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Volume 653

Target Pattern Recognition in Innate Immunity

Edited by
Uday Kishore

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DEDICATION

To Babuji and my teachers

FOREWORD

Target pattern recognition in innate immunity is responsible for the immediate, usually protective, responses shown against invading microorganisms, and it is the principal feature of self and non-self recognition by virtue of the recognition of structures on the microbial pathogens, which are not found on host cells. This is an area that has been very actively researched, over approximately the past 12 years, and therefore this volume provides a timely comprehensive, and up to date, summary of the types and range of cell surface, intracellular, and secreted, host proteins involved in the recognition of microbial products, and of the protective mechanisms triggered as a result of the recognition events.

The Toll-like receptors, first described in *Drosophila* and now well-characterised on human cells, provide an excellent demonstration of the wide range of different microbial products recognized by this family of receptors and of the signalling pathways which are triggered thus leading to induction of inflammatory cytokines and the activation of genes producing antimicrobial products. In addition, several cell surface proteins involved in target pattern recognition have been described on the surfaces of macrophages (macrophage mannose receptor and macrophage scavenger receptors), and on dendritic cells (DEC205), and to be involved with the uptake and clearance of whole microorganisms and polyanioic ligands. Pattern recognition is also utilised by intracellular receptors, with NOD-like receptors in the cytosol recognizing microbial molecules and activating the production of inflammatory cytokines or pathways that induce the production of inflammatory molecules. Secreted proteins, such as the pentraxins, which includes the acute phase reacting, C-reactive protein (CRP) and serum amyloid protein (SAP), and the collectins (mannan binding lectin, lung surfactant protein A and D) and ficolins can also readily recruit killing and clearance systems. Indeed, the serum complement system, which is one of the major defence systems in the bloodstream, is efficiently activated by CRP on its binding to the phosphocholine groups of microbial phospholipids—and the subsequent interaction of the bound CRP with C1q—to give classical pathway activation, or MBL, or ficolin, binding to arrays of mannose or N-acetyl-glucosamine residues, respectively, on the surfaces of microorganisms—to give lectin pathway activation. Also, in addition to the activation and clearance events associated with

complement activation by some of the secreted pattern recognition receptors, it is accepted that all these pattern recognition receptors can generally accelerate the uptake and clearance of microbes via phagocytic cells.

In view of the growing interest in the cross-talk between innate and adaptive immunity, a thorough understanding of the initial recognition and triggering events, mediated via innate immune receptors, as addressed in this volume, is clearly very useful in helping to also fully understand the mechanisms of activation and control of the adaptive immune system—and to allow a full assessment of the relative roles played by innate immunity and adaptive immunity against a particular infection in higher organisms.

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PREFACE

Pattern recognition in innate immunity has always fascinated me. The dramatic rise in the popularity and intensity of this area of research interestingly coincided with my time as a postdoctoral fellow at Oxford. When I was asked by Ron Landes from Landes Bioscience if I would be interested in editing a book on the title, I readily agreed. It is to the credit of Ron, Cynthia and their excellent team, and outstanding contributions by some brilliant scientists in the field, that this book is a reality.

The first chapter discusses the importance of the macrophage, its pattern recognition receptors and its relevance to innate immunity. The subsequent chapters have been loosely grouped as receptors and sensors (Toll-like receptors and NOD-like receptors) and soluble factors (collectins, pentraxins and complement). There are additional chapters that collectively address the molecular and structural organisations of these innate immune molecules. The role of target pattern recognition in the clearance of apoptotic cells has also been discussed, followed by *Drosophila* innate immune mechanisms as a model for studying innate immunity. Finally, two chapters discussing the involvement of innate immunity in dealing with two devastating pathogens, *Plasmodium* and Mycobacteria, have been included. The book chapters have been written by well established and respected scientists in their respective fields of research. It was a real delight to learn more about such an exciting field while editing such beautifully crafted chapters.

The field of innate immunity has expanded dramatically in last decade. Its impact and importance on human health and disease is being understood better than ever. The understanding of the ability of target pattern recognition molecules to modulate and link with adaptive immune processes via a range of mechanisms and pathways has been a genuine revelation. Not surprisingly, this aspect of Immunology is increasingly becoming a major component of medical and medicine-related curriculum. It is largely hoped that this book will be a useful start point for students, teachers and active researchers.

During the course of planning and organising this book, I have greatly benefitted from my interaction with two of my mentors (Ken Reid and Bob Sim), my colleagues (Britta Urban, Rohit Ghai, Trinad Chakraborty, Tony Tsolaki and Patrick Waters), collaborators (Alok Aggrawal and Taruna Madan), and a huge number of

my students. In fact, my undergraduate and postgraduate students have been a great source of inspiration to undertake this project. I would like to thank Ron Landes for providing me with this great opportunity to edit a book in the area that is so close to my heart.

I sincerely hope that the readers will find this book interesting, comprehensive and well organised.

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DR. UDAY KISHORE is currently a University Lecturer in Immunology and Medical Microbiology at Division of Biosciences within School of Health Sciences and Social Care, Brunel University, London, UK. He earned his BSc from S.P. Jain College, Sasaram, Bihar, India and MSc from Hindu College and Department of Zoology, Delhi University. He did his PhD on gene synthesis and expression of human EGF jointly from CSIR Institute of Genomics and Integrative Biology, Delhi and University of Delhi. He spent most of his postdoctoral time working at MRC Immunochemistry Unit and Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford funded by Wellcome Trust, Medical Research Council and European Commission. He has also been recipient of NASA fellowship (Salk Institute for Biological Studies, San Diego, CA, USA), Wellcome Trust International fellowship (Laboratory of Molecular Biophysics, Oxford), MRC Inventor's award, and Alexander von Humboldt fellowship (Institute of Medical Microbiology, Giessen, Germany (2004)).

Dr. Kishore's research interests include structure-function relationships within human C1q, the first subcomponent of classical complement pathway, role of hydrophilic lung surfactant proteins in modulating allergy and infection, host-pathogen interaction involving an opportunistic fungal pathogen, *Aspergillus fumigatus*, and generation of therapeutic inhibitors of complement cascade.

Dr. Kishore's hobbies include singing Indian classical and light music.

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CONTENTS

CHAPTER 1

Macrophage Pattern Recognition Receptors in Immunity, Homeostasis and Self Tolerance

Subhankar Mukhopadhyay, Annette Plüddemann and Siamon Gordon*

Abstract

Macrophages, a major component of innate immune defence, express a large repertoire of different classes of pattern recognition receptors and other surface antigens which determine the immunologic and homeostatic potential of these versatile cells. In the light of present knowledge of macrophage surface antigens, we discuss self versus nonself recognition, microbicidal effector functions and self tolerance in the innate immune system.

Introduction

The common basic features of any functional immune systems are: (i) ability to distinguish self tissues from microbial invaders (self vs nonself recognition), (ii) mount an appropriate effector response to kill or contain microbial infection, and perhaps most importantly, (iii) spare the host tissues from potentially hazardous effector responses (self tolerance). All known immune systems, however primitive or advanced, show these three basic characteristics.[1] However, the ability of the immune system to recognize and respond to foreign materials or tolerate self components is not absolute; these functions operate robustly within a given range, as normal physiology, beyond which dysfunction leads to immune pathology. Susceptibility to infection, immunopathology and autoimmunity are probably extreme examples in a spectrum of immune failure.

The molecular basis for immune recognition, response and tolerance is relatively well-studied in adaptive immunity of vertebrates, but such research was neglected in innate immunity until recently, despite the fact that most species rely solely on innate immunity to achieve these core immune functions. Innate immunity lacks most of the basic molecular machinery employed by the adaptive system such as somatically rearranged high affinity antigen receptors used by lymphocytes and thymic education of T cells for central tolerance, indicating the presence of a fundamentally different mechanism in innate immunity. The first major conceptual breakthrough came when Janeway proposed that cells of the innate immune system express a large repertoire of germ-line encoded receptors which recognize invariant molecular structures on pathogens, which are essential and unique to pathogens and not present in the host. He coined the term pathogen associated molecular patterns (PAMPs) for such molecular structures, recognised by pattern recognition receptors (PRR).[2,3] Since Janeway's proposal a large number of PRRs have been identified. Similar to adaptive Ag recognition receptors, innate PRRs can be defined as humoral and cell associated. Cellular

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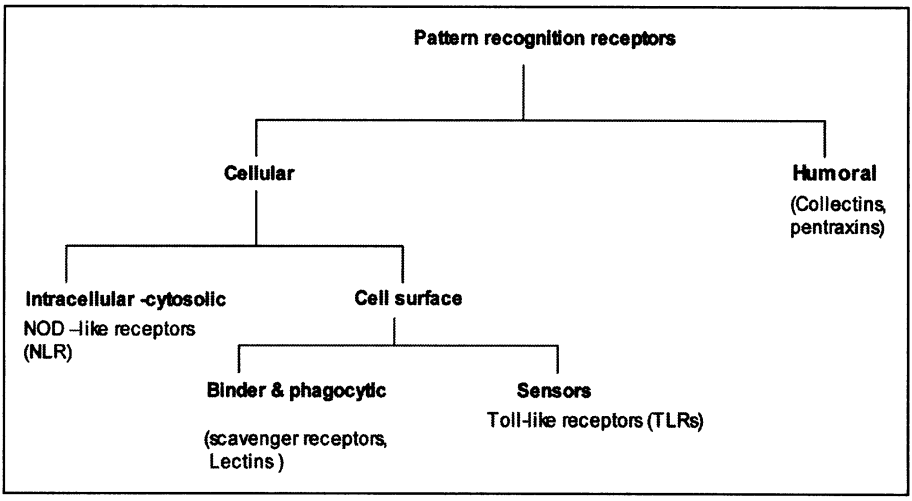

Figure 1. Schematic representation of different classes of pattern recognition receptors.

PRRs are further subdivided into intracellular or cell surface molecules. Humoral PRRs generally recognise pathogens from various body fluids and form aggregates which are subsequently cleared by phagocytes. Apart from this opsonising ability they also have many immunomodulatory properties. Cell surface PRRs are either phagocytic/endocytic or sensor in nature. Phagocytic receptors bind and internalise ligands directly and display temperature dependent, saturable and inhibitable ligand binding kinetics of classical receptors. On the other hand sensors do not bind or internalise ligand directly, but recognise PAMPs and induce a proinflammatory signalling cascade which leads to many antimicrobial effector responses. It is important to note that many intracellular PRRs are also sensing molecules.[4] A schematic diagram of different classes of PRRs and selected examples are presented in Figure 1. There is recent evidence that components of the cellular and humoral arms of the innate immune system collaborate to induce and maintain host defence.[5,6]

Although amendments have been recently proposed to the original concept of pattern recognition, this remains fundamental to our present understanding of innate immunity. "Molecular patterns" are not restricted to pathogens, but are also expressed by commensals; it has been argued that microbial ligands should be characterised molecularly, in preference to introducing the term PAMP.[7] Although not clearly stated, it could be extrapolated from Janeway's proposal that self tolerance is achieved in innate immunity through "ignorance" as PRRs only recognize microbial molecules. This is in contrast to adaptive immunity where tolerance is achieved by "education". This theory of ignorance is challenged by recent observations that many PRRs recognize modified host molecules (generated during normal or aberrant metabolism) as well as naturally occurring host molecules, other than microbial structures.[8] It is conceivable that during evolution multipurpose PRRs were selected to recognize microbial molecules to counter infectious challenges, modified host molecules for clearing, homeostasis and natural host molecules for immunomodulation, thus minimizing the genetic resources invested in immunity.

In this chapter we will discuss how PRRs differentially recognize microbial, modified host molecules as well as natural host molecules using examples from two major classes of PRRs, class A scavenger receptors and C type lectin families. We also speculate how tolerance is achieved in innate immunity.

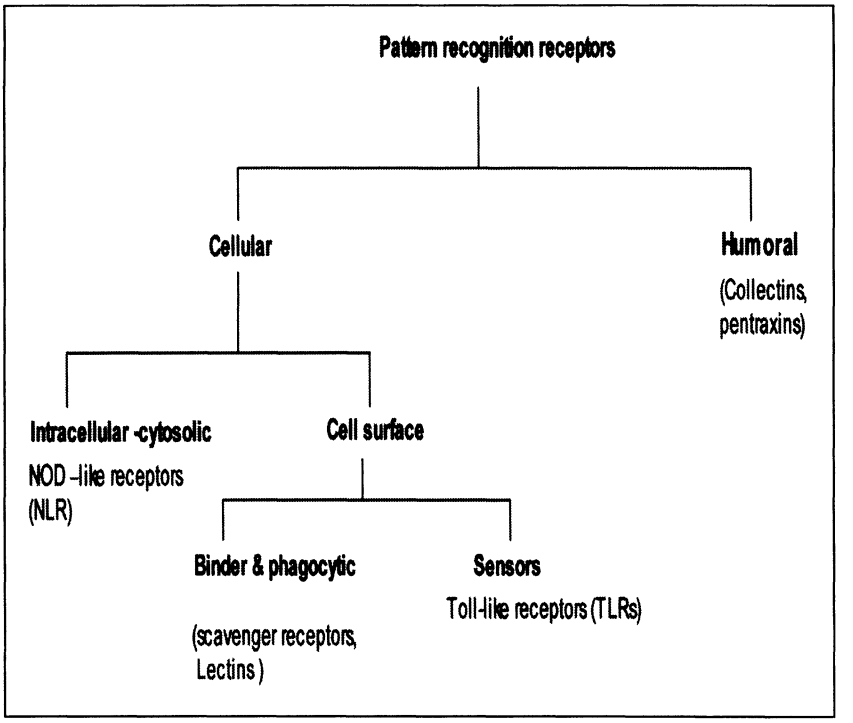

Figure 2. Different members of the class A scavenger receptor family. The scavenger receptor AI/II (SR-A), macrophage receptor with collgenous structure (MARCO) and scavenger receptor with C-type lectin-I/II (SRCL) all are three members of the class A SR family. Both SR-A and SRCL have two functional isforms, where the C terminus of SR-AII and SRCL-II are truncated. SR-A was the first member identified in this family, subsequently MARCO and SRCL were included in this family due to their structural similarities with SR-A. SR-A is a Type II trans-membrane glycoprotein which shows multi-domain protein structure with a short cytoplasmic tail, transmembrane region, followed by an extracellular spacer region, an α helical coiled coil region, a collagenous domain and a C terminal scavenger receptor cysteine-rich (SRCR) domain. Although MARCO and SRCL share a similar domain organization to that of SR-AI, the only differences are that MARCO lacks an α_helical coiled coil domain and possesses a longer collagenous domain; in the case of SRCL-I, the SRCR domain is replaced by a C-type lectin domain. Functionally, all three receptors show overlapping ligand binding properties and contribute to varied functions.

The Scavenger Receptor Family

The scavenger receptors were functionally defined by Brown and Goldstein for their ability to bind and internalise modified low density lipoprotein (mLDL) such as oxidised LDL (Ox-LDL), acetylated LDL (Ac-LDL), but not native LDL.[9] Subsequently a variety of artificial and natural polyanionic ligands including many micro-organisms and apoptotic cells were identified as ligands for SRs. After Brown and Goldstein's first proposal a large number of unrelated distinct gene products were identified which bind mLDL. Krieger and colleagues classified these molecules (classes A-F) according to their similarities in multi-domain protein structure.[10] Recently several new molecules have been identified and two new classes (G and H) added to this list to accommodate novel structural features, totalling 8 independent structural classes of SR which possess common functional criteria.[11] In this chapter we will restrict our discussion to class A SR family (Fig. 2 and refer to Table 1).

Table 1. Class A scavenger receptor recognition of endogenous and exogenous ligands

Receptor	Microbial Ligand	Refs	Endogenous Ligand	Refs	Modified Self Ligand	Refs
SR-A	LPS and LTA	15	Unidentified protein ligand in serum	25	β amyloid protein	23
			Activated B cells	26	Apoptotic cells	38
					Ox-LDL and Ac-LDL	9
					AGE modified protein	24
MARCO	LPS	39	UGRP-1	34	Ac-LDL	40
SRCL-I	G^+, G^- bacteria, Yeast	35,36	T and Tn antigen	37	Ox-LDL	35,36

Class A Scavenger Receptors

SR-A was the first molecule in this class to be cloned. Three alternatively spliced variants (SR-AI/II and III) of the same gene have been identified which are collectively called SR-A. Among these three splice variants SR-AIII is non functional and trapped in the endoplasmic reticulum. So far, no functional difference has been observed between SR-AI and II. SR-A is a Type II trimeric transmembrane glycoprotein molecule with a cytoplasmic tail, transmembrane region, followed by an extracellular spacer region, an α helical coiled coil region, a collagenous domain and a C terminal scavenger receptor cysteine-rich (SRCR) domain. The SRCR domain is absent in SR-AII and III. The collagenous domain is responsible for ligand binding, but the cytoplasmic tail is required for endocytosis and phagocytosis of ligand or adhesion to ligand-rich substrata.[12] Macrophage receptor with collagenous structure (MARCO) and scavenger receptor with C type lectin I (SRCL-I) are the other two members of the class A SR family which share a similar domain organization to that of SR-AI. The only differences are that MARCO lacks an α helical coiled coil domain and possesses a longer collagenous domain; in the case of SRCL-I, the SRCR domain is replaced by a C-type lectin domain. It has been proposed that similarly to SR-AI, SRCL-I binds Ox-LDL through its collagenous region, but exhibits additional sugar binding properties through the C-type lectin domain. In contrast, MARCO may recognise ligands through its SRCR region.[13,14]

SR-A (SR-AI/II)

The first evidence that SR-A can recognise nonself microbial components came with the observation that SR-A could bind the lipid-A portion of lipopolysaccharide (LPS) and lipoteichoic acid (LTA).[15] Furthermore, different LTA structures showed differential specificity depending on their exposed negative charge available to SR-A.[16] Another bacterial component CpG DNA, has also been reported to be recognised by SR-A, but its immunostimulatory effect is independent of SR-A.[17] Finally, Dunne et al confirmed that SR-A also binds intact Gram-positive and negative organisms.[18] In a direct binding assay, use of different strains of *Escherichia coli* and *Staphylococcus aureus* and primary macrophages (Mϕ) from wild type (WT) and SR-$A^{-/-}$ mice confirmed that the contribution of SR-A to bacterial recognition depended on the source of Mϕ and the strain of bacteria.[19] Several in vivo infection models showed that SR-$A^{-/-}$ animals are more susceptible to *Listeria monocytogenes* and *S. aureus* infection, underscoring the importance of SR-A in antibacterial host defence.[20,21] Screening of several pathogenic and nonpathogenic bacterial strains in an in vitro binding assay showed that SR-A contributes to the majority of *Neisserial* recognition by Mϕ, but the contribution is minimal to recognition of *Haemophilus sp*, indicating strain-specific recognition (Peiser & Gordon, unpublished observation). Furthermore, although LPS has been shown to be a ligand for SR-A, use of an LPS deficient mutant strain

confirmed that SR-A mediated recognition of *Neisseria meningitidis* is LPS-independent, indicating the presence of nonLPS ligands for SR-A.[22] Recently we have identified several surface proteins on *N. meningitidis* which are ligands for SR-A, providing evidence for unmodified protein ligands for SR-A (Peiser and Gordon submitted).

As mentioned earlier, SR-A was first identified by its ability to bind mLDL. Subsequently, several other modified self molecules were identified as SR-A ligands which are generated during normal or aberrant metabolism. SR-A recognises β amyloid proteins, a hallmark of Alzheimer's disease, and contributes to the inflammatory nature of this disease by recruitment and adhesion of Mϕ. Similarly, SR-A contributes to inflammatory pathology by recognising advanced glycation end products (AGE) generated during diabetes. In contrast, SR-A contributes to phagocytosis of apoptotic cells inducing a profound anti-inflammatory response.[23,24]

Fraser et al first described the presence of a natural ligand for SR-A in human and bovine serum which allows divalent cation-independent adhesion of Mϕ to tissue- culture plastic through SR-A.[25] Presently our group is involved in identifying the chemical nature and physiological role of this serum ligand. Similarly, another unidentified natural ligand for SR-A has been reported on activated B cells, but its physiological relevance is unknown.[26]

MARCO

Functional binding studies confirmed that SR-A and MARCO not only share very similar structural features, but also show similar ligand binding properties. Similarly to SR-A, MARCO also binds several Gram-positive and negative organisms or their isolated products such as LPS or CpG DNA.[27] Infectious challenge with the lung pathogen *Streptococcus pneumoniae* confirmed that MARCO$^{-/-}$ mice display an impaired ability to clear pneumococcal infection, resulting in increased pulmonary inflammation and reduced survival, confirming a role of MARCO in antibacterial protection.[28] Recently we have reported that like SR-A, MARCO also recognises *Neisseria* independent of LPS.[29] Furthermore, we identified several *Neisserial* surface proteins as potential nonLPS ligands for MARCO, distinct from those that bind SR-A (Mukhopadhyay & Gordon, unpublished observation). Other than microbial pathogens, MARCO also binds and protects the host from a range of nonself environmental pollutants such as TiO_2 and asbestos.[30] MARCO has been reported to bind modified self ligands such as Ox-LDL and Ac-LDL. MARCO expression is induced in murine atherosclerotic plaques, but its exact role in the pathogenesis of atherosclerosis remains to be determined.[31] The presence of natural or self ligand(s) for MARCO has been reported on subsets of splenic marginal zone B cells. Blockade of this cell-cell interaction confirmed that it is critical for development and retention of the marginal zone microarchitecture in rodent spleen.[32] Similarly, MARCO$^{-/-}$ animals showed defects in splenic microarchitecture, with significant immunological consequences.[33] Uteroglobin related protein -1 (UGRP-I), a secreted protein expressed by lung clara cells, has been shown to be another endogenous ligand for MARCO. UGRP-I also binds to bacteria and therefore may act as an opsonin which increases MARCO-mediated clearance of bacteria.[34]

SRCL-I/ CLP-I

Recently two groups independently cloned a novel member of the class A SR family from a human placental cDNA library, designated as SRCL-I (scavenger receptor with C-type lectin) and CLP-I (Collectin from placenta-1), respectively. As mentioned earlier, this molecule differs from SR-AI by its C terminal C-type lectin domain in place of a SRCR domain. One group also identified a C terminal truncated form of this molecule which lacks the C-type lectin domain and displays a very similar structure to that of SR-AII. These studies showed that SRCL-I transfectants bind Gram-positive and negative bacteria, as well as yeast and Ox-LDL in a polyanion sensitive manner. Fungal recognition by SRCL-I is not observed with other class A SR family molecules and possibly occurs through the lectin domain.[35,36] The C-type lectin domain showed specificity for GalNac type glycoconjugates which is inhibitable by free GalNac, L-D-fucose and D-galactose. SRCL-I has also been shown to bind T and Tn antigens, two carcinoma associated autoantigens which display distinct modified glycosylation.[37]

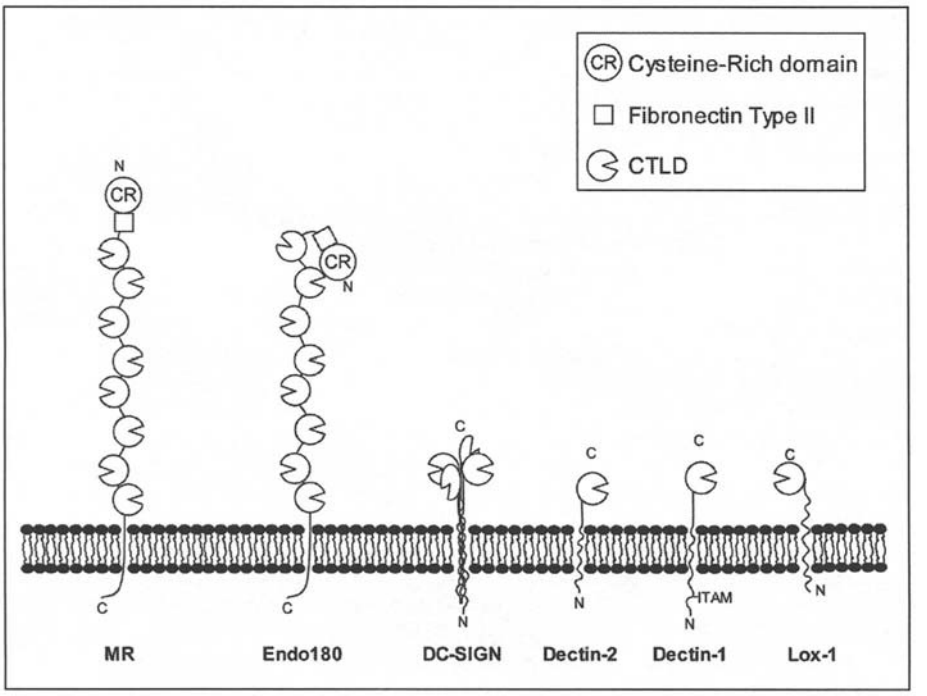

Figure 3. C-type lectin receptors. The C-type lectin receptor (CLR) family is made up of a wide range of receptors that are defined in part by their ability to bind carbohydrate molecules. Some C-type lectins contain multiple lectin domains (Mannose receptor and Endo180) while others contain a single lectin domain (Lox-1, Dectin-1). In the case of Lox-1 and Dectin-1, the lectin domain is called NK like C-type lectin like (NKCL) domain, the major difference between NKCL and the classical C-type lectin domain is the sugar binding in the former is calcium independent. The mannose receptor and Endo180 is expressed by most macrophages, dendritic cells (DCs), tracheal smooth muscle cells and endothelial cells. Endo180 is predominantly expressed by fibroblasts, endothelial cells and macrophages. DC-SIGN is highly expressed by mature and immature DCs and by subpopulations of macrophages in the lung and placenta. Dectin-1 and Dectin-2 are predominantly expressed on myeloid cells, including macrophages, DCs and neutrophils. Lox-1 is expressed on vascular endothelial cells, smooth muscle cells, fibroblasts, platelets and macrophages.[4,92]

C-Type Lectin Receptors

The C-type lectin receptor (CLR) family is made up of a wide range of receptors that are defined in part by their ability to bind carbohydrate molecules (Fig. 3).[4] They can be divided into three groups, (1) the C-type lectins containing a single carbohydrate recognition domain (CRD), (2) the C-type lectins containing multiple CRDs and (3) the NK-like C-type lectin-like receptors (NKCL) which have a single CRD. The classical C-type lectins require calcium for binding, however the NKCL receptors differ from the other two groups in that their C-type lectin domains (CTLD) lack the residues involved in calcium binding. Members of the C-type lectins with a single CRD are Type II membrane receptors and include DC-SIGN and Dectin-2. C-type lectins containing multiple CRDs are Type I membrane receptors and include the mannose receptor (MR), Endo180, DEC-205 and the phospholipase A_2 receptor.[41] The Type II membrane receptors termed NK-like C-type lectin-like receptors include Dectin-1, CD69 and LOX-1 (reviewed in ref. 4). Most of these receptors recognise both endogenous and exogenous molecules, self and nonself ligands. A few examples of CLRs and their binding properties will be discussed in the following section.

C-Type Lectins with a Single CRD

This group of receptors is made up of DC-SIGN and related molecules. DC-SIGN (CD209) was originally described to be involved in the adhesion of T-cells to dendritic cells via the intercellular adhesion molecule 3 (ICAM-3) and therefore the receptor was designated dendritic cell-specific ICAM-grabbing nonintegrin.[42] The receptor has since also been shown to play a role in DC migration via ICAM-2, a molecule that is highly expressed on vascular and lymphoid endothelium.[43] DC-SIGN is a tetrameric endocytic receptor consisting of a single CTDL, a stalk region, a transmembrane domain and a cytoplasmic tail containing an internalisation motif.[4] It generally recognises *N*-linked high mannose structures as well as fucose-containing glycans, but also discriminates between ligands on the basis of secondary binding sites.[44] The key to selective interaction with pathogens may be in the close proximity of the four CRDs which bind closely spaced glycans, as clusters of either mannose-type or fucose-type ligands are not common in endogenous molecules. This receptor has been shown to be involved in the recognition of various pathogens including viruses such as HIV-1, HCMV, Hepatitis C, Dengue and Ebola, as well as *Mycobacterium tuberculosis, Candida albicans, Leishmania mexicana, Helicobacter pylori* and *Schistosoma mansoni* (Table 2).[45,46] The carbohydrate ligand on *H. pylori* and *S. mansoni* was found to be the nonsialylated Lewis^x antigen.[45,47] Dengue virus that infects dendritic cells has been shown to utilise DC-SIGN for enhanced cell entry.[48] Interestingly, an association between a variant in the *CD209* promoter and severity of dengue disease was shown recently.[49] In some cases the pathogens modulate the immune response by binding to DC-SIGN and examples of this are HIV-1 and *M. tuberculosis*.[50] HIV-1 exploits the antigen presenting properties of DC-SIGN to gain access to T-cells that are the primary target of the virus. The binding of mycobacterial lipoarabinomannan to DC-SIGN blocks DC maturation and triggers inhibitory signals that allow this bacterium to evade the immune response.[51] Dendritic cells play a pivotal role in antitumor responses where DC-SIGN interacts with colorectal cancer cells via Lewis^x carbohydrate moieties on carcinoembryonic antigen produced by the cancer cells during malignant transformation.[52] Recently it has been shown that DCs interact with neutrophils via DC-SIGN-mediated binding to the nonsialylated Lewis^x antigen on Mac-1 of these cells.[53] Since activated neutrophils induce DC maturation which in turn triggers the T-cell response, this indicates that neutrophils may contribute to adaptive immune responses via their interaction with DCs, providing a cellular link between innate and adaptive immunity.

C-Type Lectins with Multiple CRDs

The structure of these receptors includes an N-terminal cysteine-rich domain, a domain containing fibronectin Type II repeats, multiple extracellular CTLDs, a transmembrane domain and a short cytoplasmic tail.[41] The best characterised receptor in this group is the mannose receptor (MR) (CD206). This receptor is a 180-kDa Ca^{2+}-dependent lectin that functions as an endocytic receptor and has been shown to bind bacteria, viruses and yeasts (Table 2).[54] MR specifically binds terminal mannose, fucose, *N*-acetylglucosamine or glucose residues which allow it to distinguish nonself from self as these moieties are commonly found on microorganisms, but not in terminal positions on mammalian cell surface oligosaccharides or serum glycoproteins.[55] On alveolar macrophages, for example, the mannose receptor has been identified as a pattern recognition receptor capable of NF-κB activation in response to the fungus *Pneumocystis*.[56] The ligand on *Pneumocystis carinii* mediating interaction with the mannose receptor was shown to be the major mannose-rich surface antigen complex termed glycoprotein A (gpA).[57] This receptor has also been implicated in nonopsonic binding of another pathogenic fungus, *C. albicans*, most likely via mannose residues on the fungal surface.[58,59] The cysteine-rich domain has been shown to bind endogenous glycoproteins via their sulphated *N*-acetylgalactosamine or galactose moieties[60] and this receptor has been implicated in the clearance of serum glycoproteins to maintain homeostasis.[61] On dendritic cells, the mannose receptor plays a role in binding to MUC1, an aberrantly glycosylated membrane protein that is highly expressed on tumour cells and is released into the circulation.[62]

Table 2. C-type lectin recognition of endogenous and exogenous ligands

Receptor	Microbial Ligand	Ref.	Endogenous Ligand	Ref.	Modified Self Ligand	Ref.
DC-SIGN	HIV-1 Envelope glycoprotein gp120	68	Intercellular adhesion molecule (ICAM)-3	42	Tumor-associated carcino-embryonic antigen	52
	Hepatitis C virus envelope glycoproteins E1 and E2	69	ICAM-2	43		
	Dengue virus	48	β2-integrin Mac-1 on neutrophils	53		
	Marburg virus glycoprotein	70				
	Severe acute respiratory syndrome coronavirus S protein	70				
	Helicobacter pylori LPS containing Lewis^x antigen	45				
	Schistosoma mansoni glycan antigen (Lewis^x) of soluble egg antigen	47				
	Leishmania mexicana mannose capped surface lipophosphoglycan	45				
	Mycobacterium tuberculosis lipoarabinomannan	51				
	Candida albicans	71				
	Aspergillus fumigatus	72				
SIGNR1	*Streptococcus pneumoniae* capsular polysaccharide	73	ICAM-2	43	NR	
	Candida albicans	74	ICAM-3	42		
	HIV-1 gp120	75				

continued on next page

Table 2. Continued

Receptor	Microbial Ligand	Ref.	Endogenous Ligand	Ref.	Modified Self Ligand	Ref.
Dectin-2	*Mycobacterium tuberculosis*	50	ICAM-3	42	NR	
	HIV-1	50				
Mannose	*Pneumocystis carinii* Glycoprotein A	57	CD45	76	MUC1	62
receptor	*Candida albicans*	58	Sialoadhesin	76		
	Klebsiella pneumoniae lipopolysaccharide	77	Lysosomal acid phosphatase	78		
	Streptococcus pneumoniae Capsular polysaccharide	77	myeloperoxidase	79		
	Influenza A virus	80	Serum glycoproteins	61		
	HIV-1	81	Lutropin hormone	82		
Endo180	NR		Collagen	83	Denatured collagen	4
Dectin-1	*Candida albicans* β-glucan	63	T-cell ligand	67	NR	
	Saccharomyces cerevisiae β-glucan	63				
	Pneumocystis carinii	66				
LOX-1	*Staphylococcus aureus*	84	Activated platelets	85	OxLDL	86
	Escherichia coli	84	Hsp70	87	Apoptotic cells	85

* NR: not reported.

NK-Like C-Type Lectin-Like Receptors

These receptors typically possess a single extracellular carbohydrate binding domain (CTLD), a stalk region, a transmembrane domain and a cytoplasmic tail with or without signalling motifs.[4] Some receptors in this family contain cysteine residues in the stalk region which are involved in homo- or heterodimerization. Dectin-1 is a small (~28 kDa) Type-II membrane receptor with a single extracellular C-type lectin-like domain and a cytoplasmic domain with a tyrosine-based activation motif.[63,64] Carbohydrate recognition is independent of calcium. It recognises a variety of β-1,3-linked and β-1,6-linked glucans and thus binds and promotes phagocytosis of yeasts such as *Saccharomyces cerevisiae* and *C. albicans*.[63,65] In alveolar macrophages, Dectin-1 has been shown to bind the fungus *P. carinii*.[66] In contrast to the mannose receptor, it does not recognise monosaccharides or carbohydrates with different linkages. Dectin-1 has also been shown to interact with an endogenous ligand on activated T-cells, although the identity of this ligand is as yet unknown.[67]

Self Tolerance in Innate Immunity

In recent years there has been significant progress in the field of innate recognition and antimicrobial host defence. However, our knowledge concerning induction of tolerance in the innate immune system is still rudimentary and the role of PRRs in tolerance induction is not clear. It is proposed that microbes express ligands for both phagocytic and sensing PRRs which simultaneously engage these two classes of receptors and induce full scale antimicrobial responses. However, phagocytic receptors possibly recognise natural or modified self molecules in the absence of TLR stimulation, resulting in a tolerogenic outcome. Recognition of modified self molecules by phagocytic receptors can also lead to inflammatory responses such as recognition of mLDL or β amyloid protein by SR-A. It is conceivable that in such aberrant metabolic conditions, TLR agonists are also produced which promote a dual signal through SR-A and TLR. Other than PRR mediated recognition several other possible mechanisms for tolerance induction have been proposed in innate immunity.

Our first mechanistic insight concerning tolerance induction in the innate system came from studies on activatory and inhibitory natural killer (NK) cell receptors. NK cells express a range of ITAM containing inhibitory receptors which recognise MHC class I molecules which are present in all nucleated cells of the body as a marker of self, sparing them from killing. On the other hand, if MHC-I expression is absent or reduced, as in the case of viral infection or tumour cells, NK cells recognise them as foreign. However, "missing self" alone does not determine the target cell killing and virus-infected or tumour cells also express ligands for many activatory receptors present on NK cells, which initiate the killing machinery. Therefore absence of MHC-I as a self marker and presence of ligands for activatory NK cell receptors together act as a switch for cytotoxic activity of NK cells.[88]

Inhibitory receptor-mediated self tolerance is also observed in the Mϕ system. SIRP-α (CD172a) is a predominantly myeloid restricted molecule of the immunoglobulin superfamily (IGSF), whereas its ligand CD47 is more broadly expressed, including on myeloid cells. CD172a contains three extracellular Ig-like domains; its intracellular domain contains several tyrosines and has been shown to interact with the tyrosine phosphatases SHP1 and SHP2. This inhibits Mϕ activation, such as the response to growth factors or phagocytosis via Fc or complement receptors. Recently Oldenborg et al showed that $CD47^{-/-}$ red blood cells are rapidly cleared by splenic red pulp Mϕ after infusion in WT animals. CD47 expression on WT RBC prevents such elimination by binding to the inhibitory molecule, CD172a. Thus Mϕ rely on the presence or absence of CD47 to distinguish self from foreign.[89]

CD200 and CD200R are both members of the IGSF and contain two Ig domains each in their extracellular region. CD200 has a very short cytoplasmic domain and is unable to signal. In contrast, CD200R contains several tyrosine phosphorylation sites in its relatively longer cytoplasmic tail. CD200 is reported to be expressed by a broad range of cells including neurones, but not by myeloid cells. On the other hand CD200R expression is restricted to myeloid cells,

particularly Mϕ. Interaction between CD200 and CD200R induces an inhibitory signal through CD200R to Mϕ.[90] Knowledge of the physiologic relevance of CD200-CD200R interaction in vivo came from studies of $CD200^{-/-}$ animals. Naïve $CD200^{-/-}$ animals constitutively show some degree of myeloid expansion and Mϕ activation. However, $CD200^{-/-}$ animals show much faster disease progression and significantly more susceptibility to several autoimmune diseases, such as collagen induced experimental allergic encephalomyelitis (EAE), which is a mouse model for the human disease, multiple sclerosis.[91]

Conclusion

In recent years our understanding has improved significantly about how innate PRRs recognise nonself, modified self and self molecules and how an appropriate inflammatory response is mounted against microbes. Our knowledge has grown, concerning how initial recognition by PRRs instructs the shape and nature of protective adaptive responses against microbes. However, knowledge is still sketchy about how discriminatory responses are induced against self and nonself molecules and how successful pathogens evade innate recognition and responses by PRRs. Future research should study the mechanistic differences between tolerance induction in innate and adaptive immunity. Why is autoimmunity predominantly associated with adaptive rather than innate immune responses? Does innate immunity instruct the regulatory functions of adaptive immunity? Recent advances in molecular and cellular biology make it possible to study new aspects of innate immunity and to understand the causes of many infectious and immune pathologies.

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CHAPTER 2

Pattern Recognition by Toll-Like Receptors

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Abstract

The mammalian immune system senses pathogens through pattern recognition receptors and responds with activation. The Toll-like receptors (TLRs) that are expressed on antigen presenting cells such as macrophages and dendritic cells play a critical role in this process. Their signaling activates these cells and leads to an innate immune response with subsequent initiation of an adaptive immune response. Each TLR recognizes specific structures and induces common inflammatory cytokines. However, some TLRs have specific functions, such as induction of Type I interferons. The TLR dependent cytokine response is reflected in the induction of common and specific signaling pathways leading to adequate immune responses for different pathogens. Some TLRs are also activated by endogenous structures that are released during infection, but these structures may promote or sustain autoimmune diseases under certain circumstances. In addition, TLRs directly shape adaptive immune responses of T and B cells and play an important role in homeostasis of gut epithelium and lung repair after injury.

Introduction

In vertebrates the immune system can be broadly categorized into an adaptive and innate system. Adaptive immunity relies on clonally distributed T and B cells which confer a specific and memory response against pathogens. The innate immune system has developed germ-line encoded pattern recognition receptors (PRR) that promote rapid responses to microbial pathogens during the invading phase. These receptors recognize conserved pathogen associated molecular patterns (PAMP) which are not present in the host and which are usually important for pathogenicity and/or survival of the pathogens. Sensing these patterns by innate immune cells activates and directs the emanating response against pathogens.[1] Two classes of PRR on antigen presenting cells (APC) have been identified: One class of receptors facilitates uptake of pathogens leading to its internalization with subsequent presentation of antigen via major histocompatibility complex (MHC) molecules. This process does not activate the APC. In contrast, Toll-like receptors (TLR) as member of the second class of PRR activate APC after engagement of their cognate PAMP to express costimulatory molecules and to secrete proinflammatory Tumor-necrosis factor- α (TNF)-α, interleukin-6 (IL-6) and regulatory cytokines (IL-12, IL-18). Overall the innate immune system is central in sensing an infection and directing the adaptive immune response.[2]

Toll-Like Receptors and Their Ligands

Toll receptors are Type I transmembrane proteins which are evolutionary conserved between insects and vertebrates.[3] In Drosophila, Toll was first identified as essential molecule for

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Table 1. Pathogen derived ligands for TLRs

Receptor	Ligand	Origin of Ligand	References
TLR1/TLR2	Triacyl lipopetide	Bacteria	19,21
TLR2	Lipoprotein/lipopetide	Various pathogens	
	Peptidoglcycan	Gram-positive bacteria	18,24
	Lipoteichoic acid	Gram-negative bacteria	17,18
	Glycoinositolphospholipids	Trypanosoma cruzi	99
	Atypical lipopolysaccharide	Legionella, Leptospira, Porphyromonas,	13-15
	Zymosan	Fungi	88;89
	Hemagglutinin	Measles virus	54
	Structural viral proteins	CMV, HSV	55-57
	Glycoinositolphospholipid	Trypanosoma cruzi	103
TLR3	Double-stranded RNA	Viruses	81
TLR4	Lipopolysaccharide	Gram-negative bacteria	10
	Zymosan	Fungi	89
	Fusion protein	Respiratory syncytial virus	48,52
	Envelope protein	Mouse mammary-tumour virus	50
TLR5	Flagellin	Bacteria	36
TLR6/TLR2	Diacyl lipopetides	Mycoplasma	22
TLR7	Imidazoquinoline	Synthetic compounds	73,74
	Loxoribine	Synthetic compounds	75,76
	Single-stranded RNA	Viruses	65-67
TLR8	Imidazoquinoline	Synthetic compounds	74
	Single-stranded RNA	Viruses	65,69,70
TLR9	CpG-containing DNA	Bacteria, viruses	44,45,47, 59-62
	Hemozoin	Plasmodium	106
TLR10	?	?	
TLR11	Profilin	Toxoplasma gondii, uropath. bacteria	35,104

dorso-ventral patterning of the embryo and subsequently as key molecule for the antifungal immune response in the adult animal.[4,5] A homologous family of toll receptors, termed TLRs exists in vertebrates.[3] In vertebrates 13 members (TLR1-13) have been reported so far which are fundamental in recognition of PAMP.[6,7] The family of TLRs recognizes various PAMPS from different pathogenic origin such as bacteria, viruses, fungi or protozoan parasites[7] (Table 1).

Bacterial Recognition by TLRs

Bacteria contain unique structures that are absent from the host and are suitable targets for recognition by the innate immune system. In addition, most of these structures are strictly conserved since they are important for pathogenicity or survival of the pathogens. These characteristics render these structures to preferred targets for the recognition by the immune system because mutations or modification to evade immune recognition are very unlikely.[8]

Due to different staining characteristics of the cell wall bacteria can be classified into Gram-positive and Gram-negative bacteria. The endotoxin lipopolysaccharide (LPS), a compound of the outer cell membrane of Gram-negative bacteria is a very potent PAMP among the cell wall components. The lipid portion of the LPS, termed Lipid A, is responsible for the immune stimulating activity that can potentially lead to endotoxin shock, a life threatening complication during

infection with Gram-negative bacteria (sepsis).[9] TLR4 is the key molecule of LPS induced signaling and utilizes several cofactors for efficient recognition.[10] LPS associates first with LPS binding protein (LBP) and then with CD14, a glycosylphosphatidylinositol (GPI) anchored protein.[11] This complex binds to MD-2, an additional cofactor that is associated with TLR4, and this interaction results in TLR4 aggregation and subsequent signaling.[10,12] LPS from different bacteria varies in number and length of acyl chains which influences its biological activity. Furthermore, LPS from certain bacterial strains such as *Legionella pneumophila*, *Leptospira interrogans* and *Porphyromonas gingivalis* has been described not to activate TLR4 but instead act as a ligand for TLR2.[13-15] However, these results must be viewed with caution since impurities in the LPS preparation could account for a TLR2 activation which is independent of LPS.[16]

Apart from LPS, other components of the cell wall found in Gram-positive and Gram-negative bacteria can stimulate innate immune cells. For example, lipoteichoic acid (LTA), an amphiphilic negatively charged glycolipid and lipoproteins are potent immune stimulators and TLR2 is involved in the recognition of these compounds.[17,18] TLR2 associates with TLR1 and TLR6 and this interaction allows discrimination of differences within the lipid part of lipoproteins. Accordingly the TLR2/TLR1 heterodimer recognizes triacylated lipopetides, whereas the complex consisting of TLR2 and TLR6 is activated by diacylated lipopeptides.[19-21,22] A recent report challenges this view, since it demonstrates that a triacylation pattern is necessary but not sufficient to render a lipopeptide TLR1-dependent. In addition a diacylation pattern is necessary but not sufficient to render a lipopeptide TLR6-dependent. Therefore contrary to the current model, distinct lipopeptides are recognized by TLR2 in a TLR1- and TLR6-independent manner.[23]

The recognition of peptidoglycan (PG), a large molecular structure composed of alternating N-acetyl glucosamine (GlcNac) and N-acetyl muramic acid (MurNac) sugar chains that are interlinked by peptide bridges has also been attributed to TLR2, but this observation is still controversial.[18,24] Again the contribution of TLR2 in the recognition of PG must be viewed with caution since impurities in the biochemically purified PG such as lipoproteins or LTA could account for a PG independent TLR2 activation.[25] Supporting the latter view, synthetic components of PG are not recognized by TLR2, but instead two cytoplasmic proteins termed NOD1 and NOD2 sense synthetic structures such as γ-D-glutamyl-meso-diaminopimelinsäure and muramyl-dipeptid, respectively.[26-29]

The importance of TLR2 and TLR4 in the host defense against bacteria has been demonstrated using TLR2 and TLR4 deficient mice. TLR2 deficient mice ($TLR2^{-/-}$) are highly susceptible to infection with *Staphylococcus aureus* and *Streptococcus pneumonia*[30,31] whereas $TLR4^{-/-}$ or TLR4 mutated C3H/HeJ mice succumb easily to infection with *Salmonella typhimurium* or *Neisseria meningitis* compared to wildtype mice.[32-34] Uropathogenic bacteria are recognized by TLR11 in mice (the human gene is nonfunctional) although the specific ligand for TLR11 has not been yet identified.[35]

Many pathogens are motile and use a flagellum as the motility apparatus. The major component of the flagellum is flagellin, a potent activator of TLR5.[36] TLR5 recognizes the constant domain D1 of flagellin that is relatively conserved among various species.[37] TLR5 is expressed by immature dendritic cells, monocytes and by epithelial cells.[38-40] Since epithelial cells express TLR5 on the basolateral side, flagellin is only recognized when flagellated pathogens have crossed the epithelium.[38] A recent report demonstrates that monomeric flagellin produced by salmonella during infection of intestinal epithelial cells was not derived from polymeric cell wall-associated flagellum but instead was synthesized and secreted de novo after direct sensing of host-cell derived lysophospholipids.[41] A TLR5 associated polymorphism within the ligand binding domain is associated with susceptibility to *Legionella pneumophila* induced pneumonia and therefore underscores the importance of this receptor ligand interaction.[42] Some flagellated bacteria such as *Helicobacter pylori*, *Bartonella bacilliformis* and *Campylobacter jejuni* escape TLR5 mediated recognition by producing flagellin with mutations in the D1 domain that presumably abolish binding to TLR5.[43]

TLR9 recognizes bacterial genomic DNA. Studies by Tokunaga et al first demonstrated that bacterial DNA itself was the component of bacillus Calmette-Guerin (BCG) which promoted immunostimulatory and antitumor effects.[44] The stimulatory effects of bacterial DNA is due to the presence of unmethylated CpG dinucleotides in a particular base context named CpG motif.[45] Vertebrate DNA is not stimulatory due to methylation of CpG dinucleotides, their low frequency (CpG suppression) and the presence of possibly inhibitory sequences.[45] The immunostimulatory effects of bacterial DNA can be mimicked by synthetic oligodeoxynucleotides containing a CpG-motif (CpG-ODN). CpG-DNA activates immune cells and leads to production of IFN-α, proinflammatory cytokines such as TNF-α, IL-1 and IL-6 as well as the regulatory cytokines IL-12 and IL-18 promoting Th1 differentiation.[46] The role of TLR9 in bacterial infection has not been studied in detail. Interestingly, one study shows that TLR9 activation is critical for the production of IFN-α during infection with *Propionibacterium acnes* (formerly *Corynebacterium parvum*) that is part of the human flora and associated with several human pathologies. TLR9 dependent activation via *P. acnes* primes enhanced resistance to murine typhoid fever which is abolished in TLR9 deficient mice.[47]

Viral Recognition by TLRs

Viruses consist of structural proteins and nucleic acid (DNA or RNA, but not both) that encodes for viral proteins and ensures replication of the virus. The innate immune system utilizes various TLRs to sense viral components such as glycoproteins or nucleic acids.

Some viral-envelope proteins such as the fusion protein F from respiratory syncytial virus (RSV) and the envelope protein of mouse mammary tumor virus (MMTV) activate TLR4 and induce cytokine production.[48-51] In general this activation does not lead to Type I IFN secretion, but favors secretion of proinflammatory cytokines. Accordingly, RSV infection in TLR4$^{-/-}$ mice resulted in a reduced rate of viral clearance due to reduced IL-12 production.[52] MMTV activates B cells via TLR4 and induces maturation of bone marrow-derived dendritic cells that up-regulate expression of the MMTV entry receptor (CD71) and therefore facilitate infection and may attenuate the antiviral response.[50,53] TLR2 is activated by hemagglutinin from measles virus and structural proteins from cytomegalovirus (CMV) and HSV-1.[54-57] The induction of a proinflammatory cytokine response may be responsible for morbidity and mortality associated with HSV-1 infection.[56]

In contrast to the recognition of structural proteins, the recognition of viral nucleic acid leads to a different immune response. Sensing of viral nucleic acid induces the production of Type I IFN such as IFN-α and IFN-β which are indispensable for an antiviral immune response and control of viral infection.[58] Since the viral genome can consist of single-stranded (ss) RNA/DNA or double-stranded (ds) RNA/DNA, various TLRs are involved in its recognition.

DNA viruses such as murine cytomegalovirus (MCMV), herpes-simplex virus 1 and 2 (HSV-1/HSV-2) are recognized by TLR9 and induce production of inflammatory cytokines and Type I IFN. The TLR9 mediated IFN-α response to HSV-1 and HSV-2 is limited to a subtype of dendritic cells, called plasmacytoid dendritic cell (pDCs) or natural interferon-α producing cells (NIPCs).[59-62] This cell type is characterized by their ability to secrete high amounts of IFN-α in response to viral infection.[63] Cellular activation does not require viral infection since live, heat and UV-inactivated HSV-1/HSV-2 produce high levels of IFN-α. In contrast, macrophages produce IFN-α upon HSV infection in a TLR9 independent manner suggesting that pDC and TLR9 independent redundant mechanisms exist that induce an effective response against DNA-viruses.[61,64]

Single-stranded (ss) guanosine and/or uridine rich RNA and ssRNA viruses such as influenza, vesicular stomatitis virus (VSV), Newcastle disease virus (NDV), coxsackivirus and human parechovirus 1 are recognized by TLR7 and/or TLR8.[65-70] Both genes are homologous to each other and are located on the X chromosome.[71,72] Both receptors also recognize synthetic antiviral nucleoside analogs such as imidazoquinolines (R848 or imiquimod) or loxoribine (7-allyl-7,8-dihydro-8-oxo-guanosine).[73-76] Murine TLR8 is expressed, but seems nonfunctional.[74]

In analogy to DNA-virus recognition by pDCs, the same cell type utilizes TLR7 to sense influenza, NDV and VSV.[66,67] Other cell types such as conventional dendritic cells and fibroblasts also produce Type I IFN upon infection with ssRNA viruses in a TLR7 independent manner.[68] This observation suggests that alternative pathways must exist that sense viral infection. These TLR-independent receptors in non pDCs are the RNA helicase termed RIG-I and MDA-5 that confer the recognition of viral RNA and subsequent Type I IFN production.[77-79] Accordingly, the resistance of patients that are deficient in IRAK-4, a signaling molecule necessary for TLR7-9 mediated IFN-α production (see below), to numerous RNA and DNA viruses underscores that alternative and redundant pathways exist for the recognition of viral RNA and DNA.[80]

TLR3 recognizes dsRNA and the synthetic analog polyinosine-polycytidilic acid (polyI:C) and induces Type I IFN.[81] The dsRNA can be generated as an intermediate during the replication cycle of ss RNA or DNA viruses.[82] Since dsRNA seems to be a universal viral PAMP, TLR3 was believed to be the key receptor in an antiviral immune response. However, viral infection experiments with various viruses such as MCMV, VSV, lymphochoriomeningitis virus (LCMV) and reovirus in wildtype and TLR3$^{-/-}$ mice revealed that TLR3 is not required for the antiviral response.[83] In contrast, West Nile virus (WNV), a ssRNA flavivirus that can cause neuronal injury in man, utilizes TLR3 mediated proinflammatory cytokine production such as TNF-α. Since TNF-α leads to the disruption of the blood-brain barrier, virus induced TLR3 activation facilitates the entry into the brain. Accordingly, TLR3$^{-/-}$ mice were resistant to peripheral WNV infection compared to other wildtype littermates.[84] In addition, TLR3 has been reported to promote cross-presentation of virus-infected cells through engagement of virus-derived RNA. Immunization with virus-infected cells or cells containing polyI:C lead to a increase in TLR3 mediated cross-priming against cell-associated antigens.[85] The use of poly IC as mimic for dsRNA has been questioned by Löseke et al who demonstrated that in vitro generated dsRNA fragments of genomic sequences of NDV induced IFN-α production in plasmacytoid DCs whereas polyI:C did not.[86]

Recognition of Fungi and Protozoan Parasites by TLRs

Cell wall components of fungi such as zymosan and phospholipomannan are recognized by TLR2 and TLR4.[87-91] The outcome of the recognition can vary depending on the receptor triggered. Since a Th1 immune response is important for clearance of fungal infection, the Th1 promoting activity of TLR4 enhances an effective immune response against fungi. Accordingly, TLR4$^{-/-}$ mice show increased susceptibility to disseminated *Candida albicans* infection.[88] In contrast, TLR2 induces less Th1 inducing inflammatory cytokines and promotes, under certain conditions, production of IL-10 that can support Th2 immune responses and also downregulate immune responses. Thus, TLR2$^{-/-}$ mice are more resistant to fungal infection. Infected TLR2$^{-/-}$ mice show normal production of proinflammatory cytokines, but IL-10 secretion is severely impaired suggesting that *C. albicans* induces immunosupression via IL-10.[88,92] *Aspergillus fumigatus*, an opportunistic fungal pathogen, uses a similar mechanism for immune evasion. The noninfectious conidia are recognized by TLR2 and TLR4 whereas the more virulent hyphae, are only recognized by TLR2 leading to immunosuppressive IL-10 production.[93]

TLR2 collaborates with dectin-1, a C type lectin which binds to β-glucan and carries an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic tail for signal initiation.[94-96] On phagocytes, dectin1 is the predominant receptor for the uptake of fungi.[96] In conjunction with TLR2, the dectin mediated recognition of *C. albicans* leads to the induction of proinflammatory cytokines via the protein tyrosine kinase Syk.[97] *C. albicans* possibly evades the dectin-1 mediated recognition by growing into a filamentous state which is characterized by the loss of β-glucan expression.[98]

Protozoan parasites are also sensed by TLRs. TLR2 recognizes certain glycosylphosphatidylinositol (GPI) anchors from *Trypanosoma cruzi*, *Toxoplasma gondii*, *Leishmania major* and *Plasmodium falciparum*, whereas TLR4 is activated by glycoinositolphospholipid

(GIPL)-containing ceramide from *T. cruzi*.[99-103] In addition, *Toxoplasma gondii* contains a profilin-like molecule of unknown function that activates murine TLR11 and induces proinflammatory cytokines.[104] Blood-stage schizonts from *P. falciparum* are recognized by TLR9. One study claimed a yet undefined molecule which is heat labile and can be precipitated with ammonium sulfate induces IFN-α in wildtype pDCs but not TLR9$^{-/-}$ pDCs.[105] Another report identified hemozoin, a heme degradation product, as TLR9 ligand that induces various cytokine except IFN-α.[106]

Cellular Localization of TLRs

TLRs involved in the recognition of structures unique to bacteria or fungi are expressed on the cell surface (TLR1, 2, 4, 5 and 6), whereas TLRs that recognize viral or bacterial nucleic acids (TLR3, 7, 8 and 9) reside within intracellular compartments.[107] In nonactivated immune cells TLR9 is expressed in the endoplasmic reticulum (ER). Upon cellular activation, TLR9 traffics to endosomal and lysosomal compartments where it interacts with endocytosed CpG-DNA at an acidic pH, a condition that is thought to be necessary for DNA recognition.[108-110] Compounds that interfere with endosomal acidification such as the weak base chloroquine and bafilomycin A1, an inhibitor of the ATP dependent acidification of endosomes, consequently prevent CpG-DNA driven TLR9 activation.[111,112] In analogy to TLR9, the trafficking of TLR3, 7 and 8 is considered to be very similar, although extensive studies have not been performed. In addition, the mechanism underlying TLR3, 7, 8 and 9 trafficking is poorly understood, although the membrane region has been implied in trafficking. Studies involving random mutagenesis and chimeric fusion proteins have identified a role of the cytoplasmic linker of TLR3 and the transmembrane domain of TLR7 and 9 in the regulation of intracellular receptor localization.[113-116] The mutation of a conserved charged residue in the membrane proximal region that is conserved in all intracellular expressed TLRs renders the receptor inactive and inhibits trafficking form the ER to endosomes (Thomas Müller, unpublished observation).

For TLRs located in the endosome, the nucleic acid has to enter these vesicles for activation. For certain viruses that enter the cell by receptor mediated endocytosis (such as influenza virus) the encounter is mediated during enzymatic degradation of some virus particles in these vesicles. Because nucleic acid is not unique to pathogens, the specificity of the nucleic acid recognizing TLRs has been attributed to structural differences between eukaryotic and prokaryotic/viral nucleic acid. Accordingly, suppression of the CpG-DNA motif in eukaryotic DNA and frequent base modifications such as 5'methyl-cytosine for DNA and 6'methyl-adenosine or 5'methyl-cytosine for RNA have been shown to be responsible for impaired recognition of eukaryotic nucleic acid by TLRs.[45,117,118] However, structural differences among eukaryotic and prokaryotic DNA are presumably not the only mechanism to distinguish self from nonself DNA, because eukaryotic DNA and RNA can stimulate B cells and pDCs in a TLR dependent manner under certain natural and experimental conditions which possibly reflect pathological conditions in autoimmune disease such as systemic lupus erythematosus (SLE) (see below).[119,120] Apart from the modification of nucleic acid the compartmentalization of the receptors (expression in the endosome) is important to avoid recognition of self-DNA. Thus, the intracellular localization of the nucleic acid recognizing TLRs provide a 'safety' mechanism for avoiding recognition of self-DNA that would occur if these TLRs were expressed at the cell surface.[116]

Recognition of Endogenous Ligands by TLRs and Involvement in Autoimmunity

The recognition of viral, bacterial, fungal and protozoan structures by TLRs supports the notion that TLRs distinguish between self and foreign. The idea of endogenous TLR ligands challenges this view and is still highly controversial[121,122] (Table 2). Generally speaking, endogenous ligands are either molecules released from necrotic or apoptotic cells or molecules such as lipid or matrix proteins that may be altered due to unnatural inflammatory or metabolic

Table 2. Endogenous derived ligands for TLRs

Receptor	Ligand	References
TLR2	Gp96,Hsp60, Hsp70	123,124,126
	Hyaluronic acid	142
	HMGB1	135
TLR4	Gp96, Hsp60, Hsp70	123,124,126
	Hyaluronic acid	133,142
	Heparan sulphate	132
	Fibronectin	131
	Fibrinogen	129
	Surfactant-protein A	130
	HMGB1	135
	β-defensin	134
TLR7	Endogenous RNA	149-151
TLR8	Endogenous RNA	149
TLR9	Endogenous DNA	119,148

conditions. To incorporate the view of endogenous ligands in TLR recognition Polly Matzinger has recently proposed that TLRs sense danger in form of hydrophobic portions that are derived from pathogens or endogenous structures released during infection. Therefore, the ability of biological molecules to engage these TLRs does rely on the exposed hydrophobic portions displayed by self or nonself targets.[122]

Extensive work has suggested that heat shock proteins (Hsp), such as Hsp60, Hsp70 and gp96 are potent activators of the innate immune system. Hsp from bacterial and mammalian source induce proinflammatory cytokines such as TNF-α, IL-1 and IL-6 and upregulate costimulatory molecules on APC.[123-128] Similar cytokine effects have been also reported for various molecules of mammalian origin such as fibrinogen,[129] surfactant-protein A,[130] fibronectin,[131] heparan sulfate,[132] oligosaccharide of hyaluronan,[133] β-defensin[134] and high-mobility group protein 1 (HMGB1).[135] All compounds are ligands for TLR2 and / or TLR4, respectively (see Table 2). Since the cytokine effects of these endogenous ligands are similar to the cytokine pattern induced by LPS and lipoproteins, the contribution of TLR2 and TLR4 in the recognition of endogenous structures has been viewed with caution: LPS or lipoprotein contamination within biochemically purified endogenous ligands could contribute to observed effects. Studies using extensively purified Hsp have failed to demonstrate induction of cytokines and support the concern that contamination in the Hsp preparation are responsible for cytokine production.[136,137] In contrast, a recent report has demonstrated DC activation by transgenic expression of cell-surface gp96 suggesting that contamination-free Hsp stimulates the innate immune system and leads to autoimmunity.[138] Accordingly, the TLR mediated recognition of endogenous Hsp with subsequent inflammatory immune activation may contribute to the pathogenesis of a number of autoimmune diseases and chronic inflammation such as Type I diabetes,[139] artherosclerosis[140] or juvenile arthritis.[141]

However, it is unlikely that all the reported effects of endogenous ligands on TLRs are due to LPS contamination since in vivo models have demonstrated a role for the endogenous TLR2/4 ligand hyaluronan in lung repair.[133,142] Interestingly, TLRs act as mediators of injury or repair in the inflamed lung and the balance is dependent on the integrity of hyaluronan, a sugar polymer in the extracellular matrix. During inflammation hyaluronan is degraded by hyaluronidases generating fragments that activate TLR2 and TLR4 on macrophages. Accordingly, double deficient cells produce no cytokines, whereas TLR2 or TLR4 single deficient cells respond to these fragments. During repair after acute lung injury hyaluronan is produced and

signals through TLR2 and TLR4 on epithelial cells. This prevents apoptosis and lung injury during inflammation. Therefore, epithelial cell-surface hyaluronan is protective against apoptosis, in part, through TLR-dependent basal activation of NF-κB. It is hypothesized that hyaluronan-TLR2/TLR4 interactions provide signals that initiate inflammatory responses, maintain epithelial cell integrity and promote recovery from acute lung injury and that the type of response depends on the integrity of hyaluronan.[142]

In addition to hyaluronan, Hsp and other TLR2/4 ligands, endogenous nucleic acids such as RNA and DNA can activate TLRs and promote or sustain autoimmune diseases such as systemic lupus erythematosus (SLE). SLE is a human autoimmune disease in which increased serum levels of Type I IFNs correlate with disease activity and severity.[143] Due to loss of tolerance to nuclear self-antigens, autoantibodies against DNA, histones, RNA and RNA-binding proteins such as Sm/RNP are produced which form immune complexes (ICs) with DNA or RNA.[144] These immune complexes are deposited in the kidney and lead to glomerulonephritis. The antibodies recognizing nucleic acid and/or associated proteins are produced by autoreactive B cells.[145] Autoreactive B cells are activated if their immunoglobulins have a specificity to recognize autologous IgG2a antibodies, termed rheumatoid factor-positive (RF+), via sequential engagement of B cell receptor (BCR) and TLR9.[119,146] In this system, the BCR first recognizes the isotype of the autoantibody and triggers the endocytosis of the ICs into the endosomes where TLR9 resides. The same paradigm has been reported for RNA-containing ICs and TLR7 activation.[147] The pDC activation and secretion of IFN-α is mediated through endocytosis of RNA or DNA/IC via FcγRIII (mouse)[146] or FcγRIIa (human).[148] This uptake translocates the nucleic acid to endosomes where IFN-α is induced in a TLR7[149-151] and TLR9-dependent[146] fashion. Interestingly, lupus-prone mice on a MRL/lpr background that lack TLR9 fail to generate anti-dsDNA antibodies although severity of glomerulonephritis is not affected.[152] In contrast, a report by Wu et al demonstrated that the loss of TLR9 in a similar autoimmune model aggravates the autoimmune disease.[153]

As mentioned earlier, the exposure of immune cells to eukaryotic DNA should not activate immune cells due to low numbers of CpG-motifs and 5'-methylation of the cytosine in CG dinucleotides.[45] Accordingly, in vitro exposure to ssDNA oligonucleotides (ODN) containing methylated CpG motifs or nonfunctional inverted GpC-motifs do not stimulate innate immune cells. However, if these altered and inactive DNA molecules are complexed to cationic lipids such as N-[1-(2,3-Dioleoyloxy)propyl]-N,N,Ntrimethylammonium methylsulfate (DOTAP), which enhances endosomal translocation and retains the nucleic acid in the endosomal compartment, these DNA molecules induce IFN-α in a strictly TLR9 dependent manner.[154] These observations suggest that DNA sequences which are poor ligands for TLR9 due to methylation of cytosine or due to the absence of a CpG-motif can activate TLR9 upon increased ligand concentration in the endosome. Binding studies utilizing surface plasmon resonance technology (Biacore) support this finding by showing that binding of a DNA ODN lacking a canonical CpG-motif to TLR9 is strongly enhanced by increasing the concentration of the respective ODN.[154]

It is evident that TLRs are likely to have a role in autoimmunity. However, these studies have also raised a fundamental question: Is TLR activation critical for both the initiation and perpetuation of autoantibody production, or is the initiation independent of TLR7 and TLR9? The mammalian DNA or RNA must enter endocytic compartments in order to interact with TLR3, 7, 8, and 9 mediated uptake of nucleic acid is a prerequisite for activation. Apart from the artificial use of transfection with cationic lipids, uptake and endocytosis is achieved through engagement of IgG-nucleoprotein complexes with RF+ B cells or with Fc-γ receptors on DCs.[146,148] Therefore it is hypothesized that after the production of autoantibodies, when tolerance has already been broken, the TLR mediated activation presumably sustains immune activation and autoimmunity. Certain scenarios for the TLR dependent initiation of autoimmunity are also conceivable. Since low affinity antibodies specific for self-DNA or RNA circulate in the periphery,[155] the avidity of interaction between the BCR and nucleoprotein

could influence the uptake as well as the downstream BCR mediated signaling and therefore lead to initiation of autoimmunity. In addition, exposure to inflammatory cytokines or defects in rapid clearance of apoptotic cells could influence the production of pathogenic autoantibodies. A recent report suggests that the expression levels of TLR7 can influence autoimmune responses. The B cells on an autoimmune background (deficient in the inhibitory Fcγ receptor, Fcγ RIIB) that contained the Y-linked autoimmune accelerator (Yaa) locus showed an increased expression of TLR7 which correlated with enhanced production of autoantibodies against nucleolar antigens.[156]

TLR Signaling

The engagement of TLRs by their cognate ligands leads to the activation of a signaling cascade with subsequent induction of genes that are involved in the immune response against pathogens. In general, three major pathways are activated, the first culminates in the activation of the transcription factor NF-κB, which acts as a master switch for inflammation. The second leads to activation of the MAP kinases p38 and Jun amino-terminal kinase (JNK), which also participate in increased transcription and the third pathway leads to Type I IFN production via IFN regulatory factors (IRFs)[157] (Fig. 1).

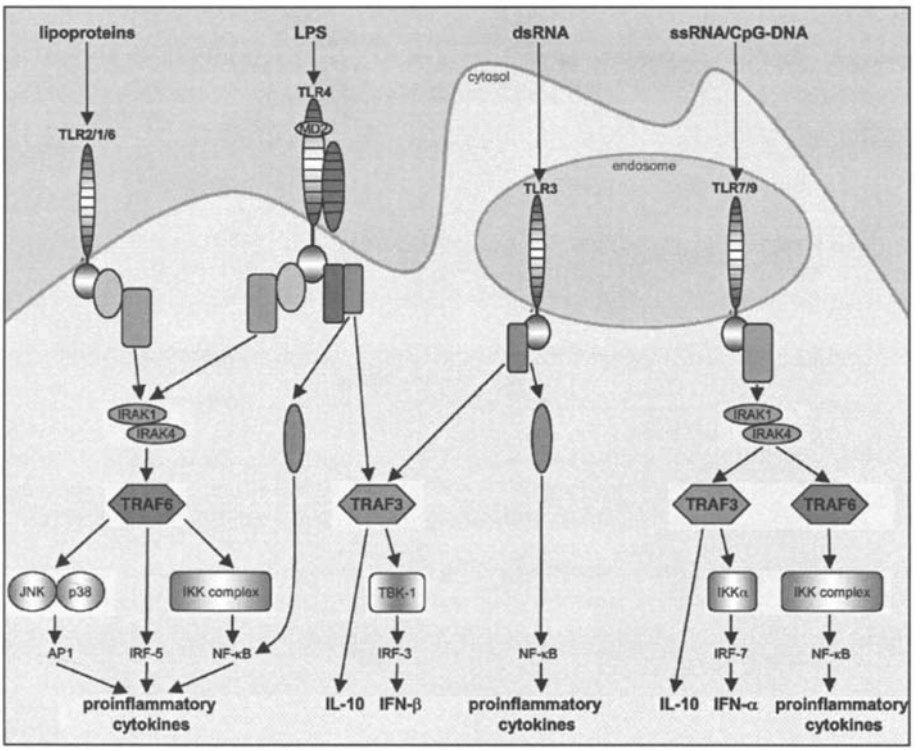

Figure 1. TLRs and various signaling pathways. Activation of TLRs induces secretion of proinflammatory cytokines, Type I IFN and anti-inflammatory IL-10 depending on a specific TLR, cell type and the adaptors used for signal transduction (MyD88, TIRAP, TRAM and TRIF). Expression of proinflammatory cytokines is induced via IRAK1, IRAK4, TRAF6 and the transcription factors NF-κB, IRF-5 and/or AP1. NF-κB activation is also mediated via the TLR3-TRIF pathway and RIP-1. TRAF3 controls the IL-10 and Type I IFN production. IFN secretion is also critically dependent on the transcription factors IRF-3 and IRF-7.

It has been shown that all TLR mediated signaling are initiated after ligand binding which presumably leads to receptor dimerization. The receptors undergo conformational change and recruit adapter molecules to its intracellular domain termed Toll/IL1-receptor like domain (TIR domain) which is shared by the TLR and IL1-receptor signaling pathway.[158,159] Four different adapter molecules have been identified: MyD88 (Myeloid differentiation protein 88),[160-163] TIRAP (TIR-associated protein) / MAL (MyD88-adaptor like),[164,165] TRIF (TIR-domain containing adaptor protein -inducing IFNβ/TICAM1 (TIR domain containing molecule 1)[166,167] and TRIF-related adaptor molecule (TRAM).[168] In general, MyD88 and TRIF are important for the activation of distinct signaling pathways and lead to the production of proinflammatory cytokines and Type I IFN, respectively. However, some TLRs such as TLR7 and 9 also utilize MyD88 to induce Type I IFN. For TLR2/4 signaling, TIRAP/MAL is required for recruiting MyD88 to the receptors and TRAM bridges TLR4 and TRIF[157] (Fig. 1).

MyD88 is the master adapter protein utilized by all TLRs except TLR3. MyD88 is recruited to the cytoplasmic TIR domain through interaction with its TIR domain. Recruitment of MyD88 is followed by engagement of IL-1 receptor associated kinase 4 (IRAK-4) and IL-1 receptor associated kinase 1 (IRAK-1) which is phosphorylated by IRAK-4. Phosphorylated IRAK-1 associates with TNF receptor associated factor-6 (TRAF-6).[169-171] Oligomerization of TRAF6 which acts as an ubiquitin protein ligase (E3) leads in conjunction with a ubiquitination complex E2 to polyubiquitination of TRAF-6 itself and IKK-γ/NF-κB essential modulator (NEMO) and IKKB kinase (IKK) complex resulting in activation of the transcription NF-κB.[171] The stress kinases like c-Jun N-terminal kinase (JNK) and p38 are activated by a complex composed of TGF-β activated kinase-1 and the TAK binding proteins TAB1-3. This complex influences the activity of NF-κB by phosphorylation of IKK-β.[172] TRAF3 is recruited along with TRAF6 and is essential for the induction of Type I IFN and the anti-inflammatory cytokine IL-10, but it is dispensable for expression of pro-inflammatory cytokines.[173,174] The fact that TRAF3 is also recruited to the adaptor TRIF and is required for activating the protein kinase TBK1 (also called NAK) explains its unique role in activation of the IFN response.[173] Recently the transcription factor IRF-5 was described as important component in the TLR-MyD88 signaling pathway for gene induction of proinflammatory cytokines, such as IL-6, IL-12 and TNF-α. Accordingly, the induction of these cytokines in DCs from IRF-5 deficient mice was severely impaired, whereas IFN-α induction was normal.[175]

Interferon Production by TLRs

TLR3 and TLR4 utilize TRIF to produce IFN-β and IFN inducible genes.[176,177] TRIF interacts with receptor-interacting protein-1 (RIP1) and TANK binding kinase 1 (TBK1) to initiate NF-κB activation and IRF-3 and IRF-7 phosphorylation, respectively.[178-180] Phosphorylated IRF-3 and IRF-7 form homodimers, translocate into the nucleus and regulate the expression of IFN-inducible genes by binding to interferon stimulated response element (ISRE). IRF-3 and IRF-7 are essential for the production of Type I IFN since $IRF7^{-/-}$ and IRF-3 and -7 double deficient cells are partially or completely impaired in their IFN Type I response to viral infection, respectively.[181,182]

MyD88 is the key adaptor for IFN-α production in pDCs that are known to produce high amounts of IFN-α upon viral stimulation via TLR7 and TLR9. This dependency is in contrast to TLR3 and TLR4-mediated IFN production which is TRIF, but not MyD88 dependent. IRF-7 is the key component in IFN-α induction which is recruited to a complex consisting of MyD88, IRAK-4, IRAK-1 and TRAF-3.[183,184] The pDCs deficient in one of these components fail to produce IFN-α, although in IRAK1 deficient cells, the production of proinflammatory cytokines is still functional. This suggests a specific role of IRAK-1 in the IFN-α inducing pathway such as phosphorylation of IRF-7.[185] Furthermore the kinase IKK-α has been recently identified as an important component in TLR7/9 mediated IFN-α production since IKKB-α deficient pDC fail to secrete IFN-α upon TLR stimulation.[186]

TLR Structure

The extracellular domain of TLRs contains leucine-rich repeat (LRR) motifs which are defined by a consensus sequence of 24 to 29 amino acids (aa) in length and a highly conserved core region of 11 aa (LxxLxLxxN/CxL).[3,187] The number of LRRs among the members of the TLR family varies from 19 to 26 as determined by pattern analysis.[188] These tandem arrays of LRR have been found in the primary structure of various proteins that participate in biological processes like cell adhesion, signal transduction, enzyme inhibition, ribosome and DNA-binding.[187] The crystal structure of ribonuclease inhibitor (RI), one member of the LRR protein family, reveals that LRRs correspond to structural units which consist of a β-strand and an α-helix. The structural units are arranged in a way that all the β-strands and the α-helices are parallel to a common axis, resulting in a horseshoe-shaped molecule with curved parallel β-sheet lining the inner circumference of the horseshoe and the α-helices flanking its outer circumference.[187]

Initial analyses of human TLR sequences and molecular models suggested that the TLR ectodomains would fold as an uninterrupted solenoidal array of 19-23 LRRs with a pronounced curvature.[188] Recently two independent groups reported the crystal structure of human TLR3 ectodomain at 2.1 and 2.4 Å resolution, respectively.[189,190] The X-ray crystallographic data revealed the expected horseshoe shaped structure arranged by 23 LRRs, flanked by characteristic cysteine-rich N- and C-terminal capping modules (Fig. 2). There are two main protrusions from the regular TLR3 fold in LRR12 and 20 and numerous N-glycosylations (8 or 11 of 15 possible) were identified. Interestingly, the inner cavity of the TLR3 horseshoe and part of the convex surface area are covered with sugars and therefore no obvious binding site could be identified. Therefore, the interaction site of TLR3 and its ligand ds RNA or polyI:C is still

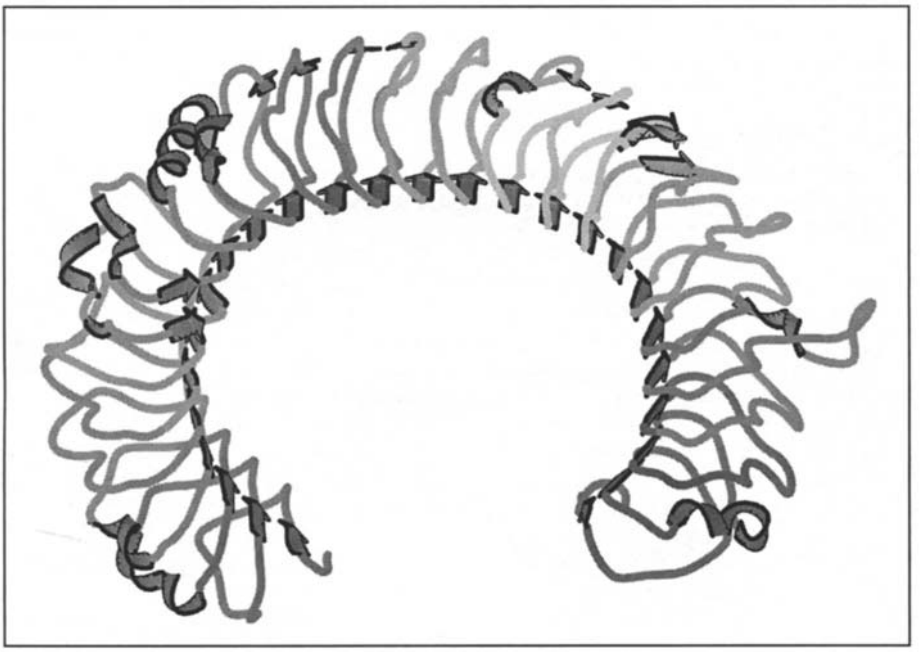

Figure 2. TLR3 ribbon structure using KiNG viewer at <http://www.rcsb.org/pdb> and the structure file 1ZIW.pdb. All 23 leucine rich repeats (LRRs) are depicted. A LRR is formed by a β-sheet and an extension (usually an α-helix) that connects to the next LRR. Overall, the TLR3 structure resembles a horseshoe shaped molecule. The exact binding site for dsRNA is not yet defined.

controversial. While Bell et al hypothesize a binding groove for dsRNA within the inner cavity of the TLR3 monomer in conjunction with two sulfate ions,[189] Choe et al[190] propose that the binding site for dsRNA is not on the concave LRR surface. Instead, the ligand interaction site is suggested to be formed by a V-shaped valley between dimerized TLR3 ectodomains. The latter view is appealing since it would help explain how TLRs in general can recognize structurally different PAMPs. Divergent loops, variable in sequence and length on the convex top and flat sides of the horseshoe could create diversity in its recognition repertoire and would allow different TLR homo- or heterodimers to bind different pathogen ligands. However, a cocrystal structure of TLR3 in complex with dsDNA should reveal further details on the interaction sites. Recently using mutational analysis, Bell et al have identified the glycan-free lateral surface of TLR3 toward the C terminus as the TLR3 ligand binding site.[191]

A similar structure can be expected for TLRs with 23 or fewer number of LRR. TLR7, 8 and 9 contain 26 LRR and a 40-50 amino acid insertion after LRR14. The structure of these TLR might be different in respect to TLR3, because in proteins that contain more than 23 LRRs and retain the curvature of the structure TLR3, the overall organization of the repeats may be different to prevent the N- and C- termini from colliding. Therefore TLR7, 8 and 9 probably form 2 independent domains with a horseshoe structure that are connected via the insertion that could be defined as a non structured hinge region (Bauer et al, unpublished results). Furthermore, it is intriguing to speculate that the CXXC domain located in domain one and the MBD motif located in domain two may associate to form a binding pocket for DNA.[110] Crystallization and 3-D structure analysis of the extracellular domain of TLR7-9 will be important to understand the structure-function relation of nucleic acid recognition by these receptors.

TLR Expression in Cells of the Adaptive Immune System

TLR expression has been detected in many cell types. Innate immune cells such as antigen presenting cells (macrophages and DCs),[192] natural killer cells,[193] neutrophils,[194] eosinophils[195] and mast cells[196] express various TLRs and TLR induced cytokine production is important for fighting various infection and regulate innate immune responses. For example, in human pDCs only TLR 7 and 9 are expressed and nucleic acid mediated stimulation of these receptors induces secretion of large amounts of Type I IFN. Therefore pDCs seem specialized in the recognition of viruses and the production of antiviral cytokines such as Type I IFN. In contrast, macrophages do express a variety of TLRs and respond to lipopetides, LPS, polyI:C, flagellin and RNA or synthetic nucleoside analogs. These cells respond to various pathogens and mainly produce proinflammatory cytokines. Human NK cells are activated by CpG-DNA and dsRNA resulting in the induction of cytokine release and increased cytotoxicity against tumor cells. Neutrophils that are among the first immune cells to arrive at the site of infection express all TLRs except TLR10. The corresponding agonists trigger cytokine release and superoxide generation, while inhibiting chemotaxis. Eosinophils constitutively express TLR1, TLR4, TLR7, TLR9, and TLR10 mRNAs, but only the TLR7 and TLR8 ligand resiquimod regulates adhesion molecule (CD11b and L-selectin) expression and prolonged survival. In addition, TLRs have also been detected on cells of the adaptive immune system, such as T and B cells. In this case, TLR activation directly controls the adaptive immune response.

In B cells TLR triggering directly influences B-cell activation and antibody production in humans and mice.[197,198] According to the current model, naive B cell activation is dependent on the sequential integration of two signals: B cell receptor (BCR) cross-linking by antigen and cognate interaction with helper T (Th) cells. BCR stimulation and T cell help induces initial cell division but this is not sufficient to promote survival and differentiation of naive B cells. B cell proliferation, isotype switching and differentiation to immunoglobulin (Ig)-secreting cells are induced by TLR activation (in human B cells with TLR2 and TLR9 ligands) and required irrespective of the nature of the Th cells. These results clearly demonstrate that naive B cell activation is critically dependent on innate stimuli acting directly on B cells.[197,198]

TLR2 and TLR4 expression have been reported for activated $CD4^+$ T cells as well as memory T cells. The TLR2 ligand lipoprotein acts as costimulus for T cell activation combined with enhanced cytokine production (IFN-γ, IL-2 and TNF-α), whereas TLR4 stimulation has no such effect implying that TLR4 is not functional on these cells. Expression of TLR2 on memory T cells ($CD45RO^+$) is constitutive and its triggering induces proliferation and secretion of IFN-γ.[199]

Previous work by Medzhitov's group has demonstrated an indirect effect of TLR-mediated activation on regulatory T cells (Treg, $CD4^+$ $CD25^+$ $foxp3^+$). The Treg cells regulate T cell immune responses and induce immune tolerance by suppressing host immune responses against self or nonself antigens. The TLR stimulation of DCs blocks the suppressive effect of $CD4^+CD25^+$ Treg cells by IL-6 production (and probably other soluble mediators), allowing activation of pathogen-specific adaptive immune responses.[200] Recently, a direct effect of TLR activation on Treg cells has been observed. Wang et al demonstrated that synthetic and natural ligands for human TLR8 can reverse Treg cell function. This effect was independent of DCs but required functional TLR8-MyD88-IRAK4 signaling in Treg cells.[201] This Treg suppressive function of TLR8 was underscored using in vivo adoptive transfer experiments of TLR8 ligand-stimulated Treg cells into tumor-bearing mice which dramatically enhanced anti-tumor immunity.

Sutmuller et al recently identified TLR2 as an important PRR on Tregs.[202] Since the $CD4^+CD25^+$ Treg subset in $TLR2^{-/-}$ mice is significantly reduced compared to wildtype mice a link between Tregs and TLR2 has been proposed. Interestingly, TLR2 triggering on Tregs with the synthetic lipopetide Pam3Cys augmented—with combined costimulation—Treg proliferation in vitro and in vivo and resulted in a temporal loss of the suppressive Treg phenotype. Importantly, wildtype Tregs adoptively transferred into TLR2 deficient mice were neutralized by systemic administration of TLR2 ligand during the acute phase of a *Candida albicans* infection which resulted in a strongly reduced outgrowth of C. albicans.[202]

Conclusion

Overall, the recent years in TLR research has illuminated how the innate immune system senses invading pathogens and initiates the adaptive immune response. Current research focuses on their direct function on cells of the adaptive immune system and their role in autoimmunity, tissue homeostasis and tissue repair. Future work will exploit all this knowledge to efficiently manipulate immune responses or treat autoimmune diseases aided by the generation of 'small molecules' with agonistic and antagonistic TLR function.

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CHAPTER 3

NOD-Like Receptors—Pivotal Guardians of the Immunological Integrity of Barrier Organs

Philip Rosenstiel and Stefan Schreiber*

Abstract

NOD-like receptors (NLRs) exert pivotal roles in innate immunity as sensors of exogenous or endogenous cellular danger signals. The NLR protein family has a characteristic domain architecture comprising a central nucleotide binding and oligomerization domain (NOD), an N-terminal effector binding domain and C-terminal leucine-rich repeats (LRRs). Mutations in *NLR* genes are genetically associated with a number of chronic inflammatory diseases of barrier organs. In this chapter, we focus on the influence of NLR regulation and function in the complex pathophysiology of mucosal homeostasis. The understanding of NLR biology may guide our future understanding of how the interaction between the human genome and the metagenome of transient and resident microbiota precipitates into chronic inflammatory disorders, such as Crohn's disease or atopy.

Introduction

Barrier organ integrity is maintained by interplay between mechanical factors (e.g., epithelial cells, mucus layer) and the organism's defense mechanism, which includes innate and adaptive immunity. Most barrier organs such as the skin, gastrointestinal, lung and urogenital mucosa share common structures: (i) a tight lining of epithelial cells builds up the first line of defense against physical and microbial stress and (ii) a specialized barrier-associated immune system, e.g., the mucosa-associated lymphoid tissue (MALT), which is located in the underlying connective tissue. Maintenance of barrier integrity is pivotal to survival. Consequently, many evolutionarily "old" functions of the barrier maintenance have been retained and, and thus, the genes involved are highly conserved.

The insights into the molecular genetic basis of barrier disorders have strongly influenced our understanding of receptors and effectors of the innate immune system in the context of human pathophysiology (reviewed in ref. 1). Indeed, the interest, which was raised when *NOD2/CARD15* was described as the first disease gene for Crohn's disease,[2-4] a chronic relapsing-remitting inflammatory disorder of the intestine, has significantly contributed to the basic understanding of the biological function of the group of NOD-like receptors (NLRs/ NOD-LRR proteins). Subsequently, mutations in other NLRs have been genetically associated with a number of rare autoimmune disorders (e.g., Muckle-Wells syndrome, familial cold urticaria,[5] early-onset sarcoidosis

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syndrome[6] or the infantile chronic inflammatory syndromes NOMID and CINCA[7,8]). In this review, we will use NOD1 and NOD2 as a molecular guide to summarize the current knowledge on the structure and function of NLRs. Implications for our understanding of molecular genetics and pathophysiology of barrier diseases will be discussed. The role of NLRs in the complex biology of the barrier organs will also be used as a model for the interaction between the human genome and the metagenome of transient and resident microbiota on body surfaces.

NOD-Like Receptors—Phylogenetically Ancient Molecular Platforms of the Innate Immune System

NLR genes encode for a family of proteins with a modular tripartite domain structure, characterized by a central nucleotide-binding and oligomerization domain (NOD), C-terminal leucine-rich repeats (LRR) and a N-terminal effector binding domain , e.g., a Pyrin (PYD) or caspase recruitment domain (CARD)[9] (Fig. 1). The activated proteins serve as molecular platforms by promoting the activation of downstream effector molecules through self-association and induced proximity of binding partners. This tri-modular structure can be also observed in cytosolic resistance *(R)* genes in plants. *R* gene products are important components of the plant defense response, which appears macroscopically as a rapid localized host cell death at the site of pathogen ingress. This form of programmed cell death is termed "hypersensitive response" (HR) and is thought to inhibit further infection. The HR is induced only after R-specific recognition of pathogen-derived or -modified molecules (avirulence *(avr)* gene products) followed by the activation of complex cellular signals including kinase cascades, alteration of membrane conductance and the generation of reactive oxygen species.

Despite their clear structural similarity, the evolutionary relationship between *R* genes and NLRs is not yet fully understood. In certain plants (*Oryza)*, as many 600 distinct *R* genes comprising >1% of the individual genome have been identified. Interestingly, in the animal kingdom NLR genes seem to arise for the first time at the level of the teleost fish, whereas in *Drosophila* and *Caenorhabditis* no complete *NLR* gene can be observed. It will be interesting to

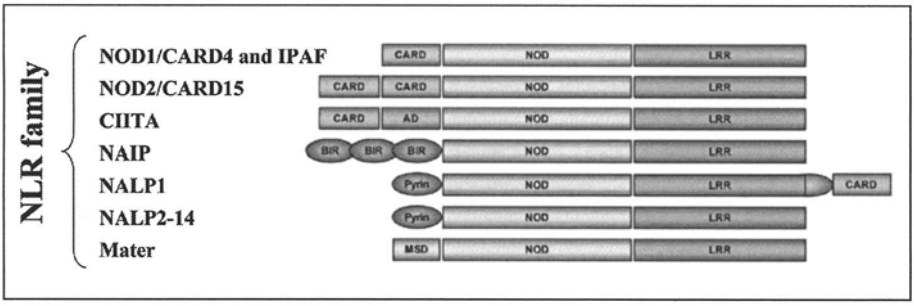

Figure 1. Domain architectures of selected NLR proteins from different eukaryotes. All NLR proteins share a tripartite domain structure with a C-terminal ligand recognition domain consisting of leucine rich repeats, a central nucleotide binding and oligomerization domain (NOD) and the N-terminal effector-binding domain (EBD), which determines subgroups of the NLR family. CARD (caspase recruitment domain family) can be found in NOD1, NOD2 and CLAN. PYD (pyrin domain) domains characterize the NALP (NACHT, leucine-rich repeat and PYD containing) proteins. Note that the central NOD domain is structurally similar to the NB/ARC domain of the apoptosis-promoting protein APAF1, an intracellular sensor for cytochrome c released from damaged mitochondria. The individual number of leucine-rich repeats (LRRs) varies within the NLR proteins. The other abbreviations are: AD, activator domain; BIR, baculovirus inhibitor of apoptosis repeat; FIIND, an interaction domain that is involved in inflammasome formation; NB/ARC, nucleotide binding/found in APAF1, R-genes and CED-4; WD40, a domain with a length of approx. 40 amino acids that typically ends with trytophane ("W") and aspartic acid ("D"), sensor domain for cytochrome c.

investigate a comprehensive set of genomes from lower animal phyla to demonstrate a parallel versus sequential evolution across kingdom boundaries.

Nevertheless, not only the domain structure, but also details of the function of *R* gene products reveal striking parallels to NLR signaling networks:

i. Recognition of pathogen-derived or -modified structures.
It is thought that pathogenic structures are recognized via the LRR sensor domain in a direct or indirect fashion. The term "ligand sensing" rather than "ligand binding" must be stressed in this context as there is only weak evidence for a direct interaction of NLRs or R proteins with their cognate ligand.

ii. Association of protein kinases/Ubiquitin ligases to effector binding domains.
Both NLRs and R proteins have been demonstrated to mediate cellular activation via the recruitment of kinases and/or other adaptor molecules via their N-terminal effector binding domains. NOD1 and NOD2 recruit the serine-threonine kinase RIP2 and activate the NF-κB signaling pathway via a process called "induced proximity signaling".[10-12] Interestingly, the activation of NF-κB via NOD1/NOD2/RIP2 seems to be independent of the kinase activity of RIP2 as the process is only critically dependent on the presence of the intermediate domain between the CARD and the kinase domain. RIP2 has been demonstrated to ubiquitinylate the subsequent effector kinase NEMO/IKKγ, which is part of the canonical NF-κB activation pathway.[13] This ubiquitinylation on residue lysine 285 is necessary for full activation of NF-κB. These findings parallel results from plant *R* genes, where homologues of the ubiquitin ligase-associated proteins SGT1 and RAR1 are required for disease resistance in plants.[14] Furthermore, the E3 Ubiquitin ligase ACRE276 seems to be required for the *R* gene-induced hypersensitivity response in tobacco.[15]

iii. Complex formation by heat shock protein.
The modulation of R protein function by complex formation with endogenous heat shock proteins is a well-recognized phenomenon in plants. Hsp90 associates with the resistance protein N that confers resistance to tobacco mosaic virus. This interaction governs a macromolecular complex with other effectors (e.g., RAR1 and SGT1) and modulates preactivationm, R protein accumulation and signaling competence.[16] Similarly, NOD1 is bound by a Hsp90 chaperone complex containing a mammalian RAR1-related protein CHP1 and protein phosphatase 5 (PP5).[17] These findings suggest a strictly conserved modulatory pathway dependent on a Hsp90 chaperone complex conserved across kingdoms.

iv. Splice variants.
NLR and *R* genes share a striking abundance of splice transcripts. There is increasing evidence to suggest that alterations in splicing patterns of genes may be involved in the regulation of gene functions by generating endogenous inhibitor or activator molecules. In innate immunity signalling, a LPS-inducible short form of MyD88 appears to play a major role in LPS-tolerance induction and inhibits TLR/IL1 signalling.[18-20] A short isoform of NOD2 is a negative regulator of MDP-activated pathways.[21] The existence of splice variants has also been described in other *NLR* genes, e.g., *PYPAF1/NALP3/CIAS1*, but these variants lack a detailed functional characterization.[22,23] The importance of different isoforms generated by alternative splicing in humans is paralleled by findings in *R* genes in plants, where the dynamic regulation of the ratio of alternative transcripts has shown to be critical for pathogen resistance from a single gene locus.[24]

Modular Domain Structure

Ligand Binding Domain of NLRs—Leucine Rich Repeats (LRR)

It has been suggested by bioinformatical modeling that the LRRs of the NLRs form a horseshoe-like structure capable of interacting with specific protein, carbohydrate or lipid moieties of pathogenic or cellular origin. The prediction is based on the crystal structure solution of the prototypical LRR-containing ribonuclease inhibitor protein.[25] LRRs are formed by short repetitive leucine-rich protein motifs with a length of 20-29 amino acids and may serve

as protein interaction platforms or regulatory modules of protein activation.[26] The LRRs of NLRs are homologous to those seen in plant disease resistance proteins (R proteins) and TLRs, which form the sensor module for the recognition of PAMPs.

It is now well established that the minimal structures recognized by NOD1 and NOD2 are the peptidoglycan-derived peptides γ-d-glutamyl-meso-diaminopimelic acid (iE-DAP)[27,28] and muramyl dipeptide (MDP),[29,30] respectively. Albeit the evidence for a direct binding of the NOD1 and NOD2 ligands to the LRR domains is still missing, the sensing of MDP and iE-DAP is very specific and can be abolished by either mutations of critical residues within the LRRs or minimal changes to the ligand (e.g., stereoisomers MDP-LD/MDP-DD). Evidence is emerging that in most NLR genes, LRR exons may be subjected to extensive alternative splicing thus creating a variety of different transcripts encoding for different LRR regions. It is tempting to speculate that the encoded protein isoforms may recognize different PAMPs. Alternatively, the cellular ratio of the different isoforms may play a regulatory role in the signaling efficacy of NLR oligomers upon ligand stimulation.

NOD Domain

The NOD domain, also designated as NACHT (domain present in neuronal apoptosis inhibitor protein (NAIP), the major histocompatibility complex (MHC) transactivator (CIITA),HET-E and TP1),[31] belongs to the recently defined STAND family of P-loop NTPases. It has a sequence homology with the nucleotide-binding motif of apoptotic protease activating factor-1 (APAF-1), which is responsible for the dATP/ATP dependent oligomerization of APAF-1 upon cytochrome c sensing during intrinsic apoptotic processes. The oligomerized APAF-1 serves as a molecular platform inducing the recruitment and activation of pro-caspase-9, a process called induced proximity signalling (reviewed in ref. 32). It is thus tempting to speculate that the NOD domain of the NLR proteins is pivotally involved in the initiation of a cellular signal upon binding of the respective ligand. An intramolecular complex formation between the LRR and NOD domain has been proposed to inhibit autoactivation of NLRs.[33] This concept has recently been verified in the potato resistance protein Rx, which confers resistance to the potato virus X.[34] Constructs encoding for forms of NOD2, IPAF and NAIP without LRRs or point mutations of putative interaction sites render the proteins constitutively active, whereas small truncations within the LRR of NOD1 and NOD2 that may interfere with the muropeptide recognition lead to inactive protein species.[35] Disease-associated sequence variants in the NOD domain of NLRs are in close vicinity to conserved regions, e.g., certain NTPase motifs (Walker B Box), which may interfere with the cycle of nucleotide-binding, -hydrolysis, and -release and/or conformational changes induced by NTP-hydrolysis.[33] Albeit the profound biological consequences of NLR autoactivation (sustained inflammation) or inactive NLRs (impaired recognition of pathogens), NLR protein function must be tightly controlled to provide a delicate balance between the initiation and perpetuation of immune responses and anti-inflammatory mechanisms. Regulatory mechanisms may include negative feed-back loops abolishing NF-κB activation (NOD2)[36] or the induction of splice variants encoding for inhibitory "dominant-negative" protein isoforms (NOD1/NOD2).[37,21]

Effector Binding Domains

The signals of NLR activation are transferred into the cell via three distinct N-terminal effector binding domains, which also constitute the NLR subfamilies: NODs carry the caspase recruitment domain (CARD), NALPs are characterized by a pyrin domain (PYD), whereas baculovirus inhibitor of apoptosis protein repeats (BIR) are characteristic of NAIP. PYD and CARD are members of the death domain-fold superfamily that also includes death domains (DD) and death effector domains (DED), which are found in receptors and adaptors of death-ligand pathways.

The diseases linked to the NLR family members NOD1 and NOD2 are discussed below. The genes, polymorphisms and associated diseases have been summarized in Table 1.

Table 1. Selected NLRs and associated diseases

NLR Protein	Synonyms	Locus	Associated Disease	First Description in	Region Affected by Mutation	Assumed Defect
NOD1	CARD4	7p14	Atopic eczema/Asthma	Hysi et al, 2005, Weidinger et al., 2005	Intronic SNP	???
			Crohn's disease	pos association.:McGovern et al., 2005		
NOD2	CARD15	16q12	Crohn's disease	Hugot et al., 2001, Ogura et al., 2001, Hampe et al., 2001	LRR	Impaired NF-κB activation by MDP
			Atopy-related traits	Kabesch et al., 2003; Weidinger et al., 2005 Miceli et al., 2001	LRR	
			Blau syndrome Early- onset sarcoidosis	Kanazawa et al., 2005	NBD	Enhanced basal NF-κB activation
NALP3 CIAS1	PYPAF, Cryopyrin,	1q44	Muckle- wells syndrome CINCA=NOMID FCU Feldmann et al. 2002	Hoffman et al., 2001 Dode et al.,2002	NBD	Increased basal NF-κB activation and IL-1β release (by dysregulated Caspase-1 activation) Cold-triggered activation

CINCA, Chronic infantile neurological cutaneous and articular syndrome; NOMID, neonatal-onset multisystem inflammatory disease; FCU, familial cold urticaria.

NLRs and the Recent Rise of Barrier Diseases

Changing living conditions have led to the appearance of numerous mucosal inflammatory diseases in industrialized nations which, before the 20th century, were either rare or completely absent from these populations.[38] The most striking example is Crohn's disease, which was unknown until 1920[39] and currently has a life-time prevalence of up to 0.15% in North-West Europe and North America,[40] and atopic eczema and asthma, which affect up to 20% of children in the same countries. It is now widely recognized that inflammatory diseases of barrier organs share some key characteristics including an onset during childhood or early adolescence, a relapsing inflammatory pattern, and an overlap between organ manifestations. Furthermore, regions of genetic linkage that have been identified through independent studies of these diseases are often found to overlap. The extraordinary role of NLRs in innate immunity is emphasized by the remarkable association of polymorphisms in *NLR* genes with human barrier diseases. Polygenic diseases associated with variations in NLRs include Crohn's disease, atopic disease and asthma, which are characterized by chronic relapsing-remitting inflammation of barrier organs (intestine, lung, skin). Other rare autoinflammatory diseases caused by mutations in *NLR* genes (Blau syndrome, CINCA, Muckle-Wells syndrome, familial cold urticaria, early-onset sarcoidosis) or immune defects (Bare Lymphocyte syndrome) follow classical Mendelian modes of inheritance and have contributed significantly to our current understanding of NLR physiology. Interestingly, the suggested association between certain forms of cancer (breast and colonic carcinoma, gastric MALT lymphoma) and germline mutations in *NOD2* further corroborates the notion that chronic inflammation induced by perturbed epithelial barrier function may contribute to the etiology of malignant diseases. These studies on *NLRs* have shown that genetic factors affecting barrier function may precipitate into different human diseases and have led to the realization that the traditional, organ-based schemes of clinical classification in chronic inflammation and autoimmunity may become questionable in the era of molecular medicine. It will be interesting to define environmental conditions and polygenic interactions involved in the different pathophysiologies (e.g., *NOD2* in Crohn's disease, atopic dermatitis and asthma).

NOD2

Single nucleotide polymorphisms (SNPs) within the LRR (L1007fsinsC, R702W and G908R) are associated with an increased risk for the development of Crohn's disease (CD).[2-4] CD is a human chronic relapsing-remitting inflammatory bowel disease characterized by granulomatous discontinuous inflammatory lesions in the whole gastrointestinal tract.[41] The symptoms include abdominal pain, (bloody) diarrhea and complications such as growth retardation in children, anemia, toxic megacolon and stenosis and fistulae. The genetic component of CD has been shown by consistent familial clustering and the phenotype concordance of the disease among monozygotic twins (concordance rates of ~50%) as compared to dizygotic twins (concordance rates of 4%). However, how impairment of the NOD2 sensor domains results in a widespread NF-κB activation within the mucosa of CD patients is not completely elucidated. Cells expressing the CD-associated mutations lack an appropriate NF-κB activation upon MDP-stimulation.[3,42] A long recognized phenomenon is the immunostimulatory role of the NOD2-ligand MDP. A pro-inflammatory cytokine bias after stimulation of *NOD2* defective mononuclear cells with TLR2 stimuli[43] (i.e., an inhibitory influence of NOD2 on TLR signaling) as well as the synergistic activation of pro-inflammatory pathways by TLR2 and wild type NOD2 in primary monocytes has been described.[44] An alternative mechanistic hypothesis to explain the inflammatory pathophysiology of CD in *NOD2* deficient individuals has recently been introduced by a transgenic murine model in which a variant equivalent to the human 1007fsInsC was introduced.[45] In this model a direct augmentation of NF-κB signaling and an impact on processing of IL-1β by the truncated NOD2 was observed. However, it is likely that the CD-associated NOD2 variants cause a defect in early (epithelial) immune recognition and pathogen clearance. This view is supported by the increased presence of culturable bacteria in draining lymph nodes from CD patients and abnormal adaptive immune responses to microbial antigens (Fig. 2).

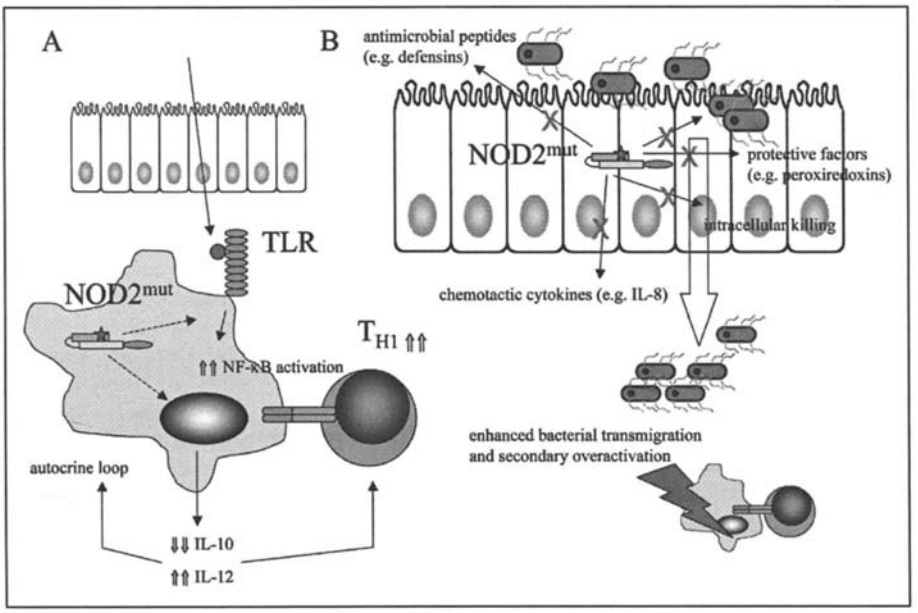

Figure 2. NOD2 and barrier organ function Different hypotheses how mutant NOD2 may to overactivation of the immune system are shown. A) This hypothesis focuses on the role of NOD2 as a negative immune regulator in professional antigen-presenting cells. A lack of inhibitory signals (direct or indirect via IL-10) leads to increased inflammatory signaling (e.g., via TLRs) and (over-) activation of adaptive immune responses. B) The barrier hypothesis suggests that the primary defect is within the epithelial defect to recognize and fight invading bacteria.

Blau syndrome (BS) is a rare autosomal dominant disorder characterized by early-onset granulomatous inflammation (arthritis, uveitis, skin), visceral involvement and camptodactyly.[46] CD and BS share the histopathological hallmark of epithelioid granulomas. Miceli-Richard et al[47] investigated the mutational spectrum of *CARD15* in families with BS. The identified mutations R334Q, L469F and R334W are in close vicinity to the Mg^{2+} binding sites of the NOD/NACHT domain and cause an increased basal activation of NF-κB ("gain of function").[48]

The closely related EOS (early-onset sarcoidosis) shares with BS the distinct triad of skin, joint, and eye inflammation. Sarcoidosis is an inflammatory disease characterized by noncaseating epithelioid granulomas that may follow an acute or chronic clinical course. Two distinct types of infantile sarcoidosis have been described. In older children the clinical manifestations are comparable to adult sarcoidosis and often comprise the classical triad of lung, lymph node, and eye involvement. However, EOS can be found nearly exclusively in children younger than 4 years of age, is quite rare and affects skin, joint, and eyes, without apparent pulmonary involvement. EOS is progressive, and in many cases, causes severe complications, e.g., destructive arthropathy or blindness. Interestingly, the majority of the analyzed cases also had heterozygous mutations in the *NOD2* gene, whereas no association of *NOD2* mutations could be detected for adult sarcoidosis. Thus, EOS shares with BS a common genetic etiology of mutations that cause constitutive NF-κB activation.[6,49]

Intensely pruritic skin lesions of typical morphology and distribution are the characteristic clinical manifestation of atopic dermatitis (AD), which is a frequent chronic inflammatory disease of the cutaneous barrier. In industrialized countries, a steep increase of the prevalence of AD in childhood (presently approx. 15%) has been observed over the last decades. AD is an important manifestation of atopy, which characterized by the formation high IgE titers specific for ubiquitously present antigens ("allergens") and also comprises asthma and allergic rhinoconjunctivitis.

It was shown that the CD-associated polymorphisms in the *NOD2* gene are also significantly associated with a increased risk for atopic diseases and high serum IgE levels (Hyper IgE syndrome).[50-52] The data emphasize the view that NOD2 may play a crucial role as a guarding molecule for host/bacterial interactions on many body surfaces, including the gut. It is tempting to speculate that the precipitation into a given NOD2-associated disease in individual patients depends on the dynamics of the individual immunological interaction with the resident bacterial metagenome. Therefore, further studies are required in order to identify disease-specific bacterial signatures in NOD2-mutated individuals at different body surfaces.

Loss-of-function mutations in the *NOD2* gene have been associated with an increased risk for the development of different types of cancer (colonic adenocarcinoma, breast and lung cancer and *H. pylori*-induced MALT lymphoma).[53-58] It has become increasingly clear that chronic inflammatory reactions, e.g., those induced by persistent mucosal colonization by *H. pylori* or physicochemical noxa, are among the strongest inducers of cancers in humans. A sustained activation of pro-inflammatory transcription factors, e.g., NF-κB, can promote malignant transformation via the induction of anti-apoptotic proteins or increased cellular proliferation.[59] Individual virulence factors of pathogens as well as the type of the inflammatory response may further contribute to clinical outcomes. Deficient NOD2-signalling due to genetic or nongenetic factors may contribute to the etiopathogenesis of malignant disease not only via impaired pathogen recognition, but also via a perturbed bacterial clearance, as NOD2 has been shown to serve as a direct and indirect anti-bacterial factor. It will be important to gain more insight into the role of NOD2 in malignant diseases, especially since the involvement of NOD1 and NOD2 in apoptosis is still controversial.[10,60]

NOD1-Associated Diseases

A systematic haplotype-based report could demonstrate no significant association of the *NOD1/CARD4* region with CD in a French population.[61] Recent reports demonstrated the association of an intronic SNP of unknown function with an increased risk for CD in a cohort from the North of Great Britain.[62] The same polymorphism was shown to confer susceptibility to childhood asthma and high IgE levels.[63] No coding SNPs have shown to be associated with either CD or UC.

This seemingly contradictory findings can probably be explained by regionally different bacterial signatures encountered (e.g., Scotland vs. Southern Europe) during the priming period of the host immune system in early adulthood, which is critical for induction of tolerance to commensal bacteria.[64] In line with these findings, Norwegian and German CD patients have been found to exhibit strong heterogeneity in the variants of the *NOD2* locus. No significant association of the described SNPs with CD in the Norwegian samples was detected and, except for a cohort from Iceland, the population attributable risk percentage (PAR%) for *NOD2* variants in the Norwegian cohort is the lowest reported for a European population (1.88%). These results appear to suggest an emerging pattern of a low frequency of the *NOD2* variants in Northern countries, which nevertheless have the greatest prevalence of CD in Europe. As different parts of the armament of the innate immune system could be necessary to perform the same task in response to varying microbial metagenomes, mutations in different NLRs could lead to the same disease phenotype under different environmental conditions.

Plasticity of NLR Function

NLRs are archetypal molecules for the understanding of the dynamic evolutionary remodeling of the innate immune system in barrier organs under selection pressure. Epithelial barriers shielding complex metazoan organisms against environmental stress or invading microbiota are a pivotal step in the diversification of life forms. Plasticity of the interface structures and interspecies signaling allow a complex interaction of different organisms, such as biofilm formation, quorum sensing and commensalism. The maintenance of epithelial barrier integrity is a key element for the survival of higher organisms, and probably no other physiological system has undergone more stringent selection during evolution.

The epithelial lining of barrier organs has long been regarded as a passive defense component, merely providing a physical barrier and controlling transcellular transport. Over the last years, it has become imminent that epithelial cells build up the first line of defense against invading pathogens and therefore constitute an integral part of the innate immune system of the barrier organs (reviewed in refs. 65,66). Intestinal epithelial cells express PAMP receptors and, although physiologically tolerant to commensals, recognize invading and adherent pathogens.[67-71] This recognition activates complex cellular programs necessary for a full immune response against the pathogenic threat.

Lessons from NOD1 and NOD2

The extent of evolutionary conservation of NLR genes is contrasted by their high degree of sequence variability in humans. A proof that plasticity in NLRs at the sequence level contributes has been advantageous, as it has been described for major histocompatibility loci in geographical regions with documented epidemics of bubonic plague, is yet to be described. However, it has been estimated that the SNPs in *NOD2* that are associated with CD have arisen some 40,000 years ago.[72] It is very unlikely that such functional variability would have been retained over such a long period of time without any evolutionary pressure favoring it.[73] Therefore, the specific sequence variability observed in genes encoding NLR proteins may be the result of specific evolutionary challenges and, paradoxically, some of the adaptive changes may have become detrimental through a drastic change in today's life conditions. The characterization of the variation in barrier genes and of their role in the pathophysiology of different species under different evolutionary pressures will thus provide a new way of understanding the mechanisms of disease.

The highest degree of plasticity of NLR function is not achieved at the level of sequence variation. NLRs display a complex regulation through the generation of different protein isoforms from a given nucleotide sequence, modulation of protein expression and protein-protein interaction. Regulated expression of barrier genes contributes to the generation of unique cell identities and function. The low expression level of NOD2 in colonic IECs can be upregulated by pro-inflammatory cytokines both in vitro and in vivo. NOD2 has been implicated as a direct anti-bacterial factor in IECs.[74] NOD2 over-expression sensitizes intestinal epithelial cells to bacterial cell wall components and induces the release of the chemotactic cytokine IL-8,[71] which is the strongest known activator of neutrophils. An impairment in NOD2 function, as in CD, would therefore lead to a complex defect including facilitated entry of bacteria into epithelial cells through defective regulation of antimicrobial peptides,[75] impaired bactericidal capacity and reduced epithelial immune defense. Only a small part of this complex program, also including with antioxidant properties (peroxiredoxins), has been characterized in detail.[76]

Differential recruitment of adaptor proteins and the formation of isotypic and heterotypic protein complexes may be pivotal to the modulation of biological responses. A prominent feature of *NLR* genes is the existence of multiple alternatively spliced transcripts and thus these genes seem to be good candidates to answer the question how genetic variability and variable splicing patterns contribute to disease pathophysiology. Context-specific regulation of mRNA splicing has been recognized as one of the key elements of proteome plasticity.[77] In *R*-genes of plants, for example, ratios of certain splice isoforms change dramatically upon pathogen invasion and the isotypic interaction of the encoded proteins seems to be mandatory for effective immune responses.[24,78] SNPs in conserved donor or acceptor sites may cause the skipping of whole exons or the unmasking of cryptic splice sites, thereby leading to profound changes in protein structures.[79] We have recently described tandem splice acceptors and donors (NAGNAG and GYNGYN, where N stands for any nucleotide), which provide a mechanism for subtle changes of the proteome by generating single amino-acid insertions or deletions. Tandem acceptor and donor sites can be found in 30% of human genes, including NLR genes (e.g., *NOD1, NOD2, NALP1, NALP2* and *NALP6*)[80,81] and affect functionally relevant protein domains more often than expected by chance alone. SNP-dependent splicing, which may lead to small or drastic changes at the protein level, may represent an evolutionary mechanism to

increase proteome plasticity. The strong selective pressure assumed for innate immune genes such as NLRs renders them ideal candidates for the investigation of this intriguing hypothesis.

Conclusion

The identification of the *NLR* gene family has led to exciting new insights both into fundamental cellular processes involved in innate immunity and the etiopathogenesis of chronic inflammatory barrier disorders. The association of SNPs in *NLR* genes with seemingly distinct disease entities such as CD and asthma has also helped realize that conventional organ-based classifications of human diseases may require reconsideration in the post-genome era. Disease-associated genetic variations in NLRs may have been important elements to provide plasticity to antigen recognition and host defense in the past. It has been demonstrated that genetic variations leading to defects of barrier function in humans play a pivotal role in the pathogenesis of acute and chronic inflammatory disorders. The broad spectrum of diseases linked to barrier dysfunction from acute bacterial infections to atopy and CD poses a major burden for healthcare systems worldwide. Thus, it may be expected that the field of NLRs may serve as an prototype how a comprehensive understanding of an element of the immunological barrier will eventually lead to the development of targeted diagnostic, therapeutic and/or preventive strategies.

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CHAPTER 4

Toll-Like Receptors and NOD-Like Receptors: Domain Architecture and Cellular Signalling

Tanja Langefeld, Walid Mohamed, Rohit Ghai and Trinad Chakraborty*

Abstract

The innate immune system forms the first line of defense against pathogens. The Toll-like receptors and the Nod-like receptors are at the forefront of both extracellular and intracellular pathogen recognition. They recognize the most conserved structures of microbes and initiate the response to infection. In addition to the microbial stimuli, they are now also being implicated in the recognition of danger-associated stimuli, making them pivotal in disorders unrelated to microbial pathogenesis. Toll-like receptors and the Nod-like receptors share commonalities in structure, ligands and downstream signalling but they differ in their localization, and extent of influence on a wide variety of cellular processes including apoptosis. Here we discuss the common ligand recognition and signalling modules in both these classes of receptors.

Introduction

Vertebrate immunity is broadly made up of two complementary structures, the evolutionarily ancient, fast responding, innate immune system that utilizes genetically fixed receptors, and the delayed adaptive immune system based upon clonal selection of antigen-specific receptors. The innate immune system recognizes invariant features of pathogens, and is crucial in initiating the adaptive immune response when the first line of defense fails to eliminate the infection. Only the adaptive immune system is able to provide lasting immunological memory that provides protection against re-infection. However, adaptive immune responses fail if the innate immune system is compromised. Both the innate and the adaptive immune systems employ a variety of receptors and effector mechanisms, in a controlled, temporal fashion to detect, control, and destroy a huge variety of pathogens seeking to inhabit the body. The Toll-like receptors (TLRs) and the NOD-like receptors (NLRs) are the key receptor molecules of the innate immune system, providing for constant surveillance of the extracellular and intracellular environments respectively. Here we dwell on domain organization and signalling pathways that involve TLRs and NLRs (Fig. 1).

Toll-Like Receptors

In 1997 the discovery of human Toll-Like Receptors (TLRs) based on toll receptors in fruit fly *Drosophila melanogaster* has substantially changed the knowledge of pathogen recognition.[1] "Toll" was first identified as a protein important in early stages of development in Drosophila. Later on it was discovered that Toll signals to Dorsal (like mammalian NF-κB) and is required for coordinating antifungal and antibacterial responses.

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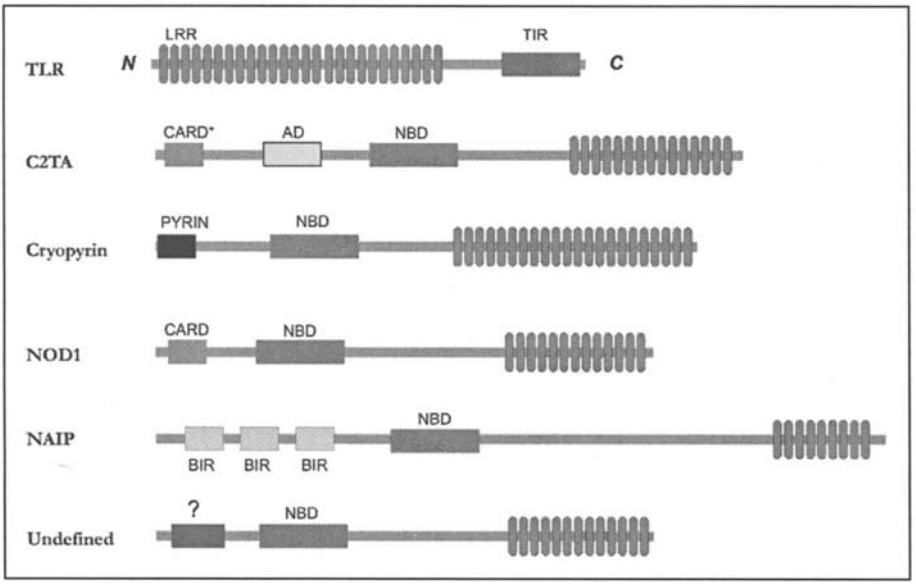

Figure 1. Domain structures of pathogen recognition receptors, TLR and NLR. All TLRs have an extracellular Leucine rich repeat (LRR) domain at the N-terminus and an intracellular Toll-interleukin receptor like (TIR) domain at the C-terminus. TLR4 is shown here as a representative of this family. Members of the NLR family are characterized by the presence of a nucleotide-binding domain (NBD) and a LRR domain at the C-terminus. The N-terminal region of these proteins is highly variable and may have various domains like CARD, PYRIN, or BIR. * C2TA contains an atypical CARD domain. TLR: Toll-like receptor; C2TA: Class II MHC transactivator; NOD1: nucleotide-binding oligomerization domain containing 1; NAIP: neuronal apoptosis inhibitory protein.

TLRs are Type I transmembrane receptors that activate innate immunity by sensing for Pathogen-Associated Molecular Patterns (PAMPs), conserved molecular structures consistently found on pathogens.[2] TLRs can thus discriminate the harmful microbial pathogens from "self" which is an integral feature of the innate immune system. TLRs are genetically fixed molecules, in contrast to the B and T cell receptors of adaptive immunity. They constitute the first line of defense against many pathogens. Therefore, they are mainly distributed among cells involved in the first line of defense against pathogens like dendritic cells, macrophages, neutrophils, dermal endothelial cells and mucosal epithelial cells. A few, TLRs (TLR2 and TLR4) are also expressed on B and T cells, thus directly affecting the development of the adaptive immune response.[3] In all, 11 members of the TLR familiy have been recognized in humans till now (named simply TLR1 to TLR11) with common molecular features[4](Fig. 1): (1) N-terminal multiple leucine rich repeats (LRRs), (2) a short transmembrane region, and (3) a conserved carboxy-terminal cytoplasm domain, highly homologous among the individual TLRs and contains a Toll/Interleukin-1 receptor (TIR) domain, similar to the cytoplasmic domain of the interleukin-1 receptor (IL-1 R).

As both the IL-1R and the TLRs share the cytoplasmic signalling domain, this was one of the first clues that led scientists to think that both molecules might be involved in the highly conserved signalling pathway for inflammation in eukaryotes, from the mitogen-activated protein kinases (MAPK), leading to the activation of the transcription factor nuclear factor kappa B (NF-κB). This was subsequently shown to be the case, and TLRs were recognized as crucial receptors for initiating the inflammatory response.[5]

Table 1. Toll-like receptors

TLR	Localisation	Ligand	Activated Cascade	Mutation/ Polymorphism
TLR1/TLR2	extracellular	Triacyl lipopeptides, lipoarabinomannan (LAM) from Mycobacterium, zymosan of yeast	Myd88 dependent	Susceptibility to tuberculosis and acute rheumatic fever
TLR2/TLR6	extracellular	Diacyl lipopeptides, Lipoteichoic acid (LTA), peptidoglycan, zymosan of yeast, glucosylphosphatidyl inositol linked proteins		
TLR3	endosomal	poly (I:C) double stranded RNA	Myd88-independent TRIF	
TLR4	extracellular	Gram-negative bacterial LPS, heat shock proteins, fibrinogen	Myd88-dependent TIRAP, Myd88-independent TRIF/TRAM	LPS hyporesponsiveness, susceptibility to malaria and meningococcal diseases, osteomyelitis, Crohn's disease and ulcerative colitis
TLR5	extracellular	flagellin	Myd88-dependent IRAK	linked to heightened Legionnaires' disease
TLR7	endosomal	Single-stranded RNA	Myd88-dependent IRAK	
TLR8	endosomal	Single-stranded RNA	Myd88-dependent IRAK	
TLR9	endosomal	CpG motifs DNAs, bacterial and viral, (unmethylated cytidine-guanosine dinucleotides)	Myd88-dependent IRAK	
TLR10	extracellular	Unknown		
TLR11	extracellular	Protozoan profilin-like protein, uropathogenic bacteria	Myd88-dependent IRAK	

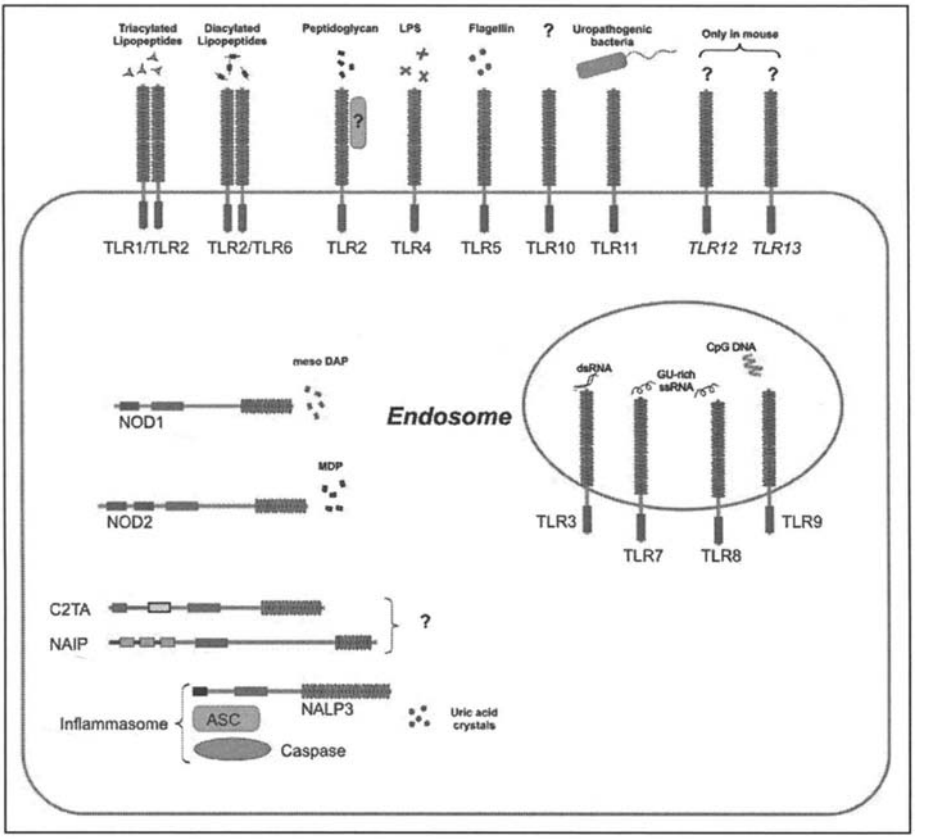

Figure 2. Known ligands for members of the TLR and the NLR family. TLRs sense pathogens extracellularly or in endosomes, while the NLR family members patrol the cytosol. LPS: lipopolysaccharide; mesoDAP: meso-diaminopimelic acid; MDP: muramyl dipeptide; ASC: also called Pycard. See Table 1 for more details on ligands.

It is believed that the leucine rich repeats provide the scaffold for the recognition of the various PAMPs. The known PAMPs for each TLR are listed in Table 1. PAMPs recognition can be achieved by individual TLRs as homodimers or by heterodimerised TLRs like TLR2 which forms a heterodimer with TLR1 or TLR6, each dimer having a different ligand specificity. It has been shown that the diacylated bacterial lipopeptides are recognized by a TLR2/TLR6 heterodimer whereas triacylated lipopeptides are sensed by a TLR2/TLR1 heterodimer.[6] TLRs constantly survey extracellular fluids and endosomal compartments with their ligand binding domains, the leucine rich repeat motifs (Fig. 2).

In addition to known microbial/pathogen derived ligands, TLRs also sense other endogenous factors produced by stressed or damaged cells like heat-shock proteins (Hsps) or fibrinogen, which may be termed collectively as "danger-associated" signals.[7,8] These are also listed in Table 1.

TLR4 was the first human TLR for which a ligand was identified, lipopolysaccharide (LPS), which is present in cell walls of all Gram-negative bacteria. The potent inflammatory effects of LPS had been known long before its actual receptor was found. However, the recognition of LPS by TLR4 required two additional proteins, CD14, a glycosylphosphatidylinositol (GPI)-anchored protein that binds to LPS with extremely high affinity, and MD2, a small secreted protein that associates with the LRR domain of TLR4.

Myd88 Dependent TLR Signalling

TLR-dependent responses may be classified broadly into two types, Myd88 (myeloid differentiation primary response gene 88) dependent, and Myd88 independent (see Fig. 3). Myd88 dependent pathways were identified first. Myd88 is an adaptor protein molecule with two protein domains, a TIR domain and a death domain (DD). The TIR domain interacts with the cytoplasmic TIR domain of the TLRs, while the DD is responsible for downstream signalling. Several other TIR domain-containing adaptors as well as Myd88 independent pathways have subsequently been discovered (Fig. 3). All TLR molecules, except TLR3, have the ability to signal through Myd88. The Myd88 dependent pathway leads to the activation of NF-κB that

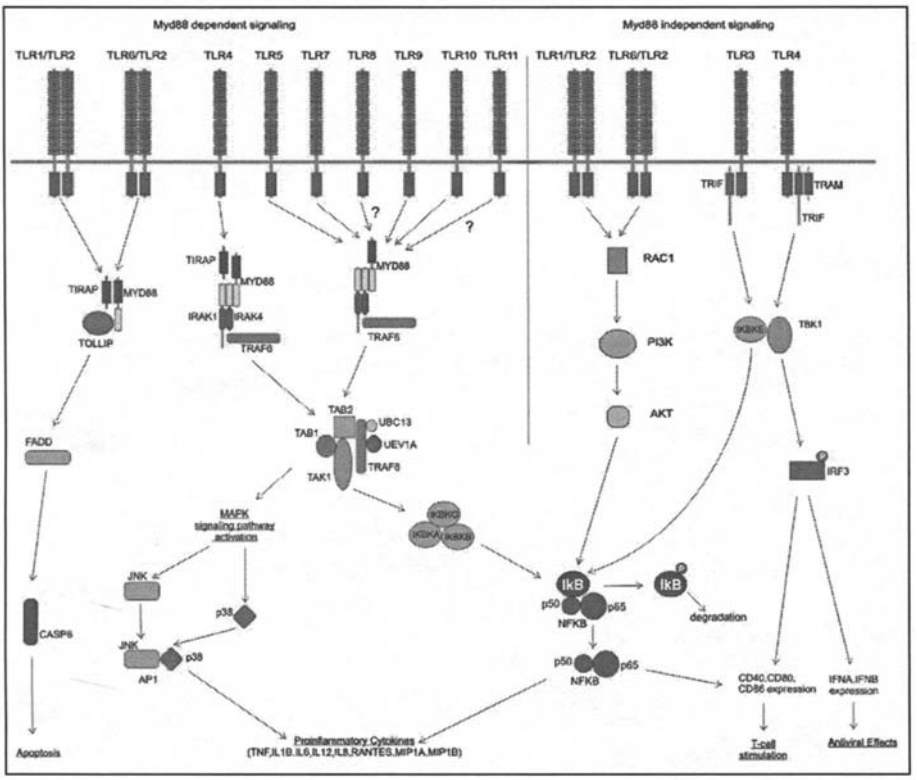

Figure 3. Myd88-dependent and Myd88-independent TLR signalling pathways. AKT-protein kinase B; CASP8: caspase 8; FADD: Fas (TNFRSF6)-associated via death domain; IKBκA: NF-kappa-B inhibitor kinase alpha; IKBKE: inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon; IRAK4: interleukin-1 receptor-associated kinase 4; IKBKB: NF-kappa-B inhibitor kinase beta; IKBKγ: NF-kappa-B inhibitor kinase gamma; IRAK1: interleukin-1 receptor-associated kinase 1; IRF3: interferon regulatory factor 3; MIP1α: macrophage inflammatory protein 1 alpha; MIP1β: macrophage inflammatory protein 1 beta; PI3K: phosphatidylinositol 3-kinase; RAC1: ras-related C3 botulinum toxin substrate 1; RANTES: chemokine (C-C motif) ligand 5; TAB1: mitogen-activated protein kinase 7 interacting protein 1; TAB2-: mitogen-activated protein kinase 7 interacting protein 2; TAK1: mitogen-activated protein kinase kinase kinase 7; TBK1: TANK-binding kinase 1; TIRAP: toll-interleukin 1 receptor (TIR) domain containing adaptor protein; TOLLIP: toll interacting protein; TRAF6: TNF receptor-associated factor 6; TRAM: toll-like receptor adaptor molecule 2; TRIF: toll-like receptor adaptor molecule 1; UBC13: ubiquitin-conjugating enzyme E2N; UEV1A: ubiquitin-conjugating enzyme E2 variant 1.

culminates in the production of proinflammatory cytokines like interleukin 1-B (IL-1B) and tumour necrosis factor alpha (TNF-α). Myd88 dependent signalling in many cases converges to the activation of a complex of TRAF6, TAB1, TAB2, TAK1, UBC13 and UEV1A (see Fig. 3 legend for full names). The TIR domain of Myd88 binds to the cytoplasmic TIR domain of the TLRs. TRAF6, IRAK1 and IRAK4 are recruited to this complex, where IRAK4 and Myd88 associate with their death domains. IRAK1 is phosphorylated by IRAK4. Subsequently, the phosphorylated IRAK1 dissociates from the receptor along with TRAF6, and then TRAF6 interacts with TAK1, TAB1 and TAB2. UBC13 and UEV1A are also recruited to this complex, and TAK1 is activated. The activated TAK1 phosphorylates the IKK complex, and can also activate the MAP kinase signalling pathway. These pathways lead to the activation of transcription factors NF-κB and AP-1, and the production of proinflammatory cytokines such as TNF and IL6.

Myd88 Independent TLR Signalling

TLR3 uses an alternative pathway using an adaptor molecule different from Myd88. TLR4, TLR1/TLR2 and TLR6/TLR2 can also signal without requiring Myd88, and indeed, without any other adaptor molecule like Myd88. In addition, TLR3 and TLR4 are unique among TLR proteins in that they can initiate a second signalling through a Myd88-independent pathway. This alternative pathway uses an adaptor protein named TRIF (Toll-like receptor adaptor molecule 1) to activate the TBK-1 (Tank Binding Kinase 1), which can activate NF-κB and interferon regulatory factor-3 (IRF3). IRF3 controls the production of interferon alpha (IFN-α) and interferon beta (IFN-β) that are known to enhance antiviral immunity.

These pathways are also discussed in more detail in the earlier chapter. Since TLRs are such crucial connections between innate and adaptive immunity, it is not surprising that mutations in these molecules lead to increases susceptibility to a variety of microbial diseases.[9-16] Table 1 summarizes the currently known diseases caused by single nucleotide polymorphisms in TLR genes.

Nod-Like Receptors

After the discovery of TLRs an intracellular surveillance system for pathogen recognition was detected in early 2000, based on receptors resembling disease resistance proteins in plants.[17,18] Following the identification of additional components, a general picture has emerged regarding the domain structures of these proteins. They all possess a leucine-rich-repeat motif (LRR) at the C-terminus, a nucleotide-binding domain (NBD) in the middle, and a third, N-terminal effector domain, which forms a basis for classification of this family (Fig. 1).[19] Four known N-terminal domains are an acidic domain, a pyrin domain, a caspase activation and recruitment domain (CARD) and a baculovirus inhibitory repeat (BIR) domain. A minority of family members also contain undefined domains.

Nod-like receptors (NLRs) sense pathogen-associated microbiological patterns (PAMPs) in the cytoplasm of mammalian cells (Fig. 2). They are present in an inactive, autorepressed form, as the LRRs are folded back onto the NBD, inhibiting spontaneous oligomerization and activation of the NBD. The C-terminal LRR domain is believed to be the ligand-sensing motif, as in the case of the TLRs, with the ability to recognize conserved patterns or other ligands. Following direct or indirect binding of a PAMP to the LRR, the molecule undergoes a conformational rearrangement, exposing the NBD and thereby triggering oligomerization and activation. The downstream signals generated are dependent upon the type of the effector domain in the N-terminus. Upon activation the N-terminal effector domain is exposed, and downstream signalling is initiated through homophilic or heterophilic interactions.[20]

NLRs are crucial in the control of the cytokines inflammatory response, NF-κB-activation and likely cell death and survival. This indicates that these proteins are important regulators of the innate immune system. The NLR family has nearly 20 known members.[21] A few examples of some important NLRs, classified by their N-terminal activation domains, which link them to multiple pathways, are given below.

1. Acidic domain: As an activation domain, it forms a part of a transcription factor making contact with the initiation complex. The master regulator of major histocompatibility complex (MHC) Class II genes, C2TA (MHC Class II transactivator), is an NLR with an acidic activation domain. The ligand of C2TA has not been defined till now. It interacts with multiple transcription factors (TF) including RFX5 (regulatory factor X, 5), NF-Y (nuclear transcription factor Y) and CREB (cAMP responsive element binding protein), which leads to MHC gene transactivation.[22]
2. Pyrin domain (PYR) is involved in homophilic protein-protein-interactions. Pyrin domains belong to the death domain-fold family. Numerous proteins that are thought to function in signalling pathways of inflammation and those pathways causing cell death by apoptosis contain this domain. One important member is cryopyrin (NALP3/PYPAF1). Cryopyrin senses peptidoglycan (PGN), an essential structure in bacterial walls. After activation cryopyrin interacts through its pyrin domain with an apoptosis-associated speck-like protein (ASC) containing a CARD and a pyrin domain. In vitro studies have shown that this interaction leads to an activation of NF-κB. Cryopyrin can also interact through its NBD with CARD8 that activates caspase-1. Caspase-1 leads to a splitting of the immature form of IL-1β to produce the active form of the molecule. Once IL-1β is activated, it can be secreted out of the cell where it binds to the IL-1β receptor on other cells to trigger an immune response. The pyrin and the NBD domain are also involved in a protein-protein-interaction leading to a complex called *inflammasome* that regulates post-translational cytokine processing.[23]
3. CARD domain is also engaged in homophilic interactions with the prodomains of cysteine proteases (caspsases) and serves to recruit the inactive polypeptide precursors (Zymogens) of these enzymes to the receptor-adaptor protein. CARD proteins have been shown to activate transcription factor NF-κB. Two most well-known representatives of this subgroup are NOD1 and NOD2. They recognize PGN-derived products. NOD1 recognizes meso- diaminopimellic acid (meso-DAP). DAP is an amino acid, that can be found in PGN structures of all Gram-negative bacteria and a few Gram-positive bacteria such as *Listeria* and *Bacillus*. NOD1 can also recognize certain bacterial toxins. NOD2 recognizes muramyl dipeptide (MDP) present in Gram-negative and Gram-positive bacterial cell walls. NOD2, thus is a general bacterial sensor. If NOD1 and NOD2 are activated through binding to a ligand, they themselves bind to RICK (RIP2, receptor interacting serine-threonine kinase 2), a CARD-containing protein kinase, through a CARD-CARD-interaction. Now RICK links to the phosphorylation of IκB-a (inhibitor nuclear factor of kappa light chain gene enhancer in B-cells). The IKK (inhibitor of nuclear factor kappa B) complex converges and the bound, inactive NF-κB can translocate to the nucleus, where the transcription of inflammatory cytokines begins.[24]
4. BIR domain (BIR) is essential for inhibitory activity of IAP (inhibitory apoptosis proteins). It has been suggested that proteins with a BIR domain may also have other functions. NAIP (Neuronal apoptosis inhibitory protein) is an NLR with three BIR domains. It is reported that NAIP inhibits cell death in response to serum withdrawal, menadione and tumour necrosis factor (TNF). However, the exact activities and the ligands of NAIP proteins are unclear.[25]

Gene mutations in NLR proteins are predicted to be associated with an increased susceptibility to infection and therefore seem to lead to inflammatory diseases:[19]

1. CIITA: Mutations in the CIITA gene lead to the bare lymphocyte syndrome (BLS). This disease is characterized by a MHC-II deficiency that is followed by severe primary immunodeficiency in early childhood. Single nucleotide polymorphisms (SNPs) in the promoter of the gene are associated with multiple disorders with an immunological component, like rheumatoid arthritis and multiple sclerosis.[24]
2. Cryopyrin: Here mutations can lead to three autosomal-dominant diseases—familial cold autoinflammator sysndrome (FCAS), Muckle-Wells syndrome (MWS) and chronic infantile neurological cutaneous and articular syndrome (CINCA). Patients with these diseases show recurrent episodes of fever, tissue inflammation and skin rashes.[24]

3. NOD1 and NOD2: Genetic variation in NOD1 seems to predispose asthma in childhood and inflammatory bowel disease.[26] Mutations in the NOD2 gene have been implicated in inflammatory diseases such as Crohn's disease, a chronical inflammation of the intestinal tract; Blau syndrome, an early-onset granulomatosis with arthritis, uveitis and skin rashes; Atopy related traits and early onset sarcodiosis.[24]
4. NAIP: Mutations in NAIP have been linked to spinal muscular dystrophy.[27]

Conclusion

TLRs and NLRs have multifunctional role in pathogen-triggered immune response and represent an important connection between innate and adaptive immunity. They are part of an extensive surveillance system recognizing conserved, invariant features of pathogens, both extracellularly and intracellularly. Both types of receptors contain leucine-rich repeat motifs, which are believed to be responsible for ligand recognition. They also converge on the conserved inflammatory signalling pathway of NF-κB activation. In addition, there are examples of cross-talk among both systems (Fig. 4). Our knowledge of this extensive system of receptors is constantly increasing. Recently, a family of DexD/H box RNA helicases have been identified as cytoplasmic sensors of virally derived dsRNA, which also lead to activation of NF-κB and IRF3 activation. Although a number of questions remain to be answered, the elucidation of

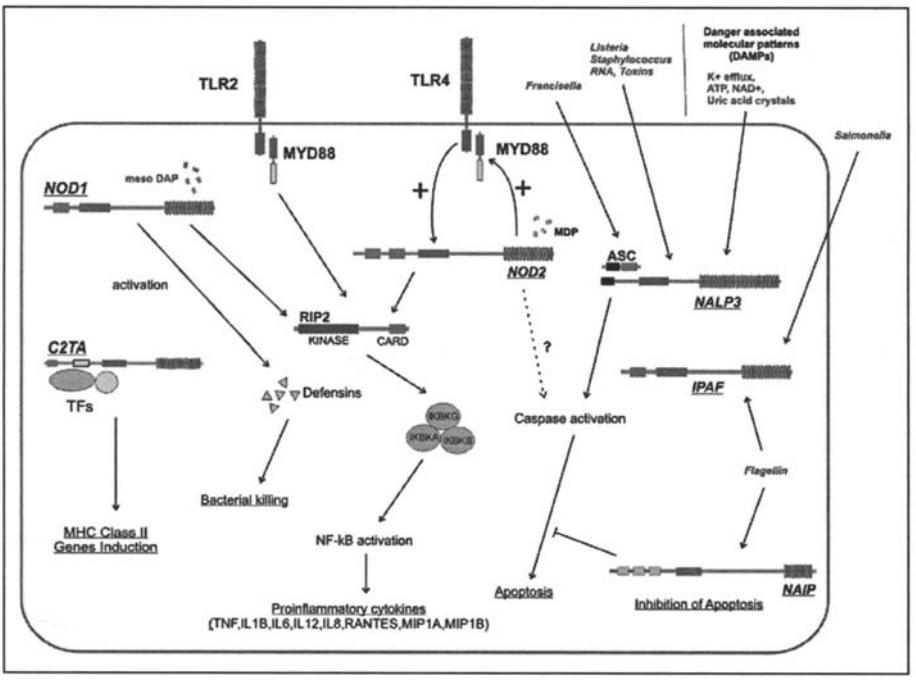

Figure 4. NLR pathways and cross talk with TLRs. NOD1 and NOD2 sense meso-DAP and MDP respectively. Both can activate the kinase RIP2 which leads to NF-κB activation. NOD1 can also activate defensins (short antimicrobial peptides), that can directly act upon intracellular pathogens. TLR and NLR pathways can also cross-link to each other, e.g., TLR2 can activate RIP2, while TLR4 expression is also known to enhance the expression of NOD2, which in turn increases the expression of Myd88 in a positive feedback loop. Other NLR family members are known to be involved in important processes, e.g., C2TA is the master regulator for MHC Class II gene induction. NALP3 associates with ASC and can activate caspases on recognition of Uric acid, while NAIP, activated by various stimuli, performs a protective, anti-apoptotic role in the cell (IPAF: ICE-protease activating factor).

these immune sensors and their downstream signalling mechanisms promises to provide us with new targets for treating several inflammatory disorders.

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CHAPTER 5

Humoral Pattern Recognition Molecules:
Mannan-Binding Lectin and Ficolins

Steffen Thiel and Mihaela Gadjeva*

Abstract

Innate immunity comprises a sophisticated network of molecules, which recognize pathogens, and effector molecules, working together to establish a quick and efficient immune response to infectious agents. Complement activation triggered by mannan binding lectin (MBL) or ficolins represents a beautiful example of this network. Both MBL and ficolins recognize specific chemical structures on the surface of antigens and pathogens, thus bind to a broad variety of pathogens. Once bound further complement deposition is achieved through a cascade of proteolytic reactions. MBL and ficolin induced complement activation is critical for adequate anti-bacterial, anti-fungal and anti-viral responses. This is well illustrated by numerous and convincing studies that demonstrate associations between MBL deficiency and infections. Recent work has also highlighted that MBL and ficolins recognize self-structures, thus extending the role of these molecules beyond the traditional view of first line defense molecules. It appears that MBL deficiency may modulate the prognosis of inflammatory and autoimmune diseases. What is known about the mechanisms behind this broad scope of activities of MBL and ficolins is discussed in this chapter.

Introduction

Complement activation is induced by a variety of pathogens and their ligands and proceeds through a series of enzymatic reactions that lead to the formation of lytic membrane attack complex (MAC) formation. There are three defined pathways of activation: the classical, the alternative and the lectin pathways. Each pathway is triggered by unique combination of initiating molecules that recognize a variety of different targets. The initiating molecule of the classical pathway is C1q. The classical pathway activation occurs when C1q binds to immunoglobulin coated antigens, whereas mannan-binding lectin (MBL) and ficolins are the initiating molecules of the lectin pathway. MBL and ficolins are highly promiscuous molecules, which recognize variety of bacteria, viruses, fungi or altered-self structures. MBL and ficolins recognize oligosaccharide-based or acetyl-based molecular patterns ("micropatterns"), specific for pathogens. All the initiating pathways converge at the level of C3 deposition on the target. This is followed by the assembly of MAC and subsequent target destruction (Fig. 1).

In addition to the complement-initiating activity, MBL exerts profound biological effect through MBL receptors. Several receptors are suggested so far. These are complement receptor 1 (CR1), the C1qRp phagocytic receptor and calreticulin.[1] Through these interactions MBL stimulates phagocytosis and modulates cytokine synthesis. In this chapter we will describe our

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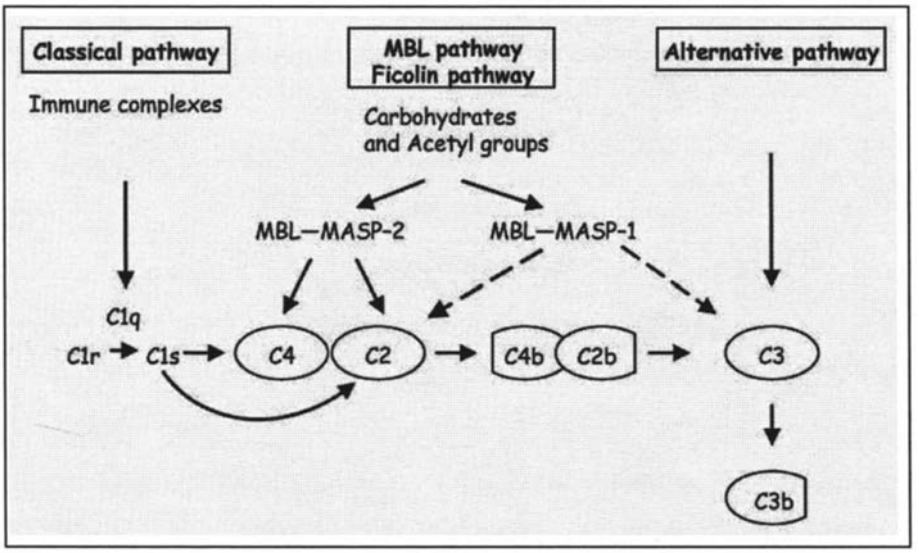

Figure 1. Schematic presentation of the initiating pathways of the complement system (adapted from Sorensen et al ref. 125). The classical pathway is activated by the binding of the C1 complex to deposited antibodies. MBL and Ficolin-dependent recognition of patterns of carbohydrates or acetyl groups trigger activation of complement through MASPs. This leads to cleavage of complement factors C4 and C2 and translates in activation of C3.

recent understanding of the biology and significance of MBL and ficolins as pattern recognition molecules. We will illustrate the importance of MBL and ficolins by few examples of MBL and ficolin interactions with bacteria, fungi, or viruses and the consequences hereof.

Biochemistry

MBL and Ficolins Are Pattern Recognition Molecules

MBL is a plasma protein with the ability to distinguish between self and non-self by recognising certain patterns of carbohydrate structures.[2] The MBL polypeptide chain is composed of collagen-like region linked to a carbohydrate recognition domain (CRD) via the neck region (Fig. 2). Three individual MBL polypeptide chains form a collagen triple-helix, stabilized by inter-chain disulphide bonds, resulting in a MBL monomer. Each MBL monomer comprises three extending CRDs. The individual monomers can associate to form higher oligomeric structures with different number of subunits. MBL dimers, trimers, tetramers and hexamers have been described.

Each CRD recognizes a minimal structural motif of a pair of adjacent equatorial monosaccharide hydroxyl groups. This "micropattern" is present in, e.g., mannose, N-acetyl-glucosamine (GlcNAc) or fucose, but not in galactose or sialic acids. Binding to the micropattern is metal ion-dependent. The MBL-bound calcium ion directly contacts both carbohydrate hydroxyls. Apart from the hydroxyl groups, the calcium ion is coordinated by a small set of conserved amino acid side chains. The mature MBL molecule is an oligomer of identical polypeptide chains (Fig. 2), and many CRDs will at the same time have the chance to react with "micropatterns". MBL, thus, binds to carbohydrates at two levels. Firstly, each CRD will recognise single oligosaccharide structures on the ligand. The dissociation constant of the binding between a single recombinant rat MBL-A CRD and a target monosaccharide indicates a very weak binding, i.e., estimated to be 1.5×10^{-3} M.[3] Secondly, ligands with multiple possible

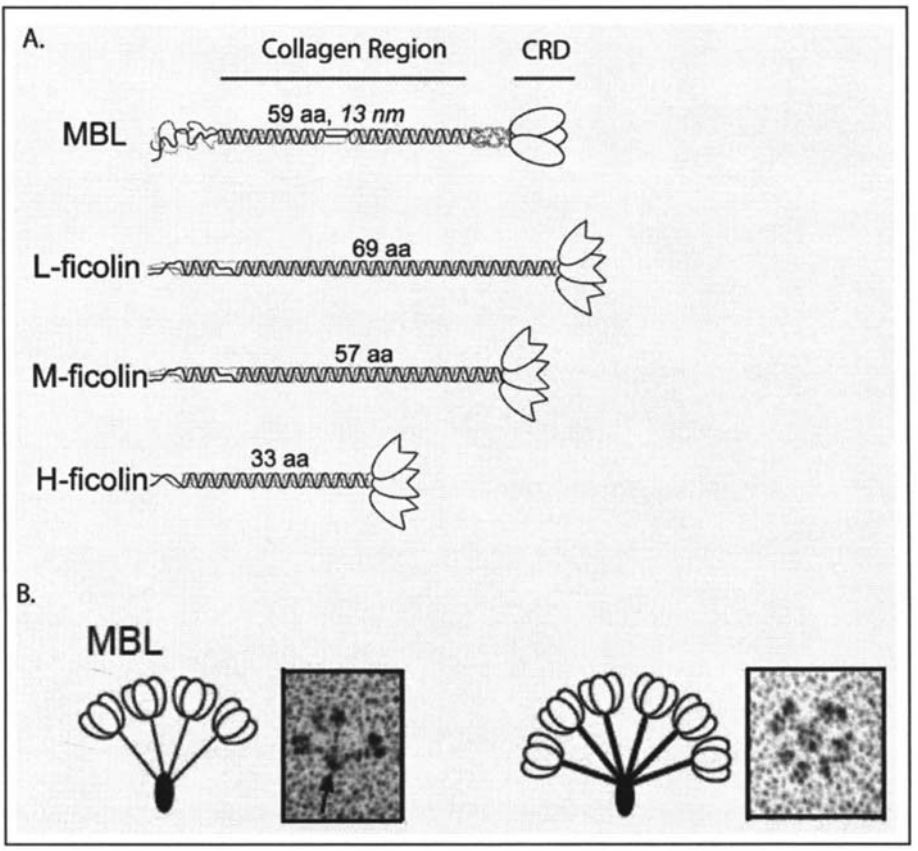

Figure 2. MBL and ficolins (adapted from Holmskov et al ref. 126). Panel A) The individual subunits of MBL and ficolins are schematically presented comprising the collagen region and the CRD. Panel B) High molecular weight MBL oligomeric structures: MBL tetramers and MBL hexamers. The schematic drawing and electron microscopy images are presented.

binding sites will have to fit the geometry of oligomeric CRDs. This will lead to a clustering of several CRDs onto a surface. The scatchard plot analysis demonstrated that the binding between native MBL and polyvalent carbohydrates on microorganisms occurs with a dissociation constants in the range of 10^{-9} M, i.e., which is comparable to the binding of antibodies to antigen.[4] It is important to realize that the combination of "micropatterns" and "macropatterns" (a combination of several micropatterns) allows for high affinity MBL binding to the ligand. MBL obtains sufficient binding strength only when a pattern that corresponds to the spatial orientation of the CRDs is present, e.g., as in mannan (a carbohydrate rich structure found in fungi).

Similar to MBL, ficolins were initially described as pattern recognition molecules recognising carbohydrate structures. It is today realized that a more complex set of ligands is preferred by ficolins. Three ficolins exists in primates: L-, M- and H-ficolin. Interestingly other animals only have two ficolins, with similarity to L- and M-ficolin. Ficolin molecules consist of collagen- and fibrinogen-like domains (named so due to its similarity to the C-terminal globular domains of fibrinogen polypeptide chains) (Fig. 2). The ligand binding activities of ficolins is assigned to the fibrinogen-like domain. L-ficolin and M-ficolins bind to acetylated

monosaccharides, e.g., GlcNac and Man NAc.[5,6] The binding specificity is due to the presence of the acetyl groups, rather than carbohydrate structures, thus begging the question if ficolins are, in fact, lectins. Since human L-ficolin is a tetramer, where each monomer is composed of three identical polypeptide chains, it binds to the targets by 12 individual CRDs. While H-ficolin is 49% identical to L-ficolin in primary structure, its tertiary structure is that of a hexamer of trimers. Recently, it has been proposed that M-ficolin possesses a similar structure to that of H-ficolin, based on the molecular size of the M-ficolin multimer (about 610kDa, or 18mer of 35 kDa). Much like in the case of MBL, the multiple binding of CRDs dramatically increases the affinity of interaction. A lectin binding activity of H-ficolin is even less absolute although it was suggested by its ability to agglutinate human erythorocytes coated with LPS from *Salmonella typhimurium* and inhibition by fucose and GlcNac.

The binding of ligands to L- and M-ficolin may be quite different from the recognition by H-ficolin (L-ficolin and H-ficolin only shares 53% sequence identity in their recognition domains). New crystal structures of ficolins in complex with ligands have been recently reported and will bring valuable structural information.[7]

MASPs Are Associated with MBL and Ficolins

MBL in plasma exists in complex with MBL associated serine proteases (MASPs), MASP-1,[8] MASP-2,[9] and MASP-3[10] and with a smaller splice variant of MASP-2 called MAp19 (sMAP).[11-13] Interestingly these associated proteins are not equally distributed among the different oligomers of MBL. High oligomeric structures of MBL are often found in association with MASP-2 and MASP-3 whereas lower oligomeric forms are found in complex with Map19. As MBL, L-ficolin, H-ficolin and M-ficolin are capable of activating complement via MASP-2 associations and recombinant L-ficolin and M-ficolin have been shown to form complexes with MASP-1, MASP-2 and MAp19.[14,15,16]

When MBL or ficolins bind to a fitting carbohydrate pattern, the MASPs are activated. The polypeptide chain of the MASP molecule is cleaved into A and B chains, resulting in an active enzyme.[17] Apart from MASP-2, it is not yet clear what the preferred substrates for the different MASPs are. However, it is believed that complement factors C4 and C2 are the physiologically relevant substrates for MASP-2. Activated MASP-2 cleaves C4 twenty times better than activated C1s, the other known efficient C4 cleaving enzyme, which is present in plasma in complex with C1q and C1r (C1 complex).[18] MASP-1 has been shown to be able to cleave with low activity complement factors C3 and C2, coagulation factor XIII, and fibrinogen.[19] All of the three MASPs cleave various synthetic substrates.[18,20] The activities of MASP-1 and MASP-2 are inhibited by C1 esterase inhibitor.[20-22]

The initiation of the complement system cascade subsequently leads to clearance of microorganisms via formation of a membrane attack complex (MAC) in the bacterial membrane, opsonophagocytosis, and/or enhanced attraction/activation of inflammatory cells. As MBL interaction with cells has been found to trigger a number of functions, several receptors for MBL were suggested, but there is no concordance as to the most relevant molecules (for further details see refs. 23,24).

MBL Genetic Polymorphisms Result in MBL Deficiency

The level of MBL in the blood is influenced by single nucleotide polymorphisms (SNPs) in the MBL gene (*mbl2*).[2] As illustrated in Figure 3, there are polymorphisms in the promoter region (X or Y) and in exon 1 (A (wild type), B, C and D). The mutations in exon 1 lead to disruption of the Gly-Xaa-Yaa pattern of the collagen region. Such a disordered collagen helix appears to act like a dominant feature and results in decreased circulating levels of MBL. The SNPs within exon 1 are: D52C (alleles A/D), G54D (alleles A/B), G57E (alleles (A/C) (Fig. 3). In addition, SNPs located at the 5' regulatory region of the MBL gene at -550 g/c (alleles H/L), - 221 c/g (alleles X/Y), and +4 c/t (alleles P/Q) are in linkage disequilibrium with exon 1 SNPs. Overall seven distinct haplotypes exist in humans, four of which

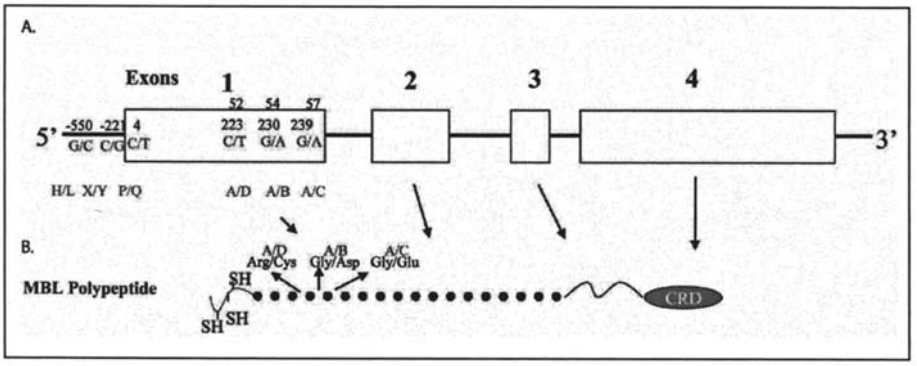

Figure 3. MBL genetics. Panel A) MBL gene. The location of the individual SNPs is pointed out. There are SNP located in the 5´region at positions (-550 and -221), one -in the untranslated region (at position 4) and three in the translated region of exon 1 (at positions 223, 230 and 239). The latter three SNPs lead to exchange of amino acids at positions 52, 54 and 57 respectively. Panel B) MBL polypeptide chain. The polypeptide chain is drawn as it is translated from the corresponding exons, the mutated amino acids are indicated.

(YB, YC, YD and XA) dictate low serum levels.[25] Haplotype occurrences vary in different human populations.[26,27] The different polymorphisms on structural and promoter levels strongly influence MBL protein levels. In plasma MBL may vary between 1 ng to 10 000 ng/ml. A total lack of MBL does not exist as a substantial amount of low oligomeric forms of MBL are detected in individuals with low MBL levels, however the low oligomeric forms of MBL fail to activate complement.[28] This broad variability of MBL plasma concentration poses the question how to define MBL deficiency. Today, humans with MBL levels less than 100 ng/ml are considered to be MBL-deficient.

Unlike humans, who have only one gene for MBL, there are two genes encoding MBL in mice: *Mbl1* and *Mbl2*.[29] They give rise to two isoforms: MBL-A and MBL-C. Both isotypes are capable in triggering of complement activation, yet MBL-A appears to be five fold more efficient than MBL-C. This difference in activity is balanced by MBL-C's higher levels in serum. 2 to 10 fold higher concentrations of MBL-C are detected.[30] No polymorphisms that modulate MBL-A or MBL-C structure and activity are described so far.

Ficolins Genetics and Polymorphisms

Ficolins were first discovered as TGF-β1-binding proteins on the uterine membranes of pigs. The cDNAs encoding two types of ficolins (α and β) were isolated and reported to share 83% identity.[31] Since then ficolins have been identified in humans, mice, hedgehog, *Xenopus* and *Ascidian*. The phylogenetic analysis of ficolins suggested that each ficolin might have diverged after the emergence of the respective lineage by gene duplication. This implies that evolutionary pressure has led to generation of two types of ficolins (serum type and nonserum type) within each species.[32] Three ficolin genes are identified in humans: *FCN1, FCN2* and *FCN3*. They encode ficolin-1 (synonymous to M-ficolin), ficolin-2 (synonymous to L-ficolin) and ficolin-3 (synonymous to H-ficolin). *FCN1* and *FCN2* are both located on chromosome 9q34, and are 80% homologous at the level of amino acid sequence. *FCN3* is assigned to chromosome 1 and is only 40% homologous to *FCN1* and *FCN2*.[33] *FCN2* is predominantly expressed in the liver. *FCN3* expression is found in the liver and lung, whereas *FCN1* is detected on the surface of monocytes.[34] Similar to humans, three ficolin genes are identified in mice. One of them is a pseudogene, whereas the rest encode for Ficolin A and Ficolin B. Ficolin B is the mouse orthologue of M-ficolin.[35]

As compared to the 10.000 fold difference in MBL levels the L-ficolin serum levels vary much less—only 10 fold from 1-14 μg/ml. Three SNPs located within the promoter region of L-ficolin gene (*FCN2)* are involved in lowering the circulating levels. Two additional SNPs, positioned within the coding region, lead to amino acid substitutions Thr^{236}Met and Ala ^{258}Ser, and are associated with either increased or decreased GlcNAc binding.[33,36] The M-ficolin gene contains more than 10 SNPs in the promoter and structural part of the gene.[32] No deficiencies or disease associations are currently reported to be associated with these polymorphisms. More than 100.000 individuals have been screened for presence of H-ficolin and no deficiencies have been found (except for a few cases where deficiency was due to the presence of anti-H-ficolin antibodies).[37]

Biology

Binding of MBL and Ficolins to Bacteria

In vitro experiments have identified a very long list of bacterial targets for MBL. These are different strains of Gram-positive and Gram-negative bacteria including but not limited to *Escherichia coli, Salmonella typhimurium, Salmonella typhi, Staphylococus aureus, Pseudomonas aruginosa, Haemophilus influenzae, Neiserria meningitides, N. gonorrhoea.*[38-45] While the functional importance of MBL-dependent recognition of bacteria is illustrated by animal infection studies and correlative clinical analysis for a few bacteria (see below), for many other bacterial targets the importance of MBL binding is less clear. For more comprehensive lists of MBL interaction with bacteria we refer to Eisen et al[46] and Turner.[2] The molecular nature of the ligands on the various MBL-binding bacteria has only been studied in very few cases, but it has been described that binding may be hindered by encapsulation of the bacterium or the addition of sialic acids to structures on the bacterial surfaces. Thus, various strains of one bacteria species may vary substantially with regards to binding of MBL.

Abundant number of studies suggested that MBL has a critical role in triggering antimicrobial responses. It was demonstrated that low MBL protein levels or low-coding MBL alleles may correlate with increased susceptibility to infection.[43] The initial observation was that an opsonic defect in serum, due to lack of MBL, correlated with a broad phenotype of recurrent infection in young children. Jensenius modified this concept and suggested that the phenotype of susceptibility to infection was more obvious when low MBL levels were present in conjunction with other immune deficiencies.[47]

The importance of MBL as a risk factor for recurrent bacterial infections is illustrated by the examples of cancer patients undergoing chemotherapy. Patients with suppressed cellular immune system, e.g., due to chemotherapy treatment, have higher number of infections and some of those patients tend to suffer from uncommon bacterial infections. It was speculated that under such conditions deficiencies in the innate immune system would be an important risk factor. In line with this bacterimia and pneumonia were associated with decreased MBL levels in chemotheraphy-treated hematological adult patients.[47] A correlation between longer time-of-fever and lower levels of MBL where noted among children with hematological malignancies.[48] Similarly, the presence of alleles giving rise to low MBL levels, were associated with bacterial, viral and fungal infections in patients after treatment with myoablative bone marrow transplantation conditioning regimes.[49]

In contrast, patients undergoing therapy for acute myeloid leukemia showed no association between infections and MBL levels.[50,51] When comparing the different cancer patients it becomes apparent that not only the underlying diseases are heterogenous, but also the chemotheraphy regimes are very different. Thus, no association was found when multiple myeloma patients undergoing induction treatment were analyzed for presence of variant MBL alleles and recurrent bacterial infections,[52] in contrast to when the same patient group were analyzed after bone marrow transplantation (and, thus, a more aggressive chemotherapy) where an association was found between severe infections and the presence of variant MBL alleles.[52]

The observations suggest that chemotherapy-induced neutropenic patients with low MBL levels appear more susceptible to infection in only certain treatment regimes. There are continuously ongoing prospective studies trying to pinpoint the specific groups of treatments and patients that show the highest dependence of MBL with regards to infections. MBL may be especially important with regards to bacteria that are normally not pathogenic, but can appear so if the individual is immunocompromised.

Another group of patients, where the immune system may be suppressed, is critically ill patients admitted to ICUs. Systemic inflammatory response syndrome (SIRS) may render the patients partly immunocompromised and may in some cases progress to sepsis and septic shock. When the frequencies of variant alleles were compared in adult SIRS patients it was prominent that higher levels of MBL may be important for avoiding sepsis and septic shock,[53] a finding that was also reflected in children with SIRS.[54] Lower MBL levels were observed in the group of nonsurvivors as compared to those who survived admission to ICUs.[55,56] MBL thus appears to predispose to serious infection and SIRS, but further larger studies are warranted.

MBL deficiency affects prognosis during septicemia. A frequent cause of mortality in burn victims are bacterial infections often caused by *P. aeruginosa*.[57] MBL null mice undergoing burn injury and *P. aeruginosa* infection had significantly higher titers of bacteria in circulation, kidneys and livers compared to wild types. These results indicate that MBL plays a role in bacterial clearance during sepsis. Similar observations were made for *S. aureus* infections. If MBL double KO mice were infected with *S. aureus i.v.*, they have faster death rate than the wild type controls.[58]

Like MBL the ficolins bind to various bacteria. The accumulated data reveal a long list of bacterial targets. In the case of L-ficolin these targets include Gram-positive bacteria such as serotype III of Group B Streptococci, and *S. aureus*, and Gram-negative bacteria such as *E. coli*, and *S. typhimurium*. M-ficolin, like L-ficolin, recognizes *S. aureus* and *S. typhimurium*, whereas H-ficolin does not. These observations suggest that ficolins share common bacterial targets. However, their biological functions are likely not redundant due to their tissue distribution. The L-ficolin is largely considered as serum ficolin, whereas the M-ficolin is a nonserum ficolin, found in the intracellular granules of monocytes, neutrophils, alveolar epithelial cells or on the cellular surface of peripheral blood monocytes.

MBL Binds to Viruses

Viruses have developed numerous ways to avoid being labeled as non-self, however, some of them are easily recognized by MBL (e.g., herpes simplex virus (HSV), human immunodeficiency virus (HIV), influenza A virus (IAV), etc). In most cases the interaction between MBL and viral lysates or virally infected cells is mediated through recognition of high -mannose glycans by MBL. For instance, it was shown that MBL recognizes an enveloped glycoprotein gp120 of HIV-1 in a sugar specific manner and that the interaction could be inhibited by mannan or N-glycanase treatment.[74,75] Moreover, the binding of MBL to gp120 or gp110 (the corresponding enveloped glycoprotein on HIV-2) can trigger complement activation, independently of C1q or antibodies.[76] Virus interaction and subsequent complement activation may be beneficial for anti-viral immunity, since it can lead to a better viral neutralization. Alternatively, complement receptor bearing cells may be better targets for viral infection. Thus, a huge interest exists towards clinical studies which attempt to correlate MBL protein levels and/or genotype with HIV infection. However, conflicting data is published showing either significantly more cases of MBL deficiency among HIV infected patients[77-79] or lack of correlation between MBL levels and susceptibility to HIV infection.[79-81]

MBL Binds to Fungi

Mannan is the major component of fungal cell wall. MBL binds avidly to fungi like *Candida albicans, Saccharomycis cerevisiae* and *Aspergillus fumigatus* and contributes to anti-fungal responses. The role of MBL in innate responses to Candida infection was indicated in 1995[82]

when MBL transgenic mice carrying the human MBL transgene were infected with *Canidida*. MBL may modulate anti-*Candida* responses by stimulating phagocytosis, respiratory burst and TNF-α synthesis by monocytes.[83,84] The TNF-α mediated effects are of vital importance for anti-*Candida* immunity, since the TNF deficient mice are highly susceptible to *Candida* infection.[85] MBL mediated opsonisation of *Candida* affects dendritic cell activation. MBL or C3 increases uptake of *Candida* through complement receptor 3 (CR3) and prevents upregulation of costimulatory molecules and IL-12 production.[86] Depending on the context of infection (MBL levels, presence of protective antibodies) MBL may modulate anti-*Candida* responses. Indeed, clinical correlative studies suggested that reduced MBL levels correlated to recurrent vaginal candidiases.[87]

The binding of MBL to bacteria may be influenced by changes in the growth conditions of the microorganisms, as also indicated by the inhibition of binding by the composition of capsule structures (see above). An influence of growth conditions is also observed for the binding to *C. albicans*, e.g., *C. albicans* grown at 37°C showed high binding as compared to low binding seen if the fungi were grown at 23°C.[88] MBL avidly binds *Aspergillus fumigatus*.[38] In fact, chronic necrotizing aspergillosis, which is a rare progressive infection seen in individuals without recognizable immunodeficiency, has been shown to associate with polymorphisms of MBL gene as there was a significant increase in MBL-D allele carriers among the patient group.[89] In support of a role of MBL therapeutic administration of MBL in a murine model of pulmonary invasive aspergillosis rescued mice from death.[90-92] Another aspect of *A. fumigatus* infections are allergic bronchopulmonary aspergillosis, a hypersensitivity disease induced by *A. fumigatus*. A higher MBL level was found in such patients indicating that MBL is part of the response towards the pulmonary hypersensitivity.[93] Further correlative clinical analyses are pertinent to verify the importance of MBL–*A. fumigatus* interaction and its biological significance.

MBL and Ficolins Recognize Variety of Endogenous Ligands

The ability of MBL and ficolins to recognise altered-self or self molecules that are not normally accessible suggest that these molecules have roles that extend beyond the first line host defence (Table 2).

Is MBL Deficiency or Low Ficolin Levels a Predisposition Factor for Autoimmunity?

The similarity between MBL, ficolins and C1q has led investigators to propose that they, like C1q, may be involved in clearance of apoptotic debris. Since impaired "clearance" of immune complexes and apoptotic material through the classical complement pathway was suggested to be a predisposition factor for SLE development, it was proposed that MBL deficiency may predispose to spontaneous autoimmunity. Several independent studies have indicated an association between low MBL producing haplotypes and SLE.[94-96] MBL reacts with apoptotic cells, which may expose GlcNAc as terminal sugars of cytoskeletal proteins.[97] It has also been suggested that tissue deposition of IgGs with exposed GlcNAc (the so called IgG(0)), IgA, IgM or C3, all of which have high mannose structures) will render enough ligands to form a good binding pattern.[98,99] Nauta et al reports that MBL/MASP complexes formed on late apoptotic/necrotic cells promoted phagocytosis by macrophages and thus contributed to non-inflammatory sequestration of dying cells.[97] Ogden et al demonstrated that MBL and C1q bound to apoptotic cells and facilitated phagocytosis via calreticulin and CD91.[100] This raised the possibility that MBL null mice will develop SLE-like features spontaneously. However, Stuart et al reported that MBL null mice did not develop anti-nuclear antibodies and characteristic kidney damage, associated with SLE-like disease with age.[101] Importantly a slower clearance of apoptotic cells was actually seen in the MBL null mice. These observations suggest that MBL deficiency is a weak genetic modifier. Meta-analysis has reviewed studies in this area and found MBL variant alleles to be SLE risk factors.[102] Both L-ficolin and H-ficolin have been shown to bind to late apoptotic/necrotic cells.[103,104] No clinical studies are yet published.

Table 1. Ficolins

Ficolins	Tissue Expression	Molecular Targets on Pathogens "Micropatterns"	Function
Human			
L-ficolin (ficolin-2)[5]	Liver[5,59]	GlcNAc[60,61] N-acetylated groups[6] 1,3-beta D glucan[62]	Complement activation[61,62] Opsonophagocytosis[5] Clearance of apoptotic debri[63]
H-ficolin (ficolin-3)[64]	Liver and Lung	GlcNAc	Complement activation[65] Opsonophagocytosis Clearance of apoptotic debri[63]
M-ficolin (ficolin-1)[59,66,67]	Monocyte and Lung[67]	GlcNAc[68] N-acetylated groups[69]	Complement activation[68] Opsonophagocytosis[70]
Mouse			
Ficolin-A[71]	Liver and Spleen[71,72]	GlcNAc[73] GalNAc[73]	Complement activation[73]
Ficolin-B[71]	Bone Marrow and Spleen[71,72]	GlcNAc[73] GalNAc[73] Sialic acid residues[73]	Complement activation[73]

Table 2. Endogenous ligands recognized by MBL and ficolins

Endogenous Ligands	MBL	Ficolins
Dying cells	Apoptosis	Apoptosis
	Necrosis	Necrosis
Ischemic tissue	Myocardial reperfusion	Potential binding expected
	Renal reperfusion	
	Gastrointestinal reperfusion	
Anoxic cells	Endothelial	Potential binding expected
Transformed cells	Colon adenocarcinoma	Potential binding expected
	Colorectal carcinoma	
Nucleic Acids	DNA, RNA	
Phospholipids	Phosphatydilserine	
	Phosphatydilcholine	

What Are the Consequences of MBL Recognition of Self-Structures?

MBL has the potential to react with altered structures within the body if GlcNAc, fucose, or mannose is exposed in a fitting pattern. Modifications of self surfaces following ischemia-reperfusion injury lead to tissue damage, partly mediated by complement.[105] While it is not yet clear what the MBL targets on ischemic tissues are, changes in O- or N-linked glycosylation patterns of both extracellular and intracellular proteins may contribute to formation of MBL specific neo-epitopes. A series of studies by G. Stahl's group have challenged the notion that the ischemia reperfusion damage is triggered mainly by classical and/or alternative pathway activation and instead suggested that tissue destruction may be mediated by the lectin pathway depending on the organ type.[105-107] For instance, inhibition of MBL-A activity reduced post-ischemic myocardial injury in a rat myocardial ischemia (MIR) model. The effect was thought to be attributed to reduction of neutrophil infiltration and attenuation of pro-inflammatory responses (e.g., IL-1, IL-6 mRNA levels).[108,109] Ongoing research showed that the tissue injury in a mouse MIR model is C1q independent, while the infarct size and C3 deposition were comparable between C1q knock out and wild type mice undergoing myocardial I/R.[110] In contrast, MBL double knock out mice showed a full protection. Recently it has been suggested that MBL can bind to deposited IgM that targets self antigens.[111-114] The binding is responsible for complement mediated damage during myocardial infarction. This observation questions the nature of the MBL epitope on IgM and how these observations fit with the reports that claim that human deposited IgM fails to bind MBL.[115,116]

Although the nature of self-targets on ischemic tissues remains to be validated, it is clear that deficiency in MBL-dependent complement activation is protective during different types of IR injury.[117] We recently extended these observations to kidney I/R injury model[118] suggesting that MBL inhibitors may be therapeutically relevant in IR.

Another example of MBL neo-generated ligands is different cancer cells. Aberrant glycosylation happens essentially in all types of cancers as a result of oncogenic transformation and is a key event in induction of metastasis.[119] It is intriguing to speculate that glycosylation-dependent inhibition of tumor growth may be mediated by complement. In fact, several independent groups have shown MBL binding to a number of cancer cell lines like Colo 205, Colo 201 (colon adenocarcinoma cell line) and SW1116 (human colorectal carcinoma cell line).[120,121] MBL recognised highly mannosylated Lewis structures (Le^a and Le^b) on Colo 205. Interestingly, MBL induced C4 and C3 deposition but not complement mediated lysis of cancer cells.[120] Ma et al demonstrated that MBL bound to cancer cells can mediate cytotoxic effects: MBL-dependent cell mediated cytotoxicity (MDCC).[122,123]

Whether MBL polymorphisms can affect susceptibility or progression of cancer was addressed in a number of studies but a correlation was only established for MBL plasma levels and susceptibility to acute lymphoblastic leukemia (ALL),[124] where children with low MBL plasma levels appear to be at increased risk for development of ALL.

Conclusion

MBL and ficolins are proteins that consist of N-terminal collagen-like domain and C-terminal carbohydrate (MBL) or fibrinogen-like domain (ficolins). The collagen-like domain allows the monomers to assemble in different multimeric structures, whereas the ligand recognition ability resides within the N-terminal region of the molecule. The most important feature of MBL and ficolins is their ability to recognize carbohydrate- and acetyl-containing structures on the surfaces of variety of pathogens. Since MBL and ficolins form complexes with MASPs, when pathogen recognition occurs, MASPs activation triggers subsequent complement activation.

Our understanding of the chemistry and genetics of MBL and MASPs have expanded immensely during the last 20 years. Many disease association studies have been performed and more are to come. Many of these have concluded that there is a role for MBL in infective, inflammatory and autoimmune diseases and it is suggested that MBL replacement therapy may be a treatment in immunocompromised patients, e.g., patients undergoing chemotherapy treatments. Unlike MBL, our current knowledge of ficolin biology is rather limited and future experiments will undoubtedly illuminate further the importance of these patter recognition molecules.

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CHAPTER 6

Lung Surfactant Proteins A and D as Pattern Recognition Proteins

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Abstract

Lung surfactant proteins A and D belong to a group of soluble humoral pattern recognition receptors, called collectins, which modulate the immune response to microorganisms. They bind essential carbohydrate and lipid antigens found on the surface of microorganisms via low affinity C-type lectin domains and regulate the host's response by binding to immune cell surface receptors. They form multimeric structures that bind, agglutinate, opsonise and neutralize many different pathogenic microorganisms including bacteria, yeast, fungi and viruses. They modulate the uptake of these microorganisms by phagocytic cells as well as both the inflammatory and the adaptive immune responses. Recent data have also highlighted their involvement in clearance of apoptotic cells, hypersensitivity and a number of lung diseases.

Introduction

Nonclonal germline encoded pattern recognition receptors (PRR) that bind common essential constituents of pathogenic organisms such as lipopolysaccharide, peptidoglycan, lipoteichoic acid, mannan, bacterial and viral DNA or RNA, and fungal glucans were first proposed by Charles Janeway in the early 1990s.[1,2] These broad-spectrum innate immune molecules are thought to be a first line of defense against invading pathogens, involved in controlling pathogen number and minimizing tissue damage caused by the inflammatory response, while allowing time for activation of the adaptive immune response. Since then, a large number of PRR's have been described and can be classified either functionally as endocytic or signaling, or by their subcellular localization: cytosolic, membrane-bound or soluble.[2-6] One family of predominantly soluble PRRs is called collectins, which bind carbohydrate and lipid structures found on microbial surfaces.[5-8] These oligomeric molecules are characterized by the presence of four structural elements: a short cysteine containing N-terminus, a collagen domain, a coiled-coil domain and a C-terminal Ca^{2+} dependent C-type lectin domain that, apart from collectin placenta-1 (CL-P1), have a preference for mannose type monosaccharide subunits. At present nine mammalian collectins have been described: mannan-binding lectin (MBL), collectin liver-1 (CL-L1[9]), collectin placenta-1 (the only membrane-bound collectin; CL-P1 [10,11]), collectin kidney-1 (CL-K1[12]), surfactant protein A (SP-A[13]), surfactant protein D (SP-D[14,15]), conglutinin, collectin of 43 kD (CL-43[16]), and collectin of 46 kD (CL-46[17]).

SP-A and SP-D are two of the best characterized of these collectins. Apart from their homeostatic role in the regulation of lung surfactant, they are effective PRRs, employing a variety of mechanisms to kill, or reduce the infectivity of different pathogens. Initially these include

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bacteriostatic and fungistatic mechanisms, agglutination of bacteria, fungus, and viral particles, opsonization, and enhancement of phagocytosis. As the adaptive immune response matures the collectins direct the helper T-cell polarization, and can alter the activity of a variety of other immune cells depending on the pathogen involved.[18]

In this chapter we will briefly describe the domain structure of mammalian surfactant proteins A and D and look at the details of the active sites which give the molecules their fine specificities that enable them to carry out their different effector functions. We will highlight the range of microorganisms and immune receptors they interact with and illustrate the different mechanisms by which they help clear pathogens and carry out their roles as pattern recognition receptors.

Collectin Structure Overview

Members of the collectin family have specific single chain architecture (Fig. 1). They have a short N-terminal segment of 7 to 25 amino acids that includes 1 to 3 cysteine residues followed by a Gly-X-Y rich collagen-like sequence of between 66 and 177 residues long. A single exon encodes the short 30 amino acid neck region which is followed by the carbohydrate recognition domain (CRD; ~125 amino acids). These individual chains form trimers in all collectins and can form higher order multimers via disulphide bonds. They are divided into two groups based on their multimeric structure, MBL and SP-A have a sertiform or 'bouquet-of-tulips' structure with six trimers, while conglutinin, CL-46 and SP-D form a second group that assembles into a cruciform-like structure (Fig. 2). SP-D can form even higher-order multimers, so-called fuzzy balls, with a mass of several million kDa.[19]

N-Terminal Region

The N-terminal noncollagenous sequence of collectins shows limited similarity within the family and no homologous regions have been found in other proteins. However the conservation of the cysteine residues indicates that they may play an important role in collectin structure and function. Mutation of the cysteine residues within the N-terminal region of SP-D to serine residues led to the formation of trimers only. However, more importantly, the transgenic mice with these two mutations developed a phenotype similar to the SP-D-/- mice with emphysema and foamy macrophages, highlighting not only their importance in the multimerization of the trimers of SP-D, but also the vital role the quaternary structure of SP-D plays in lung homeostasis.[20]

It is thought that in order to form multimers of the trimeric subunits, at least two cysteine residues have to be present in the N-terminal domain. This view is supported by the fact that CL-L1, which has only one cysteine residue in this domain, exists as a trimer.[9] However, CL-43 is also secreted as a trimer, despite having two N-terminal cysteine residues that are found in exactly the same positions as in the highly multimerized SP-D. Others have demonstrated with chimeric collectins that the N-terminal cysteine rich domain along with the collagen domain determines their level of mutlimerization.[21-24] This was confirmed by the SP-A allele SP-A1. It contains a cysteine residue in its collagen region (residue 85) that permits inter-trimer disulphide bond formation and the creation of larger multimers than the SP-A2 allele, which lacks this essential cysteine.[25]

Collagen Region

The collagen regions of SP-A and SP-D, which form triple helices, are thought to be aligned by their trimerising neck regions.[26,27] Each of the three chains forms a left-handed polyproline II like helix that are coiled in a right-handed manner with the glycine residues in the interior of the helix. Interchain hydrogen bonds between N-H groups of glycine and the C=O groups of the amino acid in the X position of the Gly-X-Y motif stabilize the structure. Interruptions in the Gly-X-Y triplet within the collagen domain results in a destabilization of the collagen region, and can introduce a hinge point as can be seen in SP-A (see Fig. 2).[28,29]

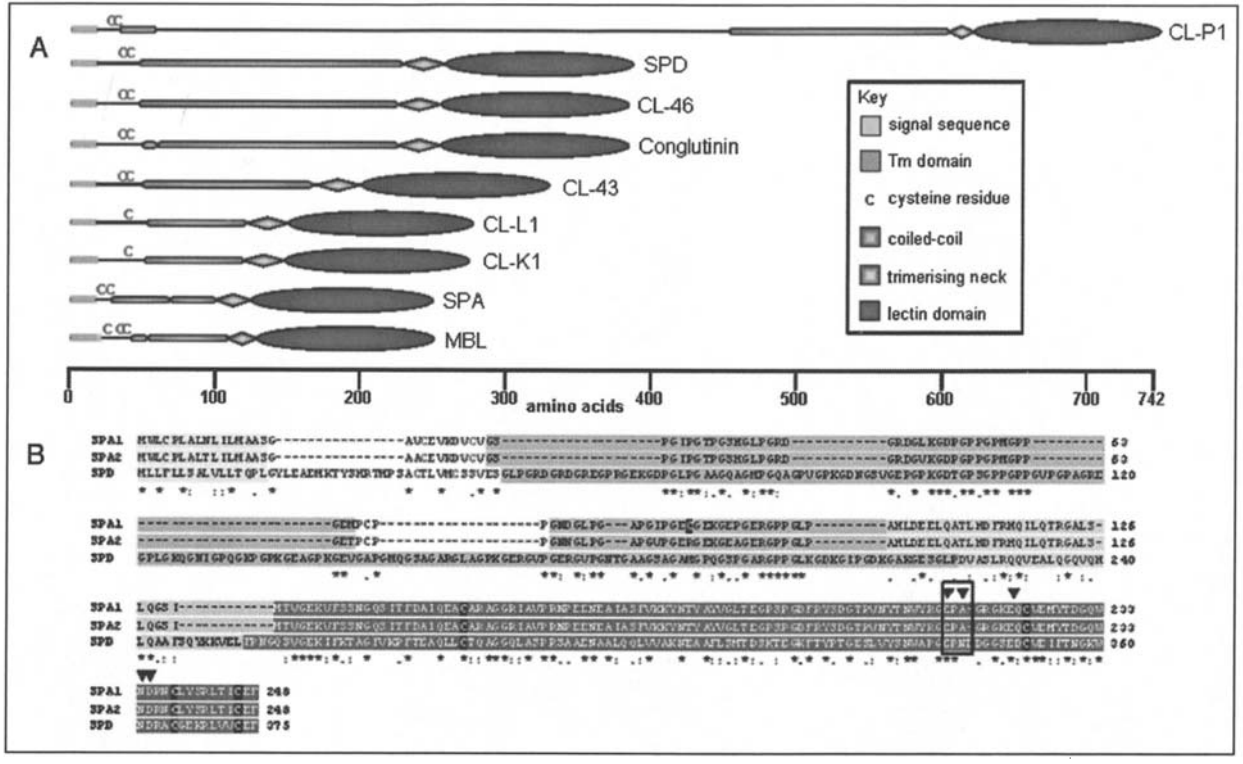

Figure 1. A) Schematic overview of the domain architecture of the mammalian collectins. The conserved N-terminal cysteine residues are coloured red, the collagen domain is blue, the neck domain is green and the lectin domain is red. B) A ClustalW alignment of SP-A1, SP-A2 and SP-D, colour coded in a similar manner to (A). The five residues involved in the coordination of the calcium ion are marked with an arrow-head (▼), the four conserved lectin domain cysteine residues are coloured in dark red and the tripeptide involved in saccharide specificity is boxed. A color version of this figure is available online at www.landesbioscience.com/curie.

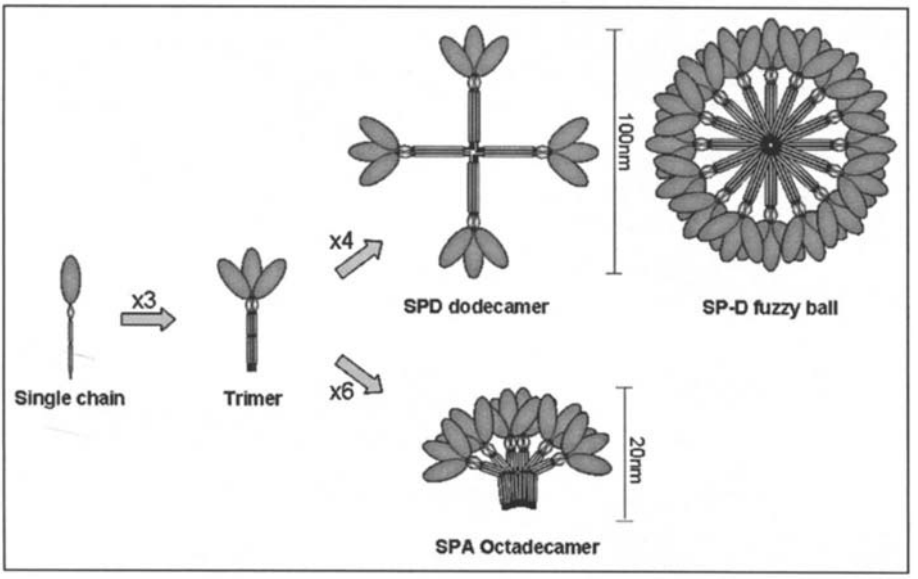

Figure 2. The single chain architecture of the collectins is composed of a cysteine containing N-terminus, a collagen-like region, a trimerising neck domain and spherical calcium dependent sugar binding domain. All collectins can form trimers. SP-D can further multimerize into dodecamers and fuzzy balls, while SP-A has a sertiform shape.

The collagen domain of collectins is thought to have several distinct functions. As previously mentioned, it is involved in determination of the mutlimericity of the different collectins, which has significant effects on its role in lung homeostasis. It also separates trimers to allow the cross-linking of microorganisms, leading to their subsequent aggregation and neutralization.[21] Another interesting structural feature of the collagen domain is their glycosylation,[26,27] which has important immune implications as the carbohydrate moiety of SP-A provides sialic acids that act as competitive targets for Herpes simplex virus (HSV) and Influenza A virus (IAV) binding, reducing viral infectivity and expediting their removal by macrophage and neutrophils.[30] Finally, CL-P1 was shown to bind negatively charged molecules including oxidized LDL via the exposed positive charges of the collagen domain.[31] It is not clear if the soluble collectins can bind other molecules in a similar fashion.

Neck Region

The collagen region is attached to the CRD via a short sequence of approximately 30 residues.[32] This sequence is characterized by conserved hydrophobic residues in a pattern oXooXoooXooXoooXooXoooXooXooo, where o denotes any residue; X denote hydrophobic residue like Leu, Val, Phe or Tyr. Each copy of this sequence forms an alpha helix and three alpha helices form a coiled-coil bundle structure that is held together via interhelix hydrophobic interactions.[32] As previously mentioned the neck domain is considered to assist in formation of collagen like triple helices and also trimerises the CRD's. Recombinant proteins consisting only of the neck and CRD region are still assembled as trimers, whereas isolated CRDs lacking the neck domain are secreted as monomers[33,34] or in the case of MBL as dimers.[35]

Carbohydrate Recognition Domain (CRD)

The highly conserved calcium-dependent lectin domain found at the N-terminus of all collectins is composed of about 125 amino acids and has a characteristic motif that includes 3

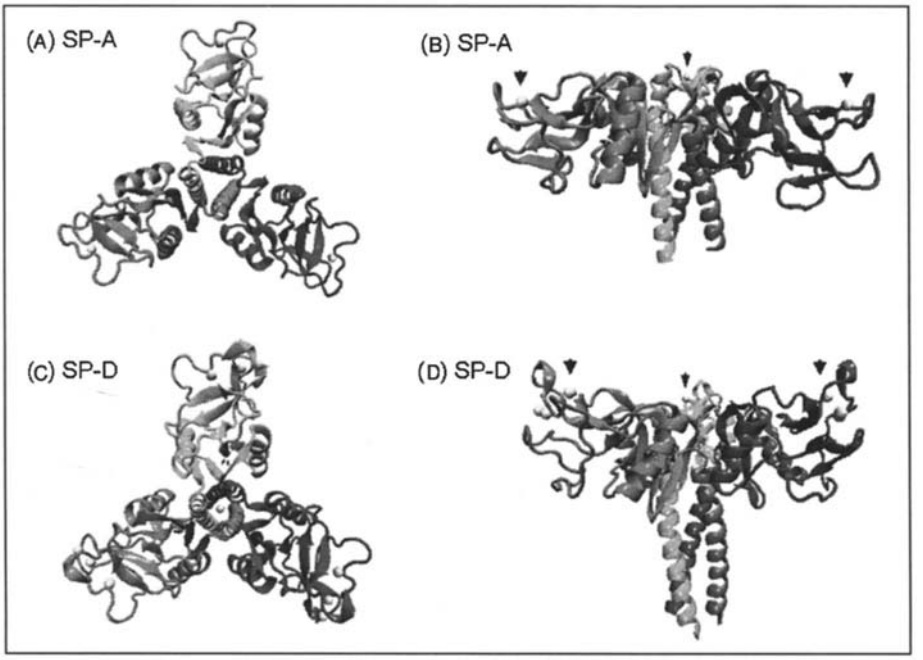

Figure 3. Ribbon diagrams of the neck and CRD domains of SP-A (A,B) and SP-D (C,D). Figures B and D are rotated 90° from figures A and C respectively. The individual chains are coloured red, green and blue, with the calcium ions, one in SP-A and 4 in SP-D coloured yellow. The sugar binding sites are highlighted with arrow-heads. A color version of this figure is available online at www.landesbioscience.com/curie.

of the 4 cysteine residues that form the intradomain disulphide bonds (see Fig. 3 for X-ray crystal structures of the CRD and neck region of SP-A and SP-D).[36] Within this motif is a tripeptide, which despite their broad monosaccharide specificity is thought to divide C-type lectins into mannose/glucose-type or galactose-type lectins (see Table 1 for a summary of the relative carbohydrate affinities of the collectins). These conserved residues are Glu-Pro-Asn at positions 239-241 in SPD and Glu-Pro-Ala at positions 212-214 in human SPA. Although Ala is found at position 3 in this motif in human SP-A or Arg at the same position in rat SP-A, they still have a preference for mannose over galactose, indicating that the conservation of the last amino acid of the triplet is not critical in determining relative saccharide affinity. In fact apart from the membrane bound CL-P1, which has a motif predicted to bind galactose (Gln-Pro-Asp), all collectins have mannose-type CRDs.[31] The specificity of CL-P1 was confirmed by showing a soluble form of its CRD bound GalNAc-conjugated gel in a Ca^{2+}-dependent manner, and this binding was inhibited by free GalNAc, L-, D-fucose, D-galactose, and lactose.[37] In addition to these studies, substitution of the motif of SP-A or SP-D with Gln-Pro-Asp changes their CRD specificity from mannose-type to galactose- type.[36,38] CL-L1 has Ser at position 3 of the triplet, but the effect on its monosaccharide specificity is not known.

The molecular basis on which the CRDs discriminate between mannose- and galactose-type ligands lies in the presentation of two vicinal hydroxyl groups on the 3' and 4' position of the sugar ring of hexoses. For ligand binding in mannose-type CRDs, these hydroxyl groups need to have an equatorial position, whereas for high affinity binding by galactose-type CRDs, at least one of the hydroxyl groups have to be placed axially. Mannose-type CRDs are thought to bind fucose in a slightly different manner, as this molecule has equatorial hydroxyl groups on

Table 1. The mammalian collectin family gene names, chromosomal location, size, expression pattern, carbohydrate preferences and structure

Collectins*	Gene Name Chromosome	Residues in Monomer	Mol. wt (Da)	Expression and Distribution	Monosaccharide Preference	Oligomeric Structure
Conglutinin (bovine)	CGN1 28q1.8-1.9	371	37,973	Liver, plasma	GlcNac>Man, Fuc Glc>ManNac, Mal	Tetramer of trimers (cruxiform)
Collectin-43 (bovine)	CL-43 28q1.8-1.9	321	33,607	Liver, plasma	Man>ManNac>Fuc, glcNac>glc, Mal>Gal	Monomeric and trimeric
Collectin-46 (bovine)	CL-46 28q1.8-1.9	371	37,450	Liver, thymus, plasma	Man>ManNac>Fuc, GlcNac>Glc, Mal>Gal	Monomeric and trimeric
MBL	COLEC1 10q11.2-q21	248	26,143	Liver, plasma and amniotic fluid	GlcNac>Fuc, Man, ManNac>Mal>Glc>Gal	Hexamer of trimers (C1q like)
SP-A	SFTPA (2 expressed genes, 1 pseudogene) 10q22.2-q23.1	248	26,242	Type 2 alveolar cells, clara cells, lungs, amniontic fluid, GIT, generalized mucosal occurrence	ManNac>Fuc, Mal> Glc>Man>Gal, GlcNac	Hexamer of trimers (C1q like)
SP-D	SFTPD 10q22.2-q23.1	375	37,728	Type 2 alveolar cells, clara cells, lung, amniotic fluid, GIT, generalized mucosal occurrence	Mal>Man, Glc>Lac, Gal>GlcNac>Fuc	Tetramer of trimers (cruxiform)
Collectin-L1 (Liver)	COLEC10 8q23-q24.1	277	30,733	Ubiquitous, mainly liver, placenta adrenal gland, cytosolic only.	weakly binds Gal, Man, Glc, GlcNac	At least trimeric
Collectin-K1 (Kidney)	COLEC11 (4 splice variants) 2p25.3	271	28,660	Ubiquitous, high in kidney, liver, small intestine, thymus, sc, placenta, adrenal gland, placenta, plasma	Fuc> weak Man	Trimers and larger on nonreducing PAGE.
Collectin-P1 (Placenta)	COLEC12 18pter-p11.3	742	81,516	Mainly placenta, retina, and also mammary gland, heart, skeletal muscle, lung, tonsils, predominantly cytosolic	GalNac>weak Fuc>Gal	Likely to be trimeric

*Homo sapiens unless otherwise indicated. GlcNac: N-acetyl glucosamine; Fuc: Fucose; Glu: Glucose; Man: Mannose; Gal: Galactose; GalNac: N-acetyl galactosamine Lac: Lactose; ManNac: N-acetyl mannosamine; Mal: Maltose.

its 2' and 3' positions of the sugar ring which in molecular models can be superimposed on the hydroxyl groups at the 3' and 4' position of the sugar ring of mannose.[39,40] In addition to fucose, α-D-glucose also appears, by molecular modeling, to dock into the SP-D CRD via vicinal equatorial hydroxyl groups on the 2' and 3' position of the sugar ring.[41] Even though MBL has a low affinity for galactose, there are X-ray crystal structures of MBL complexed with galactose. They show that the sugar is coordinated via its equatorial hydroxyl groups at positions 1' and 2'. This excludes the possibility of MBL binding to galactose residues that are part of galactosides, as the hydroxyl group at the 1' position is involved in glycosidic bonding.[40]

SP-A and SP-D Bind Lipid via Their CRDs

The pulmonary surfactant molecules also recognize specific lipids via their CRDs. SP-A binds to DPPC[42] and galactosylceramide, while SP-D associates with phosphatidylinositol[43,44] and glucosylceramide.[45,46] The CRD of SP-A appears important in the association of SP-A with tubular myelin, a unique structure formed by secreted surfactant phospholipids.[47] The lipid binding capabilities of the surfactant proteins may be of greater importance in their homeostatic role in lung surfactant rather than as PRRs.[48,49]

Calcium Ions Are Required for CRD Function

Although the CRDs are calcium dependent, both for their correct folding and physiological function, the number of calcium ions bound to each CRD under physiological conditions is as not clear. A different number of $Ca2^+$ ions are present in X-ray crystal structures of SP-A and SP-D depending on the concentration of $Ca2^+$ employed during their crystallization.[50] Three $Ca2^+$ ions are commonly found in X-ray crystal structures of the CRD of SP-D while a fourth calcium ion has been reported in the presence of 2.5 mM calcium in the funnel formed by the three CRDs and close to the neck–CRD interface.[51] It is known that the Ca2+ ion coordinated by Glu321, Asn323, Glu329, Asn341 and Asp342 is the active site calcium involved in binding sugar residues,[35,39,40,50] but the function of the other two calcium ions located near the active site is not known (see Fig. 4 for the location of the calcium ions and the carbohydrate binding site of SP-A and SP-D). There are indications that at least one of them is involved in the correct folding of the CRD, while others have attempted to show by computer simulation that they may be involved in binding extended ligands. Docking studies of SP-D showed that flanking saccharide residues in trisaccharides form additional hydrogen bonds with amino acids outside the CRD binding pocket, and thereby contribute to overall binding energy.[41,51,52] But the majority of the X-ray crystal structures show interaction solely between the two equatorial hydroxyl groups on a single monosaccharide, even though many of them are extended structures.[50] Shrive et al showed that the fourth calcium ion, located at the top of the funnel between the three monomers causes a change in charge distribution between Glu232 and Lys246, which allows a conformational change that opens the cleft between the CRDs and the neck. They hypothesized that this may be important in binding either ligands or immune cells.[51]

Factors Affecting Interaction with Polysaccharides

Collectins bind the (poly)saccharide ligands present on the surface of microorganisms via their CRDs. The affinity of an individual CRD binding a monosaccharide is low (in the order of 10^{-3} M), hence the concerted binding of two or more CRDs is necessary for biologically relevant interactions. Collectin trimers and higher-order multimers bind to polyvalent ligands in the order of 10^{-8} or 10^{-11} M, respectively.[34] These interactions depend on the density of carbohydrate ligands on the microbial surface, the degree of oligomerization of the collectin and the flexibility of both.[23] As collectins predominantly bind terminal saccharides, their position within the carbohydrate structure and their chemical bonding are also important. The collectins can bind either multiple monosaccharides on a single glycolipid or glycoprotein, or individual saccharide moieties present on different surface ligands.

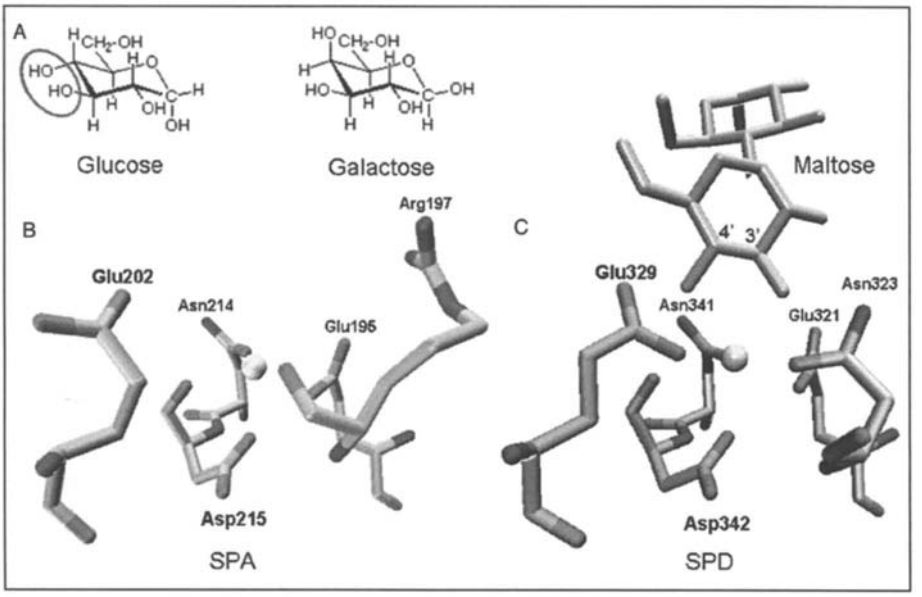

Figure 4. A) The equatorial 3′ and 4′ hydroxyl groups that are bound by SP-A and SP-D are highlighted in red in the glucose molecule. Galactose has an axial hydroxyl group at the 4′ position. Line diagrams of the sugar binding site of SP-A (B) and SP-D with a maltose in the active site (C) highlight the residues involved in coordinating the active site calcium ions. There is no difference in the position of the side-chains with and without bound sugar in SP-D, but water molecules complete the calcium ion coordination in the unbound structure (not shown). A color version of this figure is available online at www.landesbioscience.com/curie.

As we have described in the structure of the collectins, SP-D and SP-A form multimeric structures with at least 12 or 18 chains respectively, and can form much higher order polymers that allow higher affinity binding to pathogens. Genetic studies have highlighted allelic variations that can influence the quantity and mutlimericity of SP-D produced in the serum. These changes have differential effects on binding. A change at position 11 in the coding region of SP-D from methionine to threonine resulted in the production of low levels of only monomeric SP-D in the serum of homozygotes. As predicted by their structure their SP-D binding to intact influenza A virus, Gram-positive and Gram-negative bacteria was weaker, but surprisingly it did bind isolated LPS better than native SP-D.[53] Polymorphisms in receptor molecules that bind SP-A or SP-D are associated with an increased risk of infection as well as variety of disorders including autoimmune diseases, asthma, and atherosclerosis.[54-56]

The X-ray crystal structures of SP-A and SP-D show no interaction between individual CRDs, but there is substantial interaction between the CRD and neck regions, hinting at limited mobility of this region. However, electron microscopy pictures of dodecameric SP-D and conglutinin revealed great flexibility of the trimeric subunits within higher-order multimers.[57] It is thought that the kink in the collagen stalk of SP-A provides it with additional flexibility to bind microbial surfaces and is necessary for lung lipid homeostasis.[58] Also, it is well known that carbohydrates are relatively mobile structures. They are generally removed for crystallography studies, and NMR studies have shown them to have considerable flexibility, which may be of importance for collectin binding.

Finally, carbohydrate binding of collectins is usually to terminal carbohydrate residues. However, recently SP-D has been shown to bind to nonterminal glucosyl residues of polysaccharides, and binding was shown to be dependent on the nature of the glycosidic linkage

between monosaccharide units, as the hydroxyl groups on the 2' and 3' or on the 3' and 4' position had to be available to dock into the CRD.[41,59] It is not known if the ability to bind internal saccharide units is a property of all collectins, or specific to SP-D.

Directing the Immune Response

As innate pattern recognition molecules SP-A and SP-D are first line defense molecules that attempt to regulate the immune response in an appropriate manner against a multitude of pathogens (see Table 2 for a list of microbes that SP-A and/or SP-D have been shown to interact with). They are capable of stimulating or dampening the inflammatory response.

They can reduce the inflammatory response in many ways: by binding to membrane bound PRR and altering their activity or surface expression, by binding inhibitory regulatory proteins, reducing C1q mediated complement activity and aiding in the removal of cellular debris. SP-A binds CD14 on alveolar macrophages and inhibits its binding to smooth LPS, reducing the production of the pro-inflammatory cytokine TNF-α.[60] Similarly, by binding to TLR2, SP-A can inhibit its activation by peptidoglycan and zymosan.[61,62] Both SP-A and SP-D bind the signal inhibitory regulatory protein alpha (SIRPα), which stops pro-inflammatory mediator production via the activation of the tyrosine phosphatase SHP-1 and its subsequent blockade of signaling through src-family kinases and the p38 MAP kinase. Recent studies suggest that SP-D, and SP-A to a lesser extent, binds to the DNA on the surface of apoptotic cells as well as bacterial cellular fragments. They act as opsonins speeding up the clearance of dying cells and cellular debris by phagocytic cells.

As activators of the inflammatory response the lung collectins act similarly, by binding to surface receptors and changing their activity or expression. Although SP-A inhibits the activation of CD14 by smooth LPS, it enhances the inflammatory response induced by rough LPS through the same receptor. It binds rough LPS via its CRD and can interact with CD14 via its neck domain forming an LPS-SP-A-CD14 complex, which is thought to activate the receptor and increase the expression of TNF-α. Although, a direct interaction between SP-A and TLR-4 has not been reported, a study by Guillot et al, 2002, demonstrated that SP-A induced the NF-κB pathway and induced TNF-α secretion both of which were critically dependent on a functional TLR-4 complex.[63] The collagenous tails of SP-A and SP-D were also shown to induce pro-inflammatory responses including stimulating phagocytosis via an interaction with calreticulin/CD91 after binding to foreign material or damaged cells. SP-A and SP-D are involved in increasing the number of PRR on the surface of different immune cells including the mannose receptor on monocyte derived macrophage, and the scavenger receptor A (SR-A) on alveolar macrophage after exposure to S. pneumoniae, which increases their phagocytic capabilities.[64-66] A list of receptors or binding proteins that interact with SP-A and SP-D can be found in Table 3.

Thus, the lung surfactant proteins interact with many different microorgamisms and can activate or dampen the immune response depending on the pathogen type, quantity and the state of activation at the site of infection. The roles of SP-A and SP-D as PRRs against three different pathogens are described in more detail below in order to highlight differences both in their functions and the specific surfactant-pathogen interactions, which can lead to different outcomes.

Specific Examples of SP-A and SP-D as PRRs

Bacteria

There is a wealth of information on the interaction of the pulmonary surfactant proteins with different pathogenic organisms (Table 2). They predominantly bind surface glycoproteins via their CRDs. For example, SP-A and SP-D bind both rough and smooth forms of LPS although to different sites on the rough LPS molecule: SP-A interacts with the lipid-A moiety of LPS, whereas SP-D binds to the LPS core saccharides. SP-A binds to *Haemophilus influenza*

Table 2. SP-A and SP-D bind a variety of fungi, bacteria, yeast and viruses

	SP-A	SP-D
Fungus/Yeasts		
Acapsular Cryptococcus neoformans	√	√
Aspergillus fumigatus	√	√
Candida albicans	+/-	+/-
Encapsulated Cryptococcus neoformans	+/-	√
Histoplasma capsulatum	√	√
Pneumocystis carinii	√	+/-
Saccharomyces cerevisae	x	√
Gram-negative bacteria		
Enterobacter aerogenes		
Klebsiella pneumoniae	√	√
Chlamydia trachomatis	√	√
Chlamydia pneumoniae	√	√
Escherichia coli	√	√
Haemophilus influenzae	√	x
Helicobacter pylori	x	√
Pseudomonas aeruginosa	+/-	√
Gram-positive bacteria		
Alloiococcus otitis	√	x
Bacillus subtilis	x	√
Group A Streptococcus	√	x
Group B Streptococcus	√	x
Staphylococcus aureus	√	√
Streptococcus pneumoniae	+/-	√
Type 25 pneumococci	√	x
Mycobacteria		
Mycobacterium avium	+/-	√
Mycobacterium bovis (BCG)	√	x
Mycobacterium tuberculosis	√	√
Mycoplasma		
Mycoplasma pneumoniae	√	√
Mycoplasma pulmonis	√	x
Viruses		
Adenovirus	√	x
Herpes simplex virus	√	x
Human immunodeficiency virus	√	√
Influenza A virus	√	√
Respiratory syncytial virus	+/-*	√
Rotavirus	x	√

√: The collectin in question binds the microorganism. *: Binding occurs here, but there is a question over its effect on RSV infection. x: No information on binding between the collectin and the microorganism, or they do not interact. +/-: Disagreement on whether binding between the collectin and the microorganism occurs or not.

not via its LPS, but instead via its glycosylated major outer membrane protein P2,[59] while lipoteichoic acid (LTA) of *Bacillus subtilis* and peptidoglycan of *Staphylococcus aureus* represent ligands on Gram-positive bacteria for SP-D, but not for SP-A. SP-D also binds to *Mycoplasma pneumoniae* via interactions with its membrane glycolipids.[67]

Table 3. Receptors and binding proteins for SP-A and SP-D

Receptor/Binding Protein	Interacting Protein and Domain	Cells Expressing the Receptor	Proposed Functions
56 kDa	SP-A	Monocytes, macrophages, neutrophils, endothelial cells, platelets, microglia	Phagocytosis
C1qRp (CD93)	SP-A (collagen region)	Monocytes, macrophages, neutrophils, endothelial cells, platelets, microglia	Originally proposed to have a role in phagocytosis
SPR210	SP-A (36 residue long collagen region containing RGD motif)	Bone marrow derived macrophages, alveolar macrophages, Type II cells, peripheral blood mononuclear cells	Phagocytosis, regulation of phospholipid secretion, regulation of cytokine production, inhibition of T-cell proliferation, antibody to the receptor inhibits T cell mediated responses against Mtb
55 kDa	SP-A	Alveolar Type II cells	Phospholipid uptake
SIRP_α (CD 172)	SP-A, SP-D (CRD)	Myeloid cells, neurons, endothelial cells, B cells, antigen presenting cells, macrophages	Inhibition of pro-inflammatory cytokine production, suppression of clearance of apoptotic cells
Calreticulin and CD91	SP-A, SP-D (collagen region)	Ubiquitous	Phagocytosis and production of pro-inflammatory cytokines
gp340	SP-A, SP-D (CRD region)	Soluble opsonin	Stimulation of alveolar macrophage migration; an opsonin
CD14	SP-A (neck region) binds the peptide portion containing the leucine-rich repeats of CD14; SP-D (CRDs) binds the carbohydrate moiety of CD14	Alveolar macrophages	Modulation of the CD14-LPS interaction and probably release of pro-inflammatory cytokines

continued on next page

Table 3. Continued

Receptor/Binding Protein	Interacting Protein and Domain	Cells Expressing the Receptor	Proposed Functions
TLR-2	SP-A, SP-D	Monocytes/macrophages, dendritic cells, epithelial cells, B cells	Inhibits activation by peptidoglycan and zymosan
TLR-4/MD-2	SP-A	Monocytes/macrophages, dendritic cells, epithelial cells, B cells	Attenuated cell surface binding of smooth LPS and smooth LPS-induced NF-kappaB activation
TLR-2/TLR-4	SP-D	Monocytes/macrophages, dendritic cells, epithelial cells, B cells	-
TLR-2/TLR-4	SP-A	Macrophages	Up-regulate TLR2 expression; dampens TLR2 and TLR4 signaling by decreasing the phosphorylation of IkappaBα
Desmin and Vimentin	SP-A	Myometrial cells	Inhibits in vitro the polymerization of desmin filaments
$\alpha_2\beta_1$ integrin	SP-A	Peritoneal mast cells	
p63 (CKAP4/ERGIC-63/CLIMP-63)	SP-A	alveolar Type II cells	Inhibition of ATP-stimulated phospholipid secretion

The effect of these different interactions depends both on the pathogen and the collectin involved. To highlight both the different roles played by SP-A and SP-D as innate immune molecules as well as some unique mechanisms that pathogens have developed to avoid their elimination we attempt to collate the information available on their role in the protection against three pathogens: Mycobacterium tuberculosis (Mtb), *Pneumocystis carinii* and IAV.

Mtb, the airborne etiological agent of pulmonary tuberculosis, is one of the leading causes of death in the developing world due to an infective agent.[68] This pathogen of mononuclear phagocytes attempts to attach to, and enter alveolar macrophage as an initial step in its life cycle. It encounters this cell within the complex mixture of lipids (90%) and proteins (10%) that make up pulmonary surfactant. The two major hydrophilic surfactant proteins, SP-A and SP-D, bind the bacterium when it enters lung surfactant, but these interactions lead to very different outcomes.

Mtb attempts to enter AM through an interaction of its surface glycoproteins e.g., lipoarabinomannan (LAM) or alanine- and proline-rich antigenic glycoprotein (APA) with the macrophage mannose receptor.[69] It is believed that SP-A, the most abundant surfactant protein, binds Mtb via α1-2 linked mannosyl residues on two of its major envelope lipoglycans, LAM and lipomannan (LM), with a Kd of 10^{-7}-10^{-9} M.[68,70] SP-A is thought to act as a chemoattractant for macrophage, and to upregulate the mannose receptor (MR; CD14) on the macrophage surface, facilitating the attachment of LAM on the Mtb surface to the MR. Entry via this receptor is thought to prevent phagosome-lysosome fusion, providing a permissive environment for bacterial growth. It does not stimulate the production of intracellular oxidative species.[71] However, when SP-A is bound to Mtb, the effective reduction in free SP-A may reduce its interaction with SIRPα and activate inflammation via the production of the proinflammatory cytokines IL-6, IL-8, IL-1b and TNF-α.[72,73] The SP-A-mtb complex may also promote inflammation by presenting clustered collagen tails to the calreticulin receptor (CD91).

At the same time the second surfactant PRR, multimeric SP-D, also binds Mtb via the terminal mannosyl residues of LAM, but with a greater affinity than SP-A (in the order of 10^{-10} M).[74] This binding leads to agglutination of the bacteria, and a reduction of Mtb uptake and growth within AM.[75] Both binding and agglutination have different protective effects. SP-D binding to both Mtb and to the MR reduces infectivity by reducing the bacteria-macrophage interaction. This leads to a reduction in bacterial uptake and a decline in bacterial multiplication within AM. However, Mtb can be taken up via other macrophage receptors e.g., gp340 or complement receptors (CR1 or CR3), permitting phagosome-lysosome fusion and its degradation.[76] This still does not lead to the production of intracellular oxidative species. The agglutination is not thought to enhance phagocytosis, but may aid mucociliary clearance.

The level of SP-A in the lungs may be a key regulator of inflammation upon Mtb infection. Patients with HIV have increased SP-A levels, which is thought to reduce pulmonary inflammation and increase pathogen burden, while patients with pulmonary tuberculosis have reduced SP-A levels and initiate an inflammatory response augmenting pathogen clearance.[77]

Fungus and Yeast

The number of immunocompromised individuals worldwide is increasing due to HIV infection, transplants and treatment with steroids or chemotherapy.[78] These patients cannot clear opportunistic pathogens such as *Pneumocystis carinii*; hence there has been a renewed interest in the molecules involved in regulating the immune response to these pathogens.[79-81] It is known that SP-A and SP-D bind a variety of fungi and yeast via at least two different types of surface molecule. Structural polysaccharides that consist of repetitions of the same oligosaccharide, for example the *Saccharomyces cerevisiae* cell wall component mannan and glycosylated proteins like gp55 and gp45 found on the surface of *Aspergillus fumigatus*.

Here we examine the role of SP-A and SP-D against one of these opportunistic pathogens: *P. carinii*. After entry into the upper airways, it forms a proteinaceous foamy exudate that includes lipids and surfactant proteins, which can restrict respiration and lead to pneumonia.

SP-A (Kd: 10^{-9} M) and SP-D bind to *P. carinii* via the surface glycoprotein gp120 (also known as gpA, gp95 or major surface glycoprotein).[78,82,83] SP-D aids its attachment to alveolar macrophage, but does not seem to increase its uptake,[84] while SP-A reduces *P. carinii* binding to AM and hence its phagocytosis,[85] but may potentiate its binding to alveolar epithelium.[83] Their effects in vivo have been examined in two mouse models: SP-A-/- mice and SP-D-/- mice, both of which required the depletion of CD4+ cells to develop disease.[80,81] Without $CD4^+$ depletion only the SP-A-/- mice showed some lung burden after *P. carinii* infection. These SP-A-/- CD4-depleted mice did show a greater lung burden, an increase in numbers of alveolar macrophage, inflammation and lung injury.[80,81] The SP-D-/- CD4-depleted mice showed a more rapid onset in disease, with increased lung burden and inflammation, but the levels were similar to wt CD4-depleted mice after 4 weeks. Both SP-A and SP-D modified the production of oxidative species. The higher oxides, which are involved in pathogen killing, were reduced in the lungs of the SP-A-/- mice, while the higher levels of all oxidative species in the lungs of the SP-D-/- animals was thought to increase lung injury. These models also highlight the interdependence of SP-A and SP-D. A reduction in the post-infection level of SP-D in the lungs of the SP-A-/- mice was thought to be due to the lower IL-4 and IL-5 levels, while the SP-D-/- mice had a 40-50% decrease in SP-A levels at baseline. These observations emphasize the difficulty in separating the effects of these two proteins during *P. carinii* infection, while demonstrating that they are involved in its clearance both during the early stage of infection, modifying the inflammatory response, and later regulating the adaptive immune response.

Viruses

Generally, SP-A and SP-D are thought to bind viruses or virally infected cells in a manner that involves an interaction between their CRDs and surface-exposed glycoproteins that contain oligosaccharides of the high-mannose type. Their different modes of binding and their roles in defense against viral pathogens are highlighted here in their interaction with IAV.[86]

The IAV enters the human host via mucosal surfaces, predominantly the lungs. Its envelope protein hemagglutinin binds to sialic acids that are typically linked α2-6 or α2-3 to galactose residues on surface glycoproteins of lung epithelia. After their uptake and replication the majority of particles bud-off the apical surface and reenter the alveolar airspace without killing the epithelial cell. As the inflammation develops, the release of cytokines enhances a profound infiltration of neutrophils and macrophage into the lungs. The neutrophils release defensins and reactive oxidative species in an attempt neutralize and kill the pathogens, but their large numbers and the quantity of oxidative species released are thought to cause lung damage.

SP-A and SP-D strongly agglutinate and neutralize viral particles via different mechanisms. SP-D binds via its CRD to the oligosaccharide moieties of viral envelope proteins including hemagglutinin and neuraminidase, while IAV binds the sialic acid residues on the carbohydrate moiety of SP-A.[86,87] Although SP-A2 has greater activity than SP-A1 in almost all aspects of SP-A function, both isoforms perform equally well as soluble competitive targets for hemaglutinin binding.[88] Since SP-A and SP-D interact with IAV in different ways, they agglutinate and neutralize different strains of IAV. Further highlighting the roles of SP-A and SP-D in host defense against IAV infection the SP-A-/- and SP-D-/- animals have increased viral titres, increased neutrophil influx, inflammation and increased reactive oxidative species in their lungs compared to their wild-type counterparts, with SP-D-/- animals more strongly affected.[89,90] IAV inhibit neutrophil activity and augments its apoptosis, but SP-D reduces this inhibition.[91,92] It also increases IAV uptake by neutrophils, without altering the release of reactive oxidative species.

Although we still do not fully understand the mechanism that has led to the death of millions of people due to the different influenza pandemics, the research on SP-A and SP-D is shedding some light on these key innate molecules and the mechanisms they use to clear viral pathogens (Table 4).

Table 4. Effector Mechanisms of SP-A and SP-D as pattern recognition molecules

Mechanism	Effect
Microbiostatic	SP-A and SP-D increase the surface permeability of bacterial pathogens. They increase the lag time of fungal growth and inhibit hyphal and pseudohyphal outgrowth. They inhibit bacterial, fungal and viral entry into host tissue.
Aggregation/Agglutination	SP-A and SP-D bind to the surface glycoproteins and cross-link pathogens.
Opsonization and Phagocytosis	The binding of SP-A and SP-D on the surface of bacteria, fungus, yeast and viral particles enhances the respiratory burst and pathogen uptake by macrophage and neutrophils.
Adaptive immune modulation	SP-A and SP-D bind allergens, which has the effect of inhibiting IgE binding, reduces B and T lymphocyte proliferation, suppresses histamine release from basophils and mast cells, and directs the polarization of T cells towards Th1. Modulate the maturation of dendritic cells. SP-A binds C1q, blocking its binding to C1r and C1s, reducing complement mediated damage.
Chemotaxis	The chemotactic domains (for phagocytes) can be localized to the CRD for SP-D and the collagen tail for SP-A.

SP-A and SP-D in Protection against Allergens and Pulmonary Hypersensitivity

Owing to their presence in the lung, SP-A and SP-D, were investigated for their role in allergic hypersensitivity. Being lectins in nature, initial studies explored their affinity towards glycosylated allergens. SP-A and SP-D can bind via their CRD region to allergenic extracts derived from pollen grains,[93] house dust mite,[94] and *Afu*.[95] This binding resulted in inhibition of specific IgE binding to allergens, allergen-induced histamine release from sensitized basophils[94,96] and proliferation of PBMC's isolated from mite-sensitive asthmatic children.[97] Madan et al observed that Asp f1, a nonglycosylated major allergen of *A. fumigatus*, showed binding to SP-A and SP-D leading to inhibition of ribonuclease activity of Asp f1 (unpublished data). Recently Deb et al, 2007, showed that two mite allergens with cysteine protease activity degrade SP-A and SP-D.[98]

Another set of studies are on levels of SP-A and SP-D in murine models and allergic patients. Cheng et al, 2000, reported increased levels of surfactant protein A and D in bronchoalveolar lavage fluids in patients with bronchial asthma.[99] However, Wang et al, 2001 showed that murine model of asthma had decreased levels of SP-A and SP-D.[100] Atochina et al, 2003 associated attenuated AHR of C57BL/6 mice in comparison to Balb/c mice to the 1.5 fold increased levels of SP-D in C57Bl/6 mice.[101] Schmeidl et al, 2005 showed that SP-A specifically decreases in allergen sensitized and provocated rat lungs.[102] Erpenbeck et al, 2006, showed that allergen challenge of patients resulted in increased BAL levels of SP-D and correlated with eosinophil numbers but not with levels of IL-5 and IL-13.[103] These patients however, showed a decrease in SP-A on allergen challenge.

In vivo therapeutic trials of SP-A, SP-D, and rhSP-D in murine models of *Afu*-, mite- or ovalbumin- induced pulmonary hypersensitivity yielded interesting results.[100,104-106] *Afu* is an opportunistic fungal pathogen that is most commonly implicated in causing both

IgE-mediated and non IgE-mediated hypersensitivity in immunocompetent human subjects, leading to development of allergic bronchopulmonary aspergillosis (ABPA). ABPA is clinically characterized by episodic bronchial obstruction, positive immediate skin reactivity, elevated *Afu*-specific IgG and *Afu*-specific IgE antibodies in serum, peripheral and pulmonary eosinophilia, central bronchiectasis, and expectoration of brown plugs or flecks. Other important features of ABPA are activated Th2 cells and asthma, and patients may develop localized pulmonary fibrosis at later stages of the disease. The murine model resembles the human disease immunologically, exhibiting high levels of specific IgG and IgE, peripheral blood and pulmonary eosinophilia, and a Th2 cytokine response.

Intranasal administration of SP-A, SP-D, and rhSP-D using 3 doses on consecutive days significantly lowered eosinophilia and specific IgG and IgE antibody levels in the mice. This therapeutic effect persisted up to 4 days in the SP-A-treated ABPA mice and up to 16 days in the SP-D- or rhSP-D-treated ABPA mice. Lung sections of the ABPA mice showed extensive infiltration of lymphocytes and eosinophils, which were considerably reduced following treatment with SP-D or rhSP-D. The levels of IL-2, IL-4, and IL-5 were decreased, while IFN-γ levels increased in supernatants of the cultured spleen cells, suggesting a shift in the cytokine profile from pathogenic Th2 to protective Th1 response.[107] Thus, SP-A and SP-D appear to suppress the Th2 responses, probably via their ability to modulate functions of antigen-presenting cells, such as macrophages[106] and dendritic cells,[108-110] which may eventually lead to an induction of IL-12-dependent Th1 responses. In addition, a significant inhibition of nitric oxide production has been reported when alveolar macrophages, derived from Derp mice, are preincubated with rhSP-D, resulting in reduced levels of TNF-α in the rhSP-D treated Derp mice.[111]

Very recently, Mahajan et al, 2008, have reported that SP-D induces apoptosis in activated eosinophils and significantly increased phagocytotic clearance of the apoptotic eosinophils.[112] Since IgE cross-linking, histamine release, lymphocyte proliferation, persistent activated eosinophilia and antigen presentation are central steps in the development of allergic asthma, the possibility of using exogenous SP-A and SPD (or their recombinant fragments) as therapy for allergic disorders merits further investigation.[34,107]

Phenotype and Susceptibility of SP-A and SP-D Gene Deficient Mice

The distinct phenotypes and variable susceptibility of SP-A-/-, SP-D-/- and SP-A-/-SP-D-/- mice to allergens and pathogens have elucidated the distinct roles of these two collectins in airway remodeling, inflammation and host defense.

SP-A Gene Deficient Mice

Mice lacking SP-A mRNA and protein in vivo, generated by gene knock-out technology, survive and breed normally. They have normal levels of SP-B, SP-C, and SP-D, phospholipid composition, secretion and clearance, and incorporation of phospholipid precursors. Although there is a complete absence of tubular myelin in SP-A-/- mice, it does not appear to have a significant physiologic effect.[113,114] SP-A-/- mice show increased bacterial proliferation and systemic dissemination following intratracheal inoculation with Group B *Streptococci*, and defective clearance of *S.aureus*, *P. aeruginosa* and *K. pneumoniae*.[115] These mice also show increased susceptibility to RSV, *M. pneumoniae*, and pneumocystis than the wild-type mice. Killing of group B *Streptococcus* and *H. influenza* is significantly reduced in SP-A-/- mice, which is accompanied by increased inflammation of the lung, decreased oxidant production, and decreased macrophage phagocytosis.[79,89] SP-A-/- mice exposed to LPS have elevated levels of TNF-α and these levels get normalized on exogenous administration of SP-A, confirming an association of SP-A and the control of inflammation.[116,117] Zhang et al 2005, reported two SP-A-sensitive *P. aeruginosa* mutants exhibited reduced susceptibility in SP-A gene deficient mice.[118] Later in 2007, they showed that a flagellar-deficient *P. aeruginosa* mutant harbors inadequate amounts of LPS required to resist membrane permeabilization by SP-A and was

preferentially cleared by the SP-A+/+ mice, but survived in the SP-A-/- mice.[119] These studies on SP-A-/- mice essentially reaffirm the role of SP-A as an important regulator of pulmonary infection and inflammation.

SP-D Gene Deficient Mice

Mice, bred after disruption of the SP-D gene (SP-D -/- mice), have shown remarkable abnormalities in surfactant homeostasis and alveolar cell morphology. The SP-D-/- mice exhibit a progressive accumulation of surfactant lipids and apoproteins in the alveolar space, hyperplasia of alveolar Type II cells with massive enlargement of intracellular lamellar bodies, and an accumulation of foamy alveolar macrophages.[120] These mice spontaneously develop emphysema and fibrosis of the lungs, which suggests continuous inflammatory reaction associated with abnormal oxidant metabolism and MMP activity.[49] Expression of SP-D in adult lungs restored alveolar SP-D levels and corrected pulmonary lipid abnormalities but emphysema persisted.[121] The SP-D-/- phenotype appears to share selected features of both GM-CSF-/- as well as GM-CSF over-expressing phenotypes.[122-124] Experiments involving cross out-breeding of compound heterozygous mice have suggested that the mechanisms underlying the alveolar surfactant accumulation in the SP-D-/- and GM-CSF-/- mice are different, and that GM-CSF might mediate some of the changes associated with macrophages and Type II cells seen in SP-D-/- mice.[125-127] SP-D deficiency results in a low SP-A pool size, rapid conversion from large-aggregate to small-aggregate surfactant, increased uptake into alveolar Type II cells and recycling.[49,120]

When exposed to 80% or 21% oxygen, SP-D-/- mice had 100% survival vs. 30% in SP-D+/+.[128] Biochemically, in contrast to SP-D+/+, SP-D-/- mice had higher levels of surfactant phospholipid and SP-B accompanied by a preservation of surfactant biophysical activity. From a multiplex assay of nine cytokines, we found elevated levels of IL-13 in BAL fluid of normoxic SP-D-/- mice compared with SP-D+/+. SP-D-/- mice infected with bacteria or viruses have increased lung inflammation compared with infected wild-type strains, suggesting an anti-inflammatory role for SP-D. SP-D-/- mice show a decreased clearance of RSV, an increase of recruitment of inflammatory cells, and a reduced level of phagocytosis and oxygen radical production by alveolar macrophage.[89]

When wild-type, C1q-/-, SP-A-/-, and SP-D-/- mice were compared for their ability to clear exogenously instilled apoptotic PMN, only SP-D altered apoptotic cell clearance from the naive lung, emphasizing a major role for SP-D in the clearance of apoptotic and necrotic cells in vivo.[129] Thus, SP-A-/- and SP-D-/- mice have distinct phenotypes with respect to microbial challenge and the inflammatory response. This is further evident by the distinct response of SP-A and SP-D gene deficient mice to allergen challenge. SP-D-/- mice show higher intrinsic hyper-eosinophilia and a several fold increase in levels of IL-5 and IL-13, with a lowering of the IFN-γ to IL-4 ratio in the lungs in comparison to SP-A-/- and wild type mice.[130] Similarly, Haczku et al, 2006 also showed that mice lacking SP-D had increased numbers of CD4+ cells with elevated IL-13 and thymus- and activation-regulated chemokine levels in the lung and showed exaggerated production of IgE and IgG1 following allergic sensitization.[131] The hyper-eosinophilia and Th2 predominance is partly reversible by treating SP-A-/- or SP-D-/- mice with SP-A or SP-D, respectively.[130] SP-D-/- mice are more susceptible than wild-type mice whereas SP-A-/- mice have been found to be nearly resistant to *A. fumigatus* sensitization. Intranasal treatment with SP-D or rhSP-D can rescue the *A. fumigatus* sensitized SP-D-/- mice, while SP-A treated *A. fumigatus* sensitized SP-A-/- mice show several fold elevated levels of IL-13 and IL-5, resulting in increased pulmonary eosinophilia and damaged lung tissue. This suggests differential mechanisms involved in SP-A and SP-D mediated resistance to allergen challenge. Hypereosinophilia exhibited by both SP-A-/- and SP-D-/- mice, probably due to significantly raised levels of IL-5 and IL-13 in these mice, suggests that SP-A and SP-D have a role in regulating eosinophil infiltration

and modulation in the lung in response to environmental stimuli. It is interesting to note that similar to SP-D-/- mice, IL-13 over-expressing mice have characteristic foamy macrophages, Type II cell hypertrophy, fibrosis, massive inflammation involving eosinophilia, protease-dependent acquired emphysema, and AHR.[132] Given the involvement of IL-13 in processes such as mucus production and AHR, as well as eosinophil survival, activation and recruitment, it is likely that certain physiological effects in SP-A-/- as well as SP-D-/- mice arise due to over-expression of IL-13.[130,132]

In another study involving a murine model of lung inflammation induced by ovalbumin, SP-D-/- mice have been shown to express increased BAL eosinophils, IL-13 and IL-10, and lowered IFN-γ at early time points (1–3 days) compared with wild-type mice.[133] Ovalbumin-induced TLR- 4 expression in the lungs has been shown to be increased in the wild-type, but not in SP-D-/- mice. Spleen cells, when stimulated in vitro, showed increased lymphocyte proliferation and reduction in IFN-γ in the SP-D-/- mice. These studies highlight that SP-D is involved in the early resistance to allergen challenge and its deficiency leads to default Th2 response. A recent study by Brandt et al, 2008, showed that SP-D gene deficient mice have increased CD4+ T cell, macrophage and neutrophil levels in bronchoalveolar lavage fluid, increased large airway mucus production and lung CCL17 levels. However, 4- to 5-week-old SP-D-/- mice showed significantly lower levels of IgG1 and IgE and splenocytes of these mice on anti-CD3/CD28 stimulation released significantly less IL-4 and IL-13 ($P < .01$)[134]. After intranasal allergen exposures, a modest decrease in bronchoalveolar lavage fluid eosinophilia and IL-13 levels was observed in association with decreased airway resistance.

SP-A-/-SP-D-/- Mice

Mice deficient in both SP-A and SP-D genes (double knock-out) show a progressive increase in BAL phospholipid, protein, and macrophage content through 24 weeks of age.[135] The double knock-out phenotype is characterized by the excessive accumulation of surfactant lipid in the alveolar space, increased numbers of foamy alveolar macrophages with up-regulation of MMP-12, and emphysema.[135] The absolute increase in macrophage number and the extent of MMP up-regulation by macrophages was greater in the double knock-out mice compared with the SP-D-/- mice. Jung et al 2005 showed that A-/-D-/- mice in comparison with wild-type, have fewer and larger alveoli, an increase in the number and size of Type II cells, as well as more numerous and larger alveolar macrophages.[136] More surfactant-storing lamellar bodies are seen in Type II cells, leading to a threefold increase in the total volume of lamellar bodies per lung, but the mean volume of a single lamellar body remains constant. These results demonstrate that chronic deficiency of SP-A and SP-D in mice leads to parenchymal remodeling, Type II cell hyperplasia and hypertrophy, and disturbed intracellular surfactant metabolism. Using SP-A-/-, SP-D-/- and double knock-out mice, Hawgood et al, 2004, have shown that SP-D, but not SP-A, is important in restricting IAV replication and spread in vivo, which is crucially dependent on the glycosylation of residue 165.[137]

Conclusion

The pulmonary collectins SP-A and SP-D not only function as regulators of lung surfactant, but also as innate pattern recognition molecules. They bind pathogenic microorganisms predominantly via their multimeric low-affinity Ca^{2+}-dependent lectin domains that cause their agglutination, opsonization and clearance via phagocytosis. They modulate the immune system via interactions with membrane bound PRRs and other receptors on immune cells, which can lead to an increase or decrease in inflammation depending on the microbe involved. They also act to clear up cellular debris and dying cells in order to reduce inflammation and help avoid autoimmune pathology.

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CHAPTER 7

Pattern Recognition by Pentraxins

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Abstract

Pentraxins are a family of evolutionarily conserved pattern-recognition proteins that are made up of five identical subunits. Based on the primary structure of the subunit, the pentraxins are divided into two groups: short pentraxins and long pentraxins. C-reactive protein (CRP) and serum amyloid P-component (SAP) are the two short pentraxins. The prototype protein of the long pentraxin group is pentraxin 3 (PTX3). CRP and SAP are produced primarily in the liver while PTX3 is produced in a variety of tissues during inflammation. The main functions of short pentraxins are to recognize a variety of pathogenic agents and then to either eliminate them or neutralize their harmful effects by utilizing the complement pathways and macrophages in the host. CRP binds to modified low-density lipoproteins, bacterial polysaccharides, apoptotic cells, and nuclear materials. By virtue of these recognition functions, CRP participates in the resolution of cardiovascular, infectious, and autoimmune diseases. SAP recognizes carbohydrates, nuclear substances, and amyloid fibrils and thus participates in the resolution of infectious diseases, autoimmunity, and amyloidosis. PTX3 interacts with several ligands, including growth factors, extracellular matrix component and selected pathogens, playing a role in complement activation and facilitating pathogen recognition by phagocytes. In addition, data in gene-targeted mice show that PTX3 is essential in female fertility, participating in the assembly of the cumulus oophorus extracellular matrix. PTX3 is therefore a nonredundant component of the humoral arm of innate immunity as well as a tuner of inflammation. Thus, in conjunction with the other components of innate immunity, the pentraxins use their pattern-recognition property for the benefit of the host.

Pentraxins

Pentraxins are a family of phylogenetically conserved, pattern-recognition proteins and a host-defense-related component of the innate immune system.[1-5] Based on the length of their primary structure, the pentraxins are divided into two groups: short pentraxins and long pentraxins. Short pentraxins include C-reactive protein (CRP) and serum amyloid P component (SAP). The first discovered long pentraxin, known as pentraxin 3 (PTX3), serves as the prototype protein of the long pentraxin group. The term pentraxin was first assigned to CRP for its pentagonal appearance of five subunits in electron micrographs.[6] Pentraxins (Table 1) recognize a wide range of altered-self and nonself pathogenic substances and lead to protection of the host.

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Table 1. The pattern-recognition property of pentraxins involved in host-defense

Pentraxin	Recognition	Pathologic Implication
CRP	Bacteria	Microbial infection
	Parasites	Parasitic infection
	Apoptotic cells	Autoimmunity
	Necrotic cells	Autoimmunity
	Damaged cells	Autoimmunity
	Nuclear materials	Autoimmunity
	Modified LDL	Atherosclerosis
	Fibronectin	Cancer
SAP	Bacteria	Microbial infection
	Apoptotic cells	Autoimmunity
	Nuclear materials	Autoimmunity
	β-amyloid	Amyloidosis
PTX3	Bacteria	Microbial infection
	Apoptotic cells	Autoimmunity
	Oophorous matrix	Female fertility

Short Pentraxins: CRP and SAP

CRP, the most characteristic acute phase protein in humans, was discovered in 1929.[7] The plasma concentration of CRP rises in both chronic and acute inflammatory conditions.[7,8] SAP was discovered in 1965 as an amyloid protein of plasma (P) origin and is not an acute phase protein in humans.[9] SAP is associated with the deposits that characterize amyloid fibrils in systemic amyloidosis, Alzheimer's disease and the transmissible spongiform encephalitis.[10] In mouse, which is a widely used animal to determine the in vivo functions of short pentraxins, SAP is the acute phase protein.[11] CRP is only a trace serum protein in mice and not an acute phase protein.

Phylogeny

Both CRP and SAP have been found in all vertebrates where they have been sought.[12,13] CRP is also found in the hemolymph of invertebrates such as the arthropod *Limulus polyphemus* and the mollusc *Achatina fulica*.[14,15] At least one short pentraxin is present in all vertebrates as well as in some invertebrates. Humans have got both CRP and SAP. The pattern-recognition property of the short pentraxins towards a wide range of substances of biological importance has been conserved throughout evolution although their acute phase nature is species-specific. For example, in contrast to humans and mice, in rats CRP is constitutively expressed at relatively high levels and is only a minor acute phase protein.[16]

Metabolism

The major site of CRP synthesis is liver.[17] In vitro, in human hepatoma cells, cytokines IL-6 and IL-1 are the main inducers of CRP expression.[18-20] Nitric oxide has been shown to reduce the induction of CRP production by cytokines.[21] Expression of CRP mRNA in the tissues other than the liver has also been reported.[22,23] In mice transgenic for human CRP, the CRP gene has been shown to be under hormonal control while in hamsters, SAP gene is under hormonal control.[24,25] In healthy persons, the median concentration of CRP is 0.8 mg/L but, following an acute-phase stimulus, this may increase to more than 500 mg/L. The half-life of CRP is about 19 h in humans.[26] SAP is also produced exclusively by hepatocytes and turnover rapidly with plasma half-life of 24 h.[27] SAP and CRP are both resistant to proteolysis. SAP, which has localized to the deposits, persists there for prolonged periods without being catabolized.[10]

Structure

CRP has five identical, 206 amino acid long subunits of 23 kD each.[28,29] A single internal disulfide bond is present in each subunit.[29] There is no carbohydrate present in human CRP and there are no potential N-glycosylation sites either. The subunits are held together through noncovalent interactions and are arranged in pentameric symmetry.[30] Each subunit is made up of two antiparallel β-sheets and a single short α-helix, and is characterized by the presence of a cleft that extends from about the center of the subunit to its edge at the central pore of the pentamer. The overall dimensions of the CRP pentamer are about 102 Å outside diameter with a central pore diameter of 30 Å and a subunit diameter of 36 Å.[30] SAP, on the other hand, is a glycoprotein made up of five noncovalently attached subunits of 23 kd each.[31,32] SAP is a single pentamer in serum but under certain conditions, SAP forms decamers by stacking of two pentamers.[33] The complete glycoprotein structure is important for the functions of SAP.[10]

Binding to Calcium and Phosphocholine

CRP is called so because it reacts with C-polysaccharide of the cell wall of *Streptococcus pneumoniae*.[7] The reaction of CRP and C-polysaccharide requires calcium ions.[34] CRP binds two Ca^{2+} through the two overlapping Ca^{2+}-binding sites present on each subunit.[30] The two Ca^{2+} in CRP are coordinated by Asp60, Asn61, and by residues (Glu138, Gln139, Asp140, Glu147 and Gln150) in a loop. This loop, in the absence of bound Ca^{2+}, moves away from the main body of the CRP molecule exposing an otherwise hidden site of proteolysis. Bound Ca^{2+} are integral structural elements of CRP and protect CRP from proteolytic cleavage.[35]

Phosphocholine (PCh) is the principal Ca^{2+}-dependent ligand of CRP.[36] The PCh-containing substances, to which CRP binds, are present in many prokaryotes and in all eukaryotes and include pneumococcal C-polysaccharide, damaged, necrotic and apoptotic cells, altered lipid bilayers, and pulmonary surfactant lipids.[2-4,37] CRP also binds in a Ca^{2+}-dependent manner to phosphoethanolamine and other phosphate monoesters.[2-4] A PCh-binding site, located next to the Ca^{++}-binding sites, is present on each CRP subunit. Since the subunits have same orientation in the assembled pentamer, all five PCh-binding sites fall on the same face of the CRP pentamer. The PCh-binding site consists of a critical hydrophobic pocket formed by residues Leu64, Phe66 and Thr76, and two Ca^{2+} that are bound to CRP.[38] The phosphate group of PCh directly coordinates with the two Ca^{2+}. Phe66 and Glu81 in CRP provide contacts to the choline moiety of PCh that lies within the hydrophobic pocket.[38-41]

Like CRP, SAP also binds two Ca^{2+} through the two overlapping Ca^{2+}-binding sites present on each subunit.[32] Ca^{2+}-bound SAP is also protease-resistant.[42] Since SAP binds to phosphoethanolamine, it can bind to necrotic and late apoptotic cells in Ca^{2+}-dependent manner.[43] SAP pentamers are also capable of interacting with CRP pentamers to form decamers but only in Ca^{2+}-free conditions, indicating that CRP and SAP do not interact in vivo.[44,45]

Binding to Bacteria and Protective Role against Microbial Infections

CRP binds a wide variety of bacteria including several serotypes of *S. pneumoniae*, *Haemophilus influenzae*, and *Neisseriae spp.*[46-49] In vitro, CRP has been shown to promote phagocytosis of PCh-expressing bacteria and to block the attachment of such bacteria to the receptors for platelet-activating factor (PAF) on the host cells.[48,50]

Human CRP protects mice against fatal infection with *S. pneumoniae*.[51-53] Employing complement C3 knockout mice and complement depletion using cobra venom factor, it has been shown that a functioning complement system is required for full CRP-mediated protection.[51-53] However, the protection is independent of naturally occurring anti-pneumococcal antibody.[54] CRP also protects mice from infection with *Salmonella typhimurium*, a pathogen to which CRP is not known to bind.[55]

SAP binds to several bacteria via lipopolysachharide (LPS) and prevents LPS-mediated complement activation.[56] SAP binds LPS and thereby protects the host from LPS toxicity.[57,58] However, for certain organisms to which SAP binds, such as *S. pyrogens* and rough strains of

E. coli, SAP enhances virulence by protecting the bacteria against phagocytosis.[59] SAP is not involved in resistance against TNFα-induced lethal hepatitis shock.[60] SAP enhances the ability of mouse macrophages to kill *Listeria monocytogenes* and this activity is not associated with the binding of SAP to the pathogen.[61] It has been shown that SAP is protective in infection with organisms to which it does not bind.[59]

Binding to Lipoproteins: Implications for Atherosclerosis

Another major CRP-ligand of tremendous biological significance is modified low-density lipoprotein (LDL). CRP readily gets complexed in a Ca^{2+}-dependent manner to modified (oxidized and enzymatically-treated) LDL but not to native LDL.[62-65] Binding of CRP to LDL is mediated by the PCh-binding site in CRP that interacts with the PCh and cholesterol moieties present on LDL.[64,65] Consistent with the interaction between CRP and LDL in vitro, CRP has been found deposited and colocalized with LDL in human atherosclerotic lesions.[66,67] CRP is thus capable of covering certain properties of modified LDL such as complement activation. It has been shown that CRP protects the host from complement activation by LDL.[68] However, CRP has not been found to be either atheroprotective or proatherogenic in mouse models of atherosclerosis.[69-71] Although the denatured CRP has been shown to be atheroprotective in mice, the role of intact CRP in the development of atherosclerosis is not clear.[72]

The serum level of CRP is raised in atherosclerosis. Measurement of serum CRP is recommended for use as an indicator of arterial inflammation and predictor of future cardiovascular events.[73] Statins that lower cholesterol levels have also been shown to lower CRP levels.[21] Recent data have indicated that the measurement of serum CRP levels alone, at least in individuals on statin-therapy, is not beneficial.

Binding to Nuclear Constituents: Role in Protection against Autoimmunity

The presence of CRP in the nuclei of synoviocytes and histiocytes in the patients with rheumatoid arthritis led to the finding that CRP binds nuclear materials such as chromatin, histones, small nuclear ribonucleoproteins, nuclear envelope proteins, and nucleosome core particles.[74-77] The interaction is Ca^{2+}-dependent and involves the PCh-binding site of CRP.[75-77] CRP, however, does not bind chromatin in serum suggesting that this interaction occurs only if CRP or chromatin is deposited at sites of inflammation.[78]

SAP binds to histones and chromatin, and also to DNA. The binding is Ca^{2+}-dependent and does not occur in serum.[78,79] SAP binds chromatin in vivo also; it has been detected with the nuclear deposits in skin biopsies from lupus patients.[80] CRP and SAP have a nuclear localization signal and thus their transport into nuclei is possible.[81] The primary role of CRP and SAP is believed to be the disposal of nuclear materials released in the extracellular environment by apoptotic and necrotic cells, thereby preventing the hazard of autoimmunity.[77-79]

In lupus-prone mice, CRP delays the onset of nephritis and increases clearance and prevents accumulation of immune complexes in the renal cortex. It also decreases autoantibody levels that reduces autoimmune manifestations and thus prolongs the survival.[82-84] Another important aspect of the role of short pentraxins in autoimmunity is their ability to bind immobilized IgG and immune complexes.[85,86] SAP-deficient mice degrade chromatin more rapidly than normal, have enhanced antibody response to exogenous chromatin, and develop anti-chromatin autoimmunity and glomerulonephritis, a phenotype resembling lupus.[87,88] Thus SAP, by controlling chromatin degradation, prevents glomerulonephritis although anti-nuclear antibodies are formed.

Binding to Polycations and Extracellular Matrix Proteins

For binding to certain ligands, CRP does not require Ca^{2+}. Such ligands include polycations like protamine, leukocyte cationic proteins, and a variety of arginine-rich and lysine-rich cationic molecules, and extracellular matrix (ECM) proteins like fibronectin (Fn).[89-92] Instead, Ca^{2+} inhibits these interactions at the physiological pH. SAP also binds to Fn.[93] The Ca^{2+}-binding

site of CRP participates in binding to Fn and polycations.[92] On Fn, the C-terminal domain including the cell-binding and heparin-binding regions is involved in binding to CRP.[91] The CRP-Fn interaction may explain in part the selective deposition of CRP at sites of tissue injury and may play a role in the formation of ECM needed for tissue repair.

No binding occurs between soluble CRP and Fn. The maximum interaction between CRP and Fn occurs at pH 5.0 and this interaction is not inhibited by Ca^{2+}.[90,92] Since CRP circulates in the blood in its Ca^{2+}-bound form, it can interact with Fn exclusively at the ECM of the inflammatory sites including carcinomas where the pH goes down.[90,92] Because CRP, Fn and the ECM have been implicated in cancer, it has been proposed that CRP-Fn interactions may change the architecture of ECM to modify the course of the disease progression.[92] In mouse models of melanoma, CRP is tumoricidal and inhibits metastases.[94]

Binding to Carbohydrate Structures on the Parasite Surface

CRP also reacts with bacterial polysaccharides that do not contain PCh, with several galactans through galactose and phosphate residues, and to substances derived from fungi and yeast.[85,95-98] CRP also binds, in a Ca^{2+}-dependent manner, to certain parasites such as *Plasmodium falciparum, Hymenopepis diminuta*, and *Leishmania donovani* through either PCh or carbohydrates on their surfaces.[99-102] Binding of CRP to *L. donovani* induces their developmental transformation.[103] The PCh-binding site of CRP participates in binding of CRP to carbohydrate moieties.[41]

The carbohydrate-binding property of SAP is shown by the binding of SAP to agarose, microbial polysaccharides, aggregated IgG, Type IV collagen, calumenin, shiga toxin 2, lactate, influenza virus hemagglutinin, heparin, 6-phosphorylated mannose, 3-sulfated saccharides, and glycosaminoglycans.[104-108] Most of these interactions require Ca^{2+}. The interaction of CRP and SAP with carbohydrates occur best at mildly acidic pH.[85,109]

The binding of SAP to glycosaminoglycans neutralizes its anticoagulant effect.[110] Heparin, in the presence of SAP, is a better inhibitor of thrombin-catalyzed conversion of fibrinogen to fibrin than heparin alone, and thus SAP also inhibits fibrin polymerization.[111] Heparin and lactic acid prevent SAP self-association, however, lactic acid does not dissociate SAP-heparin complex. Finally, SAP has been shown to enhance refolding of denatured lactate dehydrogenase and thus SAP also acts as a chaperone.[112]

Binding to Platelet-Activating Factor

CRP binds to PAF, -O-alkyl-sn-2-O-acetyl-n-glycero-3-phosphocholine, probably through the PCh group in PAF.[113,114] CRP inhibits PAF-induced platelet-aggregation, inhibits binding of PAF to platelets, and prevents capture of neutrophils by platelets.[115-118] In vivo, CRP protects mice from lethal challenge with PAF.[114,119] The platelets treated with CRP have been shown to kill immature form of *Schistosoma mansoni* in vitro and confer protection against *S. mansoni* in rats.[120]

Binding to Complement C1q and Modulation of the Classical Complement Pathway

Ligand-complexed CRP binds C1q, the first component of the classical pathway of complement, and activates the classical pathway of complement.[121,122] CRP-initiated activation of complement leads to the assembly of an effective C3 convertase and generates anaphylotoxins C3a and C4a and the opsonins C4b, C3b, and iC3b. CRP-complexes do not result in the formation of an effective C5-convertase, and therefore prevent generation of pro-inflammatory molecules like C5a and also avoid the assembly of membrane-damaging, terminal membrane attack complex of the complement pathway.[123]

The C1q-binding site on CRP is located in the cleft regions present one on each subunit and is formed by Tyr175, Asp112, Glu88, His38, and Asn158. Residues Asp112 and Tyr175 appear to provide contacts with C1q.[124,125] The CRP-binding site on C1q is located on the globular region of C1q.[126-128] SAP, in addition to binding to C1q, also binds to C4b-binding

protein.[129] Aggregated SAP and ligand-bound SAP activate the classical pathway of complement.[130,131] The C1q-binding site on SAP is not known.

Complement activation by CRP-complexes participates in phagocytosis of apoptotic cells.[132] CRP attenuates the formation of the membrane attack complex on the surfaces of apoptotic cells, thereby protecting the cells from lysis. This effect is achieved by the recruitment of factor H, a complement regulatory protein that accelerates the decay of the C3 and C5 convertases.[132] The factor H-dependent inhibitory effect of CRP on activation of the alternative pathway of complement by *S. pneumoniae* and artificially sensitized erythrocytes has been observed.[133-135] Interestingly, the property of CRP to activate the classical pathway of complement is irrelevant for the protection of mice from pneumococcal infection.[136]

Binding to the IgG Receptors FcγRI and IIa and Interaction with Phagocytes

CRP interacts with phagocytic cells through the IgG receptors, FcγRI (CD64) and FcγRII (CD32), in heat-inactivated plasma, in cell culture medium, and in buffered solutions.[137-143] The exact physiological conditions in which this interaction can occur in vivo are unclear but it is reasonable to believe that either CRP should be ligand-complexed or the phagocytic cells should be immobilized. FcγRIIa, the low affinity IgG receptor providing stimulatory signals in the cells, is the high affinity receptor for CRP.[139] In contrast, FcγRI, the high affinity IgG receptor providing inhibitory signals in the cells, is the low affinity receptor for CRP.[138] The binding of CRP to FcγR is Ca^{2+}-dependent, specific, saturable, reversible, and rapid with a half-life of 3 min.[137-143] PCh does not inhibit CRP-FcR interaction.[142] Because IgG inhibits binding of CRP to FcγR, it is likely that the sites on FcγR that bind IgG and CRP are similar.[141] A hydrophobic region present in the cleft on the CRP molecule provides the contact amino acids for FcγR. These amino acids are Thr173, Asn186, Lys114, and Leu176.[144] Thus the binding sites on CRP for FcγR and for C1q are discrete but overlapping.

CRP affects the functions of the phagocytic cells such as augmentation of FcR-dependent aggregated IgG-mediated respiratory burst activity.[145] Upon binding to monocytic cells, CRP is internalized and degraded.[146] Although CRP enhances the phagocytosis of a variety of Gram-negative pathogens in vitro, it has been shown that the binding ability of CRP for FcγR is not required for protection of mice from pneumococcal infection.[147] However, binding of CRP to FcγR is required for the protection of mice from LPS toxicity.[148] CRP induces macrophage tumoricidal activity but it is not known whether CRP-FcγR interactions are involved.[149] CRP also binds mouse FcγR.[150-152] SAP also binds to human and mouse FcγR.[153,154]

Binding of SAP to Amyloid Fibrils and Role in Amyloidosis

SAP binds to β-amyloid peptide in a Ca^{2+}-dependent manner and inhibits the formation of amyloid fibrils.[155,156] SAP has been shown to induce cell death in primary cultures of rat cerebral cortex suggesting that SAP may play a role in the development of Alzheimer's disease.[157] Binding of SAP to amyloid fibrils prevents proteolysis of the amyloid fibrils in vitro and thus enhances induction of amyloidosis in vivo as shown by delayed and reduced amyloid deposition in SAP-deficient mice.[158,159] The interference with binding of SAP to amyloid fibrils in vivo may promote regression of the deposits.[160] SAP-deficient mice do not necessarily develop severe autoimmune disease; they have high antinuclear antibodies but no glomerulonephritis.[88]

Long Pentraxin: PTX3

In the 1990s a new member of the pentraxin family was cloned in endothelial cells stimulated with IL-1 or in fibroblasts treated with TNFα.[161,162] The prototypic long pentraxin 3 (PTX3) is characterized by a C-terminal domain sharing a high degree of homology with short pentraxins, associated to an unrelated long N-terminal domain. In spite of the sequence homology, PTX3 differs from CRP and SAP in terms of gene organization and chromosomal localization as well as cellular source, inducing-stimuli, and ligand-binding properties.[1,163] PTX3

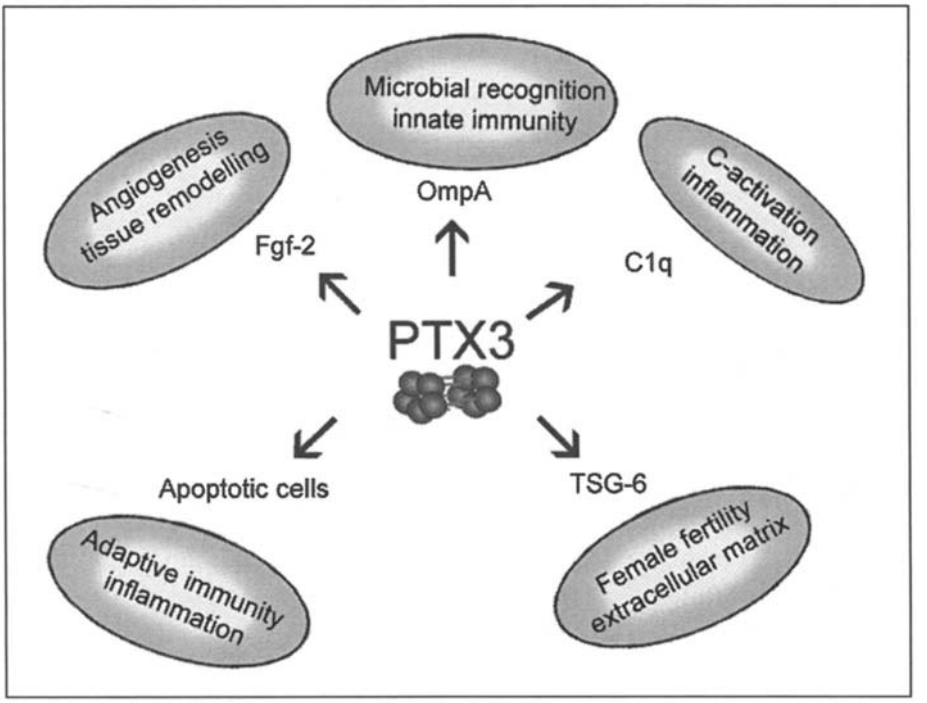

Figure 1. The long pentraxin PTX3 acts as a soluble multifunctional protein. The multifunctional properties of PTX3 involve interaction with a number of different ligands such as the complement component C1q, the growth factor FGF2, the extracellular matrix component TSG-6, late apoptotic cells and outer membrane proteins of Gram-negative bacteria. Interaction of PTX3 with its ligands is essential for the multifunctional properties exerted by this pentraxin in microbial recognition, discrimination between self and nonself, tissue remodelling, and tuning of the inflammatory response. Binding of PTX3 to plastic-immobilized C1q induces activation of the classical complement pathway and the angiogenic activity of FGF2 in vivo and in vitro is blocked by PTX3. In addition PTX3 is an integral component of the extracellular matrix and plays a crucial role in female fertility.

interacts with C1q, the growth factor FGF2, the ECM component TSG-6 and selected pathogens (Fig. 1).[164-169] Recent data in gene-targeted mice show that PTX3 has complex nonredundant functions in vivo, ranging from female fertility and innate immune response against diverse microorganisms, to the assembly of ECM.[1,167,168,170-173] PTX3 is highly conserved in evolution suggesting that the results obtained in animal models are likely to be informative for the function of PTX3 in humans. In humans, PTX3 plasma levels are very low in normal condition (≤2 ng/ml) but increase rapidly in several pathological conditions raising the possibility that PTX3 may have a diagnostic and prognostic role.

Gene and Protein Organization

The human *ptx3* gene is located on chromosome 3, band q25 and is organized in three exons coding respectively for the leader peptide, the N-terminal domain and the C-terminal pentraxin like-domain of the protein. PTX3 protein is 381 amino acids long, including a signal peptide of 17 amino acids, and it has a predicted molecular weight of 40,165 Da. The C-terminal domain contains the pentraxin signature, two conserved cysteines (Cys210 and Cys271) and a N-linked glycosylation site at Asn220. PTX3 protomers are assembled to

form multimers predominantly of 440 kDa apparent molecular mass, corresponding to decamers. In contrast to what was observed for CRP and SAP pentamers, the decameric form of PTX3 is dependent upon interchain disulfide bonds, as demonstrated by nonreducing SDS-PAGE.[164]

A murine *ptx3* has been also described: the murine gene is located on chromosome 3, in a region (q24-28) homologous to human chromosome 3q, and has the same genomic organization in three exons and two introns as the human counterpart.[174] Human and murine PTX3 are highly conserved: both proteins are 381 amino acids long and share 82% of identical amino acids and 92% of conserved amino acids.

A model for PTX3 tertiary folding has been proposed based in part on the sequence homology with CRP and SAP. According to this model, PTX3 pentraxin domain well accommodates on the tertiary fold of SAP, with almost all of the β-strands and the α-helical segments conserved.[174]

PTX3 is produced by a variety of cell types, including mononuclear phagocytes, dendritic cells (DC), fibroblasts, endothelial cells, smooth muscle cells, adipocytes, synovial cells, chondrocytes and cells of epithelial origin, such as renal and alveolar cells.[161,162,175-182] Resting cells generally do not release appreciable levels of the protein but they can be triggered to produce PTX3 by primary inflammatory signals, such as TNFα and IL-1β, toll-like receptor (TLR) ligands and microbial moieties, such as LPS, lipoarabinomannans and outer membrane proteins.[1,169]

Myelomonocytic DCs are major producer of PTX3 in vitro in response to TLR engagement while plasmacytoid DCs do not produce PTX3.[183] On the other side, PTX3 can regulate the maturation program of DCs as well as the secretion of soluble factors such as IL-10 and TNF-α, behaving as a flexible regulator of the function of this cell population.[184] IFNγ and IL-10 play divergent effects on regulation of PTX3 production by DCs: IFN-γ, which has generally a synergistic effect with LPS, inhibits LPS induction of PTX3 while IL-10 amplifies the response to LPS, TLR ligands and IL-1β.[185,186] Production of PTX3 by IL-10-treated DCs is likely to be associated with matrix deposition, considering the role of IL-10 in the induction of a genetic program related to tissue remodeling.

Role in Female Fertility

Ptx3-deficiency in mice is associated with a severe defect in female fertility.[168,173] Infertility is due to fertilization failure in vivo and is associated with an abnormal cumulus oophorus expansion. The oocyte develops normally in the absence of PTX3 and can be fertilized in vitro, but the unstable cumulus ECM, in which cumulus cells are uniformly dispersed instead of radiating out from a central oocyte, accounts for fertilization defect in vivo. Cumulus granulosa cells express PTX3 mRNA during the preovulatory period, upon the induction by hormonal ovulatory stimuli as follicle stimulating hormone or human chorionic gonadotropin, and by oocyte derived soluble factors, in particular a member of the TGFβ family, growth differentiation factor-9.[167,173] The protein localizes in the ECM, where it plays a crucial role in the assembly of the hyaluronic acid (HA)-rich matrix of the cumulus oophorus.[167,187] PTX3 does not interact directly with HA, but it binds TSG-6, which participates in the assembly of the HA-rich matrix. PTX3/TSG-6 complexes might thus serve as an anchoring site for multiple HA molecules, thereby substantially forming a multi-molecular complex that acts as a 'node' for cross-linking HA chains. Therefore, PTX3 plays a nonredundant role as structural constituent of the cumulus oophorus ECM essential for in vivo fertilization.

Human cumulus cells express PTX3 as well, and PTX3 protein is present in human cumulus matrix, suggesting that this molecule might have the same role in human female fertility.[167,187] Studies have been conducted to assess the potential role of PTX3 as diagnostic marker for oocyte quality: real-time PCR data showed a relatively higher abundance of PTX3 mRNA in cumulus cells from fertilized oocytes compared with cumulus cells from unfertilized oocytes;[187] by contrast, PTX3 levels in follicular fluids did not correlate with oocyte quality.[188]

Results collected over the years on PTX3 levels in patients with a series of inflammatory and infectious disorders (see below) outlined a role of this protein as marker of pathology and prognostic factor, in particular, for conditions reflecting the involvement of the vascular bed. Endothelial dysfunction is a prominent feature of preeclampsia, an important cause of maternal as well as perinatal morbidity and mortality, which may arise in the third trimester of gestation. The pathogenesis of this disease, still not completely defined, is characterized by an excessive maternal inflammatory response; accordingly, significantly higher levels of PTX3 have been found in preeclampsia compared to normal pregnancies.[189]

Role in Innate Immunity, Inflammation and Apoptotic Cell Clearence

Pathogen recognition is a common feature among the members of the pentraxin family including PTX3 and efforts have been made in order to identify the molecular moieties recognized on bacterial surface. PTX3 does not bind LPS as well as lipoteichoic acid, N-acetylmuramyl-L-alanyl-D-isoglutamine, exotoxin A and enterotoxins A and B. However, it binds with high affinity to recombinant outer membrane protein A from *Klebsiella pneumoniae* (KpOmpA). KpOmpA binds and activates macrophages and DCs in a TLR2-dependent way, activating a genetic program that includes induction of PTX3. PTX3 in turn binds KpOmpA, and plays a crucial role in the amplification of the inflammatory response to this microbial protein, as demonstrated by the impairment of the inflammatory response induced by KpOmpA in *ptx3*-deficient mice.[169]

PTX3 plays an important role in defence against selected pathogens such as *Aspergillus fumigatus*. This can be explained, at least in part, by an opsonic effect of PTX3, facilitating ingestion of conidia by macrophages.[168] Macrophages from PTX3-overexpressing mice have an improved phagocytic activity towards zymosan and *Paracoccidioides brasiliensis*.[170] Moreover, recombinant PTX3 binds to zymosan and *P. brasiliensis*, and functions as an opsonin, thereby increasing the phagocytic activity of peritoneal macrophages from wild-type animals. These findings provide evidence for a role of PTX3 as a functional ancestor of antibodies and imply the existence of a receptor for this molecule. Accordingly, a binding site has been observed on murine macrophages as well as human mononuclear phagocytes and DCs (Bottazzi, unpublished observations).

Studies in *ptx3*-deficient mice suggest that the role played by PTX3 in innate resistance is nonredundant and relevant in selected fungal and bacterial infections (*A. fumigatus, P. aeruginosa, S. typhimurium*) and irrelevant in others (*L. monocytogenes, S. aureus*, polymicrobic intra-abdominal sepsis) (Garlanda, unpublished observations).[168] In particular, extreme susceptibility was observed to invasive pulmonary aspergillosis which was associated with the lack of development of appropriate and protective Th1 anti-fungal responses and to an unbalanced cytokine profile skewed towards a Th2 response.[168] The specificity of the defect and the therapeutic potential of PTX3 could be demonstrated by the complete protective effect following treatment with recombinant PTX3.[168,190] Variable susceptibility to different pathogens suggests that PTX3 deficiency does not cause a generalized immunodeficiency, and that PTX3 is involved in recognition and resistance against specific microorganisms.

Ptx3 overexpressing and deficient mice were used to evaluate the role of PTX3 in inflammatory conditions. *Ptx3* overexpression increases resistance to LPS toxicity and cecal ligation and puncture,[171] but induces an exacerbated inflammatory response and reduces survival rate following intestinal ischemia reperfusion injury.[172] In a model of kainate-induced seizures, *ptx3*-deficient mice had more widespread and severe IL-1-induced neuronal damage. In this model PTX3 confers resistance to neurodegeneration, possibly by binding to dying neurons and rescuing them from otherwise irreversible damage.[191]

Like other members of the pentraxin family,[132,192] PTX3 binds apoptotic cells inhibiting their recognition by DCs.[184,193] Binding occurs late in the apoptotic process and modulates cytokine production by DC. In addition, preincubation of apoptotic cells with PTX3 enhances C1q binding and C3 deposition on the cell surface, suggesting a role for PTX3 in the complement-mediated clearance of apoptotic cells.[165] Moreover, in the presence of dying cells,

PTX3 may contribute to editing recognition of apoptotic self versus infectious non self and restricts the cross presentation of antigens derived from dying cells.[184] These results suggest that PTX3 has a dual role in the protection against pathogens and in the control of autoimmunity.

Role in Human Pathology

The results obtained in gene-modified mice together with the similarities to a widely used marker of inflammation such as CRP, have given impetus to efforts aimed to investigate the role of PTX3 in diverse human pathologies. PTX3 is expressed at very high levels in the heart of rodents, after systemic administration of microbial products and inflammatory cytokines or ligation of the left coronary artery to model acute myocardial infarction (AMI) (Latini, unpublished observations).[174] PTX3 is present in atherosclerotic lesions and is induced by oxidized LDL in smooth muscle cells, moreover PTX3 increases tissue factor expression by mononuclear cells, potentially playing a role in thrombogenesis and wound healing.[178,194-196] In this context a pilot study was conducted in a small group of patients with AMI, showing that PTX3 plasma levels increase rapidly reaching a peak 6-8 hours after the onset of symptoms.[196] In the same context, plasma CRP increased, but it peaked much later, between 24 and 48 hours after symptom onset. Because CRP is produced mainly by the liver in response to IL-6 and PTX3 by the heart and vasculature in response to primary inflammatory stimuli, it was hypothesized that PTX3, rather than CRP, could be an acute-phase reactant more closely related to cardiac injuries such as AMI and therefore could be a sensitive and specific prognostic indicator in this context.[197] This hypothesis was confirmed in a recent prospective study on a large group of patients with AMI where it has been shown that PTX3 is an earlier and stronger prognostic marker of death compared to other accepted markers of myocardial necrosis such as creatine kinase and troponin T.[198]

PTX3 blood levels, barely detectable in normal conditions (1-2 ng/ml), increase rapidly and dramatically (200-800 ng/ml) during a range of pathological conditions others than AMI. Plasma levels of PTX3 are increased in diverse infectious disorders, including sepsis, *A. fumigatus* infections, active pulmonary tuberculosis and dengue virus infection.[168,199-201] The higher levels of PTX3 observed in patients with pulmonary tuberculosis or dengue are associated with disease severity and possibly with clinical outcome. Patients with active vasculitis have significantly higher plasma levels of PTX3 than patients with quiescent disease.[202] PTX3 inhibition of macrophage phagocytosis of late apoptotic cells could in part explain the phenomenon of leukocytoclasia observed in small vessel vasculitis. PTX3 is also expressed at high levels in patients with systemic juvenile idiopathic arthritis.[203]

A general feature common to all these pathologies is the rapidity of PTX3 increase compared to CRP: CRP is made in the liver in response primarily to IL-6 while PTX3 is produced locally by a number of different cells in response to proinflammatory signals and thus representing a rapid marker for local activation of inflammation and innate immunity.

Data collected so far in a number of different pathologies indicate a correlation between PTX3 plasma levels and severity of disease, suggesting a possible role of PTX3 as marker of pathology. It remains to be elucidated whether the impressive correlation with outcome and severity actually reflects a role in the pathogenesis of damage, for instance by amplifying the complement and coagulation cascades.

Conclusion

Most suggested functions of short pentraxins CRP and SAP are based on their pattern-recognition characteristics seen in vitro (Table 1). The data on the exact physiological situations in vivo where the short pentraxins would interact with their ligands are beginning to emerge. Likewise, the functional consequences of the recognition properties of CRP and SAP are subjects of current research interests (Fig. 2). Of most importance are the values of CRP in bacterial infections, atherosclerosis and autoimmunity and the value of SAP in amyloidosis. The contribution of the two known effector functions of short pentraxins, that is complement activation and phagocytosis, is under intensive investigation in mouse models of human diseases where these pentraxins have been implicated.

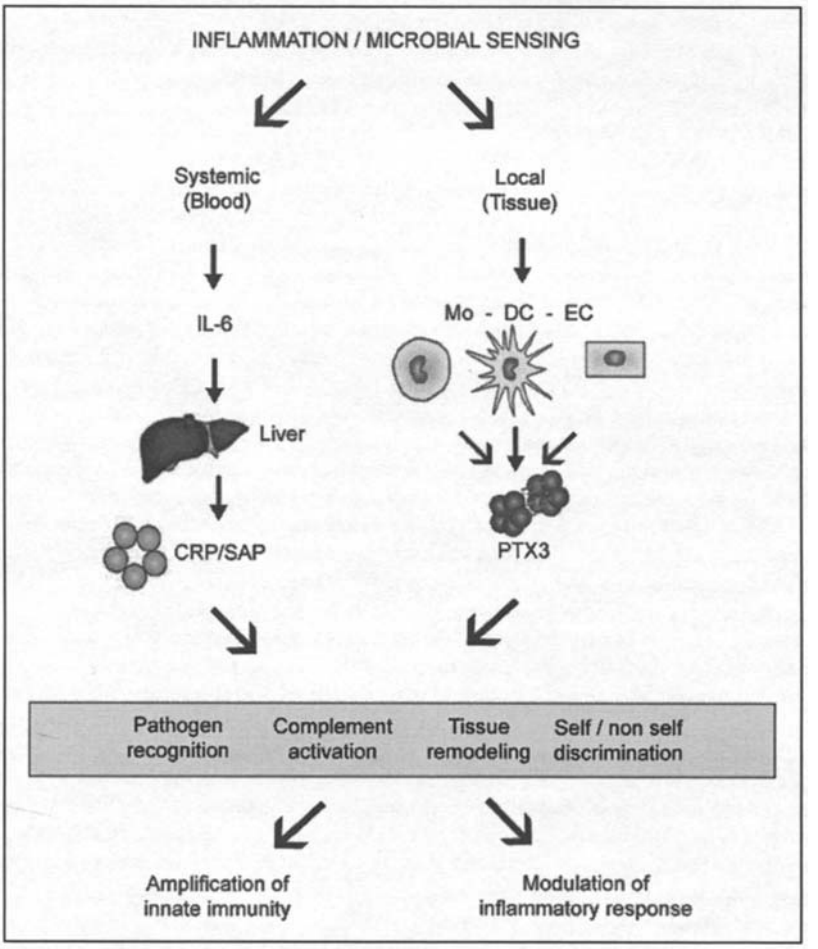

Figure 2. Pentraxins in innate immunity. Inflammation and microbial sensing induce both local and systemic responses characterized by the production of different members of the pentraxin family. Systemic response involves production by the liver of the prototypic short pentraxin CRP and SAP while local response involves production by macrophages, dendritic cells and endothelial cells of the long pentraxin PTX3. Both short and long pentraxins recognize pathogens, activate the classical complement cascade, participate in tissue remodelling and in self/non self discrimination, outlining the role of these proteins in the amplification of innate immunity and in the modulation of inflammatory response.

Gene targeting of the prototypic, evolutionarily conserved, long pentraxin PTX3 has unequivocally defined the role of this molecule at the crossroad of innate immunity, inflammation, matrix deposition and female fertility (Fig. 1).[1] Moreover, recent progress has further defined the structure, regulation, microbial recognition and in vivo function of PTX3.

PTX3 is a component of the complex and complementary network of cellular and humoral pattern recognition receptors involved in the recognition and response to microbial elements and damaged tissues, in tuning inflammatory reactions, in discriminating between infectious nonself and apoptotic self. Translational efforts suggest that PTX3 may represent a new marker of innate immunity and inflammation, rapidly reflecting tissue and vascular bed involvement.

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CHAPTER 8

Target Pattern Recognition by Complement Proteins of the Classical and Alternative Pathways

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Abstract

The complement system is a major component of the innate defence of animals against invading microorganisms, and is also essential for the recognition and clearance of damaged or structurally-altered host cells or macromolecules. The system is activated by three different pathways, each of which responds, using different recognition molecules, to a very wide range of activators. The recognition protein of the complement classical pathway, C1q is described in detail here, with comparisons to the alternative pathway.

The Complement System

A major component of innate immunity in vertebrate animals is the complement system. Found in plasma, complement is a collection of over 30 proteins that are capable of recognising and initiating a response against a wide range of microorganisms and damaged or altered host components.[1-4] Complement makes use of three recognition pathways: the classical, lectin and alternative pathways (Fig. 1). Each of these activates a proteolytic cascade that converges at the C3 convertase—a serine protease complex which cleaves C3, leading to the deposition of C3b on the surface of the microorganism. From this point there are three possible responses against the complement-activating particle: lysis (through formation of the membrane attack complex), opsonisation (marking of the microorganism for phagocytosis) and inflammation (through the release of small inflammatory peptides which recruit phagocytes and other inflammatory cells).

The classical pathway uses the C1 complex (made up of the glycoprotein C1q bound to 2 molecules of each of the serine protease proenzymes C1r and C1s ($C1qr_2s_2$)) as its recognition molecule. C1q is able to bind and recognise a wide range of targets including immune complexes.[5,6] On binding a target, C1q undergoes a conformational change which causes C1r to autoactivate.[7] C1r then cleaves C1s which is able to continue the proteolytic cascade by cleaving C4 and C2. The lectin pathway (Fig. 1) relies on complexes similar to C1 to initiate activation. In the lectin pathway, there are proteins similar in shape and function to C1q: these are mannose-binding lectin (MBL) and the ficolins (H-, L- or M- ficolin). MBL binds to surfaces via neutral sugar arrays (eg mannose, glucosamine) on the target. Ficolin binding specificity is less well-defined, but seems to involve N-acetyl sugars and other acetylated species.[8] MBL and the ficolins circulate in complex with proteases called the MASPs

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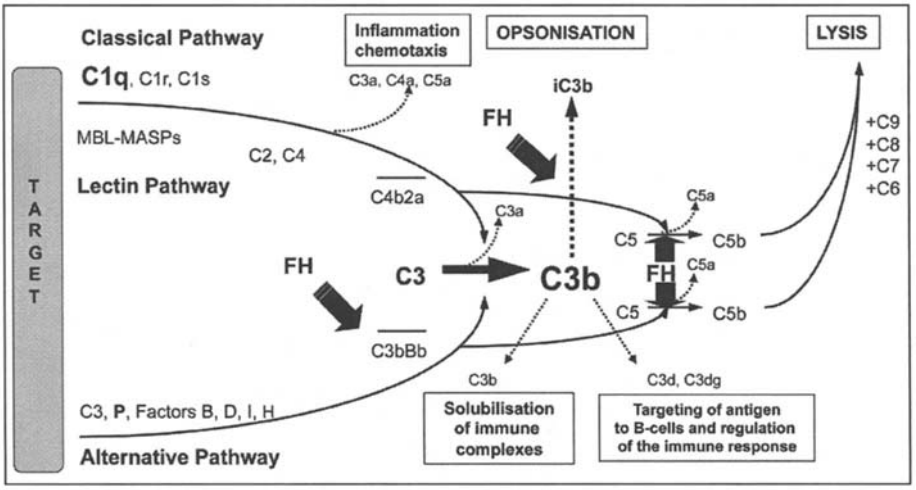

Figure 1. Overview of the human complement system. The complement cascade can be activated by three distinct pathways on recognition of a target. All three pathways lead to the formation of multicomponent serine proteases (C3 convertases), which allows these three routes to converge to the activation of C3. The covalent binding of C3b to activating surfaces is responsible for initiating many of the effects of the system.

(MBL-associated serine proteases) which are homologous to C1r and C1s.[9] On binding of MBL or ficolins to a target, MASP-2 autoactivates, and then, like C1s, cleaves C2 and C4. The mechanism of activation of the alternative pathway is less well understood, but relies on spontaneous cleavage and activation of C3, together with recognition of surface properties of target particles by proteins such as properdin and Factor H.[10,11]

C1q and C1 Structure

Human C1q (460 kDa) is composed of 18 polypeptide chains (six of each of the homologous A, B and C polypeptides). The N-terminal region of each chain has collagen-like sequence of repeating Gly-X-Y triplets, and like collagen contains hydroxylysine and hydroxyproline residues. The C-terminal approx. 140 amino acids of each chain forms a globular domain, called a C1q domain. The A chain (223 residues), B chain (226 residues), and C chain (217 residues) associate to form a subunit consisting of a collagen triple helix at the N-terminal end, and a three-lobed globular head (termed gC1q) at the C-terminal end (Fig. 2). The A and B chains are disulphide bonded at the N-terminal end and the C chain is disulphide bonded to the C chain of another ABC subunit. Six such subunits associate to form a structure shaped like a bunch of tulips as viewed under the electron microscope.[12,13] C1r and C1s are both serine protease proenzymes (mol. wt. about 85kDa). C1r forms a dimer, and a C1s molecule binds to each monomer in the dimer. This C1s-C1r-C1r-C1s complex binds via C1r to the collagen-like region of C1q to give a proenzyme C1 complex.[5,14] When C1q binds to a target, C1r and C1s are activated, then after a short period (tens of seconds) are inactivated by the serpin, C1-inhibitor. This reaction causes C1r and C1s to dissociate from C1q, leaving the collagen region free to interact either with cell-surface receptors, or to capture more $C1r_2s_2$ (Fig. 3).[15,16]

Biosynthesis of C1q

C1q circulates in plasma at a concentration of about 80ug/ml as the calcium ion- dependent C1 complex. In humans and other mammals investigated, C1q is not made in hepatocytes. Many different tissues and cell types secrete functionally active C1q in vitro. Theses

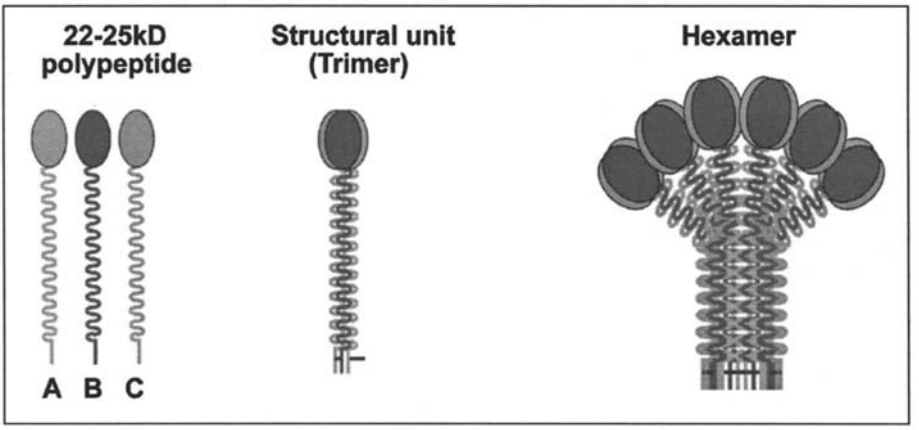

Figure 2. The assembly of C1q from three polypeptide chains. C1q is a macromolecule of 460 kDa and is made up of 18 polypeptide chains, six of each of three types (A, B and C). Each polypeptide is approx 25 kDa and contains a short N-terminal region, a collagen-like region and a C-terminal globular region (three of which form a globular head). Three polypeptides interact to form a collagen triple helix and a three-lobed globular head. Disulphide cross-linking at the N-terminus is shown by horizontal bars.

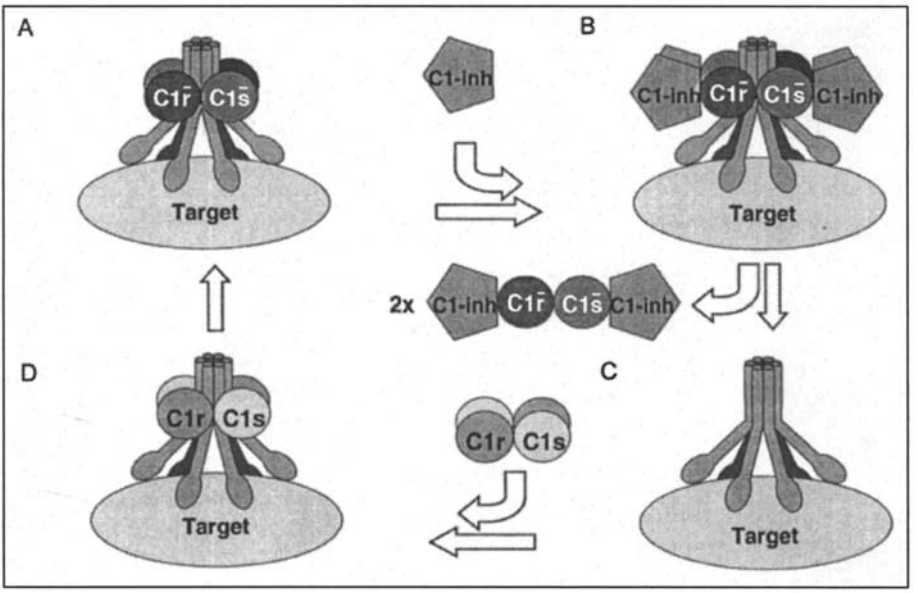

Figure 3. The interaction between activated C1 and C1-inh. When the C1 complex binds to an activator, C1r becomes activated and in turn activates C1s (A). C1-inh then forms equimolar covalent complexes with activated $C1r_2s_2$ and weakens the interaction between C1q and $C1r_2s_2$, thus releasing two molecules of C1-inh-C1r-C1s-C1-inh from each complex (B). C1q left on the activating surface can interact with a C1q receptor or it can recruit more proenzymic $C1r_2s_2$ from the circulation, which can then be activated to complete the cycle (C and D). In this way, a small quantity of bound C1q can activate many $C1r_2s_2$. C1q not occupied by $C1r_2s_2$ can interact with C1q receptor. Based on reference 15.

include epithelial cells in the intestine,[17] mesenchymal cells[18] and cells of monocyte-macrophage lineage.[19,20] C1r and C1s, however, like most soluble complement proteins, are synthesised and secreted mainly by hepatocytes in the the liver.[21] Thus C1r and C1s associate with C1q to form the C1 complex after secretion from different cell types. The association has very high affinity.[22]

Target Recognition by C1q

C1q binds to a wide range of charge/hydrophobicity patterns on the surfaces of targets that act as classical pathway activators. Multivalent binding (the engagement of two or more globular heads) is required to provide high-avidity binding, and for activation of C1r.[23-26] Thus the target will normally present an (ordered) array of repeated motifs to which all six heads of each C1q molecule can bind. Until the 1970s, it was widely assumed that C1q bound only to antibodies in antibody-antigen complexes. This opinion is still often expressed in reviews, but in fact C1q binds to a very wide range of targets in the absence of antibodies.[5,6,27] Initial binding of the C1q "head" to a target is mainly an electrostatic interaction, but further reorientation occurs as more heads of C1q bind to the target, and this secondary binding is stabilised mainly by nonpolar interactions.[28]

Interaction of C1q with Immunoglobulins

In humans five distinct classes of immunoglobulin occur, IgG, IgM, IgA, IgE and IgD which share similar structures. Each immunoglobulin molecule is composed of two identical 50-77 kDa class-specific heavy chains (γ, μ, α, ε and δ), joined by one or more disulphide bridges. Each heavy chain is disulphide bridged to a 25 kDa light chain of which there are two isoforms named κ and λ. Both heavy and light chains are composed of regions called immunoglobulin domains. The immunoglobulins are split into Fab (antigen binding fragment) and Fc (crystallisable fragment) regions, separated by a flexible region which has no defined secondary structure. This flexible hinge in IgM and IgE is replaced by an additional Constant Heavy (CH) domain, which greatly restricts the motion of the Fab heads relative to the Fc.[29]

C1q binds to Fc regions of antigen-bound IgG and IgM. There are occasional reports of C1q binding to other classes of immunoglobulin, but in general IgA, IgE and IgD do not bind C1q. Binding of C1q to IgG or IgM in immune complexes via the globular head domain activates the classical pathway of complement. The binding of C1q to nonaggregated (monomeric) IgG is very weak but the binding to multiple, closely-spaced IgG (ie an array of IgG attached to the surface of a particulate target, or artificially heat-aggregated IgG) is enhanced 10^3-10^6-fold.[23-26] C1q binds to the Cγ2 domain of IgG and Cμ3 domain of IgM. The binding affinity of C1q to different subclasses of IgG varies, with the strongest binding to human IgG1 and IgG3 but weaker interaction with IgG2 and no interaction with human IgG4. A site-directed mutagenesis study of a mouse Ig2b isotype identified three charged residues which are involved in the binding of C1q to IgG.[30] These residues, in the IgG heavy chain, are E318, K320, and K322 located in the Cγ2 domain and are highly conserved in different IgG isotypes and in different species. However, these binding sites for C1q are not relevant to all IgG. A study by Idusogie et al[31] showed that C1q binding sites in a human IgG1 are different from that of murine IgG2b. Using Rituximab (a chimeric monoclonal antibody with human IgG1 constant domains), they demonstrated that alanine substitution at positions E318 and K320 in human IgG1 had no effect on C1q binding. K326 and E333 in this human IgG1 are important in the C1q binding and complement activation.

C1q binds very weakly to soluble IgM, but when IgM is bound to an antigen, IgM undergoes a conformational change, into a bent form referred to as the "staple" form (from its shape) and C1q binds (multivalently and with high avidity: potentially one C1q to one IgM pentamer or hexamer).[23] The charged residues Asp 417, Glu 418, and His 420 in the Cμ3 region of IgM are implicated in the binding of C1q.[32]

Interaction of C1q with Nonimmunoglobulin Activators

In in vitro experiments on complement activation or in assays of complement classical pathway protein activity, immune complexes or aggregated IgG are nearly always used as activators, as this is easy to do. However many nonimmunoglobulin activators have also been described (Table 1). The classical pathway, therefore, can be 'antibody-independent' and is activated by direct interaction of C1q with nonimmunoglobulin substances. These include a variety of polyanions (or negatively-charged clusters) presented generally as an array, or as a large polymer, such that more than one head of C1q can be engaged. Very recently, it has been recognised that C1q has additional modes of target recognition in that it can bind some neutral sugars in a lectin-like interaction. Arlaud and colleagues[33] have shown that a region of C1q heads binds to arrays containing galactose, N-acetylglucosamine, or deoxy-D-ribose. Such lectin-like activity of C1q may be important in binding to DNA,[33] and recognition of this new property of C1q may lead to reinterpretation of the mechanism by which C1q interacts with some other known targets.

Nonimmunoglobulin ligands for C1q include (Table 1) lipid A in lipopolysaccharide on the surface of Gram-negative bacteria,[34,35] porins[36] on Gram-negative bacteria, nucleic acids,[33,37] cardiolipin and other negatively-charged phospholipids such as phosphatidylserine.[38-40] The pentraxins, C-reactive protein (CRP) and serum amyloid P component (SAP) themselves recognise invading bacteria, and host cell breakdown products such as chromatin, and bind to them. The bound pentraxins then bind C1q via its globular heads leading to activation of the classical pathway of complement.[41-45] High molecular weight nonimmunoglobulin salivary agglutinins also bind some viruses and bacteria, and can subsequently bind C1q.[46] Some viruses including Moloney murine leukaemia virus and HIV also activate the classical pathway by direct binding of C1q.[27,47] Lipoteichoic acids on some Gram-positive bacteria also bind C1q, and this is dependent on the charge-density of the lipoteichoic acid.[48] C1q also binds directly to Mycobacterium bovis in the absence of antibodies.[49]

Host proteins which become altered by polymerisation also bind C1q and activate complement: these include several amyloids and polymerised prions,[50] and also cross-linked fibrin clots (Y. Kang and R.B. Sim, unpublished). C1q also recognises many "foreign" synthetic polymers, such as carbon nanotubes,[51] plastics.[52,53] C1q also interacts with many

Table 1. C1q binds to many types of charged surfaces

Activating Ligands of C1q
Gram-negative bacteria
Lipid A of lipopolysaccharide, Porins
Gram-positive bacteria
Lipoteichoic acid
Viruses
Moloney virus, Vesicular stomatitis virus, HTLV-1, HIV-1, DNA polyoma virus
Polyanions
Heparin, Chondroitin-4-sulphate
Single-stranded and double-stranded DNA, Polynucleotides
Cardiolipin and other anionic PL in vesicles (or on apoptotic cells)
Other proteins
Ligand-bound CRP and SAP
Immunoglobulins
Fc portion of IgM, IgG (IgG3, IgG1, IgG2)
Amyloids, Prions, Fibrin Clots

glycosaminoglycans (GAGs) and proteoglycans, but not all such interactions promote activation of the complement classical pathway.[27,54-57]

Many of the nonimmunoglobulin targets to which C1q is known to bind appear likely to be involved in homeostasis—the maintenance of host cells and tissues—while others are involved in innate immune resistance to invading microorganisms. C1q recognizes directly many particles (chromatin, mitochondria via cardiolipin, membrane vesicles with exposed anionic phospholipids) which might be formed from host cells which undergo apoptosis or may be damaged by physical injury. Thus complement activation via C1q has a major role in clearing (by opsonisation) damaged and dying host cells. C1q has also been suggested to play a similar role in neuronal remodeling and tissue development in the central nervous system.[58,59]

C1q and Apoptotic Cells

There are many studies on the recognition of apoptotic cells by C1q. C1q binds directly to apoptotic blebs of peripheral blood mononuclear cells, vascular endothelial cells and other cell types in apoptosis via the gC1q (head) domain.[60,61] and enhances the uptake of apoptotic cells by macrophages and dendritic cells.[62,63] It is not clearly established what surface feature C1q recognises on apoptotic cells. Exposed anionic phospholipid is a candidate, as is DNA.[33,40] Binding of C1q to apoptotic cells may also occur via the pentraxins CRP and SAP.[64]

Deficiencies in early classical pathway complement components, particularly C1q, are associated with an increased risk for the development of systemic lupus erythematosus (SLE).[65] SLE is an autoimmune disease characterised by the presence of autoantibodies and high levels of circulating immune complexes. In C1q-deficient mice, immune deposits and multiple apoptotic cell bodies are present in the kidney associated with the spontaneous development of autoantibodies and glomerulonephritis.[66] Apoptotic cells have been suggested to be a major source of autoantigens of SLE.[67] Failure in the clearance of apoptotic cells due to complement deficiency may explain the strong correlation between complement deficiency and the development of SLE.

Structural Basis of C1q Binding to Targets

A major breakthrough in understanding the very versatile binding properties of C1q came when the crystal structure of the C1q "head" was solved and refined to 1.9 Å resolution.[68] Each head is an almost spherical heterotrimer made up of three modules, one from each of the A, B and C chains. The modules are held together by nonpolar interactions, with a central Ca^{2+} ion coordinated by six ligands. This heterotrimeric structure has direct implications in explaining the versatile recognition properties of C1q. Each of the three modules (A or B or C) exhibits particular surface patterns in terms of charged and hydrophobic residues and may, thus, be expected to have specific individual independent binding functions (i.e., A or B or C chains may show selectivity in the binding of a specific complement activator).[5] The homology of the globular regions of the A, B and C chains is shown in Figure 4. Modelling of the binding of activators to the C1q crystallographic structure indicates that IgG binds via charged residues of the B module, while CRP occupies a larger binding site involving A, B and C modules. Crystallographic studies are currently limited by the difficulty in making recombinant C1q. C1q is an oligomer of three separate gene products, so it has not yet been possible to make whole recombinant C1q, or the recombinant heterotrimeric heads. However Kishore and colleagues[70,71] took an alternative approach to making recombinant C1q, and made recombinant homo-oligomeric heads, so that the binding specificity of the A, B and C modules could be studied separately, and mutagenesis could be used to investigate binding interactions. Maltose binding protein fused to globular head region A or B or C domains were made and used to bind to heat-aggregated IgG or IgM or other targets.[71] MBP-ghA bound both heat-aggregated IgG and IgM, while MBP-ghB bound preferentially to aggregated IgG, and MBP-ghC showed preferential affinity for binding IgM rather than IgG.[72] Similar constructs showed preferential binding to peptide targets: A showed a preference for an HIV target peptide, B for a beta-amyloid

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C1qA        KDQPRPAFSA IRRN..PPMG GNVVIFDTVI TNQEEPYQNH SGRFVCTVPG YYYFTFQVLS
C1qB        KATQKIAFSA TRTINVPLRR DQTIRFDHVI TNMNNNYEPR SGKFTCKVPG LYYFTYHASS
C1qC        KQKFQSVFTV TRQTHQPPAP NSLIRFNAVL TNPQGDYDTS TGKFTCKVPG LYYFVYHASH
Consensus   K.....aFsa tR....Pp.. ...irFd.Vi TN....Y... sGkFtCkVPG lYYFtyhass

C1qA        QWEICLSIVS SSRGQVRRSL GFCDTTNKGL FQVVSGGMVL QLQQGDQVWV EKDPKKGHIY
C1qB        RGNLCVNLMR G.RERAQKVV TFCDYAYN.T FQVTTGGMVL KLEQGENVFL QATDKNSLLG
C1qC        TANLCVLLYR S....GVKVV TFCGHTSK.T NQVNSGGVLL RLQVGEEVWL AVNDYYDMVG
Consensus   ..nlCv.l.r s.r....kvv tFCd.t.k.t fQV.sGGmvL .L.qGe.Vwl ...dk....g

C1qA        QGSEADSVFS GFLIFPSA..
C1qB        MEG.ANSIFS GFLLFPDMEA
C1qC        IQG.SDSVFS GFLLFPD...
Consensus   ..g.adSvFS GFLlFPd...
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Figure 4. The amino acid sequences of the globular head domains (gC1q) of human C1q A, B and C chains. The alignment was produced by Multalin version 5.4.1 (Corpet, 1988).[69] The consensus sequence shows residues which occur in all three chains (capital letters) or in 2 out of 3 chains (lower case).

peptide, and C for an HTLV peptide.[72] Mutagenesis of the recombinant heads implicated B module arginine residues in IgG binding, and B and C residues in CRP binding.[73,74] Studies with further mutants and single-chain antibodies provide evidence for reorientation after initial binding.[75] C1q binding has long been considered to be independent of divalent metal ions, as binding to many targets occurs in the presence of EDTA, but it appears that the bound Ca^{++} ion may subtly alter binding interactions.[76] Thus recognition of targets by C1q is heterogeneous and complex, and as noted earlier, initial mainly ionic binding "matures" to involve hydrophobic interaction.

C1q Receptors

The collagen-like region of C1q interacts with cell surface proteins expressed by many cell types. Several cell surface proteins which may bind to C1q have been tentatively identified. Studies of C1q receptor activity show that the structurally-related proteins mannose-binding lectin (MBL), lung surfactant proteins A and D (SP-A, SP-D), adiponectin, conglutinin generally share the same receptor, binding to the receptor via a charged region of their collagen domains.[77-79] A protein on the surface of leukocytes that binds to the collagen-like region of C1q (and of MBL and SP-A) was designated cC1qR, and was shown to be (almost) identical to calreticulin (CRT).[77,78] CRT is a highly abundant, mainly intracellular protein which is proposed to have numerous biological functions. It is probably constitutively expressed on the surface of most cells. However, CRT lacks a transmembrane domain. Therefore, for signal transduction, it is likely that CRT must associate with a transmembrane receptor. CD91, also known as the α_2 macroglobulin receptor or LRP1 serves as this receptor at least on cells of monocyte lineage, hepatocytes and fibroblasts.[62] CRT in association with CD91 acts in the phagocytic clearance of C1q-opsonized apoptotic cells.[62] On cell types which do not express CD91, other cell surface proteins may serve as anchors for CRT. HLA class I heavy chain and CD59 have been reported to have this property. A protein related to CD91, named megalin or LRP2, can bind C1q (but not MBL or SP-A) directly and mediate its uptake into cells.[77] Megalin is expressed mainly on absorptive epithelia, and the physiological relevance of its interaction with C1q has not yet been explored.

There are many papers describing a protein called C1qRp or CD93 as a putative C1q receptor expressed mainly by monocytes and macrophages, which was suggested to enhance phagocytosis in the presence of C1q.[80] C1qRp/CD93 is also described in many reviews and databases as a C1q/MBL/SP-A receptor, but it has now been shown that C1qRp/CD93 is not a receptor for any of these proteins but is instead an adhesion receptor.[81]

Complement receptor 1 (CR1or CD35) present on leukocytes and erythrocytes binds the complement activation fragments C3b and C4b, and has also been reported to bind C1q, MBL and SP-A.[82] CR1-C1q interactions have not yet however been confirmed by other labs.

The interaction of C1q with cell surface proteins/receptors present on various cell types can trigger an array of cellular responses, including phagocytosis, enhanced microbial elimination and removal by phagocytes, induction of chemotaxis and production of cytokines[83] Thus, after C1q has bound to a target and activated the classical pathway, it has other roles in interactions with cells: these occur only if C1r and C1s have been dissociated from C1q by the action of C1-inhibitor, as noted above. The C1 complex does not bind to C1q receptor (CRT) as CRT occupies the same binding region as does C1r.

C1q across the Animal Kingdom

The discussion above is based on human C1q, which is the most intensively investigated. However C1q from other mammals, including mouse, rat, cow, rabbit has been isolated and is of similar structure and function to human C1q. There have been no extensive comparisons of target recognition specificity, although there is limited information about cross-species incompatibilities in the binding of C1q to immunoglobulins. As summarised in reference 84, C1q has also been identified, at the protein level, in a few species representing birds, amphibians, bony and cartilaginous fish, and the jawless fish (Agnatha). Agnathans have no immunoglobulins, so lamprey C1q cannot have evolved to recognise IgG or IgM. It appears to recognise GlcNAc (N-acetyl glucosamine)-containing structures.[85] This appeared at the time it was first reported (2004) to be a specificity profoundly different from mammalian C1qs, but as noted above, Arlaud and colleagues have subsequently detected lectin-like activity in mammalian C1q.[33] A protein with C1q-like structure (collagen region and head-region modules homologous to mammalian C1q) and possible innate immune function has recently been identified in the cephalochordate Amphioxus.[86] This does not bind GlcNAc, but does bind bacterial lipopolysaccharide. C1q-like DNA sequences have been identified in two invertebrates, the ascidian (sea squirt) *Ciona Intestinalis* and the echinoderm *Strongylocentrotus purpuratus* (purple sea urchin).[84]

Properdin and the Alternative Pathway

The alternative complement pathway has no recognition proteins similar in structure to C1q, collectins or ficolins, and it has been generally accepted that there was no protein in the alternative pathway which had the same type of recognition function as C1q: that is, to bind with at least moderate specificity to a target, and stimulate protease activation or activity.[1-4] In the blood, constant turnover of C3 to form C3b occurs, by hydrolysis or low-level proteolysis of C3. The C3b formed reacts harmlessly with water, or binds randomly to any nearby surface, by reacting with amino or hydroxyl groups on the surface. On host cell surfaces, it is inactivated by complement regulatory proteins, such as CD35, CD46, CD55, which bind C3b and prevent it from forming a C3-activating enzyme (C3 convertase, C3bBb, Fig. 1) . Nonhost surfaces may be able to bind Factor H, a soluble regulatory protein which has similar activity to CD35, and Factor H will also inhibit C3b from forming C3bBb. Factor H binds surfaces via poorly-defined charge clusters (rather like C1q) so it confers specificity on the activation of the alternative pathway, in that activation occurs if Factor H (and CD35, 46, 55) are absent. When C3b is deposited on a surface with no regulators, it may form a complex with the soluble protease proenzyme, Factor B, which is activated by Factor D, to form C3bBb, which activates C3b, resulting in binding of many C3b molecules to the surface, opsonising it. Another complement regulatory protein, Properdin, appeared to stimulate C3b deposition, and it was determined that it binds to C3bBb, stabilising this two-subunit protease, and increasing the time period over which it is active.

Recently, there has been evidence that properdin can bind directly to some types of surface, and there act as a focus for the assembly of C3bBb.[10] If this is the case, it is acting as a recognition protein, binding to some feature of a surface, and promoting protease activity.

As reviewed in reference 10, Properdin binds directly to several well-characterised alternative pathway activators, such as rabbit erythrocytes and zymosan. It has recently been shown to bind to apoptotic T cells, via sulphated glycosaminoglycans, and to the bacterium Neisseria gonorrhoeae. Studies of direct binding of Properdin to potential alternative pathway activators is at an early stage, but it is possible that Properdin will be shown to have the capacity to recognise a wide range of targets, as does C1q.

Properdin is an oligomeric protein, made up of small oligomers (trimers to pentamers) of a single 45kda polypeptide chain type. Each polypeptide is made up of six homologous thrombospondin Type 1 domains (TSRs).[87] Like the A, B, C chains of C1q, the TSRs of Properdin may have independent or overlapping binding specificities, and oligomer formation provides the potential for multiple low-affinity interactions (eg binding of one TSR to a target) to form a high-avidity binding. Again superficially like C1q, Properdin is synthesised mainly in cells of myeloid lineage, and not in liver hepatocytes. Release of Properdin from neutrophils has been suggested as a major determinant of localised alternative pathway activation.[88]

Dedication

This chapter is dedicated to Kenneth B.M. Reid, FRS, on his retirement in 2008 as director of the MRC Immunochemistry Unit. In his laboratory the sequences and structures of C1q and Properdin (and many other innate immune proteins) were revealed.

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CHAPTER 9

Pattern Recognition in Phagocytic Clearance of Altered Self

Yoshinobu Nakanishi,* Peter M. Henson and Akiko Shiratsuchi

Abstract

Cells that are unnecessary or harmful to our body emerge in substantial numbers throughout our life. Such "unwanted" cells need to be promptly and selectively removed for tissue homeostasis to be maintained. Most of those cells are induced to undergo physiologic cell death, i.e., apoptosis, and subsequently eliminated by phagocytosis. Target selectivity in this phagocytosis reaction comes from the specific cell-cell interaction between phagocytes and dying cells. The surface structure of apoptotic cells is altered during the death pathway so that they become pattern recognizable as "altered self" by phagocytes, and such surface structures are sometimes called ACAMPs for apoptotic cell-associated molecular patterns. ACAMPs arise either from the exofacial exposure of endogenous molecules or the modification of preexisting surface molecules. Pattern-recognizing phagocytosis receptors present at the surface of phagocytes specifically bind, either directly or indirectly with an aid of bridge molecules, to ACAMPs and transmit signals to induce phagocytosis of bound apoptotic cells. Phagocytes often evoke subsequent actions, rather than simply digesting engulfed apoptotic cells, for a finer tuning of tissue homeostasis. In contrast, precise mechanisms and consequences of cells undergoing nonapoptotic death, i.e., necrosis or autophagy-related death, are less well understood.

Roles and Mechanisms of Phagocytosis

Throughout the life of multi-cellular organisms, cells of particular types are eliminated in certain places in the body and at certain developmental stages. Prompt and selective removal of such unwanted cells is prerequisite for morphogenesis, establishment of tissue functions, tissue renewal, avoidance of diseases, and effective progress of tissue functions (Table 1). Cells to be removed are either those cells that are foreign to our body or own cells that have become dispensable. The former includes invading microbes and transplanted tissues, and the latter, which might be called "altered self", is exemplified by cells that are either structurally or functionally used, unwanted, aged, or harmful. Removal of those cells creates space for morphogenesis; eliminates obstacles to tissue functions; avoids excessive cellular action; exterminates pathogens; and prevents noxious contents of dying cells from leaking into the tissues. Failure in the expeditious removal of altered self sometimes leads to the development of diseases such as inflammation and autoimmunity.

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Target Pattern Recognition in Innate Immunity, edited by Uday Kishore.

Table 1. Maintenance of tissue homeostasis by phagocytic clearance of altered self

Consequences	Cells to Be Cleared	Phagocytes
Morphogenesis		
formation of digit	interdigital cells	macrophages
Establishment of tissue function		
establishment of immune system	self-reacting lymphocytes	macrophages
formation of neural network	neurons connected with inappropriate tissues	microglia, macrophages
Tissue renewal		
renewal of various cells	aged cells	macrophages, others
renewal of photoreceptor	aged outer segment of photoreceptor	retinal epithelial cells
Avoidance of diseases		
inhibition of autoimmune disease	apoptotic cells	macrophages, others
inhibition of excessive immune response	activated T cells	macrophages
inhibition of excessive inflammation	neutrophiles	macrophages, Kupffer cells
removal of microbial pathogen	microbe-infected cells	neutrophiles, macrophages, others
Progress of tissue function		
differentiation of reticulocyte	nuclei of erythroblast	macrophages
involution of mammary gland	aged epithelial cells of mammary gland	macrophages, epithelial cells
involution of corpus luteum	corpora lutea with no fertilization	macrophages
production of sperm	apoptotic spermatogenic cells	Sertoli cells

Removal of unwanted cells is accomplished by phagocytosis, an event where cells are engulfed and digested by other cells.[1,2] Cells that possess phagocytic activity are termed phagocytes, and several types of phagocytes exist in our body (Fig. 1). Phagocytes are classified into two groups, professional and amateur cells. Professional phagocytes, macrophages as a representative, are full-time executors of unwanted cells. In contrast, amateur phagocytes, which exert functions other than phagocytosis most of the time, exhibit phagocytic activity only when it is needed. There is another classification of phagocytes; that is, those phagocytes that circulate through our body and are responsible for phagocytosis in various places, and the others that are localized in certain tissues and engaged in phagocytosis only in there. Monocytes/macrophages and neutrophils, which travel around via the blood vessel, are representative of the former, and the latter include osteoclasts, microglia in the brain, nurse cells in the thymus, Kupffer cells in the liver, Sertoli cells in the testis, and tissue-restricted macrophages in the lung and the kidney. Phagocytosis is induced when receptors present on the surface of phagocytes are activated by target cells. Upon binding of target cells, receptors transmit a signal in phagocytes, which in most cases leads to rearrangement of the actin cytoskeleton. As a result, portions of the plasma membrane of phagocytes extend and surround the targets. There are other modes of phagocytosis where extension of the membrane does not occur—the target particles appear to "sink" into the phagocyte. It is presumed that the mode of engulfment varies depending on which receptors are responsible for the induction of phagocytosis.[1]

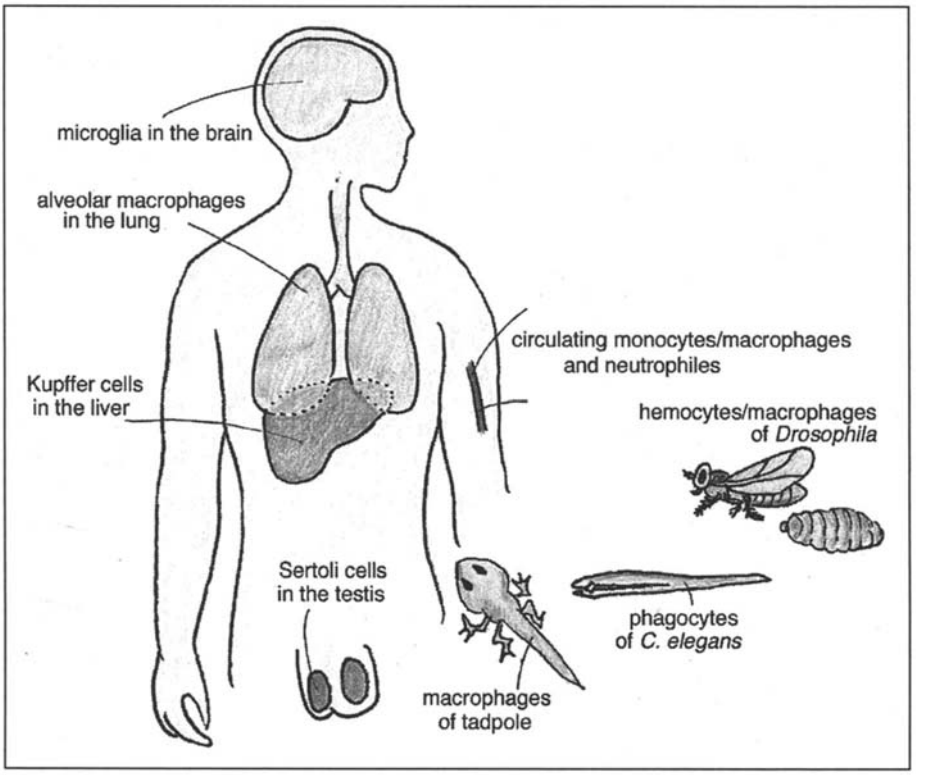

Figure 1. Phagocytes of human and other species. Names and localization of phagocytes of human, frog, *Drosophila*, and *C. elegans* are shown.

Target selectivity in phagocytosis reactions is defined by specific molecular interaction between ligands and receptors, which are present at the surface of target cells and phagocytes, respectively.[1-3] Phagocytosis ligands are either molecules that preexist at the cell surface or soluble molecules that afterward bind to target cells. The latter type of phagocytosis ligands are called opsonins, and representatives known to be involved in host defense include serum proteins such as immunoglobulins, complement components, and collectins. Bacteria are phagocytosed by macrophages and neutrophils mostly in a manner dependent on opsonization by immunoglobulin and complement, which are respectively recognized by Fc receptors and complement receptors of phagocytes.[4] The signaling pathway located downstream of Fc receptors is the best characterized, in which a small G protein activated at the end of the protein phosphorylation relay induces rearrangement of the actin cytoskeleton. Preexisting surface molecules of bacteria, i.e., components of the cell wall or the outer membrane, serve as ligands to trigger humoral innate immune responses in macrophages.[5] However, it is uncertain whether or not such ligands, called PAMPs for pathogen-associated molecular patterns, also act as ligands in phagocytosis, a cellular immune response. It also remains to be determined exactly how bacteria are phagocytosed in organisms that are not equipped with acquired immunity. On the other hand, altered self seem to be phagocytosed in either an opsonin-dependent or -independent manner, as noted below.

Recognition of Altered Self by Phagocytes

Apoptosis-Dependent Phagocytosis of Altered Self

As described above, bacteria are phagocytosed mostly in a manner dependent on the opsonization with antibodies or complement components. This means that acquired immunity plays a more important role than innate immunity in cellular responses against invading bacteria. In contrast, the phagocytosis of altered self may be categorized into an innate immune response,[6,7] because it does not require genes that undergo rearrangement after fertilization. This phagocytosis partly depends on the opsonization of targets, but the opsonins used are often different from those involved in the phagocytosis of bacteria.

Cells that have become dispensable or harmful are induced to undergo various forms of programmed cell death, including apoptosis, itself involving a variety of different signaling pathways within the cell. Upon the induction of apoptosis, a variety of structural and functional changes occur in those cells, including loss of microvilli, blebbing of plasma membranes, condensation of chromatin, DNA cleavage, fragmentation of nuclei, inactivation of mitochondria, and disintegration of cell itself, i.e., formation of apoptotic bodies.[8] Although as many as 10 billion of cells die daily by apoptosis, only a small number of apoptotic cells are detected when organs are histochemically analyzed. This can be explained by the high efficiency of the removal process, so that apoptotic cells are eliminated by phagocytes immediately after they are formed. In fact, the number of detectable apoptotic cells increases when phagocytosis is inhibited in vivo or in animals with defects in the removal process. In addition to the above-described apoptotic changes, finer structures at the cell surface are altered during apoptosis. Such structures serve as the molecular pattern, sometimes called ACAMPs standing for apoptotic cell-associated molecular patterns,[9,10] to designate apoptotic cells so that immune cells discriminate these from viable cells, i.e., as altered self. Apoptotic cell-selective phagocytosis is defined by specific recognition of this molecular pattern by phagocytes. ACAMPs are generated either by the exofacial exposure of endogenous molecules or the modification of preexisting surface molecules. ACAMPs are bound by specific receptors residing at the surface of phagocytes, either directly or indirectly with an aid of serum proteins as bridge molecules, and this molecular interaction subsequently activates a signaling pathway for the induction of phagocytosis (Fig. 2).[9-11]

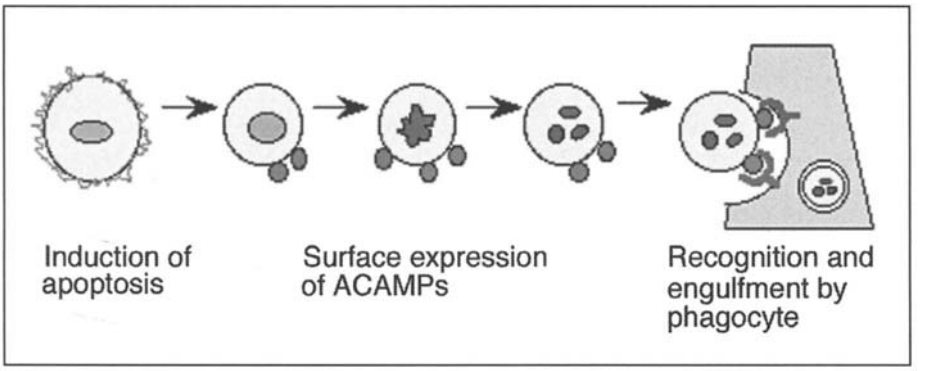

Figure 2. Apoptosis-dependent phagocytosis of altered self. Cells to be eliminated are induced to undergo apoptosis, and ACAMPs are expressed at their surfaces. Phagocytes recognize ACAMPs using specific receptors or bridge molecules and engulf the apoptotic cells.

Externalized Phosphatidylserine as a Molecular Pattern to Designate Apoptotic Cells

Any structural alterations that occur at the cell surface upon the onset of apoptosis could mark those cells as altered self. Such changes are caused by either the externalization of molecules that exist intracellularly in viable cells or the modification of preexisting surface molecules. A representative of the former type alteration of surface structure is redistribution of phospholipids in the plasma membrane. In viable cells, phospholipids are unevenly distributed between the inner and outer leaflets of the plasma membrane bilayer. This is attributed to the sum of action of phospholipid transport: phospholipid flip-flop that results in movement of phospholipids in both directions, aminophospholipid translocase activity that helps maintain the asymmetry by moving phosphatidylethanolamine and phosphatidylserine from the outer to the inner leaflet, and trasport mediated by the ATP-binding cassette transporter such as outbound movement of phosphatidylcholine.[12,13] As a consequence, phospholipids are asymmetrically distributed in the two layers; some phospholipids such as phosphatidylcholine are enriched in the outer leaflet while others, including phosphatidylethanolamine and phosphatidylserine, are mostly confined to the inner leaflet. However, the effects of these transport processes change in apoptotic cells so that asymmetrical distribution of phospholipids is disrupted; flip-flop increases, and aminophospholipid translocase activity contrarily drops.[14] As a result, phosphatidylserine that had been restricted to the inner leaflet becomes distributed in the outer leaflet and appears at the surface of apoptotic cells (Fig. 3). It remains to be clarified how the level of activity of phospholipid transporters is altered after the onset of apoptosis; indeed, the precise identity of the transporters that are responsible are still not assured.

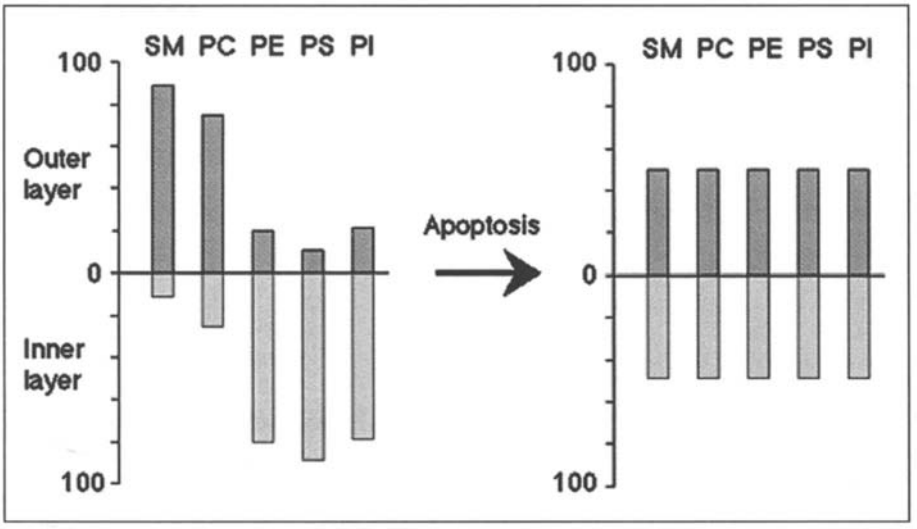

Figure 3. Redistribution of phospholipids in plasma membrane bilayer during apoptosis. Phospholipids are asymmetrically distributed between the inner and outer layers of the plasma membrane of viable cells. This asymmetry is disrupted upon the induction of apoptosis, and phospholipids like phosphatidylserine, which are usually maintained at the inner leaflet, become distributed in the outer layer and exposed on the surface of apoptotic cells. SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol.

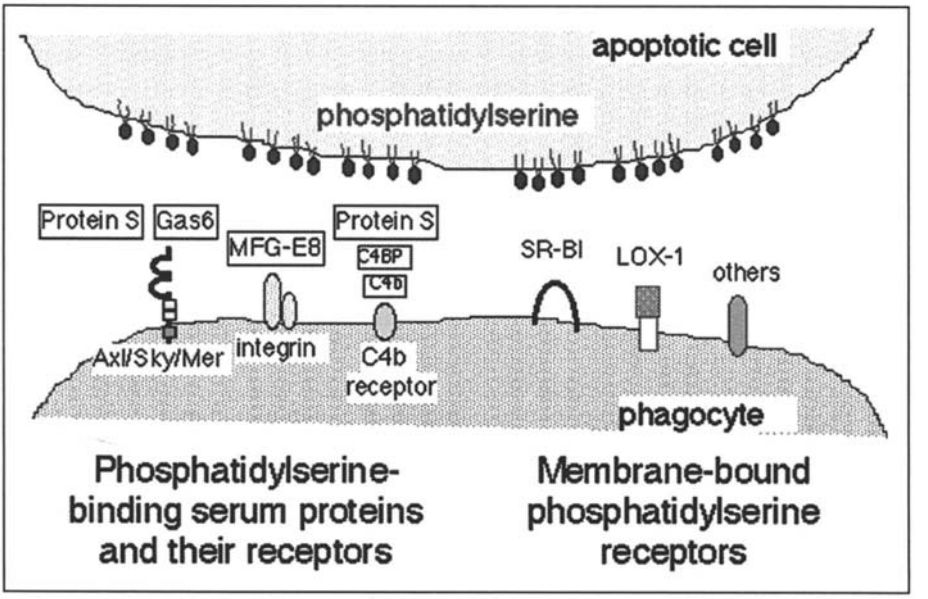

Figure 4. Recognition of phosphatidylserine by phagocytes. Phagocytes recognize phosphatidylserine present at the surface of apoptotic cells either directly using membrane-bound receptors or indirectly with an aid of serum opsonins and their receptors. Gas6, product of growth arrest-specific gene 6; MFG-E8, milk fat globule epidermal growth factor protein 8; SR-BI, class B scavenger receptor Type I; LOX-1, lectin-like oxidized low-density lipoprotein receptor 1.

Any phospholipids that are restricted to the cytoplasmic side of viable cells and have become distributed in the outer leaflet during apoptosis may serve as markers to designate apoptotic cells. Among such phospholipids, phosphatidylserine has been known as a general ACAMP,[15] which is present at the surface of a variety of cell types undergoing apoptosis as a molecular pattern. It is unclear if phosphatidylethanolamine, another amino phospholipid that externalizes during apoptosis, plays a role as an ACAMP. Phagocytes either directly or indirectly recognize phosphatidylserine present at the surface of target apoptotic cells (Fig. 4). Several membrane-anchored phosphatidylserine receptors have been reported, including class B scavenger receptor Type I (SR-BI) of testicular Sertoli cells, lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) of endothelial cells, and a putative, but so far undefined, phosphatidylserine receptor on a variety of cells including macrophages. On the other hand, some serum proteins such as the product of growth arrest-specific gene 6 (Gas6), protein S, and milk fat globule epidermal growth factor protein 8 (MFG-E8, also known as lactadherin) bind to phosphatidylserine and bridge apoptotic cells and phagocytes. Such serum proteins thus act like opsonins, as do antibodies and complement components.[6] In this mode of recognition, membrane proteins of phagocytes that bind to the serum opsonins function as phagocytosis-inducing receptors. It is presumed that the Axl/Mer/Tyro3 receptor tyrosine kinase family of proteins is the phagocytosis receptors for Gas6 and protein S, and that α_v integrins serve as similar function for MGF-E8. The molecular basis for binding of the receptors or the bridge molecules to phosphatidylserine remains to be solved.

Phagocytosis-inducing signaling pathways, which are presumably activated in phagocytes upon the binding of phosphatidylserine to its receptor or bridge molecules, have not been clearly delineated. Two partly overlapping signaling pathways for phagocytosis have been

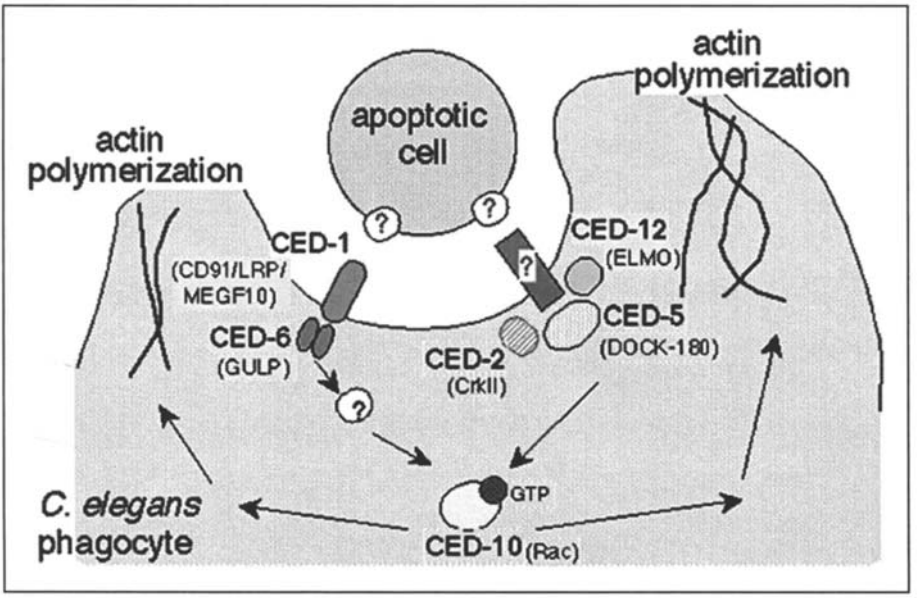

Figure 5. Phagocytosis-inducing signaling pathways in phagocytes of *C. elegans*. Two partly overlapping signaling pathways for the induction of phagocytosis of apoptotic cells, which were genetically identified in *C. elegans*, are schematically shown. Shown in the parentheses are names of mammalian counterparts of the signal mediators.

genetically identified in phagocytes of *C. elegans* (Fig. 5).[16] These pathways are presumably conserved, because the signal mediators involved possess counterparts in insects and mammals. The receptor residing the furthest upstream of one of the pathways is CED-1, but its *Drosophila* homologue does not seem to bind to phosphatidylserine.[17] Furthermore, it is still uncertain if phosphatidylserine serves as an ACAMP in *C. elegans* and *Drosophila*. It is necessary to determine the molecular identity of phagocytosis ligands and receptors of *C. elegans* for understanding not only the role of phosphatidylserine but also a general view of signaling pathways for the induction of phagocytosis.

Other Molecular Patterns Involved in Phagocytosis of Apoptotic Cells

Phosphatidylserine-independent recognition of apoptotic cells by phagocytes has been reported (Fig. 6). Several molecules other than phosphatidylserine are translocated from the intracellular space to the cell surface during apoptosis. These include nuclear autoantigens such as chromatin proteins, but whether or not they participate in the recognition of apoptotic cells by phagocytes is unknown. Structural alteration of preexisting surface molecules occurs in apoptotic cells: the best characterized is the desialylation of sugar moieties. A change in the distribution of preexisting surface molecules might be another way of marking apoptotic cells. The membrane protein CD43 and the endoplasmic reticulum chaperon calreticulin form surface aggregates during apoptosis. Calreticulin has recently been proposed to be another general ACAMP that binds to phagocytes and induce phagocytosis of many types of apoptotic cells.[18] Calreticulin levels increase on the surface of many cell types during apoptosis (or cellular stress) and also appear to redistribute into patches. This molecule can directly interact with low density lipoprotein receptor-related protein 1 (LRP-1), a known internalization molecule whose intracellular signaling domain is homologous with that of *C. elegans* CED-1. Here again, bridge molecules may serve to enhance the recognition and signaling

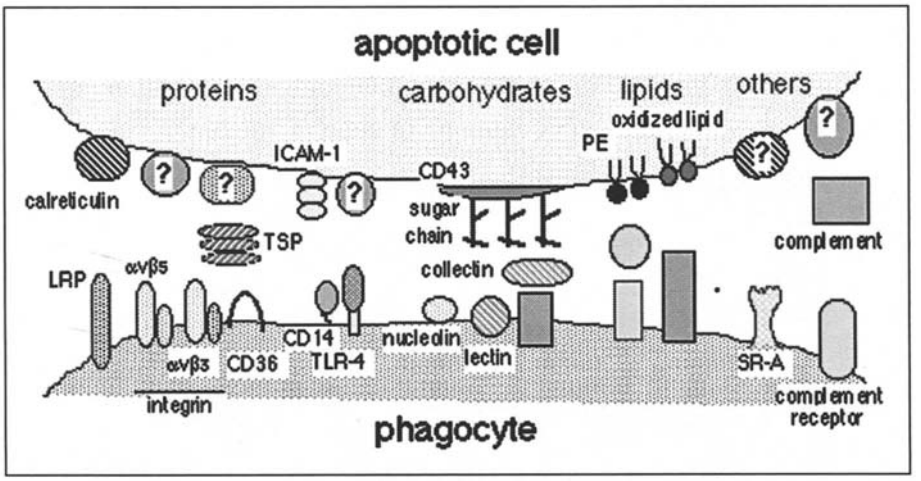

Figure 6. Phosphatidylserine-independent recognition of apoptotic cells by phagocytes. Proposed ACAMPs and their receptors, which are respectively present at the surface of apoptotic cells and phagocytes, are schematically shown. ICAM-1, intercellular adhesion molecule 1; PE, phosphatidylethanolamine; LRP, low density lipoprotein receptor-related protein; TSP, thrombospondin; TLR-4, Toll-like receptor 4, SR-A, class A scavenger receptor.

potential. Thus, members of the innate immune system collectin family of molecules (and probably ficolins as well) can bind to apoptotic cell surfaces and then interact with calreticulin and LRP-1 on the phagocyte to induce phagocytosis.

It has been suggested that the formation of distinct bridges between apoptotic cells and phagocytes is necessary for efficient induction of phagocytosis; one for tethering and the other for activation of a signaling pathway.[19] It is therefore important to determine not only what ACAMPs are responsible but also which one of the two roles, tethering or signaling (or both), they play for the induction of phagocytosis.

Signaling to Inhibit Phagocytosis

Apoptosis appears to bring about another change in addition to the formation of ACAMPs, that is, cancellation of phagocytosis inhibitory signals. A membrane protein named CD31 or PECAM-1 (for platelet endothelial cell adhesion molecule 1), which is expressed in a variety of blood cells and endothelial cells, functions as a cell adhesion molecule. When macrophages bind to viable cells, homophilic association of CD31 is created between the two cell types and activates a signaling pathway involving phosphorylation of tyrosine residues in viable cells. As a result, macrophages are repelled from viable cells, and phagocytosis does not occur.[20] However, apoptosis endows CD31 with quantitative or qualitative changes so that it no longer transmits a signal for repulsion. Another membrane protein involved in inhibition of phagocytosis is CD47 or integrin-associated protein, which is expressed on a wide variety of cells. When CD47 on the surface of viable cells binds to its receptor, named SIRPα (or SHPS), on the phagocyte, an inhibitory signaling pathway is activated to prevent phagocytosis.[21] Apoptosis seems to cause a reduction in CD47 activity and reversal of the inhibitory signal.[18] The relative roles contributed by loss of inhibitory signals, in addition to stimulatory signals provided by the binding of ACAMPs to phagocytosis receptors, are at this point unclear.

Consequences of Phagocytic Clearance of Apoptotic Cells

Cells that are induced to undergo apoptosis in vitro exhibit necrotic changes with time, including a notable increase in the permeability of their plasma membranes. When the same cells are maintained in the presence of phagocytes, they are usually phagocytosed before the onset of such changes. A primary role for the phagocytic clearance of altered self is, therefore, presumed to be to prevent the leakage of materials contained in dying cells. Engulfed apoptotic cells are taken up into phagosomes, i.e., are surrounded by the former plasma membranes of phagocytes. These then fuse with lysosomes, and the apoptotic cells and their contents are rapidly digested by lysosomal enzymes.[1] Materials that broken cells spill include noxious contents and autoantigens. The former may damage surrounding tissues and induce inflammation, and the latter may contribute to autoimmune diseases.[22] In fact, the inhibition of phagocytosis in mice has been shown to cause increased levels of inflammation and autoantibody production. This is rather a passive way to protect organisms from damage caused by dead cells.

Phagocytosis of apoptotic cells also acts to maintain tissue homeostasis in a more active way.[23,24] Engulfment of apoptotic cells brings about a change in the repertoire of proteins in phagocytes, much of it at the level of transcription. A typical example is alteration in the balance of pro- and anti-inflammatory cytokines produced by the phagocytes upon engulfment of apoptotic cells; the expression of the former (such as interleukin 8) is decreased and that of the latter (such as transforming growth factor β) is stimulated. Therefore, phagocytes appear to act to repress inflammation in dual ways by directly eliminating inflammatory substances and also by producing anti-inflammatory mediators. It is not yet clear, however, exactly how phagocytosis of apoptotic cells leads to a change in the level of transcription of genes coding for inflammation-related cytokines. It seems likely that phagocytosis receptors transmit two distinct signals, one for the induction of phagocytosis and the other for the activation of a transcription factor(s) that regulates expression of the cytokines. A second example is the elimination of pathogenic microbes; cells infected with viruses or bacteria are often induced to undergo apoptosis and cleared by phagocytosis. This is the typical cellular innate immune response involving direct phagocytic removal of bacteria. Antigens contained in apoptotic cells, such as viral proteins, might be processed and presented with class I major histocompatibility antigen by phagocytes, such as dendritic cells, that possess the activity of presenting antigens. This way of antigen presentation is called cross-presentation, in which antigens not de novo expressed in antigen-presenting cells are used to stimulate CD8-positive T lymphocytes. A controversy remains, however, as to which type of dead cells, apoptotic or necrotic, become a source of such antigens. There is a report showing that phagocytes can be transformed with DNA contained in engulfed apoptotic cells such as viral genome, but this phenomenon itself needs to be confirmed. Finally, phagocytosis of altered self is also involved in a wide variety of normal physiologic processes, such as differentiation of reticulocytes, involution of mammary glands and corpora lutea, and production of gametes. Removal of unnecessary organelles or cells seems to be essential for normal differentiation, reproduction, and tissue homeostasis.

Fate of Necrotic Cells or Cells Dying by Other Modes

When apoptotic cells are not promptly phagocytosed, they eventually become necrotic; permeability of the plasma membrane increases, and cell contents start to leak out.[25] Dead cells at this stage are also recognized and engulfed by phagocytes. While some of the molecules involved in the recognition of necrotic cells by phagocytes seem to be the same as those for apoptotic cells, others are likely different and can lead to different consequences to the tissues. When stress stimuli continue to be applied to cells where caspases are dysfunctional, those cells sometimes undergo cell death in modes other than apoptosis or necrosis; including forms of death resulting from autophagy.[26] However, little is known as to the fate of cells undergoing this type of death, including whether or not phagocytes target those cells.

Conclusion

The phagocytosis of apoptotic cells seems to be accomplished as a cellular innate immune response, and players that participate in this phenomenon are mostly distinct from those in the humoral innate immune response against invading microbes. The identification and characterization of the molecular patterns and their receptors have not been finalized, and physiological and pathological roles of these important biological processes require further clarification.

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CHAPTER 10

Structural Basis of Pattern Recognition by Innate Immune Molecules

Eamon P. McGreal*

Abstract

The importance of the innate immune system as a first line defence against pathogenic challenge has long been recognised. Over the last decade the identity of many of the key molecules mediating innate host defence have been clarified and a model of self/nonself discrimination by families of pattern recognition receptors (PRRs) has emerged. Although a large amount of information is now available concerning the action of these innate immune molecules at the level of the cell and organism, little is known about the molecular interface between pathogens and innate immune recognition molecules. In this chapter the molecular basis for innate immune discrimination of a wide variety of pathogen derived molecules is discussed in the context of the emerging literature.

Introduction

It is almost 100 years since Ehrlich and Metchnikoff shared the Nobel Prize in Medicine for highlighting the importance of humoral factors such as complement and cellular components such as macrophages in antimicrobial responses. However, it is only in the past decade that the molecular details underlying pathogen recognition have come to light, revealing a complex, intersecting and multi-layered approach to discriminating potentially dangerous microorganisms from harmless self antigens.

The proposed existence and subsequent discovery of the family of pattern recognition receptors (PRRs) known as toll-like receptors (TLRs) and their importance as proximal mediators of inflammatory responses to pathogen associated molecular patterns (PAMPs) has tied together many previously unexplained immune phenomena, and has bridged the gap between innate and adaptive immunity. Janeway originally defined PRRs as nonclonal receptors which have coevolved with pathogens such that they recognise microbial but not host derived molecular patterns.[2] Beyond the TLRs, there are other cell surface, cytoplasmic and soluble molecules which play important roles in binding and clearing pathogens and pathogen associated antigens. Specific aspects of many of these molecules including C-type lectins, scavenger receptors,

Note: The molecular structures of most molecules discussed in this chapter have been resolved. The reader may find it useful to visualise these structures using the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) website (http://www.rcsb.org/pdb/Welcome.do).[1] Direct web links to individual structures are given in parenthesis throughout the text.

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nucleotide-binding oligomerisation domain (NOD) molecules, pentraxins, collectins and components of the complement system are dealt with in detail in other chapters of this volume. This chapter is concerned with structural aspects of ligand recognition by some of these innate immune molecules. Advances in recombinant protein production, biophysical methodology and bioinformatics over the last decade or so has seen a dramatic increase in the quality and quantity of available data regarding the structural basis of molecular interactions in the innate immune system. Here the structure of several well described families of innate immune molecules will be discussed in the context of their ability to detect a wide variety of PAMPs, from lipids and carbohydrates to peptides and nucleic acids. In addition to this I will detail how the structure of pathogen associated molecular patterns (PAMPs) impacts on ligand recognition by the innate immune system.

Molecules Containing Leucine Rich Repeats (LRRs)

Genes encoding LRRs are present in the genomes of many organisms including plants, insects and mammals. LRRs are composed of a characteristic repeated 20-29-residue leucine-rich motif characterised by a consensus sequence of specifically spaced hydrophobic residues with a unique α/β-fold.[3] Each LRR forms a loop and the tandem arrangement of sequential LRRs results in a coiled 'horse-shoe' or 'solenoid' shaped secondary structure which has been observed in the crystal structures of several LRR containing molecules. These structural characteristics appear to place receptors with LRRs as highly effective ligand binding molecules. Although these molecules have diverse functions many, including TLRs, NOD-LRRs and CD14 are known to play important roles in innate recognition of a wide variety of pathogen derived ligands. Despite the apparent structural similarity of these molecules they appear to be able to recognise a diverse spectrum of ligands and one of the most intriguing questions in the post TLR-world is how this diversity of ligand specificity is achieved at the molecular level.

Toll-Like Receptors

Ligand induced agonism of TLRs on a variety of innate immune cells leads to the assembly of intracellular signalling cascades resulting in the activation of a protective inflammatory transcriptome.[4] Despite the obvious importance of these receptors in pathogen induced inflammatory responses, it is not immediately obvious how TLRs can specifically bind such structurally diverse ligands. TLR agonists include lipids (lipopolysaccharide (LPS)/TLR4), nucleotides (viral and bacterial CpG DNA/TLR9, ds viral RNA/TLR3, ss viral RNA/TLR7 and 8) and proteins (flagellin/TLR5, bacterial porins/TLR2). In addition, individual TLRs are known to respond to diverse ligands; TLR4 has been implicated in responses to LPS, viral glycoproteins and endogenous heat shock proteins; TLR2 is implicated in responses to lipoproteins of Gram-negative bacteria, peptidoglycan and lipoteichoic acid (LTA) from Gram-positive bacteria, trypanosomal glycoinositolphospholipids, fungal zymosan and outer membrane porins from *Neisseria* sp. The extracellular domains of all 13 mammalian TLR family members are composed of between 19 and 25 tandemly arranged LRRs (Fig. 1). Whilst the majority of these domains resemble the canonical 20-29-residue LRR, distinct insertions of up to 16 residues are evident at positions 10 or 15 in specific LRRs within individual TLRs.[5] It is believed that these insertions may define the ligand specificity of individual TLRs.

The Structural Basis of Direct TLR/Ligand Interactions

The crystal structures of several non-TLR LRR-containing molecules including ribonuclease inhibitor,[6] Nogo receptor[7] and follicular stimulating hormone receptor[8] show a clear preference for the concave inner surface of the 'horse-shoe' as the main ligand binding region. Although we still await the full structural detail of a TLR/ligand complex the crystal structure of TLR3 ectodomain[9,10] (http://www.rcsb.org/pdb/explore.do?structureId=1ZIW and http://www.rcsb.org/pdb/explore.do?structureId=2A0Z) has offered some surprising insights into the potential ligand binding mechanism of this dsRNA receptor. In keeping with other LRR-molecules TLR3 forms a horse-shoe shaped secondary structure. An unexpected feature

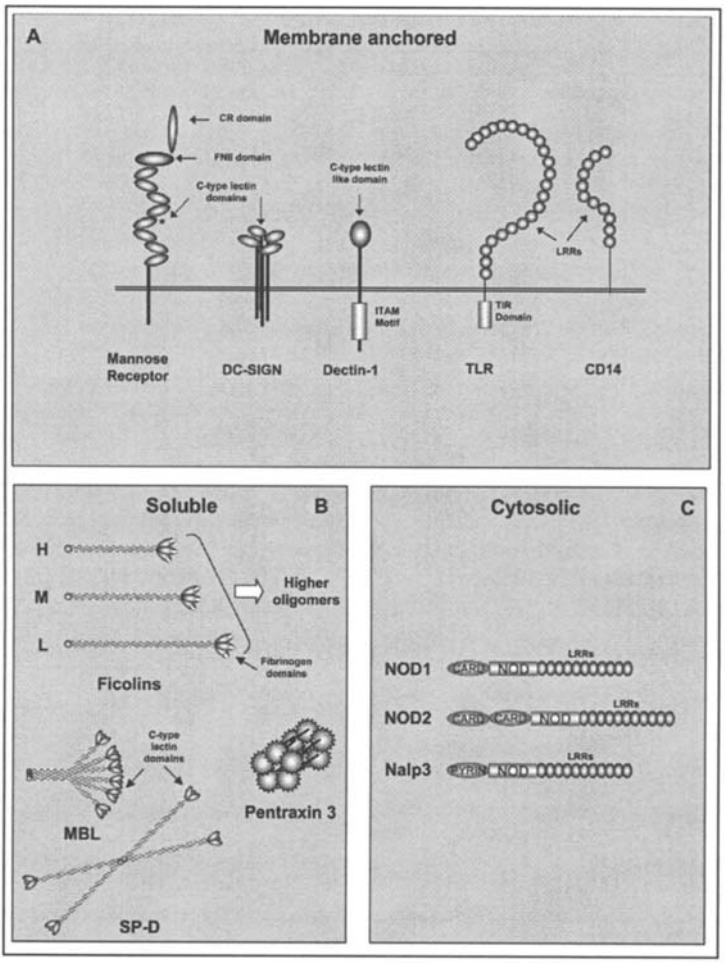

Figure 1. Representatives of the major soluble, membrane anchored and cytosolic innate immune recognition molecules. A) Membrane anchored; C-type lectin domains are found in both DC-SIGN and Mannose receptor (MR). Of eight C-type lectin domains found in MR, only that at position four (*) makes a major contribution to Ca^{2+} dependent ligand binding. Further ligand binding activities are mediated by both the cysteine rich (CR) and fibronectin Type-II (FNII) domains. Dectin-1 is an unusual receptor in that it binds a carbohydrate ligand although it does not possess a Ca^{2+} dependent C-type lectin domain. The mode of ligand binding by dectin-1 is as yet unresolved. Both TLRs and CD14 are composed of repeating leucine rich repeats (LRRs) which form a horseshoe shaped structure. Direct ligand binding by CD14 and certain TLRs has been demonstrated and can be localised to specific LRRs. B) Soluble; MBL, SP-D and the ficolins all contain extended intertwined collagen like regions which play important role in molecular oligomerisation. Both MBL and SP-D interact with carbohydrate ligands through multiple C-type lectin domains, the ficolins utilise fibrinogen like domains to interact with DNA and acetyl groups. Pentraxin-3 is thought to exist as a decamer with subunits linked together through di-sulphide bonds and higher oligomers have also been observed. The representative structure of pentraxin-3 was adapted from a structure originally presented in reference 136. The structure of MBL and SP-D was adapted from a structure originally presented by Kishore et al, in Molecular Immunology 2006;43(9):1293-315. C) Cytosolic; LRRs are also found in the family of cytosolic molecules known as NOD LRRs. LRR dependent ligand binding induces molecular oligomerisation via the NOD allowing interactions with further cytosolic effector molecules via the CARD and pyrin domains.

of this molecule is the decoration of much of its surface with N-linked glycans. It would appear that glycosylation of TLR3 is important not only for correct trafficking of the receptor to the cell surface but also for efficient signalling through this molecule (independently of any effects on cell surface expression).[11] Crowding of the concave inner face of TLR3 with carbohydrates, in addition to an overall negative charge in this area seems to rule this out as a ligand binding site for nucleic acids. In contrast, one of the flat faces of TLR3 is conspicuously devoid of glycosylation and also contains patches of basic amino acids which may form an attractive positively charged ligand binding region. Interestingly, one of these regions lies in close proximity to a TLR3 specific insertion in LRR12 which may indicate the evolutionary development of specific ligand binding activity. As with many other innate immune receptors, it is likely that ligand induced activation of TLR3 occurs through receptor oligomerisation. The two current crystal structures demonstrate crystal packing in a conformation which is suggestive of dimerisation through a site close to the C-terminus of the glycan free face of the receptor. This site is proximal to LRR20 which also contains a TLR3 specific stretch of additional residues, which may play a role in receptor dimerisation. Such an arrangement would also permit ligand interactions within the positively charged pocket close to LRR12.

Although frustratingly devoid of the molecular detail offered by X-ray crystallography, other methods have been used to demonstrate direct interactions between several of the TLRs and their ligands. dsRNA and the model ligand poly I:C can alter the mobility of a recombinant TLR3 extracellular domain in both gel filtration columns and mobility shift assays.[9,10] This can be taken as evidence of a direct interaction or at least of ligand induced aggregation of TLR3. In the case of TLR5, its interaction with bacterial flagellin is sufficiently strong to enable affinity purification of cell surface expressed and soluble recombinant TLR5 from cell lysates using tagged flagellin constructs.[12,13] As these assays did not use purified TLR5 constructs, it is not clear whether this interaction depends on the presence of other binding 'cofactors' present in cell lysates, as is the case for TLR4 recognition of LPS. Whatever the nature of the interaction, it depends on a stretch of amino acids (386-407) within LRR14.[12] Similar to the proposed ligand binding region of TLR3, LRR14 of TLR5 contains an idiosyncratic six residue insertion which may represent evolutionary tailoring of a specific ligand binding site.

Recent developments have seen the application of in silico modelling to questions of molecular structure and interaction.[14] Indeed such methodology has been used to predict an interaction between residues within LRR20 of TLR5 and highly conserved residues present in more than half of 723 flagellin sequences available in the public databases.[15] Mutation of many of these flagellin residues disrupts bacterial motility (required to efficiently infect host cells) and significantly reduces TLR5 recognition of this adjuvant.[13] Such observations fit neatly into the original definition of PAMPs as molecular structures which are essential to pathogen survival and are therefore likely to remain highly conserved even under immunological pressure.[2] Flagellin however, is not a strict adherent to this scripture. Several clinically important pathogens including *Helicobacter pylori* and *Campylobacter jejuni* encode forms of flagellin not recognised by TLR5[16] but retain flagellar motility through compensatory amino acid changes in other regions. Interestingly TLR5 recognises only monomeric flagellin and filamentous forms of this molecule, the main form present on motile bacteria, are much less potent inducers of inflammatory activity.[13] It is thought that monomeric forms of flagellin may be liberated from bacteria as a result of physical or chemical stress encountered during infectious episodes. Furthermore, it is likely that flagellin dissociation occurs within acidified intracellular compartments following phagocytosis of bacteria.

Three TLRs (TLR3, TLR7 and TLR9) are devoted to the recognition of pathogen derived nucleic acids. TLR9 recognises unmethylated 2'-cytidine-phosphate-guanidine (CpG) DNA found in bacteria and DNA viruses. Surface plasmon resonance has been used to verify that unmethylated CpG DNA binds directly to the murine form of this receptor[17,18] with a dissociation constant (K_D) of 200nM. This compares favourably with the K_D of 82 nM calculated for *Drosophila* Toll interacting with its endogenous ligand, Spaetzle[19] although such comparisons may not be relevant given the molecular differences between both ligands

and receptors. In contrast to the recognition of monomeric flagellin by TLR5, model systems demonstrate that DNA oligomerisation is required for effective TLR9 mediated recognition.[20] The fact that TLR9/ligand interactions are enhanced at lower pH[18] may reflect the natural presentation of CpG DNA to TLR9 within acidified endosomes. Although the evidence that acidification enhances this interaction is not universally supported[21] it is likely that a more acidic environment would sufficiently dissociate native pathogen structures to make the CpG DNA accessible for TLR9 recognition. A potential ligand binding region within murine TLR9 has also been proposed. The region demonstrates sequence similarities with a distinct family of methyl-CpG-DNA binding domains (MBDs)[22] which have roles in DNA methylation-dependent gene silencing and chromatin remodelling. Point mutation of amino acids in TLR9 corresponding to residues in MBDs known to physically contact ligand CpG DNA results in diminished ligand binding as well as reduced NF-κB activation following cellular stimulation.[18]

Molecular Complexes Mediating TLR Responses to Ligands

For some members of the TLR family, ligand interactions depend on the cooperation of several molecules in heteromeric binding/signalling complexes. The best studied example of this is the TLR4/MD2/CD14/LBP (lipopolysaccharide binding protein) receptor complex which has been extensively studied in the context of cellular responses to LPS. The sequential interaction of all four components is required for optimal responses to very low doses of LPS, which may be present at the early stages of an infection by Gram-negative bacteria. It is at this point that innate immune responses can be most effective.

Even before a point mutation in TLR4 was identified as the cause of LPS hypo-sensitivity in the C3/HeJ strain of mouse,[23] CD14 had been recognised as a vital mediator of the response. CD14 deficient mononuclear cells display seriously blunted responses to LPS when pro-inflammatory cytokine production is measured, and such mice are resistant to endotoxic shock.[24] CD14 bears structural similarity to the TLRs, as it is composed of multiple LRRs (Fig. 1) forming a horseshoe shaped dimer. The crystal structure of CD14 exhibits a conspicuous, deep, hydrophobic ligand binding pocket at its NH_2 terminus which likely accommodates the lipid A portion of LPS[25] (http://www.rcsb.org/pdb/explore.do?structureId=1WWL). The identity of this ligand binding pocket is supported by the presence in this area of a cluster of residues which can be mutated leading to reduced LPS binding or responsiveness.[26-29] In addition to the lipid A portion of LPS, highly variable carbohydrate structures may also be important for innate immune recognition.[30] Completely de-lipidated LPS still retains affinity for CD14[31] and several grooves identified within the crystal structure of CD14 may allow interactions with the variable hydrophilic carbohydrate portion of LPS.[25]

In addition to CD14, LBP (an acute phase serum LPS binding protein) also plays a key role in effective early responses to LPS. Low doses of LPS (up to 100ng/ml) fail to induce TNF-α secretion in whole blood from LBP deficient mice although normal responses are seen at doses of 1μg/ml.[32] The failure of LBP deficient mice to effectively respond to early infectious cues is reflected in their inability to control bacterial dissemination in vivo, although they are resistant to endotoxic shock.[33] Collectively these data point to an essential role for LBP in amplifying innate immune responses to low doses of LPS by catalysing its transfer to CD14.[34,35] This fits well with the higher affinity of LBP for LPS when compared to CD14 (K_D=3.5 nM and 30-74 nM respectively).[27,34] Structural analysis of LBP[36] and comparison with the crystal structure of the closely related bactericidal/permeability increasing protein[37] (http://www.rcsb.org/pdb/explore.do?structureId=1BP1) reveals a lipid binding pocket within the N-terminal barrel structure. Peptide mapping in this region has more specifically localised LPS binding activity to a stretch of 18 amino acids[38,39] and point mutation of a number of these basic residues severely reduces direct LPS binding and the ability of LBP to transfer LPS to CD14.[40] Notably, these residues had independently been predicted to form an extended LPS binding amphipathic loop in comparative modelling experiments using the crystal structure of related molecule, Limulus-anti-LPS factor.[41,42]

The final component of the LPS recognition complex, MD-2, binds directly to LPS with an apparent K_D of 100nM. This interaction is an absolute requirement for effective innate immune responses to LPS[43] and MD-2 deficient mice do not respond to LPS in vivo which corresponds to enhanced survival following endotoxic shock.[44] Although we still await structural resolution of the MD-2 molecule, predictions have been made based on primary amino acid sequence and by comparison with a so called MD-2 related lipid-recognition domain common to several other lipid binding proteins.[45,46] This model depicts a molecule with an overall positive charge, ideally suited to binding hydrophilic lipid A molecules. The existence of a specific binding pocket is supported by mutagenesis studies focusing on a region spanning residues 119-132[47] but specifically dependent on residues 90 and 120.[45] Disulphide bonds involving cysteines 95 and 96 completely disrupt MD-2 interactions with TLR4, presumably through gross conformational changes whilst more subtle mutation of tyrosine 102 and serine 103 also disrupts this interaction.[47]

Together these cell surface and soluble molecules cooperate in the recognition of LPS. The current model suggests that LBP facilitates enhanced recognition of LPS by CD14 which in turn presents LPS to TLR4 and its apparent coreceptor MD-2. Although all four molecules have been shown to bind directly to LPS, TLR4 is the terminal signalling receptor in this complex. In light of this, it is ironic that TLR4 is the least well characterised receptor with respect to LPS binding. It is likely that any interactions with LPS occur through one of the 'variant' LRRs containing extra inserted residues and some studies have highlighted candidate regions. An area of TLR4 which includes most of LRR10, 11 and 12 is required for effective discrimination of host specific LPS modifications in *Pseudomonas aeruginosa*[48] and a relatively common single nucleotide polymorphism (SNP) in LRR10 (D299G) of human TLR4 leads to LPS hyporesponsiveness and also correlates with enhanced susceptibility to infection.[49] Although these data do not conclusively prove LRR10 to be the LPS binding region, these effects are unlikely to be due to disruption of interactions with MD-2 as TLR4/MD-2 interactions seem to be exquisitely sensitive to perturbation of a stretch of nine amino acids[25-33] at the amino terminus[50] which is located some distance from LRR10. Perhaps the most striking example of a TLR cooperating with other receptors for ligand recognition is that of TLR2 and its interaction with TLR1 and TLR6.[51] A complex of TLR2 and TLR6 is needed for innate immune responses to a wide range of mycoplasma derived lipoproteins including MALP-2 (macrophage activating lipopeptide-2 kD)[52,53] and the synthetic lipopeptide FSL-1 (fibroblast-stimulating lipopeptide-1).[54] TLR2 additionally interacts with TLR1 for responses to a wide range of bacterial and mycobacterial lipopeptides[55,56] as well as the synthetic lipohexapeptide $Pam_3C\text{-}SK_4$[57] and the meningococcal outer membrane protein, PorB.[58] The direct interaction described between PorB from *Neisseria meningitides* and TLR2 appears to be of high affinity (K_D=10nM)[58] and is the first protein ligand described for this receptor. A region containing LRRs 9-12 in both TLR1 and 6[59] is required for lipopeptide discrimination and the existence of TLR specific insertions within LRR 12 points to this as a strong candidate for the ligand binding site. Interestingly, although TLR2 alone can bind PorB, TLR1 is needed for effective cellular responses to this molecule, in common with bacterial lipopeptides.[58]

A rational molecular explanation for the differential recognition of lipopeptides by TLR2/TLR1 or TLR2/TLR6 appears to lie in the structure of the lipid moiety of these molecules which is the primary determinant of immunostimulatory activity. All microbial lipopeptides are modified at the N-terminus by linkage of fatty acids to the N-terminal cysteine of the variable polypeptide chain.[60] Many mycoplasmal lipopeptides recognised through TLR2/TLR6 are diacylated (having two ester bound fatty acid chains) with a free amino terminus.[52,53] By contrast, bacterial lipopeptide and the synthetic lipopeptide $Pam_3C\text{-}SK_4$, which act through TLR2/TLR1 contain a third fatty acid chain linked to the amino group. This difference is probably explained by the absence of an *N*-acyl transferase in the genome of those microorganisms expressing di-acylated lipopeptides.[61] In support of the TLR specificity for these two types of molecules, replacing the lipid portion of MALP-2 with that of $Pam_3\text{-}Cys\text{-}K_4$ switches the

TLR dependency from TLR1 to TLR6.[62] It is of interest to note that the immunostimulatory activity of a diacylated lipopeptide is ten times more potent than a synthetically produced, tri-acylated analogue.[62] Further evidence for the cooperative nature of TLR2 and TLR6 in the recognition of diverse molecular structures has been provided by the finding that functional interactions with the novel C-type lectin-like receptor Dectin-1 mediate cellular responses to carbohydrates in the form of fungal β-glucans.[63]

A large amount of information concerning the mode of TLR recognition of pathogenic ligands has been discovered over the last few years. The first crystal structures are now available and are lending clues to other members of the TLR family. The increasingly complex interactions between different members of the TLR family and other types of PRRs are of great interest and clearly permit greater discrimination of a wide spectrum of pathogens by the innate immune system. In the next section the role of cytoplasmic LRR containing molecules in pathogen recognition will be assessed, adding another layer to cellular innate host defence mechanisms.

Intracellular LRR Containing Molecules

In recent years there has been increasing interest in a family of more than 20 cytosolic LRR-containing molecules known as the nucleotide-oligmerisation domain (NOD)-LRR proteins.[64] NOD-LRRs are found in a wide range of organisms from plants to humans where they play important roles in pathogen detection and host defence. The best characterised vertebrate NOD-LRRs are the Nod-1 and -2 molecules although a number of other members including IPAF (ICE-protease-activating factor) and Nalp-3 (cryopyrin) are also emerging as major players in host defence. All members of this family share common architectural features (Fig. 1). They generally consist of three distinct functional domains; a carboxy-terminal region composed of multiple LRRs determines ligand specificity. A centrally placed NOD domain induces molecular oligomerisation upon ligand binding and finally an N-terminal effector domain links ligand recognition to intracellular effector pathways through homotypic interactions with other cytosolic molecules resulting in activation of specific 'inflammasomes' within the cytoplasm. Although IPAF and Nalp-3 share overall architectural homology with Nod-1 and -2, Nalp-3 differs in that its C-terminal effector domain is a pyrin rather than a CARD (caspase recruitment domain). This has important implications for interactions with and activation of other CARD/Pyrin containing molecules within the inflammasome, leading to processing and secretion of pro-inflammatory factors such as IL-1β[65] as well as regulating apoptosis[66] and impacting on NF-κB signalling pathways.[67]

As NOD-LRRs have been recognised as important mediators of pathogen resistance in plants for many years, it is also useful to draw functional and molecular comparisons with these molecules.

Nod-1 and -2

To date the only PAMPs known to act through Nod-1 and -2 are conserved molecular features of bacterial peptidoglycan (PGN).[68] PGN is composed of repeating simple glycan chains containing two alternating saccharides, *N*-acetylglucosamine (GlcNac) and *N*-acetyl muramic acid (MurNac). Parallel glycan chains are crosslinked to each other by short stem peptides linked to MurNac residues. Nod-2 recognises muramyl dipeptide (MDP) which is composed of MurNac with two linked amino acids. Nod-1 specifically recognises a GlcNac-MurNac-L-Ala–γ-D-glutamyl-*meso*-DAP tripeptide (GM-Tri_{DAP})[69] although the minimal active structure appears to be the γ-D-glutamyl-*meso*-DAP dipeptide (iE-DAP).[70] Interestingly there is some species specific ligand specificity and murine Nod-1 will not respond to GM-Tri_{DAP}, requiring instead the tetrapeptide, GlcNac-MurNac-L-Ala-γ-D-glutamyl-*meso*-DAP-D-Ala.[71] mDAP (*meso*-diaminopimelic acid) is an amino acid unique to PGN from most Gram-negative and certain Gram-positive bacteria. The importance of mDAP in innate immune activation is illustrated by certain Gram-negative spirochetes including *Borrelia burgdoferi* and *Treponema* spp. which contain L-ornithine rather than *meso*-DAP[72] and correspondingly exhibit weak pro-inflammatory activity.[68]

In contrast to the precise molecular identity of Nod-1 and -2 ligands the nature and structure of the ligand binding domain within these molecules remains unknown. Furthermore, it is not clear whether ligand interactions are direct or require additional binding cofactors. Evidence from TLRs and CD14, as outlined above, points to the LRR as an obvious ligand binding site. Indeed amongst the numerous and diverse NOD-LRR molecules encoded by plants, the regions of greatest variability are seen in the LRR domains which probably reflects selective pressure imposed on a ligand binding domain by pathogens[73] and points to this as a key domain for ligand differentiation. Most convincingly, recombinant chimeric molecules in which the LRRs of Nod-1 are replaced by those from Nod-2 results in an analogous switch of ligand specificity from mDAP to MDP.[74] Systematic mutation of residues within the LRRs of Nod-1 and -2 has also more closely identified residues mediating responses to PGN.[74,75] Mutation of key residues within LRRs 5-7 of Nod-1 abrogates ligand sensing activity.[74] Importantly these residues are highly conserved among Nod-1 orthologues from other species, reinforcing the importance of this region in ligand sensing. Likewise in Nod-2, mutation of specific residues in LRRs 6-11 results in a loss of ligand sensing capacity. Two of these mutations corresponded to SNPs causing loss of in Nod-2 function in patients with Crohn's disease highlighting the importance of microbial sensing in normal gut homeostasis.[76]

Ipaf and Nalp-3

Given the importance of NOD-LRR molecules in pathogen sensing throughout many organisms, there has been much interest in identifying microbial ligands for many of the 'orphan' NOD-LRRs in the human genome. Recently ligands acting through both Ipaf and Nalp-3 have been identified, extending the spectrum of pathogens recognised by these cytoplasmic pattern recognition molecules.

A variety of structurally diverse compounds, including bacterial RNA, ATP, and gout associated uric acid crystals act through Nalp3 to induce caspase-1 activation and pro-IL-1β processing to active IL-1β.[77-79] Responses to cytosolic flagellin have recently been shown to depend on the presence of this Ipaf, which mediates the activation of caspase-1 and IL-1β secretion.[80,81] For both Nalp-3 and Ipaf, it is unclear whether ligand sensing occurs directly through LRRs or as a result of interactions with other binding cofactors. The manner in which ligands interact with LRRs of these cytoplasmic molecules is not yet known, however evidence from similar systems in other organisms lends clues as to possible mechanisms of interaction.

Models for NOD-LRR Recognition of Pathogens in Plants

In the case of the NOD-LRRs encoded by plant resistance genes, another interesting mechanism of action has been proposed and partially proven. The 'guard hypothesis'[82] is a conceptual relative of the danger model of innate immune activation[83] and the notion of altered self as an innate immune stimulus. It has been suggested that plant NOD-LRRs do not detect the pathogens themselves but rather the evidence of their presence. Many plant pathogens manipulate a limited number of host molecules to gain entry to and proliferate within the host. Whilst different pathogens may use a variety of strategies to cause the same host modifications, it may therefore be more economical and efficient for the host to be alerted by the presence of an altered aspect of its own molecular structure. This certainly appears to be the case with detection of pathogens by *Arabidopsis* and the tomato plant.[84,85] In the case of *Arabidopsis*, the *Pseudomonas syringae* virulence factor is a cysteine protease which cleaves the host molecule RIN4. This cleavage results in the activation of the protective NOD-LRR, RPS2.[84] In the tomato plant a slightly different mechanism has been discovered whereby the pathogen delivered virulence factor is an inhibitor of a host cysteine protease and this inhibition is sufficient to activate a host defence pathway via a NOD-LRR molecule.[85] The guard hypothesis may present a more precise model of innate inflammatory activation as it depends on the capacity of an agent (biological, physical or chemical) to

cause damage. The host therefore responds to evidence of damage to itself in the form of altered self molecules. Whilst the 'guard hypothesis' has been partially proven in plants and is essentially analogous to the manner in which *Drosophilla* Toll responds to a fungally induced cleavage of an endogenous molecule Spaetzle,[86] it is not yet apparent that such a model operates in vertebrate innate immune systems.

Carbohydrate Recognition by C-Type Lectin and C-Type Lectin-Like Molecules

Carbohydrates represent one of the most diverse targets for molecular recognition in a wide range of biological systems including innate immunity. A number of different families of lectin molecules concerned with the detection of carbohydrate moieties are present across numerous species from worms to man. Of these, the C-type lectin and the structurally related C-type lectin-like family are the best characterised in terms of innate immune function. In keeping with other innate host-defence systems, these molecules link pathogen recognition by one domain to induction of anti-pathogen responses via an effector domain.

C-type lectin domains are found in a wide variety of animal host defence molecules and exist as both soluble oligomeric molecules (Fig. 1) (including the collectins, mannose-binding lectin (MBL) and surfactant proteins-A (SP-A) and -D (SP-D)) and membrane bound molecules (including the macrophage mannose receptor (MR) and dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN)). Membrane bound receptors can be further sub-divided into Type-I and Type-II receptors with most of the Type-I receptors such as MR (Fig. 1), DEC-205 and Endo-180 conforming to a multi-lectin architecture. All Type-II receptors on the other hand consist of just one membrane distal C-type lectin domain separated from the membrane by a neck region which is variable in length, commonly as a result of alternative splicing, and appears to play an important role in receptor oligomerisation. Such oligomerisation is essential for effective ligand binding by many members of this group of receptors, a subject which will be returned to later in this chapter.

In contrast to many of the LRR containing PRRs, carbohydrate recognition by the majority of C-type lectin containing molecules does not appear to lead directly to an inflammatory transcription programme (Dectin-1, a C-type-lectin-like molecule may be an exception to this). Furthermore, although many C-type lectin molecules have been reported to alter cellular activity, this does not seem to occur through common intracellular signalling pathways. Indeed C-type lectin containing molecules are largely distinguished based on differences in effector functions. MBL can activate the complement cascade through an associated serine protease, MASP-2.[87] MR, DEC-205 and Endo-180 seem to be predominantly involved in the delivery of antigens to distinct subcellular compartments of antigen presenting cells for antigen processing and presentation.[88] Other Type-I molecules including DC-SIGN and Dectin-1 are emerging as interesting modulators of inflammatory pathways, and the details of the molecular events governing this are now becoming clear.

The diverse function of C-type lectins is reflected in the often un-expected phenotypes of many knockout mice already available for these molecules. Many C-type lectin knockout animals demonstrate a susceptibility to infectious disease although other phenotypes are less predictable and may relate to an interesting feature of many (although not all) C-type lectin type molecules, namely their capacity to interact with host antigens in addition to pathogen derived ligands. In some cases it may be that host molecules are the primary ligands for these receptors with pathogens having coopted this function to hijack cells. The less discriminate ligand specificity of the C-type lectins compared to other PRRs points to their importance as first line identifiers of potentially pathogenic structures. It is likely that these molecules are important in the capture, internalisation and processing of antigens enhancing their availability to other innate immune molecules for complete discrimination which may conclude with activation of an inflammatory programme.

The Carbohydrate Recognition Domain (CRD) and C-Type Lectin Specificity

Whereas the term 'lectin' is used to define any molecule which can bind carbohydrate ligands, C-type lectins are so named as they use calcium ions to coordinate interactions between residues stabilising the binding pocket as well as mediating interactions with key hydroxyl groups on the sugar ring. C-type lectin carbohydrate binding activity resides within the carbohydrate recognition domain (CRD), a region of approximately 120 amino acids which is conserved among a variety of C-type lectin molecules with divergent ligand specificity.[89] In addition to a number of conserved cysteine residues, other groups of conserved residues required for effective and discriminate ligand binding by the CRD have been identified.

The description of the crystal structure of MBL in complex with an oligomannose ligand[90] (http://www.rcsb.org/pdb/explore.do?structureId=2MSB) explains two features of ligand binding by C-type CRDs, firstly the dependence of these interactions on calcium and secondly, the ability of different CRDs to discriminate specific carbohydrate structures. Multiple interactions between amino acids, carbohydrate and Ca^{2+} ions have been revealed in this crystal structure. The terminal mannose (Man) residue is the only component of the oligomer to interact with the CRD, with the remainder protruding away from the ligand binding pocket. Hydroxyl groups -3 and -4 of the terminal Man ring directly ligate Ca^{2+} at position 2. This is one of three Ca^{2+} ions found in the CRD and the only one to directly contact the ligand. This Ca^{2+} also forms coordination bonds with Glu185, Asn187, Glu193, Asn205 and Asp206 which further stabilises the binding pocket. Additional hydrogen bonds occur between hydroxyl groups -3 and Glu185 and Asn187 and hydroxyl group -4 and Glu193 and Asn205.

Another Man specific C-type lectin which demonstrates an unusual mode of ligand binding is that found in DC-SIGN and its related receptor DC-SIGNR. DC-SIGN and its related receptors are capable of recognising endogenous glycoproteins such as ICAM-2 and -3 as well as a range of viral, bacterial, fungal and parasitic glycans.[91-93]

The crystal structure of both molecules in complex with a $GlcNAc_2$-Man_3 pentasaccharide revealed striking differences in the mode of ligand binding compared to that seen for MBL[94] (http://www.rcsb.org/pdb/explore.do?structureId=1K9I and http://www.rcsb.org/pdb/explore.do?structureId=1K9J). Unlike the straightforward interaction between the CRD of MBL and the 3- and 4- hydroxyl groups of a single terminal monosaccharide unit, DC-SIGN/R forms hydrogen bonds with the 3- and 4-hydroxyl groups of an internally α1-3 linked Man residue as its primary ligand. The interactions between this primary ligand, the coordinating Ca^{2+} ion and pairs of Glu and Asn residues within the main binding pocket are similar to those seen in other Man type CRDs however additional hydrogen bonds and van der Waals interactions occur between the CRD and other ligands within the pentasaccharide. The most striking of these are a series of classical C-type lectin bonds formed by a terminal GlcNac residue and the CRD of an adjacent DC-SIGN monomer which bridges and presumably crosslinks both monomers.

The use of glycan array technology has revealed that the ligand specificities of DC-SIGN and DC-SIGNR are strikingly different.[95] Whereas both receptors bind to N-linked high Man structures in a manner similar to that described for MBL, only DC-SIGN is capable of binding a number of other carbohydrate structures with similarity to $Lewis^x$ and $-^a$ trisaccharides. These structures are characterised by a terminal branched pair of fucose (Fuc) and galactose (Gal) or *N*-acetyl galactosamine (GalNac) residues linked by a variable monosaccharide residue. The 3- and 4- hydroxyl groups of Fuc interact with the CRD at the primary binding site. This interaction differs from Fuc interactions with MBL where the 2- and 3-hydroxyl groups interact with the CRD, however this novel orientation allows additional van der Waals contacts between a Val351 and the 2-OH group of the Fuc ring. This interaction does not occur in DC-SIGNR as the Val is replaced by a Ser residue. The resulting diminished affinity of DC-SIGNR for Fuc is a key determinant of the restricted ligand profile of this receptor and experimental substitution of Val for this Ser residue confers DC-SIGNR with an ability to bind both $Lewis^x$ and $-^a$ glycans.

C-type lectin CRDs can be divided into two broad categories; those with affinity for Man type ligands, such as that of MBL, the macrophage mannose receptor and DC-SIGN, and those with specificity for Gal type ligands as typified by the CRD of the asialoglycoprotein receptor. This division is reflected in the structure of Man type monosaccharides which are all characterised by at least two equatorially aligned hydroxyl groups; at positions 3 and 4 in the case of Man, and 2 and 3 in the case of L-Fuc. The orientation of these groups permits interactions with the CRD as described above. D-Gal and its related ligands contain an axial 4-hydroxyl group and equatorial 2- and 3- groups which although similar to L-Fuc are in the opposite isomeric conformation. This conformation is unable to interact with mannose type CRDs due to steric hindrence by a His residue at position 189.[90]

The structural basis of Gal specificity by the family of Gal specific CRDs has also been determined.[96,97] Analysis of the primary sequence of these CRDs reveals many similarities to Man type CRDs and those residues found at positions equivalent to Glu193, Asn205 and Asp206 of MBL are conserved. However, both Glu185 and Asn187, which form hydrogen bonds with the equatorial 3-hydroxyl group of Man, are replaced by Gln and Asp respectively. Although substituting these residues into MBL confers Gal binding activity, these residues are not the determinants of CRD selectivity as the interaction with Gal is of relatively low affinity[96] and Man binding activity is retained. High affinity interactions with Gal depend on a conserved Trp residue at position 189 (replacing the His residue found in the Man type CRDs) with additional selectivity conferred by a glycine rich stretch of seven amino acids which immediately follows this.

C-Type Lectin Oligomerisation and Receptor Avidity

In addition to a highly specific ligand binding domain, oligomerisation of C-type lectin domains has been shown to play an important role in mediating high affinity interactions between a number of receptors and their ligands. Pathogens commonly present multiple, widely spaced terminal mannose residues at the cell surface or, in the case of viruses, on the envelope. The density and spatial arrangement of these ligands is thought to present a conformation optimal for recognition by molecules such as MBL which have CRDs arranged in trimers with individual CRDs separated from each other by approximately 50 Å.[98] Such an arrangement is also seen in the related pulmonary collectins SP-A and SP-D (Fig. 1) and occurs in all three molecules through the interaction of elongated α-helical coiled-coil 'neck' regions below the main C-type lectin 'head' regions (http://www.rcsb.org/pdb/explore.do?structureId=1M7L). It is likely that this geometric arrangement helps to preclude interactions with glycan structures present on endogenous glycoproteins which tend to be shorter and more closely spaced.[99] Although examples of endogenous molecules interacting with MBL are detailed below, the prevalence and conformation of high mannose groups on these molecules do not seem sufficient to support interactions of comparable affinity to those seen on pathogen surfaces. Further avidity of these molecules towards pathogenic ligands is also achieved by the formation of higher oligomers of the trimeric clusters.

Oligomerisation of CRDs is not limited to the collectins and is also seen in the cell surface receptors, DC-SIGN and DC-SIGNR. DC-SIGN exists as a tetramer at the cell surface (Fig. 1),[100] a conformation which has also been observed for recombinant purified DC-SIGN and DC-SIGNR extracellular domains molecules.[101] Tetramerisation is mediated by conserved repeat neck domains and confers a dramatic enhancement in affinity towards viral ligands in particular compared to monomeric CRDs alone.[102]

Recognition of Endogenous Ligands by C-Type Lectins

The ability of C-type lectins to effectively discriminate different carbohydrate structures is important as these receptors fulfil different functions in vivo. All four galactose specific C-type lectins present in human and mouse genomes appear principally concerned with the recognition of endogenous glycoproteins. The asialoglycoprotein receptor (ASGPR) efficiently

delivers glycoproteins terminating in Gal or GalNac to intracellular compartments for degradation, regulating serum glycoprotein homeostasis.[103] Despite its original description as a receptor for glycoproteins from which sialic acid has been removed, glycoproteins with sialic acid capped α2,6GalNAc have recently been identified as endogenous ligands for ASGPR[104,105] and may represent major ligands for this receptor in vivo. Gal specific receptors also contribute to the detection of altered self molecules as evidenced by the strong interaction between tumour derived Tn antigen and two Gal specific receptors, human macrophage galactose lectin (MGL)[106] and the scavenger receptor C-type lectin (SRCL).[107] Another form of altered self, the apoptotic cell, can also express ligands for Gal specific receptors[108,109] and a defect in the clearance of neural-tube apoptotic cells has been observed following ablation of one of two highly homologous genes encoding MGL in mice.[107] Although some examples of pathogen derived ligands for Gal specific receptors have been demonstrated[110] pathogen recognition by C-type lectins is largely achieved by Man type receptors.

As with most efforts to classify families of molecules, the division of C-type lectins into Man specific receptors dealing with pathogens and Gal specific receptors dealing with endogenous molecules is an over simplification. Several C-type lectins with Man type CRDs are known to interact with both pathogen derived and endogenous carbohydrate ligands. Some receptors such as the macrophage MR achieve this through the use of distinct ligand binding domains. MR uses a Ca^{2+} CRD to bind Man-type ligands on both pathogen derived and endogenous molecules. Glycoproteins bearing sulphated N-terminal GalNac residues including sialoadhesin, CD45 and lutropin are recognised by a membrane distal cysteine-rich domain[111,112] and collagen like molecules are recognised by a fibronectin Type-II domain.[113]

MBL uses its CRD to interact with as yet undefined ligands on B-cells, macrophages and dendritic cells[114,115] suggesting a potential immunomodulatory role for this lectin. Certain types of immunoglobulins have also been identified as endogenous ligands for MBL, including a subset of antigen free IgM[116] and glycoforms of polymeric IgA.[117] The differentially glycosylated agalactosyl IgG,[118] which is over represented in inflammatory diseases such as rheumatoid arthritis, can activate the MBL pathway of complement and may be one source of inflammatory activation in these patients. Oligomannose ligands capable of supporting MBL interactions are also found in the protease inhibitor α_2 macroglobulin.[119] Although this interaction is of a relatively low affinity compared to that seen with pathogen derived ligands,[119] and is unlikely to support complement activation in the fluid phase it may represent a suitable opsonin if targeted to pathogen cell surfaces. Another interesting feature of MBL which has come to prominence over the last number of years is its role in the opsonisation and clearance of apoptotic cells.[120-122] The ability to recognise apoptotic cells is shared with the other closely related collectins SP-A and SP-D[121,123,124] and whilst carbohydrate ligands for these molecules are present on certain apoptotic cells detailed studies have identified DNA as a novel ligand.[125,126] All three collectins have the capacity to bind free and synthetic single- and double-stranded DNA from a variety of sources, although interactions with SP-A are relatively weak. C1q, which has a similar oligomeric Gly-X-Y collagen like region has previously been shown to bind DNA through this regions[127] as well as through the globular 'head regions'. The collectins also bind DNA through two distinct sites. Evidence supports the existence of charge-charge interactions between DNA and N-terminal collagenous regions of SP-D. In addition, free D-pentoses and deoxy nucleotide tri-phosphates efficiently compete for SP-D binding to mannan through the C-type lectin CRD. As these pentoses contain free hydroxyl groups similar to those found in monosaccharide ligands for these CRDs, it is thought that the mechanism of interaction might be similar for both molecules.

The best studied example of a mannose type C-type lectin binding both endogenous and pathogen derived ligands is that of the dendritic cell and macrophage expressed Type-II receptor DC-SIGN and the related receptor DC-SIGNR. In addition to interactions with bacterial and yeast derived ligands[91,92] DC-SIGN also recognises several viral envelope glycoproteins including HIV gp120.[128] It is of interest to note that DC-SIGN was originally identified (and

named) during a screen for molecules which could mediate activation of T-cells through interactions with ICAM-3.[128] An additional interaction with endothelial cell expressed ICAM-2 has also been identified[129] although the affinity of interactions between DC-SIGN and ICAMs are 50-100 fold weaker than those with gp120.[130] This most likely reflects a greater prevalence of high mannose glycans on gp120 compared to either of the ICAMs. The relative importance of interactions with these different ligands is not yet clear. Our understanding of this has partly been hampered by the absence of a knockout model due to the lack of consensus on which of the eight mouse genes (designated SIGNR1-8) should be regarded as the true homologue of human DC-SIGN and DC-SIGNR. Recent studies have gone some way to clearing this up by defining the ligand specificity of all mouse molecules,[131-135] concluding that SIGNR3 is the closest functional homologue in terms of ligand specificity.[133]

Alanine scanning mutagenesis also revealed that different residues within the CRD of DC-SIGN are responsible for interactions with ICAM-2, -3 and HIV gp120.[130] It has been suggested that protein-protein as well as protein-carbohydrate interactions are involved in ICAM binding whilst interactions with gp120 conform to the more standard CRD-carbohydrate binding model.

Emerging Innate Immune Pattern Recognition Molecules

In the last number of years, several exciting and novel innate pattern recognition molecules have been identified. Although little information is available regarding the mechanism underlying their interaction with specific ligands it is appropriate to highlight these new molecules and consider the currently available information.

The Pentraxins; Multimeric, Multifunctional Pattern Recognition Molecules

The pentraxins are a group of multimeric proteins with important roles in innate immune host defence.[136] C-reactive protein (CRP) and serum amyloid-P (SAP) are members of the 'short' pentraxin family and have long been recognised as important innate immune recognition molecules. Both proteins interact with a variety of pathogens, endogenous glycoproteins as well as apoptotic and necrotic cells.[136] Although many of these interactions are Ca^{2+} dependent, these molecules do not have features characteristic of the C-type lectin CRD. SAP in particular utilises a structure reminiscent of other lectins such as that found in Concanavilin A which are arranged as a pentamer around a five fold symmetry.[137]

Recently, a newly discovered member of a family of 'long' pentraxins, pentraxin-3 (PTX3) (Fig. 1), has been identified as a major nonredundant contributor to innate host defence to fungal pathogens.[138] In addition to this PTX3 also organises the extracellular matrix of the oocyte and is essential to normal female fertility in vivo.[139] In common with several other innate immune recognition molecules discussed in previous sections, PTX3 has the capacity to interact with apoptotic cells[140] although in contrast to CRP, SAP and other innate immune opsonins, PTX3 inhibits apoptotic cell clearance by phagocytes.[140,141] Although PTX3 shares certain ligand binding specificity with CRP and SAP, it does not bind phosphorylcholine or phosphoethanolamine and it is not known whether the mode of ligand interaction is the same among the molecules. All three molecules bind microbial ligands in a Ca^{2+} dependent manner although PTX3 interactions with complement component C1q are Ca^{2+} independent (in contrast to SAP and CRP). Interestingly CRP residues which are involved in C1q binding are not conserved in PTX3 although the interaction is thought to be electrostatic in nature.[142]

Dectin-1 a Nonclassical C-Type Lectin Like Receptor Interacting with Carbohydrates

The discovery of dectin-1 as the major receptor for fungal β^{1-3} and β^{1-6}-linked glucans has been a surprising and important finding in the field of innate immunity.[143] β-glucans are major components of fungal cell walls and have been recognised for many years as the major

Table 1. Key innate immune molecules and their ligands

Molecule	Ligand Binding Domain	Expression	Ligands/Agonists		Mode of Interaction
			Macro-Molecular	Molecular	
Collectins					
MBL SP-A SP-D	Multiple C-type lectin CRDs.	Soluble Liver (MBL) Lung epithelial cells (SP-A & -D) Other epithelial cells (SP-D)[159].	Bacteria, fungi, viruses, parasites, apoptotic cells[159].	Carbohydrate moieties in glycolipids (LPS, LTA), peptidoglycan[159].	Primary Ca^{2+} dependent interaction between free -OH groups and CRD.[90] Enhanced binding by spatially arranged glycans interacting with multiple CRDs.[98]
C-type lectins					
Mannose Receptor	C-type lectin CRD FN-II Cysteine Rich.	MΦ, DC, hepatic endothelium.[160]	Bacteria, fungi, viruses, parasites.[160]	Mannose type-sugars via CRD 4.[161] (Collagen via FN-II domain.[113]	Primary Ca^{2+} dependent interaction between free -OH groups and CRD.[90]
DC-SIGN	C-type lectin CRD.	MΦ, DC.[160]	Bacteria, fungi, viruses, parasites.[160]	Sulphated Gal*Nac* via CR domain.[111,112] Mannose type sugars via CRD.	Receptor oligomerisation enhances interactions with glycan structures.[98,99]
Dectin-1	C-type lectin 'like' CRD.	MΦ, DC, Neutrophil.[160]	Fungi.[143]	β1,3- & 1,6-linked glucans.[162]	Dectin-1 binding is Ca^{2+} independent but not fully defined.

continued on next page

Table 1. Continued

Molecule	Ligand Binding Domain	Expression	Ligands/Agonists		Mode of Interaction
			Macro-Molecular	Molecular	
NOD-LRRs					
NOD-1 NOD-2	Mediated through LRRs.	MΦ, DC[64], paneth[163] & epithelial cells.[64]	Bacteria[64]	Peptidoglycan[68]	Not yet defined, unclear whether the interaction between ligand and LRR is direct or via an intermediary.
Nalp3				RNA, ATP, Uric acid crystals.[77-79]	
Ipaf				Cytosolic flagellin.[81]	
LRRs					
CD14	LRR at NH_2 terminus.	Myelomonocytic, & soluble.	Bacteria,[166] apoptotic cells.[167]	LPS,[26] LTA,[169] peptidoglycan.[27]	Charge-charge,[26] LPS binding to CD14 catalysed by LBP.[34,35]
TLRs	LRRs aided by distinct amino acid insertions.	MΦ, DC, neutrophil, B-cells, T-cell subsets,[164] epithelium.[165]	Bacteria, fungi, viruses, parasites.[168]	Diverse; pathogen derived lipids, nucleic acids, proteins.[168]	Evidence for direct interactions between certain TLR LRRs[9-13]and ligands but not shown for all family members.
Pentraxins					
PTX3	Not yet defined.	MΦ, DC, epithelial fibroblast, endothelial, smooth muscle, adipocyte, chondrocyte, synovial cells.[170]	Select bacteria[170] & fungi,[136] apoptotic cells.[140]	*Klebsiella* outer membrane proteins.[171] C1q, fibroblast growth factor, TNF-α induced protein 6.[170]	Electrostatic, Ca^{2+} independent.[142,172]
Ficolins					
L-, H- & M-ficolin	Globular fibrinogen β- & γ-chain-like domains	Liver (L-, & H-). Alveolar and bronchial epithelial cells (H). Monocytes (M).[159]	Bacteria, fungi, viruses, protozoa, apoptotic cells.[156,159]	DNA,[155] LTA,[154], acetyl groups.[150,151]	Not yet defined.

inflammatory component of the yeast particle zymosan. The identification of dectin-1 as the principle receptor for the β-glucans places it as the first C-type lectin like PRR with the capacity to directly signal for cytokine and chemokine expression in myeloid cells. As mentioned earlier, dectin-1 can collaborate with both TLR2 and TLR6 for the production of certain pro-inflammatory cytokines[51,144] and this is the first description of such collaboration between C-type-lectin like receptors and TLRs. It remains to be seen whether other receptors also collaborate with TLRs in a similar manner.

Dectin-1 is a member of the C-type lectin-like family of receptors. Unlike the classical C-type lectins discussed previously, these molecules do not retain conserved residues involved in Ca^{2+} coordination within the CRD and do not bind carbohydrates in a Ca^{2+} dependent manner. The majority of these receptors are involved in the regulation of NK-cell function through the recognition of endogenous proteinaceous counter-receptors.[145] Dectin-1 is the first of these receptors known to recognise carbohydrate structures. Clearly it is of great interest to understand how this molecule achieves highly specific, high affinity interactions with this ligand. Furthermore, as dectin-1 also binds an endogenous ligand on T-cells,[146] it is of interest to know whether this occurs through the same binding site as that of the β-glucans. Currently however, very little information is available concerning the mechanism of ligand binding. Recent work by Adachi et al has used mutagenesis to identify the residues within the Dectin-1 binding site essential for ligand recognition.[147] Two amino-acids within a predicted β-sheet close to the fourth cysteine of the CTLD appear essential to either contacting the ligand directly or in formation of the ligand binding site. Mutation of either Trp221 or His223 results in reduced binding of 1-3 linked β-glucan and when coexpressed these mutations completely abolish binding. Unsurprisingly, these mutations also diminish NF-κB activation in cells expressing both TLR2 and Dectin-1.[147] The importance of these residues to ligand binding is supported by preliminary crystallographic data which places both residues in close proximity to two grooves with potential involvement in ligand recognition.[63] We await the full crystallographic structure of dectin-1 in complex with its ligand which will not only deliver insights into a novel mode of ligand binding in the innate immune system but will also aid in the prediction of other PAMPs which may interact with this receptor.

The Ficolins; Novel Carbohydrate Binding Molecules Which Can Activate the Complement System

The ficolins were first described in the pig as transforming-growth factor-β binding molecules.[148] Three molecules, L-, H- and M-ficolin, have been described in humans. With similarity to the collectins, all three molecules contain collagen like regions which are important for molecular oligomerisation (Fig. 1).[149] Both L- and H- forms act as carbohydrate binding molecules and were originally thought to have specificity for GalNac residues. Carbohydrate binding activity in all cases is achieved through a globular domain similar to the fibrinogen β and γ chains. More recent studies have suggested that both L- and M-ficolin interact with patterns of acetyl groups[150,151] which is of interest as the fibrinogen like domains of a horse shoe crab lectin which also interacts with acetyl groups have been resolved by X-ray crystallography[152] (http://www.rcsb.org/pdb/explore.do?structureId=1JC9). The mode of ligand binding is very different to that of C-type lectin-CRDs but may offer some insights into the mechanisms of ficolin/ligand interactions.

Whatever the molecular nature of the ligands for these novel molecules they are capable of recognising and opsonising a wide range of pathogens.[153,154] In addition they can bind apoptotic cells and other dying cells through the recognition of DNA.[155,156] Another interesting feature of L- and H- ficolin is their ability to activate the lectin pathway of complement through interactions with MASPs.[150,157,158] Further details regarding the specific interactions between ficolins and their ligands are as yet unavailable however it is clear that these molecules have evolved to play an important role in innate immune defence.

Conclusion

This chapter has attempted to survey the current knowledge of molecular interactions at the interface between pathogens and major PRRs of the innate immune system. Clearly this is a rapidly moving field and in many cases we are at an early stage of understanding, however, the use of crystallography and nuclear magnetic resonance as well as developments in in silico and comparative modelling are revealing a great deal of detail about these interactions.

Our new understanding of collaboration between different receptors in innate immune sensing has opened up a new avenue of investigation for those seeking to understand how the innate immune system achieves such high definition from a limited repertoire of molecules. It is sensible to think that the innate immune system will use all available resources when discriminating between potentially dangerous pathogens which require a vigorous inflammatory response and those innocuous self antigens which need to be cleared without an ensuing immune reaction. Collaboration between receptors such as C-type lectins with specificity for both self and nonself ligands and the more discriminatory TLR family appears to allow the immune system to effectively clear many types of antigens whilst maintaining control over the inflammatory status of the host. A much greater understanding of the molecular detail of host-pathogen interactions is important if we are to capitalise on the remarkable progress made in understanding the contribution of many of these innate-immune molecules to both effective host defence as well as inflammatory disease. Manipulating these pathways for the improved design and delivery of vaccines as well as novel anti-inflammatory therapies represents an important therapeutic avenue for the near future.

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CHAPTER 11

Lessons from the Fly:
Pattern Recognition in *Drosophila melanogaster*

Subhamoy Pal and Louisa P. Wu*

Drosophila have a variety of innate immune strategies for defending itself from infection, including humoral and cell mediated responses to invading microorganisms. At the front lines of these responses, are a diverse group of pattern recognition receptors that recognize pathogen associated molecular patterns. These patterns include bacterial lipopolysaccharides, peptidoglycans, and fungal β-1,3 glucans. Some of the receptors catalytically modify the pathogenic determinant, but all are responsible for directly facilitating a signaling event that results in an immune response. Some of these events require multiple pattern recognition receptors acting sequentially to activate a pathway. In some cases, a signaling pathway may be activated by a variety of different pathogens, through parallel receptors detecting different pathogenic determinants. In this chapter, we review what is known about pattern recognition receptors in *Drosophila*, and how those lessons may be applied towards a broader understanding of immunity.

Introduction

In order to effectively prevent disease, the immune system needs to robustly identify dangerous foreign microorganisms or abnormal host cells, and eliminate them. The process of recognition of friend versus foe is thus central to a successful immune response. Families of Pattern Recognition Receptors (PRRs) have been identified that mediate this distinction. Many of them have been discovered through the study of immunity in insects such as fruit flies, silk worms, and moths, and in some cases homologues have been identified in mammals. These PRRs recognize conserved microbial determinants such as bacterial lipopolysaccharide (LPS), peptidoglycan, or fungal β-1,3 glucans. Upon recognition of these Pathogen Associated Molecular Patterns (PAMPs), the PRRs initiate an immune response conferring protection to the host. In mammals, these receptors are responsible for initiating signaling cascades that lead to the production of immune effectors such as antimicrobial peptides and cytokines, and trigger the activation of phagocytosis and proteolytic cascades.[1]

The fruit fly *Drosophila melanogaster*, has been a particularly attractive model system to study PRRs because it presents researchers with a simpler immune system to study conserved signaling pathways. Flies are also a well established genetic system and have a short generation time of 14 days. This enables the use of forward genetic screens to identify mutants necessary for the immune response. The Drosophila genome has been sequenced, which also expedites identification of genes and comparative genomics analyses.[2] It is also relatively easy to incorporate and regulate the expression of transgenes in flies, providing valuable opportunities for characterizing the role of genes in vivo.[3] Thus, the ability to combine genetic and molecular

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approaches has made fruit flies a powerful system to study innate immune recognition. This chapter will focus on some of the knowledge gained from the study of Drosophila pattern recognition receptors.

The Immune Response in the Fly

Drosophila respond to infection using a combination of three strategies:

1. Proteolytic cascades and melanization. When the fly cuticle is damaged, a set of proteolytic reactions involving its blood proteins cause a rapid clotting and deposition of melanin around the site of the breach in order to heal the wound. This involves a cascade of events marked by the proteolysis of Prophenoloxidase (PPO) and generates reactive oxygen species (ROS).[4] Both ROS and melanin are toxic to invading pathogens.[5,6] Curiously, flies with mutations in the PPO cascade alone, are not more susceptible to bacterial infection, which suggests that other cell mediated and humoral responses may be playing a more crucial role to *Drosophila* immunity in vivo.[7]
2. Phagocytosis and cellular responses. *Drosophila* have three major kinds of blood cells, as characterized by morphology and function. Nearly 90% of all blood cells are plasmatocytes, which demonstrate a macrophage-like behavior by phagocytosing invading bacteria.[6,8] Lamellocytes work together to encapsulate larger invaders that cannot be phagocytosed, e.g., the eggs of endoparasitoid wasps. Finally, crystal cells provide enzymes required for melanization reactions. Embryos and larvae produce these hemocytes until the pupal stage, after which no evidence of hematopoiesis has been found.[6] Adult flies have a fixed population of sessile blood cells localized mainly on the anterior portion of the dorsal side of their abdomens. These hemocytes are capable of phagocytosing bacterial invaders to protect the host.[8,9]
3. Antimicrobial peptides and humoral response. There are seven known classes of inducible antimicrobial peptides (AMPs) in *Drosophila*: Attacin, Cecropin, Defensin, Diptericin, Drosocin, Drosomycin, Metchnikowin.[10,11] Several of the peptides work by disrupting bacterial membranes.[12] They are primarily produced by the fat body, the flies' functional analog of the mammalian liver, within hours of an immune challenge. The AMPs are then secreted into the hemocoel where they block the proliferation of microorganisms.[10] Drosomycin for example, demonstrates antifungal activity and is preferentially induced upon fungal infection. Similarly, Defensin and Metchnikowin have activity against Gram-positive bacteria and Attacin, Cecropin, Diptericin, and Drosocin are effective against Gram-negative bacterial challenge.[10,11] The transcription of these AMPs is regulated by two major signal transduction pathways: Toll and IMD (Immune Deficiency) (Fig. 1).[13] Both these pathways have elements conserved with mammalian pathways regulating NF-κB, a transcription factor important for mediating numerous immune responses.[14] The Toll pathway is central to Drosophila immune responses. It is involved in the induction of AMPs through the regulation of Dif, an NF-κB-like transcription factor. *Toll* mutant flies are particularly susceptible to fungi and Gram-positive bacteria, and fail to induce Drosomycin.[15,16] The IMD pathway on the other hand broadly detects Gram-negative bacterial determinants through its receptor PGRP-LC (Peptidoglycan Recognition Protein LC).[17-19] Mutants in the IMD pathway fail to activate Relish, another NF-κB-like protein, that is responsible for the transcription of Diptericin.[20] The selective activation of the Toll or IMD pathways appears to confer some specificity of response against Gram-positive bacteria and fungi, or Gram-negative bacteria respectively. However, some lines of evidence suggest that IMD is also important for resistance to Gram-positive *Micrococcus luteus* infection,[21,22] while Toll is also important for responses to Gram-negative *E. coli* and *Pseudomonas aeroginosa* and the virus, Drosophila X virus.[13,23,24] Thus there may be some functional overlap between the roles of these two pathways in regulating responses to different microbes.

Before any of these immune responses can be mounted, an infection first needs to be identified. Upon recognition, one or more of these responses to combat the infection can be activated. Pattern Recognition Receptors (PRRs) identify pathogens, and are thus at the front lines of immune defenses.

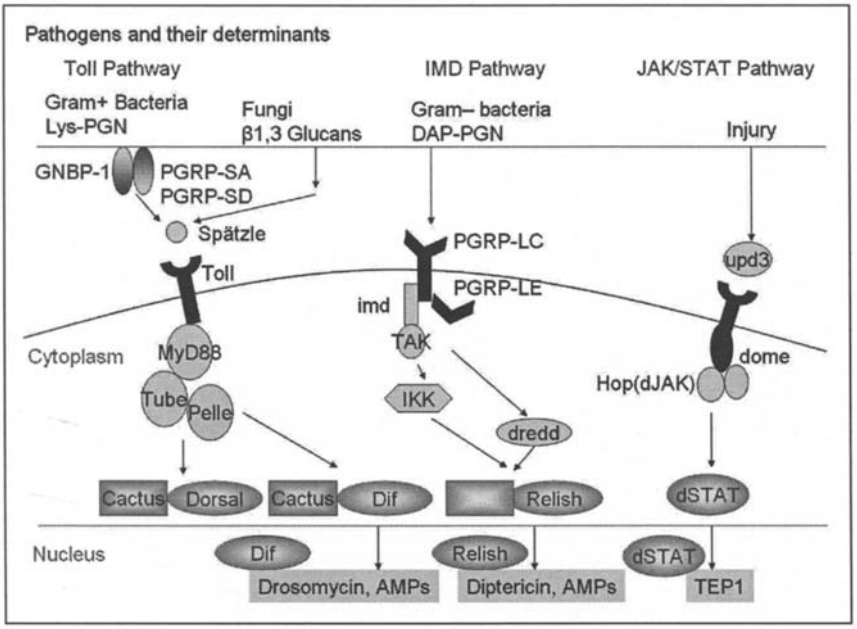

Figure 1. Immune pathways in Drosophila. Gram-positive bacteria are detected by PGRP-SA and -SD. This results in the cleavage of Spatzle, and the activation of the Toll pathway. Gram-negative bacteria activate the IMD pathway through the receptor PGRP-LC and -LE. Septic injury can lead to the activation of the JAK-STAT pathway. There may be cross talk between all these signal transduction pathways, and they are responsible for the activation of effectors that mediate an immune response.

The Pattern Recognition Receptors

Toll Receptors

Drosophila Toll was initially identified as one of 12 maternal effect genes that function in a pathway required for dorsal-ventral axis formation in fly embryos.[25,26] Interestingly, Drosophila Toll and the mammalian IL1 receptor share a conserved intracellular domain named the Toll-IL1-Receptor (TIR) domain, and regulate orthologous signaling pathways that have been discussed previously.[27]

Eleven Toll-Like-Receptors (TLRs) have been identified in mammals, and they are specialized for detection of different PAMPs, often with the help of other proteins.[28] TLR4 for example is involved in the direct detection of bacterial LPS in a complex with helper proteins CD14 and MD2.[29] TLR2 recognizes a broad array of ligands including bacterial lipoproteins, peptidoglycan, and yeast zymosan. TLR2 achieves this range of ligand specificities, by forming heterodimers with TLR1 or TLR6. The TLR 1/2 heterodimer for example, binds triacylated lipopeptides, whereas the TLR 2/6 combination is specific for diacylated lipopeptides.[30] Other TLRs appear to act alone as homodimers. For example, TLR5 recognizes flagellin, the protein that makes up bacterial flagella.[31] TLR9 recognizes unmethylated CpG DNA characteristic of bacterial genomes, and TLR3 binds to double-stranded RNA.[32, 33] Upon detection of their PAMPs, different TLRs activate combinations of downstream components, leading to potentially complex signaling outcomes. In mammalian dendritic cells for example, the TLRs 5, 7, and 8 signal through MyD88, an adaptor protein traditionally activated by mammalian Toll, to produce a pro-inflammatory reaction.[34] TLR3 on the other hand signals through MyD88

and an additional adaptor protein complex TICAM/TRIF (Toll/IL-1 Resistance containing adapter molecule/Toll/IL-1 receptor domain-containing adaptor-inducing interferon-β) to activate an antiviral response.[35] Since TLR3 detects double stranded RNA of potentially viral origin, this may represent an appropriate immune response for the host.

In contrast to mammalian TLRs, Drosophila Toll appears not to be directly involved in pathogen recognition as a PRR. It does however, play a central role in mediating responses to multiple types of infections. Fungal, Gram-positive bacterial, and viral responses require the Toll pathway.[13,15,24,36] In the case of fungi and Gram-positive bacteria, upstream PRRs recognize PAMPs and trigger a proteolytic cascade that activates Toll and results in AMP production.[16,37-39] Eight other Toll receptors have been identified in *Drosophila,* but they appear to have significant functional differences with mammalian TLRs. Foremost, Drosophila Tolls have not been shown to detect PAMPs so far. Instead some of them such as Toll-8 and Toll-2 have developmental and neural functions respectively.[40,41] Most Tolls do not appear to be upregulated upon infection, and none of them have been identified through screens for mutants with immunodeficiency.[42,43] The possible exception 18-wheeler, also known as Toll-2, is expressed in larval fat body upon infection but its role in immunity is not clear.[44] The induction of several AMPs were affected in *18-wheeler* mutants, including a 95% reduction in Attacin and 65% reduction in Cecropin expression, and these flies were susceptible to *E. coli.*[45] However, these mutants appear to have defects in fat body development which may also cause the aberrant AMP production.[41,44] Constitutive expression of another fly Toll receptor, Toll-9 has been shown to induce Drosomycin expression in Drosophila S2 cell lines. It uses components of the traditional Toll pathway like the adaptor protein MyD88, to mediate this induction.[46,47] Thus, analogous to some mammalian TLRs, Toll-9 may also be using shared signaling components to affect AMP expression in vivo. The isolation and characterization of mutations affecting the other fly Toll receptors will likely shed more light on the role and regulation of this receptor family.

Peptidoglycan Recognition Proteins (PGRPs)

Peptidoglycans are polymers of N-acetylglucosamine and N-acetylmuramic acid that are present in bacterial cell walls. In Gram-positive bacteria a Lys-type stem peptide is involved in crosslinking these monomers, and the resulting peptidoglycan constitutes nearly half of the exposed bacterial cell wall mass. Gram-negative bacteria, have a diaminopimelic acid (DAP) stem peptide connecting the monomers, and their peptidoglycan is present in a relatively thin layer underneath an LPS outer membrane.[48,49] Peptidoglycan Recognition Proteins (PGRPs) are a class of PRRs that recognize this conserved determinant of all bacteria. These receptors were first characterized in moths *Bombyx mori* and *Trichoplusia ni.*[50,51] Thirteen PGRP genes have since been identified in *Drosophila*, 7 in *Anopheles* mosquitoes, and 4 in mammals.[52-54] However, alternative splicing may generate a larger functional repertoire. In *Drosophila* alone, the 13 PGRP genes transcribe at least 17 proteins.[55]

Some mammalian PGRPs have bacteriostatic functions. PGRP-S is involved in the intracellular killing of bacteria in mouse polymorphonuclear leukocytes, though the mechanism by which they do this is not clear.[56] A bovine PGRP-S orthologue, also known as oligosaccharide binding protein (OBP) has shown activity against various types of pathogens including Gram-negative, Gram-positive bacteria and certain fungi.[57] Thus some PGRPs have evolved to recognize a very wide range of ligands, some of which are not even peptidoglycans. Drosophila PGRPs have not however been demonstrated to have intrinsic antimicrobial properties, and so far have only been shown to activate immune effectors.

Drosophila have 7 short PGRPs (SA, SB1, SB2, SC1a, SC1b, SC2, and SD) that lack a transmembrane domain, and are predicted to be secreted (Fig. 2).[55] They also have 6 long PGRPs (LA, LB, LC, LD, LE, and LF) that are predicted to be membrane bound.[55] Drosophila PGRPs can also be classified according to function, as catalytic or noncatalytic. All PGRPs share homology with N-acetylmuramoyl-L-alanine amidases, which cleave peptidoglycan at the lactylamide bond between the glycan backbone and the stem peptides.[50] Some PGRPs

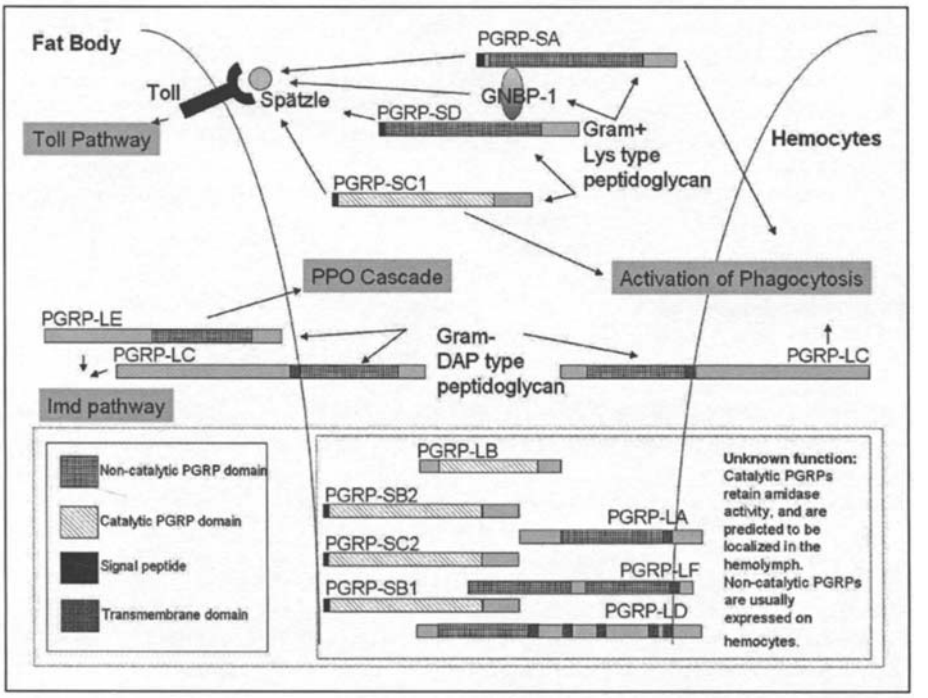

Figure 2. The peptidoglycan recognition receptors. PGRP-SA, SD, and SC1 appear to mediate recognition of Gram-positive Lys-type peptidoglycans. PGRP-LC and LE work together to recognize the DAP-type peptidoglycans of Gram-negative bacteria. PGRPs possessing a catalytic domain are capable of cleaving peptidoglycans, perhaps to neutralize its immunogenic potential or as a step in a multi-step recognition process.

such as PGRP-SC1a and SC1b, retain this function and have been demonstrated to cleave peptidoglycan in vitro.[58] However, other receptors such as PGRP-LC, LE, SA, and SD are noncatalytic due to the lack of a critical cysteine in the conserved catalytic domain.[58]

Both catalytic and noncatalytic PGRPs have been shown to play a crucial role in Drosophila immune recognition. Noncatalytic PGRP-LC (also known as ird7 or totem) responds primarily to DAP (diaminopimelic acid) type peptidoglycan found in Gram-negative bacteria, and activates the IMD pathway.[17,59-61] PGRP-LC has also been shown to activate phagocytosis because RNA inhibition (RNAi) of the gene in Drosophila S2 cells causes a reduction in the phagocytosis of Gram-negative *E. coli*, but not Gram-positive *S. aureus*.[61] PGRP-LE also appears to detect Gram-negative bacteria, and its overexpression in larvae activates the IMD pathway as well as the PPO cascade.[62] PGRP-LC, PGRP-LE (loss of function) double mutants show a more dramatic susceptibility to Gram-negative bacterial infection than either mutation alone suggesting the two PGRPs may be acting together for peptidoglycan recognition.[63] On the other hand, study of loss-of-function mutants in PGRP-SA (also known as Semmelweis) reveal its role activating the Toll pathway in response to Gram-positive bacterial, but not fungal, challenge.[37] *PGRP-SD* mutant flies are also susceptible to Gram-positive bacterial challenge and PGRP-SA and PGRP-SD appear to act together to recognize Lys-type peptidoglycan and activate the Toll pathway.[37,64] PGRPs therefore mediate specificity of immune response to Gram-positive or Gram-negative bacteria, by recognizing the bacterial peptidoglycan and differentially activating the Toll or IMD pathway respectively.[65,66]

Catalytic PGRPs also play a crucial role in immune recognition, and appear to chemically modify peptidoglycans. In vitro, PGRP-SC1b cleaves staphylococcal peptidoglycan and the resulting products exhibit a reduced ability to activate AMP genes in Drosophila blood cell lines.[58] This suggested the catalytic PGRPs may be acting as scavengers to limit an inflammatory response to free peptidoglycan.[58] Recent work with PGRP-SC1a however suggests the catalytic processing may be required for initiating both cellular and humoral responses in vivo.[67] *PGRP-SC1a* mutants are unable to activate the Toll pathway or phagocytose *S. aureus*, suggesting it may have a role in mediating both these responses. A targeted mutation in the PGRP-SC1a catalytic domain rendering it able to bind peptidoglycan but not cleave it, affects phagocytosis but not Toll signaling in vivo. This suggests that peptidoglycan cleavage products may be important for other receptors to activate phagocytosis, in a potentially multi-step recognition process.[38,60,67]

Gram-Negative Binding Proteins (GNBPs)

GNBPs are small 50 kDa proteins containing a C-terminal β-glucanase-like domain.[55] They often share structural similarity to PGRPs. They were initially isolated from immune challenged silkworm *Bombyx mori*, as binding to Gram-negative bacterial surface, but not significantly to Gram-positive bacteria.[68] Three GNBP family members have been identified in *Drosophila*, but no corresponding homologs exist in mammals.[69] They have high affinity for bacterial LPS and fungal β-1,3 glucans in vitro. While silkworm GNBPs have glucanase activity, the critical amino acids required for activity are not conserved in fly GNBPs. However, mutations in GNBP1 (also known as Osiris), the best studied of these receptors in *Drosophila*, have unexpectedly revealed that it is involved in Gram-positive bacterial recognition.[16,70] PGRP-SA and GNBP1 act in a complex together to activate the Toll pathway.[71] While the mechanism of activation is not fully understood, the hemocoel of wild type, but not GNBP1 mutant flies, can hydrolyze Gram-positive peptidoglycan.[38] GNBP1 facilitates the cleavage of peptidoglycans in vivo, to possibly generate products that are recognized by PGRP-SA. A multistep process that recognizes and processes peptidoglycan may thus be responsible for Toll pathway activation.[16]

Thiol Ester Proteins (TEPs)

In vertebrates, the complement system is an important part of innate immune responses. The C3 protein of this system binds to the pathogen surface via a thioester bond, and initiates a cascade of events leading to phagocytosis or lysis of the invader.[72] Related to mammalian C3, a class of α_2-macroglobulins has been identified in invertebrates such as horse-shoe crabs.[73] These proteins act as protease inhibitors in response to proteases secreted from tissues damaged from infection. Because of their role in the immune response, there was a search for C3-α-macroglobulin like molecules in *Drosophila*.

Four proteins of this family have been identified in *Drosophila*. They contain highly conserved thioester motifs, and have been named Thioester containing proteins (Tep 1-4).[74] They contain a signal peptide suggesting that they are secreted. Tep2 has 5 splicing isoforms, while the others have single splicing forms, and all of them have a basal level of expression throughout *Drosophila* development.[74] The Tep1, Tep2, and Tep4 genes are upregulated in the fat body during immune challenge by bacteria. The JAK/STAT pathway, important for hemocyte development and immune regulation, has been implicated in this induction; gain of function JAK components cause induction of Tep factors.[74] Gain of function *Toll* mutants also cause induction of Tep genes, suggesting that Toll may also play a role in their regulation.[74]

Evidence from RNA inhibition of these genes in S2 cell lines suggests that Tep2 is required for efficient phagocytosis of *E. coli* while Tep3 may be specifically required for *S. aureus*.[99] A related protein known as Mcr (Macroglobulin Complement Related), which has on occasion been referred to as Tep6 because of this shared structural similarity,[74] has been shown to be secreted from S2 cells into the culture media, where it binds specifically to *Candida albicans* (but not to other fungal surfaces like *S. cerevisiae*).[99] This binding appears to be crucial for

Table 1. Pattern recognition receptors in Drosophila

Gene	Type of Protein	Putative Ligand	Evidence	Refs.
18 wheeler, Toll-2	Toll-like Receptor	Unknown	Expression of Attacin affected in mutant flies.	41,44,45
Toll-9	Toll-like Receptor	Unknown	Protein activates Drosomycin in S2 cells through MyD88.	46,47
Ird7, totem, PGRP-LC	Peptidoglycan recognition protein	DAP-type peptidoglycans, G- bacteria	Activate IMD pathway in vivo. Phagocytosis of *E. coli* in S2 cell lines, affected upon RNAi.	17,59-61
PGRP-LE	Peptidoglycan recognition protein	DAP-type peptidoglycans, G- bacteria	Overexpression activates PPO cascade in cell lines. Help PGRP-LC recognize peptidoglycans.	62,63
Semmelweis, PGRP-SA	Peptidoglycan recognition protein	Lys-type peptidoglycans, G+ bacteria	Activation of Toll and phagocytosis of *S. aureus* pathway affected in mutants.	37,64, 65,67
PGRP-SD	Peptidoglycan recognition protein	Lys-type peptidoglycans G+ bacteria	Activation of Toll pathway affected in double mutants with PGRP-SA mutants.	64
Picky, PGRP-SC1a	Peptidoglycan recognition protein	G+ bacterial peptidoglycans	Activation of Toll pathway and *S. aureus* phagocytosis affected in mutants.	67
PGRP-SC1b	Peptidoglycan recognition protein	G+ bacterial peptidoglycans	Cleaves *S. aureus* peptidoglycans.	58
Osiris, GNBP1	Gram-negative binding protein	Potentially G+ bacterial determinants	Hydrolyzes G+ peptidoglycan. Acts in complex with PGRP-SA to activate Toll pathway.	16,38,70
TEPs	Thiolester containing proteins	Possibly binding to bacterial surface	RNAi of homologous mosquito gene reduces phagocytosis of Gram-negative bacteria. Plasmodia population larger in *Tep1* mutant mosquito, causing higher vectorial capacity. RNAi of Tep2 decreases uptake of *E. coli*; RNAi of Tep3 decreases uptake of *S.aureus*.	74-76, 99
Mcr	Macroglobulin complement related	*Candida albicans*	RNAi of S2 cells reduces phagocytosis of *C. albicans*	99

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Table 1. Continued

Gene	Type of Protein	Putative Ligand	Evidence	Refs.
dSR-C1	Scavenger receptor	Possibly both G+ and G- bacteria	RNAi of S2 cells reduces phagocytosis of bacteria.	77-80
Crq	Scavenger receptor, CD36 like	Apoptotic cells and possibly G+ bacteria	Phagocytosis of *S. aureus* impaired in cell lines.	81,82
Peste	Scavenger receptor, CD36 like	Mycobacteria	RNAi of S2 cells reduces phagocytosis of Mycobacteria.	83
Eater	Scavenger receptor, epidermal growth factor like	Possibly G+ and G- bacteria	Reduction in phagocytosis of *S. aureus and E. coli* in vitro in cell-line mutants and in vivo in deficiency. Increased susceptibility to natural infection in vivo.	84,85

phagocytosis of this fungal pathogen by the cells. Thus, these in vitro studies suggest that different Teps have specialized to detect specific pathogens.

No mutations in Teps have been identified in *Drosophila*, and as a result their role in the immune response in vivo is not yet clear. However, in the mosquito *Anopheles gambiae* RNA inhibition has been used to reduce Tep gene expression and examine their immune function. Tep1 appears to be important for processing *E. coli* and *S. aureus*. Teps have also been shown to bind to both kinds of bacteria in vitro and in vivo, with functional thioester bonds and promote phagocytosis.[75] Interestingly, Tep1 also binds to the rodent malaria parasite *Plasmodium berghei*, and affects the vectorial capacity of mosquito.[76] Teps thus provide the first evidence of complement-like activity in insects.[75]

Scavenger Receptors

Scavenger receptors are a class of PRRs with broad specificity. Mammalian scavenger receptors are macrophage cell surface molecules associated with the endocytic uptake of lipoproteins.[77] Some of them are membrane-bound, while others are secreted.[77] In *Drosophila*, four Class C scavenger receptors have been identified. Of these, DScr-C1 is expressed on embryonic hemocytes and is important for phagocytosis[78] of both Gram-positive and Gram-negative bacteria, but not yeast.[79] The gene loci encoding these genes have been found to be highly polymorphic between *Drosophila* species. This polymorphism may be the result of fly populations subjected to different selection pressures from exposure to different pathogens.[80]

The *Drosophila* scavenger receptor Croquemort is required for phagocytosing dying cells in the *Drosophila* embryo.[81] Its mammalian homologue, CD36 has been implicated in recognizing and internalizing primarily Gram-positive bacteria and signaling through TLR 2 and 6. As a result, mice with a critical mutation in the signaling domain, fail to clear *S. aureus* efficiently.[82] In vitro, *Drosophila* Croquemort appears to be similarly involved in the recognition and phagocytosis of *S. aureus*.[82] However, in vivo data so far is restricted to its role in the identification of apoptotic markers and phagocytosis of dying cells. Determination of whether Croquemort also facilitates phagocytosis of pathogens and the activation of Toll pathways analogous to CD36 in mammals, remains to be established. Peste, another CD36-like protein, has been identified through a genome-wide RNAi screen in Drosophila cell lines using *Mycobacterium fortuitum*.[83] Peste appears to be responsible for the

phagocytosis of mycobacteria, but not other bacteria like *E. coli* or *S. aureus*. The mechanism by which it mediates this distinction as well as its role in vivo, however remains to be characterized.

Another protein with an interesting role is a novel *Drosophila* receptor named Eater. The extracellular domain of Eater contains Epidermal Growth Factor-like repeats. These domains are similar to scavenger receptors P120 and CED-1/SREC (Cell Death Abnormality/Scavenger Receptor from Endothelial Cells), in flesh flies and humans respectively.[84,85] Like scavenger receptors, Eater seems capable of binding multiple ligands including lipids and carbohydrates. Cell line evidence indicates that RNAi silencing of this receptor causes roughly 50% reduction in binding and internalization of *E. coli* and *S. aureus*.[85] In vivo, Eater is only expressed in hemocytes and its deficiency does not appear to affect Toll or IMD pathway signaling. However, phagocytosis of *S. aureus*, and to a lesser extent *E. coli*, is impaired in Eater deficient flies. This phenotype can be reversed by the expression of an Eater transgene, suggesting that it is specifically responsible for facilitating phagocytosis of bacteria.[85] Eater-deficient flies are also more susceptible to Gram-negative *Serratia marcesens* infection (a natural pathogen of *Drosophila*) presumably due to their impaired ability to phagocytose.

Down Syndrome Cell Adhesion Molecule (Dscam)

The *Drosophila* homolog of the human Down Syndrome Cell Adhesion molecule (Dscam) has been recently identified as playing a role in immunity, and it presents the intriguing possibility for recognizing a variety of pathogens. Dscam can potentially generate a staggering 18,000 variations of its extracellular domain through alternative splicing, and these isoforms are temporally and spatially regulated.[86,87] Dscam and its human homolog, were originally studied for their role in axon guidance in the embryonic central nervous system. In *Drosophila* it interacts with the adaptor Dreadlocks (Dock) and serine/threonine signaling protein Pak.[88] Dscam is important for the pathfinding of the Bolwig nerve, and embryonic brain development. Alternative splicing leads to tremendous diversity of this protein which helps in neuronal connectivity.[87]

More recently, it was found that Dscam isoforms are produced in *Drosophila* fat body, hemocytes, and secreted into hemolymph serum. Certain splicing forms were found to bind to *E. coli* while others did not. Further, RNAi knockdown of specific isoforms of Dscams in cell lines, reduced the ability of these cells to phagocytose *E. coli* by 30%.[86] This raises the possibility that different splice-forms of Dscam might act like mammalian immunoglobulins, by detecting specific pathogenic epitopes and marking them for phagocytosis. However, it is not yet clear whether Dscams bind pathogens in vivo. Furthermore, no evidence to date has shown the clonal expansion of selected hemocytes in Drosophila in response to any pathogen. Thus, the mechanism of how an adaptive process involving Dscam diversity might work in flies, remains an open question.

Other Potential Pattern Recognition Receptors

Drosophila express classes of proteins which are predicted to be PRRs based on similarities with such proteins in other organisms. One such class is the hemomucins, some members which are secreted while others are transmembrane glycoproteins. In mammals, they are known to play a role in the immune response by enabling leukocyte attachment during inflammation. A *Drosophila* hemomucin has been isolated using affinity purification techniques, and two splicing isoforms have been identified.[89] But a specific role in pattern recognition has not been demonstrated yet. Another class of proteins, galectins have also been identified as potential PRRs.[90] In mammals, various classes of lectins work together to mark a nonself carbohydrate for attack by complement or humoral immune responses.[91] Similar genes in the flesh fly *Sarcophaga peregrine* are induced in larvae upon injury.[92] In Drosophila one galectin has been identified, and it is predicted to bind to galactose of presumeably nonself origin[93,94] but relatively little is known about its mode of action. We can look forward to better characterization of these, and other potential PRRs in the future.

Conclusion

Much of the molecular mechanisms by which PRRs detect PAMPs and mediate immune responses in Drosophila remain to be clarified. Structural analysis of more PRRs and the characterization of the mechanisms by which they detect and signal will likely yield greater insight into this mechanism.[95-97] Relatively new techniques such as RNA interference screens and proteomic analyses can help identify new components important for pathogen detection.[61,83,98] These along with traditional approaches like forward genetic screens may help identify novel genes, which can lead to a better understanding of the role of these proteins in vivo. This understanding should also shed light on complex signaling events that appear to tailor specific immune responses to different classes of pathogens. The insect immune response has historically impressed us with startling insights into its elegance and effectiveness, and this trend can be predicted to continue in *Drosophila*, its best established model system.

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CHAPTER 12

Immune Recognition of *Plasmodium*-Infected Erythrocytes

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Malaria

Over 40% of the world's population is currently at risk of exposure to malaria with children being at greatest risk of developing severe disease[1] and it is estimated that between 1.5 and 3 millions deaths per year are due to malaria.[2] Infection can result in asymptomatic parasitemia or clinical syndromes ranging from a mild febrile illness to severe malaria characterised by acidosis, severe anaemia or cerebral complications.[3] Approximately 10% of infections result in severe disease with a high fatality rate especially in nonimmune children and adults. Malaria is caused by the parasitic protozoa from the genus *Plasmodium* and is transmitted by the bite of infected female *Anopheles* mosquitoes. There are four species of *Plasmodium* that infect humans; of these, *Plasmodium falciparum* is responsible for the greatest morbidity and mortality. During the bite of an infected mosquito, sporozoites are injected into the bloodstream of the human host. They then rapidly migrate to the liver and invade hepatocytes. In the subsequent 5 to 10 days, parasites differentiate and multiply within the hepatocytes and finally 20,000 to 40,000 merozoites are released into the bloodstream that invade erythrocytes. During the intraerythrocytic phase of infection, parasites develop and multiply over 48 hours in the erythrocyte. When the infected red blood cell (iRBC) bursts 15-32 merozoites per erythrocyte are released, which invade erythrocytes to begin a new cycle. Parasite multiplication continues until it is controlled by the immune response or drug treatment and it is during this repeated intraerythrocytic cycle that symptoms of disease develop. A small proportion of iRBCs undergo differentiation into either male or female gametocytes, which are subsequently taken up in a mosquito blood meal. In the mosquito mid-gut, the male and female gametes are released and fuse to form a zygote, which then undergoes a series of complicated differentiation, and growth stages that results in the production of infective sporozoites in the salivary glands of the mosquito.

Clinical Immunity to *P. falciparum* Infection

Clinical immunity to falciparum malaria is never sterile and develops with increasing age and exposure. Mathematical modelling suggest that immunity to noncerebral disease evolves rapidly after one or two infections while the development of immunity to mild disease takes much longer.[4] In endemic areas, asymptomatic infections are common in older children and adults.

P. falciparum differs from other species infecting humans in that infection can result in higher parasitaemia and that iRBCs sequester in the vascular bed during the second half of its

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intraerythrocytic life cycle. Almost all parasite isolates bind to CD36 and some may also bind to other receptors such as CD54 (ICAM-1, intercellular adhesion molecule 1), CD31 (PECAM-1, platelet endothelial cell adhesion molecule 1) and CD35 (CR1, complement receptor 1)[5] expressed on endothelial cells, leukocytes and erythrocytes. The notable exceptions are isolates from the placentae of primigravidae women, which bind to chondroitin sulphate A and hyaluronic acid but not to CD36.[6,7]

Cytoadhesion of iRBCs to endothelial cells has long been recognised as a hallmark of malaria pathology. Children with cerebral malaria are more frequently infected with ICAM1-binding parasites whereas children with mild clinical malaria are more likely to be infected with parasites which show a high avidity for CD36.[8-10] Sequestration is mediated by the *Plasmodium falciparum*-infected erythrocyte membrane protein 1 (PfEMP-1) which undergoes clonal antigenic variation.[5] PfEMP-1 is encoded by approximately 60 *var* genes per genome, which are extremely diverse not only within one parasite genome but also between different parasite isolates.[11] Cytoadhesion, together with the cocktail of cytokines produced by cells of the innate and adaptive immune response have been associated with malarial pathology.[12] Thus susceptibility or resistance to severe malarial disease is determined not only by the infecting parasite variant but also by the genetic make-up of the host.[13]

Priming of adaptive immune responses is critically dependent on the activation of and interaction with cells of the innate immune system. Engagement of pattern recognition receptors on antigen presenting cells such as monocyte/macrophages or DCs results in their rapid activation and secretion of cytokines.[14] These in turn can act on NK cells, NKT cells and γδ-T cells and, together with pathogen-derived signals, result in cross-talk with antigen presenting cells. Myeloid DCs or monocytes, differentiating into DCs after activation via Toll-like receptors (TLRs), are the main producers of IL-12.[15] In individuals experimentally infected with *P. falciparum*, low levels of IL-12 p40, one of two subunits of the biologically active IL-12 p70 molecule, can be detected in plasma one day before the volunteers become slide positive for iRBCs, suggesting that this cytokine is produced early during blood-stage infection.[16] IL-12 can be readily detected in the plasma of children suffering from acute malaria and was negatively associated[17] with parasitaemia and severe disease. Likewise, IL-18 was detected in plasma from children with acute malaria and correlated with disease severity but not with parasitaemia.[18-20] In some studies, IFN-γ production was higher in children with mild disease than in children suffering from severe disease[21] and was associated with a reduced risk of subsequent presentation with malarial fever and longer time to reinfection.[21,22] However, other studies reported that individuals suffering from severe disease had higher circulating plasma concentrations of IFN-γ and PBMC from malaria immune donors produced less of this cytokine in response to live infected red blood cells in vitro.[23-25] By contrast, plasma levels of the anti-inflammatory cytokine IL-10 increased with increasing parasitaemia[26] and was highest in children with severe malaria,[17,27] symptomatic malaria,[28] and in adults who died with falciparum malaria.[29]

NK cells are among the first cells responding to intact iRBCs by the production of IFN-γ, followed by γδT cells and NKT cells.[30-32] Interestingly, activation of NK cells is dependent not only on IL-12 and to a certain extend on IL-18 but also is dependent on contact with iRBCs. Secretion of IFN-γ by NK cells varied between individuals and could be due to a genetic component such as expression of KIR/CD94 haplotypes by the host.[33] These studies emphasize that the balance of cytokines induced by *P. falciparum* bloodstage infection can result in detrimental or beneficial effects on the host's ability to cope with infection and combat severe disease. In addition to innate cells of the immune system, $CD4^+$ T cells may be critical for early control of parasitaemia. In malaria-exposed humans, T cells proliferate or produce cytokines in response to malaria antigens (reviewed in ref. 34). In vitro, $CD4^+$ T cells can inhibited parasite growth suggesting an effector role beyond providing help to B cells.[35-37] Further evidence for the importance of antigen-specific $CD4^+$ T cells in naturally acquired immunity to malaria comes from epidemiological studies of the interaction between malaria and HIV-1. In cohorts of adults in Uganda,[38,39] and pregnant women in Malawi[40-42] and Kenya,[43,44] HIV-1 infected

adults were more likely to suffer from clinical malaria when their CD4 count fell below a certain threshold. HIV-1-mediated depletion of $CD4^+$ T cells underlines the critical role for $CD4^+$ memory T cells for clinical immunity to falciparum malaria.

Transfer experiments of serum from immune adults into children with acute malaria have shown that control of parasites is ultimately dependent on the ability to produce and maintain antibodies against a diverse range of parasite antigens.[45] Many targets of the humoral immune response are either polymorphic or undergo clonal antigenic variation. Under conditions of natural acquisition of immunity to malaria, individuals have to acquire a diverse repertoire of antibodies against polymorphic proteins expressed by different parasite strains and antigenic variants consecutively expressed on one parasite strain.[46,47] Furthermore, antibodies to specific parasite proteins are short-lived in children and detectable only in the presence of acute or asymptomatic infection.[48,49] These observations suggest that either the induction of T-cell help or the differentiation of B-cell memory and plasma cells is perturbed during acute malaria infection.[50] The activation of NK cells, γδT cells, DCs, monocytes and macrophages early during infection determines the cytokine milieu in which adaptive immune responses are primed.

Involvement of Pattern Recognition Receptors in *Plasmodium* Infection

Recent studies have examined the role of Pattern Recognition Receptors (PRR) in the innate immune recognition of pathogen-associated molecular patterns (PAMPs) unique to Plasmodium parasites. These components can be a wide range of combinations of proteins, lipids, sugars and nucleic acids. Several ligands of PPR have been identified on iRBC including PfEMP-1, glycosylphosphatidylinositol (GPI) and hemozoin.

PfEMP-1

The extracellular portion of PfEMP-1 is composed of variable numbers of two main domains: the Duffy binding like (DBL) domain and the cysteine interspersed domain region (CIDR).[11] All PfEMP-1 molecules contain at least one CIDR domain and two DBL domains. Although domain structures can be identified, these domains show considerable sequence heterogeneity. Adhesion of specific PfEMP-1 variants to host receptors has been located within different domains.[51-53] Almost two-thirds of CIDR domains of PfEMP-1 encoded by *var* genes in the genome of the laboratory parasite line 3D7 bind to CD36, a scavenger receptor expressed on endothelial cell and myeloid cells whereas some DBL domains can bind to ICAM-1 or complement receptor (CR1).[54] Binding to CR1 expressed on erythrocytes mediates rosetting of erythrocytes by iRBCs.[52] Whether iRBCs binding to CR1 also adhere to myeloid cells i.e., macrophages in the spleen, has not been demonstrated.

GPI

Many proteins expressed on the surface of merozoites are anchored by GPI.[55] GPIs have long been associated with potent activation of innate immune cells resulting in the production of TNF-α, which makes it a good candidate PAMP.[56] Individuals living in endemic areas readily make antibodies against *P. falciparum* GPI, which have been associated with protection from severe disease in some studies.[57,58] In addition, GPI has been proposed as a ligand for CD1d, a nonclassical MHC molecule that binds glycolipids. Recognition of CD1d by invariant T cell receptors expressed on NKT cells has been shown to regulate susceptibility to severe disease in rodent models of malaria.[59]

Hemozoin

The malarial pigment hemazoin is a detoxification product of heme that is usually found in the food vacuoles of the Plasmodium parasite. Hemozoin, together with other debris, is released when mature iRBCs rupture and is rapidly taken up by neutrophils, monocyte/macrophages and DCs. However, hemozoin is not biochemically inert: Hemozoin reacts with

membrane phospholipids and is transformed into hydroxy-polyunsaturated fatty acids, which cause membrane peroxidation.[60,61] In addition, hemozoin catalysis induces the formation of prostaglandin PGE_2 and PGF_{2a}. While hydroxy-polyunsaturated fatty acids inhibit monocyte function such as phagocytosis, activation by inflammatory cytokines and generation of an oxidative burst, the release of PGE_2 and PGF_{2a} could alter T- and B-cell functions. Furthermore, monocyte differentiation into DC is impaired in the presence of hemozoin[62] and DC cocultured with hemozoin show an altered response to maturation signals. These impairments were accompanied by increased expression of the peroxisome proliferator-activated receptor-γ, up-regulation of which is known to interfere with DC maturation.[63,64]

Recognition of *Plasmodium*-iRBCs by Toll-Like Receptors

Whether or not iRBCs or their products can activate TLR has been intensely investigated in recent years. Especially the study of rodent models of malaria, using powerful tools such as knock out mice deficient in specific receptors or adaptor molecules, has considerably advanced the field. However, when comparing events in rodent models of malaria with the human disease, the differences in TLR expression on monoctye/macrophages and DC subsets have to be taken into consideration. Two major DC subsets can be detected that have distinct but overlapping functions. Myeloid DCs are the main producers of IL-12, while plasmacytoid DCs are the main producers of IFN-α. In mice, TLR2, TLR4 and TLR9 are expressed on both myeloid and plasmacytoid DCs. In humans, myeloid DCs express TLR2 and TLR4 but not TLR9 whereas plasmacytoid DCs express TLR7 and TLR9.[65]

One of the first reports of the involvement of TLR in the immune response to Plasmodium infection indicated that Myeloid differentiation factor 88 (MyD88), part of the downstream signalling cascade of TLR, was essential for responses to infection. It was demonstrated that MyD88 deficient mice failed to produce IL-12 in response to infection with *P. berghei*, preventing subsequent liver damage that is associated with infection in this model. The specific TLR mediating this response was not identified but TLR2, TLR4 and TLR6 were ruled out because knock out mice for these receptors showed normal increases of IL-12 in response to *P. berghei* infection.[66]

In vitro experiments using *P. falciparum*-iRBCs demonstrated that intact iRBCs, lysates or the soluble fractions of lysates activated human plasmacytoid DCs. Activation of plasmacytoid DCs was characterised by an increase in the expression of the costimulatory molecule CD86 but not CD40 and induction of IFN-α but not TNF-α secretion. Although the viability of plasmacytoid DCs was maintained, they never fully matured in response subsequent stimulation and induced only poor proliferation in allogeneic $CD4^+$ T cells.[67] However, plasmacytoid DCs efficiently activated γδT cells in the presence of lysate, consistent with a marked increase in circulating γδT cells observed during acute *P. falciparum* infection.[68,69] Following these observations further work was undertaken to characterise the molecule or molecules responsible for these effects. While the factors inducing activation of plasmacytoid DCs had characteristics of a protein, factor(s) activating γδT cell appeared to be lipids as had been reported before.[70] Further experiments, using mouse myeloid and plasmacytoid DCs indicated that the effect of *P. falciparum* lysate on plasmacytoid DCs in vitro was dependent on TLR9.[71]

Studies by Coban et al addressed activation of murine DCs by *P. falciparum* hemozoin.[72] They observed that proinflammatory cytokine production was not evident in MyD88 knock out mice. Both, myeloid and plasmacytoid DCs derived from wild type mice or TLR2, TLR4 and TLR7 knock out mice were activated by hemozoin, as indicated by an increase in CD40 and CD86 expression. However, neither activation nor cytokine production was observed in DCs derived from TLR9 knock out mice. Cytokine responses were the same for parasite-derived or synthetic hemozoin, indicating that in this model the effects were due to hemozoin itself rather than contaminating factors such as lipids, proteins or DNA.[72]

Plasmodium GPI has long been suspected to induce inflammatory signals. Recent studies demonstrated that GPI bound to TLR2 and to a lesser extent to TLR4 expressed by mouse and

human macrophages and induced TNF-α secretion.[73,74] Mouse macrophages also produced IL-12, IL-6 and Nitric Oxide in response to GPI when they were first primed with IFN-γ. It is therefore perceivable that GPI will also activate other myeloid cells such as myeloid DCs via TLR2 and TLR4 in humans. Of note, free GPI was very quickly inactivated in vivo by phospholipases in serum and on cell surfaces. This may explain why activation of myeloid cells by Plasmodium GPI has long been suspected but very difficult to prove.

Together, these experiments suggest that both hemozoin and as yet not identified protein(s) can activate DCs by binding to TLR9 while GPI can activate DCs by binding to TLR2 and TLR4. In mouse models of malaria, TLR9-mediated signalling will occur in both myeloid and plasmacytoid DCs. In this case, TLR9-mediated signalling can induce tolerance to TLR4-mediated signalling.[75] In human malaria however, only plasmacytoid DCs will respond to TLR9 ligands. However, their activation and production of IFN-α can induce the maturation of myeloid DCs. Whether or not myeloid DCs will be more or less responsive to TLR2 and TLR4-mediated signals remains to be investigated for *P. falciparum* infection.

Recognition of *Plasmodium*-iRBCs by Scavenger Receptors

The scavenger receptors family consists a group of receptors that bind to chemically modified lipoproteins and mediate endocytosis. These receptors are broadly expressed on macrophages DCs and some endothelial cells. The class B scavenger receptor, CD36, is the most important scavenger receptor studied so far in *P. falciparum* malaria. Recent studies suggest that CD36 is the crucial receptor for the phagocytosis of iRBCs that have not been opsonized by either complement proteins or antibodies. Macrophages from CD36-deficient mice ingested *P. falciparum*-iRBCs at a much lower rate than macrophages expressing normal levels of CD36.[76,77] Neither human nor rodent macrophages produced TNF-α in response to phagocytosis of iRBCs suggesting that nonopsonic phagocytosis does not result in the activation of myeloid cells. Likewise, monocyte-derived DCs are inert to binding and phagocytosis of iRBCs. However, their response to inflammatory signals such as LPS was modulated in that they failed to secrete IL-12, but produced high amounts of IL-10 and TNF-α compared to control DCs.[78] Parasite-modulated DCs were poor stimulators of primary and recall T cell responses. Similar modulation of DC function was observed in response to antibodies against CD36 suggesting that adhesion of iRBCs to CD36 expressed on DCs is sufficient.[79] Interestingly, it has recently been reported that TLR2 and CD36 cooperate in the recognition of microbial diaglycerides.[80,81]

The role of scavenger receptors other than CD36 in *Plasmodium* bloodstage infection is less well known. Mice resistant to *P. chabaudi* AS infection show some increase in phagocytic activity compared to susceptible mice. Subsequent blocking of scavenger receptors with polyinosinic acid inhibited phagocytosis of iRBCs and merozoites in vitro and lead to increased parasitemia in vivo in resistant mice compared to susceptible mice. However, blocking of scavenger receptors did not affect the development of protective immune responses.[82] Furthermore, Type I and II class A scavenger receptor knock out mice showed a similar course of *P. chabaudi* infection as wild type mice. The involvement of mannose receptors was tested by inhibition with mannan. Again, a decrease in phagocytosis was seen without altering the course of infection in vivo.[82] Together, these results suggest that scavenger receptors are involved in innate responses to Plasmodium infection but not critical for the control of parasitaemia or indeed survival in rodent models of malaria.

Conclusion

Studies on the role of TLRs in innate immune responses to Plasmodium infection are still in the early phases. The main TLRs that are involved in Plasmodium infection seem to be TLR2 and TLR9 and to a lesser extent TLR4. However, the involvement of multiple TLR- or MyD88-dependent signalling pathways cannot be discounted as shown for the MyD88 dependent production of IL-18 by macrophages.[83] The main *Plasmodium*-ligands for TLRs described to date are hemozoin and GPI, although there is evidence that other hitherto unknown protein(s)

may be involved in the activation of TLR9. So far the recognition of *Plasmodium* iRBCs or its products by TLRs has been determined primarily using experimental mouse models. In rodents, both plasmacytoid and myeloid DCs express TLR2, TLR4 and TLR9 and all receptors can be engaged on the same cell. Cooperation between different TLRs may have synergistic or inhibitory effects on particular signalling pathways. By contrast, in humans only plasmacytoid DCs express TLR9. Here, parasite-derived proteins induce the activation of plasmacytoid DCs resulting in the production of IFN-α. IFN-α in turn supports the maturation of myeloid DCs, the differentiation of Th1 T cells and the differentiation of memory B-cells into plasma cells.[84] Together, with GPI acting on TLR2 and TLR4 expressed on human monocytes and myeloid DCs, these signals might result in efficient activation of myeloid DCs as well as interaction with other cells of the innate immune system and T cells early during infection (Fig. 1). However, in

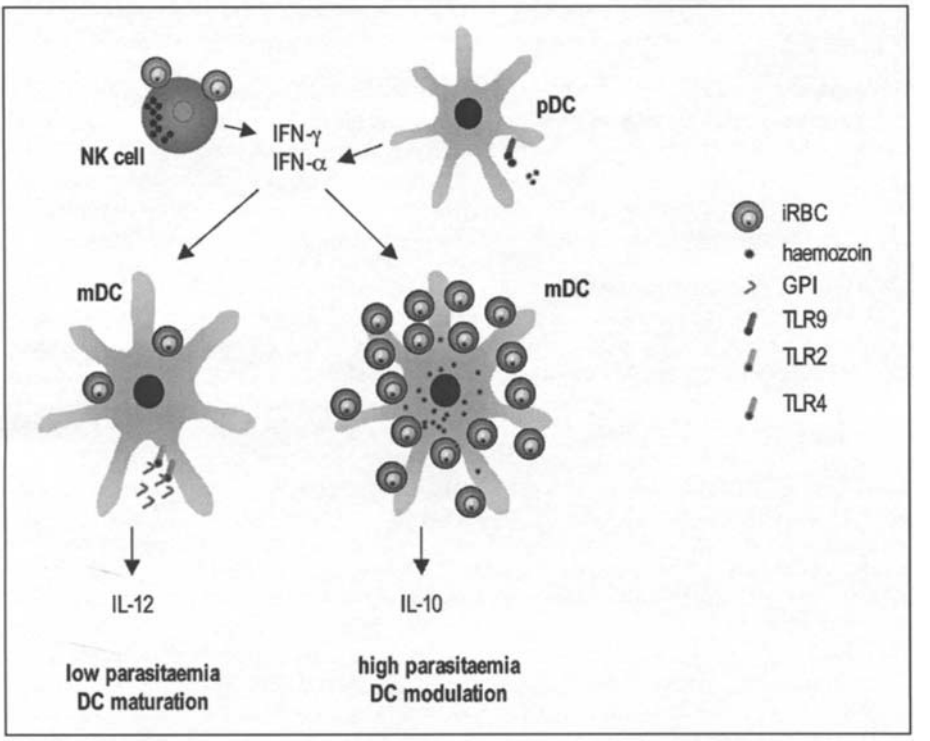

Figure 1. Schematic diagram of the interaction of infected erythrocytes and their products with DCs during early and late stages of infection. In humans, parasite-derived proteins induce activation of plasmacytoid DCs resulting in the production of IFN-α. Furthermore, NK cells may interact directly with infected erythrocytes and, in the presence of IL-12, produce IFN-γ. Both cytokines support the maturation of myeloid DCs, the differentiation of Th1 T cells and the differentiation of B-cells. Together with GPI acting on TLR2 and TLR4 these signals might result in efficient activation of myeloid DCs, secretion of IL-12 and promote interaction with other cells of the innate immune system and T cells. During late stages of the infection, the modulating effects of hemozoin and of adhesion of infected erythrocytes to CD36 on DCs function have to be taken into account. These processes are dependent on concentration or contact and TLR-mediated signalling events may be altered when parasitaemia is high or hemozoin has accumulated in myeloid DCs. More and more myeloid DCs in the spleen might be modulated either directly through interaction with iRBC and secrete IL10 or through ingestion of increasing amounts of hemozoin.

this context it is interesting to note, that nonopsonic phagocytosis of iRBCs did not result in enhanced secretion of TNF-α, suggesting that only high local concentrations of free ligands but not intact iRBCs might induce TLR-mediated signalling. In addition, the modulating effects of hemozoin and adhesion of iRBCs for monocyte and DCs function have to be taken into account. These processes are dependent on concentration or contact, indicating that during later stages of infection when parasitaemia is high, TLR-mediated signalling events may be altered. Investigation of the interaction between these different receptor-ligand pairs at different stages of the disease and under pressure from preexisting immune responses will shed further light on major questions in malaria immunology: what determines susceptibility to severe disease and how does the parasite evade immune responses.

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CHAPTER 13

Innate Immune Recognition in Tuberculosis Infection

Anthony G. Tsolaki*

Abstract

In this review, an overview of the host's innate immune response against *Mycobacterium tuberculosis* will be provided. In particular, *M. tuberculosis* interaction with Toll-like receptors (TLRs), lung surfactant proteins and the antimicrobial mechanisms in the macrophage will be discussed along with their importance in shaping adaptive immunity to tuberculosis infection.

Introduction

The bacterium *M. tuberculosis* is the cause of the most deaths as a result of a single infection in history. Every year 2 million people die of tuberculosis, with around 8 million new cases from airborne human transmission, mostly affecting the developing countries.[1] One-third of the world's population is estimated to be infected with the latent form of the disease and one at risk of reactivated tuberculosis some time in their lives.[1]

M. tuberculosis typically infects the lungs (although not exclusively), and classically is known for its ability to evade certain components of the innate immune system, thus making it an extremely efficient pathogen. *M. tuberculosis* infection begins with the tubercle bacilli being inhaled with aerosol droplets into the pulmonary alveoli. After inhalation the bacilli are initially ingested by alveolar macrophages via phagocytic receptors. At this initial encounter most bacilli are destroyed, but often some may survive and multiply in the macrophage resulting in disruption to its functions.[2] During this initial encounter, monocyte-derived dendritic cells (DCs) and macrophages are recruited from the bloodstream to the lungs that take part in the phagocytic process. However, they also do not destroy the bacilli, but allow for their growth, with minimal tissue damage, while the blood monocytes accumulate at the site of infection.[2] After two to three weeks, T-cell mediated immunity is initiated, where T cells promote granuloma formation and lysis of infected macrophages without killing the mycobacteria, consequently inhibiting extracellular growth. This adaptive immune response results in *M. tuberculosis* remaining in 'a dormant' state known as latent tuberculosis. Individuals, who are latently infected, are susceptible to disease reactivation, if their immune system is subsequently compromised, e.g., Human Immunodeficiency Virus (HIV) infection, those under physical stress, ageing, malnutrition, chemotherapy or some other unknown genetic susceptibility. In tuberculosis, latency is thought to be the result of a stand-off between the immune response and *M. tuberculosis* virulence.

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The initial stages of tuberculosis infection remain incompletely understood especially the interaction of the pathogen with components of the innate immune system. The role of innate immune molecules in tuberculosis in terms of host-pathogen interaction has become an area of considerable interest recently. For instance, TLR-mediated activation of innate immunity has been shown to be involved in host defence against *M. tuberculosis* infection. MyD88/TRIF double deficient mice, in which TLR-dependent activation of innate immunity is abolished, show high sensitivity to *M. tuberculosis* infection, indicating that innate immunity is critically involved in anti-*M. tuberculosis* responses. In another study, lung surfactant protein SP-D has been implicated in protection/development against tuberculosis.[3] The factors that govern the development of tuberculosis disease are incompletely understood. It is likely that some strains of *M. tuberculosis* are more capable of causing tuberculosis than others[4] with added contribution from innate immunity. In a recent study that involved host and bacterial genotype, where hosts were defined by single nucleotide polymorphisms (SNPs) in TLR-IL-1R domain containing adaptor protein (TIRAP) and TLR-2, the Euro-American lineage of *M. tuberculosis* strains were found less capable of extra-pulmonary dissemination.[5] Individuals with one specific TLR-2 SNP were more likely to have tuberculosis caused by the East-Asian/Beijing/W genotype of *M. tuberculosis* than other individuals.[5,6] Thus, *M. tuberculosis* genotype influences clinical disease phenotype and may modulated the host's innate immune response.

Receptor-Mediated Phagocytosis of *M. tuberculosis*

Successful infection depends on the outcome of the initial interaction between host cells and the pathogen. *M. tuberculosis* primarily targets the lungs via aerosol infection, thus the first form of defence against *M. tuberculosis* is provided by resident alveolar macrophages. Phagocytosis of the *M. tuberculosis* bacterium can transpire with or without prior opsonization and can occur via a variety of different receptors on the phagocytic cellular surface, such as complement receptors, mannose receptors, CD14, scavenger receptors and FCγ receptors. It has been proposed that the type of receptor utilised for internalisation of *M. tuberculosis* influences the host cellular response, thus implying that there is no essentiall or single preferred route of entry.

Complement Receptors

Complement Receptor 1 (CR1), CR3 and CR4 have all been implicated in playing a role in *M. tuberculosis* ingestion; however CR3 has been highlighted in particular as the major instigator of *M. tuberculosis* phagocytosis. In vitro studies performed by Schlesinger et al demonstrated the importance of CR3 when they observed an 80% reduction in phagocytosis of *M. tuberculosis* in the absence of CR3.[7] Furthermore the interaction between CR3 and *M. tuberculosis* have been characterised as preventing formation of respiratory bursts[8] resulting in phagosomal arrest of early endosomes and thus no inflammatory response.[9] In addition to using host cell receptors in this way *M. tuberculosis* is also capable of taking advantage of host cell molecules to increase the likelihood of uptake by a phagocyte. Such invasive mechanisms are shared by all pathogenic mycobacteria and involve the use of C2a to form C3 convertase, resulting in C3b opsonisation and uptake by macrophage complement receptors.[10] Although CR3 has been shown in vitro to be the primary mode for *M. tuberculosis* uptake, it is worth noting that murine models with CR3 deficient mice displayed similar bacterial burden, granuloma formation and survival patterns to wild type mice,[11] thus indicating some uncertainty over in vitro data.

Mannose Receptors

Mannose Receptors (MR) have been more associated with nonopsonised mediated phagocytosis, and are found on monocyte derived macrophages as well as tissue macrophages.[7] MRs recognise terminal mannose residues apically located on lipoarabinomannan (LAM) ligands of virulent strains of *M. tuberculosis* (Erdman strain).[12] Since LAM is also capable of associating with

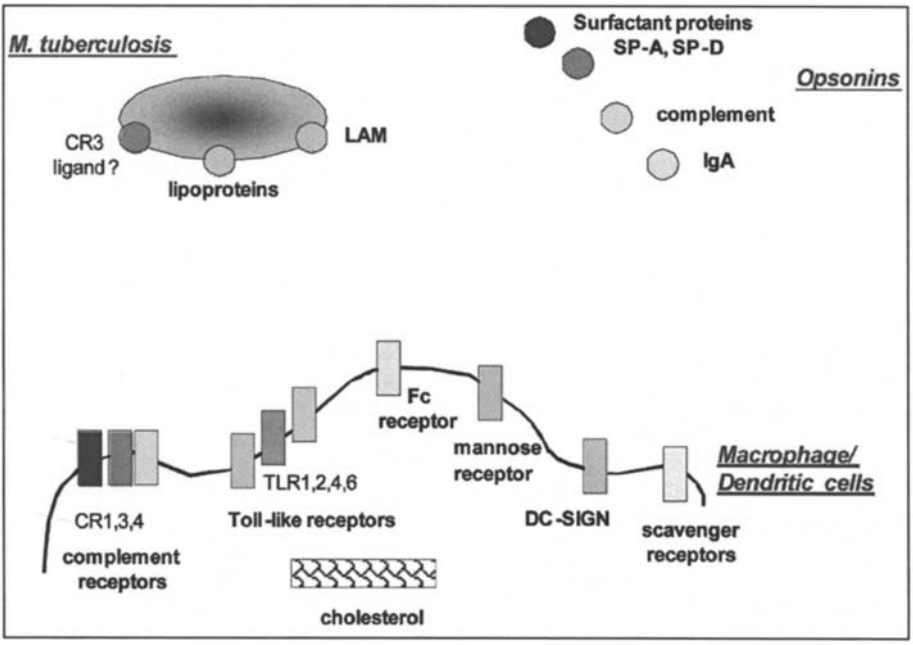

Figure 1. Molecules involved in the complex interplay between *Mycobacterium tuberculosis* and the host's innate immune response.

the receptor CD14, it is difficult to identify what biological events result from MR mediated uptake, although certain studies have indicated that the MR pathway allows mannose-capped LAM (ManLAM) *M. tuberculosis* to avoid formation of phagolysosomes, thus producing an anti-inflammatory reaction and helping it establish itself in the host cell.[13] However in contrast to complement receptors which can mediate uptake of attenuated or virulent strains of *M. tuberculosis*, MRs bind and internalise only virulent strains,[14] suggesting this route of entry is advantageous to *M. tuberculosis* pathogenesis.

CD14 receptors have a role to play after experiments by Peterson et al showed that anti-CD14 antibodies inhibited phagocytosis of *M. tuberculosis* by fetal microglia;[15] in addition, other experiments have shown that *M. bovis* preferentially infects porcine alveolar macrophages that expressed high levels of CD14, and that anti CD14 cells inhibit bacillary entry.[16] However, results from other experiments disagree with these findings, indicating that CD14 has no essential role in internalisation and instead proposes the idea that *M. tuberculosis* infection simply upregulates CD14 expression in macrophages, which in turn may facilitate the pathogens capacity to modulate the immune response.[17] Thus, the relevance of CD14 alone, in *M. tuberculosis* internalisation by macrophage is surrounded in uncertainty.

Scavenger Receptors

Scavenger receptors and Fcγ receptors (FcγR) are thought to play a less important role in tuberculosis infection. Scavenger receptors have been quantitatively implicated through experiments using competitive inhibition; thus in the case where uptake by CR's and MR's are being inhibited, *M. tuberculosis* may enter the phagocyte through Type-A scavenger receptors.[18] FcγR solicits an inflammatory response via interaction with IgG-opsonized mycobacteria, the uptake induces formation of reactive oxygen intermediates and promotes fusion between the phagosome and lysosome.[19]

As mentioned earlier, pathogenic mycobacteria have the uncanny ability to use particular host cell components to maximise internalisation, Collectins have been indicated in some studies to enhance binding of *M. tuberculosis* to alveolar macrophages. One in particular is surfactant protein A (SP-A), and can be found in abundance in alveoli. In one study, it was reported that HIV patients exhibited elevated levels of SP-A in their lungs resulting in a three-fold greater possibility of *M. tuberculosis* infection.[20] SP-A facilitates the uptake of *M. tuberculosis* by human macrophages by behaving as a ligand, which after binding, significantly decreases the level of reactive nitrogen intermediates (RNI) in the macrophage; as a result of reduced RNI levels, it is possible that macrophage cytotoxicity is reduced therefore suggesting that SP-A mediated entry aids *M. tuberculosis* survival.[21,22] It has not yet been clearly defined which receptors internalise SP-A opsonized mycobacteria, although CR1, Fcγ[23] have been proposed along with MR,[24] though it is clear that this particular area warrants further research. In contrast to SP-A, the closely related SP-D is also located in the lungs, has been found to inhibit uptake of virulent strains of *M. tuberculosis*.[25] If we take into account the proposed roles of SP-A and SP-D in the pathogenesis of *M. tuberculosis*, it is possible that the relative concentrations of each surfactant protein may correlate with risk of infection.

More recently cholesterol has been accredited with having an essential role in uptake by complement receptors after in vitro studies by Gatfield et al reported that depletion of cell plasma cholesterol resulted in complete inhibition of *M. tuberculosis* internalisation.[26] The implications from these results are important, since cholesterol accumulation around phagocytic receptors ultimately influences *M. tuberculosis* uptake rather than the nature of the receptor. In addition, cholesterol also mediates a phagosomal association with tryptophan aspartate-containing coat protein (TACO), which acts as a protective coat that may prevent phagosome-lysosome fusion.[26] However, once again the relevance of cholesterol accumulation around certain receptors needs to be studied further in vivo to determine if it is a significant factor in bacterial virulence.

Thus there are various mechanisms, innate immune molecules and pattern recognition receptors that *M. tuberculosis* utilises to gain entry into alveolar macrophages. Consequently host cellular response are most likely to be dictated by the route *M. tuberculosis* takes thus deciding whether *M. tuberculosis* will survive initial inflammatory response of the innate system. However further studies are needed in order to test how significant some of these mechanisms are under in vivo conditions in order to gain a better understanding of the series of events leading to disease.

Survival of *M. tuberculosis* inside the Phagosome

Once tuberculosis bacilli have entered the host macrophage within the phagosome compartment, it must avoid destruction by evading host microbicidal machinery in order to establish successful infection. Survival inside the phagosome is a result of the *M. tuberculosis* vacuole not fusing with lysosomal compartments.[19,27] This is an imperative step in pathogenesis as it represents a blatant evasion of innate defences, originally shown by Armstrong and Hart described on *M. tuberculosis* phagosomes did not fuse with ferretin-labelled lysosomes,[27] further research has been conducted to monitor the molecular events involved in the trafficking of the *M. tuberculosis* phagosome, its maturation and its elusion from fusion with a lysosome. Early trafficking patterns of the *M. tuberculosis* phagosome appears normal; accessibility to transferrin-bound iron[28,29] and glycosphingolipids,[30] along with trafficking of transferrin receptors[31] help to establish that the *M. tuberculosis* phagosomes are not stationary and that they associate with certain early endosomal compartments. However, it has been shown that at the arrested stage, the *M. tuberculosis* phagosome fails to acquire lysosomal hydrolases and vesicular proton-adenosine triphosphatase (ATPase) responsible for phagosomal acidification.[32] It appears that biogenesis of the phagolysosome is impeded during the phagosome maturation stage controlled by GTP-binding molecules Rab5 (localised to early endosomes and responsible for fusion between endocytic vesicles and early the endosome) and Rab7 (localised

to late endosome and important to endocytic pathway).[33] Rink et al discovery of the Rab conversion,[34] combined with their observations, showed that the *M. tuberculosis* phagosome is associated with Rab5, but not with Rab7 at the expected time for its recruitment (i.e., immediately after removal of Rab5), thus resulting in what is known as the Rab conversion block. However, the exact components mediating the Rab conversion pathway are unspecified. Rab5 is known to have an effector molecule called EEA1 (early endosomal autoantigen 1) which shows reduced recruitment by the *M. tuberculosis* phagosome.[35] *M. tuberculosis* phagosomes demonstrate reduced expression of the enzyme Type III PI3K (phospatidylinositol 3-kinase) hVPs34;[36] a critical Rab5 effector molecule which in turn generates PI3P (phosphatidylinositol 3-phosphate); a regulatory lipid, which allocates specific trafficking events. PI3P associates with EEA1 on the organellar membranes, in a process that ultimately mediates transport of lysosomal hydrolases, cathepsin and ATPases between the trans golgi network and phagosome.[35,36]

The components of *M. tuberculosis* that block maturation of the phagosome and the pathway utilised to achieve this are yet to be clearly defined, although there are various theories with supporting evidence that can be considered. One theory can be attributed to *M. tuberculosis* inhibition of the calcium pathway. The macrophage Ca^{2+} signalling pathway helps mediate phagosome maturation. *M. tuberculosis* blocks this pathway via inhibition of the sphingosine kinase enzyme and in doing so prevents recruitment of PI3K hVPs34 and subsequent PI3P to membrane organelles.[37] Also *M. tuberculosis* is capable of binding to MR via cell surface LAM, a pathway associated with prevention of phagosome-lysosome fusion.[13] LAM acts as a preformed trafficking toxin upon contact with a macrophage, ManLAM inhibits hosts phosphatidylinositol phosphorylation into PI3P[38] resulting in a lack of lysosomal hydrolases, cathepsin and ATPases.[35,36] LAM is thought to disrupt Ca^{2+} signaling. How it does so is yet to be established however its likely to be associated with *M. tuberculosis* induced inhibition of sphingosine kinase as proposed by Thompson et al.[39] Another theory suggests that disruption to phagosome actin assembly, a process that is involved in membrane fusion and is essential in phagosome maturation and may also be an important factor. In vitro studies have shown that different lipids could stimulate or inhibit actin assembly in *M. tuberculosis* phagosomes. Interestingly, certain lipids have also been shown to activate actin assembly in affected phagosomes, resulting in the killing of *M. tuberculosis*.[40] Nevertheless another study has suggested that an inadequate supply of iron during fusion with early endosomes mediates phagosomal arrest.[41] More recently, protein kinase G, which has been found to be secreted inside the *M. tuberculosis* phagosome has also been implicated after inhibition of the protein resulted in lysosomal activation and mycobacterial death of infected macrophages.[42]

DCs are important components in the innate response and are the link between innate and adaptive immunity. In contrast to alveolar macrophages, MRs and CRs have not been attributed with an essential role. Instead *M. tuberculosis* ligand ManLAM is able to facilitate uptake into DCs via a C-type lectin receptor, DC-SIGN (DC-specific intercellular adhesion molecule-grabbing integrin).[43] Interestingly a study shows that DCs intracellular climate does not promote growth of *M. tuberculosis*.[43] Although much has been learned with regards to uptake and trafficking of tuberculosis bacilli inside macrophages, little is known about its uptake and trafficking inside the DCs, clearly an area that requires further study.

Immune Recognition of *M. tuberculosis*

Interaction with TLRs is an essential step in immune recognition of *M. tuberculosis* components, a step that not only activates innate immune mechanisms, but also aids in development of antigen specific adaptive immunity. Toll-like receptors are a family of transmembrane proteins found on macrophages and DCs. They exhibit leucine-rich motifs in their extracellular domains (similar to other pattern recognition receptors of the innate immune system), which are used to detect highly conserved molecular patterns on the surface of

pathogens. They are a phylogenetically conserved family of receptors. That play an important role in mediating downstream signalling of immune reaction, in response to TB infection. It is by PAMP (pathogen associated molecular patterns) recognition that TLRs are activated. This response is pro-inflammatory and involves production of a number of effector molecules including NFκB and cytokines (principally TNFα), which in turn induce recruitment of other innate cellular components.

Of the many different TLRs identified, it seems that TLR2, TLR4 and TLR9 have been implicated as key signallers that respond to the presence of *M. tuberculosis*. *M. tuberculosis* has been accredited with expressing vast amounts of TLR2 agonists on its surface, amongst these are three mycobacterial lipoproteins, which have been identified as specific TLR2 agonist. LpqH is a 19kDa secreted *M. tuberculosis* antigen that specifically ligates with TLR2 to induce host inflammatory mechanisms, including promotion of TNFα and IL-12 secretion in macrophages and monocytes respectively, as well as stimulating inducible nitric-oxidase sythase promoter activity.[44] In addition LprA has been identified as a TLR2 agonist which stimulates similar signalling to LpqH, but additionally induces IL-10 production and DC maturation.[45] The 24kDa LprG lipoprotein (a cell wall component) also causes secretion of TNFα via TLR2 association.[46] PIM (phospatidyl-myo-inositol) and a 38kDa glycoprotein antigen have been implicated as TLR2 and TLR4 agonist with PIM-TLR4 association specifically shown to induce NFκB production[47] and a 38kDa glycoprotein associated signals shown to induce TNFα and IL-6 expression.[48] Interestingly tri-acylated lipomannan (LM) has already been identified as a potent pro-inflammatory TLR2 agonist, although recently different acylated forms of LM were found to have some anti-inflammatory stimulating properties suggesting that lipomananan acylation patterns may help regulate host innate immune response.[49] Bacterial DNA has also been suggested as agonist of TLR9 after experiments demonstrated TLR9 ability to bind with CpG dinucleotides of bacterial DNA.[50] Elucidating these *M. tuberculosis* constituents that initially interact with TLRs is imperative as further studies may help reveal important pathways, which shape host immune response (innate as well as adaptive).

Previous in vitro studies have indicated TLR2 as a chief sensor for *M. tuberculosis* induced cellular activation, and the primary promoter of TNFα secretion, assigning it with a pro-inflammatory role.[51,52] In spite of most data pointing to TLR2 positive modulator of the immune response, some experimental data have indicated that *M. tuberculosis* induced activation of TLR2 produces anti-inflammatory signalling. One experiment showed that secretion of the anti-inflammatory cytokine IL-10 was blocked in absence of TLR2.[53] However, Salgame hypothesises that IL-10 induction by TLR2 is a part of negative feedback regulatory mechanism, that reduces inflammatory response in order to limit collateral damage.[54] It has also come to light that TLR2 is not necessarily essential to mediate host resistance to *M. tuberculosis* after a series of murine experiments. *M. tuberculosis* infection was induced in $TLR2^{-/-}$ murine models, in a low dose and high dose dependant manner. The results from low dose aerosol infection revealed TLR2 deficiency had no bearing on the induction of host defence. In contrast the high dose aerosol infection indicated a definite role for TLR2 in resistance to *M. tuberculosis*.[55] Tjarnlund et al used human-like infection models to show that TLR2 and TLR4 deficient mice showed higher susceptibility to *M. tuberculosis* infection, during the acute phase of reaction, with TLR2 deficient mice displaying larger bacteria loads in lungs after 3 weeks in comparison to wild type mice.[51] After 8 weeks however, bacteria loads from all three murine sets were similar, indicating that Th1 immunity was still induced possibly via an alternative mechanisms or a TLR-independent pathway.[51] Regardless of TLR2 being deemed not essential to pathogenesis of murine tuberculosis, the data from Tjarlund et al shows that the number of *M. tuberculosis* colony forming units (CFUs) in TLR deficient mice lungs decreased from early to late infection, a trait not shown by wild type mice thus signifying the importance of TLR2 signalling as part of the innate response to early infection.[51]

It is difficult to clearly define the role of TLR4. Branger et al have accredited TLR4 with a protective role after C3H/HeJ mutant mice (having defective non functional TLR4) that had been intra-nasally infected with *M. tuberculosis* displayed a heavier bacterial burden in lungs and a higher mortality rate in comparison to wild type models.[56] Similar experiments including that of Abel et al[47] amongst others, have formed similar results with murine models, some indicating pivotal importance of TLR4. However in the dose dependant experiments carried out by Reiling et al, TLR4 defective (C3H/HeJ) mice displayed greater resistance than wild type models after low dose exposure to *M. tuberculosis*.[55] Additionally, in response to low dose *M. tuberculosis* challenge, both TLR2 deficient and TLR4 C3h/HeJ mice conferred similar resistance to that of control mice, thus dispelling theory that TLR2 or TLR4 are singularly pivotal to mediating host innate responses.[55] It is possible that activity of TLR2 and TLR4 is synergistic in modulating response to *M. tuberculosis* challenge or that activation could occur via another pathway (which maybe TLR dependant or independent). In vivo results are unfortunately inconclusive requiring further studies to establish TLR4s role.

Recently, the role of TLR9 has started to be unveiled, being found to stimulate pro-inflammatory cytokine synthesis in response to interaction with mycobacterial DNA. Bafica et al used TLR9 in conjunction with TLR2 to observe both their activities and individually.[57] The results were significant. TLR9$^{-/-}$ *M. tuberculosis* infected mice exclusively exhibited production of defective IL-12p40 and IFN-γ (Interferon-γ) in vitro, although in accordance with TLR2, TLR9$^{-/-}$ models were still able to show resistance to low dose *M. tuberculosis* aerosol challenge implicating TLR9 in IL-12 and Th1 responses to *M. tuberculosis* infection.[57] Data showing TLR2/9 double knock-out mice displayed significantly greater susceptibility to *M. tuberculosis* infection and greater defects in IL-12 response by DCs and macrophages compared to models singularly deficient in either TLR2 or TLR9 thus indicating that cooperation between these two TLRs is significant in defence against *M. tuberculosis* infection.[57]

Cytokines Induced in Response to *M. tuberculosis* Infection

Th1 Immunity is Induced by Cytokines

Although the Th1 reaction is itself an adaptive response, it is important to understand the mechanism responsible for inducing Th1 immunity which is driven by innate components and thus represent a critical pathway. The Th1 immune response is imperative to mounting critical protection against activation of tuberculosis infection (granuloma formation) as demonstrated by HIV-infected individuals.[58] IL-12, IL-23 and IL-27 have all been implicated as working in sync in shaping the Th1 response.

It has been shown that IL-12 is secreted by phagocytic cells in response to *M. tuberculosis* infection and has documented pro-inflammatory effects.[59] Giacomini et al showed that the cytokines secreted by DCs were primarily involved in inducing anti-mycobacterial T cell mediated responses, implicated IL-12 with Th1 induction.[60] In this experiment it was shown that IL-12, along with IFNγ-inducing cytokines, was exclusively secreted by monocyte derived DCs following infection with *M. tuberculosis*. Work from several laboratories has helped further characterise the role of IL-12. Flynn and coworkers showed that *M. tuberculosis* infected Balb/c mice exhibited enhanced survival after a short course therapy of IL-12 was administered, almost doubling the survival period of the control mice.[61] The work also emphasized the importance of IFN-γ presence in respect to IL-12 activity after Balb/c models with disrupted IFN-γ genes were shown to be unresponsive to the IL-12 supplementation, fundamentally highlighting that IL-12 pro-inflammatory action requires downstream signalling from IFN-γ.[61] In another experiment by Feng et al, IFN-γ secreting CD4$^+$ T cells from chronically infected murine models were transferred to *M. tuberculosis* infected models (RAG deficient mice and RAG/IL-12p40 deficient mice). The RAG deficient models presented prolonged survival times, whereas the latter IL-12p40 deficient model in contrast only displayed transient control of infection.[62]

The p40 subunit is a component shared between both IL-12 and IL-23, in addition to uniquely expressing p35 and p19 subunits respectively.[63] Incidentally previous human studies have revealed that IL-12p40 deficiency is associated with increased susceptibility to *M. tuberculosis* infection, in addition to inability to produce IL-12 and IL-23. Many studies have since taken place to explore which of IL-12 or IL-23 have a greater contributing factor leading to this susceptibility. IFN-γ secreting T cells were shown to be induced via an IL-12p70 independent pathway in an IL-23 dependant manner, after the transient Th1 response seen in IL-12p35 deficient mice was eliminated in IL-12p35/23p19 doubly deficient models, whereas IL-23p19 deficient models displayed no reduction in protection or Th1 response.[63] Furthermore IL-12p40 deficient models coinfected with *M. tuberculosis* Ag85 and IL-23 presenting plasmids, displayed strong antigen specific IFN-γ response comparable to that of IL-12/Ag85 codeliverance, partially restoring protective efficacy.[63] In respect of this information it can be seen that although IL-23 is capable of inducing moderate immunity, IL-23 is not as significant as IL-12 in mediating Th1 response, and most probably has a role in enhancing initial protection.

Il-27 has been more associated with Th1 initiation shown by Takeda et al whom establish that IL-27 induces expression of T-bet and IL-12β2 via STAT1 in a WSX-1 dependant pathway, consequently inducing IL-12p70 pathway and T-cell activity via increased expression of 12Rβ2. Il-27 is a cytokine related to IL-12, WSX-1 is a signalling component identified as part of IL-27 receptor (IL-27R) complex.[64] The role of IL-27 is in Th1 immunity and is yet to be clearly defined after it induced pleiotropic responses in similar investigations. In one investigation carried out by Holscher et al, *M. tuberculosis* challenged. WSX-1 deficient mice displayed increased resistance to *M. tuberculosis* infection, more so than wild type models,[65] whereas in another experiment, the same murine models exhibited reduced levels of IFNγ secretion per cell.[66]

TNFα

TNF-α has long been attributed the role as a protective cytokine involved in immunoregulatory pathways. Implication of its pro-inflammatory action were demonstrated in murine models, where TNFα was neutralised with monoclonal antibodies and TNFαR gene was disrupted.[67] These mice models displayed increased susceptibility to mycobacterial infection. This study helped establish that TNF-α and its corresponding 55 kDa receptor were essential in mounting protection against tuberculosis by inducing reactive nitrogen intermediates, required for the killing of bacilli by macrophages.[68]

TNF-α can exist in both soluble and membrane bound forms. The membrane bound form has been implicated as an inducer of apoptosis in *M. tuberculosis* infected macrophages.[69] TNF-α ability to induce apoptosis would appear to a be unfavourable to *M. tuberculosis* virulence, since this pathway could lead to possible destruction of bacilli by direct killing or possible induction of cross presentation of *M. tuberculosis* antigens to DCs for cross-priming of CD8 cytotoxic T cells.[70] *M. tuberculosis*s ability to disrupt this pathway suggests by suppressing TNF-α this may provide *M. tuberculosis* with an evasive tactic after it was reported that pathogenic *M. tuberculosis* strains induced release of TNFαR2 which subsequently down regulate TNF-α activity and macrophage apoptosis.[71]

Furthermore TNF-α is also one of the few cytokines with a role firmly established against reactivation of latent tuberculosis. TNF-α has been implicated in forming productive granulomas. A number of different groups have shown that latently infected, TNF-α neutralised mice exhibited disorganised granuloma and subsequent reactivation of disease.[72] Human studies have illustrated similar findings after some latently suffering, rheumatoid arthritis patients treated by anti-TNF-α antibody experienced reactivation.[73]

IL-10

IL-10 is an anti-inflammatory and immunosuppressive cytokine produced in response to *M. tuberculosis* infection of DC or macrophage. Despite in vivo studies showing IL-10 knock out

mice failing to mount an increased resistance to *M. tuberculosis* infection, a majority of data has been accumulated to suggest that increased IL-10 secretion antagonizes downstream and co-stimulatory molecules largely involved in modulating immune response. Anergic patients for instance have displayed high levels of IL-10 secretion implying that *M. tuberculosis* induced IL-10 most likely has a role in effectively suppressing a host immune response.[44] Furthermore previous studies have also found that T cells secreting IL-10 have been associated with increased susceptibility to infection.[72] In addition, in vitro studies have shown that secretion of IL-10 down regulates secretion of IL-12, consequently affecting downstream regulation of IFN-γ.[73] Interestingly, DCs, as previously mentioned, also secrete the pro-inflammatory cytokine IL-12, which is responsible for initiating and maintaining Th1 responses. The relative amounts of these contrasting cytokines and the time may determine immune response to *M. tuberculosis*.[73]

Chemokines Induced by M. tuberculosis *Infection*

M. tuberculosis infection of human macrophages and DCs stimulates the production of a large number of chemokines that play a large role in control of tuberculosis and are present from the innate response right through to the adaptive responses and are primarily induced by TNF-α activity. *M. tuberculosis* induced chemokine studies initially focused on the expression pattern following infection. CCR2 (receptor of CCL2) has been implicated as responsible for initial recruitment of immature DCs and monocytes to the site of infection.[74] Peter et al also demonstrated that CCR2 deficiency lead to reduced migration of DC to lymph nodes, and thus an increased susceptibility to *M. tuberculosis* was observed. However, subsequently depressed CCR2 models susceptibility to *M. tuberculosis* in a dose dependant manner.[75]

Following *M. tuberculosis* infection there is an influx of CCR5 expressing macrophages (including monocytes, DCs and lymphocytes) into lung tissue. In addition there is also an increase in production of CCR5 ligands.[76] Recently CCR5 has been shown to induce maturation of naïve DCs and subsequent production of IL-12 following ligation with *M. tuberculosis* antigen Hsp70.[77] Despite increase in CC5R ligands and IL-12 production, absence of CCL5 does not affect granuloma formation, however bacterial burden in draining lymph nodes was increased, thus prompting further investigation into the possibility that CCR5 signals may inhibit migration of *M. tuberculosis* carrying DCs and thus may be a *M. tuberculosis* virulence strategy to down regulate T cell priming. *M. tuberculosis* infection also results in increased expression of CCR7 on cells, and it is thought that this receptor helps in guiding DCs to draining lymph nodes.

The Role of Dendritic Cells in M. tuberculosis *Infection*

Dendritic cells (DCs) participate in both the innate and adaptive immune responses to *M. tuberculosis*, and effectively serve as a link between the two systems. They have a key role in establishing protective immunity and containing *M. tuberculosis* infection. Upon phagocytosis of *M. tuberculosis* by DCs, TLRs are activated and maturation process is initiated, a process where DCs up-regulate expression of costimulatory molecule, adhesion molecules and chemokine receptors (particularly CCR7). Up-regulation of CCR7 has a role in guiding the DCs to draining lymph nodes. Bhatt et al demonstrated this by tracking migration patterns of traceable DCs in macrophage populations. Not only was it observed that DCs migrated to regional lymph nodes, but that process was also essential for the induction of Th1 immune responses and subsequent T cell priming.[78] Following migration, DCs process and present *M. tuberculosis* antigens via MHC Class I and II molecules to naïve $CD4^+$ and $CD8^+$ cells, a process imperative to shaping the adaptive response.

It is clear that maturation and migration are key components in the innate mechanisms that induce T cell responses. It has been demonstrated that IL-1β released from antigen presenting cells have been shown to impair DC maturation, as well as reducing IL-12 secretion.[79] In addition other factors have been implicated as factors possibly disturbing DC maturation and migration, such as IL-10 possibly inhibiting migration by decreased expression of p40 homodimers.[80]

Conclusion

Why is *M. tuberculosis* able to evade innate immune destruction? Its success to persist in its host can attributed to its ability to continuously evade components of the innate immune system through the variety of different pathways it can utilize to infect cells and survive. As reviewed, *M. tuberculosis* has the ability to induce phagocytosis though a number of different receptors, with or without prior opsonisation in the case of complement receptors, therefore expressing its plasticity. Its ability to evade destruction inside macrophages via inhibition of phagosome-lysosome fusion also illustrates how the pathogen is able to evade and survive lysis. The role of TLRs, in particular TLR2, has led to greater understanding of the cell signalling involved in shaping innate and downstream adaptive responses to *M. tuberculosis* infection. However, further research regarding the signalling pathways involved and their responses to different strains of *M. tuberculosis* is needed. In conclusion, the role of the target pattern recognition receptors and hence the innate immune response in clearing *M. tuberculosis* infection is clear, but the reasons why it fails to do so remain incompletely understood. It results from a complex interplay between bacterial virulence factors and host predisposing factors. Elucidating this interplay will help us understand why some individuals develop tuberculosis upon infection and others do not.

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