Bracci-Laudiero et al. 2002). B cells also produce NGF (Santambrogio et al. 1994; Torcia et al. 1996) and antibody-mediated neutralization of NGF abrogated CGRP synthesis in resting and activated B cells (Bracci-Laudiero et al. 2002). Human monocytes synthesize basal levels of NGF and CGRP and, in response to LPS stimulation, NGF and CGRP expression are increased (Bracci-Laudiero et al. 2005). Stimulation of monocytes with LPS in the presence of $CGRP_{8–37}$ enhanced expression of CD86 and MHC class II antigens, but did not alter IL-10 secretion (Bracci-Laudiero et al. 2005). These studies support a model suggesting that endogenous NGF stimulates B and T

lymphocytes as well as monocytes in an autocrine manner to produce CGRP, which, in turn, attenuates lymphocyte activation and expression of cell surface proteins involved in antigen presentation.

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The Effects of Cannabinoids on Immune Cells, Responses and Diseases

11

Cris S. Constantinescu and Radu Tanasescu

Contents

Cannabinoids (CB) are a group of molecules that act upon cannabinoid receptors (CBR) and are divided in three categories: phytocannabinoids (natural terpenophenolic compounds derived from the *Cannabis* plant species), endocannabinoids (endogenous) and synthetic cannabinoids.

The adventure of cannabinoid research is exciting. The herb *Cannabis sativa* (marijuana) was used for centuries as medicine and psychoactive drug. However, it

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is the progress of research in the last 40 years that led to the discovery in the late 1990s of the endocannabinoid system – a neurotransmitter network that forms the basis for these effects. This system consists of endogenous ligands and receptors that are subject to modulation by natural and synthetic cannabinoid agonists. The endocannabinoid system plays modulatory functions in the brain and the periphery (immune system, autonomic nervous system, endocrine network, gastrointestinal tract, reproductive system and microcirculation).

Through their complex endogenous effects and particular constitutive features, CB own a special place among neurotransmitter substances discussed in this book. Endocannabinoids are non-conventional neurotransmitters: they do share the defining characters of this class, but are doing it in their own way. A neurotransmitter is mainly synthesized in neurons (Levite 2008); however, endocannabinoids can be released, whenever the need arises, from non-neuronal cells as well, including immune cells. Moreover, their cellular storage is different from that of classical neurotransmitters. Due to their hydrophobicity and tendency to diffuse across membranes, they cannot be stored within synaptic vesicles, but are released in a phospholipid precursor form following calcium-dependent activation of appropriate enzymes or ion channels. The same applies to synaptic transmission. It is commonly accepted that a classical neurotransmitter is present in the presynaptic terminal and released to generate a defined action on the postsynaptic neuron or effector organs. However, endocannabinoids show retrograde transmission, being released by the postsynaptic neuron and acting on the presynaptic neuron by modulating its activity. Thirdly, exogenous administration of a neurotransmitter should precisely mimic the action of the endogenously released transmitter. This is not always the case for the endocannabinoid system. Endocannabinoids act in orchestration with other endogenous molecules and transmitting pathways, which may lead to a variety of biochemical consequences when agonists are administered exogenously. Moreover, as for all neurotransmitters, a specific mechanism exists for the CB removal from the site of action, but since this site is not only the synaptic cleft, but also the pericellular space near immune or other cells, there is no uniform mechanism of removal.

Exogenous or endogenous CB can regulate the function of both immune and nervous systems. In neurons, CB act as regulators of synaptic transmission through pre- and postsynaptic mechanisms. In immune cells, the activation of CB receptors significantly alters the metabolic activity and responses during inflammation. Although other neurotransmitters interact with immune cells, the relationship with the immune system is a special one for endocannabinoids. Immune cells are the main sites of expression for an important subtype of CBR (type 2). Although our current understanding of the role of CB on the immune system is still limited, the endocannabinoid system seems to be a revolving plate of neural – immune interactions.

A measure of the complex and biologically relevant functions of CB is given by the wide range of potential therapeutic uses for CB analogues, again unique among neurotransmitter networks: obesity, dyslipidemia, addiction, inflammation, allergies, pain and spasticity are all clinical conditions in which CB might be therapeutic. Of relevance for the linked neuronal and immune actions, immunomodulation and neuroprotection are special areas of interest for CB therapeutic use.

The aim of this chapter is to provide an overview of the complexity of neuroimmune CB – related physiology and interactions in health and disease and to convince the reader of the important role played by endocannabinoids in immune regulation.

11.1 Overview of the Endocannabinoid System in Relation to Its Function

11.1.1 Brief History of Endocannabinoid Discovery and Function

The endocannabinoid system is a complex endogenous signalling network. It can be described as a pleiotropic locally acting pro-homeostatic signalling system activated 'on demand' following perturbation of cell homeostasis (De Petrocellis and Di Marzo 2009). It includes: (1) at least two cannabinoid receptors (CBR) type 1 (CB1R) and type 2 (CB2R); (2) at least two families of lipid signalling molecules known as the endocannabinoids, the N-acyl-ethanolamines (main representative being N-arachidonyl–ethanolamine or anadamide) and themonoacyl-glycerols (such as 2-arachidonoyl-glycerol and 2-arachidonyl-glyceryl-ether); (3) proteins and enzymes for the regulation of endocannabinoid levels and action at receptors. The number of members of the endocannabinoid system is increasing and might include non-CB1R non-CB2R endocannabinoid receptors, endocannabinoid-related molecules with little activity at CB1R and CB2R level, and new enzymes for the biosynthesis and degradation of these molecules (De Petrocellis and Di Marzo 2009).

The current knowledge of the endocannabinoid system has its origin in the study of plant CB. The potential for marijuana to be both a therapeutic and a drug of abuse generated major efforts to clarify the biology and physiological role of CB in humans. The marijuana plant contains more than 60 distinct chemical phytocannabinoids. Among them, D9-tetrahydrocannabinol (D9-THC) is the main psychoactive component, first structurally described in 1964 (Gaoni and Mechoulam 1964) and giving the name for this class of compounds. The identification and chemical synthesis of D9-THC led to the discovery of the CBR (CB1R and CB2R).

CB1R was cloned in 1990 and is the most abundant G-protein-coupled receptor (GPCR) within the adult nervous system (Devane et al. 1988). CB1R is localized to a number of brain structures, regulates synaptic neurotransmission and thus mediates psychoactive effects, also providing a target for the use of CB as therapeutic agents for a number of neurological disorders (Croxford 2003). CB2R was described in 1993 (Matsuda et al. 1990; Munro et al. 1993). Not thought to be involved in psychoactive effects of cannabinoids, this receptor was initially found in the periphery, particularly in immune cells (mainly B cells and macrophages), but seems to play critical immune roles in the CNS as well (Munro et al. 1993; Cabral et al. 2008). CBR are predominantly located presynaptically rather than postsynaptically, consistent with their postulated role in modulating neurotransmitter release.

The existence of specific receptors that recognize phytocannabinoids stimulated the search for endogenous CBR ligands – the endocannabinoids. In 1992, the first

endogenous CB was isolated from porcine brain and identified as anandamide (from the Sanskrit for "internal bliss") (Devane et al. 1992). Anandamide (AEA) was found to mimic many of the actions of D9-THC (Devane et al. 1992). A second endocannabinoid, 2-arachidonoyl glycerol (2-AG) was identified later (Mechoulam et al. 1995; Sugiura et al. 1995). Both compounds are derivatives of arachidonic acid and are biosynthesised 'on demand' from membrane phospholipids. Since these two discoveries, several synthetic cannabinoid analogues have been proven to induce similar in vivo effects such as analgesia and behavioural changes.

Subsequently, several enzymes involved in the biosynthesis and degradation of endocannabinoids were described, the main representatives being fatty acid amide hydrolase (FAAH) involved in AEA enzymatic degradation and monoacylglycerol lipase (MAGL) which metabolises 2-AG (Beltramo et al. 1997; Cravatt et al. 1996; Dinh et al. 2002).

The functions of the endocannabinoid system are complex and not completely elucidated. Due to the wide distribution of CB1R expression in the CNS, the endocannabinoid system has a broad spectrum of influence on both excitatory and inhibitory neuronal circuits. Therefore it participates in the regulation of physiological functions such as movement, memory and learning, cognition, neuroendocrine secretion, appetite, emesis, regulation of body temperature, pain and immune system modulation. This broad range and ability to regulate synaptic neurotransmission is translated into a great potential of CBs as therapeutic agents in conditions of inappropriate neurotransmission. (Croxford and Yamamura 2005). In addition, in the periphery, this system is an important modulator of the autonomic nervous system, immune system and microcirculation.

Although studies have demonstrated suppressive effects on immune functions following administration of a number of different CB, the picture is far more complex and the precise function of the endocannabinoid system on immune system development remains partially unclear (Croxford and Yamamura 2005).

Endocannabinoid functions appear to be of fundamental importance, as suggested by their evolutionary conservation. Some of the components of the endocannabinoid system date back about 600 million years to when the first multicellular organisms emerged (Elphick and Egertova 2001). Within a cell, CB control basic metabolic processes such as glucose metabolism (Guzman and Sanchez 1999). As evolution proceeded, the role played by the CB system in animal life increased. It is now known that this system maintains homeostasis within and across the organizational scales of all animals. The homeostatic action of CB on so many physiological structures and processes leads to the hypothesis that the endocannabinoid system is nothing less than a naturally evolved harm reduction system (Melamede 2005). It was thus suggested that endocannabinoids fine-tune biochemical functions to maintain them within healthy functioning ranges (Melamede 2005). Endogenous CB levels increase in response to injury like trauma or stroke (Nagayama et al. 1999; Caberlotto et al. 2004; Lim et al. 2003). Moreover, CBR density and coupling efficiency increase secondary to nerve injury and neuropathic pain, but their number is reduced when tolerance to CB is induced

(Caberlotto et al. 2004). Thus, the endocannabinoid system itself is up- or down-regulated as a function of need (Melamede 2005).

Relevant to the present topic, the endocannabinoid network can be found as an ancient plant signalling system regulating the plant immunity-related genes in response to infection and stress (Chapman 2000). Moreover, stereoselective binding sites for endocannabinoid ligands have been found in invertebrate immunocytes and microglia (Stefano et al. 1996). This shows that the system has been preserved from coelenterates to man. Therefore, immune regulatory function seems not to be a new acquisition of this transmitter network, but a defining one (Salzet et al. 2000).

11.1.2 CB Receptors and Ligands: The Players

CBR. An abundant amount of literature exists on CBR. CB1R and CB2R are both GPCR. They may interact with endogenous CB, with phytocannabinoids or with synthetic agonists or antagonists. However, things are more complex. Firstly, non-CB1R, non-CB2R receptors for CB seem to exists; they include other GPCR, ligand-gated ion channels, ion channels, and nuclear receptors (PPAR); they are certainly involved in some of CB effects, but cannot be classified as "CB3R" (see below). Secondly, biochemical properties of classical CBR (partial agonism, functional selectivity and inverse agonism; or CB1R capacity to form heteromers with other GPCR) play important roles in determining the cellular response to specific CBR ligands, Thirdly, some of the CB effects may be the result of simultaneous action on classical and non-classical receptor pathways. We will further summarise some general facts on CBR subtypes and interactions, which refer to CB system in general but have relevance for the immune actions of the CB.

The pharmacology of CBR and their ligands has been extensively reviewed (Di Marzo et al. 2005; Pertwee et al. 2010; Reggio 2002; Jonsson et al. 2006a; Pertwee 2006, 2008a, 2010; Sugiura et al. 2006; Ashton et al. 2008; Hanus and Mechoulam 2010). CB1R and CB2R belong to the group of rhodopsin-type GPCR, which is composed largely of receptors for amine-type neurotransmitters and neuromodulators (e.g., serotonin, adrenaline, dopamine) (Fredriksson et al. 2003; Bjarnadottir et al. 2004). CB1R and CB2R are atypical of the group in that they are activated endogenously by the lipid-type signalling CB endogenous molecules AEA and 2-AG (Pertwee et al. 2010).

CB1R and CB2R are single polypeptides with an extracellular N-terminus, an intracellular C-terminus and seven transmembrane helices (7TM). Both activate G proteins ($G_{i/o}$ proteins) inhibitory to adenylate cyclase (AC) thus inhibiting the conversion of ATP to cyclic AMP (cAMP) (Howlett and Mukhopadhyay 2000). However, they can also activate AC through stimulating G proteins (G_s proteins) (Glass and Northup 1999), and both are positively coupled to mitogen-activated protein kinase (MAPK) (Woelkart et al. 2008). CB1R is coupled with ion channels, inhibiting D-type K+, N and P/Q-type Ca2+ currents, and activating inward and A-type rectifying K+ currents (Croxford and Yamamura 2005). For CB2R, the ion channel modulation is more variable (Mackie 2008). CB2R signalling also activates

the phosphatidylinositol 3-kinase and Akt (PI3K–Akt) pathway and increases the synthesis of the sphingolipid messenger ceramide, thus having a pro-survival and a pro-apoptotic effect, respectively (Carracedo et al. 2006; Molina-Holgado et al. 2002).

The localization of CBR is highly relevant to their functions. CB1R, first identified in mouse spleen cells (Kaminski et al. 1992), is located mainly on hippocampus and basal ganglia and highly expressed in the brain regions expected from the psychoactive effects of D9-THC (Mackie 2005a). In the forebrain, immunocytochemistry studies show that CB1R are expressed mainly by axons of GABAergic interneurons containing cholecystokinin basket cells (Bodor et al. 2005; Nyíri et al. 2005). The endocannabinoids can act instantly or their effects may be long-lasting (Bacci et al. 2004). Central CB1R can be implicated in short-term and long-term neuroplasticity (Chevaleyre et al. 2006). In peripheral tissues, CB1R is found in adipocytes, liver, pancreas, skeletal muscle – possibly implicated in the metabolic cannabinoid effects. Activated somatic CB1R receptors can result in neuronal hyperpolarisation (Bacci et al. 2004; Cota 2008; Cavuoto et al. 2007). There is also evidence that CB1R are expressed by immune cells as well, thus being also involved in immune modulation (Howlett et al. 2002; Jean-Gilles et al., unpublished observations).

CB1R, like several GPCR, may exist as homo- or heteromultimers (Milligan 2004). It has been suggested that CB1R exist as homomultimers in vivo (Wager-Miller et al. 2002; Mackie 2005b). It is not clear whether monomeric and homomultimeric forms exhibit differential signal transduction or intracellular trafficking patterns, or how interconversion is physiologically regulated (Pertwee et al. 2010). However, there is good evidence that CB1R form heteromers with certain other GPCR and that this heteromerization affects the manner in which the CB1R respond to agonists, possibly by influencing ligand selectivity or relative intrinsic activity and enhancing the receptor signalling repertoire (Mackie 2005b). This is due to an allosteric interaction, defined as an "intermolecular interaction by which binding of a ligand to one of the receptor units in the receptor heteromer changes the binding properties of another receptor unit" (Ferre et al. 2009). Several CB1R heteromers are known including [CB1-D2 dopamine receptor (brain); CB1-opioid receptor (brain, spinal cord); CB1-orexin-1 receptor heteromers (brain)] and others are under study (Pertwee et al. 2010).

CB2R are generally expressed at lower levels than CB1R, and highly selective antibodies for CB2R are difficult to generate (Van Sickle et al. 2005). CB2R receptors are expressed on immune cells (especially those that are macrophage-derived: microglia, osteoclasts) and neurons (Galiegue et al. 1995; Ofek et al. 2006). CB2R was also found in other peripheral structures, such as peripheral nerve terminals in mouse and retina in rat (Griffin et al. 1997; Lu et al. 2000). Initially, northern analysis, quantitative RT-PCR analysis and autoradiography could not show the presence of CB2R in the brain, but Western blot analysis and immunohistochemistry demonstrated its presence in astrocytes and microglia, as well as on neurons (Croxford and Yamamura 2005). Because CB2R expression on microglia is related to the cell activation status, it was suggested that it is induced by

local inflammation, infection or stress (Carlisle et al. 2002; Wotherspoon et al. 2005), but further studies demonstrated that CB2R is present in astrocytes, microglia, neural subpopulations and oligodendroglial progenitors in healthy brains (Stella 2004; Maresz et al. 2005; Onaivi et al. 2006a; Palazuelos et al. 2006; Beltramo et al. 2006). Data show that, when activated, CB2R can modulate immune cell migration and cytokine release both outside and within the brain (Cabral and Staab 2005).

In view of their distribution, a common role of CB1R and CB2R appears to be the modulation of ongoing release of chemical messengers, CB2R mainly from immune cells and CB1R mainly from neurons.

Interestingly, polymorphisms in the genes of CB1R (*CNR1*) and CB2R (*CNR2*) receptors have been identified and linked to certain disorders (psychiatric conditions such as schizophrenia, depression in Parkinson's disease for *CNR1*; postmenopausal osteoporosis for *CNR2)* (Norrod and Puffenbarger 2007; Henquet et al. 2008).

CBR have particularities of reaction to stimulation that can explain some of the discordant results of experiments using synthetic cannabinoid analogues. These particularities include partial agonism, inverse agonism and functional selectivity, and assume the model of receptors existing in equilibrium between active and inactive conformations, considering that a fraction of the receptor is in the active state even in the absence of the agonist (Mackie 2008). Partial agonism is the property of activation of the same receptor to different extents by different agonists (Mackie 2008). The effect of an inverse agonist is opposite to that of an agonist. An inverse agonist binds preferentially to the inactive state of the receptor, thus decreasing the fraction of active receptor and suppressing basal signalling (Mackie 2008). Also, for both CB1R and CB2R, different conformations are corresponding to different agonist stimuli, consequently activating different signalling pathways (functional selectivity) (Mackie 2008; Kenakin 2001). Moreover, agonist and antagonist CB analogues have different consequences on receptor activation that are related to the basal level of signalling of the receptor. Effects of CB1R and CB2R modulators are difficult to distinguish in vivo, since the difference between effects of inverse agonist and neutral antagonists cannot clearly be separated (Mackie 2008; Kenakin 2001; Yao et al. 2006).

Some classical effects of CB such as anti-emesis may not be mediated only by the CB1R–CB2R receptor system (Bueb et al. 2001; Parker et al. 2004). Evidence suggests the existence of additional CBR subtypes possibly responsible for these alternative mechanisms (Howlett et al. 2002; Breivogel et al. 2001). It is accepted today that besides the CBR pathway, endocannabinoids also interact with other GPCR (Mackie 2008), with the vanilloid receptor-type 1 (TRPV-1) activated by AEA, and also K+ channels, 5-HT3 receptors and alpha7 nicotinic receptors (Oz 2006; Szallasi and Di Marzo 2000) or peroxisome proliferator-activated receptors (PPAR) (Michalik et al. 2006). It is not clear which of these interactions are relevant for the physiologic effects of CB or just a consequence of their lipophilic character (Mackie 2008). Recent studies focus on GPR55, suggesting that this receptor can be activated by AEA and 2-AG (Ryberg et al. 2007). GPR55 is a GPCR present in both brain and peripheral organs and shows very little sequence homology with CB1R

and CB2R. Because of the large body of conflicting pharmacological data, no conclusive decision can yet be reached about whether GPR55 should be classified as a novel CBR (Pertwee et al. 2010). PPAR are ligand-activated transcription factors that constitute part of the nuclear receptor family. Because they are nuclear receptors, signal transduction at PPAR is primarily directed through alterations in gene transcription (Pertwee et al. 2010). Evidence that endocannabinoids are endogenous agonists of PPAR in vivo comes from a model of inflammatory pain (Jhaveri et al. 2008).

Therefore, the endocannabinoid system seems to interact in a significant manner with several other endogenous systems. However, a major challenge is to understand those interactions that are physiologically significant, and to separate those that do not occur under relevant patterns of endocannabinoid release. There seems to be no correlation between the ability of CB compounds to activate or block CB1R and/or CB2R and their ability to target other receptors or channels. Moreover, some receptors and channels have been found to be activated by CB1R/CB2R antagonists or antagonized or inhibited by CB1R/CB2R agonists in a CB1R/CB2R independent manner (Pertwee et al. 2010).

This raises two issues: the relevance of distinct "pharmacological fingerprint" of a considered CB compound for interpreting CB interactions in vivo; and whether any known mammalian channel or non-CB1/CB2 receptor should be classified as a novel cannabinoid "CB3" receptor or channel (Pertwee et al. 2010).

Recently, criteria for new CBR were proposed (Pertwee et al. 2010). Any receptor or channel considered a new CBR should be activated at its orthosteric site and with significant potency by an established ligand for CB1R or CB2R; it should be activated by at least one established endogenous CB1R or CB2R agonist at "physiologically relevant" concentrations; as a GPCR, it should have significant aminoacid sequence similarity with the CBR; and it should be expressed by mammalian cells that are known to be exposed to concentrations of endogenously CB molecules capable of eliciting a response. If the non-CBR receptor or channel is activated endogenously by a non-CBR ligand, but without being activated endogenously by any endocannabinoid with appropriate potency and relative intrinsic activity, the new receptor should not be considered a CBR (Pertwee et al. 2010).

Therefore, no deorphanized receptor or channel is currently classified or reclassified as a novel CBR. However, it is important to note that the TRPV1 channel is activated by endogenous AEA at the capsaicin binding site, and it is presumably exposed to endogenously produced endocannabinoids since it is co-localized with CB1R at the neuronal level (Pertwee et al. 2010). This warrants further study and also raises a hypothesis with regard to other non-classical CBR: the possibility that under certain pathological conditions, endogenous CB such as AEA are acting at these receptor sites with higher potency (reaching "physiologically relevant" concentrations) (Pertwee et al. 2010). In other words, it is not known if putative new receptors are activated only in disease, or their sensitivity of activation is influenced by the absence or presence of drugs that inhibit the metabolism or enhance the biosynthesis of the activator endocannabinoid molecule

(Pertwee et al. 2010). Further effort in research will clarify these aspects, and will determine the role played by non-classical CBR activation in immune modulation.

CBR-independent mechanisms might also involve effects on lipid-raft structure and function (Biswas et al. 2003) which are known to be important for immune-cell function (Vogt et al. 2002). Lipid rafts are membrane microdomains biochemically defined by the insolubility of their components in cold non-ionic detergents. They are enriched in cholesterol and specific lipids with saturated fatty acid chains, as sphingomyelin and sphingolipids. Evidence exists that several components of GPCR signal transduction chains interact with and/or are localized within lipid rafts (Huang et al. 1997). Data in recent years suggests that a link exists between CB1R signalling in nerve cells and raft integrity (Bari et al. 2005; Fezza et al. 2006). However, CB2R in leukemic cells has not been found to be regulated by lipid rafts perturbation (Oddi et al. 2007). Since the two classical CBR are encoded by different genes, exhibiting 44% amino-acid identity throughout the whole protein, some speculate that lipid rafts might regulate CB1R by interacting with specific regions of its tri-dimensional structure (Oddi et al. 2007). However, this was demonstrated in the nerve cells in a model which used methyl- beta-cyclodextrion (MCD), a membrane cholesterol depletor, which may block AEA-induced apoptosis in a CB1R dependent manner (Oddi et al. 2007). The implications for immune reactions need further study, but based on available evidence, it is suggested that CBR can be regulated by the rate of interlayer exchange and lateral diffusion of endocannabinoid/cholesterol complexes within lipid bilayers, thus suggesting innovative approaches for the therapeutic exploitation of the membrane component of endocannabinoid signalling (Oddi et al. 2007).

Finally, at least some of the non-CBR mediated CB effects can be attributed to lipophilicity as shown by experiments in which CB agonists have direct effects on mitochondrial function, which may explain their metabolic and anti-cancer effects (Athanasiou et al. 2007a, b). This may also be relevant in pathological states such as stroke (when AEA levels increase to levels high enough affect mitochondrial function) or neurodegenerative disease (where mitochondrial enzyme activities in the brain decrease, thus leaving the brain selectively sensitive to the effects of CB) (Athanasiou et al. 2007a).

Cannabinoid-based analogues and ligands. There are two main groups of CB ligands, with varying affinities for CBR. The first group is based on the structure of marijuana CB and includes natural compounds [D9-THC, D8-THC, cannabichromen (CBC), cannabigerol (CBG) and tetrahydrocannabivarin (D9-THCV) – all psychoactive; cannabinol and cannabidiol (CBD)– both without psychoactive proprieties] and synthetic ligands [CP55940, HU-210, HU-211, ab-cannabidiol, ajulemic acid]. The main representative members of the latter group are endocannabinoids. They include arachidonic acid metabolites [N-arachidonyl ethanolamide or anandamide (AEA), 2-arachidonoyl glycerol (2-AG), palmitoylethanolamide (PEA), 2-arachidonylglycerylether (noladin ether), 2-linoleoyl glycerol (2-LG), O-arachidonyl ethanolamine (virodhamine) (Porter et al. 2002), oleoylethanolamide (OEA)]. Several CB1R- and CB2R-selective agonists and antagonists have been developed. CB1R-selective antagonists include

SR141716A (rimonabant), AM251, AM281 and LY320135; SR144528 and AM630 are CB2R-selective antagonists (Pertwee 2005). Neutral CBR antagonists apparently lacking inverse agonist activity have also been developed (Pertwee 2006). Synthetic CB have different structure from endocannabinoids. CP55,940 and HU-210 are non-CBR selective agonists more potent than D9-THC, and WIN55,212 has agonist affinity for both CB1R and CB2R.

We discuss below some characteristics regarding CB signalling and metabolism that may be relevant also for understanding of CB-immune interactions.

Endocannabinoids. As mentioned, endocannabinoids are not stored in vesicles or cells like classical neurotransmitters. Their release from neuronal and non-neuronal cells is stimulated in a receptor dependent manner by neurotransmitters. Thus, endocannabinoid biosynthesis arises in response to elevations of intracellular calcium, from lipid precursors present in the membrane through enzyme activation. The receptor response to a specific endocannabinoid is influenced by the ligand concentration, the presence of other CB ligand molecules, the receptor density and state of activation and the quantities of signalling proteins. From the extracellular site, endocannabinoids are removed by cellular uptake processes such as simple diffusion, through membrane associated binding proteins or a transmembrane carrier protein, that still await to be defined. Degradative enzymes tightly regulate the signalling capacity of endocannabinoids. There are multiple endocannabinoid biosynthetic and degradative pathways, with multiple enzymatic chains, the selectivity of which still has to be clarified (Muccioli 2010). A classical general model for endocannabinoid-based retrograde signalling is outlined in Fig. 11.1. We will further briefly discuss AEA, 2-AG and PEA.

AEA is a member of the N-acylethanolamine (NAE) family, a large group of bioactive lipids that also includes non-endocannabinoid compounds. These lipids are present throughout the body and the balance between synthesis and inactivation finely regulates their levels – even more so than those of non-lipid transmitters (Muccioli 2010). AEA is produced by immune cells and neurons, and is more selective for CB1R than CB2R. It is found in brain, spleen, skin, kidney and uterus (Yang et al. 1999). AEA is highly produced by brain areas where CB1R is highly represented (striatum, hippocampus, cerebellum) and is implicated in cannabis-related effects like nociception and catalepsy. Evidence exists that AEA can activate TRPV-1 receptors as well as acting on CB1R and CB2R (Pertwee 2005).

The immediate precursor to AEA is *N*-arachidonoyl phosphatidyl-ethanolamine (NPAE), which is formed from phosphatidyl-choline and phosphatidyl-ethanolamine. Currently, it is accepted that there are at least three important pathways through which AEA is synthesized, and the question of their selectivity is raised. Actual understanding is that depending on the acyl chain (and, thus, the resulting NAE), a given pathway will be preferred over the others (Muccioli 2010). The classical accepted pathway for AEA biosynthesis involves the action of *N*-arachidonoyl phosphatidyl-ethanolamine phospholipase (PLD). One additional pathway has glycerophospho-N-acylethanolamine lipids (GP-NAEs) as key intermediates (Muccioli 2010). Of interest for immune implications, the third pathway was characterized in macrophage-like RAW264.7 cells and involves

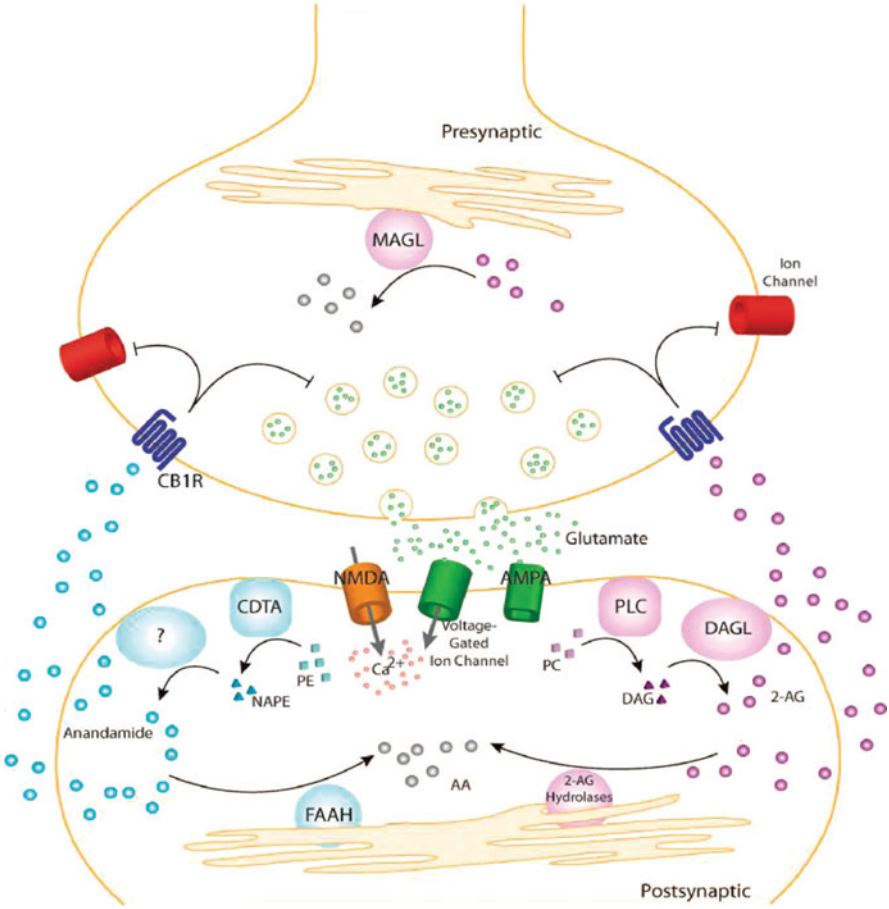

Fig. 11.1 A general outline for endocannabinoid-based retrograde signalling (reproduced with permission after (Ahn et al., 2008). Upon release of neurotransmitter (e.g., glutamate), postsynaptic receptors as AMPA (R-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), NMDA (*N*-methyl-D-aspartic acid) and voltage-gated ion channels are activated, allowing influx of Ca2+ and on-demand endocannabinoid biosynthesis (Ahn et al., 2008). Anandamide is synthesized from phospholipid precursors by a calcium-dependent transacylase (CDTA) and one or more other still uncharacterized enzymes. 2-AG is synthesized from phospholipid precursors by phospholipase C (PLC) and diacylglycerol lipase (DAGL). Endocannabinoids then migrate from postsynaptic neurons to CB1 receptors (CB1R) located on presynaptic neurons. Once activated, CB1Rs couple through the Gi/Go class of G-proteins to regulate ion channels and inhibit neurotransmitter release. The retrograde signaling of endocannabinoids is then terminated by degradative enzymes

phospho-N-arachidonoylethanolamine (pAEA) as a key intermediate in AEA synthesis (Liu et al. 2006). In the presence of lipopolysaccharide (LPS), these cells have increased AEA levels but decreased NAPE-PLD expression. Whether this pathway is more or less selective for AEA synthesis than other NAEs, and which of these networks is the main AEA source during inflammation is still not known.

Inside the cell, AEA signalling is inactivated by fatty acid amide hydrolases 1 and 2 (FAAH1, FAAH2) or N-acylethanolamine-hydrolyzing acid amidase (NAAA)-mediated hydrolysis, into arachidonic acid, or by COX2 oxidation into prostaglandin E2-ethanolamide, which can be further transformed into other bioactive lipids, such as prostaglandins and endocannabinoid-derived prostaglandins. The presence of at least three NAE hydrolases, with only partially overlapping tissue expression, raises the question of their respective roles in regulating NAE levels. Whereas it is generally accepted that FAAH1 is the principal contributor to AEA hydrolysis in the CNS, the role of FAAH-2 and NAAA is still debated. FAAH2 has limited sequence homology (20%) with FAAH1, was found in human, but not rodent, tissues (Wei et al. 2006) and is localized in cytosolic lipid droplets but not in the endoplasmic reticulum as is FAAH1 (Kaczocha et al. 2010). The high expression of FAAH2 in peripheral tissues (e.g. liver, where AEA has crucial metabolic roles) suggests that it might have a rescue role in hydrolyzing NAEs upon FAAH1 inactivation. Of interest for the immune actions of AEA, NAAA is highly expressed in immune cells, specifically in macrophages. NAAA is localized to the lysosomes, where it is activated by autoproteolytic cleavage (Zhao et al. 2007; Tsuboi et al. 2007). Thus, in contrast to FAAH, NAAA is most active at acidic pH (Wang et al. 2008). Interestingly, the preferred substrate of NAAA is PEA, whose levels are increased during inflammation. This could be relevant for CNS inflammation. Microglia respond to PEA (Stella 2009), and some suggested that NAAA is to be considered an interesting target in targeting inflammatory states. Moreover, an additional PEA-hydrolyzing activity distinct from NAAA and FAAHs was detected in a microglial cell line (Muccioli and Stella 2008).

New concepts regarding AEA intracellular transport and storage may show relevance for its immunomodulatory effects. Apart from lipophilicity, intracellular trafficking via AEA binding proteins that act as internal transporters might have a strong impact on the overall AEA signalling (Maccarrone et al. 2010a). These transporters may shuttle AEA to nuclear targets, such as PPAR, or to TRPV1 on the inner part of cell membrane. In addition, same transporters might deliver AEA to adiposomes, allowing its accumulation within these organelles. Adiposomes are a crucial site for the fate of AEA, dictating its sequestration, degradation or oxidation. AEA oxidizing enzymes are associated with adiposomes in immune cells (Weibel et al. 2009). AEA in the adiposomes may have a longer half-life and higher levels than externally. This may allow AEA to trigger PPAR which require otherwise higher concentrations and long time (hours) for an activation cycle, contrasting with the rapid activation (minutes) of CBR and TRPV1 (Howlett et al. 2010; Di Marzo and De Petrocellis 2010). Macrophages and adipocytes have a consistent adiposomic compartment, and adipocyte inflammatory responses are regulated by endocannabinoids through PPAR, suggesting that they could use AEA more as a classic hormone than as a local short-lived mediator (Maccarrone et al. 2010b). This suggests a dual endocannabinoid metabolic control levels: one acting on demand and another acting via intracellular trafficking and storage (Maccarrone et al. 2010a).

2-AG was first isolated from canine gut tissue (Mechoulam et al. 1995). Synthesis of 2-AG depends on the conversion of 2-arachidonate-containing

phosphoinositides to diacylglycerols (DAGs), which are then converted to 2-AG by the action of DAG lipase. Like AEA, 2-AG is thought to be removed from its sites of action by cellular uptake and metabolized intracellularly. Monoacylglycerol lipase (MAGL) is the principal degradative enzyme for 2-AG; two additional enzymes, ABHD6 and ABHD12, were also identified (Blankman et al. 2007). Genetic disruption of MAGL in the brain was recently shown to cause marked elevations in 2-AG levels leading to CBR desensitization, showing that excessive 2-AG signalling can functionally antagonis the brain CB system in vivo (Chanda et al. 2010).

2-AG is present in higher quantities than AEA in the immune system and has lower affinity for CB1R. 2-AG can stimulate, through CB2R, the chemotactic response of microglial cells, and these effects are antagonized by exogenous CB (D9-THC, CP55940) (Cabral et al. 2008). Interestingly, some effects of endogenously released AEA and 2-AG may be enhanced through an "entourage effect" that relies on the co-release of other endogenous fatty acid derivatives such as PEA and oleamide, which can potentiate AEA, and 2-linoleyl glycerol and 2-palmitoyl glycerol, which can potentiate 2-AG (Ben-Shabat et al. 1998; Garcia Mdel et al. 2009; Jonsson et al. 2001).

PEA is generated by neurons and immune cells. It is produced during inflammation and inhibits mast cells via CB2R and downregulates inflammation (Facci et al. 1995; Calignano et al. 1998). However, even though CB2R antagonists can counteract its CB-like effects, it has been suggested that PEA does not bind either CB1R or CB2R (Facci et al. 1995).

Phytocannabinoids. The pharmacology of plant-derived ligands was recently reviewed (Pertwee et al. 2010; Pertwee 2008b). Phytocannabinoids can stimulate the CB system via CB1R or CB2R, like D9-THC, but nonpsychotropic ligands (cannabinol, cannabidiol) can exert anti-inflammatory effects despite a low affinity for CBR (Malfait et al. 2000). D9-THC is a partial agonist which can block activation by other ligands of both CBR, but can also induce stimulatory effects, depending on the receptor expression level, coupling efficiency and endogenous CB release (Pertwee 2008b; Patel and Hillard 2006). The influence of the conversion of D9-THC into 11-OH-D9-THC, a stronger agonist, on the balance of agonist/antagonist actions is subject to discussion (Pertwee 2008b). D9-THC in vitro is a CB2R partial agonist at concentrations in the low nanomolar range, and a CB1R antagonist with tissue specificity (Pertwee 2008b). In vivo, D9-THC can act as an antagonist or, at higher doses, as an agonist for CB1R. It displays less relative intrinsic activity at CB2R than at CB1R. D9-THC possesses significantly lower CBR activity than synthetic agonists, thus suggesting that D9-THC is a CBR partial agonist (Mallat et al. 2007).

D9-THC can have modulatory effects on both cell-mediated and humoral immunity. It may suppress T cell proliferation, by inhibiting IFN-γ production and influencing Th1/Th2 balance via a CB2R-mediated mechanism that could be reversed by SR144528 (Yuan et al. 2002). CBD, by antagonizing CB1R/CB2R agonists, can inhibit immune cell migration and thus induce anti-inflammatory effects (Walter et al. 2003; Lunn et al. 2006). Furthermore, ajulemic acid – which is a derivative of THC-11-oic acid, has low affinity for CB2R but exerts anti-

inflammatory activity, which might be mediated through disruption of the arachidonic acid cascade or through activation of PPAR (Klein 2005).

Alkamides derived from Echinacea sp. have structural similarities with AEA and affinity for CB2R (Raduner et al. 2006). In low nanomolar concentrations, they can exert effects on cytokine and chemokine expression in human blood (Woelkart et al. 2006). It was shown that IL-6 produced by B cells or macrophages can be increased by alkamides and AEA in a CB2R-dependent manner (Woelkart et al. 2008). By alternative non-CB2R mechanisms, the same compounds can inhibit the LPS-stimulated expression of TNF-α, IL-1β and IL-12p70, but not IL-6 and IL-8 (Raduner et al. 2006). The immune effects (anti- or proinflammatory) of alkamides are concentration-dependent and influenced by the stimulus applied and degree of unsaturation of the lipophilic tail (Raduner et al. 2006). This higher metabolic stability compared with AEA suggests a therapeutic potential for alkamides.

11.1.3 CB Drugs Currently Registered Prescribed for Human Use

The herb marijuana has been used for centuries in countries of the Far and Middle East for pain, nausea, seizure or sleep disturbance, and against maladies as diverse as malaria, constipation and rheumatism (Booth 2008). In the Western World, the medicinal benefits of cannabis were not appreciated until the middle of the nineteenth century (Di Marzo 2006). The therapeutic applications of CB have been the subject of debate and controversy since. However, in recent years a shift in attitude concerning the therapeutic potential of CB compounds has begun to emerge. Several drugs containing CB are currently used in the therapy of emesis, pain and spasticity, but more indications are under study for this class of therapy.

The products available on this controversial yet growing market are Marinol® (dronabinol; Unimed Pharmaceuticals), Cesamet®, Nabilone® (nabilone; Cambridge Laboratories; Valeant) and Sativex® (delta-9-tetrahydrocannabinol, cannabidiol; nabiximols, GW Pharmaceuticals Ltd.). Marinol is a CB prescribed as an appetite stimulant, primarily for AIDS. It was also FDA approved as an antinauseant in patients with cancer receiving chemotherapy who have failed to respond adequately to conventional antiemetic treatments. Dronabinol, the active ingredient, is synthetic delta-9- THC. It is supplied as capsules containing 2.5, 5, or 10 mg dronabinol. The recommended doses are 5 mg/m^2 1–3 h before chemotherapy, then 2–4 h after chemotherapy for a total of 4–6 doses/day. For anorexia, doses recommended are 2.5 mg orally twice daily to a maximum of 20 mg/daily.

Nabilone is a synthetic cannabinoid that mimics D9-THC. It is approved for treatment of chemotherapy-induced nausea and vomiting that has not responded to conventional antiemetics, as well as for use in treatment of anorexia and weight loss in patients with AIDS. In Mexico, nabilone is approved as an adjunct therapy for chronic pain management. Nabilone is prescribed as an orally administered treatment, up to 2 mg twice daily.

Sativex is a cannabis-derived oromucosal spray containing approximately equal proportions of D9-THC (partial CB1R agonist) and cannabidiol (CBD; a non-

psychoactive, anti-inflammatory analgesic with CB1R antagonist and endocannabinoid modulating effects). Sativex was approved in Canada in 2005 for treatment of central neuropathic pain in multiple sclerosis (MS), in 2007 for intractable cancer pain and recently approved or in the process of approval in Canada and several EU countries for treatment of spasticity in MS. Sativex is administered oromucosally via a pump-action spray with each 100-ml pump-action actuation providing 2.7 mg of D9-THC, 2.5 mg of CBD plus other phytocannabinoids, terpenoids, and phytosterols (Cavuoto et al. 2007), in a base of 50% ethanol and 50% propylene glycol with 0.05% peppermint flavouring. The preparation has onset of activity in 15–40 min, which allows patients to titrate dosing requirements according to pain levels or other symptoms, with an acceptable profile of adverse events.

The large variety of conditions in which CB are under study as therapies reflect their complex and often promiscuous actions. Considerations on their potential role as immunomodulatory and anti-inflammatory therapies will be made in the last section of this chapter.

11.2 Cannabinoid Receptor Expression by Immune Cells

Immune cells express both CB1R and CB2R receptors, secrete endocannabinoids and have functional CB transport and breakdown mechanisms (Pestonjamasp and Burstein 1998).

T lymphocytes express CB1R, which may be involved in CB-induced T helper cell subset differentiation. Although they are expressed only al low levels in the basal state, CB1R receptors are up-regulated in T cells by stimuli such as CB themselves, an effect mediated by IL-4 (Börner et al. 2008). In addition, work by our group has shown that proinflammatory cytokines including TNF-alpha, IL-1, and IL-6 induce both CB1R and CB2R in human peripheral blood mononuclear cells (PBMC) including T cells (Jean-Gilles et al., unpublished observations).

Within the immune system, CB2R level of expression is usually higher than that of CB1R (Massi et al. 2006). This has been primarily shown in murine cells, and our data show smaller differences between mRNA expression of CB1R and CB2R on immune cells. (Jean-Gilles et al., unpublished observations). CB2R mRNA is found in decreasing amounts in human B cells, NK cells, monocytes, polymorphonuclear neutrophils and T cells (CD8 $>$ CD4) (Galiegue et al. 1995). In the immune organs, CB2R expression was demonstrated in thymus and spleen. CB2R mRNA was also detected in the cortex of lymph nodes and the nodular corona of Peyer's patches (Lynn and Herkenham 1994). Very recently, a detailed analysis of CB2R protein levels expressed by blood-derived immune cells from healthy human donors showed that NK cells, B-lymphocytes and monocytes expressed higher levels of CB2R than CD4+, CD8+ T-lymphocytes or neutrophils (Graham et al. 2010). Interestingly, NK cells had the greatest variation in CB2R expression levels, whereas for each of the other cell types CB2R levels were relatively similar between subjects. In contrast to other methods, flow cytometry revealed that

CB2R are present on resting T-lymphocytes at low abundance in some healthy subjects (Graham et al. 2010).

The presence of CB2R on dendritic cells (DC) suggests a role for CBs in modulating antigen presentation (Matias 2002). The expression of CB2R depends of the cell activation state and can be influenced by immune modulators, as shown by the studies on rodent peritoneal macrophages (Carlisle et al. 2002). Immune consequences of CB2R activation include changes in cytokine release from immune cells and migration of immune cells inside or outside the CNS (Cabral and Staab 2005).

CBR expression levels seem to correlate with the cell activation status and activating stimuli (Croxford and Yamamura 2005). The human Jurkat T-cell line and mouse macrophages express more CB1R when they are activated (Daaka et al. 1996) and splenocyte CB2R mRNA is less abundantly expressed after LPS stimulation and more expressed after anti-CD40 co-stimulation (Lee et al. 2001). In addition, inflammatory cytokines enhance expression of both receptors. TNF-alpha does this in an NF-κB-dependent manner (Fig. 11.2). (Jean-Gilles et al., unpublished observations).

Marijuana use and anti-CD40 co-stimulation can increase both CB1R and CB2R expression; LPS and phytohemagglutinin (PHA) can increase only CB1R expression, whereas PMA and IFN-γ may stimulate CB2R expression. Suppressor stimuli of CB1R expression are anti-CD3 antibody, LPS and ionomycin, and inhibitors for CB2R expression are LPS and TGF-β (Klein 2003). Conversely, the influence of CBs on immune function mediated through CBR is supported by their lack of effect in CB2R deficient T helper cells (Buckley et al. 2000). CB1R expression can be upregulated by IL-4 in T lymphocytes, which enables CB1R-mediated communication to neuronal cells (Börner et al. 2008).

A special point must be made on the presence of CB2R in the brain, which is relavant to the relation of the endocannabinoid system with neuroprotection and neuroinflammation. Microglia represent a major cell type involved in neurodegenerative and neuroinflammatory processes. Microglia also express CB1R and produce endocannabinoids (2-AG and AEA) (Carrier et al. 2004, 2005). CB2R expression is higher in activated microglia like 'primed' and 'responsive' microglia (Cabral and Staab 2005; Carlisle et al. 2002). During these activated states, CB exert a stronger influence on microglia functions. It is suggested thus that a CB2R-dependent time-window for functional modulation of microglial actions exists, and that synthetic and endogenous CB analogues have different modulatory effects at this level (Cabral et al. 2008; Walter et al. 2003). Endocannabinoids may play a modulating role between neurogenesis and neurodegeneration, via the immune system or independent pathways (Wolf and Ullrich 2008a; Fernández-Ruiz et al. 2007). Therefore, the CB2R in the CNS is an attractive target for development of drug treatment of neuroinflammation and neurodegeneration.

Studies of effects on immune competence produced by targeted disruption of CB1R and CB2R have provided various and sometimes discordant results. CBR immune functions and their modulation by D9-THC was investigated in $CB1R^{-/-}$/$CB2R^{-/-}$ mice by Kaminski and colleagues (Springs et al. 2008). Despite reports

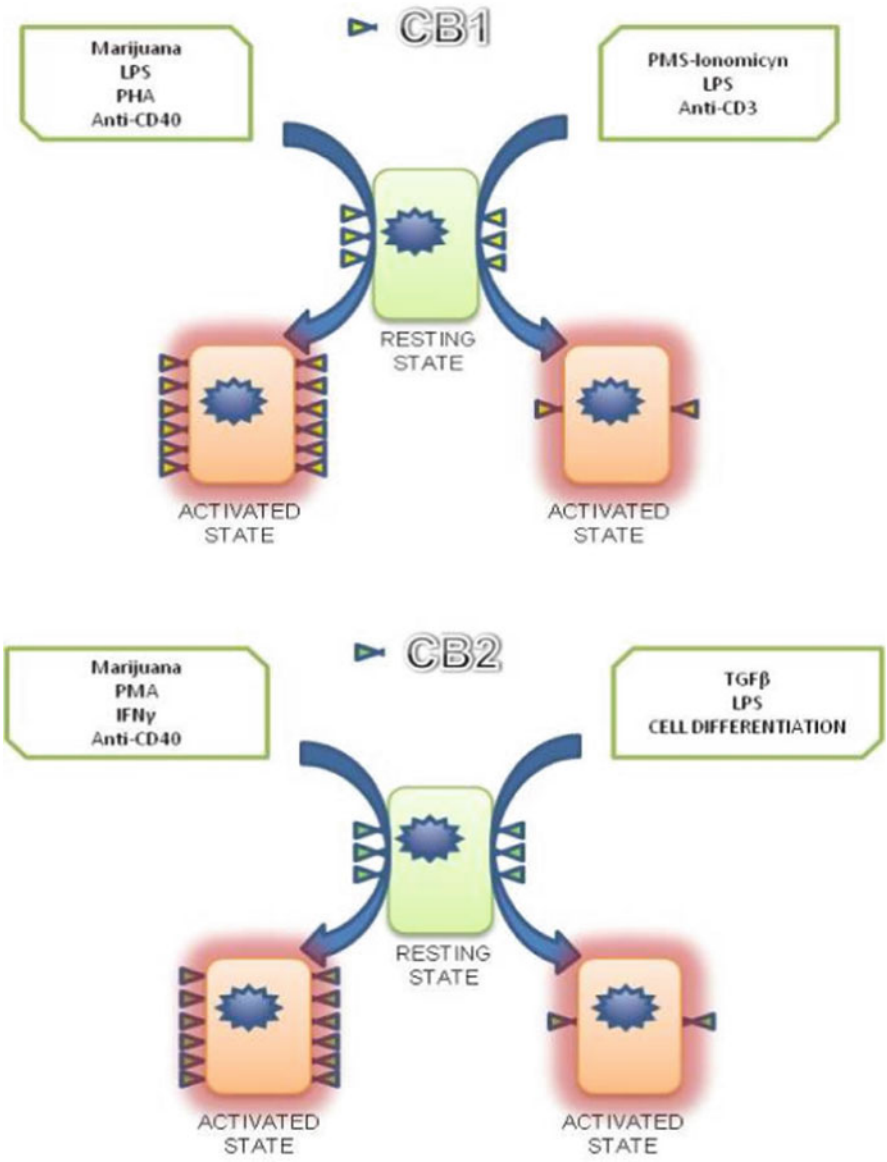

Fig. 11.2 Different stimuli increase or decrease CBR expression in function of cell activation. LPS: lipopolysaccharide; PHA: Phytohemagglutinin; IFNγ: interferon gamma; TGFß: transforming growth factor beta; PMA: phorbol myristate acetate

about enhanced T cell-mediated immune responses in $CB1R^{-/-}/CB2R^{-/-}$ mice, including delayed-type hypersensitivity and antiviral responses to influenza, targeted disruption of CBR did not produce profound effects on immune

competence, as assessed by well-established and widely used standard immune function assays (Springs et al. 2008). Also, no profound differences between $CB1R^{-/-}$/$CB2R^{-/-}$ and wild-type mice were observed in the percentages of major leukocyte subpopulations or in responses to mitogenic stimuli, the mixed lymphocyte response and the production of several cytokines (i.e., IL-2 and IFN-γ) (Springs et al. 2008). However, CBR involvement was observed in humoral responses requiring CD40-initiated signalling for suppression by D9-THC.

The CBR-associated signal transduction and the immune system. CBR stimulation regulates DNA binding of different nuclear factors in the immune cells (Massi et al. 2006). These effects are mainly achieved via down-regulation of cAMP formation and signal transduction involving AC (Koh et al. 1997). Rapid and transient bursts in AC activity are associated with preceding lymphocyte activation by mitogens, and cytokine transcription in macrophages is regulated via cAMP signaling cascade (Kaminski et al. 1994). cAMP analogues variably inhibit or stimulate immune responses in a concentration-dependent manner, and can antagonize the effect of CB on T lymphocyte-dependent production of antibodies (Koh et al. 1997).

CBR stimulation appears to antagonize the regulatory role of the cAMP pathway in the early events in immune cell activation, but these effects are probably more complex, since natural CB, unlike synthetic CB, act as inverse AC agonists, or antagonists in some circumstances (Massi et al. 2006; Bayewitch et al. 1996). cAMP regulates PKA signaling cascade, which targets multiple intracellular units such as the cAMP response element-binding protein/activation transcription factor (CREB/ATF) family. Stimulation by D9-THC inhibits IL-2 secretion and transcription after the reduction of cAMP formation via CB2R (Condie et al. 1996; Yea et al. 2000). D9-THC also inhibits PKA and CRE-specific transcription factor binding and nuclear factor binding to CRE and NF-κB in mouse splenocytes and thymocytes (Koh et al. 1997; Herring and Kaminski 1999; Herring et al. 1998). A similar mechanism was demonstrated in the macrophage cell line RAW264.7, leading to down-regulation of inducible NO synthase (Jeon et al. 1996). Besides cAMP-mediated effects, stimulation of CBR can act through Gi proteins and have a dual influence on MAPK activity depending on ligand and cell type. CB agonists can have different modulator inducing effects on MAPK signaling pathway. CB2R is probably involved in MAPK phosphorylation after stimulation with 2-AG (Kobayashi et al. 2001). Indirect evidence suggests the involvement of Gi–Go proteins, since the response induced by 2-AG is blocked by pertussis (Kaminski et al. 1994).

In conclusion, CBR stimulation generates complex cellular regulation cascades of DNA binding, mainly but not solely via cAMP pathway, involving also Gi- and Gs-binding proteins and MAPK stimulation (Fig. 11.3).

Immune effects of CB must be considered in regard to a concentration-dependent activity. There is a biphasic response associated to the CB ligand concentration. Thus, in vitro, a molecule can be stimulatory in nanomolar concentration, and have inhibitory effects in micromolar concentration range – that means more than tenfold higher than those observed in cannabis smokers' blood (Croxford and Yamamura 2005). Moreover, the interactions between the different CB ligands

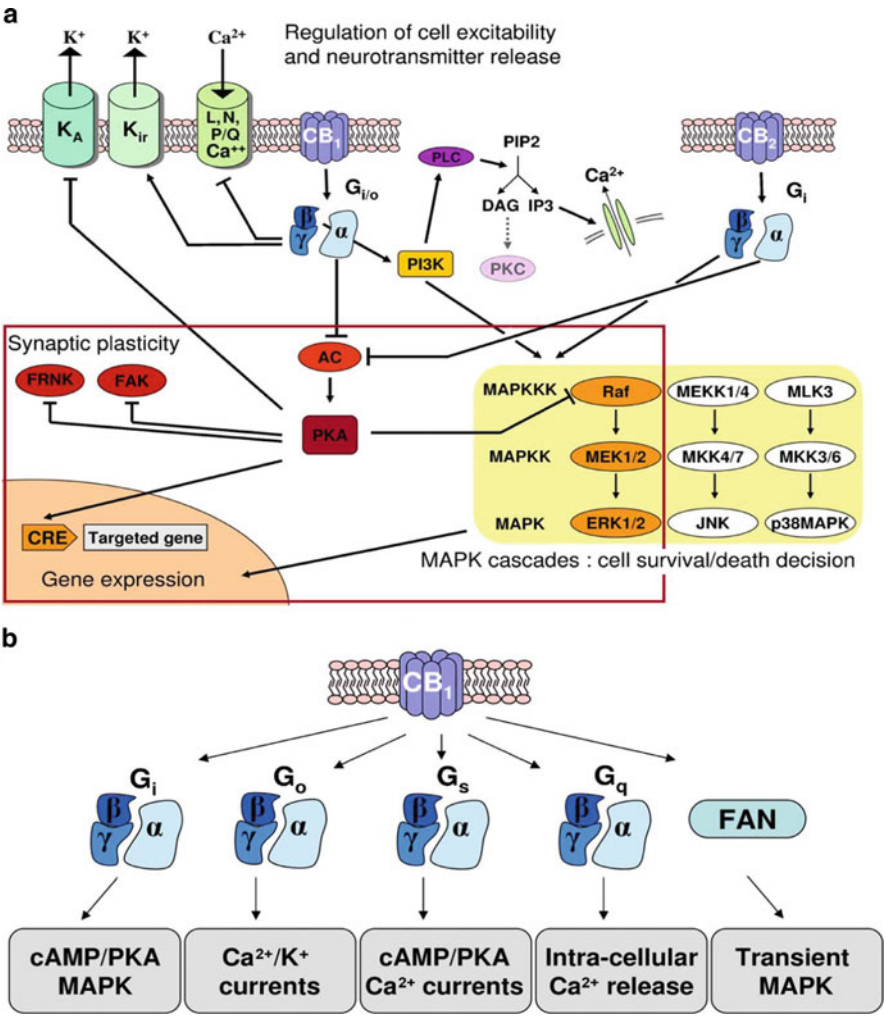

Fig. 11.3 Complexity at CBR signalling (reproduced with permission after (Bosier et al. 2010). Both CBR are associated with Gai/o-dependent inhibition of adenylyl cyclase activity and Gbg-dependent activation of the different MAPK cascades (A). In addition, the CB1R negatively regulates voltage-gated Ca2+ channels and positively regulates inwardly rectifying K+ channels and finally induces elevation of intracellular free Ca2+ through Gbg-dependent activation of phospholipase C (PLC) (Bosier et al. 2010). Cross-talks between signalling pathways are illustrated by the variety of responses requiring cannabinoid-mediated inhibition of protein kinase A (PKA). Reduction of PKA activity is related to a reduction of gene expression through decreasing cAMP response element (CRE) activity. In addition, reduction of PKA activity leads to a decrease in constitutive inhibitory phosphorylation of c-Raf and a consecutive activation of extracellular signal regulated kinase-1 and -2 (ERK1/2). Similarly, reduction of voltage-dependent K+ A channel and focal-adhesion kinase (FAK and FRNK) phosphorylations through inhibition of PKA lead to activation of these different effectors. Several of these signalling pathways are directly related to the variety of functions regulated by CBR. Activation of CB1R also leads to

makes their immune actions very complex (e.g., the partial agonist effect of phytocannabinoid D9-THC is antagonistic for the endocannabinoid agonist 2-AG). Thus, the discrepancies between many studies demonstrating inhibitory effects on the immune system and studies showing a stimulatory action on immune cells can be explained by the heterogeneity in the types and concentrations of CB ligands, methods, or experimental protocols (Tanasescu and Constantinescu 2010). On the other hand, these opposite effects may be reflecting the homeostatic activity of the endocannabinoid system on immune reactions. It can be hypothesized based on the available data that the endocannabinoid signalling in immune cells provides a tonic control of immune cell activation and limits spontaneous activation of immune cell function (Pandey et al. 2009).

Finally, the effect of CB on immune functions appears to be transient which would allow the inhibitory effects to be overcome when the immune system needs to be activated during infections. The practical consequence of this transient nature of CB effects on the immune system may be that the adverse effects of potential therapies targeting the endocannabinoid system may be low (Pandey et al. 2009).

CB immune effects have been reviewed over the years (Croxford and Yamamura 2005; Massi et al. 2006; Tanasescu and Constantinescu 2010; Hollister 1986; Cabral and Dove Pettit 1998; Klein et al. 1998; Berdyshev 2000; Roth et al. 2002). Although a clear global integrative description is still lacking, several principles can be formulated. Firstly, immune cells express endocannabinoids and CBR in immune cells are activated after infection or immune stimulation, with possible consequent regulation of cellular chemotaxis. CBR are GPCR like the receptors for chemokines and other lipid mediators, hence their activation on immune cells may regulate gene products that are required for immune-cell function (Klein 2005).

Secondly, these immune consequences are complex. On the one hand, endocannabinoids promote immune cell proliferation and induce chemotaxis and migration; on the other hand they downregulate inflammatory responses by affecting pro- and anti-inflammatory cytokines. CB drugs can suppress cytokines and cell-mediated immunity through CBR-dependent (G-protein signalling and regulation of cytokine genes) and independent mechanisms (through other receptor types, as TRPV or nuclear receptors, for example PPAR, or effects on lipid raft structure and function) (Klein 2005).

Thirdly, the reasons and consequences of endocannabinoid generation can be viewed as a complex paradigm. AEA and 2AG are each differentially linked to diverse proinflammatory stimuli, providing multiple signalling responses. CBR-inactive endocannabinoid ligands seem to play a role of independent signalling

activation or Gs and Gq proteins (B). In addition the CB1R also signals trough non-G protein partners such as the adaptor protein FAN. Preferential activation of different intracellular effectors by each G protein contributes to diversity and selectivity of responses regulated by cannabinoid receptors (Bosier et al. 2010)

molecules rather than be only simple "entourage" components of AEA-mediated signalling (Berdyshev 2000).

Finally, marijuana-based drugs can suppress the chronic inflammatory response with subsequent attenuation of disease processes and symptoms. This is achieved by the suppression of expression of cytokines and other endogenous pro-inflammatory mediators, and by increasing the production of anti-inflammatory molecules (Klein 2005).

11.3 Cannabinoid Effects on Immune Cells

Since 1970, when the first studies on the effects of smoking marijuana on immune cells were reported, the effects of CB on immune function have been extensively studied. Overall CB can have opposing actions on immune cells, depending on three key factors: (1) the type of CB, (2) the dose of CB, and (3) the type of cell that the CB is acting on. The degree of CBR expression may play role in some cases; however, a receptor-independent mechanism of action is evident for CB in some cell populations.

Both synthetic CB and phytocannabinoids inhibit T-cell mitogenesis and IL-2 production, with consequent inhibition of T cell, natural killer cell, and B cell proliferation (Tanasescu and Constantinescu 2010).

T cells. CB can influence T cell immunity in various manners: they can affect T cell number and proliferation, but may also have important effects on T helper 1- and 2-specific cytokines and TGF-β secretion (Croxford and Yamamura 2005). Initial studies done on T cells from blood of marijuana smokers showed inhibitory effects such as decreases in number or sensitivity (Nahas et al. 1977; El-Gohary and Eid 2004), but other studies failed to confirm these findings (White et al. 1975; Lau et al. 1976). This variability of results can be partly explained by the heterogeneity of the studies, with different routes of administration, type and quantity of marijuana used, D9-THC concentration, frequency of smoking and duration of inhalation. Secondly, moderate marijuana smoking has different effects on immune cells exposed directly to smoke and on cells of systemic immunity. Alveolar macrophages in smokers have less cytokine production and responsiveness and lower antimicrobial activity (Klein 2003; Shay et al. 2003). Lymphocyte recruitment to airways is decreased in D9-THC-treated mice challenged with influenza virus A/PR/8/34 (PR8). In the same model, targeted deletion of CB1R and CB2R produced enhanced inflammatory responses to influenza PR8 in the absence or presence of D9-THC, suggesting involvement of CB1R/CB2R-dependent and -independent mechanisms in D9-THC effects (Buchweitz et al. 2008).

Acute exposure-related immune effects have to be distinguished from those produced by chronic exposure to CB that may result in modulation of CBR expression, decreased T cell number and increased incidence of infection and squamous cell carcinoma of the head and neck (El-Gohary and Eid 2004; Sidney et al. 1997; Zhang et al. 1999; Nong et al. 2002).

Early studies on the D9-THC treatment of mice and rats or in animal and human immune cell cultures had shown a suppressive effect on cellular functions in T and

B cells, NK cells or macrophages (Nahas et al. 1977). Nevertheless, in some situations a biphasic effect was shown, with low (nanomolar) doses of D9-THC being stimulatory and higher concentrations (micromolar) inhibitory (Patrini et al. 1997). Non-psychoactive ligands had slightly stronger effects than D9-THC, and alternative non-CB1R/CB2R mechanisms were suggested for T cell suppression (Tashkin et al. 2002). In the Jurkat cell line, activation can induce upregulation of CB1R transcription. It was suggested that this phenomenon, together with the constitutive expression of CB2R, enables cellular responses to CB by both receptor-mediated pathways (Börner et al. 2007a).

The duration of exposure can modulate CBR response. Acute, but not chronic treatment with the synthetic CB CP55,940 inhibits PHA-induced splenocyte proliferation, possibly by CB2R down-regulation after chronic exposure (Massi et al. 1997). On the other hand, several days D9-THC treatment inhibited splenocyte proliferation induced by ConA, whereas acute injection had no effect (Patrini et al. 1997; Massi et al. 1998).

CD8 cells seem to be more sensitive to cannabinoid action than CD4 cells (Klein et al. 1991). CB can affect the cytolytic capacity of cytotoxic T lymphocytes, but apparently not inhibit the T cell binding to the target cell (Fischer-Stenger et al. 1992). T cell stimulation is reduced also via CB effects on DC, by reducing the DC surface expression of MHC class II molecules in a CB1-dependent manner (Wacnik et al. 2008).

CB also modulate the *T helper cell subsets (Th1 and Th2)*. The effects on the more recently described Th17 subset have not been extensively studied. CB alter the balance between subsets, suppressing Th1 and enhancing Th2, via both CB1R and CB2R (Yuan 2002). IFN-γ, IL-12, and IL-12 receptors are decreased by D9-THC treatment, whereas the Th2 and Th2-promoting cytokines are increased. This bias has several mechanisms. It may be partly explained by differential expression of CBR on Th subpopulations and on antigen-presenting cells. A part of this effect is due to modulation of cytokines generated by DC, involving both CBR (Lu et al. 2006). CB1R in either neural pathways or on T cells may mediate the decrease in IFN-γ and IL-12Rβ2, and CB2R on T cells, NK cells or basophils may be involved in D9-THC-induced increase in IL-4 and GATA-3 (the key transcription factor for IL-4 production) (Newton et al. 2009). Moreover, induction of Th2 associated cytokines can inhibit Th1 cells (Croxford and Yamamura 2005). Recent data also showed that CB can directly induce B cell class switch from IgM to IgE, thus biasing toward Th2 type immunity, and this involves CB2R receptors (Agudelo et al. 2008). This and other data underscore the importance of the endocannabinoid system in regulating adaptive immunity and the balance between Th1 and Th2 activity.

The effects of CB on Th cytokine production are of particular importance with regard to their therapeutic potential. D9-THC can inhibit IFN-γ secretion in a CB2R-dependent way (Yuan 2002) and CB ligands can suppress the expression of other cytokines that may potentiate inflammation, such as TNF-α, IL-1, IL-2, IL-6, IL-12 (Croxford and Yamamura 2005). Targeting and blocking Th1 associated cytokines and potentiation of Th2 type cytokine pathways have shown promising results in animal models of inflammatory conditions such as experimental autoimmune

encephalomyelitis (EAE) and experimental arthritis (Racke et al. 1991; Mageed et al. 1998; Triantaphyllopoulos et al. 1999; Croxford and Miller 2003). Regulatory T cells are involved in the attenuation of experimental autoimmune hepatitis by exogenous and endogenous CB (Hegde et al. 2008); these data potentially open the way for CB-based future therapies of inflammatory disease in humans.

As already mentioned, current data indicate that in vivo or in vitro endocannabinoids stimulate several parameters of immune function at nanomolar concentrations, while being inhibitory at micromolar concentrations (Eisenstein et al. 2007; Correa et al. 2009). Differential sensitivity to endocannabinoids and promiscuous activation of several classes of receptor appear to be involved in these effects as well (Facci et al. 1995; Alexander and Kendall 2007). Moreover, activation or inhibition in vitro can vary according to the cell lineage, medium conditions, and drug concentration, which can all explain the contradictory results found in the literature.

AEA may produce, in a concentration equivalent to those that regulate neuronal response, a dose-dependent inhibition on mitogen-induced human T and B cell proliferation (Schwarz et al. 1994). It is suggested that AEA can influence cell growth by CBR-independent mechanisms (Derocq et al. 1998). AEA was also demonstrated to have a pro-proliferative effect on hematopoietic cell lines, acting synergistically with other growth stimuli. These effects were not seen for other natural or synthetic CB ligands (Valk et al. 1997). 2-AG was shown to have strong immunomodulatory activity on mitogen-induced T cell proliferation in mouse splenocytes, enhancing it at high cell density and producing the inverse effect at low cell density conditions. Its rapid degradation to arachidonic acid may activate other pathways of lymphocyte proliferation (Lee et al. 1995). Recent data demonstrated that AEA suppresses proliferation and release of cytokines like IL-2, TNF-α and IFN-γ from activated human peripheral T-lymphocytes in a CB2R dependent-manner, without exerting any cytotoxic effect on T cells. Furthermore, AEA had an suppressive effect on IL-17 production, thus suggesting reduction of Th17 activity (Cencioni et al. 2010). Also, AEA enhances LPS/IFN-γ-induced IL-10 production in activated microglia in vitro, by targeting CB2R through the activation of ERK1/2 and JNK MAPKs (Correa et al. 2010). IL-10 is a key negative regulator of microglial activation, and microglial cells are the main source of cytokines within the brain and the first barrier of defence against pathogens by acting as antigen presenting cells. Moreover, AEA inhibits IL-12p70/IL-23 axis in human and murine microglial cells via CB2R (Correa et al. 2009). This could result in the modification of the cytokine milieu, contributing to the accumulation of anti-inflammatory microglia at lesion sites for example in the case of MS (Correa et al. 2010).

On the other hand, proving the complexity of CB effects, in vivo low dose of AEA (0.1 mg/kg) immediately prior to sensitization had stimulatory effect on cell-mediated immunity (Th1 response) in mice, by inducing both the increment of DC activation and IFN-γ production (Ribeiro et al. 2010). Concentration dependent-effects were shown in that study, as AEA at nanomolar concentrations increased the production of IFN-γ, while such production decreased at micromolar range (Ribeiro et al. 2010).

Recently it was shown that CBD, which suppresses IL-2 production by activated murine splenocytes, can suppress T cell function and may regulate the production of IL-2 in T cells, and that CBR play a role in modulating the magnitude of these effects (Kaplan et al. 2008, Börner et al. 2009).

Natural killer cells (NK): NK cell numbers are lowered by ingestion of "bhang", a form of marijuana extracted from cannabis leaves and used as a drink or smoked (El-Gohary and Eid 2004). Various animal studies showed that both proliferation and cellular cytolytic activity can be influenced by CB treatment, and that these effects can be mediated by CB1R and CB2R (Massi et al. 2006). Despite reports that in humans NK cell functions are not significantly affected by CB, in vitro investigations on purified human lymphocytes indicate that D9-THC may suppress NK activity and the constitutive expression of the chemokines IL-8, MIP1-α, MIP-1β, and RANTES, and phorbol ester-stimulated TNF-α, GM-CSF and IFN-γ at concentrations (30 mM) that were not toxic for the cells (Specter et al. 1986).

Regarding the dose-dependent effects, systemic administration of CBD repeated for 14 days at relatively low doses of 2.5 mg/kg or 5 mg/kg/day, produced bidirectional effects on lymphocyte subset distribution in peripheral blood of rats (Ignatowska-Jankowska et al. 2009). Administration of CBD at doses of 5 mg/kg resulted in clearly (lymphopenic) effects on total leukocyte number and B, T, Tc and Th lymphocyte subsets, but not NK and NKT cells. In contrast, CBD administered at dose of 2.5 mg/kg did not produce significant lymphopenia, but resulted in an increase of NK cell total number and percentage (Ignatowska-Jankowska et al. 2009). Since NK cell number in circulation has been recently suggested to be important in cancer patients' prognosis (Terabe and Berzofsky 2007) further study is needed to clarify if this may explain antitumor effects of CBD (Ignatowska-Jankowska et al. 2009).

Macrophages: Macrophages express both CBR, although predominantly CB2R (Sinha et al. 1998). As with other immune cells, the relation between CB and macrophage functions is bidirectional. CB ligands predominantly inhibit macrophage migration (CB2R mediated) (Raborn et al. 2008), antigen presentation to T cells and phagocytic capacity (Sacerdote et al. 2005). They can also influence the release of inflammatory mediators such as nitric oxide (CB1R mediated), TNFα, IL-1, IL-6, IL-10 and IL-12, and the production of arachidonic acid metabolites in macrophage cultures via CBR (Cabral et al. 1995; Berdyshev et al. 2001). On the other hand, macrophages can synthesize endocannabinoids such as AEA and 2-AG, which can modulate immune response and cell differentiation through CBR-dependent and -independent mechanisms. The pattern of CB2R expression and thus the CB effects on macrophages are dependent of their state of activation, being maximal in "primed" and "responsive" states and minimal in "resting" and "fully activated" states. In this activation window, macrophage properties include antigen processing and presentation, chemotaxis and phagocytosis and CB can influence specific proteases involved in antigen presentation.

The mechanisms of macrophage inhibition by endocannabinoids seem to be different: whereas AEA diminishes LPS-induced NO and IL-6 production, 2-AG inhibits IL-6 production but increases iNOS-dependent NO production in J774

macrophages (Chang et al. 2001). It was speculated that these discrepant results of 2-AG are due to its bioactive metabolites, AA and PGE(2), which potentiate of both iNOS and COX-2 induction, while the AEA metabolite, PGE(2)-ethanolamide, influences neither LPS-induced NO nor IL-6 production. 2-AG also serves as a substrate for COX-catalyzing PGE(2) production, which in turn modulates the action of CB2.

In a recent study in patients with coronary artery disease, it was demonstrated that the endocannabinoid system is activated with increased endocannabinoid levels in the blood and CB1R overexpression in coronary atheroma, particularly in macrophages (Sugamura et al. 2009). CB1R blockade exhibited anti-inflammatory effects on human macrophages, which might provide potential beneficial effects on atherogenesis (Sugamura et al. 2009).

Neutrophils: CBR can be expressed by neutrophils (Galiegue et al. 1995). Endocannabinoids, phytocannabinoids and related ligands are potent inhibitors of human neutrophil migration, possibly through non-CBR-dependent mechanisms, though such effects on healthy human neutrophils were not observed with low doses of D9-THC (Deusch et al. 2003). Synthetic CB such as CP55940, but not AEA, can inhibit neutrophil lysosomal enzyme release independently of CBR activation (Kraft et al. 2004). Recently, a positive relationship between AEA levels and the adhesive and phagocytic function of neutrophils in patients with fibromyalgia was demonstrated (Kaufmann et al. 2008). It is interesting to note that in these patients the effects of AEA on neutrophil functions are apparently opposite to those of endogenous glucocorticoids resulting in a restitution of neutrophil dysfunction induced by changed glucocorticoid activity under conditions of stress (Kaufmann et al. 2008).

Mast cells (MC). MC are bone marrow derived, multifunctional immune cells found in the nervous system, mucosal and connective tissue, and are involved in allergic and inflammatory responses. Despite controversy on CBR expression and CB effects on MC (Croxford and Yamamura 2005), it is accepted that both CBR can be expressed by MC, although PEA can control MC degranulation via a CB1R/CB2R-independent mechanism (De Filippis et al. 2008a; Giudice et al. 2007). CB, including PEA and related compounds, may act to control mast cell activation and degranulation early during the inflammatory response (De Filippis et al. 2008a). Also, agonists of both CBR prevent mast cell-dependent angiogenesis during granuloma formation (De Filippis et al. 2008b).

Both PEA and AEA can bind to CB2R, but only PEA could down-modulate MC activation in vitro in a rat model, while this effect is efficiently antagonized by AEA (Facci et al. 1995). On the other hand, independently of CBR but still in a Gi/o-protein-dependent manner, only the CB containing a benzopyran ring (D9-THC; D8-THC; and AEA -only in high concentrations), but not PEA or PEA derivatives have been shown to induce an energy and concentration-dependent non-lytic histamine release from peritoneal MC in a rat model (Bueb et al. 2001). In contrast, it has been demonstrated that 2-AG mediated suppression of histamine release from guinea pig mast cells can be reversed by a CB2R antagonist or an nitric oxide synthase inhibitor (Vannacci et al. 2004).

PEA has shown anti-inflammatory actions in several MC-mediated experimental models of inflammation, despite the lack of a high affinity for CBR (Jonsson et al. 2006b). It was suggested that drugs containing PEA can be efficacious as treatment for dermatitis symtoms via inhibition of nerve growth factor (NGF) release from MC (Pulvirenti et al. 2007).

Dendritic Cells (DC). DCs play a major role as antigen-presenting cells and in the development of antigen-specific T cell responses. Human DC express both CBR and the endocannabinoid system present in DCs can be regulated by cell activation, in turn playing a critical role in regulating DC growth and maturation (Matias et al. 2002). Lipid extracts from immature DC contain endocannabinoid ligands AEA, 2-AG and PEA (Matias et al. 2002). LPS can increase the levels of 2-AG in DCs, without increased CBR or FAAH expression (Maestroni 2004). In turn, 2-AG may act as chemotactic molecule capable of recruiting DC during innate immune responses (Maestroni 2004). Not all CB effects on DC are CBR-mediated, as shown by the suppression of IL-12 by D9-THC in stimulated DC cultures (Klein and Cabral 2006). Both exogenous and endogenous cannabinoids induce apoptosis in DCs (Do et al. 2004). Only AEA concentrations of 20 μM can cause marked apoptosis in murine bone marrow-derived DCs, while lower concentrations are not effective (Do et al. 2004). Thus, AEA apoptotic activity is concentration dependent and this may be linked to rapid AEA hydrolysis by FAAH. Moreover, it seems to be CBR-mediated since addition of antagonists of CB1R (SR141716A) or CB2R (SR144528) to the cultures reversed AEA effects (Do et al. 2004).

Hematopoietic stem and progenitor cells. The endocannabinoid system is involved in hematopoiesis and the CB1R/CB2R agonist axis mediates repopulation of hematopoiesis and mobilization of hematopoietic stem and progenitor cell (HSPC). Endocannabinoids have been reported as positive or negative factors in hematopoietic cell migration and differentiation (Randall 2007; Song and Zhong 2000; Miller and Stella 2008; Shoemaker et al. 2005; He et al. 2007).

The 2 major endocannabinoids AEA and 2AG, whose structural differences lie in the nature of the end-group alone, act in opposite directions, by reducing or enhancing, respectively, bone marrow cell migration. These effects are more or less independent of CBR, as for AEA (Patinkin et al. 2008). Both AEA and 2AG stimulation lead to an approximate doubling of colony forming unit: granulocyte, erythrocyte, macrophage, megakaryocyte (CFU-GEMM) colonies. The effect of AEA is considerably more potent than that of 2-AG (Patinkin et al. 2008). Very recently, it was demonstrated that CB2R mediates the retention of immature B cells in bone marrow sinusoids (Pereira et al. 2009). New data suggest that physiological levels of endocannabinoids are important for retention of HSPC in the BM niches (Jiang et al. 2011). CB1R and CB2R are expressed in human and murine HSPCs and bone marrow stromal cells express endocannabinoids, (AEA and 2AG). The agonist stimulation of CB2R, migration and enhanced colony formation of bone marrow cells were induced, mediated via ERK, PI3-kinase and Gαi-Rac1 pathways. Moreover, CB2R agonist AM1241 induced in vivo mobilization of murine HSPCs with short- and long-term repopulating abilities. A similar observation was made for CB1R, where the administration of exogenous CB1R agonists in vivo induced

chemotaxis, migration, and mobilization of human and murine HSPCs (Jiang et al. 2010). G-CSF-induced mobilization in vivo was modulated by endocannabinoids and was inhibited by specific CB antagonists. The mechanisms for endocannabinoid-mediated mobilization of HSPCs could be either changes in the expression and secretion of inflammatory cytokines in the BM niches, or activation of CXCR4 signalling and/or changes in the interactions of HSPCs with bone marrow stroma niches via integrins. Therefore, it was proposed that the endocannabinoid system regulates HSPC interactions with bone marrow niches, where endocannabinoids are expressed in HSC niches and under stress conditions, endocannabinoid levels are enhanced to induce HSPC migration for hematopoiesis (Jiang et al. 2010). Thus, CBR agonists may be therapeutically applied in clinical conditions, such as bone marrow transplantation (Jiang et al. 2011).

B cells and humoral immunity. CB compounds may affect B cell number, proliferation, migration, Ig production or isotype switching (Croxford and Yamamura 2005). In mice, 2-AG preferentially attracts unstimulated naive B cells, thus probably influencing the structure of B cell compartments in secondary lymphoid tissues (Tanikawa et al. 2007). B cells, IgG and IgM, and some complement proteins are decreased in bhang users (El-Gohary and Eid 2004) and antibody production in smokers' blood is differentially influenced by CB ingestion (Rachelefsky et al. 1976; Nahas and Osserman 1991). Also, antibody production is suppressed in splenocyte cultures by either synthetic or plant CBR ligands, possibly via a G-protein-coupled receptor mechanism (Kaminski et al. 1994). In ovalbumin-sensitized mice, CBD suppression of humoral immunity is due to impaired function of splenocytes (Jan et al. 2007). Recently, it was shown that both D9-THC and AEA induce dose-related suppression in primary and secondary in vitro plaque-forming assays of antibody formation, via CB2R (Eisenstein et al. 2007). B cell proliferation and migration can be differentially influenced by CB ligands, in a concentration-dependent manner – a biphasic effect similar to the one seen in T cell studies, with low doses acting as proliferation inducers, and in a class specific way – synthetic and phytocannabinoids being inhibitory, and endocannabinoids having positive effects (Croxford and Yamamura 2005). Interestingly, D9-THC can suppress significantly via CBR in mice the humoral immune responses involving CD40 at low micromolar concentrations, thus raising intriguing questions about why relatively high concentrations of CB are required to suppress in vitro immune responses (Springs et al. 2008). This phenomenon may be related, at least in part, to the lipophilic properties of CB ligands, which promote nonspecific binding with serum lipids and proteins.

CB2R pathway seems to be involved in some of the CB influence on B cell migration and differentiation (Jordà et al. 2002; Ziring et al. 2006). CB2R receptors can mediate B cell shift from IgM to IgE, thus contributing to the Th2 bias (Agudelo et al. 2008). It is suggested that endocannabinoids play a positive role in mobilizing B cells during immune responses, but CB effects on B cells are, at least in part, indirectly mediated through macrophages and T cells required for B cell activation (Croxford and Yamamura 2005). Moreover, the CB impact on serum Ig titres can be also via the profile of T helper-derived cytokines.

Non-CBR-mediated pathways seem to be involved as well in CB antioxidant actions which modulate cell survival and growth of B lymphocytes and fibroblasts (Chen and Buck 2000).

Human peripheral blood B cells express one CB2R transcript while mouse splenic B cells express three CB2R transcripts, with specific transcript selection occurring during B cell activation by LPS (Sherwood et al. 2009). However, further research needs to be done to verify the individual core promoter elements that are important for CB2R transcription in mouse and human B cells and to determine the extent to which transcript selection changes during B cell activation.

Cytokines: The relation between the endocannabinoid system and cytokines is bidirectional, as CB can modulate cytokine secretion, which can in turn have particular effects on CBR (Jean-Gilles et al. 2010). Some aspects have been already presented (see above), and some aspects on cytokine modulation by CB in inflammation are discussed in Sect. 11.5. Some features are emphasized in this section. Firstly, psychoactive and non-psychoactive ligands have in vivo or in vitro effects on the production and function of a variety of cytokines through CBR-dependent and independent mechanisms (Klein et al. 2000). The endocannabinoid system modulates the cytokine network and related immune interactions. Synthetic low affinity ligands and phytocannabinoids inhibit TNF-α and other acute phase cytokines, but some of these ligands have also been shown, in some conditions, to increase the expression of TNF-α and other inflammatory cytokines and chemokines (Klein et al. 2000). Moreover, depending upon the model system, the CB effects on cytokines are often conflicting. Although the current compounds offer considerable information regarding the physiological roles of the endocannabinoid system, the lack of compounds selectively interfering with the synthesis of anandamide or with the MAG lipase-catalyzed breakdown of 2-AG has made the study of endocannabinoid effects difficult. To date, information suggests that CB inducea shift in cytokine expression profile from that of proinflammatory Th1 to that of anti-inflammatory Th2, as discussed above (Klein and Cabral 2006). Secondly, immune cells with modified cytokine pattern of secretion after CB treatment can also express various humoral mediators, thus increasing the complexity and reinforcing the bidirectionality of the relationship between CB and cytokines.

A small number of studies have looked at effects of cytokines on CBR. One study investigated the effect of TGF-β on CB2R. This cytokine inhibits the activation of monocyte and T-cell subsets while enhancing the production of immunoglobulin A and fibroblast growth factor (Halttunen and Maki 1999) TGF-β seem to actively regulate lymphocyte CB2R expression in an autocrine and paracrine manner. More specifically, TGF-β-regulated CB2R expression has been suggested to occur via a negative autocrine regulatory loop as observed in in vivo experiments (Gardner et al. 2002). IFN-γ, which is produced by Th1 cells and NK cells, increases CB2R mRNA and protein in rat macrophages (Carlisle et al. 2002). IFN-γ also increases the expression of microglial CB2R in animal models of both neuropathic pain and MS (Stella 2004; Racz et al. 2008; Maresz et al. 2005). CB2 upregulation in a chronic mouse model of MS is highly correlated with the production of pro-inflammatory cytokines (Loría et al. 2008). CB1R are similarly

up-regulated by Th2 cytokine IL-4 and by CB themselves in human T lymphocytes (Börner et al. 2007b, 2008). Our own studies have provided support for the evidence of regulation of the endocannabinoid system by cytokines. We investigated the regulation of CB1R and CB2R by various pro-inflammatory cytokines in T cells, other immune cell types, peripheral blood mononuclear cells (PBMC), and whole blood collected from healthy human subjects and patients with MS. Stimulation of these different cell populations with pro-inflammatory cytokines, especially TNF-α, significantly induced CB1R and CB2R mRNA and protein levels. An inhibitor of the transcription factor nuclear factor-kappa B (NFκB), partially blocked the induction of CB1R and CB2R by TNF-α in PBMC, indicating a role for NFκB in the regulation of the CBR by TNF-α (Jean-Gilles et al., unpublished observations).

Finally, cytokines may also affect the endocannabinoid system by regulating enzymes involved in endocannabinoid degradation. IL-10 and IL-4 stimulate the activity of FAAH whereas IFN-γ and IL-12 decrease FAAH activity and protein expression (Maccarrone et al. 2001a). Such different mechanisms appear to work together in mediating the anti-inflammatory and neuroprotective effects of the endocannabinoids.

11.4 CB Production by the Immune Cells: Spotlight on Functional Consequences

Uptake and degradation of endocannabinoids can occur in immune cells, as shown extensively for macrophages and leukocytes (Pestonjamasp and Burstein 1998; Bisogno et al. 1997; Di Marzo et al. 1996) These cells can synthesize AEA and PEA, as well as take up these endocannabinoid molecules, thereby offering possibilities towards regulation of peripheral endocannabinoid system in inflammation, vascular tone and other immune interactions (Pandey et al. 2009).

The CB production by CNS and peripheral immune cells must be understood as being part of an homeostatic immunomodulatory function, being overexpressed in states of infection or inflammatory aggression. This has direct implications for innate immunity. Activation of the inflammatory response to infection depends on the release of pro-inflammatory cytokines and chemokines. In addition to cytokines, various other metabolic products of immune cells have been implicated in the inflammatory response to infection. Among them, activated immune cells can produce and release arachidonic acid and other fatty acids or chemically similar metabolites such as AEA (Di Marzo et al. 1996). As-yet-uncharacterized endocannabinoid membrane transporters may be involved both in the release and in the subsequent uptake of endocannabinoids by neurons and glial cells (Klein 2005). Moreover, stimulation with LPS in vitro increases the production of AEA and 2-AG by macrophages, PBMC, DCs and rat platelets (Klein 2005; Varga et al. 1998). LPS-activated PBMC show reduced expression of FAAH (Maccarrone et al. 2001b). Human MC also take up AEA followed by its hydrolysis by FAAH. FAAH-

dependent regulation may constitute a way by which inflammatory responses are tightly controlled to avoid extensive tissue damage (Klein 2005).

Once released, endocannabinoids can act as chemotactic agents, inducing after recognition of an invading pathogen by cells involved in the innate immune response. These effects are added to those of cytokines and chemokines, triggering an influx of lymphoid and myeloid cells from the blood to the site of infection (Moser et al. 2004). This was demonstrated in vitro for 2-AG, which can attract mouse bone marrow-derived DC, human eosinophils and Raji B cells (Klein 2005). 2-AG can induce migration of myeloid leukemic cells, which overexpress CB2R (Jordà et al. 2004). The property of chemotaxis was reported for opioids (e.g. morphine) as well, which seem to share neuroimmune functions with CB (Klein 2005; Szabo et al. 2002).

LPS increases endocannabinoid levels significantly in bone marrow stromal human and murine cells (Jiang et al. 2011). Increased levels of AEA and 2-AG may further protect HSPCs from endotoxic shock and apoptosis and induce their migration from the blood marrow niches to the peripheral blood circulation following injury (Jiang et al. 2011).

In the CNS, endocannabinoids play an important role during neuronal damage and neuroinflammation (Pandey et al. 2009). 2-AG is found at 200-fold higher concentrations in brain tissue in such conditions, being produced in response to intracellular Ca2+ and stimulation of glutamate receptors. 2-AG is produced by microglial cells and astrocytes in response to ATP released by injured neuronal cells, by stimulation of purinergic receptors (Pandey et al. 2009). In turn, released 2-AG stimulates microglial proliferation via CB2R (Carrier et al. 2004). It has been suggested that endocannabinoids such as AEA and 2-AG are thus released by CNS tissue as a mechanism that controls and limits immune response in healthy and damaged brain (Pandey et al. 2009; Eljaschewitsch et al. 2006).

In conclusion, immune cells increase the production of endocannabinoids in response to activation by LPS and other stimuli. Released endocannabinoids can interact with immune cells or be involved in cellular migration by functioning as chemotactic agents. Additional studies are needed to determine the range of immune and microbial stimuli that induce endocannabinoid production and to further define the mechanisms that regulate these effects.

11.5 CB Involvement in Immune Mediated Disease and Perspective for Therapies

The endocannabinoid system exerts immunomodulatory functions. The final goal of endocannabinoid changes is to reinstate the normal biological equilibrium by up-regulating its components in response to injury. This explains the marked increase of endocannabinoid production reported in various tissues (myocardial, cerebral, hepatic and hematopoietic system cells such as platelets, bone marrow cells and activated macrophages (Randall 2007)), which correlated with the degree of tissue injury and inflammation. CB exert their immunosuppressive properties in five main

ways: inhibition of cell proliferation, inhibition of cytokine and chemokine production, inhibition of bone-marrow-derived myeloid cell recruitment, induction of regulatory T cells and induction of apoptosis.

However, the picture is incompletely elucidated. Taking the brain for example, the level of expression of CBR or of enzymes controlling endocannabinoid levels undergo time- and brain region-specific changes during neurodegenerative and neuroinflammatory disorders, in the attempt to counteract excitotoxicity and inflammation. However, it is not clear if in neuroimmune diseases involving the brain, the endocannabinoid system activity is disturbed, increased adaptively or just not sufficiently potent in controlling the neuro-immune network. Nevertheless, by studying endocannabinoid system in disease, its dysregulation observed in certain settings may offer possibilities for therapeutic strategies.

To summarize the pathophysiological implications of endocannabinoids in immune mediated disease, we outline below CB actions in inflammation, then present several pathological conditions with immune dysregulation in which the implication of the endocannabinoid system, may provide opportunities for therapies; finally we discuss on the brain effects of CB as a paradigm for their potential dual actions: neuroimunomodulation resulting in neuroprotection versus neurotoxicity.

11.5.1 Inflammation: CB Modulates Cytokine Production and Migration of Inflammatory Cells, and Induces Immunosuppression by Apoptosis

CB can suppress the production of cytokines in innate and adaptive immune responses, in animal models and human cell cultures (Klein 2005). CB are able to inhibit the production of TNF and other cytokines in several different models and by several different mechanisms, some independent of CBR. However, in vivo, CB might either suppress or enhance the production of pro-inflammatory agents, depending on either the type of CB used or on the nature of the pro-inflammatory stimulus (Klein 2005). Consistent with this, in mice primed by infection with *Corynebacterium parvum* and injected with LPS, then treated with the synthetic CB HU-210 (a D9-THC derivative), TNF and IL-12 were both decreased in serum, while IL-10 was increased, exhibiting a probable protective role against the lethal effects of LPS (Smith et al. 2000).

In rats with closed head injury, treatment with the CB HU-211 was followed by suppression of TNF production in the brain, independently of CBR, but acting via NMDA receptors, thereby preventing excitotoxicity and neuronal death (Shohami et al. 1997). In another model of mouse myocardial ischaemia–reperfusion injury, treatment with CB agonist WIN55,212-2 decreased tissue damage while decreasing the levels of IL-1 and CXCL-8 in the injured tissue (Di Filippo et al. 2004). On the other hand, it has been shown that CB can increase the production of TNF, IL-1, IL-6 and IL-10 when administered alone or together with bacteria or other antigens

(Smith et al. 2001; Klein et al. 1993). Recently, protective effects of CBD in a rat model of cardiac ischemia were also described (Walsh et al. 2010).

In inflammation, the chemotactic effects of CB described earlier may be influenced by the overall orchestration of inflammatory reactions (Klein 2005). In EAE, the effects of the CB agonist WIN55,212-2 in suppressing disease progression and inflammatory reactions were associated with CB2R-dependent inhibition of rolling and adhesion of venous leukocytes (Ni et al. 2004). These seemingly paradoxical effects on chemotaxis, opposite to those described in Sect. 11.4 of this chapter, might result from an associated inhibition of IFN-γ, which facilitates transendothelial cell trafficking (Klein 2005).

The above examples give us a glimpse of the complexity of CB-mediated immune modulation in inflammation. A simplistic view on the therapeutic implications may assume that compounds that function either by binding CB2R or by CBR-independent mechanisms would be of benefit as anti-inflammatory drugs, since CB1R-related psychoactive side-effects would be by-passed. However, endocannabinoid actions are far more complex, and this simple approach would imply the assumption that psychoactive effects of CB are strictly CB1R mediated; that CBR independent effects are necessarily positive [this may not always be the case, for example CB can inhibit mitochondrial function with potentially deleterious consequences (Athanasiou et al. 2007a)]; and that CBR dependent and independent effects occur at similar concentrations of CB. Nevertheless, the selective suppression of Th1 and possibly Th17 immunity by CB drugs supports their potential use in the treatment of chronic inflammatory diseases.

Another way for alleviating inflammatory responses and protecting the host from acute and chronic inflammation is apoptosis. It has been suggested that the endocannabinoid system has the property of hormesis: a process whereby low-level stress induces resistance to that stress (preconditioning). These properties have been recently reviewed (Rieder et al. 2010; Nunn et al. 2010). The endocannabinoid network could thus be viewed as an endohormetic signalling system: damage to membranes releases endocannabinoids that have both local and remote effects by transmitting information about stress via redox modulation. In this light, apoptosis may be a context-driven protection: CB will protect or induce apoptosis of individual cells, depending on disposability. At optimal concentrations, CB may induce apoptosis in immune cells, and thus have a beneficial effect when there is a need for immune modulation (Rieder et al. 2010). The cumulative effect of CB on all cell populations of the immune system can regulate inflammatory states, by mediating the balance between proliferation and apoptosis. In conditions where disease is caused by activated immune cells, like MS, lupus, arthritis or septic shock, targeting immune cells via CB2R agonists may trigger apoptosis and anti-inflammatory effects (Rieder et al. 2010). However, in other instances such as in patients with breast cancer in which CBR may not be expressed by the cancer cells, CB may worsen the disease, since the immune system is weakened and the breast cancer cells are resistant to CB-induced apoptosis. It is then critical to balance the immunosuppressive actions with the anti-cancer effects. This requires dose–response studies on these outcomes (Rieder et al. 2010).

11.5.2 Stress: CB Regulation of Hypothalamic-Pituitary-Adrenal (HPA) Axis

Finally, the endocannabinoid system can interact with the immune system via the HPA axis (Tasker 2004). This may be another way in which CB modulates immune responses, protecting from exaggerated glucocorticoid effects on immune cells. Endogenous CB signalling is essential for stress adaptation, and differential regulation of AEA and 2-AG are associated with distinct HPA axis habituation (Hill et al. 2010). CB signalling constrains HPA axis activity, facilitate adaptation or habituation of HPA axis and behavioural responses to stress, reduce anxiety- and depressive-like behaviour and mediate analgesic responses to unconditioned or conditioned stress (Patel et al. 2004; Finn 2010). Lack of CB1R produces HPA axis dysregulation and exacerbates stress-induced excitotoxic and neuroinflammatory responses (Zoppi et al. 2010). Stress-induced suppression of endocannabinoid signalling in amygdala contributes to HPA axis activation (Hill et al. 2009).

Some considerations, albeit speculative, can be made on the possible role played by the immune system in endocannabinoid-mediated regulation of stress responses. The endocannabinoid system modulates the function of all of the major types of immune cells, in CNS and periphery. These cells release a range of chemokines and cytokines, which allow for bidirectional communication between the brain and immune system. Evidence suggests that cytokines directly modulate HPA axis activity (Mastorakos and Ilias 2006; Jara et al. 2006; Dunn 2000). Moreover, several studies demonstrated a role for the endocannabinoid system in regulating peripheral and brain cytokine responses to immune stress in vivo (Smith et al. 2000, 2001; Roche et al. 2006, 2008). It has, therefore, been hypothesized that modulation of cytokine signalling may mediate both the effects of endocannabinoids on HPA axis and behavioural reactions to stress (e.g. anxiety, despair, analgesia) (Finn 2010). This mechanism may be added to modulation of classical neurotransmitters or neuropeptides and has been suggested as for other psychotropic drugs like antidepressants (Leonard 2006; Griebel et al. 2005; Craddock and Thomas 2006). Supporting this idea, involvement of CB2R – present in the immune system but also on glia and neurons – has been linked to psycho-behavioural conditions such as anxiety- and depression-related behaviour or stress responses (Onaivi et al. 2006b, 2008). Further study is needed to clarify in what manner the CBR-related effects may represent a link between modulation of anxiety-, depression, or pain-related behaviour and alterations in cytokines and neuroimmune signalling (Finn 2010).

11.5.3 Diseases with Immune Involvement and Implication of CBs

1. *Multiple sclerosis:* MS is a neuroinflammatory and neurodegenerative disease. Although primarily used for control of symptoms such as spasticity and pain in MS patients, CB have the potential to exert both immunomodulatory and neuroprotective effects, as suggested by animal studies. EAE is a CD4+

T lymphocyte-mediated autoimmune disease that results from induction of primed myelin epitope-specific lymphocytes and serves as an animal model of MS (Kubajewska and Constantinescu 2010). In EAE, immunomodulation by CB was associated with reduced myelin-specific T cell responses and reduced clinical disease (Croxford et al. 2008). This implies indirect mechanisms by CB1R nerve signalling pathways controlling the systemic release of immunomodulatory molecules, and direct actions by CB2R-mediated inhibition of macrophages, microglia and lymphocyte function (Baker et al. 2007). In clinical practice, however, the relevance of these actions is unclear, since these effects only occur at high doses. On the other hand, the expression of both CBR and its upregulation by inflammatory cytokines on immune cells appears to be higher in MS than in its murine counterpart, and thus immunomodulatory effects of CB at therapeutically used current doses are not excluded. Moreover, it is suggested that lower doses of CB, non-immunosuppressive, can slow the accumulation of axonal loss and disability, acting on the glial response implicated in the neurodegenerative component of the disease. Also, potentiation of the endogenous CB signalling could be a substitute to the use of exogenously administered CB (Loría et al. 2008).

CB may have different effects in function of the phase of the disease. Very recently, it was shown that D9THC, CBD and non-psychoactive flavonoids from Cannabis sativa, may exert heterogeneous effects on chronic relapsing EAE – induced motor deficits, depending of the type of the extract and the moment of administration (Buccellato et al. 2011).

Therefore, CB can influence both pathological aspects of MS, neuroinflammation and neurodegeneration. CB2R activation can exert an anti-inflammatory effect by inhibiting the production of proinflammatory cytokines in microglial cells and by directly suppressing T-cell effectors. CB1R-mediated immunomodulatory effects, as well as CB2R mediated neurobiological effects, are also possible. The stimulation of CB1R located on presynaptic glutamatergic nerve terminals leads to inhibition of glutamate release, limiting excitotoxic damage and thus exerting a direct neuroprotective effect (Rossi et al. 2010). A role for postsynaptic CB1R signaling cannot be ruled out, since CB1R activation blocks the TNFα-induced increase in surface AMPA receptors and protects hippocampal neurons from excitotoxicity (Zhao et al. 2010). Moreover, pharmacological inhibition of endocannabinoid uptake can protect specifically against AMPA-induced excitotoxicity by enhancing the endocannabinoid tone and activating CBR as well as PPARγ (Loría et al. 2010). Other anti-neurodegenerative actions of CB can target mitochondrial dysfunction and Ca++ dysregulation occurring under pathological conditions (Ryan et al. 2009). Moreover, CB allows initiation of repair mechanisms, including the development of synaptic plasticity (Hashimotodani et al. 2007; Kano et al. 2009).

The endocannabinoid system is altered in MS, but the results of studies on these changes are controversial. CB ligands were found to have either increased or decreased levels. We found altered endocannabinoid levels in the blood of MS patients, differing between MS subtypes or when compared to normals, thus

suggesting that the endocannabinoid system may be dynamically modulated depending on the subtype of the disease (Jean-Gilles et al. 2009).

A unitary concept of CB changes in MS and EAE is lacking, one reason being the difficulty to point the time window of the changes on endocannabinoid levels in accord to the evolution and progression of these chronic inflammatory diseases. Different analytical methods for measurements of endocannabinoids may contribute as well to the heterogeneity of the results. However, selective glial expression of CBR and FAAH is induced in MS, thus supporting a role for the endocannabinoid system in the pathogenesis and/or evolution of this disease (Rossi et al. 2010; Benito et al. 2007).

2. *Atherosclerosis*: A growing body of evidence suggests that endocannabinoid signalling plays a critical role in modulating atherogenesis and its clinical manifestations (Steffens et al. 2005; Mach and Steffens 2008). CB2R activation by D9-THC inhibits atherosclerotic plaque progression in mice by inhibiting macrophage recruitment and anandamide inhibits inflammatory gene expression in endothelial cells, and consequently monocyte adhesion (Mach and Steffens 2008). CB2 may influence atherosclerosis by modulating lesional macrophage apoptosis (Freeman-Anderson et al. 2008). Endocannabinoids might also mediate pro-atherosclerotic effects by inducing platelet activation (Mach and Steffens 2008). Recently, it was demonstrated that 2-AG, PEA and OAE levels are altered in the aorta and visceral adipose tissue in a mouse model of atherosclerosis. Some of these alterations were suggested to be related specifically to the formation of atherosclerotic plaques (Montecucco et al. 2009). It was thus suggested that, since antagonists are expected to be efficacious in the presence of elevated endogenous ligands for the receptors they target, the increase of endocannabinoids in the atherosclerotic plaque may provide a molecular mechanism for the plaque reducing effect of a CB1R antagonist reported in another model of atherosclerosis (Dol-Gleizes et al. 2009). Further understanding of whether increased endocannabinoid signalling is associated with disease progression and increased risk of acute thrombotic events may result in novel pharmacological approaches to atherosclerosis.
3. *Rheumatic disease*: The CBR may become therapeutic targets for the treatment of pain and inflammation associated with ostheoarthritis (OA) and rheumatoid arthritis (RA). The basis of this approach could be the reduction in Th1 immunity, or triggering the articular CB system. This has been demonstrated in an experimental model of arthritis, where CBD had anti-arthritis effects (Malfait et al. 2000) and in patients with RA where the drug combination of D9-THC and CBD reduced disease activity (Blake et al. 2006). Non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit cyclooxygenase, have been shown to inhibit FAAH, thus interfering with the synthesis of endocannabinoids (Fowler et al. 2003). CB1R and CB2R, AEA and 2-AG are present in the synovia of patients with OA and RA, whereas PEA levels are higher in the synovial fluid of normal volunteers (Richardson et al. 2008). This suggests that the loss of PEA may contribute to arthritis and supports the role of the endocannabinoid system in these conditions. Also, CB1R and TRPV-1 seem to be important targets in

controlling OA pain (Schuelert and McDougall 2008). The attenuation of CB2R-mediated vasodilatation in acute and chronically inflamed rat joints suggests an alteration in CB2R expression or sensitivity following an arthritic insult (McDougall et al. 2008).

The CB ajulemic acid has several effects that make it attractive for future therapies in RA, systemic lupus erythematosus and osteoporosis. It suppresses macrophage IL-6 (Parker et al. 2008), inhibits osteoclastogenesis in mononuclear precursor cells and induces apoptosis in mature osteoclast-like cells (George et al. 2008).

4. *Inflammatory bowel disease (IBD)*: Both CBR are found on B cell, NK cells and MC, which are involved in immune surveillance of the gut (Klein and Cabral 2006). In vitro studies showed that endocannabinoid signalling (i.e. AEA, CBR protein expression) is increased in the inflamed intestine (Izzo and Sharkey 2010). Moreover, preclinical experiments in humans show enhanced endocannabinoid levels and/or CBR expression in intestinal biopsies of patients with ulcerative colitis, Crohn's disease, diverticulitis and coeliac disease (D'Argenio et al. 2006; Wright et al. 2008). Current data highlight the importance of both CBR in modulating inflammatory processes: CB1R promotes epithelial wound healing (Wright et al. 2005) and CB2R inhibits IL-8 release in human colonic epithelial cells, which are involved in the maintenance of intestinal immune homeostasis (Ihenetu et al. 2003). Endocannabinoids may limit intestinal inflammation via CBR, as shown in rodent models of IBD (Smid 2008). While genetic ablation of CBR or treatment with a CBR antagonist, rendered mice more sensitive to induced colitis (Massa et al. 2004), CBR agonists reduced experimental intestinal inflammation (Storr et al. 2008). Moreover, FAAH-deficient mice, which higher levels of AEA, showed significant protection against induced intestinal inflammation (Massa et al. 2004), while increased intestinal AEA levels by AEA reuptake or hydrolysis inhibitors reduced colonic inflammation in wild-type, but not in CBR-deficient mice (D'Argenio et al. 2006; Storr et al. 2009).

   The CB effect on gastro-intestinal motility in sepsis, viewed as interplay between inflammation, immune system and neuronal pathways, has recently been rewieved (De Winter and De Man 2010). Both CBR have been shown to play a role in motility in pathophysiological inflammatory conditions (Izzo and Sharkey 2010). Septic ileus in mice is associated with upregulation of intestinal CB1R but not CB2R and increase in FAAH. CBD decreases LPS-induced motility disturbances in vivo (De Filippis et al. 2008c). More recently, it was shown that LPS-induced sepsis in mice results in hyperactivation of glial cells, an increase in intestinal MC, macrophages and TNFα in the intestine. These effects are abrogated by CBD treatment and associated with a decrease in expression S100B (a marker for glial cell proliferation) (De Filippis et al. 2009). In summary, research performed on isolated epithelial cells and in vivo shows that the endocannabinoid system mediates protective effects in the inflamed gut, via CB1R and/or CB2R activation through suppression of inflammatory

mediators, intestinal motility and diarrhoea, and attenuation of visceral sensitivity (Izzo and Camilleri 2009).

5. *Transplantation*: Targeting CBR and understanding the role and use of CB in experimental models of allograft rejection may provide an exciting new approach with significant translational impact. CB may suppress the immune reaction, specifically T cell-mediated, against the engrafted organ. Future data on tolerance to allografts in $FAAH^{-/-}$ mice, on levels of endocannabinoid modulation during allograft rejection and endocannabinoid roles in the function of endothelial cells at allograft sites will help clarify the involvement of the endocannabinoid system in allograft rejection (Nagarkatti et al. 2010).
6. *Diabetes and lipid metabolism*: In diabetes, CB may protect against islet destruction by suppressing insulitis and IFN-γ, TNFα and IL-12 mRNA expression (Li et al. 2001), but also treating neuropathic pain in diabetic patients mainly via CB2R pathway (Toth et al. 2010). Rimonabant (SR141716), the CB1R-selective inverse agonist of CBR, can inhibit adipocyte function and was used in the treatment of obesity. However it has psychiatric side-effects. (Van Diepen et al. 2008).
7. *Liver disease*: Exogenous or endogenous CB, targeting CBR and the use of FAAH inhibitors may become therapeutic modalities for immune-mediated liver inflammation (Hegde et al. 2008), hepatic fibrosis and hepatic neoplastic disease (Izzo and Camilleri 2008). In a murine model of ConA-induced hepatitis, D9-THC upon binding to CBR on immune cells, inducse apoptosis in effector T cells, up-regulates Treg function, and suppresses inflammatory cytokines thus preventing ConA-induced T-cell-mediated liver injury. AEA ameliorates ConA-induced hepatitis, while FAAH reduction increases resistance to the disease (Hegde et al. 2008).
8. *Allergic asthma*: CB may be beneficial in asthma, by ameliorating cytokine profiles, decreasing overproduction of mucus in the lungs and by playing a role in bronchodilation (Croxford and Yamamura 2005).

11.5.4 CB and the Brain: Neuroinflammation, Neuroprotection and Neurotoxicity

The brain is a special example for the complex interactions between immune and neuronal systems, and between anti-inflammatory, neuroprotective and neuroregenerative activities of the endocannabinoid system. Most of the published studies support the notion that endocannabinoids act as neuroprotective agents and that a loss of such a neuroprotective tonus facilitates neurodegeneration.

CB can also influence neurogenesis. Neural progenitor cell proliferation and differentiation depends on their intrinsic properties and local environment and is reduced in conditions associated with brain inflammation (Rossi et al. 2010). Conversely, newly-formed neurones can survive despite chronic inflammation and even specifically arise within an inflammatory environment. Since the endocannabinoid system controls immune responses and influences cell proliferation, fate decision and

cell survival in the CNS, brain CB might regulate neurogenesis, directly or indirectly via the immune system (Wolf and Ullrich 2008b).

In this light, apart from MS, other neurodegenerative diseases with immune connections may be targeted by therapeutic approaches in the future. For example, in Parkinson's disease (PD), CB might provide protection against the progression of neuronal injury and influence local inflammatory events associated with the characteristic pathogenesis of PD (Lastres-Becker and Fernández-Ruiz 2006). In Alzheimer's disease (AD), CB2R expression is strongly up-regulated, particularly in the microglial cells surrounding beta-amyloid plaques in human AD brain (Benito et al. 2003; Ramirez et al. 2005). The unifying hypothesis encompassing most of the studies on CB effects in AD is that changes in endocannabinoid levels and CB2R expression are induced by the inflammatory environment that occurs in AD. Consequently, CB2R activation by up-regulated endocannabinoids is an attempt to halt microglial activation, but this innate compensation is insufficient to prevent the inflammatory damage to neurons, which may also be more vulnerable due to CB1R down-regulation. Some pre-clinical data demonstrate that CB stimuli may have therapeutic benefit by augmenting the brain's innate response (Scotter et al. 2010).

The discussion above is based upon the assumption that endocannabinoids are by nature protective. This is a simplistic view. The efforts for development of novel CB neuroprotective drugs has produced compounds that show a promising profile in experimental animals but disappointing results in clinical settings. There is thus a need for caution in the interpretation of preclinical studies.

Several issues can explain this paradox. For acute conditions like stroke, the window of opportunity for CB treatment is often neglected. In chronic conditions like AD, an important determinant of clinical outcome is the degree to which the target receptors are functional. In AD there is an impaired G protein signaling, which would greatly impact upon the efficacy of compounds targeting the receptor pathways in question (Rossi et al. 2010).

CB may produce neurotoxic effects as well. For example, CB1R activation may have neurodegenerative effects in cortical neurons by inducing JNK and caspase-3 activation, increased Bax expression, and DNA fragmentation (Downer et al. 2003). In vivo data investigating both the potential for beneficial and harmful effects of modulators of the endocannabinoid system in models of neurodegeneration have been extensively reviewed recently. It is worth stating that these effects are difficult to separate from other actions of CB, and often coexist. While direct or indirect activation of CB1R on glutamatergic nerve terminals decreases excitotoxicity, direct or indirect activation of CB1R on GABAergic nerve terminals is detrimental, by decreasing GABA release and thus the inhibitory signaling in pathological excitotoxic conditions(Rossi et al. 2010). Moreover, additional effects secondary to the blockade of endocannabinoid metabolism via non-CBR by increased levels of AEA can be both detrimental (via TRPV1) and beneficial (via PPARα). As already mentioned, CB can influence mitochondrial metabolism, promoting apoptosis (Rossi et al. 2010). This can be beneficial or harmful, depending on the context. Also, selective activation of CB2R

to target inflammatory processes may be beneficial, but strictly related to a time window for treatment (Rossi et al. 2010).

The context-dependent effects of CB have different consequences on immune interactions. Endogenous CB are released following various types of injury to the brain. The "immune economy" is different depending on the type of injury (Tanasescu and Constantinescu 2010). Immune effects of CB will be different for inflammation, stroke or various infections, making it more difficult to predict the net impact of CBR activation on complex pathological events. Further study is necessary to clarify how and when to enhance the positive anti-inflammatory and tissue protective potential of CB, without deleterious effects.

The complexity of global CB actions and relations with the immune system is far more than the simplified paradigm of immunosuppression and CBR separation in 'brain versus immune'. Advances in the understanding of the interplay between this non-conventional neurotransmitter system and the immune network may provide the basis for future treatments for conditions insufficiently alleviated by current therapies.

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