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John D. Lambris
George Hajishengallis *Editors*

Current Topics in Innate Immunity II

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Preface

Recent and emerging evidence shows that innate immunity is not simply providing first-line defense against infection or other types of insult. Innate immune mechanisms are critically involved in the development of adaptive immunity and additionally regulate diverse physiological and homeostatic processes, such as synapse maturation in the central nervous system, angiogenesis, mobilization of hematopoietic stem/progenitor cells, tissue regeneration, and lipid metabolism. Consequently, deregulation of the sensing or effector functions of innate immunity may lead to pathological disorders that are not necessarily or directly related to host defense. Leading scientists from around the world convened at the 7th International Aegean Conference on Innate Immunity in Rhodes, Greece (July 4–9, 2010) to discuss the latest advances in this rapidly evolving field. This volume represents a collection of topics on the biology of Toll-like and other pattern-recognition receptors, complement and its crosstalk with other physiological systems, inflammatory mechanisms and diseases, natural killer cells, cooperative interplay between innate and adaptive immune cells, and host-microbe interactions. Our sincere thanks to the contributing authors for the time and effort they have devoted to writing exceptionally informative chapters in a book that will have a significant impact on the field of innate immunity. We would also like to express our thanks to Dimitrios Lambris in managing the organization of this meeting. Finally, we also thank Samantha Lewis of Springer Publishers for her supervision in this book's production.

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Pentraxins in Humoral Innate Immunity

Antonio Inforzato, Barbara Bottazzi, Cecilia Garlanda, Sonia Valentino and Alberto Mantovani

Abstract Innate immunity represents the first line of defence against pathogens and plays key roles in activation and orientation of the adaptive immune response. The innate immune system comprises both a cellular and a humoral arm. Components of the humoral arm include soluble pattern recognition molecules (PRMs) that recognise pathogens associated molecular patterns (PAMPs) and initiate the immune response in coordination with the cellular arm, therefore acting as functional ancestors of antibodies. The long pentraxin PTX3 is a prototypic soluble PRM that is produced at sites of infection and inflammation by both somatic and immune cells. Gene targeting of this evolutionarily conserved protein has revealed a non-redundant role in resistance to selected pathogens. Moreover, PTX3 exerts important functions at the crossroad between innate immunity, inflammation and female fertility. Here we review the studies on PTX3, with emphasis on pathogen recognition and crosstalk with other components of the innate immune system.

Keywords Pentraxins • PTX3 • Inflammation • Innate immunity • Tissue remodeling • Biomarkers

1 Introduction

Innate immunity is the phylogenetically oldest mechanism of defence against microbes and it plays a key role in activation and orientation of the adaptive immune response. Pathogen recognition by the innate immune system is based on a set of germline-encoded receptors, known as pattern recognition molecules (PRMs), which recognize conserved microbial moieties collectively named pathogen-associated molecular patterns (PAMPs) (Iwasaki and Medzhitov 2010). Based on cellular localization and function, PRMs are classified into two major groups: (i) cell-associated receptors, localized in different cellular compartments and

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including endocytic receptors, such as scavenger receptors, signalling receptors, such as toll like receptors (TLR) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLR) and (ii) fluid phase molecules or opsonins, which represent the functional ancestors of antibodies and are involved in pathogen opsonization, complement activation and self versus modified self-discrimination (Bottazzi et al. 2010; Takeuchi and Akira 2010).

Fluid phase PRMs are essential effectors and modulators of the innate resistance in animals and humans, and comprise a heterogeneous group of molecules, including collectins, ficolins, and pentraxins (Holmskov et al. 2003; Bottazzi et al. 2010). Some soluble PRMs are stored in granules of neutrophils and rapidly released (i.e. within minutes) into the extracellular milieu upon neutrophil stimulation, others are *de novo* synthesized as early gene products in mononuclear phagocytes and dendritic cells (DCs); in both cases, these PRMs are locally released at sites of immune insult. As opposed to this, certain epithelial tissues (i.e. liver) act as a remote and delayed source of systemic PRMs (Bottazzi et al. 2010). Despite their heterogeneity in terms of molecular structure and site/time of production, soluble PRMs share basic, evolutionarily conserved functions, including opsonization, complement activation, agglutination and self versus modified self-discrimination (Bottazzi et al. 2010).

Pentraxins are essential components of the innate immune system, conserved in evolution from arachnids to humans and characterized by a multimeric structure. Based on the primary structure of the protomers, pentraxins are divided into two groups, short and long pentraxins: C-reactive protein (CRP) and serum amyloid P component (SAP) are prototypic short pentraxins, whereas pentraxin 3 (PTX3) and other subsequently identified proteins represent the long pentraxin arm of the family (Garlanda et al. 2005).

Here, we review the role of this family of proteins in innate immunity with main focus on the long pentraxin 3 and its involvement in innate resistance to pathogens.

2 The Pentraxin Superfamily

Pentraxins are characterized by the presence in their carboxy-terminal region of a ~200 amino acid long domain containing a highly conserved motif of primary sequence known as pentraxin signature (HxCxS/TWxS, where x is any amino acid). The prototype of the family is CRP, a molecule identified in the 1930s for its ability to bind in a calcium-dependent fashion the C-polysaccharide of *Streptococcus pneumoniae* (Tillet and Francis 1930). Human SAP, identified as a closely related protein (i.e. with 51% sequence identity to human CRP), is a calcium-dependent lectin originally purified for its binding to the agarose component 4,6-cyclin pyruvate acetal of *β*-d-galactose (Emsley et al. 1994). CRP and SAP orthologs in different mammal species share substantial sequence homology, with notable differences including serum basal levels and changes during the acute phase response, CRP and SAP being the main acute phase reactants in human and mouse, respectively. In the arthropod *Limulus polyphemus*, different forms of CRP and SAP were identified

as abundant constituents of the hemolymph involved in recognizing and killing of pathogens (Liu et al. 1982; Armstrong et al. 1996; Shrive et al. 1999).

CRP and SAP are 25-kDa proteins with a common structural organization that comprises five or ten identical subunits arranged in a pentameric radial symmetry (Emsley et al. 1994). Both the molecules are produced by hepatocytes in response to the proinflammatory cytokine IL-6 (Hirschfield et al. 2003). CRP is barely detectable in the plasma of healthy human adults (≤ 3 mg/l) but after tissue insult or injury its concentration increases by as much as 1,000-fold above baseline within 24 hours. This behavior makes blood CRP an ideal clinical marker of a patient's general health status, and it has thus been used for decades (Pepys and Hirschfield 2003). As opposed to this, the concentration of human SAP is substantially invariant (30–50 mg/l), even during the early acute-phase response, while the murine counterpart is the major acute phase protein in the mice (Garlanda et al. 2005).

PTX3 it the prototypic long pentraxin, first identified in the early 1990s as a cytokine-inducible gene in endothelial cells and fibroblasts (Garlanda et al. 2005). PTX3 has an unrelated amino-terminal region coupled to a carboxy-terminal pentraxin-like domain and differ from short pentraxins in gene organization, chromosomal localization, cellular source, inducing stimuli and recognized ligands. After PTX3, other members of the long pentraxin subfamily have been identified, including guinea pig apexin, neuronal pentraxin (NP or NPTX1) 1, NP2, also called NARP, and NPTX receptor, which is the only member associated to the cell through a transmembrane domain (Goodman et al. 1996). Ortholog molecules have been found so far for PTX3, NP1, NP2, and NPR in human, mouse, and rat, as well as in lower vertebrates such as zebrafish and puffer-fish (Martinez de la Torre et al. 2010). In an attempt to find new pentraxin domain-containing proteins, we have recently identified a new long pentraxin, which we named PTX4. Like other members of this family, the gene encoding PTX4 is well conserved from mammals to lower vertebrates. However, PTX4 has a unique pattern of mRNA expression in the stroma of thymus and spleen, which is distinct from that of other long pentraxins, and does not behave as an acute phase protein (Martinez de la Torre et al. 2010).

3 The Short Pentraxins in Innate Immunity

The role of CRP and SAP in innate immunity has been exploited in a variety of studies, taking advantage of gene-modified animals (e.g. *Crp*-transgenic or *Sap*-deficient mice) or exogenous administration of the proteins. While SAP is the main acute phase protein in the mice, serum levels of murine CRP are unaffected by inflammatory/infectious stimuli, thus mice may represent a natural knockdown model useful for exploiting the role of CRP.

The ability of CRP to protect mice against bacterial infection by various species, such as *S. pneumonia* (Szalai et al. 1995), *Haemophilus influenza* (Lysenko et al. 2000) and *Salmonella enterica* (Szalai et al. 2000), has been well established. Protection is mediated through CRP binding to phosphorylcholine (PC), a major

constituent of the C-type capsule polysaccharides and the most important ligand of CRP, or phospoethanolamine, a component of the cell membrane of *S. enterica*.

Similarly to CRP, SAP can bind to structures found on microbial surfaces, including lypopolysaccharide (LPS), PC and terminal mannose or galactose residues (Agrawal et al. 2009). As a consequence SAP interacts with a range of Gram-positive and Gram-negative bacterial pathogens and with human influenza A virus (Andersen et al. 1997), but these interactions have contrasting effects on innate immunity (Noursadeghi et al. 2000; Yuste et al. 2007). In fact, although *in vitro* phenotypes suggest that human SAP may protect against a variety of pathogens, including tuberculosis, malaria or influenza A (Andersen et al. 1997; Singh and Kaur 2006), *in vivo* results are controversial, with some authors showing a protective role of SAP against *S. pneumoniae* (Yuste et al. 2007), and others reporting that SAP can enable bacteria to evade neutrophil phagocytosis displaying enhanced virulence (de Haas et al. 2000; Noursadeghi et al. 2000).

The capacity to recognize a number of diverse ligands, mostly in a calcium-dependent manner, is a common feature of members of the pentraxins family (see also below for PTX3). However, the relationship between ligand binding and function of these proteins is still a matter of debate. Interestingly, CRP has been shown to protect mice from infection with *Salmonella typhimurium*, a pathogen that is not recognized by this short pentraxin, thus indicating that in some cases CRP binding to pathogens is not necessary to mediate host protection (Szalai et al. 2000). Similar observations have been reported for SAP, which exhibits a host defence function against pathogens that it does not bind. Furthermore, CRP-mediated activation of the classical complement pathway has no role in protecting mice against *S. pneumoniae* infection (Suresh et al. 2006).

Different possible mechanisms can support the mode of action of short pentraxins in their protective role to selected infections. Short pentraxins participate in activation and regulation of all the three complement pathways (i.e. classical, lectin and alternative—reviewed in (Bottazzi et al. 2010): CRP and SAP, either in aggregated form or in complex with their ligands, interact with the globular head modules of the complement component C1q (Roumenina et al. 2006). In addition CRP can interact with ficolins (Ng et al. 2007; Tanio et al. 2009), and factor H (Jarva et al. 1999; Okemefuna et al. 2009). Reduced complement activation is observed in SAP-deficient mice: this results in increased susceptibility to infection with *S. pneumoniae* due to impaired C3b-mediated phagocytosis and/or decreased complement-mediated inflammatory response to infection (Yuste et al. 2007). It has been suggested that complement activation by short pentraxins might also favour removal of the apoptotic debris, with potential implications in preventing the onset of autoimmune diseases (Nauta et al. 2003).

Specific and saturable binding to all three classes of Fcγ receptors (FcγR) has been demonstrated for both CRP and SAP, where these interactions mediate phagocytosis of apoptotic cells and microorganisms (Bharadwaj et al. 1999; Bharadwaj et al. 2001). In a recent study a 3D structure of human SAP in complex with the extracellular domain of FcγRIIa has been generated based on X-ray crystallography that suggests short pentraxins and IgG to share the same binding site on FcγR

(Lu et al. 2008). Consistent with these observations, CRP and SAP proved effective to inhibit immune-complex-mediated phagocytosis (Lu et al. 2008). Therefore, pentraxins can protect against pathogens by activating both complement and FcγR pathways, which resembles the functional properties of antibodies.

4 The Long Pentraxin PTX3

4.1 Gene Organization and Expression

The human *PTX3* gene has been localized on chromosome 3 band q25 and is organized in three exons and two introns. The first two exons code for the leader peptide and the N-terminal domain of the protein, respectively, and the third exon encodes the pentraxin domain. The murine gene has the same structural organization and is located on chromosome 3. The proximal promoters of both human and murine *PTX3* genes share numerous potential enhancer-binding elements, including Pu1, AP-1, NF-κB, SP1, and NF-IL-6 sites. The NF-κB binding site is essential for the transcriptional response to proinflammatory cytokines (i.e. TNF-α and IL-1β), whereas AP-1 controls the basal transcription of PTX3 (Garlanda et al. 2005).

PTX3 expression is rapidly induced in a variety of cell types by several stimuli, such as cytokines (e.g. IL-1β, TNF-α), TLR agonists, microbial moieties (e.g. LPS, OmpA, lipoarabinomannans) or intact microorganisms (Bottazzi et al. 2010). Myeloid dendritic cells are a major source of PTX3 that is also expressed by monocytes, macrophages, endothelial cells, fibroblasts, smooth muscle cells, kidney epithelial cells, synovial cells, chondrocytes, adipocytes, alveolar epithelial cells, granulosa cells and glial cells (for review, see Garlanda et al. 2005; Bottazzi et al. 2010). In contrast to these cell types which express PTX3 upon stimulation, both human and murine lymphatic endothelial cells constitutively express the protein (Sironi et al. 2006; Amatschek et al. 2007; Wick et al. 2007). PTX3 mRNA expression is temporally confined to immature myeloid cells, but in mature neutrophils, PTX3 is constitutively stored in the specific granules and is released in response to TLR engagement or other activating stimuli, partially localizing in neutrophil extracellular traps (Jaillon et al. 2007; Maina et al. 2009).

The inflammatory cytokine IL-1β induces high amounts of PTX3 in peripheral blood mononuclear cells, while IL-6, the main inducer of CRP and SAP, as well as typical activators of monocytes, such as monocyte chemotactic protein 1 (MCP-1/CCL2), macrophage colony stimulating factor (M-CSF), granulocyte–macrophage colony stimulating factor (GM-CSF), and interferon-γ (IFN-γ), are inactive. IFN-γ inhibits PTX3 production in dendritic cells, monocytes and macrophages both at the transcriptional level and reducing transcript stability, whereas IL-10 amplifies LPS-induced PTX3 expression (Doni et al. 2006; Maina et al. 2009). IL-4, dexamethasone, 1α, 25-dihydroxyvitamin D3 and prostaglandin E2 also inhibit LPS-induced PTX3 in myeloid dendritic cells (Doni et al. 2006).

Different signalling pathways can affect PTX3 production, depending on cell type and/or stimuli. PTX3 expression is generally controlled by the NF-kB pathway, while induction of the protein by TNF-α in lung epithelial cells involves the c-Jun N-terminal Kinase (JNK) pathway (Han et al. 2005), and in endothelial cells, PTX3 expression induced by high-density lipoproteins (HDL) requires the activation of the PI3K/Akt pathway through G-coupled lysosphingolipid receptors (Norata et al. 2008). Finally PTX3 expression can be regulated by the FUS/CHOP translocation involved in the pathogenesis of a subset of soft tissue sarcomas (Willeke et al. 2006).

Glucocorticoid hormones (GCs) induce or enhance PTX3 production in non-hematopoietic cells (e.g. fibroblasts and endothelial cells), but inhibit PTX3 production in hematopoietic cells (e.g. dendritic cells and macrophages) (Doni et al. 2008). These divergent effects are likely due to the fact that in non-hematopoietic cells, the GC receptor acts as a transcription factor, whereas in hematopoietic cells it interferes with the action of other signalling pathways, probably NF-κB and AP-1. *In vivo* administration of GCs increases the blood levels of PTX3. Indeed, patients with Cushing's syndrome exhibit increased levels of circulating PTX3 and subjects affected by iatrogenic hypocortisolism exhibit low levels of the protein (Doni et al. 2008).

4.2 Protein Structure

Human PTX3 is a multimeric glycoprotein, whose composing subunits are made of 381 amino acids, including a 17-residue signal peptide. PTX3 primary sequence is highly conserved among animal species. Like other members of the long-pentraxin family, PTX3 is composed of a unique N-terminal region coupled to a 203 amino acid C-terminal domain homologous to the short pentraxins CRP and SAP (Bottazzi et al. 1997). The N-terminal region (residues 18-178 of the preprotein) is unrelated to any known protein structure. Nevertheless, secondary structure predictions indicate that this part of the protein is likely to form four α-helices, three of which are probably involved in the formation of coiled-coil structures (Presta et al. 2007), whereas the pentraxin domain of PTX3 adopts a β-jelly roll topology (Inforzato et al. 2006). The amino acids residues that form the calcium-binding pocket in CRP and SAP are missing in the pentraxin domain of PTX3, which might explain some binding properties of this long pentraxin (i.e. PTX3 binding to C1q is calcium-independent, as opposed to short pentraxins) (Goodman et al. 1996). Among PTX3 ligands, fibroblast growth factor 2 (FGF2), inter-α-inhibitor (IαI) and conidia of *A. fumigatus* each bind to the N-terminal region (Presta et al. 2007; Scarchilli et al. 2007; Moalli et al. 2010); C1q and P-selectin interact with the pentraxin-like domain (Bottazzi et al. 1997; Nauta et al. 2003; Deban et al. 2010), whereas both domains have been implicated in the interaction with complement factor H (Deban et al. 2008) (see below).

A single N-glycosylation site has been identified in the C-terminal domain of PTX3 at Asn220 (Inforzato et al. 2006). This site is fully occupied by complex type

oligosaccharides, mainly fucosylated and sialylated biantennary sugars with a minor fraction of tri- and tetrantennary glycans. PTX3 glycosylation has been shown to affect the protein binding to a number of ligands, thus suggesting that changes in the glycosylation status might represent a strategy to tune the biological activity of this long pentraxin.

In addition to the multidomain organization, the human PTX3 protein shows a complex quaternary structure with protomer subunits assembled into high order oligomers stabilized by disulfide bonds (Bottazzi et al. 1997). Mass spectrometry and site-directed mutagenesis analysis of the recombinant human protein indicate that PTX3 is made of covalent octamers (i.e. with a molecular mass of 340 kDa), where cysteine residues in the N-terminal region form three inter-chain disulfides holding four protein subunits in a tetrameric arrangement. Two tetramers are linked together to form an octamer by additional inter-chain bridges involving C-terminal domain cysteines (Inforzato et al. 2008). A low-resolution model of the intact PTX3 molecule has been generated based on data from Electron Microscopy (EM) and Small Angle X-ray Scattering (SAXS), that shows the eight subunits of the protein fold into an elongated structure with a large and a small domain interconnected by a stalk region (Inforzato et al. 2010). This oligomerization state and the asymmetric shape of the molecule make PTX3 unique amongst pentraxins.

The structural complexity and modular nature of the PTX3 protein probably provide a molecular basis to explain the rather broad spectrum of cellular and molecular targets that are recognized by this long pentraxin and the diversity of its biological roles (Fig. 1).

4.3 Interaction with Complement and Fcγ Receptors

The first described ligand of PTX3 is the complement component C1q (Bottazzi et al. 1997; Nauta et al. 2003) (Fig. 1). PTX3 binds to plastic immobilized C1q, likely interacting with charged residues of the C1q globular heads (Nauta et al. 2003; Roumenina et al. 2006). Interaction of PTX3 with plastic-immobilized C1q, an experimental condition that could mimic the surface of microbes, results in the activation of the classical complement cascade. On the other hand, fluid-phase PTX3 inhibited C1q-dependent complement activation by competitive blocking of relevant interaction sites. These data indicate that PTX3 may exert a dual role and contrasting effects on complement activation: it supports clearance of material that is able to bind PTX3, such as microbes, while on the other hand it may protect against unwanted complement activation in the fluid phase. The interaction with C1q and subsequent activation of complement are also modulated by the extent of PTX3 glycosylation, as demonstrated by the observation that removal of sialic acid or complete deglycosylation of the protein significantly increase its binding to C1q and complement activation (Inforzato et al. 2006). The oligosaccharides attached to the naturally expressed PTX3 from TNFα-stimulated fibrosarcoma cells and LPS-stimulated DCs exhibit heterogeneity in the relative amount of bi, tri, and tetranten-

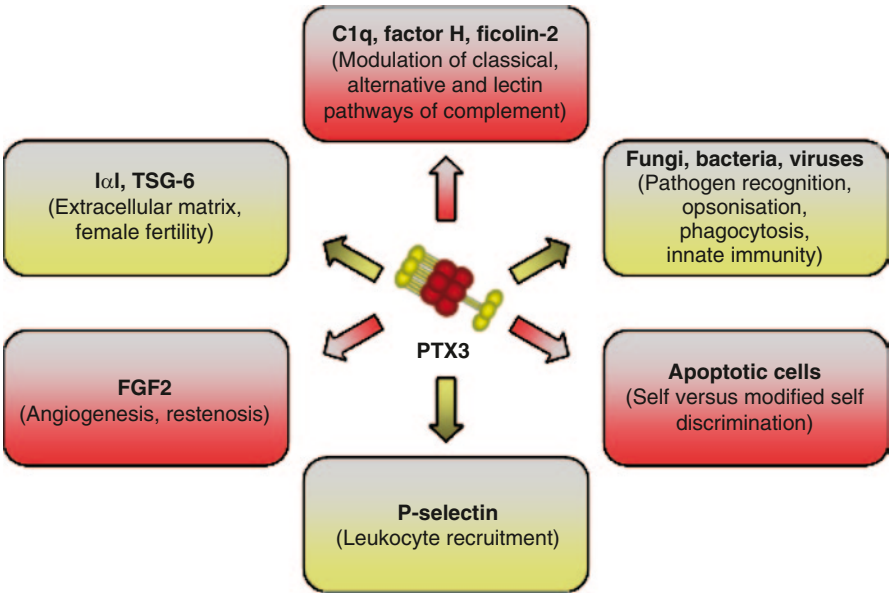

Fig. 1 Schematic representation of the most relevant PTX3 ligands, classified based on the functional implications of their interaction with the long pentraxin

nary structures (Inforzato et al. 2006). These observations suggest that changes in the PTX3 glycosylation pattern might occur depending on the cell type and inflammatory context in which the protein is produced and might contribute to modulate its biological functions.

Similarly to CRP (Jarva et al. 1999), PTX3 interacts with factor H (Fig. 1), the main soluble regulator of the alternative pathway, enhancing factor H and iC3b deposition on apoptotic cells, thus preventing exaggerated alternative complement activation (Deban et al. 2008).

Moreover, PTX3 interacts with L-Ficolin (Ma et al. 2009), similarly to CRP (Zhang et al. 2009) (Fig. 1). Ficolin-2, but not Ficolin-1 and Ficolin-3, was shown to bind *A. fumigatus* and this binding was enhanced by PTX3 and vice versa. Consistent with this, PTX3 increases Ficolin-2-dependent complement deposition on the surface of *A. fumigatus*. Other components of the lectin pathway of complement interact with PTX3: the mannose binding lectin (MBL) binds PTX3 and SAP probably via its collagen-like domain (Ma et al. 2010). Formation of the MBL/PTX3 complex recruits C1q and enhances C4 and C3 deposition on *Candida albicans* and its phagocytosis (Ma et al. 2010). Thus, components of the humoral innate immune system, which activate different complement pathways, cooperate and amplify microbial recognition and effector functions.

The interplay with key components of all three-complement pathways (i.e. C1q, Ficolin-2, MBL, factor H) points to PTX3 as an important player of the complex network of interactions that control complement functions and suggests a dual role for PTX3 in the regulation of complement-mediated immune responses.

The observation that macrophages display a binding site for PTX3 (Garlanda et al. 2002) suggested the existence of a cellular receptor for this protein. In the context of a study focused on SAP, Lu and coworkers reported recently that PTX3, similarly to CRP and SAP, can interact with FcγR, in particular FcγRIII/CD16 and FcγRII/CD32 (Lu et al. 2008), with KD 1.6 μM and 18.7 μM respectively. In parallel we observed a key role of CD32 in the opsonic activity of PTX3 versus *A. fumigatus* (Moalli et al. 2010) (see below). It remains to be established whether PTX3 receptors other than FcγR exist.

4.4 PTX3 in Pathogen Recognition and Inflammation

PTX3 interacts with a number of different pathogens including selected fungi, virus and bacteria (Fig. 1). A specific binding has been observed to *Aspergillus fumigatus* conidia, zymosan, *Paracoccidioides brasiliensis,* selected Gram positive and Gram negative bacteria, including *Staphyloccocus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium, Streptococcus pneumoniae* and *Neisseria meningitides* and with human and murine cytomegalovirus (CMV) and H3N2 influenza virus (Garlanda et al. 2002; Bozza et al. 2006; Reading et al. 2008). Among the microbial moieties tested, PTX3 binds Outer membrane protein A from *Klebsiella pneumoniae* (KpOmpA), a major component of the outer membrane of Gram negative bacteria highly conserved among the *enterobacteriaceae* family (Jeannin et al. 2005), whereas it does not bind LPS, lipoteichoic acid (LTA), enterotoxin A and B, exotoxin A and N-acethylmuramyl-L-alanyl-D-isoglutamine (MDP).

According to the binding properties, *Ptx3*-deficient mice are more susceptible than wild type mice to infection caused by these pathogens. In particular, *Ptx3*-deficient animals are susceptible to *A. fumigatus* infection as result of a defective recognition of conidia by alveolar macrophages, neutrophils and DC. This susceptibility is associated with a low protective T helper 1 (Th1) antifungal response coupled with an inappropriate Th2 response. Treatment with recombinant PTX3 restores the protective Th1 response demonstrating that PTX3 can participate in the tuning of immune responses (Garlanda et al. 2002). PTX3 interacts also with zymosan, a complex carbohydrate of the yeast cell wall that induces inflammatory signals in macrophages upon recognition by dectin-1 and the Toll-like receptors TLR2 and TLR6 (Diniz et al. 2004). PTX3 binding to zymosan promotes clearance of zymosan particles and zymosan-containing pathogens (i.e. *P. brasiliensis*) by macrophages in a dectin-1-dependent manner (Diniz et al. 2004). In accordance, *Ptx3*-overexpressing mice have an increased phagocytic index towards the fungal pathogen *Paracoccidoides brasiliensis* compared to wild type animals, and exogenous PTX3 can enhance the phagocytic activity of wild type macrophages (Diniz et al. 2004).

In the case of *K. pneumoniae* infection, over-expression of PTX3 by transgenic mice was associated with an enhanced ability to produce pro-inflammatory mediators, including NO and TNFα, and, as a consequence, with protection or faster lethality, depending on the dose of inocula (Soares et al. 2006). It is likely that,

through its interaction with KpOmpA, PTX3 might modulate the proinflammatory response triggered by *K. pneumoniae* (Jeannin et al. 2005). Indeed, KpOmpA interacts with the scavenger receptor lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) and scavenger receptor expressed by endothelial cell-I (SREC-1), activates phagocytes through TLR2 and binds to PTX3, thus leading to amplification of the innate response to this microbial moiety (Jeannin et al. 2005; Cotena et al. 2007). This cooperation amongst simultaneously engaged PRMs leads to activation of diverse cellular signaling pathways as well as of the humoral arm of the innate immune system, which results in synergy and amplification of the innate responses to pathogens. In contrast to zymosan (Diniz et al. 2004), PTX3 does not enhance the *in vitro* recognition of KpOmpA or the cell activation induced by KpOmpA. However, *in vivo*, PTX3 increases the local inflammation induced by KpOmpA, in terms of cell recruitment and proinflammatory cytokine production (Jeannin et al. 2005). This effect is complement-dependent and is abrogated after treatment with complement inhibitors (Cotena et al. 2007). However, this amplification loop is not a general mechanism, since PTX3 does not modify the inflammatory response induced by LPS, a microbial moiety not recognized by PTX3 (Cotena et al. 2007).

Recent results indicate that PTX3 has therapeutic activity in chronic lung infections by *P. aeruginosa,* which mimic lung infections of Cystic Fibrosis (CF) patients, by enhancing bacteria clearance, reducing the production of pro-inflammatory cytokines, neutrophil recruitment in the airways and histopathological lesions (Moalli et al. 2011).

Bozza et al. found that upon binding to human and murine cytomegalovirus (MCMV), PTX3 reduced viral entry and infectivity in DC *in vitro*. Consistently, *Ptx3*-deficient mice are more susceptible to MCMV infection than wild type mice and PTX3 protects susceptible BALB/c mice from MCMV primary infection and reactivation *in vivo* (Bozza et al. 2006).

Similarly, upon binding to influenza virus (H3N2), PTX3 inhibits virus-induced hemagglutination and viral neuramidase activity, neutralizes virus infectivity *in vitro* and reduces mortality and viral load *in vivo* in mice (Reading et al. 2008). However, it has been recently reported that both seasonal and pandemic H1N1 influenza A viruses were resistant to the antiviral activity of PTX3 (Job et al. 2010).

We recently investigated the molecular mechanisms underlying the opsonic activity of PTX3. The PTX3 N-terminal domain was responsible for *A. fumigatus* conidia recognition, but the full-length molecule was necessary for the opsonic activity. The PTX3-dependent pathway of enhanced neutrophil phagocytic activity involved complement activation via the alternative pathway; Fcγ receptor (FcγR) IIA/CD32 recognition of PTX3-sensitized conidia and complement receptor 3 (CR3) activation; and CR3 and CD32 localization to the phagocytic cup. Gene targeted mice (ptx3, FcR common γ chain, C3, C1q) validated the *in vivo* relevance of the pathway. In particular, the protective activity of exogenous PTX3 against *A. fumigatus* was abolished in FcR common γ chain-deficient mice. Thus, the opsonic and antifungal activity of PTX3 is at the crossroad between complement, complement receptor 3-, and FcγR-mediated recognition. Because short pentraxins (e.g. C-reactive protein) interact with complement and FcγR, these results may have gen-

eral significance for the mode of action of these components of the humoral arm of innate immunity (Moalli et al. 2010).

The relevance in humans of data obtained in animals on the role of PTX3 in infections has been demonstrated by Olesen and colleagues who recently analyzed the role of polymorphisms within the PTX3 gene in pulmonary tuberculosis (TBC) and showed that the frequency of specific PTX3 haplotypes is significantly different in TBC patients as compared to healthy individuals (Olesen et al. 2007). In addition, recent observations by Chiarini and colleagues have shown that polymorphisms in the PTX3 gene correlate with the risk of *Pseudomonas aeruginosa* infections in CF patients (Chiarini et al. 2010).

In conclusion, PTX3 is released by PMNs and produced by DCs, neighbouring macrophages, and other cell types upon TLR engagement or pathogen recognition and recognizes microbial moieties, opsonizes fungi, binds selected Gram positive and Gram negative bacteria, viruses and activates complement. Opsonization results in facilitated pathogen recognition (increased phagocytosis and killing) and activation of innate immune cells (increased cytokine and nitric oxide production); moreover, opsonization by PTX3 is likely involved in the activation of an appropriate adaptive immune response (DC maturation and polarization). Again, as discussed above, all these properties suggest that this long pentraxin behaves as a functional ancestor of antibodies.

Data obtained in different models *in vivo* demonstrated that PTX3 is involved in modulating inflammation in sterile conditions, for instance in acute myocardial infarction and atherosclerosis (Norata et al. 2010). We recently demonstrated that PTX3 selectively binds P-selectin via its N-linked glycosidic moiety, inhibiting leukocyte rolling on endothelium (Fig. 1). Moreover, exogenously administered PTX3 and endogenous PTX3 released from hematopoietic cells provide a negative feedback loop that prevents excessive P-selectin-dependent recruitment of neutrophils in a model of acute lung injury (ALI), pleurisy and mesenteric inflammation (Deban et al. 2010). Therefore, these data suggests that PTX3 produced by activated leukocytes might locally dampen neutrophil recruitment and regulate inflammation. In keeping with this, Liu and colleagues suggested that PTX3 plays a protective role in the pathogenesis of acute lung injury (ALI) and the lack of PTX3 may enhance neutrophil recruitment, cell death, and inflammatory responses in the LPS instillation-induced ALI (Han et al. 2010).

4.5 Self versus Modified Self-Discrimination

Apoptosis is the process of controlled cell death that physiologically occurs in embryogenesis, tissue turnover and resolution of inflammation. Rapid and efficient clearance of apoptotic cells by phagocytes is necessary to avoid loss of cell integrity, activation of bystander cells and tissue damage that are induced by the pro-inflammatory contents of dying cells (Jeannin et al. 2008). PRMs can opsonize apoptotic cells and modulate their recognition by phagocytes.

Both PTX3 and the short pentraxins SAP and CRP have been involved in the clearance of apoptotic cells (Fig. 1), however with opposing functional effects (Jeannin et al. 2008). While CRP and SAP opsonize apoptotic cells and promote their clearance, the binding of PTX3 to dying cells results in inhibition of their elimination (Rovere et al. 2000; van Rossum et al. 2004). In fact, the interaction between PTX3 and C1q prevents the binding of C1q to apoptotic cells, thus causing a defective recognition of these cellular targets by phagocytes (Baruah et al. 2006). Moreover, PTX3 inhibits the cross-presentation of apoptotic cell-derived antigens of self, viral or tumoral origin to autoreactive $CD8^+$ T cells (Baruah et al. 2006). It has been reported that opsonization of apoptotic cells by factor H limits the complement-mediated lysis of these cells (Trouw et al. 2008). Through its binding to factor H, PTX3 promotes recruitment of this complement component to the surface of dying cells, therefore playing a role also as a negative modulator of the alternative pathway of complement activation in injured tissues (Deban et al. 2008).

However, endogenous PTX3 translocates to the plasma membrane of late apoptotic neutrophils through a process that involves fusion of granules and apoptotic cell membrane. The translocated PTX3 accumulates in blebs of the plasma membrane, where it acts as an 'eat-me' molecule in promoting rather than inhibiting the clearance of apoptotic neutrophils by phagocytes (Jaillon et al. 2009). Therefore, membrane-associated PTX3 favours phagocytosis of late apoptotic neutrophils, as opposed to the soluble form of PTX3 that inhibits this process, as discussed above.

The apparent contradiction between the two opposing functional effects of PTX3 might be resolved if the context of the protein production is taken into account. In fact, on one hand cell-bound PTX3 might serve to enhance the elimination of apoptotic cells before loss of their cell-membrane permeability and release of self-antigens and alarmins (Jaillon et al. 2009). On the other, rapid production and secretion of PTX3 during inflammation might avoid capture of apoptotic cells in a pro-inflammatory setting that is likely to trigger an immune response against self antigens (Jeannin et al. 2008). Data obtained *in vivo* actually support the hypothesis that PTX3 plays a protective role in a murine model of systemic lupus erythematosus (Lech et al. 2011).

4.6 PTX3 Role in Tissue Remodelling

In addition to the biological activities that PTX3 exerts as a PRM of the innate immune system, new functional roles have been described for this long pentraxin in processes of tissue remodelling, including extracellular matrix deposition, angiogenesis and restenosis.

Mammalian ovulation is a highly regulated, inflammation-like process that involves extensive tissue remodelling. *Ptx3*$^{-/-}$ mice generated by homologous recombination display a severe deficiency in female fertility (Varani et al. 2002). This has been attributed to defective assembly of the viscoelastic hyaluronan (HA)-rich matrix that forms around the oocyte in the preovulatory follicle (namely, the

cumulus oophorus complex, COC), where PTX3 is produced by cumulus cells and localizes within the matrix (Salustri et al. 2004). Besides PTX3 other molecules are required for effective incorporation of HA into the cumulus matrix, including the HA-binding proteins TSG-6 and the serum proteoglycan IαI (Zhuo et al. 2001; Fulop et al. 2003). Current literature supports the hypothesis that heavy chains (HCs) from IαI become covalently attached to HA (i.e. to form HC–HA complexes) through reactions involving TSG-6, which acts as both a cofactor and catalyst (Day and de la Motte 2005; Rugg et al. 2005). The resulting HC–HA complexes are believed to be crosslinked by PTX3, where the long pentraxin can establish multiple contacts to HCs, thus providing structural integrity to the cumulus matrix (Scarchilli et al. 2007; Inforzato et al. 2008)(Fig. 1). The finding that PTX3 is an essential component of the cumulus oophorus extracellular matrix raises the possibility that this protein might have a similar localization and structural function in other HA-enriched inflammatory tissues, such as in rheumatoid arthritis, where TSG-6 and IαI are also present (Day and de la Motte 2005). This hypothesis is corroborated by the observation that TSG-6 and PTX3 colocalize in the inflammatory infiltrates and endothelial cells of inflamed tissues (Maina et al. 2009).

PTX3 recognizes with high affinity and specificity FGF2 (Fig. 1), a major angiogenic inducer that promotes cell proliferation, chemotaxis, and protease production in cultured endothelial cells (ECs) and neovascularization *in vivo* during wound healing, inflammation, tumour growth and atherosclerosis (Presta et al. 2007). Both proteins are produced by elements of the vessel wall (i.e. ECs and smooth muscle cells, SMCs) during inflammation (Rusnati et al. 2004; Camozzi et al. 2005). PTX3 binding to FGF2 results in inhibition of the FGF2-dependent EC proliferation *in vitro* and angiogenesis *in vivo* and of SMCs activation and proliferation, providing a mechanism for tuning processes of neo-vascularization and restenosis after angioplasty (Rusnati et al. 2004; Presta et al. 2007).

4.7 Diagnostic and Prognostic Potential of PTX3 in Infectious Disorders

CRP has been extensively used clinically for over 75 years as a nonspecific systemic marker of infection, inflammation, and tissue damage. Given the rapid production of PTX3 during inflammation, PTX3 is a candidate marker for inflammatory, infectious, and cardiovascular pathologies (reviewed in Mantovani et al. 2008; Norata et al. 2010). PTX3 behaves as an acute phase protein since its blood levels, low in normal conditions (about 25 ng/ml in the mouse, <2 ng/ml in humans), increase rapidly (with a maximum at 6–8 h) and dramatically (200–800 ng/ml) during endotoxic shock, sepsis and other inflammatory and infectious conditions.

Increased levels of PTX3 have been observed in diverse infectious disorders including sepsis and septic shock, meningococcal disease, tuberculosis and dengue infection (Azzurri et al. 2005; Mairuhu et al. 2005; Sprong et al. 2009; Mauri et al. 2010). Pulmonary tuberculosis patients were reported to present higher levels of

plasma PTX3 as compared to healthy controls. Moreover, at the end of treatment, cured patients showed reduction of PTX3 titers whereas the protein level increased further in patients with treatment failure (Azzurri et al. 2005). High concentrations of PTX3 were also found in dengue virus patients, where the highest titers were observed within 7 days of onset of symptoms. Interestingly, PTX3 levels were strikingly higher than those of CRP levels, suggesting that PTX3 might be a better marker of infection than C-reactive protein in dengue (Mairuhu et al. 2005). High plasma concentrations of PTX3 were seen at admission in patients with meningococcal disease, where the protein proved to be an early indicator of shock in patients with severe disease, despite its levels did not correlate with severity of the pathology (Mauri et al. 2010). In patients with severe sepsis or septic shock, high levels of plasma PTX3 over the first five days from admission have been associated with disease severity, secondary organ failures and mortality. Moreover, PTX3 levels were shown to better correlate with severity of the disease and organ dysfunctions than other measured mediators (i.e. TNFα, IL-6 and CRP) (Sprong et al. 2009).

The concentration of circulating PTX3 increases during pregnancy, a condition that has been associated with inflammation. Higher maternal PTX3 levels have been observed in pregnancies complicated by preeclampsia, which represents the clinical manifestation of an endothelial dysfunction that is part of an excessive maternal inflammatory response to pregnancy (Cetin et al. 2006; Rovere-Querini et al. 2006). PTX3 plasma and vaginal levels increase also during pregnancy complicated by spontaneous preterm delivery, in particular in cases of placenta vasculopathy (Assi et al. 2007). Furthermore, recent studies have reported the presence of PTX3 in the amniotic fluid and elevated protein concentrations in intra-amniotic inflammation and infection (Kacerovsky et al. 2010; Cruciani et al. 2010). In contrast, the increased titres of maternal plasma PTX3 observed during labor are not related to either intra-amniotic inflammation or infection (Cruciani et al. 2010).

Collectively, these studies demonstrate that measurement of PTX3 may help monitoring disease activity, efficacy of therapy and might have a prognostic value.

5 Concluding Remarks

Pentraxins are essential components of the humoral arm of innate immunity; as such, they participate to recognition of and response to pathogens and tissue damage, in coordination with the cellular arm. The short pentraxin CRP was the first identified soluble pattern recognition molecule. Yet, in spite of its widespread use as a diagnostic tool in the clinic, its *in vivo* function has not been unambiguously defined, mostly due to the diverging regulation of its expression in the evolution from mouse to man. In contrast, gene targeting of the prototypic, evolutionarily conserved, long pentraxin PTX3 has unequivocally defined the role of this molecule in innate immunity and inflammation. Recent literature has further clarified the structure, regulation, microbial recognition and *in vivo* functions of PTX3. As a component of the humoral arm of innate immunity, PTX3 plays a similar role to that

exerted by antibodies in adaptive immunity, including complement activation, opsonization and glycosylation-dependent regulation of inflammation. Pathogen sensing and elimination are major functional roles of PTX3, where this long pentraxin facilitates recognition of microbes and microbial moieties, and promotes activation of innate immune cells. Also, the available evidence suggests that PTX3 not only acts as a PRM, but also plays prominent roles at the crossroad between vascular biology, female fertility and discrimination between self, non-self and modified self. Furthermore, clinical observations point to PTX3 as an early marker of infection and inflammation that rapidly reflects the physiopathological state of tissues and vessel wall.

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Galectins as Pattern Recognition Receptors: Structure, Function, and Evolution

Gerardo R. Vasta

Abstract Galectins constitute an evolutionary conserved family of ß-galactoside-binding proteins, ubiquitous in mammals and other vertebrate taxa, invertebrates, and fungi. Since their discovery in the 1970s, their biological roles, initially understood as limited to recognition of carbohydrate ligands in embryogenesis and development, have expanded in recent years by the discovery of their immunoregulatory activities. A gradual paradigm shift has taken place in the past few years through the recognition that galectins also bind glycans on the surface of potentially pathogenic microbes, and function as recognition and effector factors in innate immunity. Further, an additional level of functional complexity has emerged with the most recent findings that some parasites "subvert" the recognition roles of the vector/host galectins for successful attachment or invasion.

Keywords Pattern recognition receptors • Galectins • ß-Galactoside • Carbohydrate recognition domain • Glycans • Structure • Function • Proto-type • Chimera • Tandem-repeat

1 Introduction

Complex carbohydrate structures encode information that modulates interactions between cells, or cells and the ECM, by specifically binding to carbohydrate-binding proteins such as galectins, formerly known as S-type lectins [Gabius 1997, and references therein]. Galectins constitute an evolutionary conserved family of ß-galactoside-binding proteins, ubiquitous in eukaryotic taxa, including the parazoa (sponges) and both protostome and deuterostome lineages of metazoans, and fungi (Cooper 2002; Vasta et al. 1999). Two properties are required in a protein for its inclusion in the galectin family: (a) a characteristic affinity for ß-galactosides, and

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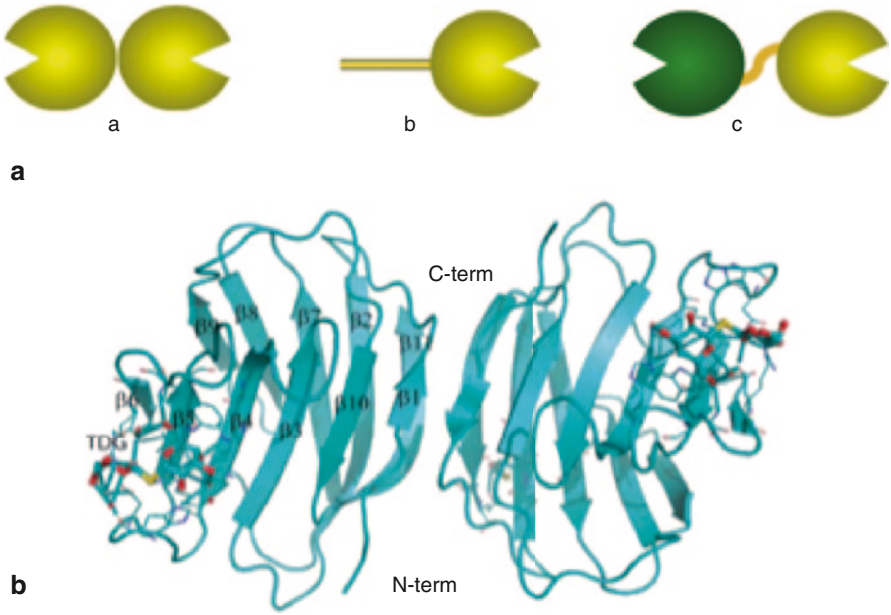

Fig. 1 Galectin types and structure of the galectin-1/LacNAc complex. a Galectins are classified in three types: "proto" (a), "chimera" (b), and "tandem-repeat" (c); **b** The structure of the galectin-1/thiodigalactoside (TDG) complex reveals a dimer, which each globular subunit binding a single oligosaccharide

(b) a conserved carbohydrate recognition domain (CRD) sequence motif. Based on structural features, galectins have been classified in three types: "proto", "chimera", and "tandem-repeat" (TR) (Fig. 1) (Hirabayashi and Kasai 1993). Proto-type galectins (Fig. 1a(a); Fig. 1b) contain one CRD per subunit and are non-covalently linked homodimers. The chimera galectins (Fig 1a(b)) have a C-terminal CRD and an N-terminal domain rich in proline and glycine. In TR galectins (Fig. 1a(c)) two CRDs are joined by a functional linker peptide. Recently, a novel TR-type galectin with four CRDs has been described (Tasumi and Vasta 2007). The dimerization of proto-type galectins is critical for their function in mediating cell–cell or cell–ECM interactions (Gabius 1997), and similar interactions via the N-terminus domain have been proposed for the chimera galectins (Colnot et al. 1997; Rabinovich et al. 2002). Proto- and TR-types comprise several distinct galectin subtypes. Galectin subtypes have been numbered following the order of their discovery, and so far, 15 have been described in mammals. Galectins-1, -2, -5, -7, -10, -11, -13, -14, and -15 are proto-type. Galectin-3 is the only chimera-type. Galectins-4, -6, -8, -9, and -12 are TR-type. Lower vertebrates and invertebrates appear to have a smaller galectin repertoire. Although galectins lack a typical secretion signal peptide, they are present not only in the cytosol and the nucleus, but also in the extracellular space (Cooper 2002) (Fig. 2). From the cytosol, galectins may be targeted for secretion by non-classical mechanisms, possibly by direct translocation across the plasma

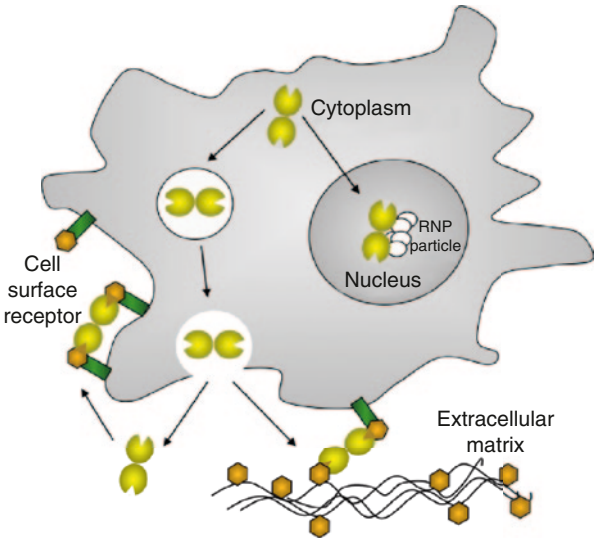

Fig. 2 Subcellular and extracellular localization of galectins. Galectins are synthesized in the cytosol, and can be translocated into the nucleus or secreted to the extracellular space, where they bind to glycans of the extracellular matrix or the cell surface

membrane (Cho and Cummings 1995; Patterson et al. 1997; Sato and Hughes 1994; Cleves et al. 1996).

2 Structure and Biochemical Properties of Galectins

The structure of galectin-1 (Liao et al. 1994; Bianchet et al. 2000) complexed with a di-galactoside (Fig. 1b) shows a jellyroll topology typical of legume lectins. The subunit of galectin is composed of an 11-strand antiparallel ß-sandwich and contains one CRD. The 3-D structure of the galectin–ligand complex allowed us to identify amino acids that participate in interactions with ligands, as well as the position and orientation of the sugar hydroxyls that interact with the amino acids (Liao et al. 1994; Bianchet et al. 2000).

Most galectins are non-glycosylated soluble proteins, although a few recently discovered exceptions have transmembrane domains (Lipkowitz et al. 2004; Gorski et al. 2002). The presence of a galectin fold in the protistan parasite *Toxoplasma gondii*, and galectin-like proteins in the fungus *Coprinopsis cinerea* and in the sponge *Geodia cydonium*reveals the early emergence and structural conservation of galectins in eukaryotic evolution (Saouros et al. 2005; Walser et al. 2005; Stalz et al. 2006). In contrast, galectin-like proteins such as the lens crystallin protein galectin-related inter-fiber protein (GRIFIN) and the galectin-related protein (GRP) (previously HSPC159; hematopoietic stem cell precursor) lack carbohydrate-binding activity, and are considered products of evolutionary co-option (Ogden et al. 1998; Ahmed and Vasta 2008). The primary structures and gene organization of mammalian galectins are substantially conserved. Prior to or during early in chordate

evolution, duplication of a mono-CRD galectin gene would have led to a bi-CRD galectin gene, in which the N- and C-terminal CRDs subsequently diverged into two different subtypes, defined by exon–intron structure (F4-CRD and F3-CRD). All vertebrate single-CRD galectins belong to either the F3- (e.g., gal-1, -2, -3, -5) or F4- (e.g., gal-7, -10, -13, -14) subtype, whereas TR galectins such as gal-4, -6, -8, -9, and -12 contain both F4 and F3 subtypes (Houzelstein et al. 2004).

Galectins are ß-galactoside-binding lectins, and their preferred ligands are N-acetyllactosamine (LacNAc; Galß1,4GlcNAc) and related disaccharides, with dissociation constants in the order of 10^{-5} M (Schwarz et al. 1998; Dam and Brewer 2008). Binding specificities of galectins for lactose (Lac), LacNAc, T-disaccharide (Galß1,3GalNAc) and the human blood group A-tetrasaccharide, together with the presence of amino acid residues that interact with the carbohydrate ligands, have enabled classification of their CRDs into "conserved" or "variable" types (Ahmed and Vasta 1994). The crystal structure of the galectin-1 (conserved type) complexed with a di-galactoside determined at 1.9 Å resolution (Fig. 1b) revealed the galectin structural fold, and allowed the identification of the amino acids involved and the hydroxyl groups of the ligands that participate in protein–carbohydrate interactions (Liao et al. 1994; Bianchet et al. 2000; Lobsanov et al. 1993). The carbohydrate-binding site is formed by three continuous concave strands (ß4–ß6) containing all residues involved in direct interactions with LacNac. Additional interactions involving a water molecule that bridges the nitrogen of the NAc group with His^{52}, Asp^{54}, and Arg^{73} explains the higher affinity of LacNAc over Lac. Unlike galectin-1, galectin-3 has an extended carbohydrate-binding site formed by a cleft open at both ends, in which the LacNAc is positioned in such a way that the reducing end of the LacNAc (GlcNAc) is open to solvent, but the non-reducing moiety (Gal) is in close proximity to residues in the ß3 strand (Seetharaman et al. 1998). The extended binding site leads to increased affinity for glycans with multiple lactosamine units, and with their substitution of the non-reducing terminal galactose moiety with ABH blood group oligosaccharides [Fucα1, 2; GalNAcα1,3(Fucα1,2); and Galα1,3(Fucα1,2)]. For the nematode *Caenorhabditis elegans* 16-kDa galectin (variable type), the shorter length of the loops connecting the three ß4–ß6 strands determines its broader binding specificity for blood group precursor oligosaccharides. Therefore, although galectins are considered a conserved lectin family, most metazoans are endowed of a complex galectin repertoire, with members exhibiting multiple isoforms and more or less subtle variations in carbohydrate specificity, which together with a certain degree of plasticity in sugar binding of each CRD, suggests a substantial diversity in recognition properties (Sparrow et al. 1987; Sato and Hughes 1992; Ahmed et al. 2002; Shoji et al. 2003; Zhou and Cummings 1990; Fang et al. 1993).

Thermodynamic approaches have been used not only to assess the galectins' carbohydrate-binding properties, but also the oligomeric organization of the protein. On microcalorimetric studies, the dissociation constants for the interactions of bovine galectin-1 with the preferred ligands (Lac, N-acetyllactosamine, thiodigalactoside) were in the range of 10^{-5} M, with two binding sites per molecule (Schwarz et al. 1998). Although galectin and legume lectins display a striking similarity in their 3-D structures, the thermal stability of the galectin is different from that of

concanavalin A (Con A). Like Con A, the bovine galectin exists as a tetramer at the denaturation temperature, but, unlike Con A, it does not dissociate upon unfolding (Schwarz et al. 1998).

3 Biological Roles of Galectins in Development and Regulation of Immune Homeostasis

Galectins were initially thought to only bind endogenous ("self") glycans and mediate developmental processes, including cell differentiation and tissue organization, and more recently, regulation of immune homeostasis (Leffler et al. 2004, Yang et al. 2008) (Fig. 3). In the past few years, however, it has become clear that galectins also bind glycans on the surface of potentially pathogenic microbes and parasitic worms, and mediate recognition and effector functions in innate immunity (Sato and Nieminen 2004). Glycans that contain N-acetyllactosamine and polylactosamine chains [(Galβ1,4GlcNAc)n], such as laminin, fibronectin, lysosome-associated membrane proteins, and mucins, are the preferred endogenous ligands for mammalian, bird, and amphibian galectins (Seetharaman et al. 1998; Sparrow et al. 1987; Sato and Hughes 1992; Ahmed et al. 2002; Shoji et al. 2003; Zhou and Cummings 1990; Fang et al. 1993). The biological function of a particular galectin, however, may vary from site to site, depending on the availability of suitable ligands. The binding properties and biological functions of galectins in the oxidative extracellular environment, however, may depend on their immediate binding to ligand, which prevents the oxidation of free cysteine residues, as well as galectin

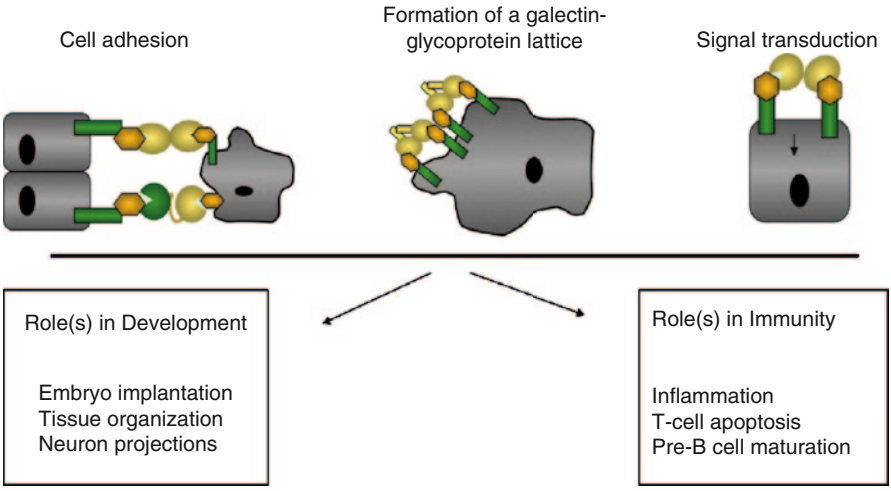

Fig. 3 Biological roles of galectins upon binding to the cell surface. Galectins can bind to glycans on neighboring cells or to cells and the extracellular matrix, leading to cell adhesion, or to the surface of a single cell resulting in the formation of lattices, and the activation of signaling pathways

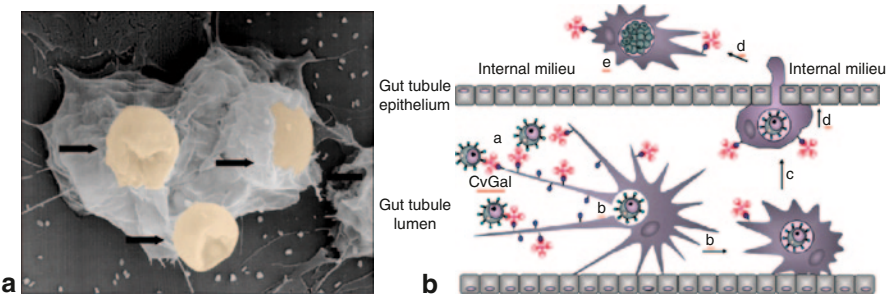

Fig. 4 The role of hemocyte galectins on parasite host invasion. a: SEM of oyster hemocytes in the process of engulfing Perkinsus marinus trophozoites (indicated by *arrows*) *in vitro* (Gauthier and Vasta; 71). **b:** Cartoon of the hypothetical process that takes place in the gut and other epithelial surfaces: The oyster galectin CvGal mediates recognition and phagocytosis of P. marinus trophozoites by hemocytes; the trophozoites survive inside the oyster hemocytes, which migrate from the gut via a trans-epithelia route and are transported to other tissues where the parasite proliferates (Tasumi and Vasta 2007)

susceptibility to proteolysis (Liao et al. 1994; Lobsanov et al. 1993). The binding of galectins to cell surface ß-galactoside-containing glycolipids and glycoproteins can lead to the formation of lattices that cluster these ligands into lipid raft microdomains required for optimal transmission of signals relevant to cell function (Rabinovich et al. 2007b; Brewer et al. 2002; Partridge et al. 2004) (Fig. 4). In solution galectins can form multivalent species in a concentration-dependent equilibrium (Morris et al. 2004). Proto-type galectins associate as non-covalently bound dimers via a hydrophobic interphase, whereas galectin-3 associates via its N-terminal domain to form oligomers that in the presence of multivalent oligosaccharides in solution or at the cell surface display binding cooperativity (Dam and Brewer 2008; Brewer et al. 2002). The bivalent TR-type galectins can recognize different saccharide ligands with a single polypeptide, although they can also form higher order aggregates that enhances their avidity. Galectin-mediated lipid raft assembly may modulate turnover of endocytic receptors, signal transduction pathways leading to T-cell activation and cytokine secretion, or apoptosis, B-cell maturation, activation and tolerance, and neutrophil activation leading to phagocytosis, oxidative burst, and protease and cytokine release. Thus, galectin-glycoprotein lattices at the cell surface have been proposed to function as an "on-an-off switch" that regulates cell proliferation, differentiation and survival, including immune cell responsiveness and tolerance (Dam and Brewer 2008; Brewer et al. 2002).

Since their discovery, galectins have been proposed to participate in embryogenesis, development, and neoplasia. This has been based on their binding to "self" carbohydrate moieties, such as polylactosamine-containing glycans, abundant at the cell surface and the ECM (Fig 3). Chicken galectins have been proposed to participate in myoblast fusion, whereas murine galectin-1 and galectin-3 would have roles in notochord development, somitogenesis, and development of muscle tissue and central nervous system (Cooper et al. 1991; Watt et al. 2004; Georgiadis et al.

2007; Fowlis et al. 1995). Despite the increasing availability of genetically modified mice, however, strains carrying null mutations for some galectins have failed to display overt developmental phenotypes (Colnot et al. 1998; Puche et al. 1996; Colnot et al. 2001). Thus, other genetically tractable model organisms endowed with a less diversified galectin repertoire such as *Drosophila* and zebrafish have become attractive alternatives for these selected galectins, with promising results (Pace et al. 2002; Ahmed et al. 2004).

In the past few years it has been shown that galectins participate in regulation of both innate and adaptive immunity (Vasta 2009; Rabinovich et al. 2002; van Die and Cummings 2010). The recently proposed roles of galectins in immune functions have been further supported by their ability to directly recognize microbial pathogens (Vasta 2009), a property well characterized for other lectin types, such as C- and F-lectins, ficolins, and pentraxins. Although the roles of lectins in non-self recognition are particularly critical in invertebrates, since these organisms lack immunoglobulins and rely solely in innate immune mechanisms for recognition of potential microbial pathogens (Vasta et al. 1999), susceptibility/resistance to several infectious diseases in humans are determined by the presence of certain lectin alleles (Dias-Baruffi et al. 2010). Galectins from both invertebrates and vertebrates recognize a variety of viral and bacterial pathogens and protozoan parasites (Reviewed in Vasta 2009).

Galectins are ubiquitously expressed and distributed in mammalian tissues, including most cells of the innate (dendritic cells, macrophages, mast cells, natural killer cells, gamma/delta T cells, and B-1 cells) and adaptive (activated B and T cells) immune system, and as in other cell types (Stowell et al. 2008; Rabinovich et al. 2007a). Since the early 1990s a growing body of experimental (*in vivo* and *in vitro*) evidence has accumulated to support the roles of galectins expressed by these cells and neighboring stromal cells in the development and regulation of innate and adaptive immunity homeostasis as well as responses to infectious and allergic challenge, and cancer. Galectins released by stromal cells in central compartments contribute to the differentiation of immune cell precursors. Immune challenge and several pathological conditions, may lead to further activation and differentiation of immune cells, and modulate the expression and release of galectins to the extracellular space where they may have autocrine or paracrine effects on immune regulation. Galectins released by immune cells can oligomerize and form lattices at the cell surface leading to activation of transmembrane signaling pathways that modulate immune cell functions, including for example, cell adhesion and migration, T-cell apoptosis, and the Th1/Th2 cytokine balance (Rabinovich et al. 2002, 2007a, 2007b). Further, galectins released into the extracellular environment under abnormal situations may constitute "danger signals", or by exerting their activities on other cells, such as mast cells, induce degranulation and release of factors (e.g., histamine) that represent the "danger signals" leading to activation of immune mechanisms in the absence of antigenic challenge (Sato and Nieminen 2004).

Galectins have diverse effects on cells involved in innate immune responses, including macrophages and dendritic cells, neutrophils, eosinophils, and mast cells. Galectin-1 participates in acute and allergic inflammation and displays anti-inflam-

matory activities by blocking or attenuating signaling events that lead to leukocyte infiltration, migration, and recruitment (Stowell et al. 2008). It also displays various other effects on innate immunity, including cell surface exposure of phosphatidylserine in activated neutrophils, a process that leads to neutrophil removal by phagocytic cells without causing apoptosis, and activation/deactivation of macrophages on a concentration-dependent manner. In contrast to the anti-inflammatory effects of galectin-1, galectin-3 shows pro-inflammatory activity. Galectin-3 is normally expressed in various epithelia and inflammatory cells, such as activated macrophages, dendritic cells, and Kupffer cells, and is upregulated during inflammation, cell proliferation, and cell differentiation. Galectin-3 also exhibits anti-apoptotic activity for macrophages and enhances their interactions with basal lamina glycans, such as laminin and fibronectin. Taken together, these observations strongly suggest that galectin-3 enhances macrophage survival, and positively modulates their recruitment and anti-microbial activity. Galectin-9 is a selective chemoattractant for eosinophils, highly expressed in various tissues of the immune system, such as bone marrow, spleen, thymus, and lymph nodes. Gal-9 released from activated T cells induces chemotaxis, activation, oxidative activity, and degranulation of eosinophils, and monocyte-derived DC maturation (Stowell et al. 2007; Zuñiga et al. 2001; Liu and Hsu 2007; Hirashima et al. 2004).

Concerning adaptive immune responses, galectins have been proposed as regulators of immune cell homeostasis (Rabinovich et al. 2002). Interactions between stromal cells from the bone marrow and thymic compartments and lymphocyte precursors are critical to their development, selection, and further progression to the periphery. In this regard, interactions mediated by galectins can modulate B-cell maturation and differentiation both at the central and peripheral immune compartments (Rossi et al. 2006) Similarly, from their early developmental stages in the thymic compartment to the removal of the mature activated T cells in the periphery, the regulation of T-cell survival is critical to a controlled immune response. Galectin-1 can regulate T-cell proliferation and apoptosis through binding and clustering of lactosamine-rich cell surface glycoconjugates into segregated membrane microdomains (Rabinovich et al. 2007b). Galectin-1 may have pro- or anti-apoptotic effects on T cells depending on the developmental stage and activation status of the cell, and the microenvironment in which the exposure takes place. The effects of galectin-3 in T-cell survival, however, are dependent on whether protein is produced endogenously (anti-apoptotic) or by exogenous exposure (pro-apoptotic) (Liu and Hsu 2007). Galectins also exert regulatory functions in T-cell homeostasis, and signaling cascades triggered by their binding and lattice formation at the T-cell surface has implications in a variety of downstream events that modulate their differentiation, functional activation, and production of pro- and anti-inflammatory cytokines. The effects of galectins on T-cell cytokine synthesis and secretion ultimately determines the Th1/Th2 polarization of the immune response. By reducing IFN-γ and IL-2 and enhancing IL-5, IL-10, and TGF-β production, galectin-1 skews the balance from a Th1- toward a Th2- polarized response, whereas by reducing IL-5 levels, galectin-3 has the opposite effect (Yang et al. 2008). Finally, given the regulatory roles of galectins on cells that mediate both innate and adaptive immune re-

sponses, their effects can be beneficial or detrimental to pathological conditions that have a basis on exacerbated or depressed immune function, such as inflammatory, allergic and autoimmune disorders, and cancer (Yang et al. 2008).

4 Galectins as Pattern Recognition Receptors

Recently, galectins have been discovered to bind glycans on the surface of viruses, bacteria, protista, and fungi (reviewed in Vasta 2009). Thus, the potential role of galectins as pattern recognition receptors (PRRs) has become an area of increased attention. Furthermore, the considerable diversity of the galectin repertoire in each organism and the substantial or subtle variations in the specificity of each galectin towards the target glycans, which are determined by oligosaccharide repeats, branchings or substitutions, suggest that there is extensive diversity and plasticity in the capacity of galectins for non-self recognition. The presence of canonical and extended CRDs, and the carbohydrate-independent binding properties of the N-terminus region of galectin-3, further suggests that galectins have a substantially diversified recognition capacity. Moreover, because galectins from all three types (proto, chimera, or TR) can form oligomers, their multivalent binding properties, including increased avidity, clearly enable galectins to participate effectively both in direct recognition of pathogens and parasites, and downstream processes that lead to modulation of innate and adaptive immune responses. Whether galectin-mediated recognition is an effective defence mechanism with a clear benefit for the host is not entirely clear, except for a few examples. It is noteworthy that a particular glycan on the surface of a microorganism or parasite can be recognized by multiple galectins, and that the outcome of the interaction differs considerably depending on the galectin type involved and the concentration of the galectin in a particular cell surface or extracellular microenvironment. This, in turn, determines the level of oligomerization and cooperative binding to ligand, and the potentially antagonistic or synergistic activation of pathogen signaling pathways (e.g., modulation of immune activation, or cytokine production and secretion) (Rabinovich et al. 2007b).

5 Some Microbial Pathogens and Parasites Subvert the Role of Galectins as PRRs

In some cases, the microbe's recognition by the vector or host galectins promote its adhesion, host cell entry, or infection persistence, in addition to modulating the host's immune responses. Thus, these pathogens and parasites would "subvert" the roles of host or vector galectins as PRRs, to attach to or gain entry into their cells. This is clearly illustrated by the participation of galectin interactions in the infection mechanisms of HIV. In contrast to the inhibitory role of galectin-1 in paramixovi-

rus-mediated cell fusion, galectin-1, which is abundant in organs that represent major reservoirs for HIV-1, such as the thymus and lymph nodes, promotes infection by HIV-1 by facilitating viral attachment to CD4 receptor, and increasing infection efficiency (Ouellet et al. 2005; Mercier et al. 2008). Recent studies showed that galectin-1 enhances HIV adsorption kinetics on monocyte-derived host macrophages, which facilitates HIV-1 infectivity by shortening the time required to establish an infection. Further, galectin-1 would also function as a soluble scavenger receptor and enhance the uptake of the virus by macrophages, which together with evidence that galectin-1 is present in the ejaculate and the heads and tails of late spermatids, led to extend the proposal that galectin-1 may also facilitate sexual transmission of HIV-1 (Mercier et al. 2008). This would take place through enhancement of viral adsorption kinetics on the target cells' surface by the galectin-1 released by sheared fibroblasts and epithelial cells following sex-related micro-abrasions. Gal-3 has no effect on HIV-1 adsorption, entry, or infection, although its expression is upregulated by the HIV Tat protein in several human cell lines, and in cells infected with other retroviruses, suggesting that it may participate in regulation of antiviral immunity (Fogel et al. 1999; Schroder et al. 1995; Hsu et al. 1996). This underscores the relevance of the subtle differences in galectin specificity and affinity that may determine very different recognition and effector outcomes. It is noteworthy that HIV also uses recognition by DC-SIGN, a C-type lectin, to enter dendritic cells, thereby underscoring the multiple adaptations of the viral glycome for host infection (Ouellet et al. 2005; Mercier et al. 2008).

Leishmania species, which spend part of their life cycle in phlebotomine sandflies that constitute vectors for transmission to the vertebrate hosts, are also illustrative examples. Upon the sandfly feeding on blood from an infected host, the ingested amastigotes mature into promastigotes, which attach to the insect midgut epithelium to prevent their excretion along with the digested bloodmeal, and undergo numerous divisions before differentiating into free-swimming infective metacyclics (Kamhawi 2006). Although the involvement of the parasite LPG in this interaction had been suspected from prior studies, the specific *Phlebotomus papatasi* sandfly midgut receptor for the procyclic *L. major* LPG was identified as a 35.4-kDa TR galectin (PpGalec) only expressed by epithelial midgut cells, and upregulated in the blood-feeding females (Kamhawi et al. 2004). Because the binding specificity of PpGalec is restricted to *Leishmania* promastigotes bearing poly-Gal(ß1-3) side chains on their LPG, it was proposed that it is the carbohydrate moiety responsible for specific binding of *L. major* to *P. papatasi* midgut linings. The assembly of polygalactose epitopes is downregulated during *L. major* metacyclogenesis, and thus, unable to bind to rPpGalec the free-swimming infective metacyclic promastigotes are released from the midgut for transmission from the sandfly to the mammalian host (Kamhawi et al. 2004).

The protozoan parasite *Perkinsus marinus* is a facultative intracellular parasite that causes "Dermo" disease in the eastern oyster *Crassostrea virginica*, and is responsible for catastrophic damage to shellfisheries and the estuarine environment in North America (Harvell et al. 1999). The infection mechanism remains unclear, but it is likely that while filter feeding, the healthy oysters ingest *P. marinus* trophozoites

released to the water column by the infected neighboring individuals. Inside oyster phagocytic cells (hemocytes), trophozoites resist oxidative killing, proliferate, and spread throughout the host. It was recently discovered that oyster hemocytes recognize *P. marinus* via a novel galectin (CvGal) that displays four canonical galectin CRDs, a domain organization unlike any of the known galectin types (Tasumi and Vasta 2007). Two amino acid residues (His^{53} and Asp^{55}) that interact with the NAc group via a water molecule are missing in all four CvGal CRDs resulting in broader carbohydrate specificity. CvGal is present in the cytoplasm of circulating granulocytes, and upon their attachment and spreading it is translocated to the periphery, secreted, and binds to the cell surface. The remaining galectin is released to the extracellular environment, where it may bind to all other circulating (non-activated) granulocytes and hyalinocytes. The most surprising observation, however, was that the soluble CvGal also binds in a carbohydrate-specific manner to a wide variety of microorganisms, phytoplankton components, and preferentially, to *Perkinsus* spp trophozoites, suggesting a direct role in recognition and opsonization of potential microbial pathogens, as well as algal food. The partial inhibition of phagocytosis of *P. marinus* trophozoites by pre-treatment of hemocytes with anti-CvGal revealed that the hemocyte surface-associated CvGal is a phagocytosis receptor for *P. marinus*. Thus, *P. marinus* may have evolved to adapt the trophozoite's glycocalyx to be selectively recognized by the oyster hemocyte CvGal, thereby subverting the oyster's innate immune/feeding recognition mechanism to gain entry into the host cells (Tasumi and Vasta 2007) (Fig. 4).

A recent study identified galectin-1 as the receptor for the protozoan parasite *T. vaginalis* (Okumura et al. 2008) the causative agent of the most prevalent non-viral sexually transmitted human infection in both women and men. As an obligate extracellular parasite, establishment and persistence of *T. vaginalis* infection requires adherence to the host epithelial cell surface. Like *Leishmania* spp, *T. vaginalis* displays a surface LPG rich in galactose and N-acetyl glucosamine, which is recognized in a carbohydrate-dependent manner by galectin-1 expressed by the epithelial cells in the cervical linings, as well as placenta, prostate, endometrial, and decidual tissue, also colonized by the parasite (Okumura et al. 2008).

6 Conclusions

Recent studies clearly indicate that galectins can function as PRRs that target lactosamine-containing oligosaccharides on the surface of virus, bacteria, protista, and helminth pathogens and parasites. A perplexing paradox arises, however, by the fact that galectins also recognize lactosamine-containing glycans on the cell surface of the host for development and regulation of immune homeostasis. According to the Medzhitov and Janeway model (2002) for non-self recognition, PRRs recognize pathogens via highly conserved microbial surface molecules of wide distribution such as lipopolysaccharide or peptidoglycan (pathogen-associated molecular patterns [PAMPs]), which are absent in the host. Hence, this would not rigorously apply

to galectins, which apparently bind the same self/non-self molecular pattern. This paradox underscores first, an oversimplification in the use of the PRR/PAMP terminology, which although it has been useful and is currently widespread, it should be use with great caution.Second, and most importantly, it reveals the significant gaps in our knowledge about the actual diversity in recognition of the host galectin repertoire, and the dynamic and mechanistic aspects of the subcellular compartamentalization and secretion of its components, as well as the detailed structural and biophysical aspects of their interactions with the microbial carbohydrate moieties. The microbial and host glycomes and their receptors continuously evolve to escape mutual recognition, a process known as the "Red Queen effect" (Varki 2006), by which the microbe avoids recognition by the host innate immune receptors (PRRs) and, the host by the microbial colonization factors (agglutinins, adhesins, and lectins). Given the key roles played by galectins in host development and immunoregulation by the recognition of "self" lactosamine moieties, strong functional constraints would prevent galectins from dramatic evolutionary changes in carbohydrate specificity, which is to some extent supported by the apparent structural conservation within this lectin family. Further, with the current evidence about how pathogens and parasites, which display a remarkable evolutionary plasticity, efficiently subvert the roles of galectins to attach or gain entrance into the host cells, it seems more plausible that instead of avoiding recognition by the host, they would have evolved their glycocomes to mimic their hosts' in a "Trojan horse" model (Tasumi and Vasta 2007), and rely on the host's self-recognition molecules such as galectins for attachment to the vector or host invasion. It is noteworthy that most of (if not all) these pathogens and parasites are endowed with diverse and powerful mechanisms to evade intracellular killing by the host, and/or down-regulate downstream immune responses. The complex strategies developed by microbial pathogens to successfully colonize, enter, proliferate, and disseminate within and among their vectors or hosts, are the products of strong selective pressures that have led to adaptations that ensure their survival in the most hostile environment of all, and thus represent a significant challenge for the development of novel strategies for intervention in human disease.

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The Role of Innate Immunity in Trafficking of Hematopoietic Stem Cells—An Emerging Link Between Activation of Complement Cascade and Chemotactic Gradients of Bioactive Sphingolipids

Mariusz Z. Ratajczak, ChiHwa Kim, Wan Wu, Dong Myung Shin, Ewa Bryndza, Magda Kucia and Janina Ratajczak

Abstract Hematopoietic stem and progenitor cells (HSPCs) circulate under steady-state conditions at detectable levels in peripheral blood (PB). The phenomenon of enforced release of HSPCs from BM into PB is called mobilization and may be envisioned as a danger-sensing response mechanism triggered by hypoxia or mechanical- or infection-induced tissue damage and is a part of stress response. It is unquestionable that the a-chemokine stromal derived factor-1 (SDF-1)—CXCR4 axis plays crucial role in retention of HSPCs in BM. However, all factors that direct mobilization of HSPCs into PB and homing back to the BM or their allocation to damaged organs are not characterized very well. In this chapter we will present mounting evidence that elements of innate immunity such as complement cascade (CC) cleavage fragments (e.g., C3a and C5a), granulocytes, generation of membrane attack complex (MAC) together with sphingosine-1 phosphate (S1P) orchestrate HSPC mobilization. On other hand some other bioactive lipids e.g., ceramide-1-phosphate (C1P) that is released from damaged/"leaky" cells in BM after myeloablative conditioning for transplant may play an opposite important role in homing of HSPCs to BM. Finally, the chemotactic activity of all chemoattractants for HSPCs including SDF-1, S1P and C1P is enhanced in presence of CC cleavage fragments (e.g., C3a) and MAC that is a final product of CC activation.

Keywords Hematopoietic stem/progenitor cells • Soluble MAC • Homing • Mobilization • Sphingosine-1 phosphate • Ceramide-1 phosphate • SDF-1

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1 Introduction

Hematopoietic stem/progenitor cells (HSPCs) circulate in peripheral blood (PB) and lymph during development, moving between major anatomical sites where hematopoiesis is initiated and/or temporarily active (e.g., blood islands in yolk sac, aorta endothelium, fetal liver) before they finally reach their final destination, which is the bone marrow (BM) (Baron 2003; Weissman et al. 1978). Later in adult life, a small percentage of HSPCs is continuously released from BM niches into the PB, which may be envisioned as a highway by which HSPCs relocate between distant stem cell niches in order to keep the total pool of BM stem cells in balance.

It has been proposed a concept of "tug of war" for chemotactic stromal derived factor-1 (SDF-1) gradient between BM and PB that decides if cells will be released/mobilized from BM into PB or home back from PB to BM microenvironment (Fig. 1a). SDF-1 which binds to the Gαi-protein coupled, seven-transmembrane-

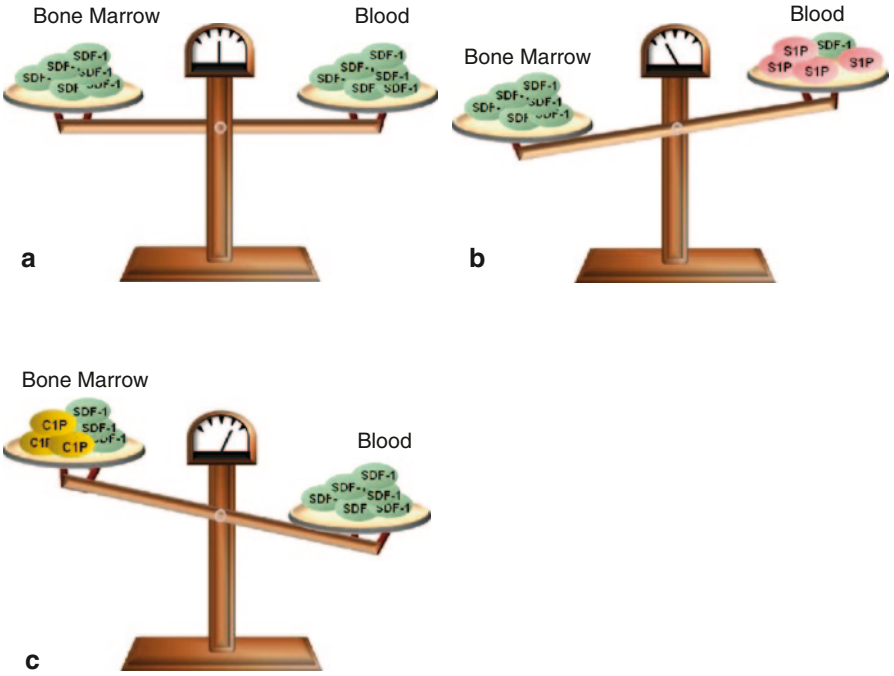

Fig. 1 Concept of chemotactic "tug of war" gradient between BM and PB to explain mobilization and homing of HSPCs. a It has been postulated that SDF-1 gradient between BM and PB regulates trafficking of HSPCs (homing vs. mobilization). In steady state conditions this gradient should be in balance. **b** A proposed regulation of homing gradient for HSPCs into BM by upregulation of concentration of C1P in BM microenvironment damaged by myeloablative therapy. **c** A proposed regulation of mobilizing gradient for HSPCs in PB by increase of plasma S1P level. Of note, what is not shown at this Figure, in addition to upregulation of bioactive lipids both during mobilization and homing, also some other "priming" molecules related to CC activation (e.g., C3a or MAC) as well as granulocyte derived cationic peptides (e.g., cathelicidin and β2-defensin) may sensitize responsiveness of HSPCs to SDF-1 and thus be involved in trafficking of HSPCs

spanning CXCR4 receptor expressed on HSPCs, plays an unquestioned role in developmental migration of HSPCs during embryogenesis and their subsequent retention in the BM (Morrison et al. 1995; Ara et al. 2003). However, changes in the SDF-1 gradient between BM and PB do not always support its having a crucial role as chemoattractant present in PB that directs egress/mobilization of HSPCs or the only chemoattractant for homing of HSPCs back into BM.

For example, as demonstrated by others (Hanel et al. 2007; Seitz et al. 2005) and us (Ratajczak et al. 2010; Marquez-Curtis et al. 2010) the plasma SDF-1 level does not always correlate with mobilization of HSPCs. On the other hand, there is increasing doubt about an exclusive role for SDF-1 in homing of HSPCs into BM. This is based on evidence that (a) $CXCR4^{-/-}$ fetal liver HSPCs may home to BM in an SDF-1-independent manner (Ma et al. 1999), (b) homing of murine HSPCs made refractory to SDF-1 by incubation and coinjection with a CXCR4 receptor antagonist (e.g., bicyclam—AMD3100) is normal or only mildly reduced (Christopherson II et al. 2004), and finally (c) HSPCs in which CXCR4 has been knocked down by means of an SDF-1 intrakine strategy also engraft in lethally irradiated recipients (Onai et al. 2000). All this strongly suggests the existence of other factors beside SDF-1 that are involved in the mobilization and homing of HSPCs. Moreover, SDF-1 that is a well-known potent chemoattractant for HSPCs is as a peptide highly susceptible to degradation by proteases that are elevated, for example, in PB during stem cell mobilization or in the BM microenvironment after myeloablative conditioning for transplantation. To support this by employing sensitive ELISA measurement we did not observe significant increases in SDF-1 plasma level during mobilization (Levesque et al. 2003a) and even observed a decrease of SDF-1 level in murine BM after myeloablative conditioning for transplantation (Kim et al. 2010a, b).

Based on this we become interested in other potential factors that in addition to SDF-1 could chemoattract HSPCs and thus play a role in their mobilization and homing. Interestingly we noticed that both PB plasma as well as media conditioned by BM cells isolated from lethally irradiated mice that show chemotactic activity against HSPCs are susceptible to charcoal extraction what suggested involvement of bioactive sphingolipids. To support this notion, it is known that sphingolipids, which are important components of cell membranes, give rise to two bioactive derivatives, sphingosine-1 phosphate (S1P) and ceramide-1 phosphate (C1P), with S1P already identified as a chemoattractant for HSPCs (Seitz et al. 2005) and C1P for monocytes (Granado et al. 2009).

We noticed that both mobilization of HSPCs as well as myeloablative conditioning for transplantation activate complement cascade (CC) in murine BM and lead to the deposition of C5b–C9 (MAC) in BM microenvironment. Interestingly, activation of CC correlated with increase of BM-level of S1P and C1P (Kim et al. 2010c). We found that while the S1P level increases in PB mainly during mobilization, the C1P concentration in BM microenvironment increases after myeloablative conditioning for transplantation. Based on these findings, we propose a new paradigm in which the S1P:C1P ratio plays an important role in mobilization and homing of HSPCs. While S1P is a major chemoattractant that directs egress of HSPCs from BM into PB (Fig. 1c), C1P is released from damaged cells in BM after myeloablative conditioning and together with SDF-1 creates a homing gradient for circulating HSPCs (Fig. 1b).

We also postulate that the S1P:C1P ratio plays a more universal role in trafficking of stem cells and is involved in regulating migration of circulating mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs), and very small embryonic-like (VSEL) stem cells (Luster et al. 2005; Ceradini et al. 2004; Kucia et al. 2008; Massberg et al. 2007). Accordingly, while S1P plays a role in egress of these cells into PB, C1P released from damaged cells (e.g., in infarcted myocardium or brain tissue after stroke) may chemoattract circulating stem cells for potential organ repair.

2 Stem Cell Mobilization

Pharmacological mobilization has been exploited in hematological transplantology as a means to obtain HSPCs for hematopoietic reconstitution. HSPCs circulating in PB are currently a preferred source of stem cells for transplantation, because they are easily accessible and—what is important from a clinical point of view—they also engraft faster after transplantation than HSPCs harvested from the BM under steady-state conditions (Bonig et al. 2007). The most important mobilizing drugs currently employed in the clinic and experimental settings are (a) cytokines (e.g., granulocyte colony stimulating factor; G-CSF), (b) cytostatics (e.g., cyclophosphamide), (c) CXCR4- or VLA-4-blocking molecules (AMD3100 or BIO4860, respectively), and (d) certain chemokines [e.g., the growth-related oncogene protein-beta (Gro-β)] (Slavin et al. 1989; Levesque et al. 2001, 2003a; Taichman 2005; Pelus et al. 2004; Ramirez et al. 2009; King et al. 2001).

2.1 Stem Cell Mobilization as Part of Stress Response

The number of circulating HSPCs increases in PB in response to (a) systemic or local inflammation, (b) strenuous exercise, (c) stress, (d) tissue/organ injury, and (e) pharmacological agents (Simón et al. 2009; Möbius-Winkler et al. 2009; Wojakowski et al. 2009; Paczkowska et al. 2009; Kucia et al. 2006; Slavin et al. 1989). Furthermore, in the process known as "pharmacological stem cell mobilization" the number of HSPCs in PB may increase up to 100-fold after administration of agents that induce their forced egress into PB.

Evidence is accumulating that mobilization varies with the mechanism that triggers or initiates it including (a) systemic inflammation, (b) tissue/organ injury, or (c) pharmacological intervention. Moreover, every mobilizing pharmacological drug may trigger mobilization by employing overlapping, yet different, mechanisms involving, for example, cytokines, chemokines, or small-molecule antagonists of BM-homing receptors. Overall, the mobilization process has been postulated to be directed by (a) a decrease in activity of SDF-1–CXCR4 axis and VLA-4 integrin–VCAM-1 interactions in BM (e.g., due to release of proteolytic enzymes or af-

ter molecular blockage after administration of small molecular antagonists against CXCR4 or VLA-4), (b) release of neurotransmitters from the synapses of the nerves that innervate BM that stimulate dopamine and β2-adrenergic receptors, (c) activation of the coagulation cascade (e.g., release of uPAR), and finally, as recently postulated, (d) activation of the CC (Levesque et al. 2001, 2003a; Lee et al. 2010; Ramirez et al. 2009; Katayama et al. 2006; Spiegel et al. 2007; Topcuoglu et al. 2004; Jalili et al. 2010a). In support of this latter mechanism, it has been observed that mice that do not activate the distal part of the CC display a profound defect in mobilization of HSPCs (Lee et al. 2009b, 2010; Ratajczak et al. 2006; Reca et al. 2007; Jalili et al. 2010b). Moreover, it is also evident that the CC becomes activated in all mechanisms leading to mobilization of HSPCs (e.g., systemic inflammation, organ injury, as well as administration of mobilizing drugs).

2.2 Activation of Complement Cascade (CC)

It should not be surprising that mobilization/circulation of stem cells is directed by the CC as an important and evolutionarily conserved regulatory mechanism for sensing and responding to inflammation and organ injury. Since the CC is activated as a result of (a) inflammation, (b) release of CC activating factors from damaged tissues, or (c) strenuous exercise as a result of hypoxia, the release of stem cells into circulation could be envisioned as part of CC-mediated immune surveillance and response to inflammation and organ/tissue damage. Work from our laboratory demonstrates that CC activation in BM is triggered by several mobilizing agents, including granulocyte colony stimulating factor (G-CSF), mobilizing polysaccharides like zymosan, as well as CXCR4 receptor antagonist—AMD3100 (Lee et al. 2009b, 2010; Reca et al. 2007). During CC activation, several bioactive cleavage fragments are released (e.g., C3a, $_{desArg}$C3a, C5a, or $_{desArg}$C5a anaphylatoxins) and finally C5b–C9 MAC is generated.

It is well known that CC is activated in BM by different yet overlapping mechanisms, depending on the mobilizing agent employed. For example, our data indicate that CC becomes activated in BM after G-CSF-induced mobilization by natural occurring antibodies (NA-Ig) that bind to the neoepitope, an antigen that becomes exposed in BM tissue damaged (Taichman 2005; Levesque et al. 2003a; Ratajczak et al. 2006), e.g., by the G-CSF-induced proteolytic microenvironment. On the other hand, zymosan activates CC by an alternative pathway. CC may also be activated directly at the C5 level by coagulation-associated proteases, such as thrombin or kallikrein or activated by proteolytic enzymes (e.g., elastase, cathepsin-G, or MMPs) that are released from granulocytes and monocytes after stimulation of these cells by the mobilizing agent (e.g., G-CSF or AMD3100) (Atkinson et al. 2006; Holers 2008; Kemper and Hourcade 2008; Lee et al. 2009b; Thiel 2007; Wagner and Frank 2010; Walport 2001).

The activation of CC during HSPC mobilization was confirmed by (a) ELISA to detect C3a and C5a cleavage fragments in plasma(Lee et al. 2010), (b) immunofluo-

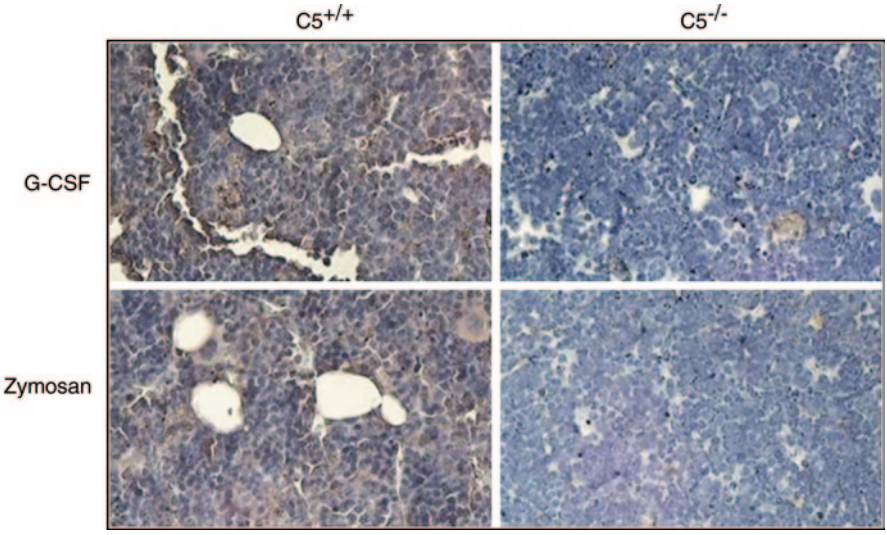

Fig. 2 Activation of complement cascade (CC) in BM during mobilization of HSPCs. MAC deposits are detectable in BM of G-CSF-mobilized wt but not $C5^{-/-}$ mice after G-CSF- and zymosan-induced mobilization. Immunolocalization of each MAC in femur was achieved using the immunoperoxidase system. Following overnight incubation with rabbit anti-murine C5b-9 Ab (1:100, Abcam # ab55811), sections were exposed to HRP-conjugated goat-anti rabbit IgG (1:1000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 60 min. Immunoreactivity was visualized using the liquid DAB Substrate Chromogen System (Sigma-Aldrich Co, St Louis MO, USA). Diffuse MAC deposition is visible in endothelial cells, osteoblasts, and interstitium. Original magnification x200

rescence showing deposition of iC3b on BM stroma and endothelial cells (Ratajczak et al. 2004, 2006; Reca et al. 2007) and (c) histochemical detection of MAC in BM tissue (Ratajczak et al. 2010) (Fig. 2).

Data from our laboratory indicate that CC cleavage fragments affect the retention/mobilization process differently (Ratajczak et al. 2004; Reca et al. 2007; Lee et al. 2009b, 2010; Wysoczynski et al. 2007). That is, while C3 (C3a or $_{desArg}$C3a) cleavage fragments increase retention of HSPCs in BM, C5 (C5a or $_{desArg}$C5a) cleavage fragments enhance their egress into PB. This was evidenced in mobilization studies performed in C3- and C5-deficient animals, which revealed that C3-deficient mice are easy mobilizers (Ratajczak et al. 2004; Reca et al. 2007; Wysoczynski et al. 2007) and C5-deficient mice are poor mobilizers (Lee et al. 2010). This nicely demonstrates that retention/mobilization of HSPCs is tightly regulated at different levels of CC activation.

2.3 Involvement of Granulocytes in Mobilization

It is well known that granulocytes that are also an element of innate immune system, are required for HSPC mobilization and the mobilization process is severely

impaired in animals that lack these cells (Pruijt et al. 2002). To explain this C5a released during CC activation stimulates granulocytes to release proteolytic enzymes into the BM microenvironment that perturb the SDF-1–CXCR4, VLA-4–VCAM-1, and KL–c-kit receptor homing signals between HSPCs and their niches (Lee et al. 2009a; Levesque et al. 2003b). On other hand C5a is a strong chemoattractant for granulocytes and we noticed that granulocytes are the first cells to egress from the BM after administration of mobilizing agent (e.g., G-CSF or AMD3100) (Lee et al. 2010). We hypothesize that in doing so, granulocytes facilitate subsequent egress of HSPCs, which follow "in their footsteps" (Lee et al. 2009b). This proposed mechanism, whereby granulocytes permeabilize the sinusoid endothelial barrier, is supported by our recent transmission electron microscopy (TEM) studies (Lee et al. 2009b). All these phenomena are directly regulated by C5a anaphylatoxin, which binds to the G-protein coupled, seven-transmembrane-spanning C5aR present on granulocytic cells and myeloid precursors (Lee et al. 2009b). At the same time our preliminary data suggest that another C5a/desArgC5a binding receptor C5L2 is not involved in the mobilization process.

2.4 Role of Sphingosine 1-Phosphate (S1P)

It has been proposed that HSPCs are released from the BM into PB in response to increasing plasma SDF-1 level (Hattori et al. 2001; Petit et al. 2002; Sweeney et al. 2002; Wright et al. 2002). However, the exact role of plasma SDF-1 level in the egress of HSPCs is not clear (Ratajczak et al. 2010; Hanel et al. 2007; Seitz et al. 2005). To support this notion we reported that plasma derived from normal and mobilized PB strongly chemoattracts murine HSPCs in SDF-1-independent manner (Ratajczak et al. 2010). Furthermore, this chemotactic effect was not dependent on SDF-1 concentration because: (a) it occurred in the presence of the CXCR4 antagonist AMD3100; (b) it was robust to heat-inactivated plasma; and (c) ELISA studies revealed negligible concentrations of SDF-1 in plasma, which did not correlate with good or poor mobilizer status (Kim et al. 2010a). This last observation is in agreement with a recent reports indicating that plasma SDF-1 levels do not correlate with G-CSF-induced mobilization efficiency in patients (Cecyn et al. 2009; Kozuka et al. 2003). Therefore, we become interested to identify other potential chemoattractants that are responsible for egress of HSPCs into PB and focused on potential role of heat resistant bioactive lipids—in particular in a role of S1P that already had been identified as a potent chemoattractant for HSPCs (Seitz et al. 2005).

Our interest on a potential involvement of S1P was based on fact that activation of CC leads to generation of MAC, that as we recently demonstrated, releases of S1P from erythrocytes (Ratajczak et al. 2010). It is well known that erythrocytes store in blood and may release S1P (Hanel et al. 2007; Ohkawa et al. 2008) that has been reported to posses chemotactic activity against HSPCs as well as regulate the egress of lymphocytes into blood and lymph (Wei et al. 2005; Pappu et al. 2007). These effects of S1P are mediated by five G-protein-coupled seven-transmembrane

span receptors (S1P1–S1P5) (Lynch 2002; Sanchez et al. 2004). While binding of S1P to S1P1 receptor promotes the chemotaxis of $CD34^+$ cells (Seitz et al. 2005), activation of the S1P2 receptor has an opposite effect.

We observed that under steady-state conditions, S1P that is present in PB is a major chemoattractant for BM-residing HSPCs and its concentration and PB gradient increase during mobilization. Furthermore, we noticed that S1P strongly chemoattracts HSPCs that reside in BM (Fig. 3a) and loses this activity against HSPCs already mobilized into PB (Fig. 3b) or umbilical cord blood (UCB) (Fig. 3c). This could be explained by desensitization of S1P binding receptors by circulating S1P released during stem cell mobilization or process of delivery.

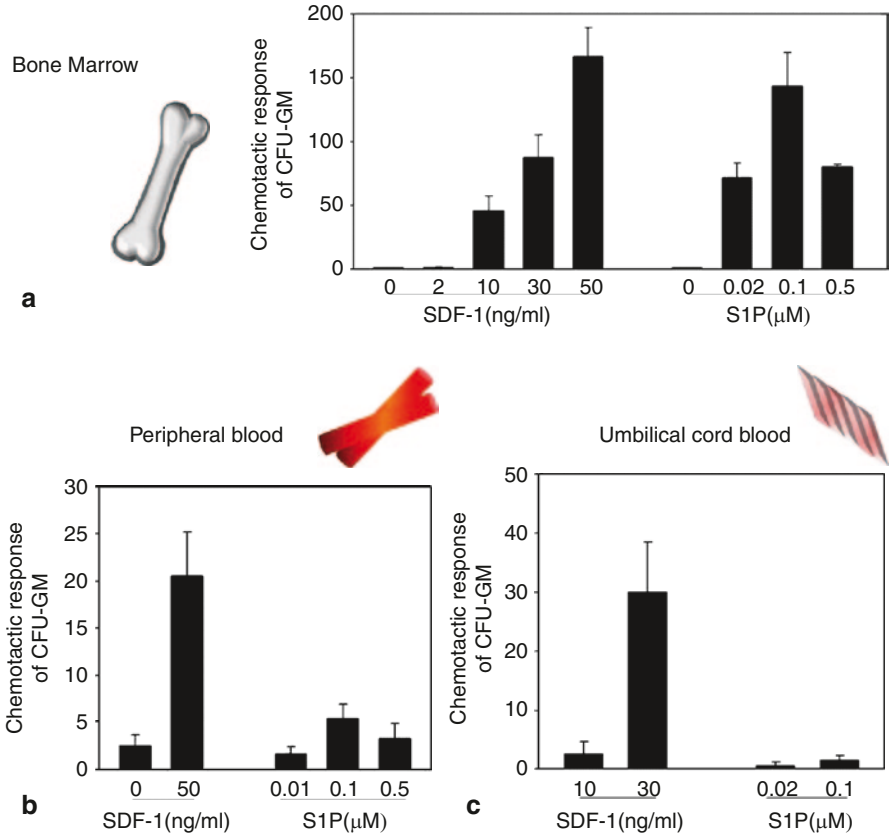

Fig. 3 Differences in responsiveness of BM-, PB- and UCB-derived HSPCs to S1P gradient. Mononuclear cells isolated from murine BM (**a**), mobilized murine peripheral blood (**b**), and human umbilical cord blood (**c**), were evaluated for chemotactic activity of CFU-GM to SDF-1 and S1P gradient. However, S1P chemoattract UCB-derived CFU-GM and is weak chemoattractant for murine PB CFU-GM, it strongly chemoattracts BM-residing naïve HSPCs. The data shown represent the combined results from three independent experiments carried out in triplicate per group ($n=9$)

Based on these data, we envision that in the steady-state condition, HSPCs are retained in BM due to active SDF-1–CXCR4/VLA-4–VCAM1 interactions that counteract the continuous chemotactic S1P gradient present in PB. To induce mobilization, a mobilizing agent (e.g., G-CSF) activates CC in BM which subsequently increases the release of proteolytic enzymes from granulocytes which in turn perturb SDF-1–CXCR4/VLA-4–VCAM1 interactions or simply block these axes (e.g., AMD3100 or BIO5192, respectively). Simultaneously, concentration of S1P in plasma increases due to the release of S1P from erythrocytes in a MAC-dependent manner, providing a more optimal gradient for HSPCs (Fig. 1c). Thus S1P accumulation in BM sinusoids, but not changes in plasma SDF-1 levels, are crucial executors of HSPC egress from BM into PB (Fig. 4a).

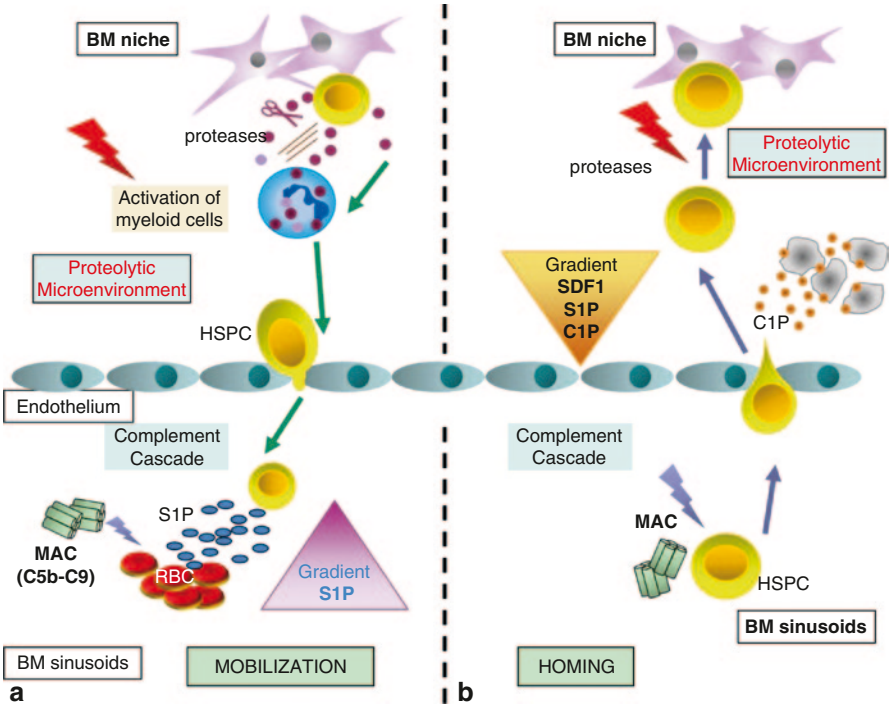

Fig. 4 Proposed scheme depicting a novel proposed role of CC activation and bioactive lipids in mobilization and homing of HSPCs. a G-CSF-directed mobilization induces in BM both proteolytic microenvironment and activates CC. Proteolytic enzymes attenuate interaction of HSPCs with homing signals (e.g., SDF-1-CXCR4 and VCAM-1-VLA-4 axes) and in addition activated CC via MAC releases form erythrocytes that are present in BM sinusoids sphingosine-1 phosphate (S1P). Released from erythrocytes S1P is a major chemoattractant that directs egress of HSPCs into PB. **b** Myeloablative conditioning for transplantation also induces in BM both (a) proteolytic microenvironment and (b) activates CC. Proteolytic microenvironment degradates SDF-1, but at the same time several bioactive lipids including C1P are released from damaged BM cells that chemoattract HSPCs. Furthermore, some elements of activated CC such as for example MAC and C3a increase responsiveness of HSPCs to SDF-1, C1P and S1P homing signals

3 Stem Cell Homing

Homing is a reverse process to mobilization in which HSPCs circulating in PB lodge back to their niches in BM microenvironment. Homing of HSPCs is followed by their engraftment in BM microenvironment that leads to reestablishment of new hematopoiesis.

If HSPCs have to be infused intravenously in large numbers for hematopoietic transplant, an important step that precede this procedure is a myeloblative conditioning by radiochemotherapy to destroy old pathological hematopoiesis and to empty stem cells niches for newly transplanted HSPCs.

3.1 Conditioning for Transplantation Induces Proteolytic Microenvironment in BM

As mentioned in the introduction section evidence accumulated that HSPCs may home into BM in SDF-1 independent manner what suggests that other factors in addition to SDF-1 must be involved in this process (Ma et al. 1999). Furthermore, we noticed that conditioning for transplantation induces highly proteolytic microenvironment in BM and several proteolytic enzymes are released that affect by proteolysis the chemotactic activity of SDF-1 (Kim et al. 2010a, b). Figure 5 shows that SDF-1 loses its chemotactic activity against clonogenic CFU-GM after exposure to

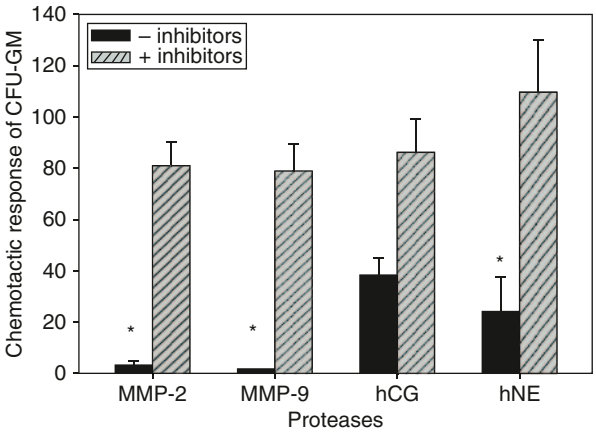

Fig. 5 SDF-1 as peptide is sensitive to proteolytic degradation by proteolytic enzymes. SDF-1 was exposed to MMP-2, MMP-9, cathepsin G (CG) and neutrophil elastase (NE) in absence (–) or presence (+) of specific inhibitors. Subsequently, these preparation of SDF-1 were employed in CFU-GM chemotactic assays. Note decrease in chemotactic responsiveness of CFU-GM to SDF-1 exposed to proteolytic enzymes. *$p<0.001$. The data shown represent the combined results from three independent experiments

different proteolytic enzymes. All this together prompt us to identify new factors that may potentiate or even replace homing functions of SDF-1. Furthermore, since our research also indicated that C3 and C5 deficient mice show delayed recovery of PB hematopoietic parameters after transplantation of HSPCs, we assumed that CC is playing also an important role in homing and engraftment of HSPCs after transplantation.

We found that media conditioned by cells recovered from murine long bones 24 hours after lethal irradiation strongly chemoattract HSPCs in SDF-1-independent manner (Kim et al. 2010a, b). Furthermore, this chemotactic effect was not dependent on SDF-1 because (a) it occurred in the presence of the CXCR4 antagonist AMD3100 and (b) was resistant to heat inactivation (Ratajczak et al. 2010). Based, on this we become again interested in bioactive lipids. While S1P is secreted from activated cells, another sphingolipid derivate—C1P is mainly released from damaged "leaky" cells. We hypothesized that this can occur in BM microenvironment during myeloablative conditioning before transplant by employing lethal irradiation. In fact, we found that C1P similarly as S1P is elevated in conditioned media harvested from BM cells after myeloblative conditioning. We also noticed and reported for a first time that C1P similarly as S1P is a potent chemoattractant for HSPCs and probably involved in SDF-1-independent homing (Fig. 1c) of transplanted HSPCs (Kim et al. 2010a, b).

3.2 Conditioning for Transplantation Activates Complement Cascade (CC) in BM

We noticed that similarly as during mobilization, conditioning for transplantation by lethal irradiation activates CC in BM. The activation of CC during conditioning for transplantation was confirmed by ELISA to detect C3a and C5a cleavage fragments in plasma(Lee et al. 2010) and histochemical detection of MAC in BM tissue (Ratajczak et al. 2010). The importance of CC in stem cell homing/engraftment is supported by fact that C3- and C5-deficient mice show delayed recovery of hematopoietic parameters in PB after transplantation of HSPCs (Reca et al. 2007). We also reported that an important role in this process play C3 soluble cleavage fragments—C3a and $_{desArg}$C3a as well as solid phase iC3b fragment deposited on stroma cells.

To explain why C3 mice poorly engraft, we demonstrated that C3a and $_{desArg}$C3a increase/prime responsiveness of HSPCs to the SDF-1 gradient (Lee et al. 2009a; Ratajczak et al. 2004, 2006; Wysoczynski et al. 2007) that decreases in BM after lethal irradiation. We recently provided a molecular explanation for this intriguing phenomenon based on the observation that actively signaling CXCR4 receptor is associated with lipid rafts (Lee et al. 2009; Ratajczak et al. 2004; Wysoczynski et al. 2005). Lipid rafts are membrane domains rich in sphingolipids and cholesterol, which form a lateral assembly in a saturated glycerophospholipid environment. The raft domains are known to serve as moving platforms on the cell surface and are more ordered and resistant to non-ionic detergents than other areas of the

membrane. These domains are also good sites for crosstalk between various cellular signaling proteins. For example, it has been recently reported that small guanine nucleotide triphosphatases (GTPases) such as Rac-1 and Rac-2, which are crucial for engraftment of hematopoietic cells after transplantation, are associated with lipid rafts on migrating HSPCs (Yang et al. 2001; Gu et al. 2003; Del Pozo et al. 2004; Palazzo et al. 2004). Accordingly, since the CXCR4 receptor is a lipid raft-associated protein, its signaling ability is enhanced if CXCR4 incorporated into membrane lipid rafts where it may better interact with several signaling molecules, including the small GTPase Rac-1 (Gu et al. 2003; Del Pozo et al. 2004). This colocalization of CXCR4 and Rac-1 in lipid rafts facilitates GTP binding/activation of Rac-1.

Thus, generation of C3 cleavage fragments in the BM microenvironment may somehow act as a mechanism to increase responsiveness of HSPCs to the SDF-1 gradient. In C3-deficient mice this phenomenon is attenuated, explaining why these animals are show delayed engraftment. In this context increase in C3a and $C3a_{desArg}$ level in BM after myeloablative conditioning promotes homing of HSPC (Ratajczak et al. 2004; Wysoczynski et al. 2009). In addition as we also demonstrated in the past (Ratajczak et al. 2004, 2006; Reca et al. 2007), C3 cleavage solid phase iC3b fragment that is deposited in the BM microenvironment tethers HSPC and increases their adhesion in BM. All this together explains why C3-deficient mice engraft poorly.

Our recent observation that C5 deficient mice also poorly engraft with HSPCs indicates that MAC C5b-9 (MAC) could be also involved in homing of HSPCs. MAC that is released at final steps of CC activation is present in PB in two forms. The first one lytic MAC forms transmembrane channels that disrupt the phospholipid bilayer of target cells, leading to cell lysis and death. However, another from the sublytic (soluble) MAC may bind to cell membranes, independent of any receptor, does not lyse cells, activates multiple signaling pathways and has wide-range effects on many cell types leading to cellular responses, such as secretion, adherence, aggregation, chemotaxis and even cell division.

Currently we proposed and reported that soluble MAC in increasing homing responses of HSPCs to both SDF-1 and bioactive lipids (Ratajczak et al. 2010) and our preliminary data lend support to this hypothesis depicted at Fig. 3b.

4 Implications of "Priming Phenomenon" that Enhances Responsiveness of CXCR4 Receptor to SDF-1 Gradient

As discussed above our recent data indicate that SDF-1 level does not significantly change in PB during mobilization and even decreases due to proteolytic microenvironment in BM after conditioning for transplantation by lethal irradiation (Levesque et al. 2002, 2003a; Pelus et al. 2004). Moreover, since for biological activity of SDF-1 are crucial few aminoacids located at N-terminus of this peptide (Fig. 5), we noticed that detection of SDF-1 protein in tissues not always correlate with its chemotactic activity. Thus, as demonstrated at Fig. 1a the concept of "tug of

war" SDF-1 gradient between BM and PB does not explain very well homing and mobilization of HSPCs.

On other hand as mentioned in previous paragraph responsiveness of HSPCs to SDF-1 could be enhanced by several molecules(Lee et al. 2009b). In addition to C3 cleavage fragments (C3a and $_{desArg}$C3a) also (a) cathelicidin and β2-defensin—cationic peptides released from activated by C5a granulocytes (Lee et al. 2009b), (b) thrombin (Huber-Lang et al. 2006; Shirvaikar et al. 2010), (c) hyaluronic acid (Avigdor et al. 2004; Shirvaikar et al. 2010), (d) membrane-derived microvesicles (Janowska-Wieczorek et al. 2001), (e) S1P (Ratajczak et al. 2010), and as we recently found MAC increase responsiveness of HSPCs to SDF-1 gradient (Kim et al. 2010c). The basis for this phenomenon as mentioned above is incorporation of CXCR4 into membrane lipid rafts that makes CXCR4 receptor more responsive to SDF-1 gradient. As result of this priming effect the lower doses of SDF-1 become "more biologically significant" in stem cell trafficking.

Further studies are needed to see if in addition to CXCR4 also S1P receptors are lipid raft regulated. More work is also required to identify receptor for C1P. Our initial data indicate that this receptor is expressed on HSPCs and is sensitive to pertussis toxin, what supports that it is probably Gαi-protein coupled type receptor.

5 Conclusions

Our recent data provide more evidence that innate immunity and the CC regulate trafficking of HSPCs by modulating the migratory properties of HSPCs by C3a and sMAC that enhance (a) S1P and C1P level in BM, (b) responsiveness of HSPCs to SDF-1, S1P and C1P and adhesion of HSPCs to BM stroma. Based on this we propose modulation of CC as a novel strategy for controlling both mobilization and homing of HSPCs. This could be achieved for example by exposure of HSPCs before transplantation to some cationic peptides (e.g., C3a or cathelicidin) that enhance responsiveness of these cells to homing factors.

We also propose a new paradigm in which the ratio between bioactive lipids (S1P:C1P) plays an important role in mobilization and homing of HSPCs. Accordingly, S1P and C1P in contrast to SDF-1 are resistant to proteolytic enzyme and while S1P is a major chemoattractant that directs egress of HSPCs from BM into PB, C1P is released from damaged cells in BM after myeloablative conditioning and promotes with SDF-1 homing of circulating HSPCs. We also postulate that the S1P:C1P ratio plays a more universal role and is involved in regulating migration of other types of stem cells, such as circulating MSCs, EPCs, and VSEL stem cells. Similar mechanisms of homing play probably role in recruitment of stem cells in other types of organ injury, e.g., heart infarct or stroke (Fig. 6).

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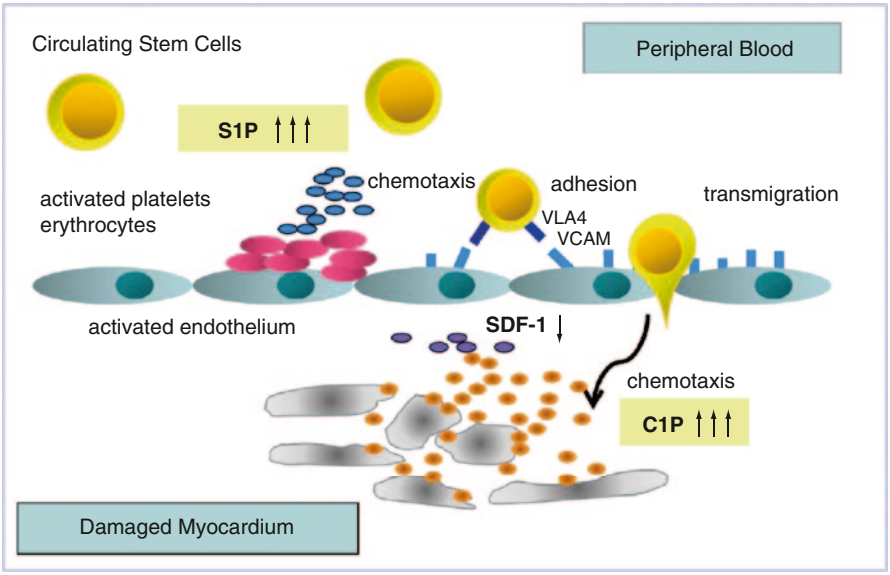

Fig. 6 Interplay of activated CC and release of bioactive lipids may direct homing of circulating stem cells to damaged organs (e.g., myocardium after heart infarct). Damaged cardiomyocytes, endothelial cells and platelets release C1P and S1P. Both bioactive lipids together with SDF-1 create chemotactic gradient for circulating in PB stem cells such as circulating mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs), and very small embryonic-like (VSEL) stem cells (Kucia et al. 2008) for potential repair. Of note bioactive lipids in contrast to SDF-1 are resistant to proteolytic enzymes released in damaged tissues

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Interaction of the Microbiome with the Innate Immune Response in Chronic Wounds

Elizabeth A. Grice and Julia A. Segre

Abstract Microbes colonizing and/or infecting chronic wounds undoubtedly play a major and interactive role in impaired healing, especially in amplifying and perpetuating the host innate immune response. The development of molecular techniques to identify and quantify microbial organisms has revolutionized our view of the microbial world. These less-biased, high throughput methods greatly enable investigations regarding host–microbe interactions in the chronic wound environment. This review focuses on the mounting evidence implicating microbes and excessive inflammation in chronic wounds, as well as the challenges associated with understanding how microbes modulate wound healing and the innate immune response.

Keywords Chronic wounds • Wound healing • Diabetic ulcers • Microbiome • 16S rRNA • Epidermal defense • Antimicrobial peptides • Chronic inflammation

1 Introduction

Our bodies are colonized inside and out by microbes, estimated to exceed the number of eukaryotic cells of our bodies 10-fold. In most cases, these microbes are harmless and provide functions that we as humans have not had to evolve on our own (Gill et al. 2006). Despite constant confrontation with the immune system, commensal microbial populations peacefully coexist with the host for the most part. The immune system strikes a careful balance between tolerance and activation. Commensal microbes occupy a niche, competing with potentially pathogenic organisms for nutrients and space.

The skin is a critical interface between the human body and its external environment, preventing the loss of moisture and barring entry of pathogenic organisms and foreign substances (Segre 2006). The skin is also colonized by myriad microbes

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(the "microbiome"), which in most cases are commensal and cause little harm to the host. However, when the skin barrier is wounded, subcutaneous tissue that is moist, warm, and nutritive is dangerously exposed to the microbial populations colonizing the skin. The presence of microbes rapidly induce the innate immune response, followed by the adaptive immune response, in an effort to combat potential invasion and infection. The immune response must be carefully calibrated to destroy invasive, pathogenic organisms yet avoid an overly exuberant response that harms the host.

This review will describe the mounting evidence suggesting that microbes inhibit wound healing, focusing on genomic approaches of characterizing microbial populations. We will also describe the innate immune response in wound healing, the aberrant innate immune response in chronic wound healing, and how the microbiota may modulate these responses. To develop a complete understanding of wound healing, the interactive nature of the host and colonizing microbiota must be considered.

2 The Burden of Chronic Wounds

Chronic non-healing wounds represent a major health care burden, cause disability, and decrease quality of life. An estimated 15–25% of persons with diabetes will develop foot ulcers (American Diabetes Association [ADA] 1999). One to two percent of the elderly suffer from venous stasis ulcers per year (Margolis et al. 2002). Current direct and indirect costs of chronic wounds are estimated to exceed US $12 billion annually in the United States and this number is expected to rise as the population ages (Bickers et al. 2006). Chronic wounds result in considerable morbidity with prolonged hospitalizations, antibiotic exposure, pain, and restricted mobility. A significant number of patients may require amputation, further worsening morbidity (ADA 1999).

Normal wound repair follows a precisely orchestrated sequence of events of coagulation and hemostasis, inflammation, cell proliferation, cell migration, and tissue remodeling, resulting in rapid closure of the wound within 3–14 days (Fig. 1) (Gurtner et al. 2008; Singer and Clark 1999). Chronic wounds fail to proceed through this series of events and are characterized by persistent inflammation (Loots et al. 1998).

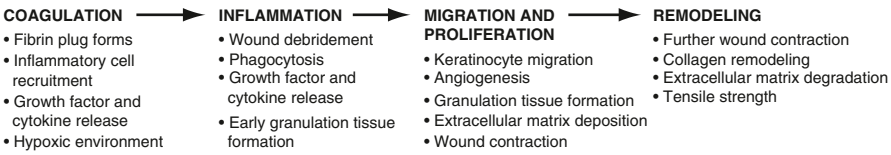

Fig. 1 The phases of wound healing and repair. Though depicted as linear, the stages of wound healing are overlapping. Chronic wounds are often characterized by a persistent inflammatory phase

Most chronic wounds occur in patients with underlying pathology or systemic disease, which includes an aberrant and/or impaired immune response (i.e., diabetes) (Fonder et al. 2008). Ischemic, necrotic, and/or hypoxic tissue provides an ideal environment for the colonization and proliferation of large microbial populations. Microbial colonization and proliferation induce neutrophil and macrophage infiltration of the wound, resulting in subsequent release of damaging free oxygen species, cytotoxic enzymes, and proteases in the wound environment. These molecules destroy cellular components and structural proteins of the extracellular matrix while amplifying persistent inflammation in chronic wounds (Eming et al. 2007).

We postulate that modulating the cycle of inflammation followed by microbial invasion and/or infection is a critical step toward better treatment strategies for chronic wounds. Although there is a general acknowledgment that microbes inhibit the normal wound healing process, their specific role remains unclear. Chronic wounds that fail to progress in healing after 2–4 weeks are commonly treated with systemic and/or topical antibiotics, yet the efficacy of these treatments is uncertain. Unwarranted use of antimicrobials is a major concern as the emergence of antibiotic-resistance bacteria poses a significant threat to public health. Development of better treatment strategies of chronic wounds is dependent on understanding the relationship between the microbiome, the innate immune system, and impaired healing.

3 Surveying the Microbial Diversity of Chronic Wounds

The microflora associated with chronic wounds, as examined by traditional culture-based approaches has been reviewed extensively (Bowler and Davies 1999; Davies et al. 2001; Gardner and Frantz 2008; Martin et al. 2010). The unifying feature among these studies is that a wide variety of bacterial species can be isolated from chronic wounds, but no convincing link between clinical phenotype and microbial colonization has been established. Staphylococci, streptococci, enterococci, and *Pseudomonas* spp., are frequently isolated from chronic wounds. However, many of those species isolated in chronic wounds are also resident commensals living on the skin of healthy individuals (Grice et al. 2009).

A major problem with traditional culture-based approaches is that only a small minority of bacteria are able to thrive in isolation (Dunbar et al. 2002). Culture-based techniques essentially select for lab "weeds", species that flourish under the typical nutritional and physiological conditions employed by diagnostic microbiology laboratories. These are not necessarily the most abundant or influential organisms in the community. Isolation of anaerobes is particularly problematic using routine culture-based approaches (Bowler et al. 2001; Davies et al. 2001). Anaerobic organisms are often slow growing and require special conditions not only for growth but also during sample transport and processing. In the case of chronic wounds, anaerobes are postulated to be particularly pathogenic, both on their own and in synergy with aerobic bacteria (Bowler and Davies 1999).

As such, the development of molecular techniques to identify and quantify microbial organisms has revolutionized our view of the microbial world. Character-

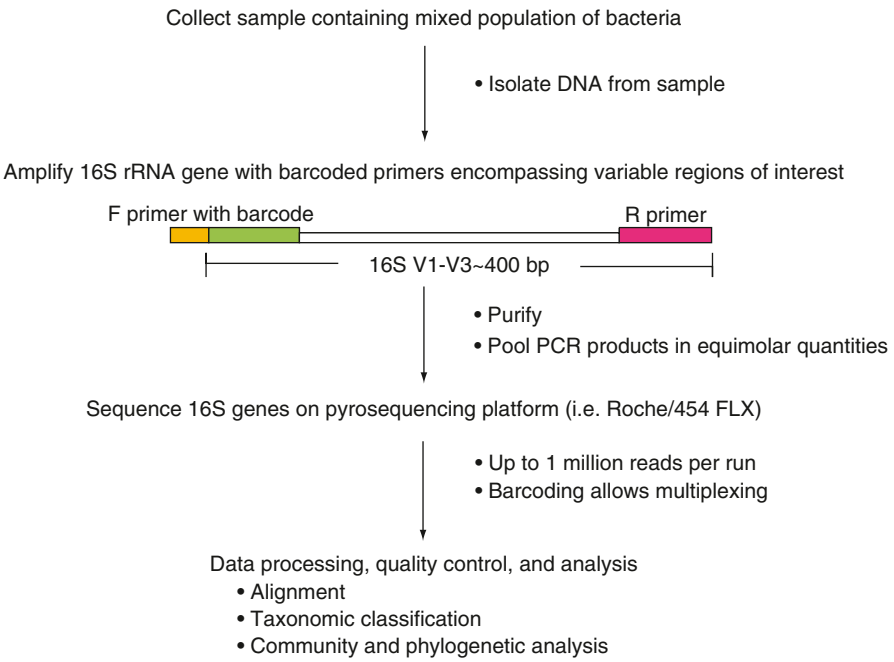

Fig. 2 Example of an experimental and analytical workflow to survey bacterial diversity of chronic wounds using a genomic approach of sequencing 16S ribosomal RNA genes

ization of the bacterial microbiome takes advantage of the 16S ribosomal RNA (rRNA) gene, present in all prokaryotes but not eukaryotes (Fig. 2). The 16S rRNA gene encodes a structurally and functionally essential component of the ribosome. The 16S rRNA gene contains species-specific hypervariable regions, allowing taxonomic classification, and highly conserved regions that act as a molecular clock and a substrate for PCR priming (Hugenholtz and Pace 1996). Following PCR amplification, 16S rRNA genes are sequenced and analyzed (Fig. 2). Classification is enabled by the enormous databases of rRNA gene sequences that have been compiled in an effort to reconstruct the Tree of Life. For example, the Ribosomal Database Project, an online database of rRNA sequences, now contains >600,000 annotated 16S rRNA sequences (Cole et al. 2007). The advent of new sequencing technologies (i.e., 454/Roche pyrosequencing, Illumina, and ABI SOLiD platforms) has massively increased throughput while decreasing the cost of sequencing per base. Importantly, an organism does not need to be cultured to determine its type by 16S rRNA sequencing. Figure 2 outlines the experimental workflow of a typical bacterial microbiome sequencing project. Approaches for analyzing fungal and viral diversity of the human microbiome are currently under development. Fungal diversity of chronic wounds is relatively unexplored but may be a contributing component to wound complications and outcome.

A handful of chronic wound analyses by 16S rRNA sequencing have been performed (Table 1), yet no single organism has been identified in wounds of the same

Table 1 Organisms associated with chronic wounds by 16S rRNA gene sequencing

Organism	Wound type	Respiration mode	Gram stain
Phylum Firmicutes			
Staphylococcus	D, V	FA	+
Enterococcus	D	FA	+
Clostridium	D, V	OA	+
Veillonella	D	OA	−
Peptoniphilus	D, V	OA	+
Streptococcus	D, P	FA	+
Anaerococcus	D, P	OA	+
Dialister	P	OA	−
Finegoldia	D, P	OA	+
Peptostreptococcus	D	OA	+
Phylum Proteobacteria			
Proteus	V	FA	−
Pseudomonas	D, V	A	−
Stenotrophomonas	D, V	A	−
Serratia	V, P	FA	−
Haemophilus	D	A, FA	−
Rhodopseudomonas	D	A	−
Citrobacter	D	FA	−
Sphingomonas	V	A	−
Acinetobacter	V	A	−
Phylum Bacteroidetes			
Bacteroides	D,V	OA	−
Phylum Actinobacteria			
Corynebacteria	D,V	A, FA	+

D diabetic ulcer, *V* venous leg ulcer, *P* pressure ulcer, *FA* facultative anaerobe, *OA* obligate anaerobe, *A* aerobe, + gram positive, − gram negative

etiology (i.e., diabetic foot ulcer or venous leg ulcer). In a 16S gene survey of debridement material from 40 diabetic foot ulcers, the most prevalent bacterial genus, *Corynebacteria*, was found in 75% of samples (Dowd et al. 2008). Other common genera (present in at least 15 of the 40 ulcer samples) were *Bacteroides, Peptoniphilus, Fingoldia*, *Anaerococcus, Streptococcus*, and *Serratia* spp. In a study of 24 chronic wounds of mixed etiology, Price et al. (2009) report *Clostridiales Family XI*, which includes many anaerobes, as being most prevalent and abundant. *Streptococcaceae* spp. were also more abundant among the diabetic ulcers as compared to the venous leg ulcers. In a study of 49 decubitus (pressure) ulcers, the microbial community was found to be highly variable with no clear significance attributed to a single bacterium (Smith et al. 2010).

The above described studies focused on characterizing the bacterial diversity of the wound (determining the relative abundance of organisms) but do not give any indication of total bacterial load. One theory put forth is that wound bioburden and wound outcome are related (Gardner and Frantz 2008). Wound bioburden refers to

three dimensions of wound microbiology: total microbial load, microbial diversity, and presence of pathogenic organisms. While some studies assert that a microbial load $> 10^5$ is related to poor outcome (Robson et al. 1999), others have challenged this, citing interactions between different species of microbes as more important (Bowler 2003; Bowler et al. 2001). Therefore, it may be valuable to develop molecular techniques to better quantify microbial load and analyze this data in conjunction with genomic microbial diversity datasets to better predict wound outcomes.

A persistent problem in wound microbiota studies, both cultivation-based and molecular-based, is the lack of uniformity in sampling method. Some studies utilize debridement or curettage material, which is essentially non-viable tissue, to analyze microbial diversity. This is problematic because non-viable tissue likely supports the growth of a greater number and different diversity of organisms. While punch biopsies of viable wound tissue would likely provide the best representation of wound microbiota, obtaining a punch biopsy is not always feasible. Gardner and colleagues have demonstrated that swab samples obtained by Levine's technique (Levine et al. 1976) provide comparable measures of wound bioburden when compared to punch biopsies of viable wound tissue (Gardner et al. 2006). Levine's technique only samples wound microbiota from viable tissue, obtained by expressing tissue fluid from deep tissue layers.

Another challenge with existing genomic surveys of wound microbiota is the lack of meticulous clinical phenotyping and metadata associated with patient samples. Therefore, rigorous analysis of microbiome datasets stratified according to precise clinical criteria is not possible. For example, blood glucose control in diabetic wounds, wound location and topography, and oxygenation of surrounding tissue are several key factors that likely modulate the microbiota and host response. Duration of wound before sampling, presence of infection, and history of antibiotic treatment also need to be considered. Since the etiologies of chronic wounds (diabetes, pressure, venous disease) are likely different and environmental and genetic factors are difficult to control, analyzing host–microbe interactions of specific wound types will likely prove to be critical to draw meaningful conclusions.

Standardizing genetics and environment can be achieved in animal models of impaired wound healing. While animal models may not fully recapitulate human phenotypes of impaired wound healing, they can provide valuable insights that inform our understanding of human diseases. Furthermore, mechanistic analyses that dissect cause and effect of microbiota on impaired wound healing are only possible in animal models. For example, our group has demonstrated that impaired wound healing in a type-2 diabetic mouse model ($Lepr^{db/db}$, deficient for the leptin receptor) is associated with a quantitative and qualitative shift in microbial colonization (Grice et al. 2010). In this model, diabetic mouse skin is characterized by a much greater bacterial load and greater representation by *Staphylococcus* spp. than healthy mouse skin. This shift in bacterial colonization is sustained throughout the entire wound healing time course. Other organisms with greater representation in the non-healing wounds included *Aerococcus, Klebsiella*, and *Weissella* spp. Diabetic mouse wounds are also colonized by a much lower diversity of organisms as compared to wounds in healthy mice. We postulate that studies in animal models

will be critical toward uncovering the mechanistic features that drive the destructive relationship between microbes and the innate immune response in chronic wounds.

4 The Skin is the First Line of Defense

The structural and functional integrity of the epidermis is the critical first line of defense against invasion by foreign and pathogenic substances. As such, the skin is a key component of the innate immune response even before injury occurs. A major challenge of maintaining the integrity of the skin barrier is modulating the immune response upon physical, chemical, or microbial insult. Response to the barrier breach must be carefully balanced between tolerance and activation, to rapidly control microbial invasion and infection without eliciting a potentially harmful, excessive inflammatory response.

The epidermal surface is both a structural and functional antimicrobial shield. Terminally differentiated, enucleated keratinocytes encased in lipid bilayers form the "bricks and mortar" of the stratum corneum, a formidable physical barrier to the exterior environment (Segre 2006). The lipid component of the epidermis, along with proton pumps, and free amino acids, render the skin surface slightly acidic (pH of approximately 5.0) (Marples 1965). The acidic and desiccated nature of the skin surface creates a hostile environment for most microorganisms (i.e., *Staphylococcus aureus*), yet allow colonization by skin commensals that are adapted to these conditions (i.e., *Staphylococcus epidermidis*). Antimicrobial substances (i.e., antimicrobial peptides [AMPs], lysozymes, RNases) are secreted in the sweat and sebum, moisturizing the skin surface while dispersing an antimicrobial shield (Elias 2007).

Keratinocytes are a rich source of innate immune sentinels and mediators (Fig. 3) (Nestle et al. 2009). Keratinocytes express pattern recognition receptors

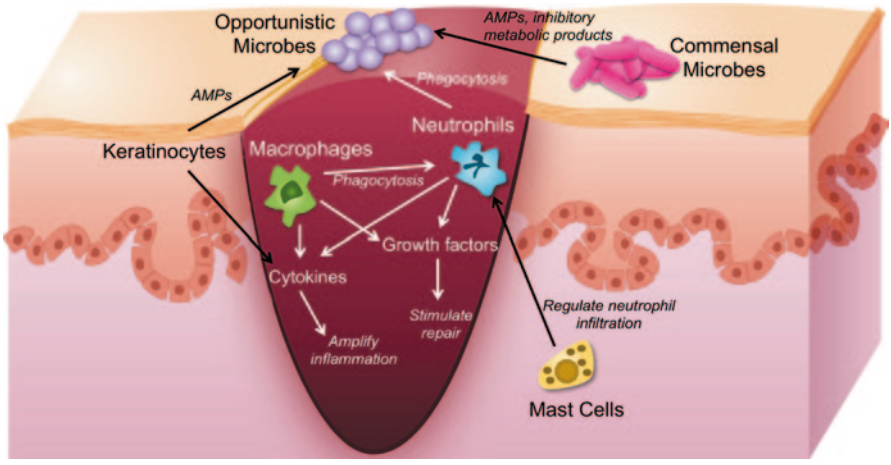

Fig. 3 Interaction of microbes with the innate immune system during wound healing. Interactions include microbe–microbe and host–microbe. *AMP* = antimicrobial peptide. Drawing not to scale

(PRRs) including Toll-like receptors (TLRs), mannose receptors, and Nod-like receptors (NLRs). PRRs recognize pathogen-associated molecular patterns (PAMPs; i.e., lipopolysaccharide, peptidoglycan, flagellin, nucleic acids) that are relatively conserved among microorganisms. Activation of TLRs induces production of cytokines, chemokines, adhesion molecules, and AMPs, ultimately resulting in killing of microorganisms and initiation of the adaptive immune response.

5 The Innate Immune Response During Normal Wound Healing

The innate immune response plays a central role in wound repair, but is especially vital during the coagulative and inflammatory phases (Fig. 3). Immediately following wounding, platelet aggregation and fibrin matrix formation triggers inflammatory and immune cell recruitment (Singer and Clark 1999). Neutrophils are the first inflammatory cells on the scene, responding to platelet degranulation, complement activation, and bacterial degradation. Neutrophils clear the wound bed of foreign debris, non-viable tissue, and microorganisms while secreting proteolytic enzymes (i.e., matrix metalloproteases [MMPs], elastase, cathelpsin G), AMPs, reactive oxygen species (ROS), and cytokines. At this stage, mast cells may help regulate neutrophil infiltration, and some evidence suggests that they are required for normal cutaneous wound healing in mice (Egozi et al. 2003; Weller et al. 2006).

Neutrophils are gradually replaced by monocytes around day 2–3 post-wounding. Monocytes differentiate and become activated macrophages. In addition to phagocytosing remaining neutrophils, activated macrophages continue to phagocytose microorganisms and clear away debris in the wound bed. Macrophages sense and respond to their environment via TLRs, complement receptors, and Fc receptors (Gordon 2003; Karin et al. 2006). A plethora of cytokines and growth factors are secreted by macrophages including TNFα, IL-1β, IL-6, IL-10, ILGF-1, PDGF, VEGF, TGFα, TGFβ, and CSF1.

Upon wounding, keratinocytes, neutrophils, and macrophages are induced to produce AMPs (Gallo et al. 1994). AMPs are multi-functional cationic peptides capable of directly killing pathogens, recruiting immune cells, and inducing cytokine production (Lai and Gallo 2009). Microbes stimulate expression of AMPs primarily through TLR signaling (Abtin et al. 2008; Buchau et al. 2007; Lai et al. 2010; Sumikawa et al. 2006). Importantly, AMPs promote wound healing. The human β-defensins hBD-2, -3, and -4 stimulate keratinocyte migration and proliferation and chemokine/cytokine production (Niyonsaba et al. 2007). Cathelicidin is expressed at high levels upon cutaneous wounding (Dorschner et al. 2001) and inhibition of cathelicidin in wounded organ-cultured human skin resulted in inhibition of re-epithelialization (Heilborn et al. 2003). Cathelicidin-deficient mice (targeted deletion of the *Cnlp* gene) display impaired wound healing and increased susceptibility to bacterial colonization and infection as compared to their wild-type counterparts

(Braff et al. 2005; Nizet et al. 2001). Furthermore, cathelicidin can promote angiogenesis and neovascularization (Koczulla et al. 2003).

The inflammatory phase of wound repair overlaps the migrative/proliferative phase where re-epithelialization and angiogenesis occur. This is followed by the remodeling phase, which can persist for up to a year. Collagen is remodeled and tissue is strengthened, though it is only restored to a fraction of its original strength before wounding (Gurtner et al. 2008). Successful wound repair also requires resolution of inflammation. This can be achieved by downregulation of inflammatory molecules by anti-inflammatory cytokines (i.e., IL-10, TGFβ1) or upregulation of anti-inflammatory molecules (i.e., IL-1 receptor antagonist). Little is known about the mechanisms regulating resolution of the inflammatory phase. As we will see in the next section, unresolved inflammation is a hallmark of chronic wounds.

6 An Aberrant Innate Immune Response in Chronic Wounds

Most chronic wounds, including diabetic, venous, and pressure wounds, are stalled in a chronic inflammatory state (Loots et al. 1998). Microbes are a critical component in amplifying and perpetuating inflammation in the chronic wound environment. Bacteria and their components can directly stimulate the influx of neutrophils and macrophages (Singer and Clark 1999). Leukocytes can be extraordinarily harmful to the wound environment. Non-viable tissue propagates the cycle of bacterial colonization/infection followed by leukocyte infiltration.

Since invading neutrophils and macrophages are a potent source of proteases, the chronic wound microenvironment is highly proteolytic. Of note, MMPs activity is upregulated and MMP-inhibitor activity is downregulated (Eming et al. 2010; Moor et al. 2009; Mwaura et al. 2006; Norgauer et al. 2002; Saarialho-Kere 1998). As a result, mediators of repair, provisional wound matrix components, and growth factors are targeted and inactivated by proteolytic cleavage (Lauer et al. 2000; Moor et al. 2009; Roth et al. 2006; Wlaschek et al. 1997).

Oxidative stress amplifies chronic inflammation in non-healing wounds. Leukocytes are a major source of ROS(superoxide anion, hydroxyl radicals, hydrogen peroxide, singlet oxygen), rendering the wound microenvironment highly prooxidant (James et al. 2003; Mendez et al. 1998; Wenk et al. 2001; Wlaschek and Scharffetter-Kochanek 2005). ROS can directly damage structural proteins of the extracellular matrix and alter signaling pathways and transcriptional regulation of proinflammatory cytokines and chemokines (Wenk et al. 2001).

While leukocytes are plentiful in the chronic wound environment, their phagocytosis, chemotaxis, and bactericidal activity appears to be diminished, at least in chronic diabetic wounds (Calhoun et al. 2002; Naghibi et al. 1987; Nolan et al. 1978; Zykova et al. 2000). This may in part be due to bacteria interference with cell–matrix interactions (Athanasopoulos et al. 2006; Chavakis et al. 2002). As a result, the wound becomes even more susceptible to increased bacterial burden and

infection. Further complicating treatment decisions, many chronic wounds do not outwardly display clinical signs and symptoms of infection despite high bacterial burden and the presence of pathogenic organisms (Gardner et al. 2001). This is likely due to population-specific factors, including tissue perfusion and oxygenation, hyperglycemia, and other aspects of immunocompetence.

Recent data indicates that deficiency of either neutrophils or macrophages is not deleterious to wound healing. In some cases, depletion of either or both cell types can enhance rate of wound repair and decrease scarring (Martin and Leibovich 2005). This would suggest that there is redundancy present in the inflammatory response and modulating recruitment and activity of different leukocyte lineages may prove to be therapeutically beneficial in chronic wounds.

Activation of TLR pathways by microbial components may be a factor in the chronic inflammation associated with non-healing wounds. Our group recently demonstrated persistent expression of several TLRs (TLR1, 2, 4, 6, 7, 8, and 13) coinciding with impaired healing and a shift in microbes colonizing the wounds of *db*/*db* diabetic mice as compared to healthy mice (Grice et al. 2010). Diabetic (streptozotocin-induced) $Tlr2^{-/-}$ mice demonstrated improved wound healing and a decreased inflammatory response as compared to $Tlr2^{+/+}$ diabetic mice (Dasu et al. 2010). Wound fluids from non-healing chronic venous leg ulcers demonstrated persistent levels of TLR-2 and TLR-4 activity over time, while healing wounds showed diminishing levels as they healed (Pukstad et al. 2010). Activation of TLR receptors ultimately leads to NF-κB-mediated transcription and production of inflammatory cytokines, amplifying the inflammatory state of the chronic wound microenvironment.

AMP production and secretion has a direct effect on microbial killing, and an indirect effect on cytokine/chemokine secretion, angiogenesis, and wound repair. Cathelicidin, an AMP normally upregulated during cutanous wound healing, has been demonstrated to be absent in chronic venous leg ulcers (Dressel et al. 2010; Heilborn et al. 2003). RNase 7, a potent antimicrobial ribonuclease, was also found to be absent, while psoriasin and hBD-2 were upregulated in chronic venous leg ulcers (Butmarc et al. 2004; Dressel et al. 2010). Proteomic analysis of wound exudates identified increased amounts of the AMPs lactotransferrin, azurocidin-1, lipocalin, and bacterial/permeability-increasing protein in non-healing wounds as compared to healing wounds (Eming et al 2010).

In a mouse model of type-2 diabetes (*db*/*db*), our group has globally analyzed the inflammatory and host defense response associated with impaired wound healing and how it correlates with colonization of microbiota (Grice et al. 2010). Non-healing wounds in *db*/*db* mice were characterized by persistent upregulation of inflammatory and host defense genes, including TLR pathway genes, complement pathway genes, and inflammatory cytokine genes. This pattern of gene expression is closely correlated with the relative abundance of *Staphylococcus* colonizing *db*/*db* wounds. While it is clear from this work and others that microbial community structure is closely associated with the host innate immune system, the mechanisms by which microbes interact and contribute to chronic inflammation in non-healing wounds remains unclear.

7 Conclusions and Perspectives

Wound healing is a complex process, further complicated by underlying pathology and systemic disease such as diabetes. The microbiome of the chronic wound undoubtedly plays a major and interactive role in impaired healing, especially in amplifying and perpetuating the host innate immune response. Technological and conceptual advances now provide unprecedented opportunities to fully delineate the role of host–microbe interactions in chronic wound healing. Recent advances in high-throughput sequencing technology allow unparalleled sampling depth for surveying microbial diversity. Generating resources to characterize the human microbiota and its role in health and disease is a major mission of the NIH Roadmap Human Microbiome Project (HMP) (Peterson et al. 2009). Yet interpreting the huge datasets generated by these studies will only provide meaningful results when the study is carefully designed and the experimental procedures meticulously validated. Teasing apart the molecular mechanisms governing host–microbe interactions in chronic wounds will also likely require studies in animal models. Tools for these studies (i.e., germ-free mice, selective colonization) have been developed for gut microbiome analysis but will need to be adapted and optimized for analyzing host–microbe interactions in a wound environment.

Gaining a better understanding of the interaction between wound microbiota and the innate immune system may provide insight into effective non-antimicrobial treatment strategies. Potential therapies could involve manipulating and/or normalizing microbiota, through inhibition of pathogenic bacteria or promotion of symbiotic bacteria, as a low-cost non-invasive target for the management of chronic wounds. Wound microbiome diversity profiling could also be utilized as a biomarker to predict wound outcomes or to identify clinical populations at risk for impaired wound healing or wound complications. On the host side of the relationship, inflammatory and/or innate immune factors may be targeted, for both control of persistent inflammation and to modulate closely associated, potentially pathogenic microbial populations. As we have shown, the cycle of inflammation triggered and amplified by microbial colonization and/or infection is highly deleterious for wound healing. Future studies aimed at dissecting the mechanisms of this relationship, though challenging, will provide a valuable foundation for clinical translation into improved diagnostics and therapeutics.

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Pathogenic Microbes and Community Service Through Manipulation of Innate Immunity

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Abstract The periodontal pathogen *Porphyromonas gingivalis* undermines major components of innate immunity, such as complement, Toll-like receptors (TLR), and their crosstalk pathways. At least in principle, these subversive activities could promote the adaptive fitness of the entire periodontal biofilm community. In this regard, the virulence factors responsible for complement and TLR exploitation (gingipain enzymes, atypical lipopolysaccharide molecules, and fimbriae) are released as components of readily diffusible membrane vesicles, which can thus become available to other biofilm organisms. This review summarizes important immune subversive tactics of *P. gingivalis* which might enable it to exert a supportive impact on the oral microbial community.

Keywords Complement • C5a • TLR • Immune evasion • *P. gingivalis* • Biofilm • Periodontitis

1 Introduction

Innate immunity is a phylogenetically ancient system of host defense that represents the inherited resistance to infection (Janeway and Medzhitov 2002). It senses pathogens through "pattern recognition" or "missing-self recognition" strategies, e.g., through Toll-like receptors (TLRs) and complement, and triggers the activation of antimicrobial and inflammatory responses (Ricklin et al. 2010; Medzhitov 2007). TLRs recognize conserved microbial structures known as pathogen-associated molecular patterns (PAMPs) and, in general, different TLRs respond to distinct PAMPs (e.g., TLR2 recognizes lipoteichoic acid, TLR4 detects lipopolysaccharide, and TLR9 binds CpG DNA) (Beutler et al. 2006; Uematsu and Akira 2008). TLRs do not

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function in isolation but cooperate with other receptors in multireceptor complexes in lipid rafts of activated cells (Beutler et al. 2006; Hajishengallis et al. 2006; Triantafilou et al. 2002). Innate receptors shown to co-cluster with TLRs include CD14, CD36, CD55 (decay accelerating factor), complement receptor 3 (CR3; CD11b/CD18), CXC-chemokine receptor 4 (CXCR4), and growth differentiation factor 5 (GDF5) (Hajishengallis et al. 2006; Pfeiffer et al. 2001; Triantafilou et al. 2001). The formation of TLR-containing receptor clusters may serve to generate a combinatorial repertoire through which the host discriminates among the abundant and diverse microbial molecules and thereby to tailor the immune response. Moreover, TLR signaling pathways crosstalk with the complement system, which is now recognized to exert functions above and beyond simple pathogen tagging and elimination (Ricklin et al. 2010). For instance, effector molecules generated during the rapidly activated complement cascade (e.g., certain opsonins, anaphylatoxins, and sublytic concentrations of the terminal complement complex) stimulate signaling pathways that intercept and modify TLR-transduced signals (Hajishengallis and Lambris 2010). An emerging body of literature indicates that the TLR-complement interplay involves both synergistic and antagonistic interactions, which could, respectively, enhance host defense or regulate it to prevent excessive inflammation or autoimmunity (Hajishengallis and Lambris 2010; Hawlisch et al. 2005; Zhang et al. 2007).

Not surprisingly, given their importance in fighting infection, both complement and TLRs are key targets of immune evasion by successful pathogens, such as *Staphylococcus aureus*, *Helicobacter pylori*, vaccinia virus, and HIV (Lambris et al. 2008; Flannagan et al. 2009). Persistent infections and disease may also ensue when microbial pathogens successfully evade or subvert complement-TLR crosstalk pathways (Hajishengallis and Lambris 2010; Hajishengallis and Lambris 2011). By subverting innate immunity, pathogens may also undermine the overall host defense system, given the instructive role of innate immunity in the development of the adaptive immune response (Medzhitov 2001).

In this chapter, we focus on immune subversion mediated by the periodontal pathogen *Porphyromonas gingivalis*, placing particular emphasis on its newly identified crosstalk sabotage tactics. Immune evasion mechanisms of specific bacteria acquire their true relevance in the context of the collective virulence of the microbial communities in which they reside. In this regard, we examine published literature and discuss the potential of *P. gingivalis* to act as a k pathogen that might impact on the entire periodontal biofilm rendering it more pathogenic.

2 Keystone Pathogens in the Periodontal Biofilm

Porphyromonas gingivalis is a gram-negative anaerobic bacterium that is strongly associated with periodontitis, a chronic inflammatory disease that affects the tooth-supporting tissues (Pihlstrom et al. 2005). Periodontal disease affects the majority of the adult population, whereas an estimated 10–15% develops severe periodontitis. This is a condition that exerts a systemic impact on the patients who thereby

run increased risk for atherosclerotic heart disease, aspiration pneumonia, diabetes, adverse pregnancy outcomes, and perhaps rheumatoid arthritis (Xiong et al. 2006; Pihlstrom et al. 2005; Awano et al. 2008; Tonetti et al. 2007; de Pablo et al. 2009; Kebschull et al. 2010; Lundberg et al. 2010).

It should be noted that periodontitis is not initiated by a single bacterium but rather by certain species of subgingival gram-negative anaerobic bacteria, and the disease represents biofilm-induced destructive inflammation of the periodontal tissue (Davey and Costerton 2006; Gaffen and Hajishengallis 2008). Using a color-coded system, Socransky and colleagues characterized the periodontal microbial communities on the basis of cluster analysis, community ordination, and associated disease severity (Socransky et al. 1998). These communities are not random collections of bacteria, but organized and dynamic consortia that have evolved through mutually beneficial interactions, ultimately forming a niche with adequate nutrients and protection against host immunity. A high prevalence of red complex members, i.e., *P. gingivalis, Treponema denticola, and Tannerella forsythia,* correlates strongly with periodontal tissue destruction (Socransky et al. 1998; Holt and Ebersole 2005). *Prevotella intermedia, Fusobacterium nucleatum*, and *Aggregatibacter actinomycetmecomitans*, all members of the orange complex, are also associated with various forms of periodontal disease (Darveau 2009; Socransky et al. 1998). Although these and other putative periodontal pathogens have received particular attention, it should be noted that a sizeable portion of the approximately 700 bacterial species in the human oral cavity are as yet uncultivable (Kolenbrander et al. 2010). Despite its perceived complexity, the pathogenicity of the periodontal biofilm might be disproportionately dependent upon a select group of so-called "keystone" species. There is circumstantial evidence to suggest that at least one such species may be *P. gingivalis* (Darveau 2010; Hajishengallis 2009).

A keystone species is an organism that plays a crucial role in maintaining the structure of an ecological community, in a manner akin to the role of a keystone in an arch. Its impact on the community is greater than would be expected from its relative abundance. The "keystone species" concept was originally proposed by the ecologist R. T. Paine in 1969 (Paine 1969). A classic example of a keystone organism is the starfish *Pisaster ochraceus*. When this starfish was experimentally removed from a particular intertidal zone, this caused a dramatic reduction in biodiversity compared to a control area. In the context of microbial ecology, Darveau, defined the keystone species as one that "serves an essential function for the entire community, similar to a differentiated cell serving a function for an entire tissue" (Darveau 2010). The implication here is not that the periodontal biofilm would necessarily collapse in the absence of a single important species. Rather, the pathogenicity of a biofilm missing a keystone member could be attenuated to the point of not being capable of driving destructive periodontal inflammation. A keystone microbe is not necessarily a pathogen. The human gut symbiont *Bacteroides thetaiotaomicron* imparts stability to the gut ecosystem and might be considered as a keystone species (Backhed et al. 2005). One of its mechanisms, in this context, involves manipulation of host gene expression. If, for example, dietary polysac-

charides become scarce, *B. thetaiotaomicron* induces gut epithelial cell production of fucosylated glycans on which bacteria can feed (Backhed et al. 2005). The modification of either dietary conditions or of immune selective pressure in ways that stabilize the microbiota could be thus considered as keystone functions.

3 *Porphyromonas gingivalis:* Interfering with Host Immunity for the Microbial "Common Good"?

Porphyromonas gingivalis is employing an array of virulence factors, such as cysteine proteinases (known as gingipains), lipopolysaccharide (LPS), and fimbriae (Table 1), through which it manipulates innate immunity in an effort to promote its adaptive fitness.

In addition to its established role in periodontitis, *P. gingivalis* is also a common isolate from aspiration pneumonia and lung abscesses (Okuda et al. 2005; Finegold 1991) and has been detected in a viable state in atherosclerotic plaques (Kozarov et al. 2005). Its role in cardiovascular disease has been the focus of a number of mechanistic and epidemiological studies (Kebschull et al. 2010). There is also intriguing, though still circumstantial, evidence that the ability of *P. gingivalis* to citrullinate bacterial and host proteins may provide a molecular mechanism for generating antigens that drive the autoimmune response in rheumatoid arthritis (Lundberg et al. 2010). Of course the potential dental and medical importance of *P. gingivalis* does not necessarily render it a keystone species. A keystone species should express virulence factors that could provide "community service". Below we discuss virulence factors of *P. gingivalis* that manipulate innate immunity in ways that could benefit additional periodontal species.

3.1 The LPS as a Tool to Antagonize TLR4

Although *P. gingivalis* is a gram-negative bacterium that expresses LPS, its lipid A moiety presents some unusual features that make it invisible to TLR4 or, even worse for the host, antagonistic to TLR4 (Fig. 1). Specifically, the organism coordinately utilizes specific lipid A 1- and 4′-phosphatases and a deacylase to generate a tetra-acylated and dephosphorylated lipid A structure (Coats et al. 2009). This modification renders the LPS biologically inert, thereby allowing *P. gingivalis* evade TLR4 activation (Coats et al. 2009). The presence of high concentrations of hemin (becomes abundant under inflammatory conditions) suppresses lipid A 1-phosphatase activity and leads to the generation of a mono-phosphorylated lipid A, which actively antagonizes TLR4 activation (Coats et al. 2009; Coats et al. 2005). Although TLR4 could be activated in periodontal tissue by LPS from other bacteria (e.g., by *A. actinomycetemcomitans*, the LPS of which is potent TLR4 ago-

Table 1 Immune subversion mechanisms of *P. gingivalis* and virulence factors involved

Mechanism	Virulence factors	References
Inhibition of complement activation through degradation of the central complement component (C3)	Gingipains (especially HRgpA and RgpB)	(Popadiak et al. 2007; Potempa et al. 2009)
Hijacking complement regulatory proteins (C4b-binding protein)	Gingipain (HRgpA)	(Potempa et al. 2008)
Inhibition of phagocyte killing via C5aR–TLR2 crosstalk	Gingipains (HRgpA, RgpB)	(Wang et al. 2010)
Suppression of TLR2-induced IL-12 by *P. gingivalis*-generated C5a	Gingipains (HRgpA, RgpB)	(Liang et al. 2010)
Degradation of TLR coreceptors (CD14), cytokines (IL-12, IL-1β, IL-6, IFN-γ), or antimicrobial peptides (e.g., LL-37)	Gingipains (HRgpA, RgpB, Kgp)	(Potempa and Pike 2009)
Inherent resistance to complement-mediated lysis	LPS with anionic polysaccharide repeat units	(Slaney et al. 2006; Rangarajan et al. 2008)
TLR4 evasion	Dephosphorylated tetraacylated lipid A LPS	(Coats et al. 2009)
TLR4 antagonism (e.g., inhibition of TLR4-induced β-defensin expression)	Monophosphorylated tetraacylated lipid A LPS	(Coats et al. 2009; Coats et al. 2005; Darveau 2010)
Upregulation of negative regulators of TLR signaling (IRAK-M) in monocytes	LPS	(Domon et al. 2008)
Inhibition of phagocyte killing via CXCR4–TLR2 crosstalk	Fimbriae	(Hajishengallis et al. 2008)
Suppression of TLR2-induced IL-12 via CR3 outside-in signaling	Fimbriae	(Hajishengallis et al. 2007)
Promotion of intracellular survival via CR3-mediated entry into macrophages	Fimbriae	(Wang et al. 2007)

nist), this activity is likely to be attenuated by *P. gingivalis* LPS. Proof-of-concept evidence was obtained in *in vitro* studies (Hajishengallis et al. 2002; Darveau et al. 2002). The underlying mechanism involves competitive TLR4 blocking mediated by the binding of the antagonistic *P. gingivalis* lipid A to MD2, a TLR4-bound protein required for TLR4 responsiveness to LPS (Coats et al. 2005). This mechanism may not only help other bacteria in close vicinity to *P. gingivalis* but also those in remoted areas of mixed species biofilms. Indeed, *P. gingivalis* releases LPS-bearing outer membrane vesicles (OMVs) that can readily diffuse in the gingival crevice (free space between the teeth and the gingiva) or even penetrate gingival tissue (Darveau 2010; Lamont and Jenkinson 1998). The *P. gingivalis* OMVs may therefore undermine TLR4-dependent innate immunity for the microbial community at

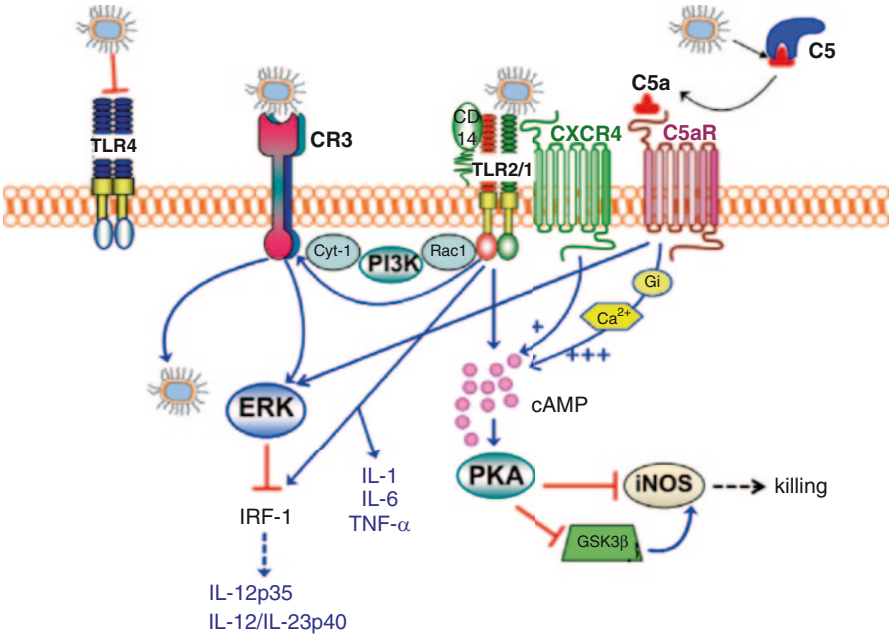

Fig. 1 Subversive crosstalk pathways exploited by *P. gingivalis*. In macrophages, *P. gingivalis* interacts with the CD14/TLR2/TLR1 receptor complex (Hajishengallis et al. 2006). Moreover, *P. gingivalis* uses its gingipains to attack C5 and release biologically C5a (Wingrove et al. 1992; Wang et al. 2010). Upon C5aR binding, C5a stimulates Gαi-dependent intracellular Ca^{2+} signaling which synergistically enhances the otherwise weak cAMP responses induced by TLR2/TLR1 activation alone. Maximal cAMP induction is achieved by the participation of another G protein-coupled receptor, the CXCR4, which interacts directly with *P. gingivalis* and coassociates with both TLR2 and C5aR in lipid rafts (Hajishengallis et al. 2008; Wang et al. 2010). The ensuing activation of the cAMP-dependent protein kinase A (PKA) pathway inactivates glycogen synthase kinase-3β (GSK3β) and impairs the inducible nitrogen synthase (iNOS)-dependent killing of the pathogen in macrophages *in vitro* and *in vivo* (Wang et al. 2010). The interaction of *P. gingivalis* with TLR2/TLR1 also activates inside-out signaling, propagated by Rac1, PI3 K, and cytohesin-1 (Cyt-1), which induces the high-affinity conformation of CR3 (Hajishengallis and Harokopakis 2007; Harokopakis and Hajishengallis 2005). CR3 then binds and internalizes *P. gingivalis*; this is a relatively safe portal of entry since CR3 is not linked to vigorous microbicidal mechanisms (Rosenberger and Finlay 2003; Wang and Hajishengallis 2008). The CR3-*P. gingivalis* interaction also leads to induction of ERK1/2 signaling. This in turn downregulates IL-12 p35 and p40 mRNA expression (Hajishengallis et al. 2007), possibly through suppression of a critical transcription factor (the interferon regulatory factor 1 [IRF1]) that is required for IL-12 expression (Hawlisch et al. 2005). This inhibitory ERK1/2 pathway is also activated downstream of the C5aR. The suppressive effects of CR3 and C5aR on TLR2-induced cytokine production are selective for IL-12 and do not affect induction of other proinflammatory cytokines (e.g., IL-1β, IL-6, and TNF-α) *in vitro* or *in vivo*. Inhibition of bioactive IL-12 by these mechanisms results in impaired immune clearance of *P. gingivalis in vivo* (Hajishengallis et al. 2007; Liang et al. 2010). *P. gingivalis* interacts with at least one more TLR, the TLR4, which, however, is proactively antagonized by an atypical lipopolysaccharide lipid A moiety (Coats et al. 2005)

large, such as inhibition of β defensin expression by epithelial cells (Lu et al. 2009). The *P. gingivalis* OMVs are not solely decorated with LPS but contain additional virulence factors including gingipains and fimbriae, both of which are implicated in immune evasion (Amano et al. 2010; Hajishengallis 2009) (Table 1). These factors are discussed below.

3.2 The Gingipains as a Means to Manipulate Complement and Leukocytes

P. gingivalis can suppress all three mechanisms of complement activation, i.e., the classical, lectin, and alternative pathways, through proteolytic degradation of key complement components such as the C3 (Potempa and Pike 2009). These subversive activities are mediated by the gingipains and particularly the Arg-specific enzymes.

When gingipain-deficient mutants are exposed to human serum, active complement fragments are readily deposited on the bacterial surface; strikingly, however, the mutants maintain full viability as does the wild-type organism (Slaney et al. 2006). This observation was conclusively attributed to the expression of a surface anionic polysaccharide (APS) which confers exquisite resistance to complement-mediated lysis (Slaney et al. 2006). This molecule was later identified as a novel type of LPS, termed A-LPS (with APS repeating units) to differentiated it from the better known O-LPS (with O-antigen tetrasaccharide repeating units) (Rangarajan et al. 2008). However, an intriguing question arising from this discovery is the following: What is the point of inhibiting complement when the pathogen is already intrinsically resistant to complement-mediated lysis? A possible explanation is that *P. gingivalis* has evolved to inhibit complement activation, not for its own protection, but for the benefit of companion species sharing the same subgingival niche.

The capacity of *P. gingivalis* to inhibit complement becomes all the more puzzling considering that it has developed ways to directly and "voluntarily" activate specific complement components. For instance, the Arg-specific gingipains of *P. gingivalis* function as C5 convertase-like enzymes (Wingrove et al. 1992) and the bacterium generates high levels of C5a *in vitro* and *in vivo* (Wang et al. 2010; Liang et al. 2010). This is quite curious for a pathogen which goes at great lengths to inhibit all three known initiation mechanisms of complement activation (Popadiak et al. 2007; Krauss et al. 2010). However, there is something unique about C5a and *P. gingivalis*. The pathogen exploits this anaphylatoxin (but not C3a) to instigate a subversive crosstalk between the C5a receptor (C5aR; CD88) and TLR2, and in doing so it escapes killing (Liang et al. 2010; Wang et al. 2010). It appears that since *P. gingivalis* does not antagonize TLR2 at the receptor level as it does with TLR4 (see above), it has instead opted to instigate crosstalk signaling between TLR2 and other innate receptors to undermine innate immunity (Hajishengallis et al. 2008; Wang et al. 2007; Hajishengallis et al. 2007; Wang et al. 2010) (Fig. 1). The crosstalk between C5aR and TLR2 may be facilitated by the ability of *P. gingivalis* to in-

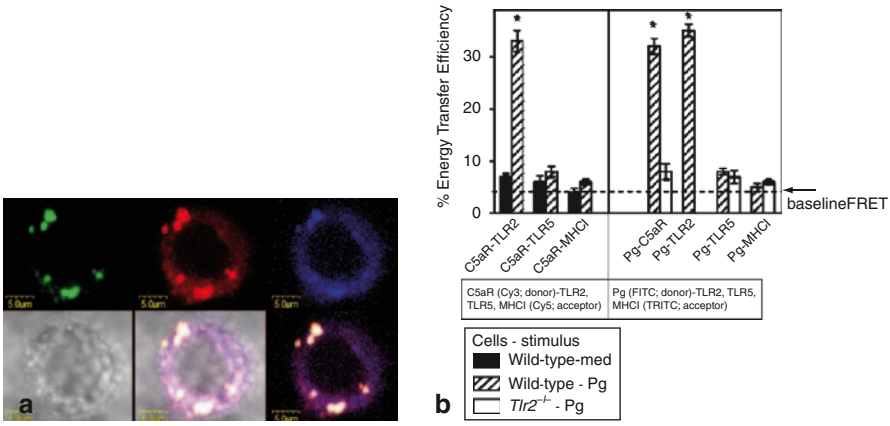

Fig. 2 ***P. gingivalis*** **induces colocalization of C5aR and TLR2 in macrophages. a** Confocal colocalization of *P. gingivalis* (*green*), C5aR (*red*), and TLR2 (*blue*). Bottom right, merged image. **b** Fluorescence resonance energy transfer between the indicated donors and acceptors measured from the increase in donor (Cy3 or FITC) fluorescence after acceptor (Cy5 or TRITC) photobleaching. Data are means ± SD ($n = 3$). *, $p < 0.01$ between the indicated groups and controls. FRET analysis has revealed significant energy transfer between Cy3-labeled C5aR and Cy5-labeled TLR2 in *P. gingivalis*-stimulated but not resting macrophages, whereas no significant energy transfer was detected between Cy3-labeled C5aR and Cy5-labeled TLR5 or MHC Class I (controls). Moreover, significant energy transfer was observed between FITC-labeled *P. gingivalis* and TRITC-labeled C5aR or TLR2 (but not TLR5 or MHC Class I). C5aR appeared to associate with *P. gingivalis* in a TLR2-dependent way since the *P. gingivalis*-C5aR FRET association was abrogated in *Tlr2*$^{-/-}$macrophages. (The data are from the reference Wang et al. 2010) (Used by permission)

duce a coassociation between these receptors. Indeed, confocal microscopy has revealed colocalization of the two receptors in *P. gingivalis*-stimulated macrophages (Fig. 2a), while fluorescence resonance energy transfer experiments have shown that C5aR, TLR2, and *P. gingivalis* come into molecular proximity(Fig. 2b) (Wang et al. 2010).

Mechanistically, upon binding the C5aR, C5a stimulates Gαi-dependent intracellular Ca^{2+} signaling which synergistically enhances an otherwise weak cAMP response induced by *P. gingivalis*-induced TLR2 activation alone. In this crosstalk pathway, sustained elevated production of cAMP leads to the activation of the cAMP-dependent protein kinase A which inactivates the glycogen synthase kinase-3β and impairs nitric oxide-dependent killing of *P. gingivalis* in macrophages (Wang et al. 2010). Crosstalk between C5aR and TLR2 also inhibits the ability of neutrophils to kill *P. gingivalis*, although the underlying signaling pathway is cAMP-independent (Hajishengallis et al. 2010). *In vivo*, the *P. gingivalis*-induced C5aR–TLR2 crosstalk regulates cytokine production in favor of the pathogen (Liang et al. 2010). Specifically, this oral bacterium proactively and selectively inhibits TLR2-induced interleukin (IL)-12p70 which is required for its immune clearance, whereas the same C5aR–TLR2 crosstalk upregulates other inflammatory and bone-resorptive cytokines (IL-1β, IL-6, and TNF-α) which do not seem to harm *P. gingivalis*. These effects would be expected to enhance the persistence of *P. gingivalis* in the host

and to create favorable conditions for inflammatory tissue damage. Indeed, this notion is consistent with observations that mice deficient in either C5aR or TLR2 are protected against *P. gingivalis*-induced inflammatory periodontal bone loss (Liang et al. 2010). Moreover, an isogenic mutant of *P. gingivalis* which is deficient in all gingipain genes (KDP128) fails to persist *in vivo*, in contrast to the wild-type organism which promotes its survival in a C5aR-dependent way (Liang et al. 2010). This difference in survival capacity may be related, at least in part, to the inability of KDP128 to generate C5a (Liang et al. 2010).

These subversive effects are unlikely to benefit only *P. gingivalis*. If neutrophils or macrophages are rendered impotent in terms of killing, the same leukocytes would also be unable to control bystander bacteria cohabiting the same niche with *P. gingivalis*. It is often assumed that biofilms are inherently resistant to phagocytosis. However, this is an unproven assumption. In fact, neutrophils can potentially recognize and respond to biofilms through phagocytosis, degranulation (e.g., release of lactoferrin and elastase) and formation of extracellular DNA traps (Meyle et al. 2010). Therefore, when biofilms successfully resist elimination, this is likely to involve proactive microbial mechanisms that evade or subvert phagocytosis or other leukocyte functions. Time-lapse video microscopy and confocal laser scanning microscopy has revealed that, depending on the nature and composition of the biofilm, neutrophils can move into biofilms and eat up bacteria as they move across, or display a relatively immobile phenotype with limited phagocytosis in their immediate vicinity (Guenther et al. 2009; Gunther et al. 2009; Meyle et al. 2010; Wagner et al. 2007). Moreover, neutrophils can detect quorum-sensing molecules of bacteria which will attract them to the developing biofilm and stimulate their killing mechanisms (Wagner et al. 2007). In the human gingival crevice, recruited neutrophils form what looks like a "defense wall" against the dental biofilm bacteria; intriguingly, however, the neutrophils largely fail to control the bacteria despite maintaining viability and capacity to elicit inflammatory responses, including release of extracellular DNA traps (Delima and Van Dyke 2003; Lange and Schroeder 1971; Newman 1980; Schroeder and Listgarten 1997; Vitkov et al. 2010; Ryder 2010). The mechanistic basis for the relative impotence of crevicular neutrophils to control periodontal infection and to become involved in non-resolving inflammation is largely unexplored, but could be due to a number of mechanisms. At least in part, this may be the result of the *P. gingivalis* capacity to generate high levels of C5a and exploit it for targeted suppression of the leukocyte killing function without affecting—in fact, enhancing—the overall inflammatory response (Hajishengallis 2010).

In addition to their role in complement manipulation, the gingipains (but not host proteases) were shown to modify the cell surface of healthy neutrophils masquerading them as apoptotic, thereby leading to their removal from active duty by fellow macrophages. The underlying mechanism involves a dual action; firstly, proteolytic cleavage of an antiphagocytic signal (CD31) and, secondly, the generation of a novel, but not yet adequately characterized, "eat-me" signal on the neutrophil surface (Guzik et al. 2007). The fact that gingipains are present in the readily diffusible OMVs released by *P. gingivalis* may allow additional periodontal bacteria to benefit from gingipain-mediated immune subversion.

3.3 *Fimbriae: Multitasking Beyond Colonization*

Fimbriae are adhesive hair-like appendages emanating from the bacterial cell surface (Lamont and Jenkinson 1998; Hajishengallis 2007). The major fimbriae of *P. gingivalis* are encoded by the *fimA* operon and are indispensable for colonization and host cell invasion (Lamont and Jenkinson 1998; Hajishengallis 2007). Moreover, recent evidence has implicated fimbriae in immune evasion. Specifically, *P. gingivalis* uses its fimbriae to bind CR3 which induces extracellular signal-regulated kinase 1/2 signaling that in turn selectively inhibits mRNA expression of the p35 and p40 subunits of IL-12 (Hajishengallis et al. 2007) (Fig. 1). IL-12 is a key cytokine involved in pathogen clearance through regulatory effects on the production of interferon (IFN)-γ, which is a potent activator of the macrophage microbicidal capacity (Trinchieri 2003). Consistent with the above *in vitro* findings, wild-type mice elicit lower levels of IL-12 and IFN-γ and display impaired clearance of *P. gingivalis* infection compared to mice that lack CR3 (Hajishengallis et al. 2007). Similar results are seen after CR3 blockade with a specific antagonist (Hajishengallis et al. 2007). Importantly, the interaction of *P. gingivalis* or its purified fimbriae with CR3 inhibits the ability of LPS from other bacteria, such as *A. actinomycetemcomitans*, to induce IL-12p70 and IFN-γ in mouse macrophages or human monocytes (Hajishengallis et al. 2007).

The fimbriae of *P. gingivalis* can additionally co-ligate the CXC-chemokine receptor 4 (CXCR4) and TLR2 in lipid rafts leading to crosstalk signaling that inhibits the killing capacity of macrophages (Hajishengallis et al. 2008). Macrophages can be found in relatively low numbers in the gingival crevice but they are consistently present in the underying junctional epithelium (Tonetti et al. 1994). In addition, macrophages can interact with invading periodontal bacteria in the connective tissue of the periodontium. When *P. gingivalis* fimbriae are released as components of OMVs, they may allow additional periodontal species evade killing through CR3 and CXCR4 exploitation. These notions, if experimentally confirmed, would substantiate the concept of community service by a single, keystone species.

4 Is the Presence of *P. gingivalis* Benefiting Other Periodontal Bacteria?

A widely used model of experimental periodontitis involves the implantation of *P. gingivalis* in the oral cavity of mice by oral gavage (Graves et al. 2008). Although sometimes regarded as a monoinfection model, this may not actually be so unless germ-free mice are used (Graves et al. 2008). The indigenous oral microbiota of mice contains bacteria with the potential to become pathogenic for the periodontium under conditions of disrupted periodontal homeostasis. For example, mice with impaired mobilization of leukocytes to sites of infection, owing to combined P- and E-selectin deficiency, display massive oral bacterial colonization and induction of

gingival inflammation and alveolar bone loss (Niederman et al. 2001). Similar bacteriological findings and periodontitis features are observed in mice deficient in lysosomal-associated membrane protein-2; in these mice, the neutrophils are readily mobilized to the periodontal tissues but display impaired bacterialkilling and are thus unable to control their indigenous oral flora (Beertsen et al. 2008). In both cases, induction of inflammation and periodontal bone loss is prevented by antibiotics, thus further confirming the involvement of indigenous bacteria in the disease (Beertsen et al. 2008; Niederman et al. 2001).

In a rabbit model of *P. gingivalis*-induced periodontitis, the implantation of *P. gingivalis* "stimulated" a shift to a more anaerobic flora in the dental biofilm and an overall increase in bacterial load (Hasturk et al. 2007). For example, significantly higher levels were seen for *Prevotella intermedia*, *Eikenella corrodens*, and *Peptostreptococcus micros*, whereas previously undetected species like *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum* were readily observed (Hasturk et al. 2007). When resolvin E1 was topically applied to inhibit the inflammatory process, this treatment also resulted in the elimination of *P. gingivalis*, possibly attributed to the inability of this asaccharolytic organism to secure nutrients under non-inflammatory conditions. In this regard, the inflammatory exudate that bathes the gingival crevice is a rich source of peptides (e.g., from degradation of host proteins or tissue breakdown products) and iron (derived from hemin) (Delima and Van Dyke 2003; Krauss et al. 2010). Interestingly, following the elimination of *P. gingivalis*, the resident oral flora returned to its baseline composition and numbers, i.e., those present before the implantation of *P. gingivalis* (Hasturk et al. 2007). We have also noticed that the introduction of *P. gingivalis* in the mouse oral cavity is associated with a dramatic increase in the total number of anaerobic oral bacteria (unpublished observations). These findings have two implications. Firstly, the observed bone loss may not be attributed solely to *P. gingivalis*. Secondly, *P. gingivalis* appears to act as a keystone species, in that its presence imparts a positive influence on the dental biofilm. However, if this action is mediated via manipulation of innate immunity, one would expect that the effect would be abrogated in animals lacking the hijacked innate receptor(s).

Although the mechanisms underlying the "keystone species" concept are poorly understood, initial observations in simple models may provide some mechanistic insight. As indicated above, *P. gingivalis* can protect itself from leukocyte killing by capitalizing on a subversive crosstalk between C5aR and TLR2 (Liang et al. 2010; Wang et al. 2010; Hajishengallis and Lambris 2011). This is not a mechanism that can be employed by just any oral bacterium and, in fact, *F. nucleatum* is susceptible to killing by leukocytes. However, *F. nucleatum* displays a dramatic resistance to leukocyte killing in the presence of *P. gingivalis* (Fig. 3). Simple co-infection models, such as the chamber model of host-microbe interactions (Graves et al. 2008), may prove helpful in identifying mechanisms of collective resistance to host defenses mediated by putative keystone species. This seemingly altruistic behavior of keystone organisms may not be quite so, considering that the benefited bacteria may reciprocate the favor. In this regard, the colonization of *P. gingivalis* is facilitated by other members of the mixed-species periodontal biofilm and *F. nucleatum* is likely

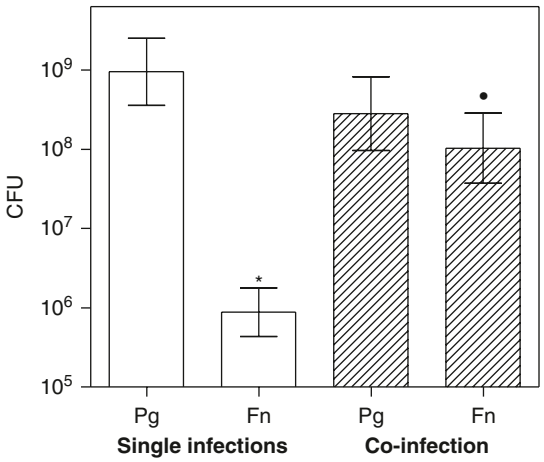

Fig. 3 The presence of *P. gingivalis* enhances the survival of other periodontal species. *P. gingivalis* ATCC 33277 and *F. nucleatum* ATCC 25586 (Fn) were injected into subcutaneously implanted chambers in mice, either alone (10^9 CFU) or together (5×10^8 CFU each), and 24 h postinfection chamber fluid was aspirated to determine viable CFU counts. The data are means ±SD ($n=5$ mice). *, $p<0.01$ between Fn and Pg CFU when inoculated alone; •, $p<0.01$ between CFU counts of the same organism recovered from single infections and co-infection

one of them (Kolenbrander et al. 2010). Interestingly, oral co-infection of mice with *F. nucleatum* and *P. gingivalis* induces increased bone loss compared to either organism alone (Polak et al. 2009). The underlying mechanisms may involve mutually beneficial interactions in addition to immune subversion, such as interbacterial adherence and metabolic cooperation.

5 Perspectives and Conclusions

In the course of evolution, successful pathogens have "learned" to breach innate defense systems such as complement and TLRs (Roy and Mocarski 2007; Lambris et al. 2008) but also, as exemplified here with *P. gingivalis*, to exploit their crosstalk pathways (Hajishengallis et al. 2008; Wang et al. 2010; Hajishengallis and Lambris 2011). These subversive strategies (Fig. 1) as well as other important evasion tactics (Hajishengallis 2009; Potempa and Pike 2009; Yilmaz 2008) (Table 1) may explain, at least in part, the ability of *P. gingivalis* to persist and establish chronic infections. There is also growing circumstantial evidence suggesting the potential of *P. gingivalis* to act as a keystone pathogen, i.e., one that could promote the survival and persistence of other members of the periodontal biofilm community (Darveau 2010; Hajishengallis 2009; Hasturk et al. 2007) (Fig. 3). Briefly stated, bacteria lacking protective or evasive mechanisms on their own may still thrive in the presence of *P. gingivalis*. A synergy of shared, communal mechanisms of immune evasion, colo-

nization, and nutrient procurement should maximize the survival potential and virulence of the whole community. In this context, various combinations of periodontal bacteria, such as *P. gingivalis*, *T. forsythia*, *T. denticola*, *F. nucleatu,* and *A. actinomycetemcomitans* become more virulent in *in vivo* models of infection than the individual organisms alone (Jenkinson and Lamont 2005; Feuille et al. 1996; Kesavalu et al. 1998; Kesavalu et al. 2007; Polak et al. 2009; Kinane and Hajishengallis 2009). *P. gingivalis*-centred ecosystems may perturb otherwise homeostatic host-bacterial interactions, thereby leading to non-protective and non-resolving chronic inflammation in the periodontium. Future research to elucidate the mechanisms by which *P. gingivalis* subverts immunity and promotes the collective virulence of the periodontal biofilm would facilitate the rational design of therapeutic interventions against human periodontitis.

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A Conserved Host and Pathogen Recognition Site on Immunoglobulins: Structural and Functional Aspects

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Abstract A common site in the constant region (Fc) of immunoglobulins is recognized by host receptors and is a frequent target of proteins expressed by pathogens. This site is located at the junction of two constant domains in the antibody heavy chains and produces a large shallow cavity formed by loops of the CH2 and CH3 domains in IgG and IgA (CH3 and CH4 domains in IgM). Crystal structures have been determined for complexes of IgG-Fc and IgA-Fc with a structurally diverse set of host, pathogen and *in vitro* selected ligands. While pathogen proteins may directly block interactions with the immunoglobulins thereby evading host immunity, it is likely that the same pathogen molecules also interact with other host factors to carry out their primary biological function. Herein we review the structural and functional aspects of host and pathogen molecular recognition of the common site on the Fc of immunoglobulins. We also propose that some pathogen proteins may promote virulence by affecting the bridging between innate and adaptive immunity.

Keywords Pathogen • Host • Antibody • Complement • Bacteria • Carbohydrate • Crystal structure • Domain junction

1 Immunoglobulins

Antibodies or immunoglobulins are central to humoral immunity and have the common goal of recognizing and defending against foreign invaders. Traditionally, antibodies are viewed as part of the adaptive immune system, but accumulating evidence demonstrates that "natural antibodies" play a key role in innate immunity (Ochsenbein and Zinkernagel 2000). As well as specifically binding antigen, anti-

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bodies interact with a range of soluble and cellular proteins to carry out a range of humoral and cellular effector functions such as complement dependent lysis, antibody-dependent cellular cytotoxicity and opsonization of pathogens for removal by phagocytosis (Nezlin and Ghetie 2004). The three-dimensional structures of human immunoglobulins have been well defined by numerous X-ray crystallographic studies of proteolytic or recombinant fragments and a few intact antibodies (Ramsland and Farrugia 2002). Similar to most extant mammals, humans have several classes of antibody (IgA, IgD, IgE, IgG and IgM) that are characterized by the type of heavy chains that are paired with the common light chains (*kappa or lambda*). In this chapter, we focus on the major classes of protective antibodies in humans (IgA, IgG and IgM) and the structural basis for their interactions with a wide variety of host and pathogen molecules through a common site located outside the antigen-binding site. Evidence that some pathogen molecules can bind to this common site on immunoglobulins and may form bridges that span adaptive and innate immune effector systems is presented.

Intact antibodies consist of two heavy chains and two light chains that interact via non-covalent contacts and covalent disulfide bridges (Fig. 1a). Two fragment antigen-binding regions (Fab) are connected to the constant region or fragment crystallizable (Fc). Depending on the class and subclass of antibody, the Fab arms exhibit a wide range of flexibility with respect to the Fc. For example, the extended hinge region in human IgG1 (depicted in Fig. 1a) allows relatively free movement of the Fab arms with respect to Fc (Roux et al. 1997), while in hinge deleted antibodies (Guddat et al. 1993) or some subclasses like IgA2 the segmental flexibility between Fab and Fc is limited (Furtado et al. 2004; Roux et al. 1998). For the IgA and IgM classes, polymeric forms consisting of dimeric (IgA) and pentameric or hexameric (IgM) further enhance the capacity of these antibodies to bind multiple epitopes or antigens resulting in high avidity interactions, even when the affinity for antigen of the individual Fab may be low. Furthermore, the multimeric Fc regions of IgA and IgM antibodies may result in enhanced interactions with effector and pathogen molecules (Czajkowsky et al. 2010).

2 A Conserved Binding Site at the Junction of Two Constant Domains

Host and pathogen molecules have been demonstrated to interact with a variety of sites on antibody including the Fab, light chain, heavy chain, hinge region, carbohydrate moieties and all constant domains (Nezlin and Ghetie 2004). It should be noted that the extensively studied inflammatory receptors for Fc (e.g., FcγRs and FcεRI) bind at a different site near the top of the two CH2 domains and the hinge region (Nezlin and Ghetie 2004), but a detailed consideration of these receptors is beyond the scope of this chapter. A junction between two constant domains on the lateral edges of the Fc is the most common target for molecular recognition of

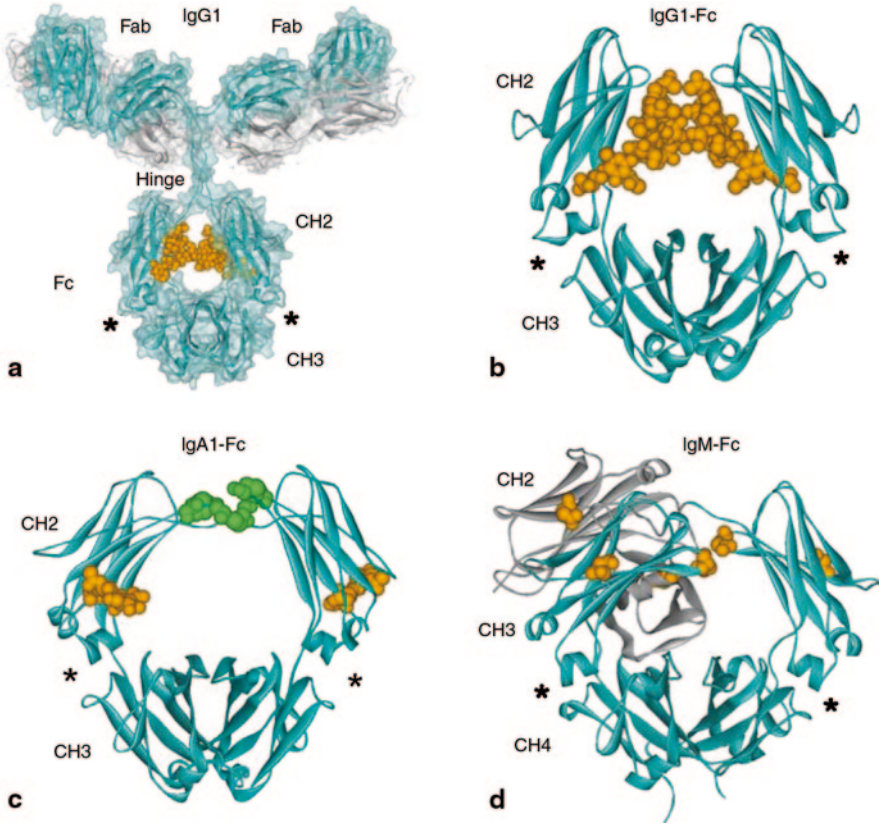

Fig. 1 Three-dimensional structures of the major protective classes of human immunoglobulin. **a** Model of intact IgG1 derived from the crystal structures of the Fc of human IgG1 (PDB code 1FC1; Deisenhofer 1981) and the Fab of a humanized anti-tumour antibody (1S3K; Ramsland et al. 2004). The IgG1 hinge region has been modelled in an extended conformation. Light and heavy chains are shown as ribbons-style representations along with the solvent-accessible surface. **b** Crystal structure of human IgG1-Fc (1FC1; Deisenhofer 1981). **c** Three-dimensional structure of human IgA1-Fc taken from its complex with a *Staphylococcus aureus* protein (2QEJ; Ramsland et al. 2007). **d** A homology model of human IgM-Fc based on its similarity with IgE-Fc as previously proposed (Czajkowsky and Shao 2009). The N-linked carbohydrates are shown as space-filling (CPK) spheres except for IgM-Fc where the putative sites are indicated by the key Asn residues (CPK spheres). Asterisks indicate the location of the common binding site for host/pathogen proteins at the junction of the CH2–CH3 (CH3–CH4 in IgM) domains

immunoglobulins (indicated with asterisks in Fig. 1). The Fc is a relatively rigid globular structure with a pseudo-twofold axis of symmetry between the two heavy chains. In IgG-Fc, the N-linked oligosaccharides attached to Asn 297 (EU index) of the second heavy chain constant domain (CH2) shields the inner surfaces of these domains and participates in some interchain carbohydrate–carbohydrate interactions (Fig. 1b). The CH3 domains interact tightly via an extended largely hy-

drophobic interface. The resultant structure resembles two arms with elbows bent and hands tightly clasped. The overall shape of the IgA-Fc resembles IgG although the N-linked oligosaccharides (Asn 263) are not sandwiched between the CH2 domains, but project outwards from the domains into the bulk solvent leaving a large opening in the middle of the IgA-Fc. The CH2 domains of IgA are held in place by disulfide linkages near the top of the two domains (Fig. 1c). The X-ray crystallographic structure of IgM-Fc is yet to be determined. However, based on sequence similarity to IgE-Fc, a three-dimensional model of IgM-Fc can be generated as previously shown (Czajkowsky and Shao 2009). In IgM (Fig. 1d), an additional domain (CH2) replaces the hinge region, while the CH3 and CH4 domain dyads resemble CH2 and CH3 of IgG-Fc and IgA-Fc. The three-dimensional locations of the N-linked oligosaccharides on IgM-Fc are not known, but the positions of the occupied asparagine residues (Arnold et al. 2005) are mainly clustered near the top of the Fc on the CH1 (Ramsland et al. 2006), CH2 and CH3 domains (Fig. 1d). Interestingly, in the CH3 domain of IgM, one Asn (OU index 402) is positioned in an analogous location to Asn 297 in IgG and the attendant oligosaccharide chain may fill the space between the CH3 domains similar to the CH2 domains of IgG-Fc. The second CH3 N-linked carbohydrate site (Asn 395) faces outwards from the sides of the IgM-Fc suggesting that this carbohydrate chain would be immersed in bulk solvent comparable to the carbohydrate associated with the CH2 domain of IgA-Fc.

The similar three-dimensional arrangements of the domains forming the Fc of IgG, IgA and IgM outlined above has resulted in the formation of homologous sites at the junction of the CH2–CH3 (CH3–CH4 in IgM) domains. This common site is primarily formed by two loops containing short α-helices from the CH2 (CH3 in IgM) domains and the F-G loop from the CH3 (CH4 in IgM) domains. A flexible linker polypeptide strand allows some flexibility of the CH2–CH3 (CH3–CH4) domain junctions. In addition, making this site suitable for recognition by multiple binding partners is the presentation of a large shallow cavity lined by multiple amino acid side-chains at the CH2–CH3 (CH3–CH4) domain junctions.

Several crystal structures have been determined for Fc regions of IgG and IgA in complex with a variety of host, pathogen and *in vitro* selected ligands. Herein we review the structures of complexes of immunoglobulin-binding molecules that bind primarily at the CH2–CH3 domain junctions of the Fc of IgG or IgA (Table 1). A wide range of protein folds have been shown to interact with the common Fc target site as illustrated by the different host and pathogen proteins, which include domains consisting of mainly β-sheet, mainly α-helix, mixed α/β-folds, monomers, dimers, glycosylated and unglycosylated proteins (Fig. 2). *In vitro* selected ligands can bind to the same target site at the CH2–CH3 domain junction of IgG and crystal structures in complex with Fc have been determined for a peptide, a peptide dendrimer and an RNA aptamer (Fig. 3).

Table 1 Crystal structures of Fc of IgG and IgA with host, pathogen and synthetic ligands bound to the conserved CH2–CH3 domain junction

PDB ID	Res. (Å)	Fc	Ligand	Reference
1FRT	4.50	IgG, mixture of subclasses (*Rattus norvegicus*)	FcRn, α-chain and β-2-microglobulin (*R. norvegicus*)	(Burmeister et al. 1994)
1I1A	2.80	IgG2a, heterodimer with one non-FcRn-binding heavy chain (*R. norvegicus*)	FcRn, α-chain and β-2-microglobulin (*R. norvegicus*)	(Martin et al. 2001)
2IWG	2.35	IgG1 (*Homo sapiens*)	TRIM21 or RO52, PRYSPRY domain (*H. sapiens*)	(James et al. 2007)
1ADQ	3.15	IgG4 (*H. sapiens*)	RF-AN Fab (*H. sapiens*)	(Corper et al. 1997)
1OW0	3.10	IgA1 (*H. sapiens*)	FcαRI or CD89 (*H. sapiens*)	(Herr et al. 2003a)
1FC2	2.80	IgG1 (*H. sapiens*)	Protein A, fragment B (*S. aureus*)	(Deisenhofer 1981)
1FCC	3.20	IgG1 (H. sapiens)	Protein G, fragment C2 (Streptococcus G148, a human group G strain)	(Sauer-Eriksson et al. 1995)
2GJ7	5.00	IgG1 (*H. sapiens*)	gE-gI (HSV-1)	(Sprague et al. 2006)
2QEJ	3.20	IgA1 (*H. sapiens*)	SSL7 (*S. aureus*)	(Ramsland et al. 2007)
1DN2	2.70	IgG1 (H. sapiens)	Peptide DCAWHL-GELVWCT (bacteriophage display)	(DeLano et al. 2000)
3D6G	2.30	IgG1 (*H. sapiens*)	Peptide dendrimer (synthetic combinatorial libraries)	(Moiani et al. 2009)
3AGV	2.15	IgG1 (*H. sapiens*)	RNA aptamer (*in vitro* selection)	(Nomura et al. 2010)

3 Functional Consequences of Host Proteins Binding to the Conserved Site on Immunoglobulins

3.1 The Neonatal Fc Receptor (FcRn) is Involved in Transplacental Transport of Maternal IgG and Maintaining Plasma IgG Levels

Transport of IgG across the placenta to the fetus in humans and from colostrum across the gut of the rodent neonate occurs through a transcytosis process mediated by FcRn, which was previously known as the Brambell receptor (Kuo et al. 2010;

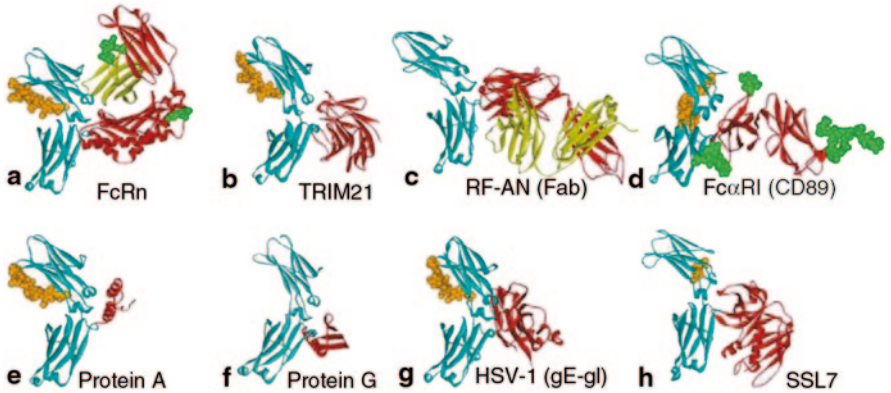

Fig. 2 Comparison of crystal structures of host and pathogen proteins bound to a conserved site on immunoglobulins. **a** Rat FcRn:IgG-Fc complex (1FRT; Burmeister et al. 1994). **b** TRIM21 (PRYSPRY domain) bound to human IgG1-Fc (2IWG; James et al. 2007). **c** Rheumatoid factor RF-AN Fab complex with human IgG4-Fc (1ADQ; Corper et al. 1997). **d** FcαRI (CD89) in complex with human IgA1-Fc (1OW0; Herr et al. 2003a). **e** Fragment B of *S. aureus* protein A bound to human IgG1-Fc (1FC2; Deisenhofer 1981). **f** Streptococcus protein G (fragment C2) bound to human IgG1-Fc (1FCC; Sauer-Eriksson et al. 1995). **g** Fc receptor of HSV-1 (gE-gI) in complex with human IgG1-Fc (2GJ7; Sprague et al. 2006). **h** *S. aureus* SSL7 bound to human IgA1-Fc (2QEJ; Ramsland et al. 2007). For clarity only one of the heavy chains (blue) from the different Fc are shown. Carbohydrates on the Fc are shown as orange space-filling (CPK) spheres and in green if the carbohydrate is on the ligand molecule. If a ligand contains two polypeptide chains these are shown in *red* and *yellow*

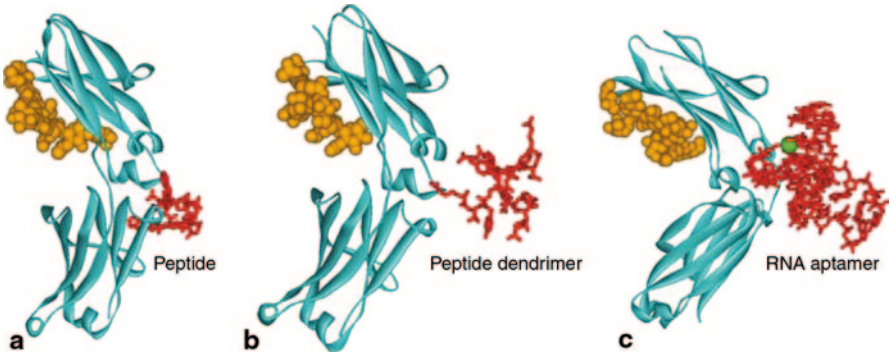

Fig. 3 *In vitro* selected ligands bound to the common site on the Fc of immunoglobulins. **a** Disulfide cyclized peptide selected by phage display in complex with human IgG1-Fc (1DN2; DeLano et al. 2000). **b** Peptide dendrimer bound to human IgG1-Fc (3D6G; Moiani et al. 2009). **c** RNA aptamer in complex with human IgG1-Fc (3AVG; Nomura et al. 2010). A calcium ion stabilizing the RNA aptamer is shown as a space-filling (CPK) sphere. A single heavy chain is shown for each complex with the carbohydrates as CPK spheres

Roopenian and Akilesh 2007). The FcRn-dependant transport across epithelial cells may also be important for IgG immunity in adults by delivering protective IgG to its various sites of action. By far the best understood role of FcRn is the maintenance of IgG levels in plasma, which is the result of FcRn expression on the recycling endosomal compartment of endothelial cells. IgG first enters the cells by pinocytosis and binds to FcRn in the endosomes at slightly acidic pH 6.0–6.5. FcRn releases IgG at pH 7.0–7.4 when the recycling endosomal compartment merges with the plasma membrane (Roopenian and Akilesh 2007). It is this FcRn-mediated recycling process that gives most subclasses of IgG a plasma half-life of >20 days in adult humans (Nezlin and Ghetie 2004). Persistence of protective IgG in the circulation is likely to maintain high levels of pathogen-specific IgG during an active infection. This view has led to significant efforts to further enhance the *in vivo* half-lives of potential therapeutic antibodies through engineering of the Fc regions for optimal FcRn binding. The efficacy of multiple therapeutic IgG monoclonal antibodies with prolonged plasma half-lives will soon be tested in clinical trials (Kuo et al. 2010; Masuda et al. 2009).

The crystal structure of rat FcRn bound IgG-Fc (Fig. 2a) from a mixture of rat IgG subclasses was first determined at a resolution of 4.50 Å (Burmeister et al. 1994). The details of the interactions between rat FcRn were confirmed and further revealed in a 1:1 complex with a heterodimeric Fc (where one heavy chain had been mutated to abolish FcRn binding) at 2.80 Å resolution (Martin et al. 2001). A crystal structure of the unliganded human FcRn has also been determined and was shown to have an overall three-dimensional structure similar to that of rat FcRn (West and Bjorkman 2000).

FcRn is a heterodimer that resembles major histocompatibility (MHC) class I molecules, consisting of an α-chain and β2-microglobulin (Simister and Mostov 1989). For MHC-peptide complexes, the peptide is located in a binding groove formed by the α1- and α2-regions of the α-chain, and bound peptide interacts with the TCR in an MHC-restricted manner (Apostolopoulos et al. 2008; Rudolph et al. 2006). In contrast, the FcRn binds IgG-Fc edge-on so that the side of the α-chain and a portion of β2-microglobulin participate in the interaction (Fig. 2a). FcRn contacts the IgG-Fc mainly at the junction between the CH2 and CH3 domains. The participation of 3 to 4 His residues in the interaction of FcRn and IgG-Fc explains the strong pH dependence of the interaction (Burmeister et al. 1994).

3.2 Tripartite Motif-Containing 21 (TRIM21) PRYSPRY Domain Binds IgG-Fc

TRIM21 is synonymous with the 52-kDa SSA/Ro protein Ro52 which has been known for many years as an autoantigen in autoimmune conditions such as systemic lupus erythematosus and Sjogren's syndrome. Antibodies to SSA/Ro are often found in these patients. In addition to its autoantigenic properties, TRIM21 was shown to form a trimer that bound specifically to the Fc region of human IgG sub-

classes 1, 2 and 4 by the C-terminal B30.2 domain (Rhodes et al. 2002; Rhodes and Trowsdale 2007). The binding site was located to the CH2–CH3 domain interface of IgG-Fc and competed with *Staphylococcus aureus* protein A. TRIM21 expression was inducible with interferon-γ. These data suggested that TRIM21 has a role in regulating IgG functions.

Interestingly, other interactions have been proposed for TRIM21. A mouse knockout indicated a function in NFκB-dependent cytokine expression (Yoshimi et al. 2009). An independent study showed that TRIM21 null mice developed inflammation due to disregulation of the IL23-Th17 pathway (Bolland and Garcia-Sastre 2009; Espinosa et al. 2009). There are marked differences in the interpretation of these two sets of data (Ozato et al. 2009). Other work showed TRIM21 interaction with IRF3 (Higgs et al. 2008; Yang et al. 2009); downregulation of active IKKβ by autophagy (Niida et al. 2010); and cytoplasmic relocation of Daxx (Tanaka and Kamitani 2010). A recent study showed the movement of TRIM21-containing cytoplasmic bodies along microtubules (Tanaka et al. 2010). These data may indicate multiple interacting partners for TRIM21, although in some studies it is possible that the IgG interaction is confounding interpretation of the data.

The IgG interaction of TRIM21 has been studied structurally in some detail. A crystal structure of TRIM21 B30.2 (PRYSPRY domain) in complex with IgG-Fc (Fig. 2b) revealed a binding interface comprised of two pockets in TRIM21 formed by six extended loops (James et al. 2007). TRIM21 interactions with IgG-Fc are primarily with the CH2–CH3 domain junction with a pocket from the SPRY-subregion recognizing the CH2 domain and a pocket from the PRY-subregion binding the CH3 domain. The crystal structure of the TRIM21 complex confirmed that the binding site overlapped with the protein A site on IgG-Fc (compare Fig. 2b–e).

The IgG-binding features of TRIM21 are conserved in different species (Keeble et al. 2008) indicating an evolutionarily important role of TRIM21 in IgG-associated immunity or turnover. Takahata et al. (2008) showed that TRIM21 through its E3 ubiquitin ligase activity can polyubiquitinate bound IgG, which is targeted to the proteosome for degradation. In addition, they identified that TRIM21-IgG complexes could interact with the chaperone p97/VCP suggesting that the endoplasmic reticulum-associated degradation (ERAD) system may have a role in IgG quality control (Takahata et al. 2008).

Mallery and coworkers (Mallery et al. 2010) have demonstrated that TRIM21 can mediate intracellular immunity by targeting antibody-bound pathogens to the proteosome for degradation. A requirement is that the antibody-bound particle (adenovirus or antibody-coated beads) must enter the cytosol for Fc-dependent TRIM21 recognition, ubiquitination and transport of the complex to the proteosome (Mallery et al. 2010). It is likely that non-enveloped viruses other than adenovirus may be neutralized by the antibody-TRIM21-ERAD pathway, but it remains unclear if enveloped viruses or non-viral pathogens are susceptible to this recently described cytoplasmic defence system.

3.3 *Autoantibodies Against IgG often Target the CH2–CH3 Domain Junction*

Rheumatoid factors (RF) are one of the most commonly occurring autoantibodies and bind to epitopes in the Fc of IgG. In rheumatoid arthritis, levels of RF have been linked to the severity of disease. However, RF are also found associated with a wide range of diseases and can also be found in healthy individuals (Bonagura et al. 1998; Mannik et al. 1988; Nezlin and Ghetie 2004). RF are normally of the IgM class, but sometimes of the IgA, IgG or IgE class. The IgM type RF displays a relatively low affinity for IgG-Fc yet high avidity IgM–IgG complexes can be formed due to the multivalency of IgM. The presence of RF-IgG immune complexes contributes to the chronic joint inflammation and erosion in rheumatoid arthritis (Mannik et al. 1988), presumably by triggering of inflammatory Fc receptors for IgG (FcγR) that are located on the surface of synovial joint effector cells (and bind to a distinct site on IgG-Fc). The reasons are unclear for the apparent lack of pathology associated with RF in healthy individuals or patients with high levels of RF such as in some cases of Waldenström's macroglobulinemia (Ilowite et al. 1991).

The crystal structure of an Fab from a monoclonal IgM (RF-AN) with RF activity was determined in complex with IgG4-Fc (Corper et al. 1997). The epitope bound by RF-AN was primarily located at the CH2–CH3 domain junction (Fig. 2c) and overlaps with, but extends further down the lateral surfaces of the CH3 domains compared to most other Fc-binding proteins (see Fig. 2). The interaction of RF-AN with IgG4-Fc is different from the majority of protein–antibody complexes since only the edge of the conventional binding site is involved in Fc recognition. This leaves the remainder of the binding site available for binding a different antigen, which may explain why many RF are cross-reactive or polyreactive with respect to antigen recognition (Corper et al. 1997).

While some RF can bind to IgG-Fc in regions outside the CH2–CH3 domain junction (Duquerroy et al. 2007), it is clear from several independent epitope mapping approaches that the majority of RF reactivity is targeted to this common site. Overlapping peptide libraries were used to establish that the linear epitopes for RF from rheumatoid arthritis patients were on solvent-exposed regions of the CH2 and CH3 domains (Peterson et al. 1995; Williams and Malone 1994). These findings confirmed earlier observations employing proteolytic fragments of IgG of various subclasses and the inhibition by protein A of most monoclonal and to a lesser extent polyclonal IgM RF (Sasso et al. 1988). The discontinuous or conformational nature of the IgG epitope recognized by most RF has been studied in detail by site-directed mutagenesis (Artandi et al. 1992; Bonagura et al. 1998). The RF IgM samples used in these studies were from Waldenström's macroglobulinemia patients and healthy blood donors immunized with mismatched human red blood cells. Despite differences in the fine specificity of RF it was clear that most of the binding occurs at the CH2–CH3 domain junction.

The propensity for human IgM to bind to peptides from the CH2 and CH3 domains of IgG may be a feature of some natural antibodies. For example, a human

IgM cryoglobulin (Mez) isolated from a patient with Waldenström's macroglobulinemia was capable of binding numerous peptides in a double-chambered binding site (Edmundson et al. 2001; Ramsland et al. 2000; Yuriev et al. 2001). Peptides recognized by Mez IgM included many of those occurring at the CH2–CH3 domain junction and the majority of the peptides were solvent-exposed in the context of intact IgG (Yuriev et al. 2002). However, Mez IgM does not bind to intact IgG suggesting that the capacity to recognize linear epitopes of IgG may be a feature of natural antibody specificity. Thus RF specificity could be readily selected in various situations from a pool of pre-existing B cells with IgM predisposed to binding IgG-Fc epitopes and particularly those residues clustered at the CH2–CH3 domain junction.

3.4 Myeloid Fc Receptor for IgA Recognizes the CH2–CH3 Domain Junction

FcαRI (also known as CD89) is a glycosylated type I transmembrane receptor with two Ig-like domains in the extracellular region, a transmembrane helix and a small cytoplasmic tail, which regulates cytoskeleton association of the receptor (Gomes and Herr 2006; Maliszewski et al. 1990). The IgA-binding α-chain associates with the common FcR γ-chain dimer, which is responsible for initiating intracellular signalling (Pfefferkorn and Yeaman 1994; Wines et al. 2006a). FcαRI is expressed on myeloid lineage cells such as dendritic cells, neutrophils, macrophages, monocytes and eosinophils (Woof and Kerr 2006) and its expression is constitutive and independent of the presence of IgA as receptor expression levels are normal even in IgA deficient patients (Monteiro and Van De Winkel 2003). FcαRI has low affinity for IgA resulting in selective binding and cross-linking of FcαRI by IgA immune complexes (Herr et al. 2003b; Wines et al. 1999), which elicit cellular effector functions such as phagocytosis, degranulation, respiratory burst and cytokine production (Wines and Hogarth 2006).

Serum and dimeric IgA opsonized bacteria activate neutrophils through FcαRI resulting in the release of leukotriene B4, a potent chemoattractant, which powerfully recruits further neutrophils. This positive feedback loop allows for a rapid cellular response to infections at mucosal sites and has been implicated in chronic inflammatory conditions such as ulcerative colitis (van der Steen et al. 2009). Indeed infection models of Gram negative bacteria, Gram positive bacteria and yeast have found pathogen specific IgA and FcαRI together confer protection against infection (Monteiro and Van De Winkel 2003). In contrast, FcαRI also has an immune-regulatory role as monomeric IgA binding to FcαRI can inhibit myeloid cell effector function (Monteiro 2010; Pasquier et al. 2005).

An early investigation of the interaction between IgA and FcαRI on monocytes demonstrated that: (1) The CH2 and CH3 domains of IgA were required for interaction with FcαRI, (2) the Fab, hinge or tail regions of IgA do not contribute to binding of FcαRI and (3) mutations around the CH2–CH3 interface disrupted bind-

ing and those away from this interface did not affect IgA interaction with FcαRI (Carayannopoulos et al. 1996). Wines et al. (2001) identified key binding residues on the first Ig-domain of FcαRI involved in binding to IgA-Fc. A model of FcαRI interacting with the CH2–CH3 domain junction of IgA was proposed (Wines et al. 2001), and the interaction fully defined at the atomic level when the crystal structure of the FcαRI:IgA-Fc complex was determined (Herr et al. 2003a).

The FcαRI:IgA-Fc crystal structure (Herr et al. 2003a), revealed that the two Ig domains of the FcαRI ectodomain are bent at approximately 90º and contain three N-linked carbohydrate chains, with two on either side of the protein–protein interface with IgA-Fc (Fig. 2d). As predicted (Wines et al. 2001), the first extracellular Ig-domain interacts predominantly with the CH2–CH3 domain junction of IgA-Fc (Fig. 2d; Herr et al. 2003a) in a binding mode that is structurally analogous to the IgG-binding proteins and bacterial IgA-binding proteins (see Fig. 2 and other sections of this chapter). The isolated IgA-Fc and presumably also monomeric IgA bind two FcαRI molecules in a proposed "standing-up" orientation with respect to the cell membrane (Herr et al. 2003a, b). It is unclear how dimeric IgA could engage FcαRI in the same orientation since one IgA molecule of the tail–tail dimer would clash with the cell membrane. Thus a lying-down orientation of IgA:FcαRI complexes on the effector cell membrane may occur in a similar way as previously proposed for FcRn-IgG complexes (Burmeister et al. 1994).

3.5 The CH3–CH4 Domain Junction of IgM is Analogous to the CH2–CH3 Site on IgG and IgA

The Fcα/μR binds IgM with high affinity and has intermediate affinity for IgA. It has been proposed that Fcα/μR has a role in endocytosis and presentation of IgM coated antigens in the early stages of the immune response (Shibuya et al. 2000). Moreover, it has been suggested that this receptor may have a role in the maintenance of IgM and IgA in the circulation similar to the role of FcRn in IgG homeostasis (Kikuno et al. 2007). Ghumra and colleagues (Ghumra et al. 2009) have located the binding site for IgM, which involves both the CH3 and CH4 domains, whereas for IgA a loop in the CH3 domain was crucial for binding to Fcα/μR. The extracellular Ig-domain of Fcα/μR resembles the polymeric Ig receptor (pIgR) and secretory component (SC, the soluble form of pIgR) was able to block IgM interactions with Fcα/μR. Furthermore, in support of the CH2–CH3 binding site on IgA, incubation with bacterial IgA-binding M proteins can inhibit interaction of IgA-coated erythrocytes with Fcα/μR (Ghumra et al. 2009).

Recently, a much sought-after high affinity receptor for IgM (FcμR) was identified as a transmembrane glycoprotein, which contains an extracellular Ig-like domain with sequence similarities to Fcα/μR and pIgR. These similarities suggest that FcμR binds IgM at the CH3–CH4 domain junction (Fig. 1d) like pIgR and Fcα/μR. In humans, FcμR is expressed on both B and T cells (Kubagawa et al. 2009), but in mice the expression appears to be restricted to B cells (Shima et al. 2010). Origi-

nally FcμR was suggested to have a role in regulating apoptosis and was designated as Fas apoptotic inhibitory molecule (FAIM3) or TOSO. However, only anti-Fas IgM (not IgG) antibody was capable of inhibiting apoptosis showing that the FcμR has no direct anti-apoptotic role (Kubagawa et al. 2009). The uptake of IgM-coated beads by FcμR expressing cells suggests a role for the receptor in IgM-mediated antigen uptake and presentation by B cells (Shima et al. 2010). Additionally, the expression of FcμR on human T cells (CD4 and CD8 positive) suggests that it may function in T cell help and IgM-dependent cell mediated cytotoxicity (Kubagawa et al. 2009). However, the proposed biological roles of FcμR require further experimental validation.

4 Hijacking of Immunoglobulins by Pathogens: Recognition of the Conserved Target Site

Several important pathogenic microorganisms are known to express Fc-binding proteins. As outlined below, these extensively studied proteins have been implicated in pathogenesis, but it should be noted that their biological role remains unclear. Since total concentrations of all antibody classes far exceeds the levels of pathogen-specific antibody, it is likely that the Fc-binding pathogen molecules would be quickly saturated with irrelevant antibody, leaving protective antibodies to directly interact with their antigens on the pathogen and elicit effector functions. However, it is possible that surface-localized Ig-binding proteins participate in "bipolar bridging", implying that the protective antibody binds a surface antigen, followed by binding of its Fc part to an adjacent Fc-binding protein located on the same surface. Alternatively, the main role of pathogen molecules in virulence might be unrelated to their immunoglobulin binding properties. Indeed, most of the well characterized proteins (e.g., staphylococcal protein A and streptococcal M proteins) bind other host factors, which include key members of the innate immune system such as complement or complement regulatory molecules (for review see Lambris et al. 2008; Smeesters et al. 2010). It is possible that the dual (or multiple) binding properties, of secreted and surface proteins of pathogens, act cooperatively with immunoglobulin binding by forming regulatory bridges between adaptive and innate immunity.

4.1 Bacterial Proteins A and G Target the CH2–CH3 Junction of IgG

Bacterial IgG-binding proteins have been isolated from a variety of infectious organisms. The best known examples are protein A from *S. aureus* and protein G from group C/G Streptococcus bacteria. Proteins A and G are cell-surface proteins with the extracellular regions comprised of several repeating domains with

binding specificity for IgG and other host ligands. Protein A has five IgG-binding domains (E, D, A, B, C) and protein G has three (C1, C2, C3), but the sequences and structures of these domains, while similar within a protein, are very different between the two proteins. Although these bacterial proteins are in wide-spread use as tools in immunochemical studies and purification of IgG, the function in bacterial pathogenesis of the IgG-binding property is still not completely understood.

A comparison of the crystal structures of the B domain of protein A (Fig. 2e) and the C2 domain of protein G (Fig. 2f) with human IgG1-Fc, reveals that the two proteins bind at the same general interface and engage many of the same residues at the CH2–CH3 domain junction. However, very different strategies are used by protein A and G for binding IgG. Protein A utilizes two α-helices from a compact 3-helix bundle to form two contact regions with IgG1-Fc (Fig. 2e). Both helices participate in the first contact that consists of residues from both the CH2 and CH3 domains. The second interaction site only involves residues of the α2-helix of protein A, which interact with residues only from the CH3 domain of IgG (Deisenhofer 1981). In contrast, the IgG-binding module of protein G consists of an α-helix lying diagonally across a four-stranded β-sheet (Fig. 2f). As an indication of the differences in the interactions of the two IgG-binding proteins, the protein G α-helix does not overlay with either helix from protein A. Furthermore, only a few residues of the CH2 domain are involved in binding to protein G with the majority of the interactions occurring with the CH3 domain. The protein G:IgG1-Fc interface constitutes three binding regions: (1) A large hydrogen bonding network involving one face of the α-helix (comprising 4 charged and 3 polar residues) that interacts with one residue from CH2 and four residues from CH3 domains of IgG, (2) a pair of residues (Glu 27 and Lys 31) also on the α-helix of protein G that form hydrogen bonds with the main-chain nitrogens of IgG residues in the CH2 domain and, (3) a region from the N-terminus of the α-helix, the connecting loop and the third β-strand of protein G, which interacts with a CH2 residue and two residues from the CH3 domain of IgG (Sauer-Eriksson et al. 1995).

As indicated above, the advantage to the bacteria of recruitment of bulk plasma IgG to the surface of *S. aureus* or group G/C streptococcal bacteria has not been fully resolved. However, the IgG-Fc-binding property has been demonstrated to dramatically boost the presentation of antigens coated with IgG and soluble protein A or G. This led to the suggestion that during an infection secreted forms of proteins A and G through enhanced antigen presentation will result in non-specific adaptive immune responses (Leonetti et al. 1999). Capture of IgG on the bacterial surface by protein A or G may also result in broad non-specific immune responses through the avid engagement of cellular receptors such as the activating FcγRs that bind to the hinge region and upper CH2 domains and are involved in antigen presentation, cytokine release and inflammation. Another possibility is the engagement/inhibition of complement receptors or regulators through IgG or bound complement proteins on the captured IgG (Nezlin and Ghetie 2004). Protein G when bound to IgG also blocks the C1q binding site suggesting it has a role

in inhibiting opsonization by complement (Nitsche-Schmitz et al. 2007). Thus it is possible that both protein A and G function by binding IgG-Fc to produce non-specific adaptive immune responses and indirectly, through IgG-complement interactions, engage or evade the innate effector arm of cellular immunity. Furthermore, in addition to binding IgG-Fc, protein A can bind to VH3 domains in the Fab region of human immunoglobulin on the surface of B cells (Graille et al. 2000), which can result in a type of "superantigen" mediated B cell death causing a defect in the antibody response to *S. aureus* (Goodyear et al. 2007). Finally, a recent study demonstrated that a protein A derivative lacking ability to bind IgG elicited antibodies protecting against *S. aureus* infection, and it was suggested that these antibodies block a toxic effect of protein A released from the bacteria (Kim et al. 2010).

4.2 An Fc Receptor for IgG from HSV-1 is Implicated in Antibody Bipolar Bridging

A receptor for the Fc of IgG (the gE–gI heterodimer) is expressed on the surface of virions and cells infected with HSV-1 (Baucke and Spear 1979; Johnson et al. 1988). Similar to other pathogen IgG-binding proteins, HSV-1 gE-gI is proposed to be involved in immune evasion by interfering with antibody-mediated effector functions. A mechanism of bipolar bridging has been demonstrated where IgG specific for HSV-1 viral antigens can bind through the Fab arms to antigen and the Fc of the same antibody is captured by the gE–gI heterodimer. Such bipolar bridging effectively prevents the binding of the HSV-1 specific IgG with FcγRs and complement component C1q and protects virions and infected cells from antibody-mediated immune clearance (Dubin et al. 1991; Frank and Friedman 1989).

The 5.0 Å resolution structure of the IgG1-Fc:gE–gI heterodimer and the 1.78 Å resolution structure of the minimal binding fragment (CgE) have revealed the structural basis for antibody bipolar bridging in HSV-1 immune evasion (Sprague et al. 2006). The gE–gI heterodimer was bound at the CH2–CH3 domain junctions of the IgG-Fc molecule (Fig. 2g). The gE part of the gE–gI heterodimer contributes all the interactions with IgG-Fc (Sprague et al. 2006). The high resolution structure of the CgE domain shows it is composed of 14 β-strands arranged into 3 β-sheets that form a variable type Ig domain (first two β-sheets) and an "inserted" three-stranded parallel β-sheet. Inspection of the low resolution structure of the IgG-Fc:gE–gI complex shows that the interactions with Fc are mainly contributed by the first two β-sheets of the variable type Ig domain of CgE. The binding site overlaps with other IgG-Fc-binding proteins (see Fig. 2). When the antigen-binding site in an IgG molecule binds HSV-1 surface antigen (such as gC or gD), the IgG molecule may therefore participate in bipolar bridging of the Fc part to the gE–gI heterodimer, which is proposed to block access of C1q and FcγRs to their binding sites on IgG (Sprague et al. 2006).

4.3 *Bacterial IgA-Binding Proteins Interact with the Common CH2–CH3 Binding Site and may be Implicated in Evasion of IgA Immunity*

Staphylococcus aureus colonizes mucosal sites like the nasal passage. *S. aureus* infections can remain asymptomatic (in carriers) or progress to cause superficial skin infections (e.g., boils) or life-threatening conditions like sepsis, toxic shock syndrome, osteomyelitis, pneumonitis and endocarditis (Dancer 2008; Jarvis et al. 2007). Among the array of proteins produced by different strains of *S. aureus* all isolates express and secrete some members of the staphylococcal superantigen-like (SSL) protein family, which are encoded by *ssl* genes located in a pathogenicity island, designated νSaα (Lindsay and Holden 2006). The prototypical member SSL7 (formally named SET1) is found in the majority of clinical isolates and is capable of binding with high affinity to serum and secretory IgA and complement component C5 (Langley et al. 2005). SSL7 is thought to block IgA-mediated effector functions by competitive inhibition of FcαRI on myeloid lineage effector cells (Wines et al. 2006b).

We determined the crystal structure of the human IgA1-Fc in complex with SSL7 at a resolution of 3.2 Å (Ramsland et al. 2007). The IgA1-Fc binds two SSL7 molecules with the interactions primarily occurring at the CH2–CH3 domain junction (Fig. 2h). Two loops from the OB-fold (oligosaccharide binding fold) of SSL7 clasp the IgA1-Fc and form the primary interface with residues from the CH2 and CH3 domains, a site that co-localizes with the primary FcαRI binding site. The dominant binding site for SSL7 is at the CH2–CH3 domain junction as confirmed by site-directed mutagenesis of this region (Ramsland et al. 2007; Wines et al. 2006b). The edge of the β-grasp domain of SSL7 participates in additional interactions with the lower parts of the IgA-Fc CH4 domains so that the SSL7 molecules shield most of the lateral surfaces of the end of the IgA-Fc. Consequently, the extended recognition site of SSL7 differs somewhat from the interaction site of FcαRI, which is focused only at the CH2–CH3 domain junction (see Fig. 2d, h).

Bacterial IgA-binding proteins were first characterized in two important human pathogens, group A and group B streptococci (Lindahl and Akerstrom 1989; Lindahl et al. 1990). The IgA-binding proteins expressed by these streptococcal pathogens bind to the common CH2–CH3 target site (Pleass et al. 2001), as also reported for the staphylococcal SSL7 protein (Ramsland et al. 2007; Wines et al. 2006b). In group A streptococci (GAS; *Streptococcus pyogenes*), the IgA-binding proteins are members of the M protein family, a family of surface-located dimeric coiled-coil proteins. These proteins are best known for their ability to inhibit phagocytosis but contribute to virulence also by other mechanisms (McNamara et al. 2008; Smeesters et al. 2010). The M proteins vary extensively in sequence among strains, in particular in the "hypervariable" N-terminal region, and all M proteins bind one or more human plasma proteins, although the binding properties vary between M proteins expressed by different strains. This recruitment of plasma proteins to the bacterial surface plays a key role for the ability of an M protein to confer phagocytosis resistance (Carlsson et al. 2003).

For this chapter it is of particular interest that many M proteins (e.g., M4, M22 and M60) bind IgA-Fc and have a separate binding site for the complement regulator C4b-binding protein (C4BP) (Thern et al. 1995). While the C4BP-binding region is located in the N-terminal hypervariable region, the IgA-binding region resides in an adjacent non-overlapping semi-variable region (Carlsson et al. 2003; Sandin et al. 2002). Both of these regions represent distinct domains that retain their ligand-binding properties when studied in isolated form (Morfeldt et al. 2001; Sandin et al. 2002) and both regions contribute to phagocytosis resistance (Carlsson et al. 2003). Extensive sequence variability characterizes not only the N-terminal C4BP-binding region but also the IgA-binding region, implying that the ligand-binding property has been retained in spite of strong evolutionary pressure (Persson et al. 2006; Schmitt et al. 2010). This conservation of ligand-binding property supports the notion that the binding to IgA enhances bacterial virulence. The ability of the M proteins to bind IgA has also been implicated in the pathogenesis of IgA-nephritis, the most common glomerulonephritis worldwide (Schmitt et al. 2010).

In group B streptococci (GBS; *Streptococcus agalactiae*), binding of IgA is conferred by the surface-localized beta protein, also known as Bac or beta C (Heden et al. 1991). The beta protein has separate (non-overlapping) binding sites for IgA-Fc and the human complement regulator factor H (Areschoug et al. 2002) and also binds human Siglec-5, an inhibitory receptor expressed on leukocytes (Carlin et al. 2009). However, the beta protein binds only weakly to secretory IgA, which is surprising considering that GBS colonizes mucosal surfaces (Lindahl et al. 1990).

4.4 A Malarial Surface Antigen can Bind Polymeric IgM by Residues in the CH4 Domain

Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) can interact with human IgM and this binding is correlated with severe placental and cerebral malaria (Czajkowsky et al. 2010). Ghumra et al. (2008) used domain-swapped antibodies, mutant IgM molecules and monoclonal anti-IgM antibodies to demonstrate that the PfEMP1 binding site on IgM is located on the CH4 domain. Only polymeric IgM bound appreciably to the PfEMP1 molecules suggesting a low affinity yet high avidity interaction. The interaction sites for PfEMP1 on CH4 of IgM are within two exposed loops, one of which (Pro-Asn-Arg-Val, residues 444–447) is structurally homologous to the solvent exposed F-G loop of CH3 in IgG and IgA, which interacts with the various host and pathogen molecules discussed above. The available data suggests that PfEMP1 binds close to the common site at the junction of the CH3–CH4 domains (Fig. 1d), but this site is not identical to that recognized by Fcα/μR and potentially the recently characterized FcμR (Czajkowsky et al. 2010; Ghumra et al. 2008).

4.5 Convergent Recognition of the CH2–CH3 Domain Junction

The adaptability of the CH2–CH3 (CH3–CH4 in IgM) site of immunoglobulins is evident from the structurally diverse host and pathogen proteins that share overlapping binding sites on the Fc (Fig. 2). A striking example is provided by the structurally unrelated protein A and protein G, both of which bind to the CH2–CH3 target site in IgG (Frick et al. 1992). Moreover, the unrelated streptococcal and staphylococcal IgA-binding proteins all target the CH2–CH3 site in IgA, as described above. These binding properties must be the result of convergent evolution favouring binding of the CH2–CH3 site in IgG and IgA. *In vitro* selection experiments with peptide, peptide dendrimer and RNA aptamer ligands further demonstrate that a wide variety of ligands can converge for recognition of a common target site on immunoglobulins. Crystal structures illustrate these convergent recognition strategies for human IgG1-Fc, which has been co-crystallized with: (1) a disulfide-cyclized peptide derived from bacteriophage display (Fig. 3a; DeLano et al. 2000), (2) a peptide dendrimer containing Arg-Thr-Tyr functionalized branches (Fig. 3b; Moiani et al. 2009) and, (3) an RNA aptamer stabilized by a calcium ion (Fig. 3c; Nomura et al. 2010). While it has been speculated that this common binding site on the Fc of immunoglobulins is promiscuous (and by implication relatively non-specific) in binding ligands, the very high affinities of some of the interactions would suggest that the specificity of many of these interactions is exquisite. Furthermore, the physical and chemical attributes of the conserved interdomain junction of immunoglobulins are obviously very suited for recognition by numerous natural and synthetic ligands (reviewed by Nezlin and Ghetie 2004).

5 Pathogen Proteins as Bridges between the Adaptive and the Innate Immune Systems

An intriguing aspect of the immunoglobulin-binding bacterial proteins described here is their ability to bind not only immunoglobulin but also other human proteins. For example, protein A binds both IgG, tumour-necrosis factor receptor 1 (Gomez et al. 2004) and von Willebrandt factor, a component of the coagulation system (Hartleib et al. 2000), and protein G binds both IgG and albumin (Akerstrom et al. 1987). Concerning interactions with the immune system, it is of particular interest that all bacterial IgA-binding proteins bind components of the human complement system, a key part of innate immunity. Thus, these IgA-binding proteins combine the ability to interact with components of the innate and adaptive immune systems. The functional significance of this bridging between innate and adaptive immunity remains unclear, but it could play an important role during infection. Concerning interactions with complement components, it should be remembered that Gram-positive bacteria (like streptococci and staphylococci) are not sensitive to the lytic function of complement, but may be eliminated through the opsonization and re-

cruitment of phagocytes that follows complement activation, effects the pathogen must evade to successfully establish an infection.

Remarkably, different bacterial IgA-binding proteins bind different complement components. In *S. pyogenes* (GAS), all IgA-binding M proteins studied so far have a separate binding site for the complement regulator C4BP. In spite of the large size of these two ligands, they do not inhibit the binding of each other (Thern et al. 1995). In *S. agalactiae* (GBS), the IgA-binding beta protein has separate binding sites for IgA and the complement regulator factor H (Areschoug et al. 2002). In *S. aureus*, the IgA-binding protein SSL7 has separate binding sites for IgA and complement component C5 (Langley et al. 2005), two interactions that have been subjected to detailed structural and functional analysis (Laursen et al. 2010; Ramsland et al. 2007).

The crystal structure of the complex of SSL7 and C5 was recently determined and showed that the β-grasp domain of SSL7 binds C5 at a site distant from the cleavage site for production of C5a and C5b by the C5 convertase (Laursen et al. 2010). Nevertheless, the binding of C5 to SSL7 blocks formation of C5a, an effect that may promote bacterial virulence (Bestebroer et al. 2010). While inhibition of C5a production can be achieved by SSL7, the inhibition is enhanced by the presence of non-immune IgA (Bestebroer et al. 2010; Laursen et al. 2010). This finding suggests that the bridging by SSL7 of C5 and IgA can effectively block access of the C5 convertase to its cleavage site in C5 (Laursen et al. 2010). Although *S. aureus* is not sensitive to lysis by the membrane attack complex (MAC), SSL7 in an IgA-dependent manner is an effective inhibitor of the final stages of the complement cascade by preventing the formation of C5b, a key component of MAC (Laursen et al. 2010). This finding suggests that SSL7-IgA-C5 complexes may confer some advantage to *S. aureus* by evading a putative non-lytic function of surface-associated MAC.

To examine the possibility of forming SSL7 bridges between IgA and C5 ligands we superimposed the crystal structures of SSL7:IgA1-Fc and SSL7:C5 by rigid-body overlays of the SSL7 molecules. A model of the complex between a single IgA1-Fc with two complexes of SSL7-C5 could be readily generated without any steric clashes between molecules (Fig. 4a). Solution structures have been reported for the various forms of human IgA using small-angle scattering, ultracentrifugation and model-building techniques (Boehm et al. 1999; Bonner et al. 2009, 2008). The molecular envelop for the best fit models for IgA were used for comparison with the model of IgA1-Fc:$SSL7_2$:$C5_2$. As with the isolated Fc, monomeric IgA1 (or IgA2) could easily accommodate two SSL7:C5 complexes (Fig. 4b). Dimeric IgA has a slightly bent planar structure where the two IgA monomers are associated tail-to-tail via their disulfide bonded tailpieces (Bonner et al. 2008). Only a single SSL7:C5 complex could be bound to dimeric IgA on the convex side of the molecule (Fig. 4c). Secretory IgA could not accommodate even a single SSL7:C5 complex (Fig. 4d) due to the apparent blocking of access to the sites by secretory component and the J-chain (Bonner et al. 2009). On the concave side of secretory IgA (or dimeric IgA) it would be possible for a single SSL7 molecule to bind with a small amount of conformational change or induced fit

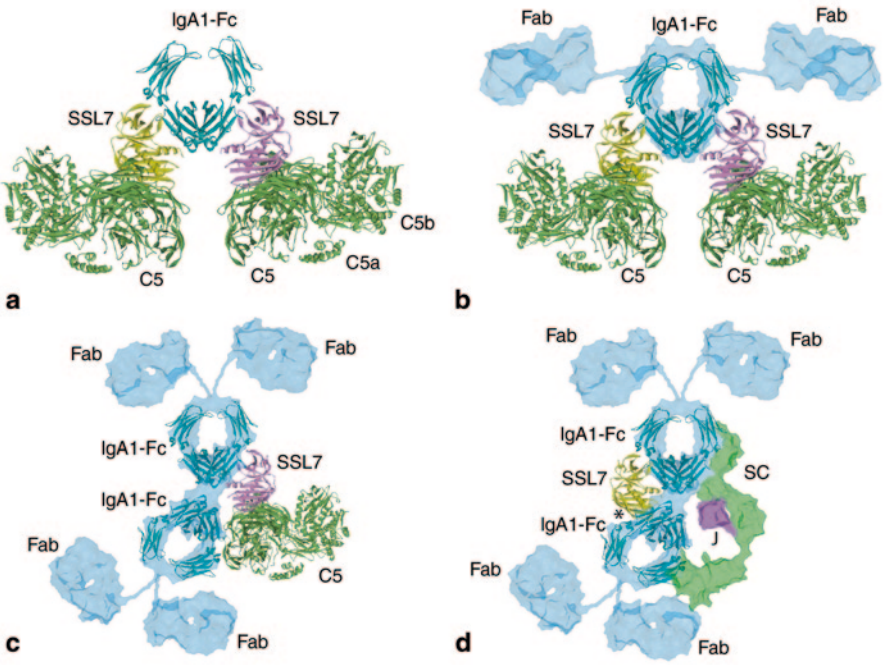

Fig. 4 The dual binding properties of SSL7 for IgA1-Fc and C5 may facilitate the formation of regulatory bridges for adaptive and innate immunity by *S. aureus*. **a** Proposed regulatory bridge where SSL7 simultaneously binds to IgA1-Fc and C5. Model was generated by the rigid-body superposition of SSL7 molecules from independent complexes with IgA1-Fc (2QEJ; Ramsland et al. 2007) and C5 (3KLS; Laursen et al. 2010). This model was used to assess the likely complexes of SSL7 and C5 with the various forms of IgA, for which structures have been predicted by small-angle solution scattering techniques. **b** Human monomeric IgA1 (and IgA2) can bind two SSL7-C5 complexes. Surface indicates the solution scattering envelope for IgA1 (1IGA; Boehm et al. 1999). **c** Dimeric IgA with a single SSL7-C5 complex bound (2QTJ; Bonner et al. 2008). **d** Secretory IgA with the attached secretory component (SC) and J-chain may only bind one molecule of SSL7 by an induced fit mechanism (3CHN; Bonner et al. 2009). An asterisk indicates a small steric clash between SSL7 and IgA-Fc in a rigid body superposition model

(Fig. 4d). This model for secretory IgA:SSL7 interaction is supported by binding data showing that SSL7 interacts strongly with serum and secretory IgA (Langley et al. 2005).

Although the cleavage site for the C5 convertase is not directly blocked in the complexes with SSL7 and IgA (see Fig. 4a), it is clear that potent inhibition of the production of C5a requires both SSL7 and IgA (Laursen et al. 2010). The C5 convertases are large multimeric complexes that in order to cleave C5 into C5a and C5b need to gain access to a large surface area around the cleavage site. Thus it has been proposed that SSL7 can block access of the C5 convertase and access can be further blocked by IgA (Laursen et al. 2010). Together these data suggest that SSL7 of *S. aureus* may form an inhibitory bridge between adaptive and innate immunity.

6 Concluding Remarks

A common site at the junction of two heavy chain constant domains is recognized by multiple host, pathogen and *in vitro* selected ligands, providing a remarkable example of convergent evolution. Because the host and pathogen molecules that bind at the common site have important roles in immune regulation and microbial virulence, further studies of these proteins promises to provide information that is of both fundamental interest and of relevance to the prevention and treatment of human disease. In particular, it will be important to identify the exact biological role of the binding of immunoglobulins to the surface of major pathogens such as staphylococci and streptococci. While it may seem obvious that these interactions allow the pathogen to inhibit the effector functions of bound immunoglobulin molecules, it remains uncertain whether this is the main function of the microbial molecules. In this context, it is of interest that most of the key pathogen molecules bind other host factors such as, but by no means limited to, complement components and complement regulatory molecules. Possibly, these interactions provide a means for the pathogens to bridge innate and adaptive immunity, as proposed for the SSL7 protein of *S. aureus*. Similarly, the ability of streptococcal IgA-binding proteins to bind both IgA and a complement regulator could promote bridging. Other examples of regulatory bridges linking adaptive and innate immunity may emerge, once the complexity of host-pathogen interactions has been further examined.

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Advances in Understanding the Structure, Function, and Mechanism of the SCIN and Efb Families of Staphylococcal Immune Evasion Proteins

Brandon L. Garcia, Kasra X. Ramyar, Daniel Ricklin, John D. Lambris and Brian V. Geisbrecht

Abstract Our understanding of both the nature and diversity of Staphylococcal immune evasion proteins has increased tremendously throughout the last several years. Among this group of molecules, members of the SCIN and Efb families of complement inhibitors have been the subject of particularly intense study. This work has demonstrated that both types of proteins exert their primary function by inhibiting C3 convertases, which lie at the heart of the complement-mediated immune response. Despite this similarity, however, significant differences in structure/function relationships and mechanisms of action exist between these bacterial proteins. Furthermore, divergent secondary effects on host immune responses have also been described for these two protein families. This chapter summarizes recent advances toward understanding the structure, function, and mechanism of the SCIN and Efb families, and suggests potential directions for the field over the coming years.

Keywords Immune Evasion • Complement Inhibitors • *Staphylococcus aureus* • Structural Biology • Mechanism • C3 Convertase • SCIN • Efb

1 Complement-directed Immune Evasion by *Staphylococcus aureus*

Few human pathogens have kept the world in suspense over the past few decades as much as *Staphylococcus aureus*. Originally considered an opportunistic bacterium associated with superficial skin infections, *S. aureus* has evolved into a major medical problem in hospital environments and, increasingly, in otherwise healthy communities (DeLeo et al. 2010). It has been reported that about one third of healthy individuals in the United States are colonized with *S. aureus*, mostly in the nostrils and skin, without showing symptoms and the bacterium may therefore

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be often considered part of the normal flora (Gorwitz et al. 2008). Yet in more and more cases, *S. aureus* strains cause severe infections, including life-threatening septicemia, which can be poorly controlled by today's therapeutic arsenal due to the bacterium's extraordinary ability to acquire resistance to antibiotics (Chambers and Deleo 2009). In 2005, the number of deaths associated with methicillin-resistant *S. aureus* (MRSA) infections in the USA was estimated to be around 19,000, thereby exceeding those associated with HIV (Klevens et al. 2007; Kluytmans et al. 2009). Although MRSA strains have been described as early as 1960 (e.g., COL strain), the current community-associated MRSA strains (e.g., USA300) appear to be much more virulent (Chambers and Deleo 2009). Although the exact driving forces behind this increased virulence are not clearly identified, immune evasion strategies are considered potential contributors to this phenomenon (Chambers and Deleo 2009). Indeed, *S. aureus* has been described to interfere at various levels of the immune system and more than 50 expressed or secreted proteins are currently considered part of its immune evasion arsenal (Chavakis et al. 2007). Interestingly, many of those molecules appear to target the human complement system, thereby moving this ancient branch of innate immunity into the spotlight of research efforts (Foster 2005; Haspel et al. 2008).

Although complement is involved in many physiological functions ranging from cell homeostasis to tissue development and metabolism, the rapid recognition and elimination of microbial intruders defines a key role of this network of plasma proteins and cell surface receptors (Ricklin et al. 2010). After recognition of bacterial surfaces by specialized pattern recognition proteins, complement can be triggered by various routes (historically termed as "classical", "lectin", and "alternative" pathways) that all result in the activation of complement component C3 to its C3b fragment, which is deposited as an opsonin on the foreign surface. Together with two enzymes, factor B (fB) and factor D (fD), C3b forms a transient yet potent C3 convertase complex (C3bBb). This convertase activates even more C3 into C3b and thereby amplifies the complement response. In addition, C3b and its degradation products (i.e. iC3b and C3d) act as ligands for complement receptors (CR) that mediate phagocytosis of opsonized particles (i.e., CR1, CR3, CR4, and CRIg) or lower the threshold for B-cell activation (CR2) and thereby bridge to adaptive immune responses. Finally, ongoing complement activation produces the potent pro-inflammatory anaphylatoxins C3a (as a byproduct of C3 cleavage) and C5a (through C5 convertases such as C3b3bBb), which attract and activate immune cells via binding to anaphylatoxin receptors (i.e., C3aR, C5aR, and C5L2).

Whereas human cells are protected by expressing cell surface-bound or acquiring soluble complement regulators, microbial cells normally lack these regulators and are therefore susceptible to complement attack (Fig. 1a) (Ricklin et al. 2010). However, an impressive body of research in recent years has revealed that *S. aureus* produces a fascinating panel of inhibitors that block complement activity at all stages from initiation and amplification to phagocytosis and inflammatory/immune signaling (Fig. 1b, c) (Laarman et al. 2010; Lambris et al. 2008; Serruto et al. 2010). Owing to its central role in the cascade, the C3 convertase appears to be a

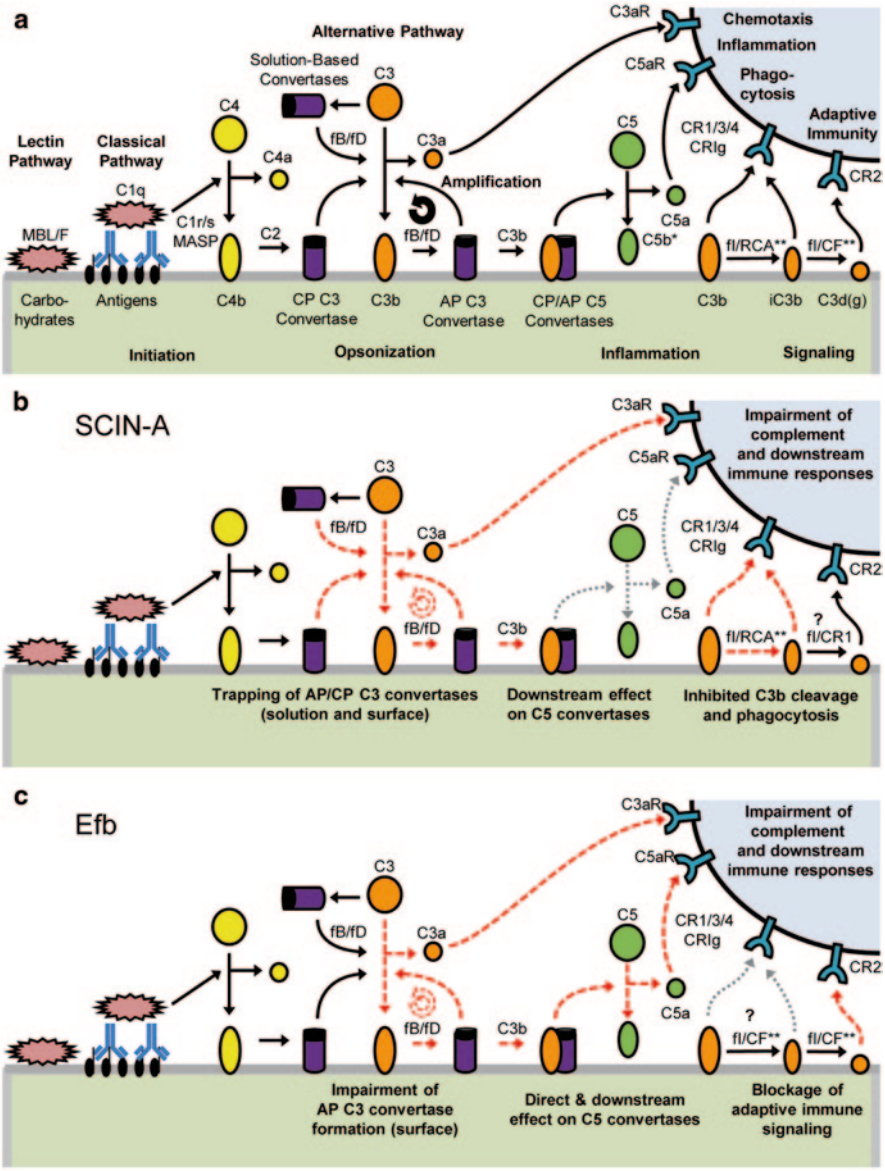

Fig. 1 Pathways of complement activation and amplification in the vicinity of an *S. aureus* cell. **a** A normal, uninhibited complement response that arises from either the classical, lectin, or alternative pathways, and which leads to changes in various immune effector cells (*top right*). **b** Disruption of the complement response by SCIN-A. **c** Disruption of the complement response by Efb. Note that red dashed lines signify processes affected directly by either of these secreted inhibitors, while dotted grey lines indicate secondary, indirect effects. Formation of the terminal complement complex is not shown, since the bacterium is resistant to its function owing to the peptidoglycan-rich structure of its gram positive cell wall (Frank 2001)

particularly attractive target for *S. aureus*. At least three different groups of inhibitory proteins, the extracellular fibrinogen-binding protein (Efb) and staphylococcal complement inhibitor (SCIN) families as well as the staphylococcal binder of immunoglobulins (Sbi), have been identified and characterized. Of these, SCIN (i.e., SCIN-A/B/C) and Efb (and its homologue Ehp; also referred to as Ecb) have proven to be particularly strong inhibitors of convertase activity. These properties not only highlight their potential roles in the overall virulence of the bacterium, they may also render these rather small, secreted proteins (~10 kDa) attractive templates for therapeutic intervention in the ever-growing list of diseases where complement-mediated inflammation is known to play a central role (Ricklin et al. 2007). For this purpose, detailed knowledge about these proteins'exact mechanisms of action, as well as key structures and residues that impart their unique activities is essential.

In view of their similar structures (trihelical bundle), their common binding partners (C3 and its activation fragments), and their identical functional targets (C3 convertases) in the complement cascade, it would seem obvious that the SCIN and Efb protein families share similar mechanisms of action. However, detailed molecular, structural, and functional studies from the past few years have surprisingly revealed that their binding modes and inhibitory mechanisms are fascinatingly distinct and include both directly competitive and allosteric strategies (Chen et al. 2010; Ricklin et al. 2009; Rooijakkers et al. 2009). There are even first indications that small differences within the same family (e.g., between Efb and Ehp) may result in slightly modified structural properties that could allow *S. aureus* to fine-tune its evasion response. As a consequence, this chapter discusses the structural and functional properties of these convertase-targeting evasion protein families, their exciting secondary effects on host immunity, and their potential implications for overall immune evasion by *S. aureus*.

2 Recent Developments Regarding the Staphylococcal Complement Inhibitor Family

2.1 Background

The founding member of the SCIN family (i.e., SCIN-A) was discovered following analysis of an uncharacterized gene on the SaPI5 immune evasion cluster (Rooijakkers et al. 2005). Further work demonstrated that the SCIN family consists of three active members (SCIN-A, SCIN-B, and SCIN-C) (Rooijakkers et al. 2007), all of which share between 46% and 48% sequence identity with one another. A putative fourth SCIN family member (typically referred to as ORF-D, but herein as SCIN-D) is also expressed by *S. aureus*, and likewise shares relatively high amino acid sequence identity (~30%) to the other SCIN family members (Rooijakkers et al. 2007). However, SCIN-D does not appear to bind directly to any C3 fragments

and thus exhibits no inhibition of complement activity (Rooijakkers et al. 2007). SCIN proteins are potent complement inhibitors and were shown to target both the classical and alternative pathway C3 convertases (C4b2b and C3bBb, respectively) (Jongerius et al. 2007; Rooijakkers et al. 2005), the latter of which is the central enzymatic component of complement amplification. Whereas many classes of complement modulators (in particular the host-derived "regulators of complement activation" and their viral homologues (reviewed in Zipfel and Skerka 2009)) possess decay accelerating activity and dissociate assembled C3 convertases (Wu et al. 2009), the active SCIN family members actually stabilize C3 convertases both in solution and at the bacterial surface (Rooijakkers et al. 2005; Rooijakkers et al. 2009). Recent work has elucidated many details about the molecular mechanism of SCIN proteins, and revealed how these bacterial inhibitors trap C3 convertases in a catalytically inactive state (Ricklin et al. 2009; Rooijakkers et al. 2009).

2.2 Structure and Active Sites of SCIN-A

A crucial first step toward understanding the complement inhibition properties of SCIN proteins was provided by the crystal structure of SCIN-A (Rooijakkers et al. 2007) (Fig. 2a). The SCIN-A structure revealed a three alpha helix bundle arranged in a compact coiled-coil, and which was most topologically similar to that described previously for the IgG-binding staphylococcal protein A (SpA) module (Gouda et al. 1992). In conjunction with multiple sequence alignment, the SCIN-A structure was used to guide construction of protein chimeras between SCIN-A and the reportedly inactive member of the SCIN family, SCIN-D (Rooijakkers et al. 2007). It was this transposition of amino acid sequences from SCIN-D into SCIN-A which subsequently identified a contiguous stretch of 18 amino acids (residues 31–48) that were critical for complement inhibition by SCIN-A, as judged by both inhibition and stabilization of the alternative pathway C3 convertase. As will be discussed shortly in Sect. 2.4 below, these peculiar stabilizing properties would prove invaluable to providing the first high-resolution structural data on the alternative pathway convertase, C3bBb (Rooijakkers et al. 2009).

2.3 Structure/Function Analysis of SCIN-A Binding to C3c/C3b

Whereas initial interaction studies suggested that SCIN proteins could only bind within the context of fully assembled C3 convertases (Rooijakkers et al. 2005), an improved surface plasmon resonance (SPR)-based approach designed to mimic the physiological orientation of covalently deposited C3b provided the first evidence for direct binding between these proteins and C3b (Ricklin et al. 2009). In this same study, direct binding of SCIN-A to C3b was also characterized by solution-based isothermal titration calorimetry (ITC) (Ricklin et al. 2009). Even though these two

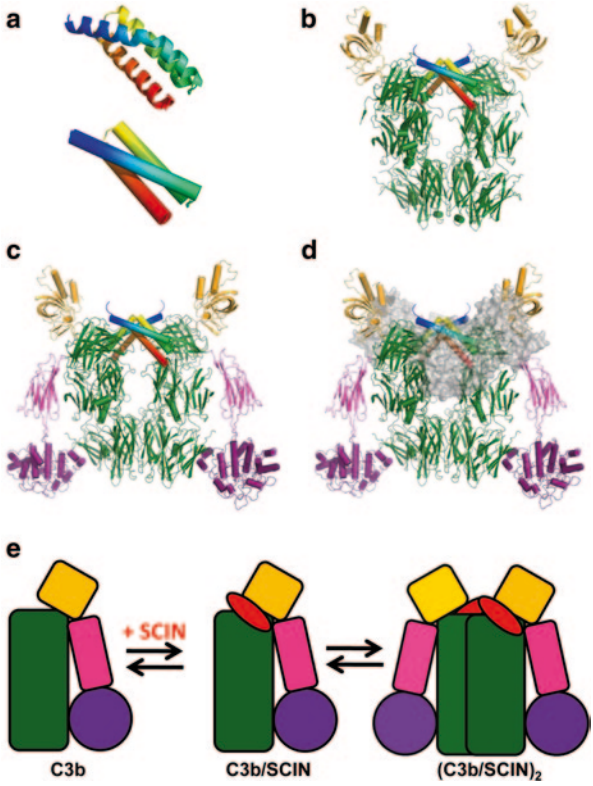

Fig. 2 Structural properties of the SCIN-A protein both free and bound to complement components. **a** Crystal structure of the unbound form of SCIN-A, shown in both ribbon (top) and tube (bottom) convention (Rooijakkers et al. 2007). The N terminus of the protein is colored blue in both images. **b** Crystal structure of SCIN-A bound to C3c, which adopts a $(C3c/SCIN\text{-}A)_2$ pseudo-dimer (Garcia et al. 2010). **c** Crystal structure of SCIN-A bound to C3b (Garcia et al. 2010). A $(C3b/SCIN\text{-}A)_2$ pseudo-dimer is found through the application of crystallographic symmetry operators. **d** Crystal structure of a dimeric, SCIN-A inhibited convertase $(C3b/Bb/SCIN\text{-}A)_2$ (Rooijakkers et al. 2009). The Bb proteins are drawn as partially-transparent grey surfaces for clarity. **e** Schematic representation of C3b-based pseudo-dimers that form upon SCIN-A binding. Such structures can form either in the presence or absence of the convertase component Bb

analytical platforms differ considerably, the results obtained were consistent with one another, yielding apparent K_D values within the range of 170–265 nM. Subsequent biochemical mapping of the SCIN-A binding site on C3b localized this interaction to the C3c fragment of the complement component, since SCIN-A failed to bind to recombinant C3d, and the C3b/SCIN-A interaction was almost completely blocked by a monoclonal anti-C3c antibody, mAb C3-9, which has previously been shown to affect the binding of key ligands of C3b, such as fH, CR1, and fB (Becherer 1992). As a consequence, identification of a direct, high-affinity interaction between SCIN-A and the C3c fragment of C3b provided an incentive for pursuing crystal structures of these binary complexes.

Structures derived from four different crystal forms of C3c/SCIN-A and C3b/SCIN-A were recently reported (Garcia et al. 2010) (Fig. 2b, c). Superposition of these structures' refined coordinates indicated that they displayed a large degree of identity with one another, and were therefore directly comparable. For example, the relative orientations of the SCIN polypeptide and nearly all C3c-derived domains were practically indistinguishable among these structures. Furthermore, this primary contact site between SCIN-A and its complement target largely agreed with the predictions made previously through the functional analysis of SCIN protein chimeras (Rooijakkers et al. 2007). It was also significant that this SCIN-A contact site localized to the seventh a2-macroglobulin-like domain (MG7) of C3c/C3b. This feature was consistent with the previously mentioned biochemical mapping studies using mAb C3-9 (Ricklin et al. 2009), and also with negatively stained electron microscopy (EM) imaging that had mapped the C3-9 epitope to this region of C3b (Nishida et al. 2006).

The significant functional differences that accompany structural transitions between C3 and its downstream activation products are a fascinating feature of complement biology that has been well described for over two decades (Alsenz 1989; Hack et al. 1988; Lachmann 1982; Lambris 1985, 1988; Nilsson 1987; Nilsson 1982; Tamerius 1985), and one that has become much more readily interpretable with the many recent advances in understanding C3 structure and dynamics (Gros et al. 2008; Janssen et al. 2005, 2006; Ricklin et al. 2010; Schuster et al. 2008). In this context, the biochemical nature of the SCIN-A binding site on C3c/C3b provided an excellent illustration of how a functionally-important neoantigen/neoepitope arises upon activation of C3 to C3b. In its native state, C3 is a large 185 kDa protein that is comprised of two polypeptide chains (α, β), which themselves are held together by two interchain disulfide bonds and an extensive non-covalent interface (Janssen et al. 2005). Proteolytic conversion to C3b causes a large number of residues that line the α/β interface to undergo structural rearrangement, many of which become surface exposed in the process (Janssen et al. 2006; Schuster et al. 2008). Comparison of the C3b/SCIN-A and C3c/SCIN-A structures to that of native C3 revealed that 15 of 23 residues that constitute the primary SCIN-A contact site were previously buried in the large α/β interface of the unactivated complement protein (Garcia et al. 2010). Consequently, SCIN-A cannot bind to native C3, yet specifically recognizes C3c and C3b with high affinity.

Aside from this, several important insights followed from correlating these new structures with various functional outcomes of SCIN-A binding to C3b. To begin, SCIN-A was shown to slightly decelerate the initial rate of convertase formation, even though it primarily stabilizes the assembled convertase complex (Ricklin et al. 2009); consistent with this, SCIN-A binding to surface-immobilized C3b partially inhibited fB binding. In addition, SCIN competitively blocked the binding the major soluble complement regulator factor H (fH), which exerts both decay acceleration of the convertase and cofactor activity for the factor I-mediated degradation of C3b to iC3b. Indeed, functional assays with a recombinant fH fragment (i.e., fH(1–4)) showed that the SCIN-stabilized convertase was essentially resistant to decay acceleration by fH, and that the presence of SCIN interfered with the cofac-

tor function of fH(1-4). Altogether, these data strongly suggest an overlapping C3b binding site for SCIN-A and the host complement proteins fB and fH. Comparative analysis of the C3c/SCIN-A and C3b/SCIN-A structures with the existing structures of both C3b/fH(1-4) (Wu et al. 2009) and cobra venom factor bound to fB (CVF/B) (Janssen et al. 2009) provided a satisfying structural explanation for these observations: the primary SCIN-A binding site on C3c/C3b partially masks that of fB, but more significantly occludes that of fH(1-4). Thus, by binding at a key functional region on C3b, SCIN-A protects inactivated forms of the convertase against host complement regulator activity (Fig. 1b).

2.4 Structural/Functional Basis for SCIN-A Inhibition of C3 Convertase Activity

In order to direct and restrict complement amplification to target surfaces, the cascade relies on a highly concerted and well-timed activation mechanism of its C3 convertase, the details of which have only been revealed very recently (Forneris et al. 2010; Rooijakkers et al. 2009; Wu et al. 2009). While it has long been shown that SCIN impairs the conversion of C3 to C3b by the convertase, the exact mode of intervention has been difficult to pinpoint and could not be readily explained by the binary structures of SCIN-A with C3c and C3b. In principle, SCIN could impair either the initial binding of native C3 to the convertase or the cleavage/activation process itself. However, previous interaction studies had shown that native C3 bound readily to surface-based, SCIN-A-inhibited convertases that had been assembled *in vitro* on biosensor surfaces, yet that bound C3 was not activated to C3b (Ricklin et al. 2009). This observation thereby excluded the possibility that SCIN-A blocks the initial binding of the convertase's native C3 substrate, and instead suggested that the bacterial inhibitor must somehow "lock" C3bBb in an inactive state by preventing access of Bb to the target scissile bond in C3 (Ricklin et al. 2009; Rooijakkers et al. 2009).

The activity of decay-accelerating regulatory proteins notwithstanding, C3 convertases are themselves intrinsically transient complexes that dissociate irreversibly with half-lives on the order of 1–2 min (Pangburn et al. 1986). While this property is vitally important in tightly regulating the overall extent of complement activation, it also had long precluded detailed structural analysis of C3 convertases. With this in mind, the unique stabilizing properties of SCIN-A were innovatively used to obtain the first crystal structure of a fully assembled C3bBb/SCIN-A (Fig. 2d) (Rooijakkers et al. 2009). In this structure SCIN-A formed an essentially identical interface with the convertase C3b scaffold to that seen in the binary complexes described above (Garcia et al. 2010). However, the C-terminally oriented residues of alpha helices 1 and 3 in SCIN-A also formed a distinct interface with the proteolytic complement component, fragment Bb (Rooijakkers et al. 2009). Whereas this additional interface did not appear to induce any conformational changes in Bb (as judged by

comparison to the unbound Bb structure (Ponnuraj et al. 2004)), it nevertheless appeared to fix the orientation of Bb relative to the remainder of the C3b molecule.

In the absence of its interactions with SCIN-A, the proteolytic Bb fragment is bound by the C345C domain of C3b and thereby remains loosely associated with its convertase scaffold. The C345C domain itself is conformationally flexible, and in many crystal structures has adopted slightly, but noticeably different positions when compared to the remaining C3c/C3b macroglobulin-like core (later referred to as "C3b core") (Garcia et al. 2010; Rooijakkers et al. 2009). The most current models of convertase activity have therefore proposed that the C345C domain might function as a swinging platform, which allows proper orientation of the Bb active site relative to its C3 substrate (Rooijakkers et al. 2009). Taken together, all of these data have suggested that the SCIN-A-dependent loss of convertase activity lies in the ability of this bacterial protein to sterically restrict critical reorientations of C345C domain, which itself harbors the Bb protease (Garcia et al. 2010; Rooijakkers et al. 2009). In this manner, SCIN-A not only slows the rate of convertase formation as described above, but once it does assemble, it holds the essential convertase complex in an inactive conformation that itself is resistant to decay by host regulators (Fig. 1b).

2.5 Structural Basis and Functional Consequences of SCIN-induced Dimerization of C3b

A distinctive feature of all SCIN-bound crystal structures of C3c/C3b determined was the presence of crystallographic or non-crystallographic symmetry-related pseudo-dimers that formed along the long axis of the complement component (Garcia et al. 2010; Rooijakkers et al. 2009) (Fig. 2b–e). In all of these cases, the pseudo-dimer inducing site consisted almost exclusively of the first alpha helix of SCIN-A in contact with residues donated by the symmetry-related C3b molecule's α'-chain. While this additional interaction may have arisen as an artifact of crystal packing forces, several lines of evidence argued against this possibility. First, the dimerization-promoting interface buried comparable surface area to that of the primary SCIN contact site described above (750 versus 900 $Å^2$, as judged by the structure of C3b/SCIN-A) (Garcia et al. 2010). Second, direct C3b/SCIN-A binding studies using either solution or surface-based methods revealed a slight deviation from 1:1 Langmuir binding that was most pronounced at higher concentrations (Ricklin et al. 2009). Furthermore, small angle X-ray scattering (SAXS) analysis of C3b/SCIN-A samples were also consistent with an equilibrium mixture between 1:1 (C3b/SCIN-A) and 2:2 $(C3b/SCIN\text{-}A)_2$ species in solution. Finally, the presence of SCIN-A in reactions that generate the alternative pathway convertase C3bBb resulted in formation of stably inhibited convertase dimers $(C3bBb/SCIN\text{-}A)_2$ when compared to controls (Rooijakkers et al. 2009). All of these observations together predicted that SCIN-A-induced dimerization of C3b or convertases may have important functional consequences.

In this regard, a recent report established a correlation between SCIN-A mediated convertase dimerization and the inhibition of phagocytosis (Jongerius et al. 2010b). In this study, a mutant form of SCIN-A was constructed that blocked convertase activity equally as well as its wild type counterpart, yet was unable to form the dimers seen in the convertase crystal structure (Rooijakkers et al. 2009). Intriguingly, while the mutant SCIN-A-inhibited convertases had no effect on the recognition of phagocytic receptors, the wild-type protein disrupted both complement receptor 1 (CR1) and CR of the Ig-superfamily (CRIg)-dependent recognition and phagocytosis of opsonized *S. aureus* cells (Jongerius et al. 2010b). While this result demonstrated a dimerization-dependent inhibition of phagocytosis, it did not address whether C3b/SCIN-A dimerization in the absence of Bb might elicit a similar effect (Garcia et al. 2010). Furthermore, though not demonstrated directly in this study, it was also proposed that SCIN-A may also interfere with CR3-dependent phagocytic responses by blocking the generation of iC3b (Jongerius et al. 2010b). This is because SCIN-A effectively competes for the C3b-binding site of fH and, in doing so, inhibits the cofactor activity needed to stimulate fI-mediated generation of iC3b (Ricklin et al. 2009).

Examining the crystal structures of both C3b/SCIN-A (Garcia et al. 2010) and C3bBb/SCIN-A (Rooijakkers et al. 2009) provided a physical explanation for the anti-phagocytic properties of SCIN-A (Jongerius et al. 2010b). When SCIN-A-dependent dimers of C3c/C3b or the C3bBb convertase assemble, a large contact interface is formed. This interface subsequently masks a large number of C3b residues, among which are those that comprise the binding site of CRIg, a receptor that is predominantly found on phagocytic Kupffer cells involved in pathogen clearance by the liver (Helmy et al. 2006; Wiesmann et al. 2006). Whereas SCIN-induced dimerization also inhibited binding of CR1, the absence of a co-crystal structure for this receptor with C3b restricts a similarly detailed analysis for this observation. However, the high structural/functional similarity with fH suggests a similar competitive mechanism as observed for this regulator (see above). Consequently, it seems quite likely that the anti-phagocytic properties of SCIN follow from steric hindrance effects that prevent efficient binding of C3b by certain classes of CR proteins (Jongerius et al. 2010b). As a result, it now appears that a secondary function of SCIN-A is to facilitate the formation of pseudo-dimeric structures of C3b at the bacterial surface. These structures not only serve to attenuate complement activation (in the case of the convertase), but also to protect *S. aureus* cells from phagocytic uptake and destruction.

3 Recent Developments Regarding the Extracellular Fibrinogen-Binding Protein Family

3.1 Background

Efb was originally identified as a 16 kDa protein present in *S. aureus* conditioned culture medium capable of binding fibrinogen (Fg) (Boden and Flock 1992). The gene

encoding Efb lies within a second immune-evasion cluster within *S. aureus* genome (Jongerius et al. 2007), which also harbors the coding regions for SCIN-B and SCIN-C. Biochemical analyses of the Efb protein suggested that it was comprised of a modular architecture consisting of two distinct structural regions (Hammel et al. 2007b; Lee et al. 2004b). While several reports ultimately concluded that the N terminal region of Efb was responsible for its eponymous Fg-binding activity (Lee et al. 2004b; Palma 1998, 2001), the function of its proteolytically-stable C terminal domain (residues 101 to 165, referred to in the literature and hereafter as Efb-C) remained inconclusive. Several years ago, the outcomes of several lines of investigation revealed that Efb-C binds directly to C3/C3b/C3d (Hammel et al. 2007b; Lee et al. 2004a, b). Interestingly, a nearby gene on the same immune-evasion cluster was found to encode a 9.8 kDa protein denoted Ehp (Hammel et al. 2007a) (for *Efb h*omologous *p*rotein; alternatively known as Ecb (Jongerius et al. 2007)) that shares 44% sequence identity with Efb-C. Although similar C3/C3b/C3d binding activities were subsequently reported for Ehp (Hammel et al. 2007a; Jongerius et al. 2007), this smaller molecule also possessed a unique ability to form ternary 1:2 complexes with C3d-containing proteins (Hammel et al. 2007a). As a result, Ehp was identified as a more potent complement inhibitor than Efb-C on a molar basis (Hammel et al. 2007a).

The C3-binding properties of both Efb and Ehp strongly suggested a virulence-promoting role during *S. aureus* pathogenesis. To this end, studies revealed that *S. aureus* strains lacking Efb were less infectious than wild-type clinical isolates in a mouse mastitis model (Mamo 1994), and functional Efb was essential for prolonged *S. aureus* survival in a rat model of wound healing (Palma 1996). Separately, inclusion of Ehp in a model of immune complex-mediated inflammation blocked the influx of neutrophils in the peritoneal cavity of experimental mice (Jongerius et al. 2007). In the latter case specifically, these *in vivo* effects arise directly from potent inhibition of C3 and C5 activity by the bacterial proteins. Whereas their inhibition at the level of C3 appears largely restricted to the alternative pathway, Efb and Ehp inhibited the turnover of C5 through all pathways (Hammel et al. 2007b; Jongerius et al. 2007). This observation pinpointed the activity of Efb-C and Ehp to the level of the C3b-containing C3 and C5 convertases (i.e., C3bBb, C3b3bBb, and C3b4b2b) (Jongerius et al. 2007). In the few brief years since these original discoveries, convertase inhibition has become a well-established mechanism of complement evasion. However, the structural and mechanistic basis for the C3 convertase-blocking activity of Efb-C has only recently been elucidated (Chen et al. 2010). Furthermore, additional and unexpected activities of Efb and its homolog Ehp have been identified that highlight the elaborate nature of the staphylococcal immune evasion arsenal.

3.2 Structure/Function Analysis of Efb-C Binding to C3, C3b, and C3d

The crystal structure of the Efb complement inhibitor domain (Efb-C) has been determined both free and bound to its cognate C3d fragment of C3/C3b (Hammel

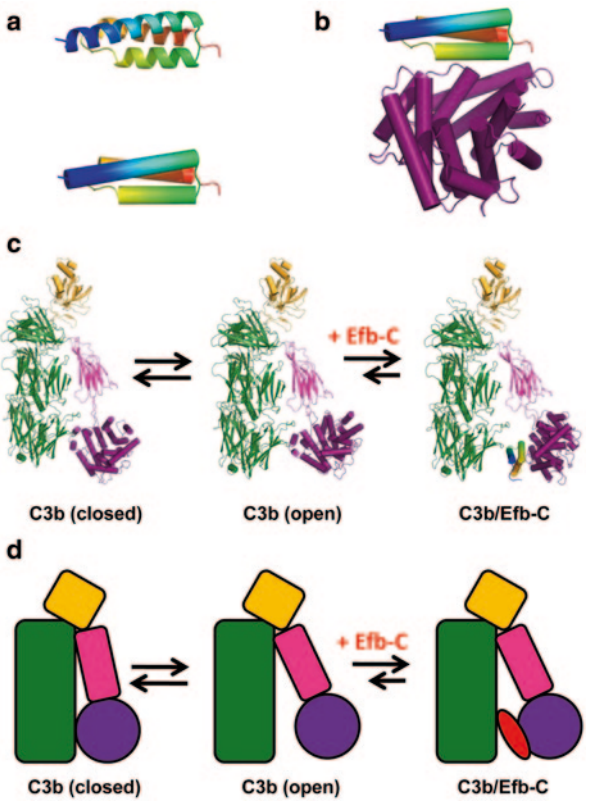

Fig. 3 Structural properties of the Efb-C protein both free and bound to complement components. **a** Crystal structure of the Efb-C protein, shown in both ribbon (top) and tube (bottom) convention (Hammel et al. 2007a). The N terminus of the protein is colored blue in both images. **b** Crystal structure of Efb-C bound to C3d (Hammel et al. 2007a). **c** Whereas solution structural transitions occur freely between the closed and open forms of C3b, Efb-C acts as a wedge to stabilize the open form (Chen et al. 2010). **d** Schematic representation of structural transitions in C3b, and the wedge effect upon Efb-C binding

et al. 2007b). In these structures Efb-C adopts a compact three helix bundle fold that is related to, but topologically-distinct from that of either SCIN-A or SpA modules (Fig. 3a, b). Superposition analysis revealed that the Efb-C structure is essentially identical in both the free and C3d-bound states, which suggested that its C3d-binding surface is preformed and requires little structural rearrangement. Further characterization of the C3d/Efb-C complex has provided a wealth of biochemical information on the nature of this interaction. First, the interaction is highly favorable thermodynamically with a K_D on the order of 1–2 nM, as judged by both solution ITC and surface-based SPR studies. Second, the C3d/Efb-C complex is also exceedingly stable kinetically, and displays a mean half-life of approximately 60 min (Hammel et al. 2007b). Finally, Efb-C binds near an acidic cleft on C3d, and the C3d/Efb-C complex is mediated by an extensive array of largely ionic interactions. These occur almost exclusively between polar residues donated by the second alpha helix Efb-C and the loops which connect helices H2–H3, H4–H5, and H6–H7 of C3d.

While the contributions of specific positions in C3d have not yet been examined by mutagenesis, this type of detailed structure/function analysis has been carried out for Efb-C (Hammel et al. 2007b; Haspel et al. 2008, 2010). This work has demonstrated that positions R131 and N138 of Efb-C are particularly critical determinants

for both the affinity and stability of the C3d complex. Studies on both single and pairwise substitutions at positions 131 and 138 revealed that interactions between Efb-C and C3d are dependent upon both the charge and polar nature of these side chains. Whereas the observed K_D rose to 26 nM and 19 nM, respectively, when either residue alone was mutated to alanine (Haspel et al. 2008, 2010), the corresponding double mutant had no residual affinity (Hammel et al. 2007b; Haspel et al. 2008). Furthermore, while loss of the side chain functionality had only minor effects on the ionic strength-dependent association phase, the rate of complex dissociation was greatly accelerated in each mutant (Haspel et al. 2008). In addition to these two residues, the results of molecular dynamics (MD) simulations and computational estimations of free energy release upon binding have suggested a supporting role for interactions involving residues K106, K110, V127, F142, and R165 of Efb-C (Haspel et al. 2008). However, this possibility has not been evaluated experimentally as of this time.

3.3 Allosteric Inhibition of C3 Convertase Function by Efb-C

Although the studies described above provided a detailed knowledge of Efb-C's structure and the molecular basis for its interactions with C3/C3b/C3d, the mechanism through which Efb-C caused specific blockade of the alternative pathway remained unclear (Hammel et al. 2007b). However, several lines of evidence demonstrated that Efb-C binding either caused or stabilized an altered structure in C3b that disrupted complement activity. To begin, wild-type Efb-C, but not a non-functional mutant, rendered C3b increasingly sensitive to mild digestion by proteases *in vitro*. Furthermore, binding of mAb C3-9, which is specific for activated forms of C3 (e.g., C3b, iC3b, and C3c), was significantly enhanced by the presence of saturating levels of Efb-C when compared to C3b alone. This observation was most unexpected, because the Efb-C binding site on the TED/C3d domain of C3b lies nearly 100 Å distant from the MG7/MG8 domains, which themselves harbor the mAb C3-9 epitope (Nishida et al. 2006). Finally, the C3b/Efb-C complex displayed a noticeably lower formation rate constant when compared to those of native C3, hydrolyzed C3, and C3d (Hammel et al. 2007b). Since Efb-C binds with similar affinity to each of these C3 derivatives, this suggested that access of Efb-C to its TED/C3d domain binding site on C3b might somehow be restricted. Indeed, analysis of the C3d/Efb-C complex in this light revealed that Efb-C binding to C3b would result in a large steric clash with the MG1 domain of the C3b core (Hammel et al. 2007b; Janssen et al. 2005, 2006); as a consequence, formation of a stable C3b/Efb-C complex could only be possible upon structural rearrangements within the C3b protein. All of these observations, together with other data which showed that Efb-C acts at the level of C3b-containing C3 and C5 convertases (Jongerius et al. 2007), suggested that Efb-C binding either resulted in or selected for an altered C3b conformation that subsequently disrupted alternative pathway convertase formation and/or function.

An investigation into the solution structure and functional consequences of these conformational changes resulted in identification of Efb-C as the first known allosteric complement inhibitor (Chen et al. 2010). In this study, conformational differences upon Efb-C binding were detected through both hydrogen–deuterium exchange mass-spectrometry (HDX-MS) and SAXS-MD-based approaches. Efb-C binding caused the greatest changes in the HDX-MS profiles of the CUB domain of C3b, which tethers C3d/TED to the remainder of the α-chain of the core. Independent analysis of SAXS data via MD methods likewise revealed that the larger-scale conformational changes in C3b/Efb-C relative to unbound C3b could largely be explained through quaternary alterations in the CUB-TED region. As a consequence, Efb-C was proposed to act as a "wedge" upon binding its C3b target that effectively separates both the CUB and TED domains from their positions relative to unbound C3b (Fig. 3c). It is interesting to note that this "open-like" state is clearly distinct from the "closed" conformations observed in crystal structures of C3b (Garcia et al. 2010; Janssen et al. 2006; Rooijakkers et al. 2009; Wiesmann et al. 2006; Wu et al. 2009). Importantly, though, previous negatively-stained EM studies implied the existence of C3b molecules with even more pronounced separation of TED from the C3b core (Nishida et al. 2006), and the SAXS-MD data mentioned above indicate an equlibrium between open and closed forms of C3b in absence of Efb-C (Chen et al. 2010). Together, these results strongly suggest that a dynamic fluctuation exists between these opened and closed forms of the C3b molecule, although the physiological implication of this conformational distribution is not yet clear. In keeping with the most recent models of protein allostery (Tsai et al. 2009), it now appears that Efb-C binds to and stabilizes the "open" form of C3b (Fig. 3d), rather than "inducing" a new C3b conformation per se.

Aside from these changes in the CUB–TED region, additional alterations were seen throughout other portions of C3b that are even more distant from the Efb-C binding site (e.g., in the MG 6–8 domains). Based on available co-crystal structures between C3b and major ligands, it was expected that these changes would modulate the affinity of C3b for at least some of its binding partners, as a large number of these structural changes mapped to functionally important areas of C3b. Most significantly among these, Efb-C binding to C3b was found to greatly reduce binding of fB and to critically impair formation of the alternative pathway C3 convertase *in vitro*. Efb-C primarily affects the fast initial binding phase of fB and impairs binding of recombinant fragment Ba (Chen et al. 2010), which indicates that Efb-C-dependent inhibition results from its effects on the Ba-mediated loading step during convertase assembly (Forneris et al. 2010; Rooijakkers et al. 2009). This observation was supported by contact site analyses, which revealed that several contact residues for Ba were close to or within segments of altered HDX; in contrast, no HDX differences overlapped with the Bb binding site (Chen et al. 2010). At the time of this report, a high-resolution structure was only available for the complex of fB with CVF, but not with C3b; since then, however, the structures of C3bB and C3bBD have been described (Forneris et al. 2010). Importantly, a good correlation is still found between available crystallographic data, HDX, and SPR analyses, when these most recent structures form the basis for contact site comparison. All of

this indicates that these independent yet complementary techniques are observing the same structure/function/mechanism relationship between Efb-C, C3b, and fB.

Aside from its effects on convertase formation, it is also noteworthy that Efb-C was found to modulate fH binding to C3b (Chen et al. 2010). Yet in this case, the molecular details of this phenomenon appear to be more confounding. Whereas Efb-C blocked binding of the regulatory fH(1–4) fragment to C3b, Efb-C clearly enhanced a second binding event that involves the fH C-terminus, as is typified by fH(19–20). Overall, this leads to a net increase of full-length fH binding in the presence of Efb-C (Chen et al. 2010). Consistent with this, very recent structural studies have lent support to the possibility of Efb-C-mediated enhancement of the C3b/fH interaction (Kajander et al. 2011; Morgan et al. 2011). Specifically, two separate structures of fH(19–20) bound to C3d show that the Efb-C binding site on C3d appears to be largely available for forming a ternary complex with this fragment of fH. Nevertheless, this raises questions on how the bacterium could benefit by tethering fH to C3b in a presumably inactive conformation, for Efb-C potently blocks binding of fH(1–4) to C3b. Given these seemingly divergent conclusions, it is clear that the functional consequences of this peculiar modulatory effect will need to be more fully investigated over the coming years.

Considering these data as a whole, the ability of Efb-C to alter the conformational/dynamic properties of regions so far distant (~100 Å) from its actual binding site is startling. Yet this "action-at-a-distance" phenomenon is highly selective as only certain ligand-binding sites are affected. Though the inhibition of convertase assembly was not complete under the conditions used in the published assay, even the observed reduction by ~80% may drastically impair the ability of complement to initiate and amplify its response on target *S. aureus* cells. The specific nature of these effects argues strongly that disruption of certain C3b activities has been selected for and optimized during the course of bacterial evolution in the face of the innate immune response. In this regard, the need to block function of C3b-containing convertases appears to be paramount.

3.4 Structure/Function Studies of the Efb Homologous Protein, Ehp/Ecb

A defining feature of the staphylococcal immune evasion arsenal is the existence of multiple, structurally related proteins that appear to have similar, and sometimes overlapping functions (Chavakis et al. 2007; Foster 2005; Geisbrecht 2008; Lambris et al. 2008). In the case of Efb and its homolog Ehp (Hammel et al. 2007a), the situation more closely mirrors the latter of these scenarios. Whereas Efb has the ability to bind both C3 and Fg (Lee et al. 2004b), Ehp appears to lack this latter activity as it lacks the disordered, Fg-binding N-terminal region of Efb (Hammel et al. 2007b). In addition, quantitative studies of complement inhibition have demonstrated that Ehp is a nearly three-fold more potent inhibitor of the alternative pathway than is either Efb or its isolated complement inhibitor domain, Efb-C. Given its relatively

high sequence identity to Efb-C, Ehp assumes a nearly identical fold when bound to C3d, as judged by a C3d/Ehp co-crystal structure. However, examination of the Ehp amino acid sequence identified a second C3/C3b/C3d-binding site that resides on the first alpha helix of Ehp. A combination of mutational, interaction, and functional analyses subsequently revealed that while this second site was of lower affinity than those found on the second alpha helices of both Ehp and Efb-C, it was required for the enhanced inhibitory potency of Ehp (reviewed in Geisbrecht 2008).

While the structure/function relationships and precise mechanism of Ehp have not been fully investigated to the same extent as Efb-C, several informative conclusions can still be drawn from the available literature. In much the same way as for Efb-C (Hammel et al. 2007b), studies using mAb C3-9 strongly suggested that Ehp binding instigates a conformational change in the C3 protein (Hammel et al. 2007a). To manifest consequences at a functional level, such changes would presumably need to occur at the level of C3b as well. So far, however, neither the monomeric C3b/Ehp nor dimeric C3b3b/Ehp complex (Hammel et al. 2007a) has been examined structurally by the SAXS-MD or HDX-MS methods described above (Chen et al. 2010). The targets of Ehp activity have been identified as the surface-bound, C3b-containing C3 and C5 convertases (i.e., C3bBb, C3b3bBb, and C3b4b2b) (Jongerius et al. 2007). Although this is consistent with its overall similarity to Efb-C, a recent study has suggested a slightly different means to this end (Jongerius et al. 2010a). While both Efb-C and Ehp inhibit formation of active solid-phase convertases, Ehp was shown to stabilize fB binding to the convertase C3b scaffold and block its fD-dependent activation to Bb. This activity stands in contrast to Efb-C, which instead prevents initial fB binding to immobilized C3b (Chen et al. 2010). It is worth noting that this stabilizing activity is vaguely reminiscent to that described for SCIN-A (Ricklin et al. 2009; Rooijakkers et al. 2005). Thus, it is tempting to speculate that the bivalent nature of Ehp may somehow contribute to this effect, much in a way that SCIN-A forms C3b-containing pseudo-dimers (Jongerius et al. 2010b; Ricklin et al. 2009). In any case, the previously described differences in their C3/C3b/C3d-binding properties, along with these more recently discovered routes to convertase inhibition represent an intriguing starting point for future studies on Efb-C and Ehp.

3.5 Disruption of Adaptive Immune Engagement by Efb and Ehp

In addition to its roles in recognizing and marking pathogens for elimination, the complement system plays a pivotal role in stimulating the downstream mechanisms of adaptive immunity. Central to this feature of innate-adaptive crosstalk is the CR2 receptor, which is expressed by and localized to the surface of B cells, follicular dendritic cells, and immature T-cells (Carroll 2004). As part of the B-cell coreceptor complex (in conjunction with CD19 and CD81), CR2 lowers the threshold of activation and affects the maturation of B cells upon binding of C3d-opsonized particles. As a consequence, the C3d/CR2 interaction is pivotal to properly engaging

the adaptive immune system following complement opsonization of pathogen-derived biomaterial. Examination of the C3d/Efb-C (Hammel et al. 2007b) and C3d/Ehp (Hammel et al. 2007a) crystal structures, along with structure/function data regarding C3d/CR2 binding (Clemenza and Isenman 2000) raised the possibility that these staphylococcal proteins might also disrupt the C3d/CR2 interaction (Ricklin et al. 2008). Consistent with this, SPR-based competition assays demonstrated that only a slight molar excess of either Efb-C or Ehp was needed to completely inhibit CR2 binding to C3d (Ricklin et al. 2008). Furthermore the presence of wild-type Efb-C, but not a non-functional double mutant, was also shown to block binding of crosslinked C3 to a CR2-expressing B-cell lymphoma line (Raji cells). Importantly, an inhibitory effect was also seen in mouse splenocytes, where inclusion of wild-type Efb-C, but not the mutant, impaired the C3d-mediated B-cell costimulatory response as judged by release of intracellular Ca^{2+} (Henson et al. 2001). Thus, even though their primary role appears to lie in disrupting formation of alternative pathway-derived convertases, both Efb-C and Ehp also inhibit CR2-mediated engagement of the adaptive immune system. Such versatility within a single protein family underscores the powerful evasion mechanisms that *S. aureus* has coevolved in the presence of the host immune system.

4 Conclusions and Future Directions

The exquisitely tailored, yet non-redundant convertase-blocking capabilities of the SCIN and Efb protein families represent only a small fraction of the overall *S. aureus* immune evasion arsenal. Yet in just a few short years, intense study of these molecules has profoundly expanded our molecular-level understanding of complement inhibition and regulation. Given these significant changes in only two segments of what has become a surprisingly large field, it is almost certain that additional and unexpected discoveries still lie in waiting. For while diverse anti-complement effects for these two protein families have been identified already, the literature also clearly underscores that these advances have been achieved primarily through study of the structure, function, and mechanism of the SCIN-A and Efb-C proteins alone. In this regard, a more detailed examination of related proteins may be more relevant now, and this may very well open new doors for further inquiry. For example, study of the remaining SCIN proteins may aid in delineating the unique interactions described here and elsewhere that are required for the two distinct, but related SCIN functions of convertase inhibition and disruption of phagocytosis. Similarly, continued investigation into the functional significance of the second C3/C3b/C3d binding site in Ehp may highlight heretofore unappreciated differences between this protein and Efb-C.

Finally, it is important to mention that nearly all studies on *S. aureus* complement inhibitors reported so far have been carried out from a reductionist perspective, where only a single proteins' function is examined at a time. While experimentally tractable, this dramatically oversimplifies the physiological situation where mul-

tiple immune evasion proteins are expressed simultaneously. Given that several of these proteins actually target the same structures (e.g., convertases), the possibility for functional synergy cannot be discounted. Regardless of the precise approaches taken, further analyses of these unique proteins will advance our understanding of *S. aureus* immune evasion and human complement regulation. In addition to this important goal, it has the potential to provide a valuable template for the design of complement-directed therapeutics as well.

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Granzyme B: A New Crossroad of Complement and Apoptosis

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Abstract In response to severe tissue trauma, several "molecular danger" sensing and signaling pathways are activated, especially the complement and the apoptosis cascade. Although possible crossroads between both systems have been proposed, little is known about the underlying molecular interactions. In this study a new interaction interface is presented for C3a and C5a generation by the pro-apoptotic factor granzyme B. *In vitro* incubation of the central human complement components C3 and C5 with the serine protease granzyme B resulted in a concentration-dependent production of the anaphylatoxins C3a and C5a. The so generated anaphylatoxin C5a was chemotactic active for isolated human neutrophils. In a translational approach, intracellular granzyme B concentration in leukocytes was determined early after severe tissue trauma. In comparison to healthy volunteers, multiple injured patients (less than one hour after trauma, Injury Severity Score > 18, $n = 5$) presented a significant increase in granzmye B levels in neutrophils and lymphocytes. Thus, tissue trauma is associated with early activation of both, the complement and apoptosis system. The present data suggest a new form of interaction between the complement and the apoptosis system on the level of granzyme B that is capable to generate C3a and C5a independently of the established complement proteases.

Keywords Apoptosis • Complement • Granzyme B • C5a • C3a

1 Introduction: Interactions of the Complement System and Apoptosis

The complement and apoptosis systems are crucially involved in the innate immune response after tissue trauma and inflammation. These evolutionary conserved systems have been described as separate cascades, which finally provoke

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the proteolysis of the target cell. However, recently some interactions between both proteolytic systems have been proposed (Cole et al. 2006; Elward et al. 2005; Fishelson et al. 2001; Guo et al. 2006; Soane et al. 1999). Central components of the complement system seem to recognize apoptotic cells and vice versa apoptotic cells may activate the complement cascade. Elward et al. found that C1q detects apoptotic cells and thereby triggers the classical complement pathway (Elward et al. 2005). Similarly, the lectin pathway, activated by mannose binding lectin (MBL), recognizes polysaccharide structures on the surface of apoptotic cells. Complement activation per se may result in the opsonization of apoptotic cells with C4b and C3b ("eat me signals") which significantly accelerate their phagocytic uptake, bypassing any inflammatory process (Elward et al. 2005). Another strategy for rendering apoptotic cells more appetizing for phagocytes was proposed by Cole at al. (2006), indicating that the expression of complement regulating protein CD46 (membrane cofactor protein) is reduced on apoptotic cells, leading to an increased C3b opsonization and final removal of these cells. In neutrophils, *in vitro* induced apoptosis was also associated with a loss of the complement regulators CD55 (decay accelerating factor) and CD59 (membrane attack complex inhibitor) (Jones and Morgan 1995). These findings indicate that the cell bound complement regulatory proteins somehow represent self-associated molecular pattern ("SAMP") signals that are lost on the surface of cells undergoing apoptosis (Elward et al. 2005). Further evidence of a link between the apoptotic and complement system may be provided by the complement activation product C5a. Upon generation, C5a has been found to suppress neutrophil apoptosis by activating several kinase pathways resulting in phosphorylation and consecutive inactivation of pro-apoptotic Bad (Bcl-2/Bcl-xL-associated death promotor). In addition, C5a is capable to induce XIAP (X-linked inhibitor of apoptosis) production and Bcl-xL expression that leads to inhibition of the intrinsic apoptotic pathway (Guo et al. 2006). Generation of the anaphylatoxin C5a can be initiated by the complement and the coagulation system (Amara et al. 2010a; Huber-Lang et al. 2006). Both cascades posses various serine proteases capable to cleave the key complement factors C3 and C5.

The various apoptotic pathways house some serine proteases (Moffitt et al. 2007), among them granzyme B. This enzyme is mainly produced by cytotoxic T- and natural killer cells to protect the host from intracellular pathogens by inducing apoptosis of the infected target cells (Lieberman 2003) by cleavage of caspase-8 and effector caspase-3. Furthermore, granzyme B is capable to activate the intrinsic apoptotic pathway by generating truncated Bid (tBid, BCL-2 Interacting Domain) (Lord et al. 2003; Wowk and Trapani 2004). Although some crossroads between the apoptotic and complement system have been proposed, little is known on the molecular interaction level, especially for the serine protease granzyme B.

Here, we present a new crossroad of complement and apoptosis regarding the cleavage of C3 and C5 by pro-apoptotic granzyme B. This enzyme was found to be enhanced in leukocytes early after multiple trauma and capable to generate the complement activation product C3a and C5a *in vitro* independent of the established complement activation pathways.

2 Material and Methods

2.1 Reagents

Unless stated otherwise, reagents were purchased from Sigma-Aldrich (Taufkirchen, Germany). Purified human C3 and C5 were obtained from Quidel (San Diego, CA). Granzyme B was purchased from Biovision.

2.2 In Vitro Cleavage of C3 and C5

In vitro experiments were performed by incubating either native C3 (100 μg/ml) or C5 (100 μg/ml) in Dulbecco's phosphate-buffered saline (DPBS) in the absence or presence of granzyme B at 37 °C for 90 min in a concentration-dependent manner, followed by Western blot and ELISA analyses.

2.3 Detection of C3a and C5a by Western Blot

Equal protein amounts of experimental samples and related controls were separated by SDS-PAGE under reducing conditions and transferred onto a polyvinylidene fluoride membrane (Schleicher et al. NH). The blots were incubated overnight at 4 °C with 1:1,000 polyclonal mouse anti-human C3a IgG (Serotec) or 1:1,000 monoclonal mouse anti-human C5a IgG (Cell Signaling). After washing, membranes were incubated for 1 h at room temperature using alkaline phosphatase-conjugated secondary 1:1,000 IgG Antibody. For development, alkaline phosphatase substrate color development buffer (Bio-Rad, Hercules, CA) was used in accordance to the manufacturer's protocol.

2.4 ELISA Analysis of C3a and C5a

For quantitative analysis of C3a and C5a, respectively, commercially available ELISA kits (Quidel and DRG Diagnostics, Marburg, Germany) were used according to the manufacturers' instructions.

2.5 Neutrophil (PMN) Isolation and Chemotaxis Assay

Human neutrophils were isolated from whole blood of healthy human volunteers (approved by the Independent Ethics Committee of the University of Ulm, Ulm,

Germany, No. 69/08, following written informed consent of all individuals). Whole blood was drawn from the antecubital vein into syringes containing the anticoagulant citrate dextrose (1:10; Baxter Health Care, Deerfield, IL). Neutrophils were isolated using Ficoll-Paque gradient centrifugation (Pharmacia Biotech, Stockholm, Sweden) followed by a dextran sedimentation step. After hypotonic lysis of residual RBCs, neutrophils were resuspended in HBSS with 0, 1% BSA and fluorescein labeled with 2′,7′-bis [2-carboxyethyl]-5-[and 6]-carboxy-fluorescein acetoxymethyl ester (Molecular Probes) for 30 min at 37 °C. Labeled neutrophils (5×10^6 cells/ml) were loaded into the upper chamber of a 96-well device (Neuro-Probe) and separated by a polycarbonate filter with a porosity of 3 mm (Neuro-Probe). The lower chambers were loaded with recombinant human C5a (1,000, 100 ng/ml, positive control) or C5 in the absence or presence of different concentrations of granzyme B (2,000, 1,000 and 100 ng/ml). After incubation at 37 °C for 60 min the number of cells migrated through the polycarbonate membrane was determined by cytofluorometry (Cytofluor II, Per Septive Biosystems, Framingham, MA).

2.6 Antibodies for the Flow Cytometry Analysis

For the analysis Phycoerythrin [RPE]-conjugated mouse monoclonal antibodies were used from the IgG1 isotype. Granzyme B-RPE was obtained from Sanquin Blood Supply Foundation (Amsterdam, Netherland). The isotype control was obtained from Serotec (Düsseldorf, Germany). Flow cytometric analysis was performed using BD FACS Canto II (BD Bioscience, Heidelberg, Germany).

2.7 Intracellular Staining for Granzyme B and Flow Cytometry Analysis

EDTA whole blood (100 µl) was incubated with 2 ml of FACS lysing solution (BD Biosciences) over night at 4 °C. Then the tube was centrifuged for 5 min at 500×g. After centrifugation, the supernatant was removed and 2 ml of DPBS was added, followed by an additional centrifugation step. After removing the supernatant, the cell pellet was resuspended in 500 µl of Perm Buffer II (BD Biosciences) and incubated 10 min at room temperature. After a further washing and centrifugation step PE-labeled fluorochrome-conjugated monoclonal antibody for granzyme B and for the Isotyp control was added and incubated at room temperature for 30 min in the dark. After the incubation period, the cells were washed with 2-ml DPBS and centrifuged (5 min 500×g). After removal of the supernatant the cells were resuspended in 200-µl Cell fix buffer (BD Biosciences) for final flow cytometric analysis. For each measurement, a minimum of 10,000 events were analyzed. For quantification of granzyme B content, mean fluorescence intensity (MFI) emitted by the RPE

monoclonal antibody was calculated by subtracting the MFI from the corresponding isotype control.

2.8 Statistics

All values were expressed as mean ± SEM. For chemotaxis assays data sets were analyzed by One-Way ANOVA; differences in the mean values were then compared using Student–Newman–Keuls test. In case of C5a ELISA and the flow cytometric measurements, the Mann Whitney rank sum test was performed to compare data versus control or healthy volunteers, respectively. Results were considered statistically significant when $P < 0.05$.

3 Results

3.1 Cleavage of C3 by Granzyme B

To investigate, whether the serine protease granzyme B is capable to cleave the central complement factor C3 and to generate the anaphylatoxin C3a, an *in vitro* co-incubation of C3 in the absence or presence of increasing concentrations of granzyme B was performed, followed by qualitative and quantitative C3a measurements. As shown in Fig. 1a, C3a could be detected by anti-human C3a antibody at the expected molecular weight in Western blot analysis. The band intensity in-

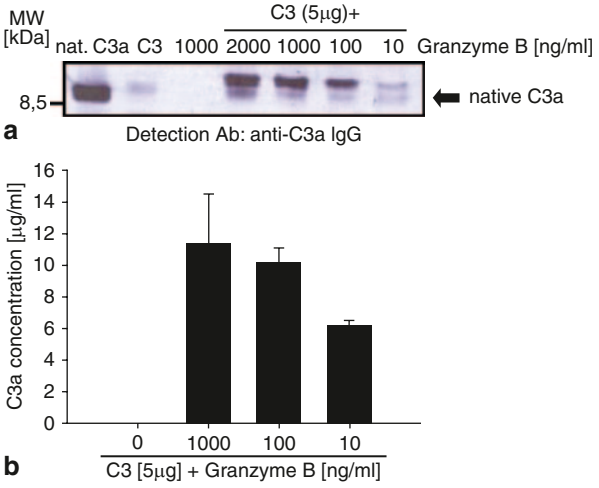

Fig. 1 Granzyme B-induced *in vitro* cleavage of complement factor C3. Human C3 was incubated with increasing concentrations of granzyme B at 37 °C for 90 min. **a** Western blot analysis for C3a. **b** C3a ELISA analysis; $n = 2$ independent experiments; *nat* = native

creased with increasing concentrations of granzyme B added to C3. The detection of the produced C3a by ELISA revealed a concentration-dependent C3-cleavage for different amounts of granzyme B (0–1,000 ng/ml) (Fig. 1b).

3.2 Cleavage of C5 by Granzyme B

To define whether the pro-apoptotic factor granzyme B is also capable to *in vitro* cleave the downstream complement component C5 and to generate C5a, human C5 (5 µg; concentration 100 µg/ml) was incubated with increasing amounts of granzyme B (0–2,000 ng/ml) at 37 °C for 90 min (Fig. 1a). Generation of C5a was subsequently determined by Western blot analysis. Granzyme B alone did not show any band, whereas C5 in absence of granyzme B revealed some cleavage activity with a faint band around 14 kDa. However, using granzyme B at higher concentrations (>1,000 ng/ml) resulted in a much stronger band lining up at the expected molecular weight for native, glycosylated C5a (ca. 14 kDa). C5a generation was further measured by ELISA technique. Here, C5 was incubated with either DPBS or different concentrations of granzyme B (0–1,000 ng/ml) at 37 °C for 90 min. As indicated in Fig. 2b, there was a concentration-dependent production of C5a in

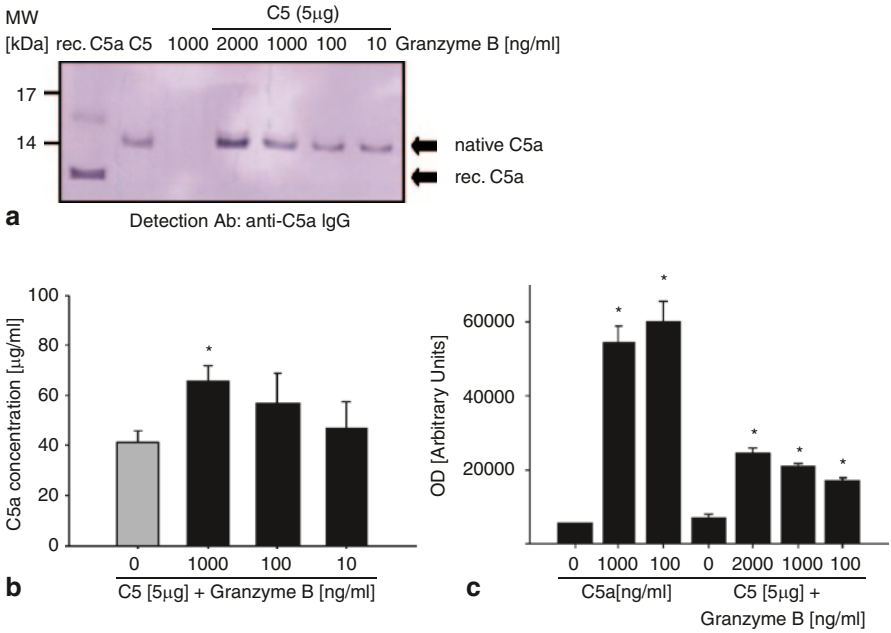

Fig. 2 Granzyme B-induced *in vitro* cleavage of C5. **a** C5a detection by Western blot; *rec* = recombinant. **b** C5a ELISA analysis for the C5 cleavage product ($n=3$, $^*p<0.05$ vs. 0-ng/ml granyzme B). **c** Chemotactic activity of C5-cleavage products and recombinant C5a as a positive control. $n=6$ independent experiments ($^*p<0.05$ vs. 0-ng/ml C5a and 0-ng/ml granzyme B)

the presence of granzyme B. To investigate, whether the detected cleavage product exhibit some biological function, human whole blood neutrophils were exposed to the cleavage products and the resulting chemotactic activity was measured. As displayed in Fig. 2c, there was a robust chemotactic response of human neutrophils toward recombinant C5a alone, and a modest but significant chemotactic response to the *in vitro* generated granzyme B-caused C5-cleavage product. The chemotactic activity was dependent on the concentration of granzyme B added to C5. Taken together, the C5 cleavage product revealed distinct chemotactic activity for human neutrophils.

3.3 Granzyme B in Leukocytes of Trauma Patients

In a translational approach we further investigated if granzyme B played a role already early after severe tissue trauma. Therefore, leukocytes from five multiple injured patients with an injury severity score > 18, as defined by the consensus criteria (Baker et al. 1974) were compared with 13 healthy age matched volunteers. As shown in Fig. 3, the amount of intracellular granzyme B as detected by flow cytometry, was significantly increased in both, neutrophils and lymphocytes, but not in monocytes (data not shown) in patients with severe tissue trauma. Interestingly, the granzyme B concentration was enhanced within less than one hour after trauma (admission to the emergency room). Present *in vitro* and *in vivo* data suggest, an early generation of granzyme B in leukocytes after severe tissue trauma, which in turn may cleave the central complement factor C3 and C5 and therefore providing a linkage between the apoptosis and the complement system.

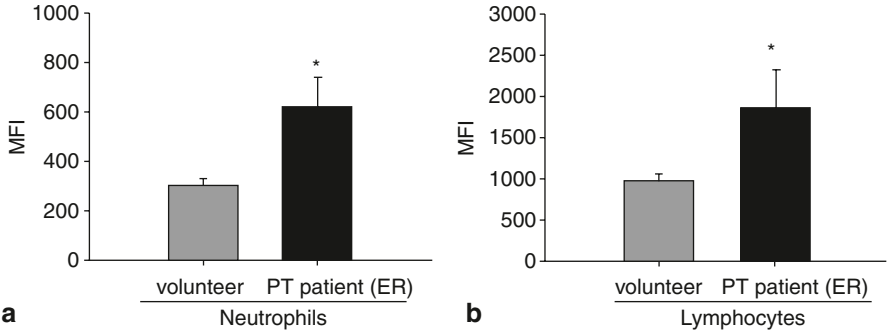

Fig. 3 Flow cytometric intracellular measurement of granzyme B in neutrophils (**a**) and lymphocytes (**b**) of polytrauma patients (ISS > 18) compared to healthy volunteers. *Light gray bars* indicate healthy donors ($n = 11$); *black bars* indicate polytrauma patients (PT); $n = 5$ (*$p < 0.05$ vs. healthy volunteers); *ER* = emergency room

4 Discussion

Here we provide evidence that after severe trauma granzyme B was increased in lymphocytes as well as neutrophils early after the insult. In addition, our data demonstrates linkage between granzyme B and the complement system. Thus, *in vitro* incubation of the complement component C3 with the apoptotic factor granzyme B resulted in C3a generation in a concentration-dependent manner. In parallel we could demonstrate the *in vitro* cleavage of C5 to C5a by granzyme B. The resulting cleavage product initiated a chemotactic response for human neutrophils.

The complement system, one of the major alarm systems of innate immunity is build around the cleavage of proteins by serine proteases. In this regard, C3 or C5 convertase are the relevant proteinases generating C3a or C5a, respectively. Human C3 and C5 are cleaved between arginine and serine at position 77 and 75, respectively, of the α chain, leading to the generation of C3a and C5a (Sahu and Lambris 2001; Sandoval et al. 2000). In the absence of C3, substitutes for the C3-dependent C5 convertase such as thrombin have been described, establishing crosstalk between the complement and the coagulation system (Huber-Lang et al. 2006; Sahu and Lambris 2001; Sandoval et al. 2000). Our recent results indicate that the complement system is early activated after severe trauma (Amara et al. 2010b), generating potent inflammatory mediators such as C3a, C5a, and the opsonin C3b. Especially C3b is considered as an "eat me signal" for phagocytes to accelerate the uptake of apoptotic cells avoiding excessive inflammatory reactions (Elward et al. 2005). To further enhance the opsonization of target cells, several strategies are proposed. Complement regulatory proteins, which inhibit the assembly of the C3 and the C5 cleaving convertases and the formation of the cytolytic membrane attack complex (MAC), are supposed to disappear from the surface of apoptotic cells, labeling them as apoptotic and making them more attractive for macrophages. In this regard, it has been shown that CD46 (membrane cofactor protein), CD55 (decay accelerating factor) and CD59 (membrane attack complex inhibitor) are down regulated on cells undergoing apoptosis giving way for the detrimental effects of complement-induced cell lysis (Cole et al. 2006; Elward et al. 2005; Jones and Morgan 1995). The expression of these costimulatory molecules is tightly regulated in response to severe tissue trauma and inflammtion. In this regard, CD55 displayed a time-dependent, elevated expression pattern on neutrophils and monocytes, but not on lymphocytes whereas CD59 expression was significantly increased on neutrophils and monocytes at the time of admission and at 5 to 10 days after trauma in lymphocytes. CD46 was significantly down-regulated in all three cell types post trauma. Hereby, CD59 and CD46 expression values on neutrophils reversely correlated with severity of injury (Amara et al. 2010b). The mechanisms leading to the reduction of these regulatory molecules is poorly understood but there exists evidence that matrix metalloproteinases (MMPs) are involved in the shedding of CD46 from the surface of leukocytes (Cole et al. 2006).

While this regulation of the apoptotic response by complement factors has recently been addressed more frequently, little is still known about the regulation of

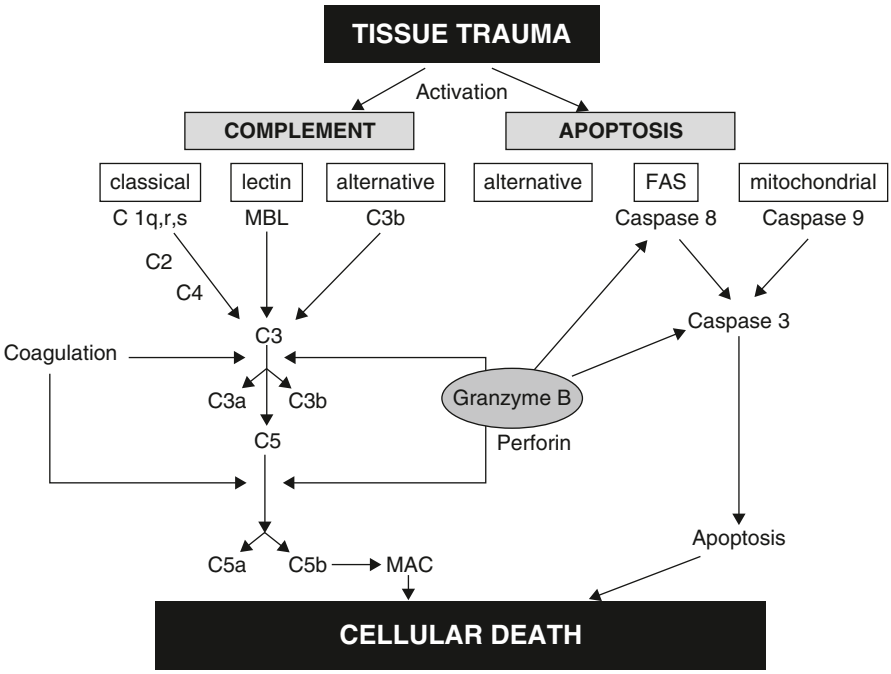

Fig. 4 Simplified scheme of the tissue trauma-induced activation of the complement and apoptosis system and possible molecular interactions regarding the pro-apoptotic serine protease granzyme B

the complement system by apoptotic responses in particular concerning direct molecular interaction. Our results now provide evidence for such an interplay by granzyme B (Fig. 4). The data presented here suggests that granzyme B was upregulated in neutrophils and lymphocytes of trauma patients early within less than one hour after the insult, when compared to healthy controls. Granzymes are a family of conserved serine proteases. Granzyme B is a protease used by cytotoxic T-lymphocytes to eliminate pathogenic target cells (Boivin et al. 2009; Lord et al. 2003). After binding to the mannose-6-phosphate/insulin-like growth factor II receptor it gets endocytosed and upon release by perforin activates caspase-8 and -3 potentially including the mitochondrial intrinsic loop and finally initiating apoptosis (Lord et al. 2003). Interestingly, granzyme B can be expressed in a variety of other cell types than cytotoxic T-lymphocytes. Among them immune cells such as $CD4^+$ cells, mast cells, activated macrophages, neutrophils (like in the present setting), basophils, dendritic cells (DCs), T regulatory cells, and non immune cell types such as smooth muscle cells (SMCs), chondrocytes, keratinocytes, type II pneumocytes, etc. have been described (Boivin et al. 2009). Until recently granzyme B has been mainly considered as an intracellular agent but there has been also some focus on extracellular granzyme B activity. In this regard, Spaeny-Dekking and colleagues have identified granzyme B in the plasma of healthy individuals at about 11.5 pg/ml,

whereas during ongoing viral infection plasma levels between 1–4,000 pg/ml were discovered. Locally in synovial fluid of patients with rheumatoid arthritis median levels of 3.183 pg/ml were measured (Spaeny-Dekking et al. 1998). In our *in vitro* experiments concentrations of 10 ng/ml lead to a robust generation of C5a indicating that the concentrations used here were rather comparable to those *in vivo*.

Granzyme B is a serine protease that has a preference for cleaving peptides immediately adjacent to aspartate residues. Bid, ICAD, PARP, DNA-PKcs, and a variety of procaspases have been demonstrated to serve as substrates for granzyme B. Interestingly, in the case of granzyme B, no single amino acid motif exists as the preferred sequence for cleavage thus yielding large numbers of potential substrates (Bredemeyer et al. 2004). In a granzyme proteomics approach Bredemeyer et al. (2004) have used recombinant murine granzymes to treat lysates from YAC-1 cells. Among the substrates for granzyme B were α-tubulin, β-actin, heterogeneous nuclear ribonucleoprotein K (hnRNP K), hnRNP A3, the Hsp70/Hsp90 organizing protein (Hop), calreticulin, and caprin-1 (Bredemeyer et al. 2004). Our data demonstrates that C5 and C3 are further substrates for granzyme B. In addition, chemotactic activity for human neutrophils with granzyme B generated C5a was demonstrated.

As opposed to necrosis, apoptotic cell death is generally considered a way to get rid of cells without alarming the immune system (Taylor et al. 2008). Hence it might be surprising that an apoptotic protease, such as granzyme B, was able to generate such a potent stimulus of the immune system as C5a. However, one has to keep in mind that under extreme situations in case of a severe tissue injury or an acute trauma that causes a systemic inflammatory response, systems can get out of control. Terms like normal physiological concentrations have to be reconsidered. It has been shown, that these exceptional circumstances also result in upregulation of apoptosis (Cobb et al. 2000; Delogu et al. 2001; Hotchkiss et al. 2000; Ogura et al. 1999; Pellegrini et al. 2000). Extensive trauma and tissue damage is attended by an enormous exposure to pathogen associated molecular patterns (PAMPs) and also endogenous danger associated molecular pattern (DAMPs) (Gebhard and Huber-Lang 2008; Kohl 2006; Levy et al. 2007). These danger signals flush the whole body resulting in production of vast amounts of pro-inflammatory cytokines that activate effector cells of the innate immune system. These cells initiate the killing program by activating the apoptosis system of infected or damaged cells in order to protect the host. One of these apoptosis inducing factors released is granzyme B. By analysing the intracellular level of granzyme B in polytrauma patients, we could detect a significant increase of granzyme B in lymphocytes and neutrophils early after trauma compared to healthy donors. Secondary necrosis of these cells, which at first might be prone to die through apoptosis, might be a scenario where granzyme B could be released after severe trauma. In addition, the presence of granzyme B in various tissues including the lung might also facilitate a trauma-induced direct release of granzyme B into the system. This might represent an alternative pathway for triggering the complement system and producing the potent anaphylatoxin C5a leading to dysfunction of blood leukocytes in critically injured patients regarding phagocytosis, chemotaxis and respiratory burst (Czermak et al. 1999; Guo et al. 2006; Huber-Lang et al. 2001; Huber-Lang et al. 2002; Riedemann et al. 2002).

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Role of C3, C5 and Anaphylatoxin Receptors in Acute Lung Injury and in Sepsis

Markus Bosmann and Peter A. Ward

Abstract The complement system plays a major role in innate immune defenses against infectious agents, but exaggerated activation of complement can lead to severe tissue injury. Systemic (intravascular) activation of complement can, via C5a, lead to neutrophil (PMN) activation, sequestration and adhesion to the pulmonary capillary endothelium, resulting in damage and necrosis of vascular endothelial cells and acute lung injury (ALI). Intrapulmonary (intraalveolar) activation of complement can cause ALI that is complement and PMN-dependent, resulting in a cytokine/chemokine storm that leads to intense ALI. Surprisingly, $C3^{-/-}$ mice develop the full intensity of ALI in a C5a-dependent manner due to the action of thrombin that generates C5a directly from C5. There is conflicting evidence on the role of the second C5a receptor, C5L2 in development of ALI. There is accumulating evidence that C5a may suppress inflammatory responses or divert them from Th1 to Th2 responses, impacting the innate immune system. Finally, in experimental polymicrobial sepsis, there is evidence that many of the adverse outcomes can be linked to the roles of C5a and engagement of its two receptors, C5aR and C5L2. These observations underscore the diversity of effects of C5a in a variety of inflammatory settings.

Keywords Complement • Anaphylatoxins • Immune complexes • Endotoxin • Sepsis • Septic shock

1 Acute Lung Vascular Injury Following Systemic Activation of Complement

Several years ago, before availability of $C3^{-/-}$ mice, purified naja naja cobra venom factor (CVF) was repetitively injected intraperitoneally over a period of 36 hr, resulting in nearly complete depletion of plasma C3 as measured quantitatively by

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immunochemical techniques. Such complement depletion resulted in greatly attenuated ischemia-reperfusion injury of hind limbs, or injury of kidney, heart, small bowel, to name a few examples (Seekamp et al. 1993; Seekamp and Ward 1993). Since later work indicated that *in vivo* neutralization of C5a had similar effects, it was assumed that C3 depletion prevented activation of C5, abolishing formation of C5a (reviewed, Collard et al. 1999; Hammerschmidt et al. 1980). This presumption was confirmed when it was shown that CVF isolated from naja haja cobra snakes (instead of CVF from naja naja cobra snakes) depleted C3 but did not activate C5 and did not cause acute lung vascular damage after vascular infusion (Till et al. 1987). In contrast, naja naja bolus CVF infusion (intravenous) caused rapid onset of extensive injury to the pulmonary vascular endothelium, leading to necrosis of endothelial cells and intraalveolar hemorrhage and flooding (Till et al. 1987). Such studies suggest that intravascular activation of complement can cause intense injury to the vascular endothelium, which is linked to PMN adherence to the endothelium associated with CD11b/CD18 activation on PMNs and rapid C5a-dependent expression of P-selectin on endothelial cell surfaces, the engagement of these adhesion molecules leading to intensification of microvascular injury due to close spatial proximity between PMNs and endothelial cells (Till et al. 1982).

In subsequent studies we demonstrated the mechanisms by which damage of endothelial cells in the presence of activated neutrophils (PMNs) occurs. Activated PMNs generate H_2O_2, which is freely permeable across the plasma membrane of endothelial cells. Production of H_2O_2 by activated PMNs is followed by $O_2^{\bullet}$ generation following conversion of xanthine dehydrogenase to xanthine oxidase in vascular endothelial cells, resulting in formation of $O_2^{\bullet}$. $O_2^{\bullet}$ can react with $Fe3^+$ from ferritin within endothelial cells, causing reduction to Fe^{2+} and release of $Fe2^+$ into the cytosol of the endothelial cell. The interaction of $Fe2^+$ with H_2O_2 within the endothelial cells results in formation of the highly-reactive and short-lived hydroxyl radical, $HO^{\bullet}$ (Gannon et al. 1987; Varani et al. 1985). Prior depletion of iron within endothelial cells using the iron chelator, deferoxamine, or addition of allopurinol which blocks the enzymatic activity of xanthine oxidase, will both greatly attenuate the ability of activated PMNs to injure endothelial cells *in vitro* (reviewed, Till et al. 1991).

2 Complement in Experimental and Clinical Acute Lung Injury

In the literature dealing with acute lung injury (ALI) or acute respiratory distress syndrome (ARDS), C5a has been found in BAL fluids along with a substantial number of neutrophils, suggesting the possibility that C5a presence in lung may be related to the buildup of PMNs in the alveolar compartment and that products of PMNs may directly cause ALI, involving both the vascular and alveolar epithelial barriers (Hammerschmidt et al. 1980; Pittet et al. 1997; Solomkin et al. 1985). Endotoxemia in mice has been linked to the appearance of C5a in plasma, but, when LPS is given intratracheally, the result is ALI with alveolar hemorrhage and fibrin

deposition together with abundant accumulation of PMNs, all of which have been shown to be associated with the requirements for migration inhibitory factor (MIF) and LTB4 receptors (Donnelly et al. 1997; Makita et al. 1998; Nishihira 2000; Rittirsch et al. 2008a). Surprisingly, in recent studies of LPS-induced ALI, no C5a could be detected in BAL fluids, although, when LPS was injected intraperitoneally, C5a appeared in the plasma (Rittirsch et al. 2008a). Furthermore, ALI after intratracheal administration of LPS was fully expressed in $C5^{-/-}$ mice, quantitatively the same as ALI developing in $C5^{+/+}$ mice. Collectively, the data suggest that LPS-induced ALI is complement-independent but requires the participation of MIF and receptors (BLT1) for LTB4. In the setting of endotoxemia, C5a appears to be required for the acute febrile response (Barton and Warren 1993; Li et al. 2005). The reason for the independence of the requirement for C5a in LPS-induced ALI may be related to the high levels in lung of C1 esterase inhibitor and surfactant A, which sharply limit activation of complement in the lung (Watford et al. 2000, 2001), or the problem could be the lack of adequate amounts of complement proteins in the alveolar compartment to generate needed levels of C5a.

It should be noted that in the setting of ALI induced by intrapulmonary deposition of IgG immune complexes, there is robust engagement of Fc receptors (FcγRI/III) (Ravetch and Clynes 1998) as well as complement activation as measured by buildup of C5a in BAL fluids and suppression of IgG-induced ALI by the use of $C5^{-/-}$ mice (Larsen et al. 1981) or by use of neutralizing antibody to C5a (see Fig. 1, Huber-Lang et al. 2006; Mulligan et al. 1996; Ward 1996). Generation of C5a within lung sets the stage for the appearance of TNFα, suppression of which reduces PMN buildup in the lung and protects from ALI, perhaps due to the lack of upregulation of vascular ICAM-1, which is needed for full adhesive interactions between endothelial cells and PMNs etc. (Warren et al. 1989).

3 Lessons Learned in $C3^{-/-}$ Mice

The genetic-based knockout of FcγRI/III, as expected, substantially reduced the intensity of IgG immune complex-induced ALI in lungs of mice (Sylvestre et al. 1996). Earlier work indicated that IgGIC-induced ALI was attenuated in $C5^{-/-}$ mice (Larsen et al. 1981). In spite of intrapulmonary neutralization of C5a greatly attenuating ALI (Gao et al. 2006), the finding of fully expressed ALI after IgGIC deposition in $C3^{-/-}$ mice led to the conclusion that this form of ALI was independent of complement activation (Sylvestre et al. 1996). However, it was subsequently shown that $C3^{-/-}$ mice undergoing IgGIC deposition had C5a in their BAL fluids and that neutralization of C5a in these mice markedly attenuated ALI (Huber-Lang et al. 2006). The absence of C3 in these KO mice inferred that no complement activation pathway could be engaged under such conditions. On the basis of earlier work, we showed that neutral proteases in PMNs could directly generate C5a in the presence of C5 (Ward and Hill 1970). More recently, we demonstrated that lung macrophages, when activated, expressed a neutral protease that would cleave C5

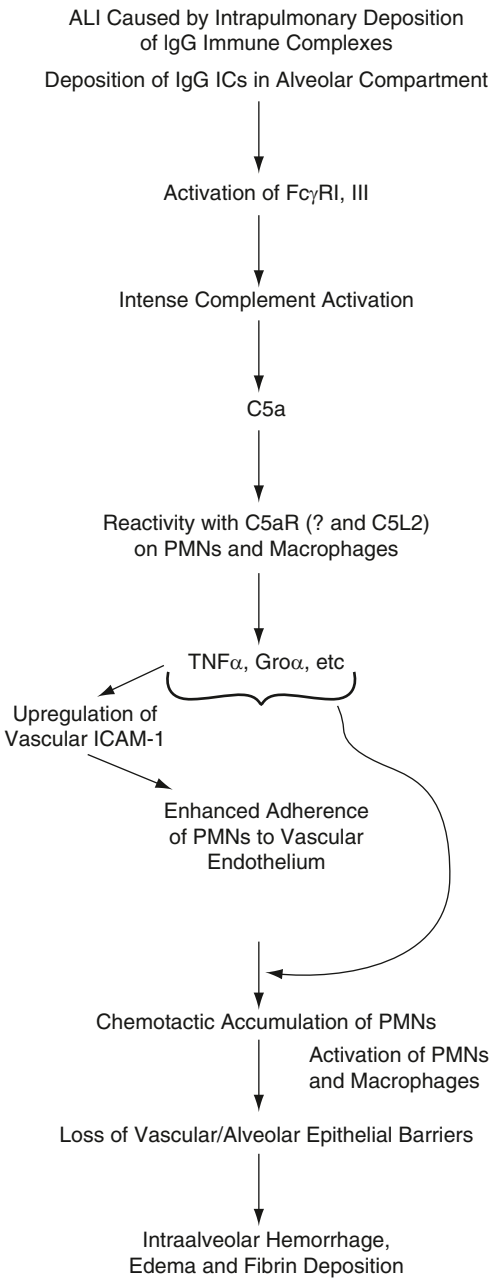

Fig. 1 Pathophysiology describing development of acute lung injury induced in rodent lung following intrapulmonary deposition of IgG immune complexes.

to form C5a (Huber-Lang et al. 2006). In the case of $C3^{-/-}$ mice, the co-instillation into lung of recombinant hirudin (a potent thrombin inhibitor) together with the IgG antibody to bovine serum albumin greatly reduced generation of C5a. In addition, it was shown that thrombin could catalyze the cleavage of C5 and amino acid sequencing confirmed that the peptide formed by this interaction of C5 with thrombin was authentic C5a. In addition, we showed that $C3^{-/-}$ mice had supernormal levels of plasma thrombin (Huber-Lang et al. 2006). All in all, these studies suggested that, in the absence of C3, mice compensate by producing supernormal levels of thrombin in liver, allowing formation of C5a at various tissue/organ sites. Recent studies also suggest that several activated clotting factors (reviewed, Amara et al. 2010; Markiewski et al. 2007) also have the ability to generate C5a from C5, emphasizing interactions between the clotting and complement cascades (see below).

A very recent study has extended the spectrum of activated human clotting factors that can generate complement anaphylatoxins (C3a, C5a) in the presence of purified human C3 or C5 (Amara et al. 2010). As described above, a few years ago it was demonstrated that thrombin (Factor IIa) can react with C5 to generate C5a (Huber-Lang et al. 2006). It has recently been shown that the following activated clotting and fibrinolytic factors (F) can generate (in rank order) both C3a and C5a when exposed to human C3 or C5: FXa > plasmin > thrombin > FIXa > FXIa. In the case of FXa, selective inhibitors (fondaparinux, enoxaparin) suppressed generation of the anaphylatoxins. Such data reinforce the existence of crosstalk between the complement, clotting and fibrinolytic cascades. Obviously, in the setting of human plasma, the ability to rank order these various factors and relate them to anaphylatoxin generation is extremely difficult especially because of various endogenous inhibitors present in human plasma.

4 Role of C5aR and C5L2 in IgGIC ALI

As indicated above, it was shown many years ago that IgGIC-induced ALI was greatly reduced in intensity in $C5^{-/-}$ mice (Larsen et al. 1981). The two receptors for C5a have been characterized over the past decade (Cain and Monk 2002; Gerard and Gerard 1991; Gerard et al. 2005; Kalant et al. 2003, 2005; Lee et al. 2008; Okinaga et al. 2003). C5aR is a traditional G-protein coupled receptor that, when ligated to C5a, proceeds through the MAPK signaling pathways, resulting in phagocyte (PMNs, macrophages) responses such as rapid $Ca2^{+}$ transients, chemotaxis, respiratory burst, oxidant production and secretion of granule enzymes. Such innate immune responses are designed to confront invading micro-organisms, resulting in their localization and destruction. The story with the other C5a receptor, C5L2 is much less well understood. C5L2 is reported to react with C5a, $C5a_{\text{des Arg}}$, and $C3a_{\text{des Arg}}$, the last being also referred to as "acylation-stimulating protein." It is clear that ligation of C5L2 does not result in appearance of $Ca2^{+}$ transients even though C5L2 has very high binding affinity to the complement-derived anaphylatoxins. The lack of $Ca2^{+}$ signaling is due to an amino acid substitution in the DRY

region of the third intracellular loop of C5L2, which prevents interactions of the receptor with G-proteins. The localization of receptor protein for C5aR is unequivocally on the outer cell membrane of phagocytes. After ligation of C5aR with C5a, the receptor is internalized, stripped of C5a, and some (<50%) of the receptor is recycled to the cell membrane. The story of C5L2 is quite complicated. It appears that, in the non-activated PMN, most of the C5L2 receptor is present within cytosolic granules (Bamberg et al. 2010; Cain and Monk 2002; Scola et al. 2009). Assuming that C5aR and C5L2 are situated in two distinctly different locales in or on PMNs, an important question is: How does C5a/C5a $_{\text{des Arg}}$ get into an intracellular position in order to bind to cytosolic C5L2 and affect cell function and is there any interaction (such as heterodimerization) between ligated C5aR and C5L2?

There is a consensus that $Ca2^{+}$ signaling does not occur after C5a interaction with C5L2. Beyond this point, there is little agreement regarding the biological and biochemical responses to C5L2. The Toronto group finds that C5L2$^{-/-}$ macrophages or PMNs exposed to C5a have defective phosphorylation of ERK1/2 (Chen et al. 2007), while the Boston group claims that ERK1/2 activation is fully intact in C5L2$^{-/-}$ phagocytic cells (Okinaga et al. 2003). C5L2 was originally described as a "default" receptor, implying that it competes with C5aR for C5a binding but in the absence of subsequent cell signaling events in C5L2$^{-/-}$ cells (Gerard et al. 2005; Okinaga et al. 2003). In the setting of IgGIC-induced ALI, it has been reported that ALI and PMN accumulation are depressed in C5L2$^{-/-}$ mice (Chen et al. 2007), while another group reports that under similar conditions C5L2$^{-/-}$ mice show substantially intensified injury (defined by increased vascular permeability) as well as enhanced PMN accumulation in lung after IgGIC ALI. On the other hand, it has been recently reported by both groups (Toronto and Boston) that asthmatic lung responses in C5L2$^{-/-}$ mice are substantially reduced when compared to Wt mice (Chen et al. 2007; Zhang et al. 2010). How to explain such divergent observations is very difficult at present, unless one assumes that the genetic backgrounds of C5aR$^{-/-}$ and C5L2$^{-/-}$ mice used in the different laboratories are disparate, although all mice are on a C57BL/6 background.

5 Evidence for Regulatory Roles of C5, C3a, C5a, C3aR and C5aR in Inflammation

As indicated above, there is abundant evidence for the proinflammatory roles of C5a and C5aR in a variety of inflammatory responses (e.g., antigen-induced arthritis, collagen-induced arthritis, acute vascular injury after systemic activation of complement, IgGIC injury of venules, ALI, ARDS, etc.). There has been recently emerging evidence that in some circumstances C5, C5a and C5aR may regulate the inflammatory response (see Fig. 2). Several years ago it was found that mice deficient in C5 developed more intense asthmatic responses when compared to C5-intact mice, under conditions using active immunization and repetitive antigenic challenge of the lung (Karp et al. 2000), suggesting that C5 or its activation prod-

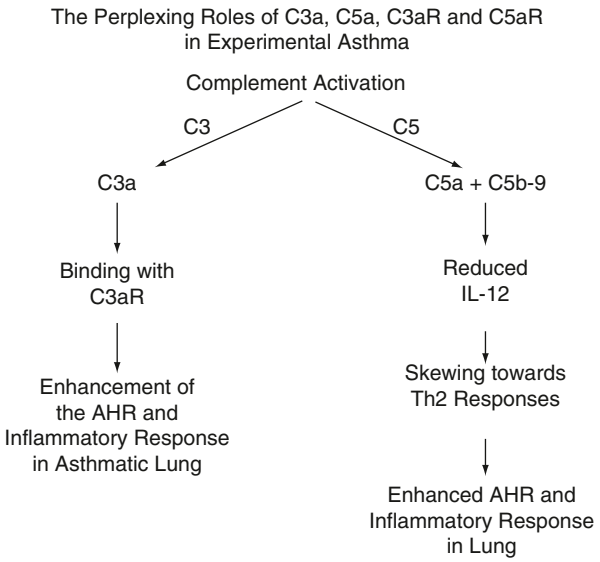

Fig. 2 Roles of C3a, C5a and their receptors in development of experimental asthma in mice

ucts somehow suppressed immune/inflammatory responses tied with development of experimental asthma. When C5aR was blocked with a mAb to C5aR, this resulted in exacerbation of airway hyperreactivity (AHR), whereas blockade of IL-17A reversed the enhanced AHR in mice in which C5aR was blocked (Lajoie et al. 2010). Such data suggested that, in experimental allergic asthma, AHR is mediated by IL-17A via enhanced IL-13-driven responses and that such responses are regulated by C5aR and C5a. Another interesting observation was that $C3aR^{-/-}$ mice had fewer Th17 cells and reduced AHR after antigen challenge (Lajoie et al. 2010). Accordingly, it appears that C5a and C5aR negatively inhibit asthmatic responses whereas C3a and C3aR have the opposite effects, suggesting a very complicated relationship between complement derived anaphylatoxins and their receptors in the setting of experimental asthma.

In work by another group, blockade of C5aR promoted Th2 sensitization and development of the asthmatic response whereas, using multi-antigenic challenges, the same type of blockade suppressed the allergen triggered acute inflammatory response in lung (Kohl et al. 2006). Other work has suggested that C5a suppresses IL-12 production (Hawlisch et al. 2005), thus skewing the immune response in lung in the direction of Th2 type responses, resulting in the asthmatic response and suppressing the cell mediated Th1 type response. Based on the complexity of the effects of C5a and C3a and their receptors, and the differences in effects of antigen based on when it was administered into lung and how often (see above), such data have greatly complicated the issue as to whether blockade of either C5a or C3a, or their receptors, would be safe and effective for the treatment of humans with asthma. Finally, there are data suggesting that IL-10 production from LPS-stimulated macrophages can be enhanced in the presence of C5a (Bosmann and Ward [unpublished results]; Zhang et al. 2007). Since IL-10 is a well known for its

anti-inflammatory properties, this could be another example in which generation of C5a results in suppression of the inflammatory response by induction of an anti-inflammatory cytokine.

6 Roles of C3, C5 and C5a Receptors in Polymicrobial Sepsis

Extensive work has been done in order to define the roles of C3, C5, C5a and C5a receptors in the setting of polymicrobial sepsis following cecal ligation and puncture (CLP) in rodents (Table 1). Several years ago (Prodeus et al. 1997) and more recently (Flierl et al. 2008), it was shown that $C3^{-/-}$ mice are extremely susceptible to CLP-induced sepsis, possibly related to the loss of protective innate immune factors such as C3b and iC3b which enhance phagocytosis and removal of bacteria from tissues. If one accepts a substantial body of literature suggesting that C5a and its receptors have major negative impacts in the setting of CLP (Ward 2010), the absence of C5 would be expected to be protective. While $C3^{-/-}$ CLP mice had a much higher mortality rate compared to Wt mice, $C5^{-/-}$ mice after CLP were largely indistinguishable from Wt mice, which raises questions as to why $C5^{-/-}$ mice could not be distinguished from $C5^{+/+}$ mice in the setting of CLP (Flierl et al. 2008). After CLP, Wt mice depleted of C6 with a neutralizing antibody, showed an almost 400-fold increase in bacterial colony forming units in blood when compared to $C5^{+/+}$ mice, suggesting that products from C5 and C6, such as C5b-9, might be regulating the level of bacteremia after CLP, perhaps related to the ability of C5b-9 to cause lysis of gram-negative bacteria. On the other hand, it has been reported that $C6^{-/-}$ rats or rats depleted of C5 with an antibody had improved survival and reduced bacterial burden in various tissues. On the basis of such conflicting data, it is difficult to determine to what extent C5b-9 plays a significant role in the outcome of polymicrobial sepsis.

It is clear that C5a and C5a receptors play a harmful role in the setting of CLP both in rats and in mice. Neutralization of C5a resulted in dramatically improved survival after CLP (Czermak et al. 1999), together with largely intact innate im-

Table 1 Examples of adverse outcomes linked to C5a and C5a receptors during polymicrobial sepsis. (Described in greater detail in Ward 2010)

1. Early in sepsis, upregulation on PMNs of CD11b/CD18 and chemokine receptors that are usually expressed at very low levels.
2. Later in sepsis, paralysis of MAPK signaling pathways in blood PMNs, resulting in loss of innate immune responses of PMNs (phagocytosis, chemotaxis, onset of respiratory burst and activation of NADPH oxidase).
3. Upregulation on endothelial cells of ICAM-1 and tissue factor.
4. Enhancement of cytokine/chemokine storm.
5. Apoptosis of lymphoid cells.
6. Activation of consumptive coagulopathy and fibrinolytic cascades.

mune responses of blood PMNs from CLP rats (Huber-Lang et al. 2001, 2002b), greatly reduced intensity of activation of the clotting and fibrinolytic cascades (Laudes et al. 2002), substantially reduced evidence of apoptosis of thymocytes (Riedemann et al. 2002), greatly reduced intensity of the cytokine storm (Rittirsch et al. 2008b), reduced evidence of defects in contractility and relaxation responses of cardiomyocytes after CLP (Niederbichler et al. 2006), and reduced levels of plasma/serum biomarkers of multiorgan failure (Huber-Lang et al. 2001, 2002a, b). Using survival of CLP mice as the main endpoint, it was concluded that both C5aR and C5L2 contribute to the adverse outcomes of sepsis. This was determined by use of receptor KO mice ($C5aR^{-/-}$, $C5L2^{-/-}$) as well as by the use of blocking antibodies to C5aR and C5L2 (Rittirsch et al. 2008b). One of the interesting facets of these studies was the observation that production of the pleiotropic mediator, HMGB1, which is known to be an important harmful mediator in CLP-induced sepsis (Lotze and Tracey 2005; Qin et al. 2006), was derived from engagement of the C5L2 receptor on macrophages (Rittirsch et al. 2008b). Based on the absence of C5a receptors or antibody-induced inhibition of C5aR and/or C5L2, the cytokine storm was dependent on both receptors, since absence or blockade of either receptor substantially reduced the cytokine storm. This observation suggested that the cytokine storm after CLP may depend on sequential engagement of C5aR and C5L2.

7 Possible Translational Applications to Human Sepsis

Taken together, these data suggest in the setting of polymicrobial sepsis that the sequences of adverse events can be collectively linked to complement activation, production of C5a and engagement of C5a with both C5aR and C5L2. The appearance of C5a (10–100 nM) in serum/plasma from septic human patients, as well as the development of the defects in blood PMNs of these patients, resulting in defective innate immune responses, apoptosis of lymphoid cells resulting in immunodeficiency and intense consumptive coagulopathy and activation of the fibrinolytic system, demonstrate striking parallels between experimental polymicrobial sepsis (CLP) and human sepsis. It is possible that the extensive data obtained in polymicrobial sepsis of rodents may have translational implications for the treatment of septic humans.

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Complement Involvement in Neovascular Ocular Diseases

Ryoji Yanai, Aristomenis Thanos and Kip M. Connor

Abstract Pathological neovascularization (NV) is a hallmark of late stage neovascular age-related macular degeneration (AMD), diabetic retinopathy (DR), and retinopathy of prematurity (ROP). There is accumulating evidence that alterations in inflammatory and immune system pathways that arise from genetic differences, injury, and disease can predispose individuals to retinal neovascular eye diseases. Yet the mechanism of disease progression with respect to the complement system in these maladies is not fully understood. Recent studies have implicated the complement system as an emerging player in the etiology of several retinal diseases. We will summarize herein several of the complement system pathways known to be involved in ocular neovascular pathologies. Current treatment for many neovascular eye diseases focuses on suppression of NV with laser ablation, photodynamic therapy, or anti-VEGF angiogenic inhibitors. However, these treatments do not address the underlying cause of many of these diseases. A clear understanding of the cellular and molecular mechanisms could bring a major shift in our approach to disease treatment and prevention.

Keywords Complement • Oxygen-induced retinopathy • Retinopathy of prematurity • Choroidal neovascularization • Age-related macular degeneration • Diabetic retinopathy

1 Eyeing the Complement System

Ocular neovascularization (NV), which can cause bleeding and retinal detachments, is a major cause of blindness in all age groups, with age-related macular degeneration (AMD), diabetic retinopathy (DR), and retinopathy of prematurity (ROP) affecting millions worldwide. Recent data indicates that retinal neovascular dis-

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ease is associated with a significant spike in immune activity. However, the role of immunity and/or inflammation in these pathologies is not fully understood. Generally, inflammation is considered a manifestation of host defense or injury and repair. Host defense is easily understood as the recognition of non-self followed by elimination (Danese et al. 2007). In the case of injury/repair, there is a finely tuned orchestration of clearance and subsequent repair of damaged tissue, which occurs at the microenvironmental level. However, dysfunction in the intensity or specificity of the inflammatory response, where abnormal self-recognition occurs, can result in the invoked inflammatory response becoming pathological. We will limit the scope of our discussion to AMD, ROP, and DR.

1.1 The Complement System

Inflammation, which is mediated in part by the complement system in innate immunity, is likely to be a critical component of retinopathy (Connor et al. 2007). Polymorphisms in the alternative complement pathway components Factor H (Cfh) and Factor B (Cfb) are linked to AMD (Edwards et al. 2005; Francis et al. 2008; Haines et al. 2005; Klein et al. 2005). However, the contribution of innate immunity in retinopathy is poorly understood. The complement system is a key component of innate immunity and plays a vital role in the defense against infection as well as in the modulation of immune and inflammatory responses (Walport 2001a, b). The complement system is an ancient mechanism of host defense, which exists in primitive form in the earliest vertebrates. In higher vertebrates, three pathways converge and lead to the activation of a central mediator, complement component-3 (C3). C3 activation leads to the entry of the final common pathway resulting in the formation of a one megadalton membrane attack complex (MAC, C5b-9). MAC can cause cellular lysis or cell death (although not all cellular destruction is mediated through MAC) (Walport 2001b). These three pathways are referred to as the classical, alternative, and lectin pathways. Complement activation can lead to several distinct downstream biological actions. Activation of complement not only helps defend the host against pathogens, but also has the potential to affect self-tissues in both a positive (protective autoimmunity) and negative manner (autoimmunity) (Markiewski and Lambris 2007) (Fig. 1).

1.2 Classical Complement Pathway

The classical complement pathway is commonly initiated by binding IgM, IgG1, or IgG3 that is associated with bound antigen (Markiewski and Lambris 2007; Walport 2001a, b). C1 is a large protein complex consisting of C1q, C1r and C1s subunits (Markiewski and Lambris 2007; Walport 2001a). Classically, the C1q subunit binds

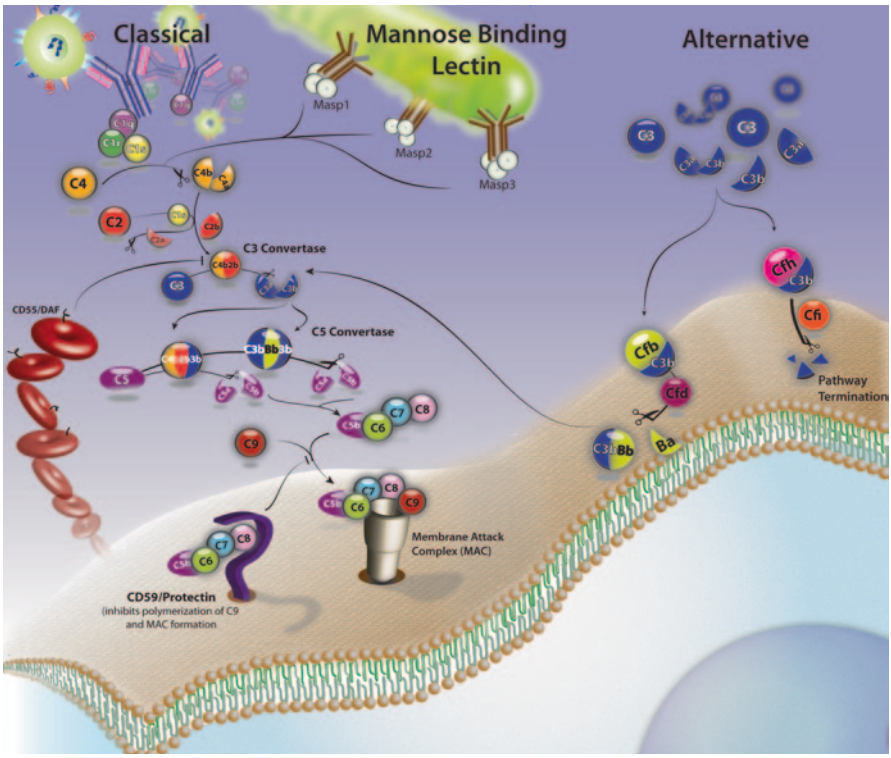

Fig. 1 Summary of the three complement cascades. The classical pathway is triggered when the protein C1, specifically C1 subunit C1q, binds to antibody and antigen complexes on infected cells. The binding of C1q activates C1s, which potentiates its action on C4, resulting in the cleavage of C4 into C4a and C4b. The mannose binding lectin cascade is triggered when the Mbl and its associate serine proteases Masp1, Masp2, or Masp3 bind to oligosaccharides on infected or foreign cell surfaces. The active product, Masp2, is able to cleave C4 and C2, resulting in the product C4b2b. C3 convertase proteolytically cleaves C3 protein into two products C3a and C3b. The active product, C3b binds with C4b2b, forming the activated C5 convertase, C4b2b3b. This C5 convertase activates the later steps of complement activation by cleaving C5 protein into two fragments C5a and C5b. C5b then complexes with C6, C7, C8, and C9, forming the membrane attack complex (MAC, or C5b6789). The alternative complement cascade is triggered by the spontaneous degradation of C3 into C3a and C3b. On healthy host tissue, which is not targeted for degradation, Cfh binds C3b fragments. This C3b/Cfh complex is cleaved into inactive products by Cfi, and the cascade is terminated. On activating cell surfaces (infected or foreign cells) Cfb optimizes cells with C3b to form the C3bB complex. Cfd cleaves the C3bB complex into C3bBb and Ba. The cleaved C3 fragment, C3b, which is formed by C3 and C3 convertase, is added to the C3bBb complex, forming the activated alternative specific C5 convertase C3bBbC3b. This convertase initiates the later stages of complement activation; C5 is subsequently cleaved into C5a and C5b. C5b complexes with C6, C7, C8, and C9, forming the membrane attack complex (C5b6789, or MAC)

to antibodies and consists of a radial array of six chains each with a globular head (Markiewski and Lambris 2007; Walport 2001a). C1r and C1s are serine proteases that form a tetramer containing two molecules of each protein that bind to C1q (Markiewski and Lambris 2007; Walport 2001a). Binding of the globular heads of C1q to two or more antibodies leads to enzymatic activation of C1r, which in turn, cleaves and activates C1s (Markiewski and Lambris 2007; Walport 2001a). Activated C1s potentiates the action of the next protein in the cascade, C4, resulting in production of C4b (Markiewski and Lambris 2007; Walport 2001a). C4b contains an internal thioester bond (similar to C3b) which allows for ester or amide linkages with the antibody complex or to cell surfaces (Markiewski and Lambris 2007; Walport 2001a). C2, the subsequent complement protein, complexes with surface bound C4b and is cleaved by C1s resulting in C2b formation (Markiewski and Lambris 2007; Walport 2001a). This C4b2b complex is the Classical pathway C3 convertase; it can bind and proteolytically cleave C3, forming C3b, which then binds antigenic surfaces (Markiewski and Lambris 2007; Walport 2001a). Some C3b molecules complex with C4b2b convertase to form C4b2b3b, also known as the classical pathway C5 convertase that initiates the formation of C5b and the later steps of complement activation (Markiewski and Lambris 2007; Walport 2001a) (Fig. 1).

The complement cascade has recently been found to regulate "self" as well as "non-self", without antibody involvement. Endothelial cells regulate vascular integrity through expression of gC1qR, a C1q binding site, which activates the classical complement cascade (Ghebrehiwet et al. 2006; Yin et al. 2007). Thus, gC1qR plays a definitive role in complement deposition and activation of the classical pathway on stressed endothelial cells, where surface bound IgG or IgM is not required (Yin et al. 2007). Fetal neurons undergoing pruning also activate complement through the classical system independently of immunoglobulins (Singhrao et al. 2000) through C1q mediated lysis. Neurons suppress membrane bound complement regulators that prevent complement deposition on host cells (Singhrao et al. 2000). C1q was recently found to regulate mature neural circuits within the developing retina (Stevens et al. 2007). Synapses to be suppressed are targeted for elimination by C1q mediated complement activation (Stevens et al. 2007).

1.3 Alternative Complement Pathway

The alternative pathway is the second major pathway by which C5b can be generated to cause cell destruction (Markiewski and Lambris 2007; Walport 2001a). The alternative pathway is activated by the spontaneous hydrolysis of the internal C3 thioester bond (Bexborn et al. 2008; Pangburn and Muller-Eberhard 1980; Tack et al. 1980) and can be further activated by other proteins, lipids, and carbohydrate moieties (Walport 2001a; Harboe and Mollnes 2008). Activation of the alternative cascade requires deposition of Cfb on targets for immune-mediated clearance (Wal-

port 2001a, b). The central enzyme in the alternative pathway is the C3 convertase, C3bBb (Walport 2001a). Its proenzyme, C3bB, is created following binding of Cfb to the activated fragment of C3, C3b (Harboe and Mollnes 2008; Walport 2001a). Bound Cfb is cleaved by the serine protease, Cfd, resulting in the liberation of Ba as well as the activation of the serine protease domain within Cfb, Bb, to form the active C3 convertase (Harboe and Mollnes 2008; Walport 2001a). The formation of the alternative pathway C3 convertase, C3bBb functions to cleave more C3 molecules setting up an amplification sequence. The net effect is the formation of hundreds of molecules of C3b on the surface of the cell where complement is being activated (Harboe and Mollnes 2008). This C3 convertase can be rapidly degraded on mammalian host cells by membrane complement regulatory proteins (discussed below) (Harboe and Mollnes 2008; Walport 2001a). Some of the C3b moieties that are generated by the C3 convertase can bind to the convertase to form the alternative pathway C5 convertase (C3bBbC3b) that will cleave C5 and initiate the later steps of complement (Harboe and Mollnes 2008; Walport 2001a). Cfh and complement factor I (Cfi) are key regulators of the alternative system. Binding of Cfh to C3b acts as a cofactor to the serine esterase (Harboe and Mollnes 2008; Walport 2001a). Cfi cleaves C3b into an inactive product, iC3b, which can no longer participate in the formation of C3 convertase (Harboe and Mollnes 2008; Walport 2001a) (Fig. 1).

It is not known what the role of the alternative pathway is in the regulation of "self". A large body of evidence links the alternative complement cascade to both the atrophic and neovascular forms of AMD in which loss of complement function is associated with more severe AMD (discussed in further detail below) (Edwards et al. 2005; Farwick et al. 2009; Haines et al. 2005; Klein et al. 2005; Magnusson et al. 2006; Sepp et al. 2006).

1.4 Lectin Complement Pathway

Mannose-binding lectin (Mbl) initiates the lectin pathway of complement activation. This pathway is triggered, in the absence of antibodies, by binding polysaccharides to circulating lectins (such as mannose or mannin) or to *N*-acetylglucosamine recognizing lectins or ficolins (Walport 2001a). Mbl's interaction with IgM contributes to the lectin pathway activation (McMullen et al. 2006). Mbl is found in a complex with Mbl-associated serine proteases, Masp-1, Masp-2, Masp-3 and a non-enzymatic protein Map19 (Stover et al. 1999a, b; Thiel et al. 2000). These soluble lectins are members of the collectin family and structurally resemble C1q. Binding of Mbl to its surface target leads to the activation of Masp-2 where it cleaves C4 and C2 through the lectin pathway (Ambrus et al. 2003; Hajela et al. 2002). The role of each of these molecules is still not fully understood. It is known that Map19 is an alternative splice variant of Masp-2 and that Masp-3 is a splice variant of Masp-1 and may be involved in competitive inhibition of Masp-2 to Mbl (Dahl et al. 2001;

Hajela et al. 2002). Subsequent events in the lectin pathway converge with those in the classical pathway (Fig. 1).

1.5 Late Steps of Complement Activation

The three complement pathways, classical, alternative, and lectin, converge at C3 convertase to form C5 convertase. During the final common pathways of complement activation, the cell associated C5 convertase cleaves C5 and generates C5b, which binds to the convertase. C6 and C7 bind sequentially, and the C5b67 complex is directly inserted into the lipid bilayer of the plasma membrane. This is followed by insertion of C8 and several C9 molecules that polymerize around the complex to form the MAC resulting in two distinct biological effects: cell lysis (when MAC is deposited at high levels) and prosurvival actions (when MAC is present at low levels on the cell surface) (Rus et al. 2006) (Fig. 1).

1.6 Membrane-Bound Mediators of Complement or Complement Inhibitors

C3 convertase can cleave many molecules of C3 to C3b thereby intensifying complement activation (Harris et al. 2007). Foreign surfaces generally are devoid of membrane regulatory proteins that inhibit this convertase, leading to complement amplification (Harris et al. 1999, 2007). In contrast, host cells are protected from the harmful effects of complement through cell surface-associated convertase regulatory proteins (Harris et al. 1999, 2007). There are several membrane-associated complement regulatory proteins in humans: decay-accelerating factor (DAF, Cd55), membrane cofactor protein (MCP, Cd46), complement receptor type 1 (CR1, Cd35), and Cd59 (Kim and Song 2006). Cd55 or DAF acts to avert opsonization and damage to self-cells at the site of complement initiation by preventing the formation of and inactivating the C3 and C5 convertase enzymes (Harris et al. 1999, 2007). Cd55 hastens the decay of the convertase by releasing Bb (Harris et al. 2007). Cd46 regulates C3 activation by acting as a cofactor for Cfi-mediated cleavage of C3b (Liszewski et al. 1991). CR1 has both Cd55 and Cd46 activities. In addition, CR1 is a major immune adherence receptor and plays a role in immune complex processing and clearance (Ahearn and Fearon 1989). Lastly, Cd59 prevents the formation of MAC at the terminal step of the complement activation cascade by binding to the complement component C8 or C9 (Meri et al. 1990; Miwa and Song 2001; Rollins and Sims 1990). Cd59 and Cd55 attach to the cell surface by a glycosylphosphatidylinositol anchor where Cd46 and CR1 are associated with the plasma membrane through their C-terminal transmembrane domain (Miwa and Song 2001) (Fig. 1).

2 Age-Related Macular Degeneration

AMD, a degenerative disease of the retina, is the leading cause of visual impairment within the elderly in the developed world (Congdon et al. 2004; Pascolini et al. 2004). Roughly eight million Americans have AMD, and its prevalence is expected to significantly increase as the population ages (Friedman et al. 2004).

The macula contains the densest concentration of photoreceptors within the retina and is responsible for central visual acuity, allowing a person to observe fine detail. The macula is located in the central, posterior portion of the retina. Damage to the macula can cause profound loss of fine vision. Genetic risk factors identified for AMD are known to involve key regulators of the complement system. The retinal-pigmented epithelium (RPE) lies beneath the photoreceptors and serves as part of the blood-retinal barrier. In addition to this function, the RPE has several additional functions including photoreceptor phagocytosis, nutrient transport, and cytokine secretion. Bruch's membrane lies posterior to the retinal pigment epithelium. Bruch's membrane serves as a semipermeable exchange barrier separating the retinal pigment epithelium from the choroidal vasculature, which supplies blood to the outer layers of the retina (Fig. 2a).

Clinically, AMD begins with the asymptomatic appearance of drusen, which consists of a focal deposition of acellular, polymorphous debris between the retinal pigment epithelium and Bruch's membrane. These focal deposits, drusen, are observed as white or yellow spots beneath the retina during funduscopic examination seen both in the macula and peripheral retina. The trigger or insult that results in the formation of drusen is not completely understood, and not all drusen formation leads to AMD. These deposits increasingly separate the RPE from the underlying choroidal vascular bed interrupting the function of the RPE and leading to photoreceptor degeneration, a hallmark of "dry" AMD. Excess drusen causing damage to the RPE can result in a chronic aberrant inflammatory response leading to large areas of retinal atrophy (called geographic atrophy), along with the expression of angiogenic cytokines such as vascular endothelial growth factor (VEGF) (de Jong 2006). In turn, this can lead to a disruption of Bruch's membrane (Fig. 2b).

In the most devastating or "wet" form of AMD, abnormal leaky blood vessels grow from the choroidal vasculature through the disrupted Bruch's membrane and RPE into the subretinal space beneath the photoreceptors causing profound visual loss. The development of choroidal neovascularization (CNV) is also associated with increased vascular permeability and fragility. CNV may extend anteriorly through breaks in Bruch's membrane and lead to subretinal hemorrhage, fluid exudation, lipid deposition, detachment of the retinal pigment epithelium from the choroid, fibrotic scars, or a combination of these findings (Fig. 2c).

A major difficulty in studying AMD clinically is the slow rate at which disease progression occurs. However, in the last few years epidemiological studies have identified family history, age, and cigarette smoking as general risk factors contributing to AMD (de Jong 2006; Seddon et al. 1997; Thornton et al. 2005).

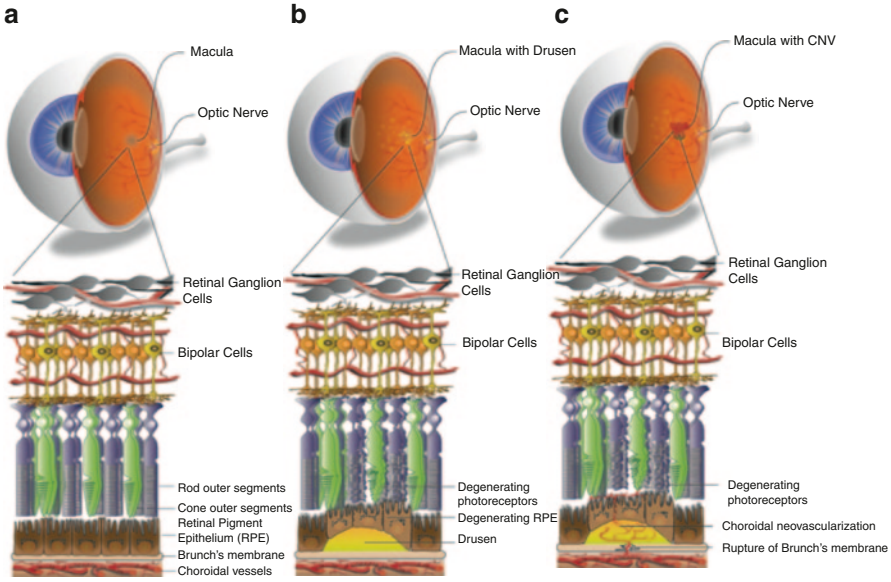

Fig. 2 Age-related macular degeneration. **a** A schematic cartoon of a normal human eye compared with an eye with AMD depicting deposits of drusen **b** (*yellow spots*) and choroidal neovascularization with subretinal hemorrhage in the macula. **c** A schematic cross-section of a normal eye **(a)**, showing retinal neuronal layers, RPE, Bruch's membrane and choroid vessels. In AMD, the intimate relation between photoreceptors, RPE and choroid is disrupted by drusen (lipid and cellular debris-containing deposits). The formation of drusen separates RPE from Bruch's membrane and the underlying choroidal vessels in association with RPE atrophy and photoreceptor degeneration (hallmarks of dry AMD) **(b)**. In wet AMD with choroidal neovascularization, abnormal leaky choroidal vessels proliferate and penetrate the altered Bruch's membrane protruding into the subretinal space, causing hemorrhage and rapid loss of vision **(c)**

2.1 The Complement System in AMD

In 2001, it was hypothesized that inflammatory components mediated the biogenesis of drusens and that this correlated with the severity of AMD (Hageman et al. 2001). Recent immunochemical and proteomic studies of drusen show an intriguing and complex protein composition that includes immunoglobulins, activated complement components (C3a, C3b, Cfb, C5, and C5a), complement regulators (vitronectin, clusterin, and Cfh, acute phase proteins and the Alzheimer's disease amyloid-β (Aβ) peptide (Crabb et al. 2002; Hageman et al. 2001; Johnson et al. 2001, 2002; Mullins et al. 2000; Nozaki et al. 2006). Additional components of drusen include oxidized lipids, and intracellular proteins (ubiquitin and crystallins) and microglia/macrophage cell (Crabb et al. 2002). The identification of immunoglobulins and complement proteins in drusen combined with the earlier findings that chronic inflammatory cells associate with atrophic RPE and neovascular lesions strongly

suggest that AMD pathogenesis has a chronic inflammatory component (Anderson et al. 2002; Hageman et al. 2001; Johnson et al. 2001; Penfold et al. 1985, 2001).

Several elegant genetic studies have shown an important linkage of AMD progression with the complement system (Edwards et al. 2005; Haines et al. 2005; Klein et al. 2005). Genetic analysis of patents with AMD identified a chromosomal region implicated by linkage analysis and further defined by association studies using single nucleotide polymorphisms (SNPs) (Edwards et al. 2005; Haines et al. 2005; Klein et al. 2005). The locus at chromosome 1q32 shows a tight association with the *CFH* gene. The *CFH* haplotype that codes for the protein variant His402 results in a several-fold increase in disease risk for those who are homozygous for this SNP relative to the risk associated with homozygosity for Tyr402. Interestingly, heterozygotes for the most common haplotype, His402/Tyr402, have an intermediate disease risk (Edwards et al. 2005; Hageman et al. 2005; Haines et al. 2005; Klein et al. 2005). Lastly the significance of *CFH* sequence variation was further underscored by the observation that in a study of AMD patients, 24% were homozygous for the His402 allele compared to only 8% of controls subjects (Hageman et al. 2005).

Expression of Cfh is the highest in liver. However, Cfh protein is also expressed by the RPE and is known to be present in drusen (Johnson et al. 2006; Mandal and Ayyagari 2006). Twenty short consensus-folding domain repeats (SCR) make up the full length Cfh protein. The Y420H SNP position resides within SCR-7 and has been demonstrated to affect the binding of Cfh, to the C-reactive protein (CRP) (Jozsi and Zipfel 2008; Meri 2007). CRP protein is a family member of the short pentraxins. The Y402H polymorphism of Cfh has been shown to significantly decrease its binding affinity for CRP (Laine et al. 2007). Complementing this observation are studies illustrating that individuals carrying the high-risk variant of Cfh have significantly elevated levels of CRP in serum as well as within the retinal RPE (Johnson et al. 2006; Schaumberg et al. 2006). Interestingly, mice in which Cfh has been deleted have decreased visual acuity with age, however, fail to form AMD like lesions (Coffey et al. 2007). This data should give us pause; while it is clear that the Y402H mutation is involved in AMD the direct effects of this mutation on AMD disease progression remains unclear.

Common sequence variants in two additional complement regulatory proteins, Cfb and complement component 2 (C2) show a two- to three-fold difference in frequency in AMD versus control cohorts (Gold et al. 2006; Jakobsdottir et al. 2008). Cfb and C2 are encoded by neighboring and highly homologous genes located in the major histocompatibility complex (Gold et al. 2006).

Other human diseases associated with chronic inflammation and mutations, deletions, or polymorphisms within the Cfh gene cluster are atypical haemolytic uremic syndrome (aHUS) and membranoproliferative glomerulonephritis type II (MPGN II) (Jozsi and Zipfel 2008). Individuals with MPGN-II develop drusen early in life that closely resemble, both appearance and composition, the drusen observed in patients with AMD (Duvall-Young et al. 1989; Leys et al. 1991; Mullins et al. 2001). The disease pathology of MPGN-II is associated with an abnormally low level of

circulating C3, implicating a chronic activation of the complement cascade thereby draining systemic complement (Zipfel et al. 2006).

Lastly, complement inhibition has been demonstrated to be protective against a mouse model of laser-induced CNV (Bora et al. 2005, 2006, 2007; Kaliappan et al. 2008; Rohrer et al. 2009). However, micro-environmental source of complement and its relevance to AMD in this model remains unclear. The mouse CNV model utilizes laser burns to rupture Bruch's membrane and injure the retina (Aguilar et al. 2008). Neovessels arising from the choroid form in response to this injury (Aguilar et al. 2008) that is largely a result of wound healing.

2.2 Anti-Complement Drugs in AMD

Therapeutic intervention in the human complement system has long been recognized as a promising strategy for the treatment of AMD. Recent FDA approval of a complement targeted drug accelerated the ongoing efforts of those wishing to target complement system as a therapeutic strategy (Mucke and Mucke 2010; Qu et al. 2009; Wagner and Frank 2010). Several compounds targeting the complement system are currently in phase-1 or -2 clinical trials.

Compstatin/POT-4 (Potentia Pharmaceuticals/Alcon) is an analog of the small cyclic synthetic peptide compstatin, an inhibitor of the central complement cascade. Compstatin was discovered more than 10 years ago by screening phage-display libraries in the search for C3b-binding peptides (Sahu et al. 1996). Compstatin functions by preventing cleavage of C3 to its active fragments C3a and C3b (Sahu et al. 2000, 2003; Soulika et al. 2003; Ricklin and Lambris 2008). To date, Compstatin derivatives have been shown to be safe and effective in a series of ex vivo and *in vivo* experiments (Fiane et al. 1999a, b; Chi et al. 2010). A sustained release formulation aims at providing therapeutic drug concentrations for several months after intravitreal injection. Here, compstatin analogs form a gel-like deposit in the eye after intravitreal injection, from which the active peptide is slowly released over time (Qu et al. 2009). This distinct pharmacokinetic behavior may prove highly advantageous for the treatment of chronic eye diseases like AMD and is expected to reduce the frequency of intravitreal injections required compared to that of anti-VEGF inhibitors. Furthermore, a prospective, uncontrolled, non-randomized, dose-escalating, pilot phase-1 safety study in AMD patients with subfoveal CNV has been completed (ClinicalTrials.gov Identifier: NCT00473928) (Charbel Issa et al. 2011). Currently phase-2 clinical trials are enrolling subjects to test compstatin/POT-4 for intravitreal use in geographic atrophy.

Eculizumab (Soliris®, Alexion Pharmaceuticals) is a monoclonal antibody, which inhibits C5 actions. The drug is approved as systemic treatment for paroxysmal nocturnal hemoglobinuria (PNH) (Rother et al. 2007) (Schrezenmeier and Hochsmann 2009) (Parker 2009), a disease characterized by the absence of CD59 expression of erythrocytes. Eculizumab is currently being investigated in a phase-2 trial (ClinicalTrials.gov Identifier: NCT00935883) in patients with non-exudative AMD (drusen

or geographic atrophy). In the randomized, double-arm, double-masked study, patients receive the drug via an intravenous infusion. Primary outcome measures are the growth of geographic atrophy and the change in drusen volume, respectively) (Charbel Issa et al. 2011; Gehrs et al. 2010; Mucke and Mucke 2010).

ARC1905 (Ophthotech Corporation), a pegylated, aptamer-based C5 inhibitor, inhibits the cleavage of C5 into C5a and C5b, thus blocking downstream complement activation (Ni and Hui 2009). Intravitreal administration of ARC1905 is currently in two registered phase-1 clinical trials. In one clinical trial, ARC1905 is used for neovascular AMD in combination therapy with either multiple doses or with only one induction dose of intravitreal ranibizumab in a non-randomized, open-label, uncontrolled, safety study (ClinicalTrials.gov Identifier: NCT00709527). The second trial (ClinicalTrials.gov Identifier: NCT00950638), a randomized, open-label, dose comparison study, aims at elucidating the safety profile of intravitreal ARC1905 application in dry AMD (drusen and/or geographic atrophy) (Charbel Issa et al. 2011; Gehrs et al. 2010; Mucke and Mucke 2010).

FCFD4514S (Genentech/Roche) is an anti-complement factor D antibody fragment that is injected intravitreally. Similarly to ARC1905, its safety is currently in evaluation in a phase-1 trial in patients with dry AMD (geographic atrophy; ClinicalTrials.gov Identifier: NCT00973011) (Charbel Issa et al. 2011).

Several other compounds are currently being tested in a pre-clinical phase that aims either at inhibiting the effect of activated complement proteins or at normalizing an increased activation state of the complement cascade in AMD. TNX-234 is a humanized antibody directed against complement factor D, TA106 is a Fab fragment of an anti-CFB antibody. Other components including CR2–CFH hybrid proteins, JPE-1375 (a small molecule C5aR peptidomimetic), anti-properdin antibody (which should, in theory, destabilize the C3 convertase), C1-INH (a protein that inhibits activation of the classical pathway), and sCR1 (a soluble form of endogenous complement receptor 1, which promotes the degradation of active C3bBb) are also currently being tested in a pre-clinical phase (Gehrs et al. 2010). Another compound in preclinical development is a recombinant, protective form of *CFH*. TT30 is the amino-terminal portion of factor H containing its C3 inhibitory activity (Huang et al. 2008).

3 Retinopathy of Prematurity

ROP is a leading cause of blindness in children. About 460,000 infants/year (11%) are born prematurely and there are ~2,000 infants/year with severe ROP, even with treatment. Furthermore, there is a high socioeconomic impact due to blindness in children with ROP as it is generally thought of as a lifelong disease (0–70 years) (Good and Hardy 2001). The prevalence of ROP has increased as a result of improved survival among the most premature infants (Good and Hardy 2001; Mechoulam and Pierce 2003). Additionally, increases in multiple births, largely due to *in vitro* fertilization, results in increased numbers of premature infants that are

susceptible to ROP (Good and Hardy 2001). More than 80% of premature babies weighing less than 1,000 g develop some ROP (Good and Hardy 2001).

Premature infants have an underdeveloped retinal vascular network (Stahl et al. 2010; Smith 2004). ROP is considered a two-stage disease. In the first stage of ROP, exposure to oxygen levels that are higher than *in utero* results in a hyperoxic retinal environment (Stahl et al. 2010). Elevated oxygen levels above that of the in utero environment can result from room air but is often exacerbated with supplemental oxygen given to premature infants with poorly developed lungs (Stahl et al. 2010). These abnormally high oxygen conditions cause the developing retinal capillaries to cease growing and regress (Smith 2004; Stahl et al. 2010) (Fig. 3a, b). However, with regressed retinal blood vessels, the retina becomes progressively more hypoxic as it becomes increasingly metabolically active with maturity. Retinal NV, the second phase of the disease, is stimulated by this hypoxia (Smith 2004; Stahl et al. 2010). The neovascular phase of ROP, which is similar to other proliferative retinopathies, is characterized by the induction of pro-angiogenic growth factors that are hypoxia-regulated. This proliferative response is regulated by alterations in many pathways involving proteases, lipids, and angiogenic factors that trigger retinal NV (Smith 2004; Stahl et al. 2010) (Fig. 3c, d). Inability to overcome this hypoxia-driven response can lead to complications such as neuronal cell death, bleeding, retinal detachment and loss of visual acuity or blindness. The severity of the NV that develops is thought to be directly proportional to the amount of avascular retina that results in the first phase of the disease. In many cases despite the formation of neovessels, the initial damage to the retinal vasculature reverses and normal physiologic vessel development continues resulting in revascularization of this pervious avascular area. This coincides with the repression of pro-angiogenic factors from the retina and subsequent regression of the pathologic neovessels (Fig. 3e).

3.1 Inflammation in ROP

Recent discoveries have demonstrated that macrophages can have many different and even opposing functions depending on the disease process, the microenvironment and the cells activation state. Currently there is emerging evidence that specific sub-populations of microglia/macrophages regulate the retinal vasculature in distinctly different ways. However, the identity of these populations and how they are regulated are still largely undefined. Microglia and infiltrating monocytes are two cell types that have been identified as having a role in ROP pathogenesis.

Several recent studies have demonstrated that microglia/macrophages co-localize with neovascular blood vessels in the mouse model of oxygen-induced retinopathy (OIR) (Banin et al. 2006; Connor et al. 2007; Davies et al. 2006; Davies et al. 2008) (Fig. 3d). A population of F4/80 positive microglia/macrophages were found to be significantly increased in the late stages of the mouse model of OIR (Davies et al. 2006). These cells were found to be closely associated with neoves-

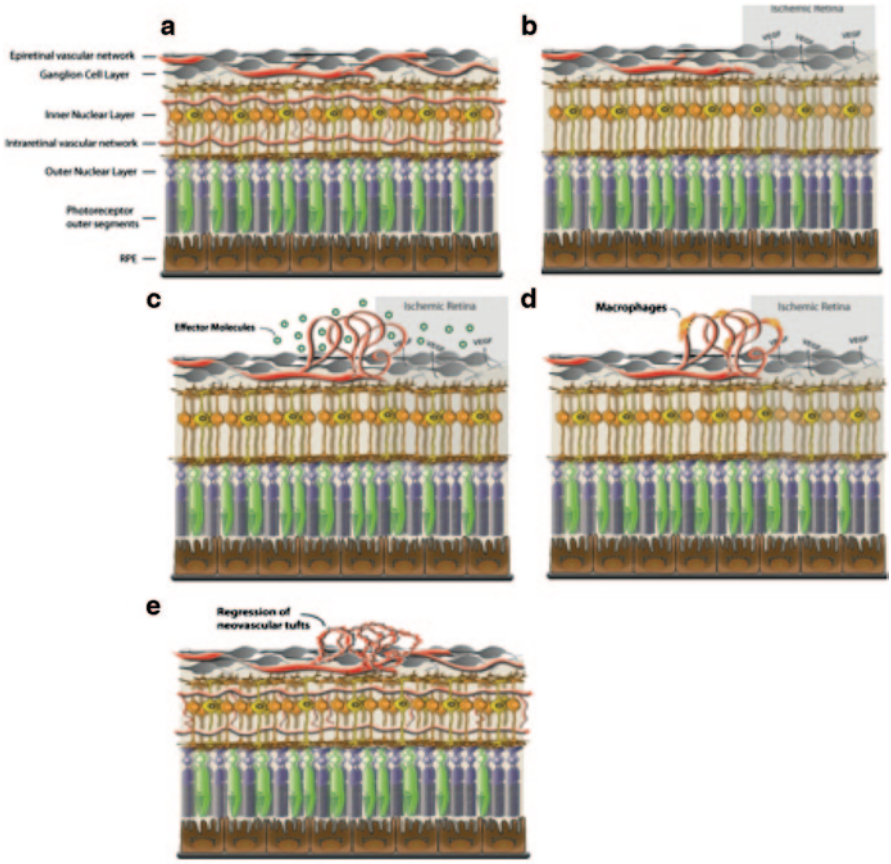

Fig. 3 Retinopathy of prematurity. **a** A schematic cartoon of a normal adult retina, in cross-section, with vascular beds intact. **b** Retinal cross-section of a premature infant with ROP at the zone of avascular and vascularized retina. Here normal blood vessel development has ceased resulting in a zone of avascular retina. As the retina continues to mature and become more metabolically active, this avascular zone will become ischemic and retinal cells within this area will produce pro-angiogenic molecules such as VEGF. **c** This sets up the second neovascular phase of the disease where pathologic neovessels are produced at the interface between vascularized and avascular retina in response to retinal ischemia. These neovessels produce effector molecules which allows them to recruit macrophages **d** to the site of neovascularization. **e** As normal physiologic vessel development revascularizes, the avascular area pro-angiogenic factors are repressed and this coincides with subsequent regression of the pathologic neovessels

sels in this model (Davies et al. 2006), and the chemokine, Ccl2, was found to be involved in disease resolution (Davies et al. 2006, 2008). Conversely, a population of bone marrow-derived myeloid progenitor cells in addition to resident retinal microglia were identified as being vital to the normalization of the retinal vasculature after mice were subjected to OIR (Ritter et al. 2006). This group later identified a CD44 (hi) expressing sub-population of myeloid cells that when introduced in-

travitreally in mice with OIR significantly promoted vascular repair (Friedlander et al. 2007).

3.2 The Complement System in ROP

A recent study using the mouse model of OIR, along with supporting *in vitro* data, suggests the complement system is a negative regulator of pathological NV in the context of vasoproliferative retinopathy (Langer et al. 2010). C3-deficient and C5aR-deficient mice revealed increased pathological retinal angiogenesis (Langer et al. 2010). However, the angiogenic inhibitory effect of complement was not mediated by a direct antiangiogenic effect of complement components on endothelial cells (Langer et al. 2010). The antiangiogenic action of complement is attributed to the presence of macrophages, as macrophage depletion reversed the proangiogenic phenotype of C3 deficiency. Macrophages are thought to act as the mediators of the antiangiogenic actions of complement and of C5a in particular (Langer et al. 2010). C5a polarized macrophages to a M1 proinflammatory phenotype by inducing secretion of inflammatory cytokines such as IL-6 and TNF-a and decreased expression of IL-10, which is recognized as the antiangiogenic macrophage signature (Gordon and Taylor 2005; Kelly et al. 2007; Lappegard et al. 2009; Ricklin et al. 2010; Schindler et al. 1990). Furthermore, monocytes/macrophages exposed to C5a *in vitro* secreted increased amounts of sVEGFR1, an established angiogenesis inhibitor (Girardi et al. 2006). Mechanistic consideration suggests that the C5a and C5aR axis inhibits pathological retina angiogenesis (Langer et al. 2010). However, the role of the second C5a receptor C5L2 (Ohno et al. 2000) expressed on macrophage (Chen et al. 2007) in angiogenesis needs further investigation.

4 Diabetic Retinopathy

Diabetic retinopathy (DR) has been classically regarded as a disease of the retinal microvasculature and is the primary cause of vision loss in working age adults in the USA (Aiello et al. 1994; Mohamed et al. 2007). The pathological manifestation associated with DR increases with duration of diabetes. Nearly all persons with type-1 diabetes and over 60% of those with type-2 diabetes have some level of retinopathy after 20 years (Aiello et al. 1994; Mohamed et al. 2007). The natural history of the disease has been divided into an early, nonproliferative stage, and a later, proliferative stage. DR is the most frequent complication of diabetes, afflicting over 90% of persons with diabetes and often progressing to a proliferative disease (Frank 2004). While tight glycemic control hinders retinopathy, strict compliance is difficult for most patients (Frank 2004).

DR is characterized by an initial and progressive decay of the retinal vasculature (Fig. 4a). The connection between hyperglycemia and retinal injury is thought to

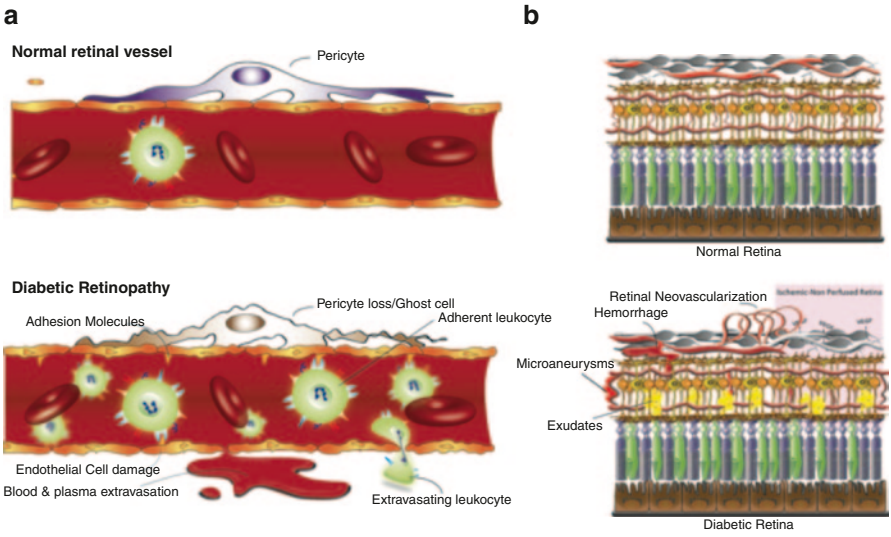

Fig. 4 Diabetic retinopathy. **a** A schematic cartoon of the normal retinal microvasculature contrasted with the retinal microvasculature of a diabetic individual. Compared to normal retinal vessels, the retinal endothelial cells of a diabetic individual significantly up regulate adhesion molecules on their surface allowing for the extravasation of circulating leukocytes. Moreover, pericytes are thought to become disassociated with the retinal vasculature leading to blood vessel dysfunction and regression. Compared to the normal retina, **b** the diabetic retina contains areas that have hemorrhages and microaneurysms. Additionally the dysfunctional retinal vasculature results in regions of ischemic non-perfused retina that can result in retinal neovascularation

be in part associated with severe risk factors that contribute to vascular disease including protein kinase C activation, buildup of advanced glycation end products, oxidative stress, and inflammatory mediators (Caldwell et al. 2003; Sheetz and King 2002). The disease pathology begins with the loss of pericytes and is increasingly thought to involve an inflammatory component involving leukocyte adhesion (Frank 2004; Joussen et al. 2004). This is followed by loss of capillaries that become acellular and nonperfused along with both vascular and neural cell apoptosis (Barber et al. 1998; Frank 2004). The destruction of retinal vessels leads to ischemia followed by expression of angiogenic growth factors as well as alterations in multiple pathways involving proteases, lipids, and angiogenic factors that trigger retinal NV (Frank 2004) (Fig. 4a, b).

4.1 Inflammation in DR

Inflammatory markers are increasingly detected in apparently healthy individuals that later go on to develop type-2 diabetes (Pradhan et al. 2001; Thorand et al. 2003; Vozarova et al. 2002). These results are indicative that inflammation occurs early

during the period of impaired glucose tolerance, prior to the diagnosis of type-2 diabetes.

Several studies have identified immunological mechanisms as playing a prominent role in the pathogenesis of DR. DR can be characterized by many features typically associated with inflammation such as tissue edema, increased blood flow, and upregulation of inflammatory mediators (Adamis et al. 1994; Aiello et al. 1994; Antonetti et al. 2006; Canton et al. 2004; Demircan et al. 2006; Esser et al. 1993; Funatsu et al. 2001; Joussen et al. 2001, 2003; Kim et al. 2005; Limb et al. 1996; Lutty et al. 1997; McLeod et al. 1995; Meleth et al. 2005; Schroder et al. 1991; Song et al. 2007; Watanabe et al. 2005; Yuuki et al. 2001; Zeng et al. 2000; Zhang et al. 2002). Direct evidence of the involvement of leukocytes in DR has come from both clinical and preclinical investigations.

Clinically elevated levels of neutrophils have been observed in both choroidal and retinal blood vessels of patients with diabetes, in conjunction with elevated immunoreactivity to intercellular adhesion molecule-1 (ICAM-1) (McLeod et al. 1995). Elevated numbers of leukocytes in the choriocapillaris of diabetic patients have been correlated with losses in viable endothelial cells and capillary dropout (Lutty et al. 1997). Supporting these observations was the finding that in animal models of diabetes elevated leukocyte numbers associate strongly with capillary damage (Kim et al. 2005; Schroder et al. 1991). Moreover, adhesion of leukocytes to retinal vessels is an early finding in diabetic animals and is associated with increased vaso-permeability and capillary occlusion changes that are linked with increases in ICAM-1 and CD18 (Joussen et al. 2004). VEGF is associated with the upregulation of ICAM and CD31 in DR, likely through nitric oxide (NO) and NF-κB dependent pathways (Joussen et al. 2002). CD4 and CD8 T-lymphocytes and macrophages have been found in elevated levels from the vitreous humor of diabetics (Canataroglu et al. 2005; Canton et al. 2004). Further preclinical evidence for leukostasis induced damage to the retinal vasculature was elucidated using the streptozotocin (STZ)-induced diabetic rat. In the STZ diabetic model increasing levels of retinal leukostasis occurred subsequent to the induction of diabetes (Miyamoto et al. 1999). Recruitment of leukocytes to the retinal capillaries lead to capillary obstruction and subsequent local nonperfusion (Miyamoto et al. 1999).

4.2 Involvement of the Complement System in Diabetic Retinopathy

Many studies have focused on the role of the complement system in relation to diabetic eye disease. Several proteomic studies have been performed analyzing protein profiles in human vitreous from patients with DR. In these studies, complement factors C3, C4b, C9 and factor B are increased in the vitreous of patients with pro-

liferative DR (Gao et al. 2008; Garcia-Merino et al. 1996; Kim et al. 2006; Koyama et al. 2003; Nakanishi et al. 2002; Ouchi et al. 2005; Yamane et al. 2003).

Increased glycosylation that occurs in diabetes mellitus has been implicated in the inactivation of important complement regulatory proteins, such as CD59, which normally serve to prevent self cells from being targeted by MAC (Qin et al. 2004). Evidence of the complement systems involvement in DR comes from the observation that membrane bound inhibitors of complement, CD55 and CD59, expression is significantly depressed or impaired due in part to nonenzymatic glycation. Supporting this are the findings that MAC (C5b-9) has been observed deposited on retinal blood vessels in diabetic rats and humans. MAC complexes have been detected in a large variety of diseased tissues: in atheromas (Bhakdi et al. 1999; Vlaicu et al. 1985); infracted myocardium (Schafer et al. 1986); and age-related and immune diseases of the skin (Sprott et al. 2000), muscle and joints (Helm and Peters 1993; Konttinen et al. 1996), neural tissues (Choi-Miura et al. 1992; Webster et al. 1997), and kidney (Couser et al. 1985; Montinaro et al. 2000; Nangaku et al. 1999). In many cases, MAC deposition has been found to bear relevance to the pathogenesis of the respective disease. Most recently, MAC has been reported to be present in drusen associated with aging and AMD (Mullins et al. 2000) in accord with the finding that C5 and vitronectin were present at these sites (Russell et al. 2000).

Increasing evidence from *in vitro* experimental studies, *in vivo* animal models and clinical data suggest a pathogenetic role of the complement system in the development of diabetic angiopathy. In a number of preclinical studies performed in diabetic MBL knockout mice, an association between the MBL pathway and diabetic microvascular and macrovascular changes has been demonstrated. Diabetic MBL knockout mice were protected against myocardial ischemia and reperfusion injury, cardiac hypertrophic (Busche et al. 2008), and diabetic kidney damage (Ostergaard et al. 2007). Normally, MBL does not bind to the body's own tissues, but changes in cell-surface glycosylation patterns in conditions such as diabetes mellitus or following tissue ischemia may dramatically increase the autoreactivity of MBL. This leads to increased immune activation, local and systemic inflammation, and a concomitant increased risk of late diabetes complications. Glycosylation pattern changes are potentially responsible for the link between the complement system and diabetic cardiovascular complications, resulting in the formation of advanced glycosylation endproducts (AGEs) of the arterial walls in patients with diabetes mellitus. These products generate new binding sites (that is, neo-epitopes) for MBL binding. While evidence is mounting, it remains unclear what the association between the MBL pathway and DR is.

Complement activation may thus have more widespread consequences in patients with diabetes mellitus than in those without the disease, and may contribute to the ongoing inflammation and microvascular and macrovascular complications including retinopathy. It remains unclear whether or not inhibition of complement could block retinal vessel drop out observed in DR.

5 Conclusions

Alterations in inflammatory and immune system pathways in neovascular diseases of the retina are emerging areas of investigation. Monocytic chemoattractants, elevated inflammatory mediators, and complement components have all been linked to neovascular eye diseases (Connor et al. 2007; Davies et al. 2008; Edwards et al. 2005; Gardiner et al. 2005; Haines et al. 2005; Jakobsdottir et al. 2008; Klein et al. 2005; Wang et al. 2009). Currently there is a fundamental lack in our understanding of the complement system, inflammatory mediators, and specific myeloid/lymphocytic subpopulations during distinct phases of neovascular disease pathology in the retina. Elucidating the underlying mechanism by which the immune system interacts and modulates retinal neovascular microenvironment could be of great clinical importance. Given the slow rate of disease progression in many of these neovascular maladies, even a slight modulation of immune activity in the retinas of these patients could extend visual acuity indefinitely.

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Platelets, Complement, and Contact Activation: Partners in Inflammation and Thrombosis

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Abstract Platelet activation during thrombotic events is closely associated with complement and contact system activation, which in turn leads to inflammation. Here we review the interactions between activated platelets and the complement and contact activation systems in clotting blood. Chondroitin sulfate A (CS-A), released from alpha granules during platelet activation, is a potent mediator of crosstalk between platelets and the complement system. CS-A activates complement in the fluid phase, generating anaphylatoxins that mediate leukocyte activation. No complement activation seems to occur on the activated platelet surface, but C3 in the form of C3(H_2O) is bound to the surfaces of activated platelets. This finding is consistent with the strong expression of membrane-bound complement regulators present at the platelet surface. CS-A exposed on the activated platelets is to a certain amount responsible for recruiting soluble regulators to the surface. Platelet-bound C3(H_2O) acts as a ligand for leukocyte CR1 (CD35), potentially enabling platelet–leukocyte interactions. In addition, platelet activation leads to the activation of contact system enzymes, which are specifically inhibited by antithrombin, rather than by C1INH, as is the case when contact activation is induced by material surfaces. Thus, in addition to their traditional role as initiators of secondary hemostasis, platelets also act as mediators and regulators of inflammation in thrombotic events.

Keywords Chondroitin sulfate • Complement • Contact activation • Inflammation • Platelets

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1 Background

1.1 The Complement System

The complement system is part of the innate immune system and one of the main effector mechanisms of antibody-mediated immunity. As many as 30 soluble and membrane-bound glycoproteins are involved in the complement system (Liszewski et al. 1996; Sunyer and Lambris 2001; Zipfel and Skerka 2009), whose central protein is complement component 3 (C3).

Complement activation is a target surface-oriented process that is centered around the activation of C3. C3 can be activated by three activation pathways: the classical pathway (CP), which in general responds to immune complexes (ICs); alternative pathway (AP), which is triggered by interaction with bacteria, viruses; and the lectin pathway (LP), which responds to the presence of certain carbohydrates on microbial surfaces. Once activated, the complement system mediates at least three traditionally described major functions: opsonization of pathogens, and thus enhancement of phagocytosis; attraction and activation of phagocytes to the site of inflammation; and lysis of foreign pathogens by damaging their cell membranes. Other more recently described functions of the complement system are elimination of apoptotic cell debris, enhancement of humoral immunity (Molina et al. 1996), modification of T-cell responses (Köhl 2006), and regulation of tolerance to self-antigens (Carroll 2000).

The complement system is strictly regulated in solution and on cell surfaces, such as endothelial cells and platelets, by both soluble and membrane-bound regulator proteins. Deficiencies in complement regulation can cause tissue damage as a result of uncontrolled inflammation and can contribute to the pathology of many diseases. Soluble complement regulators include C1 inhibitor (C1INH), factor H, C4 binding protein (C4BP), and factor I, as well as vitronectin and clusterin. The membrane-bound complement regulators include complement receptor 1 (CR1), CD59, membrane cofactor protein (MCP), decay accelerating factor (DAF), and CRIg (Liszewski et al. 1996; Meri and Jarva 1998; Helmy et al. 2006; He et al. 2008; Zipfel and Skerka 2009).

1.2 The Contact System

In the 1950s, it was observed that blood added to glass tubes clotted and the concept of contact activation was thus born (Margolis 1958; Waaler 1959). The mechanism of contact activation was initially studied in plasma or using purified proteins, with different negatively charged substances such as glass, kaolin, dextran sulfate, ellagic acid, endotoxins, and glycosaminoglycans (GAGs) to initiate the activation. More recently, studies of the interaction and activation of the contact components on endothelial cells have indicated alternative mechanisms of activation for these components.

In the presence of a negatively charged surface, factor (F) XII binds to the surface and becomes autoactivated as a result of conformational changes that uncover its catalytic serine protease region (Miller et al. 1980). This activated surface-bound form of FXII, α-FXIIa, cleaves and activates plasma prekallikrein and FXI, generating active plasma kallikrein (KK) and FXIa (Revak et al. 1978). FXIa then activates FIX, initiating the process of thrombin generation. Plasma KK enhances contact activation by cleaving both FXII, converting it to FXIIa, and surface-bound α-FXIIa, liberating soluble β-FXIIa, which produces more KK. Thus, contact activation is an autocatalytic process. KK also cleaves high molecular weight kininogen (HK), liberating the vasoactive non-apeptide bradykinin (BK) (Mori and Nagasawa 1981). HK is a required cofactor in the contact activation process, since both prekallikrein and FXI bind to negatively charged surfaces in conjunction with HK.

Multiple protease inhibitors, such as C1INH, antithrombin (AT), α1-antitrypsin, α2-antiplasmin, protein C inhibitor, and α2-macroglobulin, are able to inhibit the enzymes of the contact system (Forbes et al. 1970; Heck and Kaplan 1974; Saito et al. 1979; van der Graaf et al. 1983; de Agostini et al. 1984; Pixley et al. 1985b; Meijers et al. 1988; Wuillemin et al. 1995). Most of these proteins belong to the superfamily of serine protease inhibitors (serpins). Several studies have concluded that C1INH is the predominant inhibitor of all enzymes of the contact system, followed by α-2-macroglobulin, α-2-antiplasmin, and AT (Harpel et al. 1985; Pixley et al. 1985b; Wuillemin et al. 1995, 1996). GAGs such as heparin and heparan sulfate enhance the inhibitory effect of AT on all contact enzymes (Holmer et al. 1981; Pixley et al. 1985a; Olson et al. 1993; Gozzo et al. 2003) and have also been reported to enhance, to a lesser degree, the inhibitory effect of C1INH on KK and FXIa but not FXIIa (Wuillemin et al. 1996; Gozzo et al. 2006).

In addition to its ability to initiate coagulation, the proteins of the contact system also participate in the initiation of the inflammatory response via kinin formation and possibly also via complement activation. Contact system factors have also been reported to influence fibrinolysis.

1.3 Platelets

Apart from the traditional view of platelets as mediators of hemostasis, evidence is emerging that platelets and platelet-derived microparticles (PMPs) bring together complement and contact system activation at the site of thrombotic reactions. For instance, activated complement components have been demonstrated in many types of atherosclerotic and thrombotic vascular lesions, and platelets are involved in many of the inflammatory diseases that are mediated by complement dysregulation (Torzewski et al. 1997).

Platelets become activated when they contact any thrombogenic surface, such as injured endothelium or subendothelium, or an artificial surface such as a stent, vascular graft, or cardiopulmonary or hemodialysis equipment (Gorbet and Sefton 2004). Platelets also respond to stimulation by physiological agonists, including

thrombin, ADP, collagen, platelet activating factor (PAF), and thromboxane A2 (TxA_2). In these situations, platelet activation is initiated by the interaction of an extracellular stimulus with receptors at the platelet surface (Blockmans et al. 1995).

Under both physiological and pathological conditions, platelet activation in the human body is triggered by contact with a disrupted vascular wall and subendothelium, which exposes collagen, von Willebrand factor (vWF), and/or fibrin. Platelets adhere to the subendothelium through the binding of vWF to the glycoprotein receptor (GP) Ib/IX/V complex present on the platelet surface (Savage et al. 1996). Binding of vWF is principally mediated through the receptor GPIb (Ruggeri et al. 1983). The binding of collagen and vWF to the receptors activates an intracellular signaling pathway, which leads to contraction and release of storage granules (Holmsen 1989; Colman et al. 1994). The key feature in the extension of platelet aggregates is the presence of receptors on the platelet surface that can respond directly to some of the released agents, such as thrombin, ADP, and TxA_2 (Brass 2003). Aggregation is mediated by fibrinogen, which binds to the integrin GPIIb/IIIa on the platelet surface. When fibrinogen binds to platelets, fibrinogen forms a bridge between adjacent platelets. The affinity of GPIIb/IIIa for fibrinogen increases as a result of stimulation by ADP and TxA_2 which induce as conformational change in the fibrinogen receptor (Shattil and Newman 2004).

When platelets are stimulated by agonists such as collagen that induce secretion of granule contents, a trans-bilayer flipping of the membrane phospholipids occurs that brings procoagulant phospholipids to the platelet surface. The exposed phospholipids greatly accelerate the tenase (FIXa/FVIIIa) and prothrombinase (FXa/FVa) reactions of the coagulation pathway, resulting in the generation of thrombin, the most potent platelet agonist (Zwaal et al. 1998; Rand et al. 2003). Thrombin induces further platelet stimulation, aggregation, and secretion. Thrombin also converts fibrinogen to fibrin, which is deposited around the mass of aggregated platelets and confers stability on the formed hemostatic plug.

Human platelets express two protease-activated receptors (PAR), PAR1 and PAR4, that are proteolytically cleaved and activated by thrombin. PAR1 responds to thrombin levels of approximately 1 nmol/L, while PAR4 requires a 10-fold higher thrombin concentration (Brass 2003). Thrombin-mediated activation involves binding to the ectodomain of the PAR molecule and proteolytical cleavage between Arg^{41} and Ser^{42} (Scarborough et al. 1992). This reaction exposes a new amino-terminus, which acts as a "tethered ligand" to activate the receptor. Synthetic thrombin receptor-activating peptides (TRAP) such as SFLLRN, derived from thesequence of the new amino-terminus of the cleaved PAR,can mimic thrombin receptor activation and act as full agonists for plateletactivation (Shankar et al. 1994). TRAP acts by binding to PAR1, mimicking the N-terminal ectodomain of the receptor, and thereby activating it without the proteolytic action of thrombin.

ICs are potent activators of the complement system. Therefore, they contribute to the development of acute and chronic inflammation, which can produce the tissue damage that is associated with autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (SLE). ICs are also strong platelet agonists that bind to Fc receptors FcγRII (CD32) and FcγRIII (CD64), which are expressed on

the platelet surface (Schattner et al. 1993; McKenzie 2002; Reilly 2003). The FcRγ-chain of CD32 is a common signal transducer with GPVI and CD36, which are receptors involved in collagen-induced platelet activation. The platelet activation that occurs in response to the IC–FcγRII interaction is therefore expected to be the same as that seen in collagen-mediated activation.

1.4 Proteoglycans

In nature, all GAGs except hyaluronic acid are covalently linked to a core protein to form a proteoglycan (PG). The linkage of GAGs to the protein core involves a specific trisaccharide composed of two galactose residues and a xylose residue. The saccharide residues are coupled to the protein core through an *O*-glycosidic bond to a serine residue (Gandhi and Mancera 2008). The substituted serine residues in the core protein are adjacent to glycine, and the Ser-Gly dipeptide seems to be a basic requirement for recognition by xylosyl transferase enzymes (Kolset and Gallagher 1990). The number of GAG chain substituents on a protein core may vary from one to over 100, producing wide variation in the type and function of PGs (Kjellén and Lindahl 1991).

Serglycin is the PG, most commonly found in hematopoietic cells (Kolset et al. 2004). In the various types of blood cells, chondroitin sulfate (CS) is the major GAG, with CS-A as the dominant form (Kolset and Gallagher 1990; Kolset et al. 2004). Serglycin is stored in the granules of hematopoietic cells, where it is believed to be involved in the generation of storage granules (Abrink et al. 2004; Grujic et al. 2005; Niemann et al. 2007). In a recent study, Woulfe et al. (2008) have demonstrated defects in platelet function and aggregation in serglycin-knockout mice(Woulfe et al. 2008).

1.5 Chondroitin Sulfate (CS)

CS consists of the repeating disaccharide units *N*-acetyl-D-galactosamine (GalNAc) and D-glucuronic acid (GlcA). Dermatan sulfate (DS) differs from CS in its frequent epimerization of glucuronic acid to iduronic acid (Kolset and Gallagher 1990). Both CS and DS may be sulfated at carbon 2 of the uronic acid and at carbon 4 and/or 6 of the amino sugar (Kjellén and Lindahl 1991). The pattern of sulfation is usually used to name the CS GAGs: CS monosulfated at carbon 4 is known as CS-4 or CS-A; CS sulfated in carbon 6 as CS-6 or CS-C; and CS disulfated at carbons 4 and 6 as CS-4,6 or CS-E. DS, formerly known as CS-B, is sulfated at carbon 4 of the galactosamine and 2 of the uronic acid (Kolset and Gallagher 1990).

CS is the most abundant GAG in human plasma (≈70–80%), with CS-A being the major component (Lamari and Karamanos 2006). It has also been well established that CS-A is the predominant GAG in platelets (Okayama et al. 1986), where

it is stored in the α-granules of platelets and released during the activation and degranulation process (Barber et al. 1972; Hagen 1972). It is also rapidly expressed on the surface of activated platelets (Ward and Packham 1979). The release of CS-A in response to different agonists, including ADP, collagen, and thrombin, is very rapid and raises the concentration of plasma CS to 2–4 μg/mL, with considerably higher concentrations in the immediate vicinity of the platelets (Donato et al. 1994). Unlike the CS in blood plasma, the CS in platelets is fully sulfated, and its molecular mass has been estimated to be ~28 kDa (Okayama et al. 1986). This high degree of sulfation is thought to be linked to immune-related processes and inflammatory conditions (Uhlin-Hansen et al. 1989).

CS has been shown to bind and interact with C1q (Silvestri et al. 1981; Kirschfink et al. 1997) and has been implicated as a C1q inhibitor in several studies (Silvestri et al. 1981; Ghebrehiwet and Hamburger 1982; Galanakis and Ghebrehiwet 1994). Its binding is thought to be mediated through the globular heads of C1q and to involve ionic interactions (Ghebrehiwet and Galanakis 1993).

1.6 Platelet–Leukocyte Interactions

When platelets are activated in whole blood by various agonists, they form complexes with leukocytes, both granulocytes and monocytes (Jurk and Kehrel 2005). Such complexes are often detected in patients with stroke, unstable angina pectoris, or myocardial infections, and may thus be of pathological significance (Marquardt et al. 2009; Zhang et al. 2007). Platelets and leukocytes interact via direct cell–cell contact or indirectly via soluble mediators, following the activation of either platelets or leukocytes (Ruef et al. 2008).

Complex formation between activated platelets and leukocytes has been reported to be mediated by P-selectin, which is expressed on the surface of activated platelets, and its ligand, P-selectin glycoprotein ligand-1 (PSGL-1), on leukocytes. The interaction of P-selectin with PSGL-1 mediates the initial tethering of leukocytes to activated platelets through a mechanism resembling the interaction of leukocytes with endothelial cells (Zarbock et al. 2009). This P-selectin-mediated interaction induces the activation and up-regulation of β_2-integrin $\alpha_M\beta_2$ (CD11b/CD18) on leukocytes (da Costa Martins et al. 2006). Firm attachment is subsequently mediated by the interaction of CD11b/CD18 with various ligands and adhesion molecules on platelets (Kuijper et al. 1998). GPIIb/IIIa expressed on platelets is often considered to be essential for platelet–leukocyte interactions (Zhao et al. 2003). GPIIb/IIIa has been proposed to interact with leukocytes either by direct binding to CD11b/CD18 or through fibrinogen, which binds to GPIIb/IIIa on platelets and CD11b/CD18 on leukocytes (Lishko et al. 2004). Other proteins that are exposed on platelets and reported to take part in platelet–leukocyte complex formation are CD40L, which interacts with CD40 on leukocytes (Zhao et al. 2003), and GPIbα, which binds to CD11b/CD18 (Ehlers et al. 2003). However, Konstantopoulos et al. (1998) and Ruef et al. (2008) have shown that platelet–leukocyte attachment occurs indepen-

dent of GPIIb/IIIa. Thus, the ligand(s) for leukocyte–platelet inteaction via CD11b/CD18 are still unclear.

Upon activation, platelets shed microparticles (PMPs) from the plasma membrane. PMPs have high procoagulant activity because of the negatively charged phospholipids exposed on their surface. Various receptors that are present on the surface of activated platelets are also found on the surface of PMPs, enabling them to interact with cells (Bode and Hickerson 2000; Forlow et al. 2000). It has been well established that platelets and PMPs contain tissue factor (TF), which is the most important initiator of intravascular coagulation (Scholz et al. 2002; Siddiqui et al. 2002; Camera et al. 2003).

2 Platelet–Complement Interactions

2.1 Activation of Platelets by Complement Activation Products

Complement and platelets appear to activate each other in a reciprocal manner that may have physiological and pathological implications. Complement-induced platelet activation has been studied since the 1980s, when Sims et al. demonstrated an increased procoagulatory activity of platelets exposed to sC5b-9 complexes (Sims and Wiedmer 1991). These observations suggested that the sC5b-9 complex mimics platelet stimulation by thrombin and other agonists. However, it has been reported that platelet activation by sC5b-9 is under the control of the complement regulatory protein CD59, which is present on the surface of platelets (Sims et al. 1989). Blocking CD59 with an antibody augments the sC5b-9-mediated procoagulatory response of platelets.

2.2 Binding of Complement Components to Activated Platelets

Platelets are thought to play a significant role in the activation and regulation of complement. In agreement with this concept, several studies have shown that complement components bind to platelets (Polley and Nachman 1978; Endresen and Mellbye 1984; Sandvik et al. 1984). Platelets have also been found to store, secrete, and express complement proteins and regulators upon activation. It was recently reported that C3 is present in platelet lysates (Del Conde et al. 2005). Already in 1981, Kenney et al. had shown that platelet homogenates can inhibit the formation and accelerate the decay of the C3bBb convertase, as a result of the presence of factor H (Kenney and Davis 1981). Factor H was found to bind to washed human platelets via thrombospondin-1 (TSP-1) (Vaziri-Sani et al. 2005) or GPIIb/IIIa (Mnjoyan et al. 2008).

Complement activation has previously been reported to occur on the surface of activated platelets (Del Conde et al. 2005; Peerschke et al. 2006). Del Conde et al. (2005) reported an activation of complement and deposition of C3b on activated platelets that was dependent on P-selectin expression and mediated by the AP. Using an assay with platelets fixed to microtiter plates, Peerschke et al. (2006) demonstrated a complement activation involving CP components that was due in part to the expression of the receptor for the globular heads of C1q (gC1qR) on the surface of activated platelets. However, there is somelack of congruity between these observations and the previously demonstrated abundant expression of the membrane-bound complement regulators DAF (Nicholson-Weller et al. 1985), MCP (Yu et al. 1986), and CD59 (Morgan 1992) and the interactions of soluble regulators as C1INH (Schmaier et al. 1993), clusterin (Tschopp et al. 1993), and factor H with the activated platelet surface. One would have expected to see a well-controlled complement equilibrium on the surface of platelets under physiological conditions, as supported by the following examples that illustrate the critical importance of physiological complement regulation:

1. A potential role for the complement system in the thrombotic episodes associated with paroxysmal nocturnal hemoglobinuria (PNH) has been postulated on the basis of the increased sensitivity of platelets to activation by sC5b-9 complexes as a result of a diminished surface expression of CD59 on their surfaces (Wiedmer et al. 1993). Similarly, a correlation between complement activation and thrombotic events has been demonstrated in various autoimmune conditions, including SLE, and antiphospholipid syndrome (Peerschke et al. 2010).
2. Platelet activation and a lowering of platelet counts have also been noted in atypical hemolytic uremic syndrome (aHUS). aHUS is associated with mutations in the C-terminus of factor H (Zipfel et al. 2001); this relationship would suggest that dysregulation of the complement system plays a crucial part in the pathogenesis of aHUS. Stahl et al. (2008) have shown that aHUS patients with a mutated factor H have higher levels of deposition of C3 and C9 on platelets than do healthy controls. Combining aHUS patient sera containing mutated factor H with normal platelets results in complement activation and the activation and aggregation of platelets. These reactions can be abrogated by preincubation of the platelets with normal factor H or when normal serum is used (Stahl et al. 2008). Karpman et al. (2006) have suggested that the binding of factor H to human platelets can protect them from complement activation.

The choice of anticoagulant used in studies of the interaction between platelets and complement is of utmost importance. Most of the previous studies have been performed in citrated blood, where complement function is impaired to a certain extent. Similarly, heparin even at low doses interferes with complement. In order to avoid these side effects, we have developed a model system using blood containing the specific thrombin inhibitor lepirudin in which the plateles are activated byTRAP (Mollnes et al. 2002). By doing so, we can stimulate the platelets in whole blood via PAR1 without inducing clot formation.

In a recent study, using this system designed to elucidate how complement components bind to platelets, we used flow cytometry to monitor the binding of complement proteins to activated platelets in platelet-rich plasma (PRP) and in whole blood (Hamad et al. 2010b). C1q, C4, C3, and C9 were found to bind to the activated platelets in both PRP and whole blood. However, binding of complement components does not necessarily mean that these components are bound as a result of complement activation: In fact, blocking complement activation completely by using EDTA or at the level of C1q with an inhibitory antiglobular head C1q-85 monoclonal antibody (mAb) or C3 with Compstatin did not affect the binding of C3 or C9. Western blot analysis of platelet surface-associated C3 and flow cytometry using mAbs specific for different conformational forms of C3 showed that the bound C3 consisted mainly of C3(H_2O), with no C3b present. This result indicated that the bound C3 was non-proteolytically activated and therefore that complement activation does not take place on the surface of activated platelets under physiological conditions. The transition from C3(H_2O) to iC3(H_2O) confirmed that regulation of C3 function by factor I is fully active on the platelet surface. This form of C3 was able to bind soluble CR1 (CD35), indicating that it may act as a receptor ligand.

2.3 Complement Activation Triggered by CS-A Released from Activated Platelets

It is well known that complement is activated in clotting blood, and significantly higher levels of complement activation products, for example, C3a and sC5b-9, are found in human serum than in EDTA-, heparin-, or lepirudin-anticoagulated blood. So how does platelet activation lead to complement activation? We addressed this issue in a recent study, which showed that clotting, initiated by either the TF pathway or the contact activation system, could trigger fluid-phase complement activation, as identified by the generation of C3a and sC5b-9 (Hamad et al. 2008). The level of complement activation was highly correlated with the generation of thrombin, that is, the formation of thrombin–AT (TAT) complexes.

Our hypothesis was that CS-A released from activated platelets was acting as a trigger of complement activation. To test this hypothesis, we incubated platelet-poor plasma (PPP) with exogenously added CS-A, either commercially available or in the form of platelet supernatants. Generation of C3a and sC5b-9 increased in a dose-dependent fashion, and digestion of either CS-A or the platelet supernatants with chondroitinase ABC totally abrogated the reaction.

To further investigate the role of CS-A in complement activation, we immobilized CS-A on microtiter plates; after adding plasma or serum to the wells, we were able to detect the binding of C1q. As a functional verification of this binding reaction, we quantified the complement activation that occurred in C1q depleted-serum, with or without the addition of purified C1q, by measuring the binding of C3 fragments. No binding was seen in the C1q-depleted serum, but activation was restored

by the addition of purified C1q. These results suggest that complement is activated via the CP and that C1q serves as the recognition molecule for CS-A. CS-A has previously been suggested to act as a specific C1q inhibitor in plasma (Silvestri et al. 1981; Ghebrehiwet and Hamburger 1982). These results were obtained by measuring the ability of CS to inhibit C1q-specific hemolytic activity. We demonstrated that this inhibition is accomplished by CP activation, depleting the plasma of intact complement components and thereby inhibiting the hemolytic activity of C1q (Hamad et al. 2008).

In addition, CS-A-triggered complement activation induced the activation of monocytes and granulocytes, as indicated by the expression of CD11b and the formation of complexes between platelets and monocytes/granulocytes. Both CD11b expression and platelet–leukocyte conjugate formation were significantly inhibited by the addition of the complement inhibitors Compstatin or C5aR antagonist.

2.4 Interaction Between C1q, Complement Regulators, and CS-A

Subsequently, we further characterized the binding of C1q and the complement regulators C1INH, C4BP, and factor H to CS-A exposed on the surface of activated platelets or immobilized on various matrices (Hamad et al. 2010a). C1q was shown to be the main protein that specifically bound to CS-A. After depletion of C1q from the serum, binding of C4BP and factor H to immobilized CS-A as well as to activated platelets was increased, while C1INH binding was absent.

This binding was inhibited by soluble CS-A or by a CS-A-specific mAb, thereby linking the binding of C1q, C4BP, and factor H to exposure of CS-A on activated platelets. The need for large amounts of soluble CS-A or anti-CS-A mAb to inhibit the binding of C1q to activated platelets indicated that the binding of C1q to CS-A is multivalent. C1q contains six globular heads, each of which contains a single binding site for CS-A. The affinity between a monomeric globular head and CS-A is not known, but assuming that it is similar to that of IgG ($\approx 10^3$ M) (Kishore et al. 1998), the avidity (i.e., the combined affinities of all of the globular heads binding to CS-A) would be strong enough to explain the extremely high binding of C1q to CS-A.

In addition, CS-A-bound C1q was also shown to amplify the binding of model IC to both microtiter plate-bound CS-A and to activated platelets, suggesting a role for activated platelets in IC diseases.

2.5 Model for the Interaction Between Platelets and Complement

Physiological consequences of the platelet-mediated complement activation described in Sects. 2.2–2.4 above were an increased expression of CD11b on leuko-

cytes and an increased generation of platelet–leukocyte complexes (Hamad et al. 2008). Platelets apparently participate in this mechanism through at least two different mechanisms: the generation of C5a and subsequent activation of leukocytes, and the binding of ligands C3(H_2O)/iC3(H_2O) for the CD11b/CD18 integrin on their surface.

The steps involved in the progression from platelet activation to complement activation and inflammation, as deduced from our model, can be summarized as follows: During the activation phase (1) platelets are activated and release CS-A in the form of serglycin and PMPs; (2) CS-A binds C1q and activates complement in the fluid phase, generating C3a and C5a; (3) complement components, including C3(H_2O), are deposited on the surface of the activated platelets. During the effector phase (1) leukocytes become activated and up-regulate surface CD11b/CD18, which may enable (2) subsequent binding of platelets and PMPs via C3(H_2O) or other ligands.

3 Interactions of Cells and Cellular Components with the Contact Activation System

3.1 Cell-Induced Contact Activation

The idea that contact activation might occur in the vicinity of platelets was raised in the 1960s, when data indicating the presence of activated FXII and FXI on the platelet surface were published (Iatridis and Ferguson 1965; Iatridis et al. 1964). In the 1980s, it was reported that isolated platelets in buffer systems containing purified proteins could promote the activation of FXII (Walsh and Griffin 1981a,b). Washed platelets have been shown to bind HK and FXI in a zinc-dependent manner (Greengard and Griffin 1984; Greengard et al. 1986; Bradford et al. 1997). Apart from the observation that FXIIa binds to washed platelets via GPIb, which competes with HK (Bradford et al. 1997, 2000; Joseph et al. 1999), little has been published regarding the direct binding of FXII to platelets and platelet-triggered contact activation. Recent work using blood plasma has only demonstrated that platelets are able to amplify the contact activation induced by a negatively charged substance or material, such as high molecular weight dextran sulfate (Johne et al. 2006).

The interaction between cells and the proteins of the contact system has thus far focused on endothelial cells. Both HK and FXII bind to human umbilical vein endothelial cells (HUVECs) in a zinc-dependent manner (Reddigari et al. 1993; Schousboe 2001). The urokinase plasminogen activator receptor (u-PAR), gC1qR, and cytokeratin 1 have all been reported to be important for the binding of both HK and FXII (Herwald et al. 1996; Joseph et al. 1996, 2001; Mahdi et al. 2002). FXII is slowly auto-activated when bound to endothelial cells (Shibayama et al. 1998). Prekallikrein can also be activated, initiating bradykinin generation independent of FXII on endothelial cells (Rojkjaer et al. 1998; Rojkjaer and Schmaier 1999). The

assembly of contact proteins on the cell receptors on endothelial cell membranes has been thought to provide a physiological negatively charged surface for activation, and it has been suggested that this FXII-independent activation does not initiate coagulation but rather functions as a mediator of vascular biology, above all via the generation of BK (Schmaier 1997, 1998, 2008).

In *in vitro* systems, several negatively charged surfaces have been shown to bind and activate FXII, and many biological substances also promote FXII autoactivation, but it has been difficult to identify such activating compounds in the intravascular compartment in non-disease states. Negatively charged phospholipids and sulfatides expressed in platelets may be expected to activate FXII, but this phenomenon has never been convincingly shown *in vivo* (Schousboe 1988).

Extracellular nucleic acids, particularly RNA, enhance coagulation and have been found to be associated with fibrin-rich thrombi, thereby contributing to arterial thrombus formation in mice (Kannemeier et al. 2007). FXII binds to and is auto-activated on extracellular RNA, and therefore contact activation has been suggested as a mechanism by which RNAs can exhibit their procoagulant properties (Kannemeier et al. 2007). Thus, extracellular RNA derived from damaged or necrotic cells, particularly under pathological conditions or in severe tissue damage, may represent an *in vivo* inducer of FXII-mediated contact activation.

Another recently proposed mechanism for the *in vivo* activation of FXII is the release of inorganic polyphosphate from dense granules during platelet activation (Ruiz et al. 2004). Inorganic polyphosphate has been shown to accelerate blood clotting by triggering FXII activation and promoting FV activation in *in vitro* models (Smith et al. 2006).

FXII is also activated by mis-folded protein aggregates, and increased levels of FXIIa and KK have been found in the blood of patients with amyloidosis (Maas et al. 2008). In addition, heparin derived from human mast cells activates FXII, inducing contact activation, and is thought to be responsible for the generation of kinins in allergic reactions (Noga et al. 1999).

3.2 Platelet-Induced Contact Activation

Studies in the 1970s and 1980s have shown that isolated platelets in buffer systems containing purified proteins can promote the activation of FXII (Walsh and Griffin 1981b) and that platelets are able to amplify the contact activation induced by a negatively charged substance or material (Johne et al. 2006). Nevertheless, the contact activation system was considered to have no significance for normal hemostasis. This view has been challenged by the results of our analysis of the interaction between platelets and contact proteins (using the TRAP-mediated activation of platelets in lepirudin-treated PRP and whole blood in the system described above) and the influence of this interaction on platelet aggregation and clot formation (Bäck et al. 2010).

In our study, TRAP-mediated activation of platelets in lepirudin-treated PRP led to the formation of FXIIa–C1INH, FXIIa–AT, FXIa–C1INH, and FXIa–AT complexes in plasma in the presence of a TF-inhibitory mAb, demonstrating that activation of these proteins had occurred as a consequence of specific platelet activation. The presence of FXII, FXI, prekallikrein, and HMWK on activated platelets was then demonstrated by flow cytometry. Furthermore, using chromogenic substrates, we showed that the bound FXIIa, FXIa, and KK possessed enzymatic activity.

We also found that clotting triggered either by the TF pathway or in a shear force model (Nilsson et al. 1998) generates substantial amounts of FXIIa–AT and FXIa–AT, demonstrating that contact activation occurs in clotting blood. The amount of FXIIa–AT was found to correlate with both thrombin activity and TAT complexes, indicating that it contributes to thrombin formation. Neither FXIIa–C1INH nor FXIa–C1INH complexes were detected, suggesting that C1INH did not primarily regulate these enzymes in clotting blood. In PRP, TRAP-activated platelets exhibited shorter clotting times than did non-activated controls. Addition of the specific FXIIa inhibitor corn trypsin inhibitor (CTI) prolonged the clotting time, whereas a TF-blocking mAb had no effect.

In summary, our study demonstrated that FXII-mediated contact activation occurs on the surface, or in the vicinity, of activated platelets and contributes to platelet aggregation and clot formation. The low levels of contact system proteins on the platelet surface, combined with the substantial formation of FXIIa–AT and FXIa–AT in plasma, suggest that FXII is activated on the surface or in the vicinity of the platelets and then released into the fluid phase. Activated platelets may thus constitute a nidus for contact activation inside the blood vessels and can recruit proteins of the contact system into the clot formation process.

In a related study, we demonstrated that the regulation of FXII-mediated contact activation differs between material-induced activation and physiological activation by activated platelets (Bäck et al. 2009). When platelets were activated in lepirudin-treated whole blood and PRP by TRAP, ADP, collagen, or shear force, we detected FXIIa–AT, FXIa–AT, and low amounts of KK–AT, but no C1INH-complexes, in the resulting plasma. Thus, the platelet-induced activation of FXII resulted in activation of both FXI and prekallikrein. In contrast, contact activation triggered by kaolin, glass, or inorganic polyphosphate in PPP, PRP, or whole blood generated high amounts of FXIIa–C1INH. Regardless of which activating compound was used, low or only negligible amounts of FXIIa–AT were generated.

The effects of polyphosphate were also analyzed using the shear force model with non-anticoagulated blood. As was seen for lepirudin-treated blood, polyphosphate produced a strong activation of FXII, as detected by high levels of FXIIa–C1INH, but no further activation of FXI or prekallikrein occurred. A remarkable difference between platelet-induced and material-induced FXII activation was that CTI totally abolished the generation of FXIIa–C1INH after activation with glass, kaolin, or polyphosphate, but the generation of FXIIa–AT after platelet activation was only decreased to some extent.

Finally, we measured contact activation enzyme–serpin complexes in samples from patients treated for severe trauma (e.g., after traffic accidents). Blood was

drawn at admission to the hospital and at repeated time points for up to 10 days. At the time points closest to the trauma, the highest amounts of FXIIa–AT and FXI–AT were detected, and the levels subsequently decreased. The levels of these complexes were correlated with the amounts of thrombospondin-1 that were released by activated platelets. In contrast, FXIIa–C1INH complexes were found days after admission, when FXIIa–AT levels had disappeared. No association between FXIIa–C1INH and TSP-1 was found. FXIIa–C1INH appeared later in the treatment and may be generated on artificial surfaces such as extracorporeal circuits and after treatments involving biomaterials, for example, plasma expanders (dextran) or intravenous lipids.

In conclusion, in this study (Bäck et al. 2009) we demonstrated that contact activation triggered by activated platelets is regulated by AT and by C1INH on artificial material surfaces. Also, the inhibition of FXIIa by CTI seems to differ according to the specific activation site. CTI had minor effects on platelet-mediated contact activation, and the activation of the contact enzymes is being studied in such models, AT complexes should be assessed, rather than C1INH complexes. Since enzyme–AT complexes, which are correlated with TSP-1, are generated during platelet-mediated contact activation, FXIIa–AT should be considered an interesting new biomarker candidate for platelet activation.

4 Conclusions

Recent studies demonstrating that activated platelets trigger both complement and contact system activation have raised questions about the extent to which these cascade systems are involved in thrombotic diseases. The focus of this review article is the crosstalk between platelets and the cascade systems in clotting blood. CS-A released by activated platelets is a major mediator of complement activation, ultimately resulting in inflammation and leukocyte activation. C3 binds to the surface of activated platelets independent of complement activation and with the thiolester disrupted, enabling interaction with leukocyte CR1. In addition, activated platelets also trigger the contact system, thereby accelerating the clotting process. This process leads to the generation of enzyme–AT complexes, but no enzyme–C1INH complexes. We propose that these complexes, in particular FXIIa–AT, could be useful as markers for platelet activation. In conclusion, in addition to their traditional role in hemostasis, platelets have been shown to have many other functions, including involvement in the regulation of the complement and contact systems.

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Nuclear Factor-κB in Immunity and Inflammation: The Treg and Th17 Connection

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Abstract Although nuclear factor-κB (NF-κB) is generally considered to be a pro-inflammatory transcription factor, recent studies indicate that it also plays a critical role in the development of an anti-inflammatory T cell subset called regulatory T (Treg) cells. Two NF-κB proteins, c-Rel and p65, drive the development of Treg cells by promoting the formation of a *Foxp3*-specific enhanceosome. Consequently, c-Rel-deficient mice have marked reductions in Treg cells, and c-Rel-deficient T cells are compromised in Treg cell differentiation. However, with the exception of Foxp3, most NF-κB target genes in immune cells are pro-inflammatory. These include several Th17-related cytokine genes and the retinoid-related orphan receptor-γ (*Rorg* or *Rorc*) that specifies Th17 differentiation and lineage-specific function. T cells deficient in c-Rel or p65 are significantly compromised in Th17 differentiation, and c-Rel-deficient mice are defective in Th17 responses. Thus, NF-κB is required for the development of both anti-inflammatory Treg and pro-inflammatory Th17 cells.

Keywords NF-κB • c-Rel • Inflammation • Th17 • Treg

1 The Rel/Nuclear Factor-κB (Rel/NF-κB) Family of Transcription Factors and Immune Regulation

The mammalian Rel/NF-κB family consists of five members: c-Rel, RelA/p65, RelB, NF-κB1 (p50/p105), and NF-κB2 (p52/p100) (Barnes and Karin 1997; Beg and Baltimore 1996; Beg et al. 1995; Sha et al. 1995). These members share a highly conserved 300-amino acid Rel homology domain at their NH2-termini, which encompasses sequences required for DNA binding, protein dimerization, and nuclear localization. The C-termini of these proteins are less conserved, with

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those of c-Rel, RelA, and RelB containing transcriptional transactivation domains (Bull et al. 1990). With the exception of c-Rel, most Rel/NF-κB family members are constitutively expressed in multiple cell types including oligodendrocytes, neurons, astrocytes, and endothelial cells as well as lymphocytes and myeloid cells (Bakalkin et al. 1993; Beg and Baltimore 1996; Beg et al. 1995; Brownell et al. 1987; Grumont and Gerondakis 1990a, b; Kaltschmidt et al. 1994a, b, 1997; Kutlu et al. 2003; Kwon et al. 1995; Robbins et al. 2003; Sha et al. 1995; Vollgraf et al. 1999). By contrast, c-Rel is expressed primarily in lymphoid tissues by lymphoid and myeloid cells (Brownell et al. 1987; Gerondakis et al. 1998; Huguet et al. 1998; Wang et al. 1997). Freshly synthesized c-Rel, like other members of the family, is kept in the cytoplasm as inactive homo- or hetero-dimeric proteins in association with the inhibitory protein called IκB (inhibitor of κB). There are at least five IκBs, which act by masking the nuclear localization signal of Rel/NF-κB, preventing their nuclear translocation. Several groups of receptors including cytokine receptors for tumor necrosis factor (TNF)-α and TGF-β, T cell and B cell antigen receptors, and Toll-like receptors can activate Rel/NF-κB (Barnes and Karin 1997; Beg and Baltimore 1996; Beg et al. 1995; Sha et al. 1995; Thomas et al. 1998). Activation of Rel/NF-κB involves phosphorylation and subsequent proteolytic degradation of IκB by the specific IκB kinase (IKK) (Gerondakis et al. 1999; Zandi et al. 1998). Once activated, the free Rel/NF-κB dimers enter the nucleus, and bind to the 9–10 base pair Rel/NF-κB sites of gene promoters. The exact number and identity of Rel/NF-κB target genes in various cell types are not clear. For lymphoid and myeloid cells, Only 42 Rel/NF-κB target genes have been identified by either promoter transactivation or chromatin immunoprecipitation (Natoli et al. 2005; Pahl 1999). Of these, ~11 were previously confirmed for c-Rel (Table 1) and 30 were confirmed for NF-κB1. To date, at least 122 Rel/NF-κB target genes have been identified in various mammalian cell types by either ChIP or promoter transactivation, and the total number of Rel/NF-κB target genes in the mouse or human genome is estimated to be in the range of 250–300 (Natoli et al. 2005; Pahl 1999).

Why are five different members of the Rel/NF-κB expressed in mammalian cells? Recent studies using Rel/NF-κB-deficient mice indicate that different members of the Rel/NF-κB family play different roles *in vivo*. Thus, mice deficient in c-Rel suffer from cellular and humoral immune deficiencies, but their non-lymphoid organs are structurally and functionally normal (Gerondakis et al. 1996; Liou et al. 1999; Tumang et al. 1998). T cells derived from these mice have selective defects in response to TCR stimulation. They produce less IL-2, IL-3, and IFN-γ, but more IL-4 than c-Rel-sufficient cells (Hilliard et al. 2002; Lamhamedi-Cherradi et al. 2003). Unlike c-Rel-deficient B cells that have increased apoptosis in the culture (Tumang et al. 1998), c-Rel-deficient T cells survive equally well in the culture as compared to wild type cells (Strasser et al. 1999). Similarly, myeloid cell functions including inflammatory cytokine production by monocytes and dendritic cells are also significantly compromised in these mice. NF-κB1 (p50)-deficient mice do not have any developmental problems, although they are more susceptible to intracellular and extracellular gram-positive bacterial infections, and are partially compromised in their B cell responses to lipopolysaccharides (LPS) (Sha et al. 1995). Surpris-

Table 1 Selected c-Rel target genes in lymphoid and myeloid cells

Name	Description	Cell Type (species)	Rel/NF-κB member	Site location[b]
BfI1	BCL2 homolog A1	Jurkat T (human)	c-Rel (ChIP)[a]	–833/–823
BM2	β2 microglobulin	Jurkat T, THP-1 (human)	c-Rel, RelB, NF-κB1/p65	–106/–96
IER3	Immediate early response 3	Jurkat T (human)	c-Rel/p65, NF-κB1/p65 (ChIP)	–102/–92
IGHG4	Ig heavy constant g 4	B cell line BL-2 (human)	c-Rel	–65/–56
IL12	Interleukin 12	Macrophage, DC (mouse)	c-Rel, NF-κB1, p65 (ChIP)	p40: –132/–122 p35: –63/–54
IL1β	Interleukin 1β	U937, Jurkat T (human)	c-Rel, NF-κB1, p65	–300/–289
IL2	Interleukin 2	T cell line (human)	c-Rel, NF-κB1	–206/–195
IL2Rα	IL2 receptor α	Jurkat T (human)	c-Rel	–267/–258
IL9	Interleukin 9	T cell line (human)	c-Rel, NF-κB1	–59/–50
IRF4	Interferon regulatory factor 4	T cell line (human)	c-Rel, NF-κB1, p65	–445/–435, –517/–507
IRF7	Interferon regulatory factor 7	Monocyte (human)	c-Rel, NF-κB1, p65	–60/–50
IL23p19	Interleukin 23 p19unit	Macrophage, DC (mouse)	c-Rel (ChIP), p100, p105, p65	–95/–85, –600/–590
Foxp3	forkhead box P3	T regulatory cell	c-Rel/p65 (ChIP)	–386/–376, –331/–321
Rorgt	Retinoid-related orphan receptor gammaT	Thymocyte, Th17 cell	c-Rel/p65 (ChIP)	–166/–156, –270/–260
Rorg	Retinoid-related orphan receptor gamma	Variety of cell types	c-Rel/p65 (ChIP)	–203/–193, –290/–280

[a] ChIP, targets that have been confirmed by ChIP. Other targets are identified by promoter transactivation

[b] Site location, Rel/NF-kB site (starting/ending nucleotides) location in the respective gene promoters

ingly, they are more resistant to viral and gram-negative bacterial infections (Sha et al. 1995). By contrast, mice deficient in p65 die in utero with massive hepatocyte apoptosis (Beg et al. 1995; Doi et al. 1997), whereas RelB-deficient mice suffer from severe disorders ranging from splenomegaly to multi-organ autoimmune-like inflammatory diseases (Gerondakis et al. 1996; Weih et al. 1995). Similarly, NF-κB2-deficient mice suffer from severe developmental defects. Both their spleens and lymph nodes are bereft of B-lymphocytes, undermining their capacity to form germinal centers (Caamano et al. 1998; Franzoso et al. 1998). These findings establish that members of the Rel/NF-κB family perform non-overlapping functions, and that a loss-of-function mutation of a Rel/NF-κB gene cannot be fully compensated by other genes of this family. The mechanism underlying these member-specific effects is not entirely clear, although it is likely related to differences in the Rel/

NF-κB expression in different cell types and the nature of target genes that it activates. Understanding the member-specific and cell-specific roles of the Rel/NF-κB family will help us develop more effective strategies targeting specific Rel/NF-κB pathways for the treatment of diseases.

2 NF-κB and Treg Cell Development

Regulatory T (Treg) cells are a heterogeneous population of T cells that play crucial roles in maintaining immune homeostasis and preventing autoimmune diseases (Bommireddy and Doetschman 2007; Lohr et al. 2006; Sakaguchi et al. 2006; Shevach et al. 2006). The $CD4^+CD25^+Foxp3^+$ 'naturally occurring' Treg (nTreg) cells are generated in the thymus and are among the best-studied Treg cells (Sakaguchi et al. 2006). Unlike nTreg cells, the $CD4^+CD25^+Foxp3^+$ 'induced' Treg (iTreg) cells can be generated in peripheral lymphoid organs, or in cultures, following stimulation with antigens and Treg-inducing cytokines such as transforming growth factor (TGF)-β (Bommireddy and Doetschman 2007; Liang et al. 2005; Lohr et al. 2006). In addition to nTreg and iTreg cells, other types of Treg cells have also been described. These include $CD8^+$ Treg cells, Tr1 cells, and Th3 cells (Chen et al. 1994; O'Garra and Vieira 2004; Shevach 2006). The Foxp3 protein is the most distinct marker for nTreg and iTreg cells, and is crucial for their function. Scurfy mice that carry a *Foxp3* gene mutation present lymphoproliferation, lymphocytic infiltration, and multi-organ autoimmune diseases (Godfrey et al. 1991). Similarly, *Foxp3* gene mutation in humans causes a fatal autoimmune disorder called IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) (Bennett et al. 2001).

2.1 Treg Development is Markedly Diminished in c-Rel-Deficient Mice (Ruan et al. 2009)

The thymus is the central lymphoid organ that gives rise to both $Foxp3^+$ regulatory T (Treg) cells and $Foxp3^-$ naïve T cells (Fontenot and Rudensky 2005; Kim 2006; Kim and Rudensky 2006; Sakaguchi 2005; Ziegler 2006). c-Rel is a member of the Rel/NF-κB family that is preferentially expressed in lymphoid organs (Simek and Rice 1988). To determine the roles of c-Rel in the development of Treg and naïve T cells, we examined their frequencies in the thymus of 3-, 7-, 21-, and 42-day-old C57BL/6 mice that do or do not express c-Rel. Although the total number of thymocytes was reduced by ~20% in c-Rel-deficient mice, the frequencies of $CD4^+CD25^-Foxp3^-$ naïve T cells were comparable in the two groups. By contrast, up to 10-fold reduction of $CD4^+CD25^+Foxp3^+$ Treg cells was observed in c-Rel-deficient thymus of both neonatal and adult mice. Consistent with this finding, the frequencies of $CD4^+CD25^+Foxp3^+$ Treg cells were also markedly reduced in the

spleens of c-Rel-deficient mice, regardless of ages. Up to four-fold reduction was observed on day 3 and ~five-fold reduction was observed on day 42 in the c-Rel-deficient group. These results indicate that c-Rel is required for the thymic development of $CD4^+CD25^+$*Foxp3*$^+$ Treg cells, but dispensable for that of *Foxp3*$^-$ naïve T cells.

2.2 c-Rel Orchestrates the Formation of a Foxp3-Specific Enhanceosome that Contains c-Rel, p65, NFAT, Smad, and CREB (Ruan et al. 2009)

c-Rel may regulate Treg differentiation indirectly through cytokines such as IL-2. Several laboratories including ours also showed that it could do so directly by binding to and activating the *Foxp3* gene (Isomura et al. 2009; Long et al. 2009; Ruan et al. 2009; Zheng et al. 2010). Data from our lab has shown that c-Rel and p65 deficiency blocks *Foxp3* gene expression and Treg differentiation, whereas c-Rel or p65 co-expression increases *Foxp3* promoter activity. Although IL-2 plays an important role in Treg development (Lohr et al. 2006) and c-Rel-deficient T cells have a partial defect in IL-2 production when cultured *in vitro* (Liou et al. 1999), it seems that c-Rel regulates Treg differentiation in a cell-autonomous manner, independent of IL-2. To determine the nature of the transcriptional complexes formed at the *Foxp3* locus during Treg differentiation, we performed chromatin immunoprecipitation (ChIP) and sequential ChIP (SeqChIP or Re-ChIP). We found that in resting $CD4^+CD25^-$ T cells, the *Foxp3* promoter and enhancers were completely devoid of any transcription factors tested. Upon cultured under the Treg differentiation condition, a *Foxp3*-specific enhanceosome that contains c-Rel, p65, NFAT, Smad, and CREB was formed (Fig. 1). We propose that the Treg differentiation program consists of three sequential steps: *Initiation, Selection,* and *Fixation.* During *Initiation*, c-Rel binds to *Foxp3* CNS3 and promoter, promoting the enhanceosome formation and chromatin remodeling so that the transcriptional machinery can initiate the *Foxp3* mRNA synthesis; however, other lineage-specific genes such as T-bet may also be turned on at this stage. During *Selection*, cytokines such as TGF-β accelerate the enhanceosome-mediated *Foxp3* mRNA synthesis while inhibit the transcription of non-Treg-specific genes; TGF-β may accomplish this either through Smad (Bommireddy and Doetschman 2007; Liu et al. 2008; Tone et al. 2008), or Smad-independent mechanisms, for example, those that involve TAK1 (Lu et al. 2007). During *Fixation*, the enhanceosome together with other cytokine signals, for example, those of IL-2 and TGF-β, leads to DNA demethylation of the *Foxp3* locus by preventing the binding of the DNA methyl transferase to the locus during DNA replication. Therefore, terminally differentiated Treg cells exhibit two hallmark epigenetic changes at their *Foxp3* locus: (a) open chromatin and (b) demethylated regulatory regions.

Interestingly, also in December 2009, two other groups reported findings supporting an essential role for c-Rel in Treg cell development: (a) Isomura et al.

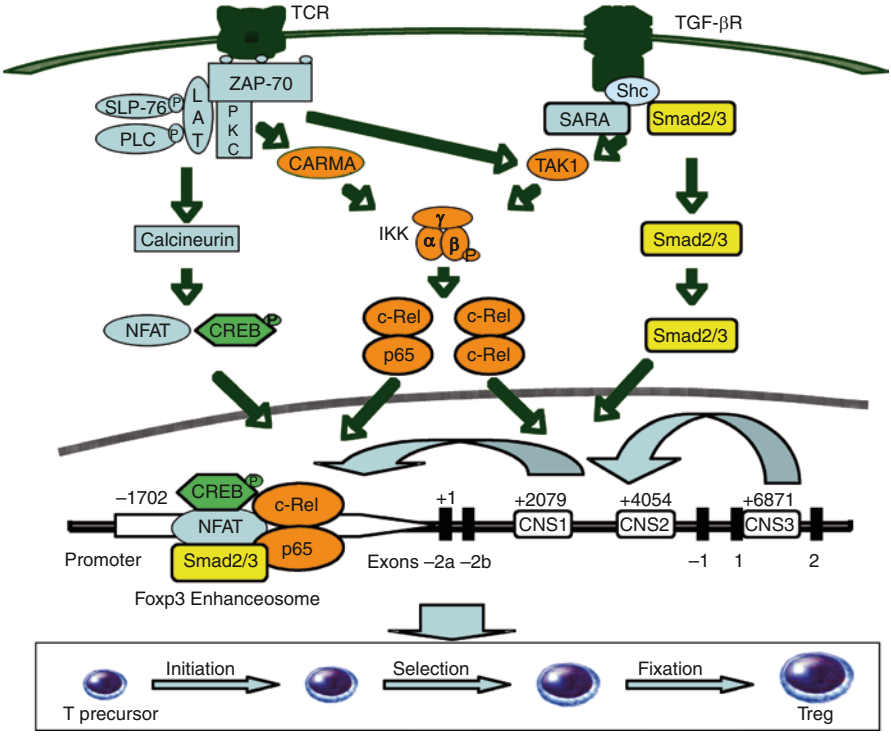

Fig. 1 The *Foxp3* enhanceosome model for Treg cell differentiation. How nTreg and iTreg cells are generated remains enigmatic. Recent findings related to *Foxp3* gene regulation enable us to propose a new model, which may apply to nTreg and/or iTreg cells. Antigen-presenting cells (APC) carrying specific peptides engage precursor T cells by TCR and CD28, in the presence of TGF-β (Chen et al. 2003; Kretschmer et al. 2006; Tang et al. 2003; Yu et al. 2008); ligation of TCR, CD28 (not shown), and TGF-β receptors leads to the activation of IKKβ (inhibitor of κB kinase β), which phosphorylates IκBα (inhibitor of κBα), releasing c-Rel and p65 (Lu et al. 2007; Zandi et al. 1997); the freed c-Rel homo- or heterodimer migrates into the nucleus, binds to the *Foxp3* regulatory elements, and induces the formation of a Treg-specific multi-factorial transcriptional complex called 'enhanceosome', which comprises transcription factors that bind to not only the promoter (such as c-Rel, p65, and NFAT) but also the distal CNS elements (such as CREB and Smad) (Ruan et al. 2009). In addition to IKKβ, TCR also activates NFAT, and TGF-β receptors activate Smad. For clarity, enhancer −1 which is located upstream of the promoter is not shown (Lal et al. 2009); other regulatory factors (such as STATs) and epigenetic changes of the *Foxp3* gene are not depicted either. It should be emphasized that although the role of TGF-β in iTreg development is well recognized, its role in nTreg development is controversial (Li et al. 2006; Liu et al. 2008; Marie et al. 2005). Lck, leukocyte-specific protein tyrosine kinase; P, phosphorylated; PKC, protein kinase C; PLC, phospholipase C; SARA, Smad anchor for receptor activation; TAK1, TGF-β activated kinase 1

(2009) reported that c-Rel is required for the development of thymic Foxp3$^+$ CD4 regulatory T cells; (b) Long et al. (2009) reported that 'nuclear factor-κB modulates regulatory T cell development by directly regulating expression of Foxp3 transcription factor'. In February 2010, Zheng et al. (2010) reported that germ line deletion of *Foxp3* CNS3 that contains a c-Rel binding site blocks Treg development, and in

March 2010, two additional groups reported that c-Rel is indispensable for Treg cell development in mice (Deenick et al. 2010; Visekruna et al. 2010).

It also has been shown that c-Rel is not only involved in the Treg cell differentiation, but also required for the homeostatic proliferation of peripheral Treg cells. But c-Rel does not appear to control the effector function of Treg cells because c-Rel-deficient Treg cells are equally effective in suppressing T cell function as compared to wild type cells (Isomura et al. 2009). Therefore, Treg cells in c-Rel-deficient mice, despite the reduction in their numbers, can still suppress immune responses (Isomura et al. 2009).

3 NF-κB and Th17 Cell Response

For many years, $CD4^+$ helper T (Th) cells have been classified into two major subsets, Th1 and Th2 cells (Romagnani 1997). Th1 cells express interferon (IFN)-γ and control cellular immunity, whereas Th2 cells produce interleukin (IL)-4, IL-5, and IL-13, and regulate humoral immunity. Recently, a different Th subset called Th17 cells has been defined (Bettelli et al. 2006; Cua et al. 2003; Ivanov et al. 2006; Langrish et al. 2005; Sutton et al. 2006; Veldhoen and Stockinger 2006; Weaver et al. 2006). Unlike Th1 and Th2 cells, Th17 cells produce IL-17A, IL-17F, and IL-6, but not IFN-γ or IL-4, and are considered to be 'pro-inflammatory' because they are involved primarily in mediating inflammatory diseases and immune defense against extracellular bacteria (Bettelli et al. 2006; Ivanov et al. 2006; Langrish et al. 2005; Sutton et al. 2006; Veldhoen and Stockinger 2006; Weaver et al. 2006). Th17 cells can be generated from naïve precursors in the presence of TGF-β and IL-6 or IL-21 (Bettelli et al. 2006), which can be further augmented by tumor necrosis factor (TNF)-α or IL-1β (inflammatory cytokines that inhibit Th1 and Th2 cell differentiation) (Chung et al. 2009; Sutton et al. 2006). Developments of Th1, Th2, and Treg cells are specified by transcription factors such as Tbet, GATA3, and Foxp3, respectively, whereas that of Th17 cells appears to depend on the retinoid-related orphan receptor (ROR) γT, RORα, and STAT3 (Ivanov et al. 2006; Yang et al. 2007, Yang et al. 2008).

3.1 T Cells Deficient in c-Rel or p65 are Significantly Compromised in Th17 Differentiation and Th17 Responses

Recent work from our lab has shown that $CD4^+$ T cells freshly isolated from c-Rel-deficient mice produced significantly less IL-17A mRNA and protein upon activation with anti-CD3 and anti-CD28. When purified naïve $CD4^+$ T cells were cultured under the Th17 cell-inducing condition, IL-17A expression was significantly reduced in the c-Rel-deficient group. Similarly, up to 70% reduction in Th17 cell numbers was observed in $p65^{-/-}$ cultures. It should be pointed out that

in addition to *Rorg,* other c-Rel target genes could also regulate Th17 responses in a cell autonomous manner. For example, both *Il2* and *Foxp3* are c-Rel target genes that are known to promote Treg differentiation and inhibit Th17 differentiation (Isomura et al. 2009; Long et al. 2009; Ruan et al. 2009; Zheng et al. 2010). Because c-Rel-deficient T cells produce less IL-2 than WT T cells, the effect of c-Rel deficiency on Th17 differentiation could be masked if anti-IL-2 was not included in the Th17 differentiation medium (Liou et al. 1999; Visekruna et al. 2010).

3.2 The Rel–RORγT Axis that Drives Th17 Differentiation

How c-Rel and p65 regulate Th17 differentiation is not known. There is no evidence that NF-κB directly regulates IL-17 expression. Also Stat3 expression was not affected by c-Rel deficiency. Instead, we found that RORγ and RORγT expression is markedly reduced in c-Rel-deficient T cells, and their reconstitution rescues the Th17 defect. Our chromatin immunoprecipitation analysis enabled us to propose the following model. Antigen-presenting cells (APC) engage naïve $CD4^+$ T cells by TCR and CD28, in the presence of IL-1, IL-6, IL-23, and TGF-β; ligation of TCR, CD28, IL-1, and possibly IL-23 receptors leads to the activation of IKKβ, which phosphorylates IκBα, releasing c-Rel and p65. We propose that the freed c-Rel-p65 dimer migrates into the nucleus, binds to the *Rorg* and *Rorgt* promoters, and initiates Th17 differentiation, with the help of other factors such as NFAT and Stat. Therefore, although *Rorg* gene specifies Th17 lineage differentiation (Ivanov et al. 2006), its own expression may not be controlled by lineage-specific factors. This principle also applies to many other lineage-specifying genes including *Foxp3* that is controlled by c-Rel. Although each factor in the enhanceosome is not unique, the complex as a whole is (Ruan et al. 2009). This concept explains how non-lineage-specific factors such as c-Rel control gene expression in a lineage-specific manner. It is to be emphasized that the transcription factors (c-Rel and p65) we identified are the only known ones that bind to and activate *Rorg* promoters. Stat3 was recently shown to bind to the first intron of the *Rorg* gene, but not the promoter (Durant et al. 2010). The nature of other *Rorg* promoter-binding transcription factors remains to be established.

3.3 c-Rel-Deficient Mice have Diminished Autoimmune Th17 Responses

Th17 cells play crucial roles in the pathogenesis of autoimmune diseases. Because c-Rel-deficient T cells have a defect in Th17 differentiation, we asked whether Th17 responses to self-antigens were affected in c-Rel-deficient mice. As we reported,

c-Rel-deficient mice were resistant to experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis (Hilliard et al. 2002). To determine whether anti-self MOG (myelin oligodendrocyte glycoprotein) Th17 response was affected by the c-Rel deficiency, we immunized WT and c-Rel-deficient mice with a MOG peptide and examined the splenic T cell response to the self-peptide two weeks later. We found that the anti-MOG Th17 response (as measured by IL-17 and IL-6 production) was significantly reduced in c-Rel-deficient mice as compared to their WT littermates.

To determine if c-Rel deficiency affects other models of autoimmunity, we crossed our c-Rel-deficient B6 mice with a NOD mouse line that carried a transgenic diabetogenic TCR called BDC2.5 (Gonzalez et al. 1997). All WT progenies developed autoimmune diabetes upon challenging with cyclophosphamide (CY) even though they were of a mixed MHC background. Remarkably, none of the c-Rel-deficient mice developed diabetes as measured by blood glucose levels. Consistent with these results, splenocytes from c-Rel-deficient mice produced significantly lower levels of IL-17A, IL-6, IL-2, and IFN-γ than WT cells upon stimulation with anti-CD3, with or without anti-CD28. Flow cytometric analysis revealed that in $CD4^+$ T cells, both Th1 and Th17 cytokines were reduced as a result of the c-Rel deficiency. Taken together, these results indicate that autoreactive Th17 responses are markedly diminished in c-Rel-deficient mice.

Differentiation of autoreactive $CD4^+$ naïve T cells into Th1 or Th17 effector cells is required for the development of autoimmune diseases. Non-differentiated autoreactive naïve T cells are not pathogenic. Our observation that c-Rel-deficient mice are resistant to EAE and T1D indicates that c-Rel-mediated T cell differentiation may play a critical role in the development of autoimmune diseases (Hilliard et al. 2002; Lamhamedi-Cherradi et al. 2003). However, since c-Rel regulates multiple gene expressions in both lymphocytes and myeloid cells (Bunting et al. 2007; Gerondakis et al. 2006), the disease resistance of c-Rel-deficient mice can be due to the defect in either lymphoid or myeloid cells, or both. Similarly, c-Rel may regulate Th cell differentiation *in vivo* through two distinct mechanisms: (a) c-Rel expressed by T cells may regulate their differentiation through an autonomous mechanism, for example, by activating the promoters of Th17-specific genes such as *Rorg*; (b) c-Rel expressed by myeloid cells may regulate Th cell differentiation through a T cell non-autonomous mechanism, for example, by regulating the expression of cytokines that drive Th17 differentiation. The autonomous theory is supported by the findings that (a) purified $Rel^{-/-}$ T cells exhibit a significant defect in Th17 cell differentiation, (b) c-Rel directly controls the RORγT expression, and (c) IL-1, IL-23, and TGF-β, cytokines that promote Th17 cell differentiation, activate Rel/NF-κB (Cho et al. 2006; Chung et al. 2009; Shim et al. 2005). Similarly, the non-autonomous theory is supported by the finding that the expression of IL-6 and IL-23, two cytokines that promote Th17 cell differentiation, is significantly reduced in c-Rel deficient myeloid cells (Carmody et al. 2007a; Lamhamedi-Cherradi et al. 2003). Interestingly, using cDNA microarrays that cover the whole mouse genome, Bunting et al. (2007) reported that the expression of ~130 genes was reduced

in c-Rel-deficient T cells as compared to WT cells treated with anti-CD3 and anti-CD28. These genes encode cytokines, chemokines, and cell surface molecules as well as intracellular signal transducers and transcription factors. However, because *Rorg* expression is induced during Th17 differentiation, Bunting et al. (2007) did not detect it in their cells, which were not of the Th17 type.

4 Future Perspective

Nuclear factor-κB (NF-κB) is a ubiquitous transcription factor that controls the expression of genes involved in immune responses, apoptosis, and cell cycle. Recent data including ours indicates that NF-κB, specifically c-Rel and p65, are involved in not only promoting inflammation, but also driving Treg cell development. However, how c-Rel controls Treg development remains to be characterized. There are four confirmed c-Rel binding sites in the *Foxp3* locus: two in the promoter (or CNS −1) as determined by us using ChIP (Ruan et al. 2009), one in CNS2 (also called Enhancer 2) as determined by Long et al. (2009), and one in CNS3 as determined by Zheng et al. (2010) through nucleotide pull-down. The roles of each of these sites in Treg development need to be established. It should be pointed out that nTreg and iTreg cells may differ considerably in their differentiation programs including the requirement for transcription factors such as c-Rel. It must be emphasized that in addition to controlling Treg differentiation, c-Rel is also essential for the normal functioning of B cells, Th1 cells, Th17 cells, and myeloid cells (but not Th2 cells) (Carmody et al. 2007b; Donovan et al. 1999; Hilliard et al. 2002; Lamhamedi-Cherradi et al. 2003; Yang et al. 2002). This may explain why c-Rel-deficient mice, despite the reduction in Treg cell numbers, do not develop spontaneous inflammatory diseases (Gerondakis et al. 1998; Liou et al. 1999). These new findings may not only help advance our understanding of the NF-κB biology, but also aid in developing new drugs targeting the NF-κB pathway for the treatment of inflammatory diseases.

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Function of Act1 in IL-17 Family Signaling and Autoimmunity

Ling Wu, Jarod Zepp and Xiaoxia Li

Abstract The maintenance of immune homeostasis requires the delicate balance between response to foreign antigens and tolerance to self. As such, when this balance is disrupted, immunodeficiency or autoimmunity may manifest. The adaptor molecule known as Act1 is a critical mediator of IL-17 receptor family signaling. This chapter will detail the current understanding of Act1's role in signal transduction as well as address the fundamental role of Act1 in autoimmunity. At the molecular level Act1 interacts with IL-17R through the conserved SEFIR domain, binds TRAF proteins and exerts E3 ubiquitin ligase activity. In *in vivo* models, Act1 deficiency provides protection against experimental autoimmune diseases, such as colitis and EAE. Yet mice lacking in Act1 develop spontaneous autoimmune diseases. Indeed, the utility of Act1 seems to rely on the specific cell type expression that may determine the pathway that Act1 mediates.

Keywords Act1 • IL-17 • IL-17 receptor • Experimental Autoimmune Encephalomyelitis (EAE) • IL-25 (IL-17E) • Asthma

1 Introduction

Autoimmune diseases result from the imbalance between self-tolerance and immune-reactivity in which auto-reactive lymphocytes that escape from selection cause damage to the body itself (Fry et al. 1989; Rocha and von Boehmer 1991; Carlow et al. 1992). The etiology of autoimmune diseases is complex, has both a genetic and an environmental component, and can be systemic like SLE (systemic lupus erythematous) or organ specific like multiple sclerosis, Sjogren's disease, and diabetes (Steinman 2001; Cooper et al. 2002; Compston and Coles 2008).

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CD4+ T helper cells play essential roles in immune responses and autoimmunity. Classically, CD4+ T helper cells were divided into two lineages, T helper 1 (Th1) and T helper 2 (Th2). Th1 cells secrete IFNγ and promote cellular immunity while Th2 cells secrete IL-4, IL-5, and IL-13 for humoral immunity. Recently, a new lineage of T helper cells that secrete IL-17, termed Th17 cells (Kolls and Linden 2004; Harrington et al. 2005; Park et al. 2005; Iwakura and Ishigame 2006), have been found to play a major role in protection against bacterial and fungal infections (Iwakura et al. 2008; Conti et al. 2009; Curtis and Way 2009). IL-17 is a pro-inflammatory cytokine that up-regulates inflammatory genes expression in various cells such as fibroblasts, endothelial cells, and epithelial cells. More importantly, IL-17 levels were elevated in various autoimmune diseases such as multiple sclerosis, asthma, inflammatory bowel disease, psoriasis, and rheumatoid arthritis (Hemdan et al. 2010; Matusevicius et al. 1999). In this chapter, we will focus on autoimmune diseases mediated by IL-17-producing cells and the current proposed mechanisms of IL-17 signaling.

2 IL-17 Family

IL-17, also known more specifically as IL-17A, is part of the IL-17 family of six cytokines that includes IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F, all of which share structural similarities. These cytokines are predicted to form homodimeric interactions, or—in the case of IL-17A and IL-17F—heterodimeric interactions prior to signaling on its respective receptors (Fig. 1) (Moseley et al. 2003; Gaffen 2009). Although much less is known of IL-17B-D, they appear to have similar signaling components to IL-17A, IL-17E, and IL-17F. IL-17A and IL-17F share the most homology and are secreted by Th17 cells. In fact, IL-17A and IL-17F can signal through the same receptor. IL-17E, also known as IL-25, is involved in airway inflammation and promotes an allergic response that includes Th2 cytokine production and eosinophilic recruitment (Claudio et al. 2009; Swaidani et al. 2009).

There are five known receptors of the IL-17 family (IL-17RA through IL-17RE) (Moseley et al. 2003; Gaffen 2009). Although the pairing of ligand to receptor is not completely clear, we know that IL-17A signals through the IL-17RA/IL-17RC complex while IL-25 signals IL-17RA/IL-17RB complex. Currently, IL-17RA and IL-17RC are found on the surface of many types of cells including epithelial cells, fibroblasts, endothelial cells, astrocytes, and macrophages. Though we know that IL-17RA is expressed on T cells due to the T cells' ability to respond to IL-17, it is unclear whether T cells also express IL-17RC (O'Connor et al. 2009). IL-25R (formed by IL-17RA/RB) more widely known for its expression on epithelial cells, is also found on the surface of effector T cells (Angkasekwinai et al. 2007) and NKT cells (Terashima et al. 2008; Stock et al. 2009), and can in fact induce the expression of Th2 cytokines (Fort et al. 2001).

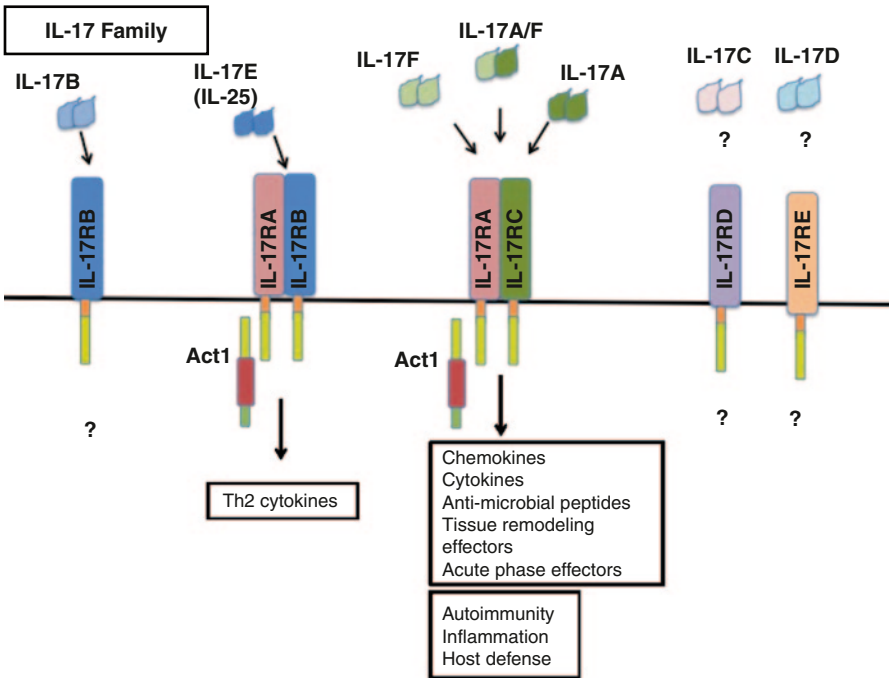

Fig. 1 IL-17 cytokine and receptor family. The IL-17 cytokine family includes IL-17 A–F, which are predicted to form homo- and hetero-dimeric interactions that are necessary for signaling. There are also five IL-17 receptor subunits, of which IL-17RA, -RC, and -RB are the best described. The receptor subunit, IL-17RA, is common for IL-17A, IL-17F, and IL-17E (IL-25) driven gene expression. IL-17A and IL-17F bind the receptor complex IL-17RA/IL-17RC to drive inflammatory gene expression. IL-25 binds to the IL-17RA/IL-17RB complex to mediate its effects on Th2 homeostasis

3 Discovery of Act1

Under the control of various inflammatory and pathogen derived stimuli, the transcription factor NFκB is a central mediator of gene expression. Upon its activation, NFκB modulates the expression of many target genes including those for cytokines, chemokines, and cell surface receptors among many others. Under normal conditions NFκB is sequestered in the cytoplasm by IkB inhibitory proteins. Upon stimulation with various extracellular stimuli (including Toll-like receptor ligands, IL-1 and TNFα), the IkB protein is phosphorylated by IkB kinase (IKK) thereby releasing NFκB and allowing its translocation to the cell nucleus. The signaling pathways that activate NFκB converge at the IKK complex that is composed of three subunits, the catalytic subunits IKKα and -β and the regulatory subunit IKKγ (Li and Verma 2002).

During a search for NFκB signaling proteins, Act1/CIKS was discovered to activate NFκB. Using an NFκB-dependent selectable marker, Act1 (NFκB activator 1) was discovered due to its ability to activate NFκB (Li et al. 2000). Over-expression of Act1 leads to constitutive activation of NFκB as well as JNK. Furthermore it was shown that Act1 activates IKK through a helix-loop-helix (HLH) domain in its N-terminal portion. Simultaneously, Act1 was also cloned by Leonardi et al. (2000), through yeast two-hybrid screening based on its interaction with IKKg. Likewise, in their study Act1 (referred to as CIKS, connection to IKK and SAPK/JNK) was also found to activate NFκB and JNK.

Initial examination of the amino acid sequence of Act1 revealed that there is an HLH domain, which was functionally important for the interaction with IKK, and two TRAF-binding domains. The TNF-receptor associated factor (TRAF) family consists of six members, TRAF 1–6. Further studies explored the interaction of the TRAF family members with Act1. CD40, a member of the TNF-receptor superfamily, is expressed on CD4 cells, B cells, and epithelial cells. Upon CD40L stimulation the recruitment of TRAF proteins is necessary for NFκB induction. It was found that upon CD40 stimulation, Act1 is recruited to CD40 and that it also interacts with TRAF3 (Qian et al. 2002). One group reported on immunoprecipitation studies with over-expression constructs of Act1 and they reported on its association with TRAF6 through its TRAF-binding domain, however functional data was not explored (Kanamori et al. 2002). The interaction between TRAF6 and Act1 was not fully elucidated until recently.

4 STIR Domain Superfamily

In 2003, Novatchkova et al. reported on the homology of a protein initially described from zebrafish known as similar expression of fibroblast-growth-factor genes, or SEF (Novatchkova et al. 2003). In the zebrafish SEF acts as an inhibitor of FGF signaling. Interestingly, they report that in mammals the closest non-orthologous homologue of SEF is found in the IL-17 family of receptors. The sequence of homology in the cytoplasmic region of IL-17R is referred to as the SEFIR (SEFs and IL-17Rs) domain. What's more is this search also revealed the SEFIR domain was present in Act1.

The SEFIR domain is closely related to the TIR (Toll/interleukin-1 receptors) domain expressed in Toll-like and IL-1 receptors. Due to this similarity, the STIR (SEFIR and TIR) domain family as it is referred now, consists of the IL-17 receptor subunits including IL-17RA, IL-17RC, IL-17RB, and Act1. The STIR domain family provided a foundation implicating the possible involvement of Act1 in IL-17R signaling. The Act1 and IL-17 interaction was predicted to be analogous to the involvement of the MyD88 adaptor protein in TIR and IL-1 signaling (Fig. 2).

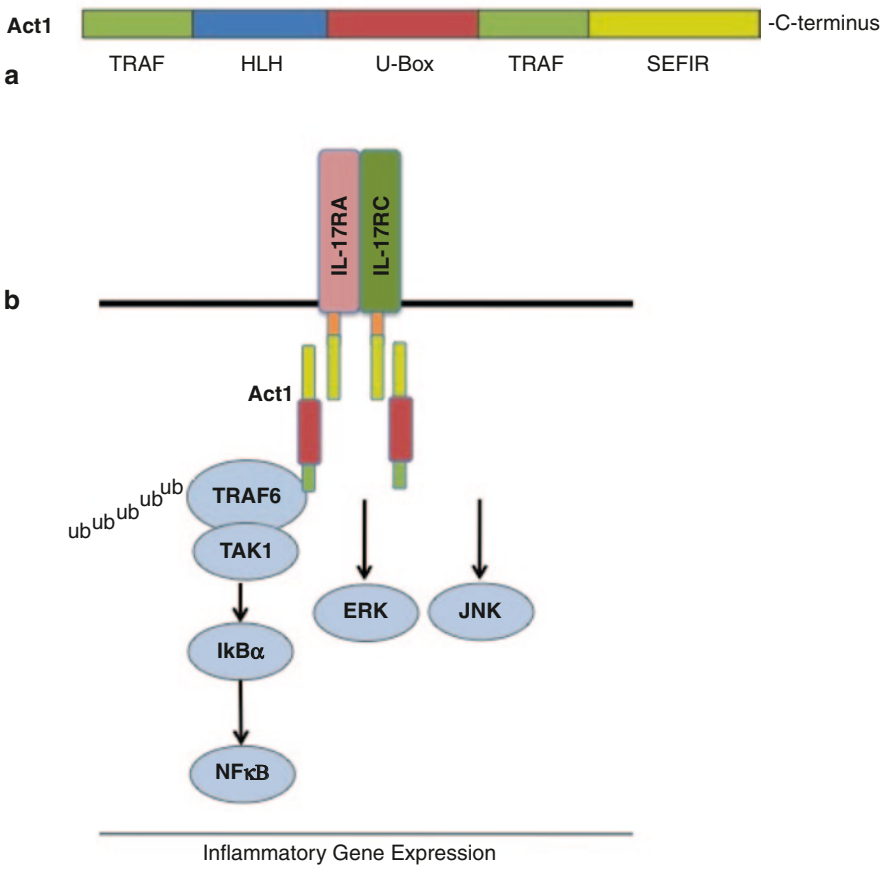

Fig. 2 Act1 structure and IL-17 signaling cascade. a The structure of Act1 consists of two TRAF binding domains that mediate TRAF6 interactions following IL-17 stimulation. Moreover, the U-Box E3 ligase domain is functionally important for mediating the ubiquitination of TRAF6. The helix–loop–helix (HLH) domain and SEFIR domain mediate Act1 protein–protein interactions. Furthermore, the SEFIR domain is necessary for Act1 interaction with IL-17R subunits and for IL-17-dependent NFκB activation. **b** The IL-17A signaling cascade depends on SEFIR–SEFIR domain (shown in *yellow*) interaction between the IL-17R subunits and Act1. Following this, Act1 exerts its E3 ligase activity by mediated K63-linked ubiquitination of TRAF6, allowing its interaction with TAK1 and subsequent NFκB activation. IL-17 activation of the ERK and JNK pathways are also Act1 dependent, however the exact mechanism leading to their activation is yet to be elucidated

5 Act1 is a U-box Type E3 Ubiquitin Ligase

Ligand-receptor binding is the initiating factor that sets a series of downstream signaling events into action. Signaling events are dependent on protein interactions and specific modifications. Following the identification of the STIR domain

superfamily, Act1 was found to be a critical mediator in the IL-17 signaling pathway. Studies conducted by Li and Dong revealed the requirement for Act1 in the IL-17 pathway (Chang et al. 2006; Qian et al. 2007). The structure of Act1 provides insight to the mechanism of signal mediation. Taken together the different domains of Act1—HLH domain at the N-terminus, two TRAF-binding domains, and a coiled-coil domain at its C-terminus—suggest protein–protein interactions. It was indeed shown that Act1 is recruited to IL-17R upon IL-17 stimulation through SEFIR–SEFIR domain interaction, followed by recruitment of the TGFβ Activated Kinase 1 (TAK1) and TRAF6, leading to NFκB activation.

Interestingly, further investigation of Act1 revealed an essential U-box domain that is common in protein E3-ubiquitin ligases. Much like protein phosphorylation, protein ubiquitination is an important modification required for many signaling events. Protein ubiquitination is a sequential process involving the activities of three types of enzymes (E1–E3): the ATP-dependent activity of the ubiquitin-activating enzyme (E1); the acceptance of the activated ubiquitin by the ubiquitin-conjugating enzyme (E2); and an ubiquitin protein ligase (E3), which binds to the E2 and facilitates the conjugation of ubiquitin on the target protein. There are three families of E3 ligases that have been described: RING (really interesting new gene), HECT (homology to E4AP C terminus), and U-box. The conjugation of ubiquitin to different lysine residues can mark proteins for either proteasomal degradation or promote protein–protein interactions if linked on Lys48 or Lys63, respectively.

The study by Liu et al. (2009) examined a region within Act1 from residues 273–338 that is homologous to the U-box domain in E3-ligases. *In vitro* assays revealed that Act1 exhibited E3-ubiquitin ligase activity. Further analysis was aimed at determining the substrate for Act1 ligase activity. It was previously reported that IL-17 signaling and activation of NFκB was dependent on TRAF6 (Schwandner et al. 2000). Upon stimulation with IL-17 ubiquitinated forms of TRAF6 are detectable in wild-type cells but not in cells lacking Act1. *In vitro* ubiquitination assays using TRAF6 as a substrate showed that Act1 catalyzed the Lys63-linked ubiquitination of TRAF6 and that this was dependent on the U-box domain of Act1. Furthermore the ubiquitination of TRAF6 by Act1 was required for the IL-17-dependent activation of NFκB.

The recruitment of Act1 to the IL-17R is through its SEFIR domain and the U-box E3 ligase activity is the second functional domain of Act1. The U-box domain of Act1 is required for IL-17-dependent activation of NFκB that occurs through the ubiquitination of TRAF6. TRAF6 may not be the only molecule that Act1 can exert its E3 ligase activity and this was suggested in the seminal work by Liu et al. (2009). Interestingly in this study it was found that in TRAF6 deficient cells, IL-17-dependent activation of NFκB and JNK was abolished; however, ERK phosphorylation was intact, but this activation was still dependent on the U-box of Act1. These data allude to another downstream component of IL-17-dependent ERK activation that relies on the E3 ligase activity of Act1.

6 Epithelial Requirement of Act1 in IL-17 and IL-25 Mediated Lung Inflammation

Allergic asthma results from a chronic inflammatory response in the lungs with prevailing $CD4^+$ T cells occupying the airways as well as eosinophils and neutrophils, excessive production of mucus and IgE/IgG production. Once activated, the $CD4^+$ T cells differentiate to distinct effector subsets. The $CD4^+$ Th2 cells produce IL-4, IL-5, and IL-13 and act to mediate the humoral and allergic immune responses. In human and in several mouse models of antigen-induced asthma, it is well established that Th2 cells are critical mediators of the immune condition of the lung. However, prior to T cell activation, resident APCs must present antigens detected at the mucosal surface. Therefore, the mucosal surface is not just a passive barrier but is actually required for the orchestration of an appropriate immune response.

Recently, lung epithelium-derived cytokines, IL-25 (IL-17E), IL-33, and TSLP have been found to promote Th2 responses. IL-25 has been demonstrated to promote the differentiation of naïve T cells to effector Th2 cells in an IL-4 and STAT6 dependent manner (Angkasekwinai et al. 2007). Additionally IL-25 can also act directly on the epithelial compartment. It was reported that allergens induce the expression of IL-25 in the epithelium and that increased IL-25 expression can promote Th2 immunity (Angkasekwinai et al. 2007). Importantly, intranasal administration of IL-25 leads to an increase in Th2-driving cytokines—IL-4, IL-5, IL-13, TSLP—and eotaxin and eosinophilia. Although IL-25 is the most divergent IL-17 family member, we and another group found that Act1 interacts with IL-17RB and is required for IL-25-induced responses (Claudio et al. 2009; Swaidani et al. 2009). In the study by Swaidani et al. (2009), Act1 was specifically deleted in the epithelial compartment, which abolished IL-25-induced cytokine production and eosinophilia. Moreover, it is important to note that in human asthmatic tissue, the expression levels of IL-25 as well as IL-25R (IL-17RB) were found to be elevated (Wang et al. 2007). These studies provide insight into the role of IL-25 signaling in epithelium on the initiation and maintenance of allergic responses.

It has also been shown that IL-17 is also involved in allergic airway inflammation. In contrast to IL-25, when IL-17 is injected to the mouse airway there is a dramatic increase in chemokine expression of KC (CXCL1) and IL-6, which is followed by an accumulation of neutrophils. The administration of IL-17 primarily acts on the epithelial compartment as shown by Act1 deletion from the epithelial compartment leading to an abrogated cytokine and neutrophil response.

Besides mediating the direct induction of distinct airway cellularity and cytokine/chemokine production by IL-17 and IL-25, Act1 is also important in antigen-induced asthma. In the asthma challenge model, mice are immunized with OVA and are subsequently challenged two weeks later with OVA aerosol. Mice that are deficient in Act1 in the epithelial compartment have reduced airway eosinophilia/neutrophilia and reduced cytokine/chemokine production. It is important to note

that there is no difference in OVA-specific IgE or IgG production or airway hyperresponsiveness in the epithelial-deleted Act1 mouse. This observation may be due to the essential role of Act1 in other immune cell types. Overall, these findings demonstrate the utility of a common signaling component, Act1, and its role in the epithelial compartment. How Act1 mediates the diverse immune response by IL-17 and IL-25 is yet to be determined, but most likely it may be explained by the specific signaling mediated by different receptor subunits.

7 Act1 in CNS Resident Cells

Multiple sclerosis (MS) is generally accepted as an autoimmune disease where pathogenic T cells specific for myelin antigens elicit damage to the central nervous system (CNS), causing demyelination of axons and subsequent axonal damage. The brain and CNS have always been deemed an immune privilege site, an area to which the immune system lacks access. The pathogenesis of MS is thus baffling and the question remains as to how T cells gain access to the CNS. Th17 cells have been proposed to act either on glial cells or directly on endothelial cells to promote blood brain barrier (BBB) breakdown and subsequent Th17 cell access to the CNS (Kebir et al. 2007). Although the exact mechanism of this BBB breakdown is still unknown, recent evidence indicate that different cellular compartments may contribute to disease progression (Benveniste 1997; Chavarria and Alcocer-Varela 2004).

Classically, MS was believed to be mediated by pathogenic Th1 cells, exerting its damage via the secretion of IFNγ. In earlier experiments, scientists hypothesized that if IFNγ is involved in the pathogenesis of this disease, then the disease should be ameliorated when this cytokine is blocked. However, the opposite was seen. In fact, blocking IFNγ actually exacerbated MS disease severity (Billiau 1995). The discovery of the involvement of Th17 cells helped to explain this phenomenon. After years of research, scientists now know that both Th1 and Th17 cells play major roles in the pathogenesis of MS (El-behi et al. 2010; Steinman 2001). It is now believed that Th1 cells act as initiators of MS, whereas Th17 cells serve to promote disease progression. In addition, the two cytokines exhibit regulatory roles on each other: IFNγ inhibits STAT3 expression, which is needed for Th17 differentiation, while IL-17 inhibits the expression of the Th1 signature transcription factor, T-bet (Komiyama et al. 2006; O'Connor et al. 2009). Thus, by blocking IFNγ, the inhibition of STAT3 is lost, allowing Th17 differentiation to occur and disease severity to progress.

In the animal model of MS, experimental autoimmune encephalomyelitis (EAE), mice deficient in IL-17 exhibit less disease severity upon disease induction, again indicating the importance of this cytokine in the pathogenesis of EAE. Likewise, in mice deficient in the IL-17 receptor, mice also exhibit decreased disease severity, indicating the importance of IL-17-mediated signaling in disease pathogenesis

(Hu et al. 2010). As indicated earlier, endothelial cells, epithelial cells, astrocytes, macrophages, and neurons all express receptors for IL-17. It seems that in IL-17-mediated EAE, both cells that are producing IL-17 and cells that are responding to IL-17 play major roles in disease progression and pathogenesis. This highlights the importance of the interaction between cells of the immune system with non-immune cells, a delicate balance that helps to maintain homeostasis.

In the absence of Act1, *in vitro* studies show that IL-17-dependent gene expression of pro-inflammatory cytokines such as KC, IL-6, and MIP-2 is markedly diminished (Qian et al. 2007). In mice deficient in Act1, mice exhibit decreased disease severity upon EAE induction, consistent with the idea that Act1 is essential in IL-17-mediated signaling. Thus, to determine which cellular compartment contributes most to MS pathogenesis, Kang et. al. deleted Act1 in specific cellular compartments. The absence of Act1 in endothelial cells, macrophages, or microglials did not alter the disease process, indicating that IL-17 signaling in these cells do not play a major role in Th17-induced EAE pathogenesis. However, mice with Act1 deleted in neuroectoderm-derived cells exhibit reduced disease severity and delayed onset of disease (Kang et al. 2010). In fact, Act1 deficiency in these cells resulted in decreased immune infiltration in the brain during the disease process, even though the number of Th17 cells remains unchanged compared to wild type mice. This suggests that IL-17 signaling through one (or more) of these cells—astrocytes, oligodendrocytes, or neurons—play a major role in immune cell recruitment and subsequent demyelination and axonal injury. Perhaps it is this feed-forward mechanism through IL-17 signaling in the neuroectoderm-derived cells, resulting in the amplification of inflammatory response that eventually contributes to disease progression.

8 Act1, The Double-Edged Sword

We have provided several lines of evidence that Act1 is necessary for IL-17-mediated inflammatory responses. But what we've lacked to mention up to this point is that Act1 deficiency actually leads mice to develop spontaneous autoimmune disease. This observation of spontaneous autoimmune disease in Act1 deficient mice is quite baffling, given that they display resistance to EAE disease induction, an autoimmune disease itself. This intriguing observation adds another layer of complexity to the role of Act1.

In fibroblasts, endothelial cells, epithelial cells, astrocytes, and macrophages, Act1 serves as a component of the IL-17 receptor-signaling cascade. However, in B cells, Act1 serves as a negative regulator of CD40–CD40L and BAFF-BAFFR signaling to control B cell maturation and survival, respectively (Giltiay et al. 2010; Qian et al. 2002, 2004). The loss of Act1 thus results in an increase in B cell population, culminating in splenomegaly, lymphadenopathy, hypergammaglobulinemia, and autoantibody production. In fact, BALB/c mice develop Sjogren-like disease

as early as 3 weeks of age, while C57BL/6 mice exhibit autoimmune phenotype by 9 months of age (Qian et al. 2008). This observation of autoimmune phenotype has also been seen in mice with a spontaneous mutation in the Act1 gene (Matsushima et al. 2010).

In addition to the increase in B cell population, loss of Act1 results in an increased number of Th17 cells. The mechanism of this hyper Th17 response is currently unclear and is still under investigation. It is exciting to note that three recent independent cohort studies of psoriasis patients found a genetic mutation in Act1 that predisposes them to develop this autoimmune disease (Ellinghaus et al. 2010; Hüffmeier et al. 2010; Strange et al. 2010). Psoriasis is a skin disease characterized by epidermal hyper-proliferation and chronic inflammation of the skin. Th17 cells have been found to be mediators in psoriasis. Future studies are required to investigate the molecular mechanisms for the precise role of Act1 in modulating Th17 cells and autoimmunity.

These observations in both mouse and human subjects suggest a dual role of Act1 in modulating the immune response. First, as a component in IL-17 and IL-25-mediated signaling cascades, Act1 serves as a positive role in carrying out inflammatory and allergic reactions. Second, as a negative regulator in B cells, Act1 serves as a keeper that prevents hyper B cell functions to eventually lead to autoimmune phenotypes. Third, due to the complexity of the immune system and the intricate interactions between different cells, receptors, and molecules, Act1's role as a positive effector in one pathway may lead to its indirect role as a negative effector in another pathway. On one hand, loss of Act1 alleviates autoimmune disease, on the other it exacerbates it. The delicate balance of Act1's role in the immune system reflects the intricate interactions that are necessary to maintain immune homeostasis and regulate a balance between protection against pathogens and tolerance to self.

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Differential Effect of Inhibiting MD-2 and CD14 on LPS- Versus Whole *E. coli* Bacteria-Induced Cytokine Responses in Human Blood

D. Christiansen, O. L. Brekke, J. Stenvik, J. D. Lambris, T. Espevik and T. E. Mollnes

Abstract *Background*: Sepsis is a major world-wide medical problem with high morbidity and mortality. Gram-negative bacteria are among the most important pathogens of sepsis and their LPS content is regarded to be important for the systemic inflammatory reaction. The CD14/myeloid differentiation factor 2 (MD-2)/TLR4 complex plays a major role in the immune response to LPS. The aim of this study was to compare the effects of inhibiting MD-2 and CD14 on ultra-pure LPS-versus whole *E. coli* bacteria-induced responses. *Methods*: Fresh human whole blood was incubated with upLPS or whole *E. coli* bacteria in the presence of MD-2 or CD14 neutralizing monoclonal antibodies, or their respective controls, and/or the specific complement-inhibitor compstatin. Cytokines were measured by a multiplex (n = 27) assay. NFκB activity was examined in cells transfected with CD14, MD-2 and/or Toll-like receptors. *Results*: LPS-induced cytokine response was efficiently and equally abolished by MD-2 and CD14 neutralization. In contrast, the response induced by whole *E. coli* bacteria was only modestly reduced by MD-2 neutralization, whereas CD14 neutralization was more efficient. Combination with compstatin enhanced the effect of MD-2 neutralization slightly. When compstatin was combined with CD14 neutralization, however, the response was virtually abolished for all cytokines, including IL-17, which was only inhibited by this combination. The MD-2-independent effect observed for CD14 could not be explained by TLR2 signaling. *Conclusion*: Inhibition of CD14 is more efficient than inhibition of MD-2 on whole *E. coli*-induced cytokine response, suggesting CD14 to be a better target for intervention in Gram-negative sepsis, in particular when combined with complement inhibition.

Keywords CD14 • MD-2 • Complement • Cytokines • LPS • TLR • *E. coli* • Sepsis • Inflammation

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1 Introduction

Sepsis is a severe condition world-wide with high morbidity and if progressing to septic shock also with considerable mortality (Annane et al. 2005). Gram-negative bacteria account for approximately one-third of all cases with sepsis, *Escherichia coli* being the single most important pathogen (Martin et al. 2003). Thus, revealing the inflammatory mechanisms and development of new treatment strategies of Gram-negative sepsis is an important scientific and medical task.

The outer membrane of Gram-negative bacteria contains lipopolysaccharide (LPS) specific to each strain giving different reactions in various species. LPS-binding protein (LBP) enhances the binding of LPS to CD14, which exist in a soluble form (sCD14) and as a glycosyl-phosphatidylinositol-anchored membrane protein without transmembrane signaling properties. CD14 presents LPS to a complex of Toll-like Receptor 4 (TLR4) and myeloid differentiation factor 2 (MD-2) and further signaling is mediated via MyD88 and/or TRIF/TRAM giving cytokine production. In addition to being important for LPS recognition by TLR4, CD14 has also been shown to be implicated in TLR2 and TLR3 signaling (Miyake 2006; Akashi-Takamura and Miyake 2008; Nilsen et al. 2008; Janot et al. 2008). Recently it was even shown that CD14 is a co-receptor for TLR7 and TLR9 and is partly responsible for their inflammatory responses (Baumann et al. 2010). This is in accordance with the assumption that CD14 is a promiscuous upstream recognition molecule, reacting with a number of ligands with low affinity, transferring the ligand to receptors with higher degree of specificity and affinity (Antal-Szalmas 2000).

MD-2 was first described by Shimazu et al. (1999), documenting the need for assembling of MD-2 and TLR4 for cells to be LPS sensitive. Furthermore, translocation of TLR4 to the cell membrane was reported to depend on glycosylation, for which MD-2 was required (Ohnishi et al. 2001). MD-2 is found both as a membrane bound and soluble protein. In contrast to CD14, MD-2 is selectively associated with TLR4 and, in contrast to CD14, does not transmit signals from danger associated molecular patterns (Chun and Seong 2010).

MD-2 and TLR4 has over the past years become key target molecules for inhibiting innate immune responses induced both by endogenous and exogenous danger signals, including LPS and other potential ligands acting in Gram-negative sepsis. Notably, although LPS has been used in innumerable experimental studies, LPS is a surrogate marker of Gram-negative bacteria, which do not sufficiently reflect whole bacteria and only partly mimics sepsis in animal models. We have previously shown, using an *ex vivo* human whole blood model particularly designed for inflammatory crosstalk (Mollnes et al. 2002), that combined inhibition of CD14 and complement efficiently attenuates Gram-negative bacteria-induced inflammatory reaction (Brekke et al. 2007, 2008; Lappegard et al. 2009).

Thus, the aim of the present study was to compare the effects of inhibiting CD14 and MD-2 in the human whole blood model challenged with phenol-extracted ultra-

pure LPS (upLPS), highly specific for TLR4, or whole *E. coli* bacteria potentially recognized by numerous pattern recognition molecules. Since whole *E. coli* bacteria activates complement potently, in contrast to upLPS (Brekke et al. 2007), we included the specific complement inhibitor compstatin in whole bacteria experiments to reveal the relative role of complement, compared to CD14 and MD-2, in the cytokine response.

2 Materials and Methods

2.1 Bacterial Preparation

Non-opsonized smooth *E. coli*, strain LE392 (ATCC 33572), was obtained from American Type Culture Collection (Manassas, VA, USA). The bacteria was grown, heat-inactivated and washed nine times in PBS without Ca^{2+} and Mg^{2+} (Sigma, St. Louis, MO, USA) to remove extracellular LPS as previously described. A stock solution of 1×10^9 bacteria/mL PBS was stored at +4°C. The final concentration of *E. coli* used in the experiments was 3×10^6/mL.

2.2 Inhibitors

The anti-MD-2 monoclonal antibody (mAb) (clone 5D7) was a kind gift from Dr. Greg Elson, NovImmune SA, Geneva, Switzerland, while the corresponding IgG2b isotype control (clone TEN/0) was purchased from Dako (Glostrup, Denmark) and dialyzed to remove azide. The anti-CD14 $F(ab')_2$ mAb (clone 18D11) and its corresponding control (clone BH1) was obtained from Diatec (Oslo, Norway). The compstatin analog Ac-I[CV(1MeW)QDWGAHRC]T, which binds to and inhibits cleavage of C3 was produced as previously described (Katragadda et al. 2006). The inactive peptide Ac-IAVVQDWGHHRAT was used as control. Compstatin and anti-CD14 have previously been titrated and found to have maximum effects at 25 μM and 10 μg/mL (not published), respectively. Anti-MD-2 mAb was titrated five-fold from 25 μg/mL in initial experiments in the present study to obtain a working dose for the further experiments.

2.3 Whole Blood Model of Sepsis

The study was approved by the regional ethics committee. Human whole blood was drawn from healthy individuals after they gave their written consent. The whole blood model of sepsis has been described in detail previously (Mollnes et al. 2002).

Briefly, blood was drawn into Nunc tubes (Roskilde, Denmark) containing lepirudin (Refludan®, Pharmion, Copenhagen, Denmark) to a final concentration of 50 μg/mL. Inhibitors, controls or PBS with Ca^{2+} and Mg^{2+} were administered to Nunc tubes prior to blood sampling and immediately after the sample was obtained, blood was added to the respective tubes. Tubes were preincubated at 37°C for minimum four minutes and then further incubated with 3×10^6/mL *E. coli* or 100 ng/mL phenol-extracted upLPS from *E. coli* (InvivoGen, San Diego, CA, USA). Samples were rotated in an incubator for two hours, then put on ice and ethylenediaminetetraacetic acid (EDTA) (Sigma) was added to a final concentration of 10 mM. Samples were centrifuged at $3220\times g$, plasma was obtained and stored at −80°C until further analysis.

2.4 Cytokine and TCC Measurements

Cytokines in plasma was analyzed using a multiplex cytokine kit from BioRad (Hercules, CA, USA). The assay of 27 interleukins, chemokines and growth-factors, was performed according to instructions from the manufacturer and comprised the following analytes: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, eotaxin, basic FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α and VEGF. Complement activation was measured as generation of the soluble terminal sC5b-9 complement complex (TCC) using an ELISA kit from Becton Dickinson (C5b-9 BD OptEIA™, San Diego, CA, USA). The assay was performed according to instructions from the manufacturer.

2.5 Transient Transfection and NF-κB Luciferase Assay

Human embryonic kidney (HEK) 293 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) from Euroclone (Siziano, Italy), L-glutamine and 10-μg/mL ciprofloxacin (Bayer AG, Leverkusen, Germany) at 37°C and 8% CO_2. Transient transfection was done using GeneJuice™ transfection reagent (Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol. In short, cells were plated at a cell density of 1×10^4 cells/well in 96-well dishes and grown to 50% confluency. Plasmids used were human CD14 in pcDNA3 kindly provided by Dr. D. Golenbock (University of Massacusetts Medical School), human MD-2 in pEFBOS kindly provided by Dr. K. Miyake (University of Tokyo), human TLR4 in pcDNA3 kindly provided by Dr. R. Medzhitov and Dr. C. Janeway (Yale University, New Haven, CT), and human TLR2 in pRK7 kindly provided by Dr. C. Kirschning (Technical University of Munic). As reporter we used the NF-κB dependent luciferase plasmid pELAM-luc (Chow et al. 1999). The pRL-TK vector

(Promega, Madison, WI, USA) encoding the sea pansy (*Renilla reniformis*) luciferase was co-transfected and served as endogenous control with a low constitutive expression. Each plasmid was transfected at a dosage of 25 ng/well and pcDNA3 (Invitrogen, Carlsbad, CA, USA) was used to adjust the total amount of plasmid to 100 ng/well. All plasmids were isolated using the EndoFree plasmid kit (Qiagen, Hilden, Germany). After incubation for 24 h, the cells were stimulated for 18 h. Cytoplasmic extracts were prepared and the Dual-Luciferase® Reporter Assay System (Promega) was used to measure sequentially the firefly and sea pansy luciferase activities with the Victor^{3TM} 1420 multilabel counter (Perkin Elmer, Waltham, Massachusetts, USA). Results from triplicate wells are given as fold induction relative to cells transfected with reporter and pcDNA3 plasmids only. Positive controls for TLR2 activation were the synthetic compounds fibroblast stimulating lipopeptide (FLS-1), Pam_3Cys-Ser-Lys_4 (P3CYS), and macrophage-activating lipopeptide 2 kD (MALP-2) from EMC microcollections GmbH (Tuebingen, Germany), and standard *S. aureus* lipoteichoic acid (LTA) and upLPS 0111:B4 from Invivogen (San Diego, CA, USA).

2.6 Statistics

Statistics were calculated with one-way ANOVA with Dunnett's multiple comparison post-test using GraphPad Prizm for Windows. $P < 0.05$ was considered significant.

3 Results

3.1 Effect of MD-2- and CD14-Inhibition on E. coli UpLPS-Induced Cytokine Response in Whole Blood

We first studied the effect of neutralizing MD-2 and CD14 on the cytokine response to phenol extracted highly purified LPS (upLPS). The anti-MD-2 mAb dose-dependently and efficiently inhibited a broad panel of the cytokines studied, including TNF-α, IL-6, IL-8 and IL-1ra (Fig. 1). Anti-MD-2 mAb at 25 μg/mL abolished the effect of upLPS and was equal to the effect of the anti-CD14 $F(ab')_2$ mAb, suggesting that the anti-MD-2 mAb and the anti-CD14 mAb had equal effects on upLPS-induced cytokine release. The release of IL-8 (Fig. 1, panel C) was, however, not completely inhibited by the anti-MD-2 mAb, in contrast to the effect observed with the anti-CD14 $F(ab')_2$ mAb. This finding is in accordance with the fact that anti-MD-2 mAb is whole IgG and activated complement in the system (see below), an event that is known to stimulate IL-8 production.

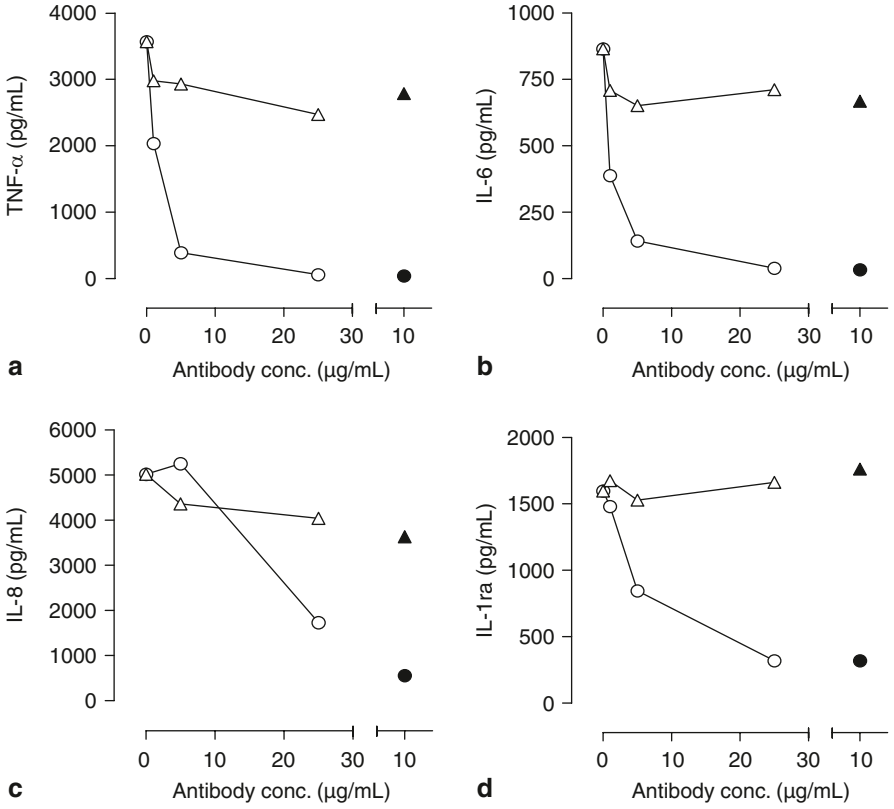

Fig. 1 Effect of MD-2- and CD14-inhibition on cytokine response to *E. coli* upLPS. To compare the effects of MD-2- and CD14-inhibition we titrated anti-MD-2 mAb and the isotype matched (IgG2b) control antibody five-fold from 25 μg/mL to 1 μg/mL, while anti-CD14 $F(ab')_2$ and control $F(ab')_2$ antibody were used at a fixed dose (10 μg/mL), known from previous studies to be inhibiting in this model. Whole blood was challenged with 100 ng/mL *E. coli* upLPS for 2 hours at 37°C. *Open circles*; anti-MD-2 mAb, *open triangles*; IgG2b-control mAb, *closed circles*; anti-CD14 $F(ab')_2$, *closed triangles*; $F(ab')_2$ control antibody. Panel A; TNF-α, panel B; IL-6, panel C; IL-8, panel D; IL-1ra. The figure displays one representative out of three experiments

3.2 Effect of MD-2-, CD14- and Complement-Inhibition on Whole E. coli Bacteria-Induced Cytokine Response in Whole Blood

We then studied the effect of neutralizing MD-2 and CD14 mAbs on whole *E. coli* bacteria-induced cytokine response, since whole bacteria are more clinically relevant than LPS and they are known to activate complement more efficiently compared to soluble LPS. Thus, we also included the complement inhibitor compstatin in these experiments.

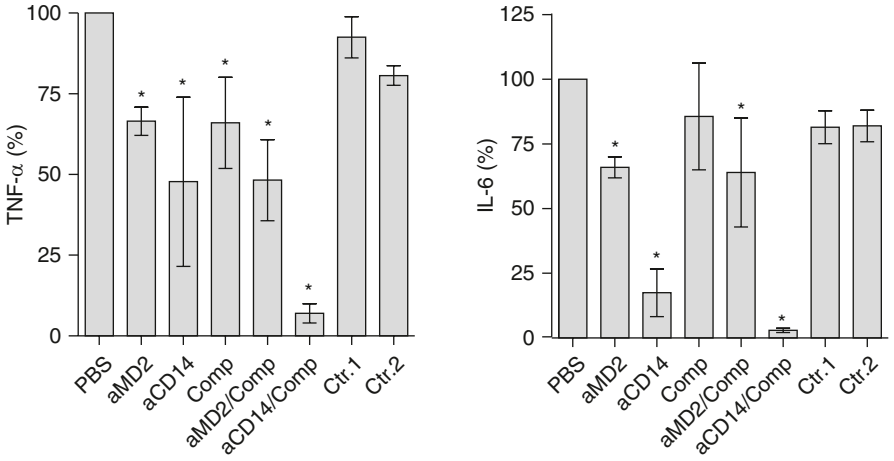

Fig. 2 Effect of MD-2-, CD14- and complement-inhibition on proinflammatory cytokine response to whole *E. coli* bacteria. Anti-MD-2 mAb (aMD-2) in the highest concentration used in the initial titration experiments (25 μg/mL) was compared to anti-CD14 $F(ab')_2$ (aCD14) (10 μg/mL), the complement C3 inhibitor compstatin (Comp) (25 μM), and combinations thereof. The first control (Ctr. 1) is the isotype matched control to anti-MD-2 mAb (IgG2b) in combination with the control peptide to compstatin. The second control (Ctr. 2) is the control antibody to anti-CD14 $F(ab')_2$ in combination with the control peptide to compstatin. Whole blood was challenged with 3×10^6 *E. coli*/mL for 2 hours at 37°C. Left panel; TNF-α, right panel; IL-6. Data (mean ± SD) are from experiments (n = 3 – 4) performed on separate days (n = 2 for Ctr. 1) and are given as % of the positive control (PBS), due to inter-individual variations. * = $p < 0.01$ compared to the positive control

The *E. coli*-induced increase in the proinflammatory cytokines TNF-α and IL-6 was partially and significantly ($p < 0.01$) inhibited by anti-MD-2 mAb and anti-CD14 $F(ab')_2$, anti-CD14 $F(ab')_2$ being the most efficient (Fig. 2). TNF-α, but not IL-6, was significantly ($p < 0.01$), though modestly, inhibited by compstatin alone. The combination of anti-CD14 $F(ab')_2$ and compstatin inhibited the TNF-α release by 93% and IL-6 by 97% ($p < 0.01$ for both). In comparison the combination of anti-MD-2 mAb and compstatin inhibited the formation of each cytokine by 52% and 36%, respectively ($p < 0.01$ for both). The less efficient effect of neutralizing MD-2 compared with CD14 in the combination with compstatin could not be explained by the complement-activation potential of the anti-MD-2 mAb since the presence of compstatin completely inhibited complement activation (see below).

3.3 Effect of MD-2-, CD14- and Complement-Inhibition on Whole E. coli-Induced Chemokine Response in Whole Blood

The chemokines IL-8 and MIP-1α were partially and significantly ($p < 0.01$) inhibited by anti-CD14 $F(ab')_2$ (Fig. 3). The effect of anti-MD-2 mAb was less pronounced

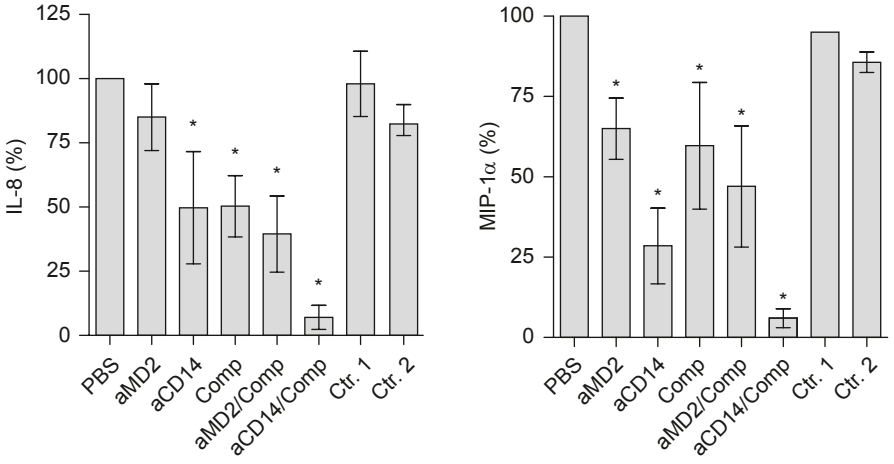

Fig. 3 Effect of MD-2-, CD14- and complement-inhibition on chemokine response to whole *E. coli* bacteria. Anti-MD-2 mAb (aMD-2), anti-CD14 F(ab′)$_2$ (aCD14), compstatin (Comp), combinations thereof, and controls, were used exactly as described in legend to Fig. 2. Whole blood was challenged with 3×10^6 *E. coli*/mL for 2 hours at 37°C. Left panel; IL-8, right panel; MIP-1α. Data (means ±SD) are presented as described in the legend to Fig. 2. *=$p<0.01$ compared to the positive control

and significant ($p<0.01$) only for MIP-1α formation. Compstatin alone significantly ($p<0.01$) inhibited both chemokines, most pronounced for IL-8, which was inhibited to the same degree by anti-CD14 F(ab′)$_2$ and compstatin (50% for each of them). The combination with compstatin markedly enhanced the effect of both mAbs. IL-8 was reduced 93% by compstatin and anti-CD14 F(ab′)$_2$ and 60% by compstatin and anti-MD-2 mAb, whereas the corresponding reduction in MIP-1α was 95% and 53%, respectively ($p<0.01$ for all).

3.4 Effect of MD-2-, CD14- and Complement-Inhibition on Whole E. coli-Induced IL-17 and IL-1ra Response in Whole Blood

The proinflammatory cytokine IL-17 and the anti-inflammatory mediator IL-1ra were then investigated (Fig. 4). IL-17 was significantly (71%, $p<0.01$) inhibited only by the combination of compstatin and anti-CD14 F(ab′)$_2$. IL-1ra was inhibited by both antibodies, but significantly ($p<0.01$) only for anti-CD14 F(ab′)$_2$. Compstatin alone did not inhibit IL-1ra, but enhanced the effect of anti-CD14 F(ab′)$_2$ when these were combined (72%, $p<0.01$).

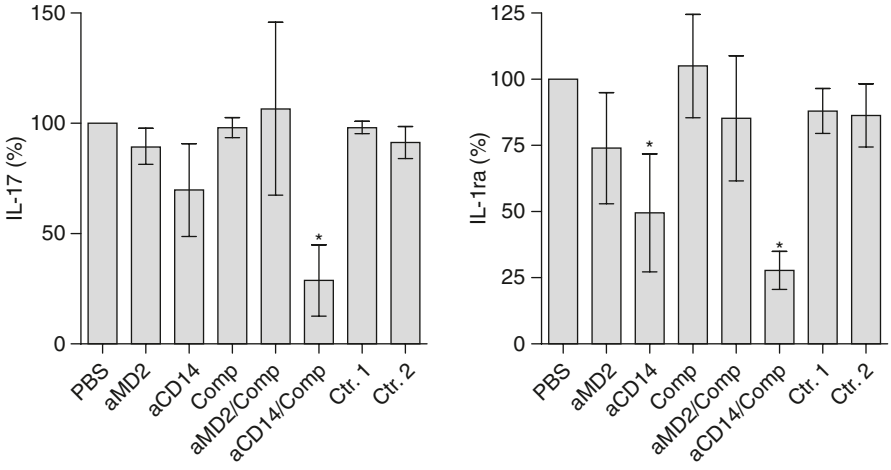

Fig. 4 Effect of MD-2-, CD14- and complement-inhibition on IL-17 and IL-1ra response to whole *E. coli* bacteria. Anti-MD-2 mAb (aMD-2), anti-CD14 F(ab′)$_2$ (aCD14), compstatin (Comp), combinations thereof, and controls, were used exactly as described in the legend to Fig. 2. Whole blood was challenged with 3×10^6 *E. coli*/mL for 2 hours at 37°C. *Left panel*; IL-17, *right panel*; IL-1ra. Data (means ± SD) are presented as described in Fig. 2. * = $p < 0.01$ compared to the positive control

3.5 *Effect of MD-2-, CD14- and Complement-Inhibition on E. coli UpLPS and Whole Bacteria Induced Complement Activation in Whole Blood*

Complement activation was measured as formation of TCC. In the experiments described above using upLPS (presented in Fig. 1.), there was a dose-dependent increase in TCC with the anti-MD-2 mAb which was not seen for the anti-CD14 F(ab′)$_2$ (Fig. 5, left panel). In the experiments described above using whole *E. coli* bacteria (presented in Figs. 2, 3, 4), compstatin abolished TCC formation, either alone or in combination with the two antibodies (Fig. 5, right panel), confirming that compstatin completely inhibited complement activation in these experiments. TCC formation was not influenced by anti-CD14 F(ab′)$_2$, but a modest increase in TCC was observed when anti-MD-2 mAb alone was used.

3.6 *Effect of Whole E. coli Bacteria and TLR Ligands on NFκB Activity*

HEK-cells transfected with TLR2 or TLR4/MD-2 in combination with or without CD14 were used to investigate the effect of whole *E. coli* bacteria and TLR4 and

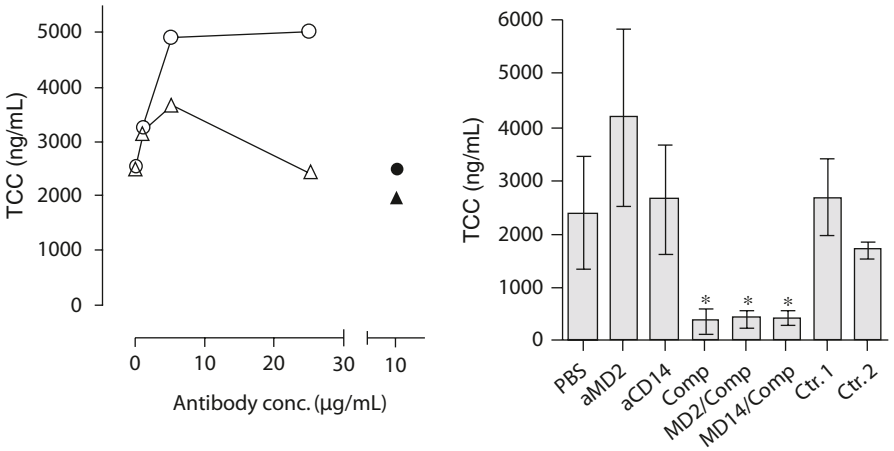

Fig. 5 Effect of MD-2-, CD14- and complement-inhibition on upLPS and *E. coli*-induced complement activation induced by upLPS and whole *E. coli* bacteria. Complement activation was measured as formation of the terminal complement complex (TCC). *Left panel:* TCC formation was examined in the experiment described in Fig. 1 (upLPS). Anti-MD-2 mAb (*open circles*) and the isotype matched (IgG2b) control mAb (*open triangles*) were titrated from 25 µg/mL to 1-µg/mL while anti-CD14 F(ab′)2 (*closed circle*) and control $F(ab')_2$ antibody (*closed triangle*) was used at a fixed dose (10 µg/mL). *Right panel:* TCC formation was examined in the experiment described in Fig. 2. Anti-MD-2 mAb (aMD-2), anti-CD14 $F(ab')_2$ (aCD14), compstatin (Comp), combinations thereof, and controls, were as described in the legend to Fig. 2. Data (means ±SD) are presented as ng/mL. Statistics was calculated between the positive control and the groups containing the complement inhibitor. * = $p < 0.01$ compared to the positive control

TLR2 ligands on NFκB activity (Fig. 6). The TLR4 effect of upLPS was largely dependent on CD14 (Fig. 6, upper left). Whole *E. coli* bacteria induced a significant response in the absence of CD14, compared with upLPS, but the signal was markedly enhanced in the presence of CD14. The TLR4 effect of FLS-1 and TLR2 effect of upLPS was nil, both in the presence and absence of CD14 (Fig. 6, upper left and right). CD14 did not influence the TLR2 response to whole *E. coli* bacteria (Fig. 6, upper right). In contrast, the TLR2/1 ligand P3CYS and the TLR2/6 ligands (FSL-1, MALP-2, and LTA) were enhanced by CD14 (Fig. 6, lower panels), although not as much as the TLR4-response toward *E. coli* and upLPS.

4 Discussion

This study is the first to show the different effect of specific inhibition of two key TLR-related molecules, CD14 and MD-2, on upLPS- versus whole *E. coli* bacteria-induced inflammatory response. The experiments were performed in an established ex vivo human model designed for inflammatory crosstalk (Mollnes et al. 2002) and are thus regarded to be relevant for human sepsis. The data emphasize that

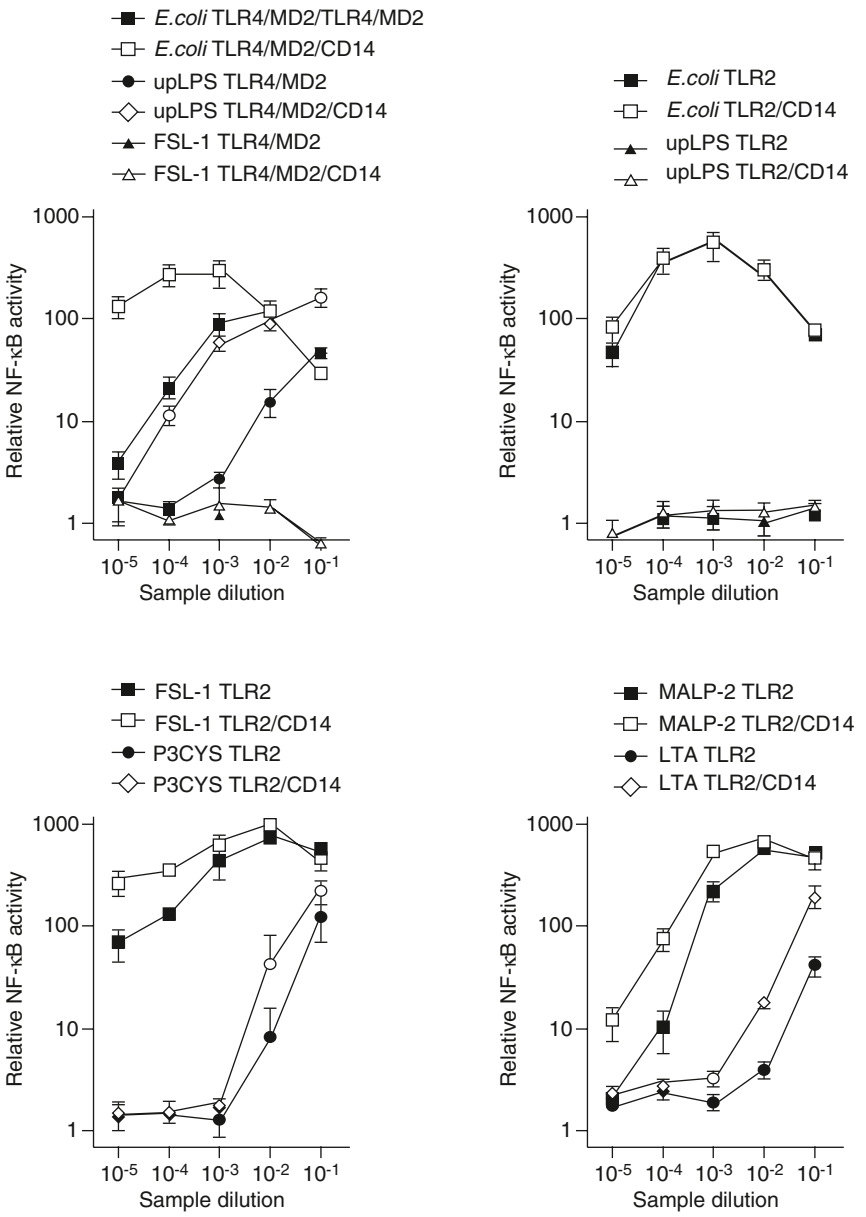

Fig. 6 Effect of CD14 on TLR4 and TLR2 mediated NF-κB activation by whole *E. coli* bacteria, upLPS and TLR2 ligands (MALP2, FSL-1, P3CYS). Relative induction (means ± SD) of NF-κB in HEK293 cells transiently transfected with NF-κB luciferase and TLR4/MD-2 alone or in combination with CD14 (*upper left panel*). NFκB induction through TLR2 with or without CD14 (*upper right and bottom panels*). The highest sample concentration during treatment (10^{-1} dilution) was 1×10^8 bacteria/mL, 5 μg/mL LTA and 1 μg/mL of the other ligands

the multifunctional CD14 molecule, acting very upstream by being co-receptors for several TLRs, is a more promising target to attenuate whole Gram-negative bacteria-induced inflammation, than the more specific LPS-binding molecule MD-2 (Antal-Szalmas 2000).

LPS from Gram-negative bacteria is regarded as the main inducer of the host inflammatory response to these bacteria. It should be noted, however, that although LPS is definitely of importance, the bacteria contain a number of other molecules recognized by the human innate immune system. Notably, CD14 is a promiscuous recognition molecule reacting with numerous ligands of both Gram-negative and Gram-positive bacteria, as well as fungi (Pugin et al. 1994) and dozens of specific ligands have been identified (Antal-Szalmas 2000). This is in contrast to MD-2, which seems to be specific for LPS in the interaction with TLR4. Our data are in accordance with this concept. Inhibition of MD-2 efficiently abolished upLPS-induced cytokine release, whereas inhibition of CD14 proved much more efficient in inhibiting the whole bacteria response. Our findings support the importance of using whole bacteria instead of LPS in experimental models of Gram-negative sepsis, as detailed in a recent experimental study in pigs (Thorgersen et al. 2009). The assumption of using whole bacteria instead of LPS in experimental models is further supported by the fact that neutralizing LPS has not proven effective in treatment of sepsis (Nahra and Dellinger 2008).

CD14 is known to play a role not only in TLR4, but also TLR2 and TLR3 signaling (Miyake 2006; Akashi-Takamura and Miyake 2008; Nilsen et al. 2008; Janot et al. 2008). We therefore investigated whether the MD-2-independent CD14 effect could be explained by TLR2/CD14. Our data using transfected HEK cells do not support this assumption, as *E. coli* activated TLR2 in a CD14 independent fashion. Still, CD14 increased signaling of TLR2 in response to low levels of TLR2/1 and TLR2/6 ligands, as has also been shown by others (Nakata et al. 2006; Erridge et al. 2008). The reason for this difference between whole *E. coli* and soluble TLR2 ligands is not known. It is likely that the TLR2 ligands in *E. coli* are immobilized and activate TLR2 by aggregating the receptor and that this activation process is unaffected by CD14. In any case, our data, consistently observed with all inflammatory readouts, collectively point to CD14 as a key target molecule for inhibition of whole *E. coli* bacteria-induced inflammation.

Complement activation can take place in blood when antibodies bind to their epitope. The anti-MD-2 antibody is IgG2b with an intact Fc-region. In contrast, the anti-CD14 antibody used was a well established $F(ab')_2$. As documented in the present study, anti-MD-2 activated the complement system, revealed by increase in TCC. Thus, the results obtained with anti-MD-2 alone should be interpreted with caution. In our experiments, however, we always included the complement inhibitor compstatin in parallel, both in order to neutralize the complement activation potential of the antibody, and to reveal the effect of complement activation by the bacteria. The results unequivocally documented the effect of the anti-MD-2 antibody *per se,* that is, in the presence of a complement inhibitor. Unfortunately we did not have access to large scale anti-MD-2 mAb to produce $F(ab')_2$ fragments. By using the complement inhibitor, however, we circumvented this problem. Our data highlight

the importance of using inhibitory agents that do not activate complement, unless a combined treatment with a complement inhibitor can be used.

The present study included a number of inflammatory read-outs including the biomarkers in the 27-plex cytokine assay. Principally, all these mediators responded similarly to the combined inhibition of CD14 and complement. Of particular interest is IL-17, which recently has been reported to play a critical role in protection against polymicrobial sepsis (Freitas et al. 2009). In contrast, Flierl et al. (2008) reported increased survival when blocking IL-17A, reported IL-17A to be responsible for the production of TNF-α, IL-1β and IL-6 in a cecal ligation and puncture model and, thus, postulated IL-17 to be a novel target for treatment of sepsis. Recently it was shown that C5a was responsible for the increase in IL-17 in this model (Xu et al. 2010). In our study we showed that combination of anti-CD14 and compstatin effectively reduces the formation of IL-17, consistent with a previous observation in our laboratory (Brekke et al. 2008). In the present study we show that inhibition of MD-2 had no effect on *E. coli*-induced IL-17. We therefore suggest that the combined upstream treatment regimen targeted at CD14 and complement attenuates a number of mediators potentially contributing to *E. coli*-induced systemic inflammatory response, including IL-17 release. Although in our model we found inhibition of CD14 to be generally more efficient than inhibition of MD-2 with respect to the cytokine response, others have documented an MD-2-dependent, CD14-independent, LPS-mediated influx of neutrophils into the lung alveoli during experimental pneumonia in mice (Cai et al. 2008). Thus, it cannot be excluded that MD-2 may be a target for inhibition also in humans under conditions where signaling is CD14-independent and specific targeting of MD-2 is desired.

The effect of blocking CD14 and complement in the case of Gram-positive bacteria or other Gram-negative bacteria including rough strain, still needs to be investigated. Due to the diversity of potential invaders, a therapeutic for sepsis may well consist of a cocktail of inhibitors targeting essential upstream recognition molecules. In our opinion, targeting downstream single mediators of inflammation is not a rational strategy for treatment of sepsis. Accordingly, it was recently shown in a phase II clinical trial that treatment with eritoran, an LPS antagonist interacting with the CD14/MD-2/TLR4 might improve the clinical condition in sepsis (Tidswell et al. 2010), in contrast to the disappointing results observed in a number of clinical studies targeting single cytokines like TNF-α, IL-1β and IL-6 (Remick 2007). We recently documented a profound effect on *E. coli*-induced inflammation in a pig model of sepsis by blocking CD14 (Thorgersen et al. 2009). Furthermore, we have recently shown in a baboon model of sepsis that complement-inhibition substantially improved the disturbed pathophysiolgy, including the fall in systemic blood pressure (Silasi-Mansat et al. 2010). It remains to be shown whether combination of CD14- and complement-inhibition will improve the outcome in experimental and clinical sepsis studies.

In conclusion, inhibition of CD14 and MD-2 was equally effective in attenuating upLPS-induced cytokine response, whereas inhibition of CD14 was substantially more effective in attenuating the inflammatory response to whole *E. coli* bacteria. This effect was further markedly enhanced by combining CD14- and complement-

inhibition, which virtually abolished the whole cytokine response. The marked effect of inhibiting CD14 is consistent with its broad pattern recognition of a number of danger ligands, and its position as co-receptor for numerous TRLs as well.

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Zebrafish: Model for the Study of Inflammation and the Innate Immune Response to Infectious Diseases

Beatriz Novoa and Antonio Figueras

Abstract The zebrafish (*Danio rerio*) has been extensively used in biomedical research as a model to study vertebrate development and hematopoiesis and recently, it has been adopted into varied fields including immunology. After fertilization, larvae survive with only the innate immune responses because adaptive immune system is morphologically and functionally mature only after 4–6 weeks postfertilization. This temporal separation provides a suitable system to study the vertebrate innate immune response *in vivo*, independently from the adaptive immune response. The transparency of early life stages allows a useful real-time visualization. Adult zebrafish which have complete (innate and adaptative) immune systems offer also advantages over other vertebrate infection models: small size, relatively rapid life cycle, ease of breeding, and a growing list of molecular tools for the study of infectious diseases. In this review, we have tried to give some examples of the potential of zebrafish as a valuable model in innate immunity and inflammation studies.

Keywords Zebrafish (*Danio rerio*) • Inflammation • Innate immunity • Infectious disease • Ontogeny

1 Introduction

The zebrafish (*Danio rerio*) has been extensively used to study vertebrate development and hematopoiesis but interest in this model organism has gradually expanded in recent years into the fields of human disease, cancer, and immunology (Dooley and Zon 2000; Trede et al. 2001, 2004; Yoder et al. 2002; Traver et al. 2003; Stern and Zon 2003; de Jong and Zon 2005; Langenau and Zon 2005; Sullivan and Kim 2008). Concerning immunology and infectious diseases research, interestingly, there is a clear temporal separation between both innate and adaptive immune responses in zebrafish. Only the innate immune system is present until several weeks after fertiliza-

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tion; larvae must survive until that time solely on the strength of their innate immune system. The innate immune system is detectable and active at day 1 of zebrafish embryogenesis (Herbomel et al. 1999, 2001), whereas the adaptive immune system is morphologically and functionally mature only 4–6 weeks after the fertilization of the egg (weeks postfertilization, wpf) when the lymphocytes become functional (Willett et al. 1999; Davidson and Zon 2004; Trede et al. 2004; Lieschke and Currie 2007). This temporal separation provides a suitable system to study the vertebrate innate immune response *in vivo*, independently from the adaptive immune response (Stockhammer et al. 2009). The transparency of early life stages is another advantage that allows useful real-time visualization. Moreover, adult zebrafish which have complete (innate and adaptative) immune systems, may have certain advantages over other vertebrate infection models such as mice: their small size, relatively rapid life cycle, and ease of breeding permit a large number of genetic screens to be performed.

Like those of amphibians, fish immune systems present almost the full repertoire of lymphoid organs and immune cell types found in mammals (Trede et al. 2004; Zapata et al. 2006). However, unlike that of mammals, fish development occurs in an open environment. Therefore, the immune system may be exposed early to a large number of pathogens. Zebrafish larvae hatch 2–3 days after fertilization, suggesting that their immune system must develop quickly to produce a heterogeneous immune repertoire (Du Pasquier 2000; Poorten and Kuhn 2009).

Yoder et al. (2002) have pointed out that the zebrafish can be employed as a new immunological model system. However, these authors also enunciate a key question "what will studies using this species offer that cannot be realized using other models?" Trede et al. (2004) have discussed the advent of the zebrafish as a powerful vertebrate model organism that may have an impact on immunological research based on the important role that innate immunity plays in orchestrating immune responses. The review by Traver et al. (2003) provides an overview of the value and potential of zebrafish as a model organism to study the development and function of the immune system. These authors propose to "use the zebrafish as a model organism for immunology as an alternative to study humans or mice". Fish are phylogenetically lower vertebrates and rely more than mammals on innate immune mechanisms. The use of the whole animal in studies that utilize zebrafish can complement research on components of immunity that is based on *in vitro* experiments utilizing isolated or cultured cells (which are very useful for understanding specific pathways but may not reflect the cellular interactions that occur in the whole animal).

In this chapter we have tried to summarize advantages that zebrafish can offer for immunological research.

2 Main Cells Involved in the Innate Immune Response in Zebrafish

Zebrafish leukocytes, even in embryos, function in host defense. By direct microscopy, it is possible to observe that neutrophils rapidly accumulate at wounds (Lieschke et al. 2001; Renshaw et al. 2006) and bacterial foci (Le Guyader et al. 2008)

and that primitive macrophages also phagocytose particles and bacteria (Herbomel et al. 1999; Lieschke et al. 2001; Hall et al. 2007). Although recent studies have reported the presence of eosinophils and mast cells in zebrafish (Bertrand et al. 2007; Dobson et al. 2008; Balla et al. 2010), larvae innate immune system comprises primarily of neutrophils and macrophages. Neutrophils are the first to respond to an injury; macrophages are subsequently recruited to inflamed tissues to phagocytose pathogens and tissue debris.

Neutrophils: Zebrafish possess cells analogous to neutrophils in adults and larvae. Neutrophils rapidly accumulate at wounds (Renshaw et al. 2006)and this chemotactic activity is critical in responding to tissue injury and infections (Fig. 1).Today, a range of tools for labeling neutrophils has been developed in zebrafish using transgenic lines such as the zMPO:GFP which expresses GFP under the control of the myeloperoxidase promoter (Renshaw et al. 2006; Mathias et al. 2006) and the transgenic line CLGY463 which has an enhancer detection insertion near a novel *myc* transcription factor (Meijer et al. 2008). Moreover, Anne Huttenlocher and colleagues have identified mutants with increased neutrophil numbers. The first such mutant, in the *Hai1* gene, results in damage to the epithelium and is associated with increased neutrophil retention at the site of an epithelial injury (Mathias et al. 2007). The second, in the *Fad24* gene, leads to muscle damage and is also associated with increased tissue neutrophilia (Walters et al. 2009).

These tools permit real-time visualization of the response of neutrophils to inflammation and infections making it possible to visualize the neutrophil migration in three-dimensional (3D) tissue environments *in vivo* (Yoo et al. 2010). Until now, these studies were difficult to accomplish because few systems were available to

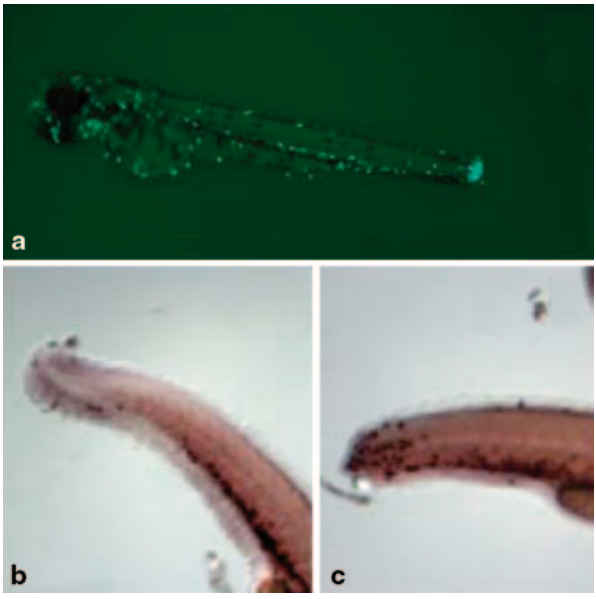

Fig. 1 Migration of zebrafish neutrophils to the injury site at the tail. **a** shows transgenic fish (Renshaw et al. 2006) expressing GFP under the control of the myeloperoxidase promoter with a cut in the tail. Panel **b** (normal fish) and **c** (fish with a cut in the tail) correspond to a whole mount *in situ* hybridization of zebrafish embryos using a myeloperoxidase probe which also labels neutrophils

permit high-resolution imaging of the signaling dynamics in living cells within multicellular organisms.

Zebrafish are also being used to understand the mechanisms that regulate the resolution phase of the inflammatory response. One of these mechanisms is the regulation of apoptosis (Haslett 1999) which is being studied in zebrafish by using pan-caspase inhibitors and by blocking or overexpressing candidate regulators of apoptosis (Renshaw et al. 2007). Moreover, *in vivo* time-lapse imaging has been used to demonstrate that neutrophils subsequently display directed retrograde chemotaxis back toward the vasculature. These findings implicate retrograde chemotaxis as a novel alternative mechanism that regulates the resolution phase of the inflammatory response (Mathias et al. 2006).

Macrophages: Although several genes have been suggested as markers for the monocyte/macrophage lineage in zebrafish, including l-plastin and lysozyme C (Herbomel et al. 1999; Liu and Wen 2002), subsequent studies have indicated that these genes can also be expressed in other leukocytes (Su et al. 2007; Meijer et al. 2008). Only *CSF1R* or *c-fms* has become an accepted marker for zebrafish macrophages (Herbomel et al. 2001). Phagocytically active macrophages are the first leukocytes to appear in the zebrafish embryo (Herbomel et al. 1999; Lieschke et al. 2002) and exhibit avid motility andphagocytosis of cellular debris and bacteria (Herbomel et al. 1999; Redd et al. 2006).Several different subsets of the macrophage/monocyte lineage have been described, including those recently described as "inflammatory macrophages" which are involved in the inflammatory responseto wounding in zebrafish larvae (Mathias et al. 2009). Recently, a macrophage-specific marker has been identified (*mpeg1*) and its promoter has been used in *mpeg1*-driven transgenes. Using these lines, researchers have followed the different behavior of neutrophils and macrophages after wounding (Ellett et al. 2010).

3 Immune Genes Characterized in Zebrafish

An important requirement to use the zebrafish as a model to study human immunity (Trede et al. 2004) is the knowledge of the genes that encode components of the mammalian immune system that are also found in fish (Purcell et al. 2006). This knowledge would also aid our understanding of the evolution of immunity. We must also consider that a whole genome duplication occurred early in the teleost lineage. It has been proposed that the availability of additional gene copies facilitated the evolution of the highly diverse morphology and behavior of teleost fish (Venkatesh 2003; Volff 2005).

Many protein and gene families involved in innate immune mechanisms have been described in zebrafish, suggesting that many components of the innate immune signaling pathways known from mammals are conserved in teleost fish. Stein et al. (2007) have searched the fish genomes for genes encoding components of the immune system. Although most of the components known in mammals have

clearly recognizable orthologous in fish, class II cytokines and their receptors have diverged extensively, obscuring their orthologies. In the opinion of Stein and colleages, the main innate immune signaling pathways (kinases, adaptors in the TLR signaling pathway, interferon response factors, signal transducers and activators of transcription) are conserved in teleost fish. Whereas the components that act downstream of the receptors are highly conserved, components that are known or assumed to interact with pathogens are more divergent. These observations agree with those of Carradice and Lieschke (2008) who have reported that zebrafish intracellular cytokine signaling pathways are more conserved overall than their ligands and receptors.

Aggad et al. (2010) have studied the conditions under which Ifn-γ is induced in fish larvae and adults and have also identified also the receptors for class II helical cytokines (IFNs and Il-10 and its related cytokines). Infection studies using two different pathogens have shown that IFN-gamma signaling is required for resistance to bacterial infections in the young embryo (Sieger et al. 2009).

Concerning to the complement system, C3, C4 and factors B and H have been identified to date in zebrafish (Sun et al. 2010). It has been shown that complement components such as C3 and Bf can be transferred from mother to offspring and play a protective role in developing embryos. Their expression increases in zebrafish embryos and larvae in response to lipopolysaccharide (LPS) (Wang et al. 2008a, b, 2009). Multiple copies of mannose binding lectin (MBL) which is involved in the activation of the lectin pathway of the complement system, have been detected in zebrafish. Polymorphisms within MBL may be critical in determining fish susceptibility or resistance to various pathogenic organisms, as has been reported in humans (Jackson et al. 2007).

Other genes related to the immune response have been described in zebrafish. Yoder et al. (2001) have described a highly diverse, multigene family of novel immune-type receptor (NITR) genes in zebrafish. These genes are predicted to encode type I transmembrane glycoproteins which consist of extracellular variable (V) and V-like C2 (V/C2) domains, a transmembrane region and a cytoplasmic tail. All of the genes examined encode immunoreceptor tyrosine-based inhibition motifs in the cytoplasmic tail. NITRs have been proposed to be “functional orthologs” of mammalian natural killer receptors (NKRs) (Yoder 2009).

Antimicrobial peptides (Zou et al. 2007) and peptidoglycan recognition proteins (PGRPs) with peptidoglycan-lytic amidase activity and a broad spectrum of bactericidal activity (Li et al. 2007; Chang et al. 2007) have also been identified in zebrafish.

Zebrafish have been investigated for the presence of Toll-like receptor (TLR) proteins which function as sentinels against infection, participating in the earliest innate immune responses. Purcell et al. (2006) have characterized the key components of the TLR-signaling pathway, including MYD88, TIRAP, TRIF, TRAF6, IRF3 and IRF7 in zebrafish. It has also been reported that the main receptor for LPS, the TLR4, is expressed in zebrafish during early stages of infection (Meijer et al. 2004; Jault et al. 2004). However, zebrafish appear to respond to LPS through a mechanism that is independent of the mammalian TLR4-MD2 LPS receptor com-

plexes. Zebrafish TLR4 fails to respond to LPS due to differences in its extracellular domains (Sepulcre et al. 2009; Sullivan et al. 2009). The zebrafish genes *tlr4a* and *tlr4b* appear to be paralogous rather than orthologous to human *TLR4* but they probably play a role in zebrafish immunity, supporting the hypothesis that alternative LPS induction pathways predominate in fishes (Sullivan et al. 2009).

4 Functional Ontogeny of the Immune System

Excellent studies have been performed on the ontogeny of the lymphoid system during the embryonic period of the zebrafish (Willett et al. 1997, 1999; Trede and Zon 1998; Trede et al. 2001), but little is known about the maturation of its immune system with regard to form and function, which occurs later in development. Lam et al. (2004) have observed a humoral response to T-independent antigen (formalin-killed *Aeromonas hydrophila*) and T-dependent antigen (human gamma globulin) in immunized zebrafish at 4 and 6 wpf, respectively, indicating that immunocompetence had been achieved. The findings confirm previous studies that have reported that the zebrafish adaptive immune system is morphologically and functionally mature by 4–6 wpf. The function of the embryonic zebrafish immune system before maturation has not been addressed in detail. Dios et al. (2010) have investigated the expression levels of several antiviral and inflammatory genes (IL-1β, iNOS, TNF-α, TLR3, IFN-I, IFNγ, IRF3, MDA-5, Mx) both constitutively and after viral stimulation during early development. Most of the genes involved in the antiviral response reached a positive reaction threshold as early as 5 days postfertilization (dpf). This finding is not surprising because oviparity requires a rapid development of the immune system. The same authors have determined how the expression of these genes is affected by changes in the temperature. Whereas the expression of most of the antiviral genes was almost completely inhibited at 15°C, inflammatory genes such as IL-1β, iNOS and TNF-α showed not obvious differences between 15 and 28°C. After treatment with poly I:C (which mimics a viral infection), larvae showed significant differences in the gene expression, especially for of the interferon-induced protein Mx. In adults, however, poly I:C treatment led to a smaller increase in gene expression compared to larval Mx levels. Thus, Mx apparently plays an important role in viral immunity in larvae, in which the adaptive immune response is not fully functional.

5 Zebrafish as a Model for Infectious Diseases

Sullivan and Kim (2008) have published a comprehensive review of the capabilities and potential of the zebrafish model system with an overview of information on zebrafish infectious disease models. The advantages of the zebrafish system are particularly relevant during the embryonic and larval stages (Fig. 2) and are very useful in the study of host–microbe interactions (Kanther and Rawls 2010).

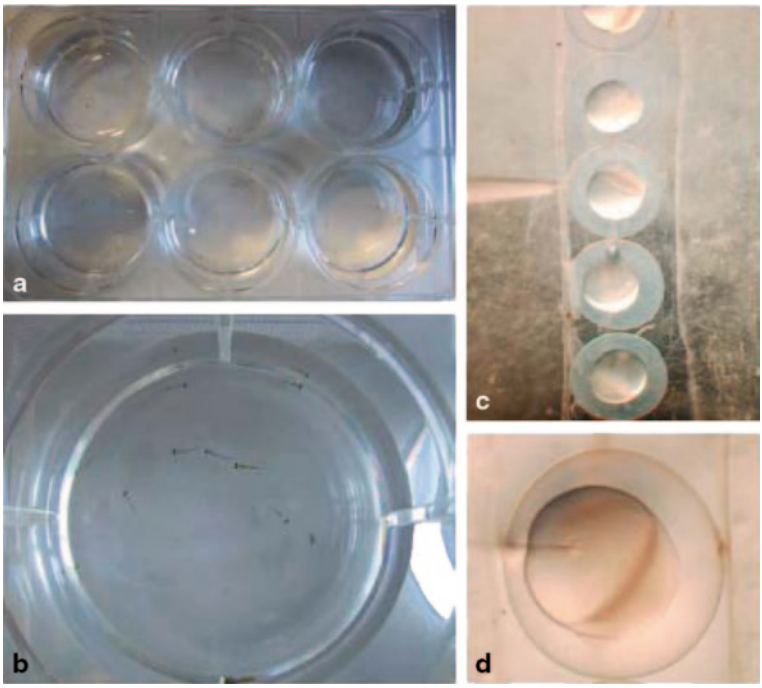

Fig. 2 Zebrafish embryos and larvae are useful to study innate immune functions and interaction with pathogens, numbers of animals can be high in a reduced space. **a** A multiwell plate where it is possible to conduct experimental infections with zebrafish larvae. **b** shows one of the wells at higher magnification in which the larvae can be seen. **c** and **d** describe the microinjection of zebrafish embryos

Viral diseases. The zebrafish has been proposed and used as a laboratory model fish species to study fish viral diseases. To date, most studies of viral infections in zebrafish have been related to viruses affecting aquacultured fish (Fig. 3). Vaccine and treatment trials, which are sometimes highly expensive to perform with commercial species, can be conducted at a reduced cost using this model.

LaPatra et al. (2000) have infected hematopoietic precursors from the zebrafish, with the rhabdovirus infectious hematopoietic necrosis virus (IHNV) and the birnavirus infectious pancreatic necrosis virus (IPNV). Infection of whole fish with viral supernatants demonstrated infectious replicants for both viruses, indicating that the virus host range includes the zebrafish. In other species, infection with these viruses leads to prominent hematopoietic necrosis of the head kidney, the major site of adult hematopoiesis. The kinetics of hematopoietic defects differed between IHNV and IPNV infection; however, fish infected with either virus recovered by 6 days postinfection. Other experimental infections have been conducted with other rhabdoviruses, for example, Sanders et al. (2003) have shown that zebrafish are susceptible to another rhabdovirus adapted to higher temperatures, spring viremia of carp virus (SVCV). Mortality exceeded 50% in fish exposed to the virus, which

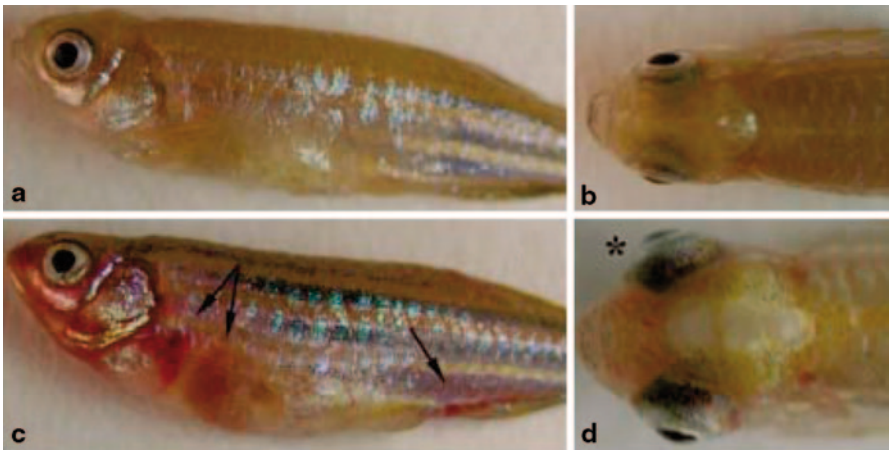

Fig. 3 Aspect of the external clinical signs of adult zebrafish infected with viral hemorrhagic septicemia virus (VHSV), a serious rhabdovirus caused disease affecting aquacultured fish. **a** and **b** show uninfected fish and **c** and **d** correspond to infected fish with the characteristic symptoms of the disease: hemorrhages (*arrows*), exophthalmia (*) and a distended visceral cavity

exhibited epidermal petechial hemorrhages followed by death. Histological lesions included multifocal brachial necrosis and melanomacrophage proliferation in the gills, liver and kidneys. López-Muñoz et al. (2010) have also found that zebrafish larvae are unable to mount a protective antiviral response to waterborne SVCV. Nevertheless, zebrafish larvae appear to possess a functional antiviral system since ectopic expression of the cDNA of both groups I and II IFN was able to protect them against SVCV via the induction of IFN-stimulated genes (ISGs).

Novoa et al. (2006) have proposed to use zebrafish as a model to study vaccination against viral hemorrhagic septicemia virus (VHSV) (Fig. 3). Even at low temperatures, fish were protected by a vaccine generated by reverse genetics against the virulent virus.

Lu et al. (2008) have successfully infected zebrafish with a nodavirus, nervous necrosis virus (NNV) that induces high mortalities in the larval and juvenile stages of infected marine fish. The disease caused by this virus is characterized by lethargy, abnormal spiral swimming, loss of equilibrium and neurological lesions characterized by cellular vacuolization and neuronal degeneration mainly in the brain, retina, spinal cord, and ganglia of the affected fish. In zebrafish, infected animals exhibited typical NNV symptoms, showing brain lesions similar to those observed in natural hosts.

Fungal diseases. Chao et al. (2010) have developed a zebrafish model for *Candida albicans* infections. They have shown that *C. albicans* can colonize and invade zebrafish at multiple anatomical sites and can kill the fish in a dose-dependent manner. They monitored the progression of the *C. albicans* yeast-to-hypha transition, the gene expression of the pathogen and the early host immune response. Experimental infections with different *C. albicans* strains were conducted to determine

each strain's virulence, and the results were similar to findings reported in previous mouse model studies. Using zebrafish embryos, the interaction between pathogen and host myelomonocytic cells can be visualized *in vivo*. Chao et al. (2010) conclude that zebrafish are a useful model host to study *C. albicans* pathogenesis and other invasive fungal research.

Bacterial diseases. A number of studies on bacterial diseases have been conducted using zebrafish. For instance, *Streptococcus iniae,* which causes a systemic invasive infection in fish resembles human infections by several streptococcal species (Neely et al. 2002; van der Sar et al. 2004; Phelps et al. 2009). Kizy and Neely (2009) have determined the role of several *Streptococcus* pyogenes virulence genes using zebrafish as a host.

Zebrafish infection with *Mycobacterium marinum* has been proposed as a model for tuberculosis (Davis et al. 2002). Swaim et al. (2006) have shown that zebrafish are naturally susceptible to *M. marinum*, a close genetic relative of the causative agent of human tuberculosis, *Mycobacterium tuberculosis*. They have also developed a zebrafish embryo-*M. marinum* infection model to study host–pathogen interactions in the context of innate immunity. Zebrafish tuberculous granulomas undergo caseous necrosis, similar to human tuberculous granulomas. In contrast to mammalian tuberculous granulomas, zebrafish lesions contain few lymphocytes, calling into question the role of adaptive immunity in fish tuberculosis. However, like rag1 mutant mice infected with *M. tuberculosis*, they found that rag1 mutant zebrafish are hypersusceptible to *M. marinum* infection, demonstrating that the control of fish tuberculosis is dependent on adaptive immunity.

Lin et al. (2007) have studied the zebrafish immune response to infections with *Aeromonas salmonicida* and *Staphylococcus aureus*, a Gram-negative and a Gram-positive bacteria, respectively. Many of the identified genes induced upon infection (IL-1, fibrinogen, haptoglobin, complement components and hepcidin) are related to the acute phase proteins (APPs), with induction patterns similar to those observed in mammals. This observation implies evolutionarily conserved mechanisms among fish and mammals. Lin et al. (2007) also discovered some novel APPs, suggesting different immune strategies adopted by fish species. Notably, LECT2 was induced by up to 1000-fold upon infection, shedding new light on the function of this gene.

Rodríguez et al. (2008) have reproduced *A. hydrophila* disease symptoms similar to those present in humans and mortality in fish after experimental infection by intraperitoneal injection or by immersing wounded fish (Fig. 4). Fish showed clinical symptoms such as hemorrhaging and abdominal swelling. However, histological lesions were not observed perhaps because the peracute form of the disease killed the fish before any changes could become evident.

Vojtech et al. (2009) have established a zebrafish/*Francisella* (a highly virulent and infectious pathogen) model of pathogenesis and host immune response. Adult zebrafish are susceptible to acute *Francisella*-induced disease and suffer mortality in a dose-dependent manner. Zebrafish mount a significant tissue-specific proinflammatory response to infection, as measured by the upregulation of IL-1, interferon gamma and TNF mRNA beginning by 6 h postinfection and persisting for up to 7 days postinfection.

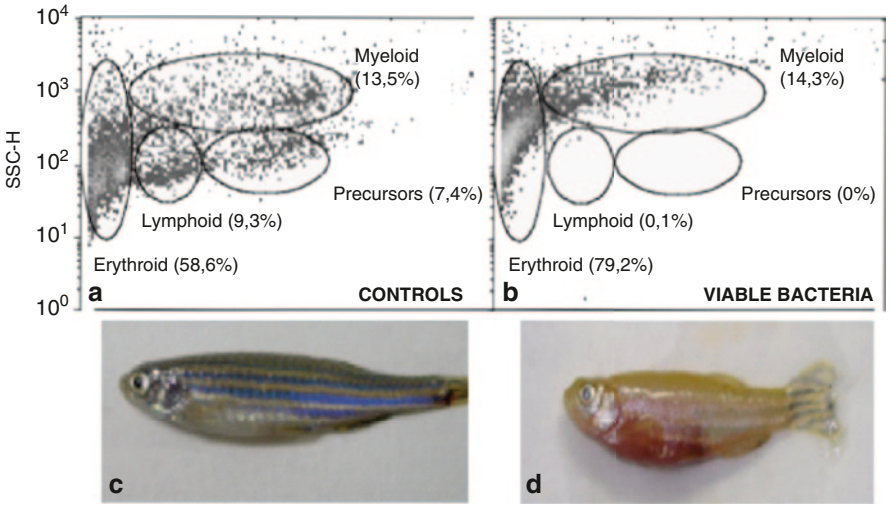

Fig. 4 Adult zebrafish were susceptible to the *Aeromonas hydrophila* infection. Flow cytometry of zebrafish kidney cell populations analyzed by size (forward scatter; FSC) and granularity (side scatter; SSC) shows important changes after infection related with the hemolytic activity of *Aeromonas*: The kidney cells treated with viable bacteria showed a drop in the populations of **a** lymphoid cells and precursor immature cells compared with **b** uninfected cells. **c** and **d** show the aspect of control fish or infected fish with symptoms characterized by a distended visceral cavity and abdominal hemorrhages

Infections with *Salmonella typhimurium* and *Vibrio anguillarum* have also been conducted in zebrafish (van der Sar et al. 2003; O'Toole et al. 2004). To characterize the embryonic innate host response at the transcriptome level against *Salmonella*, which causes a lethal inflammatory infection in zebrafish embryos, Ordas et al. (2010) have extended and validated previous microarray data through Illumina, next-generation sequencing analysis. Their report describes infection-responsive genes in zebrafish embryos, which include genes encoding transcription factors, signal transduction proteins, cytokines and chemokines, complement factors, proteins involved in apoptosis and proteolysis, proteins with antimicrobial activities and many known or novel proteins not previously linked to the immune response.

6 Application of Genomics, Transgenesis and Other Tools in the Study of Infectious Diseases

Powerful genetic approaches can be conducted in zebrafish to ascertain the roles that particular genes play in disease resistance.

6.1 Mutagenesis

One of the main advantages of the zebrafish is the ability to easily perform forward genetic screens (Streisinger et al. 1981; Solnica-Krezel et al. 1994; Knapik 2000). Target induced local lesions in genomes (TILLING) methodology is being employed routinely to generate "knock-out" zebrafish (Deiters and Yoder 2006). Together with several mutants described above, one of the most interesting examples of the application of these techniques to immunological research is the disruption of the *rag1* gene by an ENU-induced point mutation that creates a premature stop codon in the *rag1*^{t26683} allele thus encoding a truncated Rag1 protein (Wienholds et al. 2002). Although homozygous fish (*rag1*$^{-/-}$) are more susceptible to an injected dose of *M. marinum* and their immunoglobulin genes fail to undergo V(D)J recombination, they are able to reach adulthood and are fertile. Jima et al. (2009) have hypothesized that *rag1*$^{-/-}$ zebrafish may possess an enhanced innate immune response to compensate for the lack of an adaptive immune system. Using microarrays, these authors have compared the expression profiles of rag1 deficient zebrafish and controls. The majority of the differences between wild type and mutant zebrafish were found in the intestine, where *rag1*$^{-/-}$ fish exhibited an increased expression of innate immune genes, including those of the coagulation and complement pathways. Petrie-Hanson et al. (2009) have shown that in comparison to wild-type zebrafish, *rag*1 mutants have a significantly reduced lymphocyte-like cell population (lacking functional T- and B-lymphocytes) but have a similar macrophage/monocyte population and a significantly increased neutrophil population. These zebrafish have leukocyte populations comparable to those of severe combined immunodeficient (SCID) and *rag* 1 and/or 2 mutant mice.

Although the development of zebrafish model systems for many medical problems is in its early stages, large-scale genetic screening programs have been successfully applied to blood research and other developmental problems (Patton and Zon 2001). Today, these methods are being used for several diseases, including epilepsy (Hortopan et al. 2010).

6.2 Microarrays and Next-Generation Sequencing Methods

Van der Sar et al. (2009) have conducted microarray studies to analyze the transcriptome responses of zebrafish to two *M. marinum* strains that produce distinct disease outcomes (acute disease with early lethality or chronic disease with granuloma formation). The transcriptome profiles involved in acute versus chronic infections and in embryonic versus adult infected fish partially overlapped, even though the strains induce profoundly different disease phenotypes. The strongest differences were observed at the initial stage of the disease. Stockhammer et al. (2009) have used microarrays to perform a time-course transcriptome profiling study and gene ontology analysis of the embryonic innate immune response to infection by

two *Salmonella* strains that elicit either a lethal infection or an attenuated response. These authors have confirmed a conservation of the host responses similar to that detected in other vertebrate models.

Wu et al. (2010) have used a commercial zebrafish microarray to identify alterations in gene expression in zebrafish injected with *Streptococcus suis*, an important pathogen in swine. At least 189 genes showed differential expression.

The immune response of zebrafish has been studied not only using microarrays but also using Solexa/Illumina's digital gene expression (DGE) system, a tag-based transcriptome sequencing method. This method has been used to investigate the changes in zebrafish transcriptome profiles induced by *Mycobacterium* and *Salmonella* (Hegedus et al. 2009; Ordas et al. 2010).

6.3 Transgenesis and RNAi

Morpholino-modified antisense oligonucleotides ("morpholinos") are routinely used in zebrafish to transiently block genes and reduce protein expression. Levraud et al. (2008) have provided a protocol to generate zebrafish embryos deficient in a protein of interest for innate immune signaling using antisense morpholino oligonucleotides.

Chang and Nie (2008) have used RNA interference (siRNA) and real-time quantitative PCR to explore the effect of zebrafish peptidoglycan recognition protein 6 (zfPGRP6) on the TLR signaling pathway. The expression of beta-defensin-1 was downregulated in embryos silenced by zfPGRP6. In challenge experiments to determine the antibacterial response to Gram-negative bacteria, RNAi knock-down of zfPGRP6 markedly increased susceptibility to *Flavobacterium columnare*. Aggad et al. (2010) have used morpholino-mediated loss-of-function analyses to screen candidate receptors and identify the components of their receptor complexes. They found that Ifn-γ1 and Ifn-γ2 bind to different receptor complexes.

Overexpression of a protein of interest is another strategy to investigate gene functions. In some cases, zebrafish can express genes from other animals: Yazawa et al. (2006) have established a transgenic zebrafish strain expressing a chicken lysozyme gene under the control of the Japanese flounder keratin gene promoter and have investigated its resistance to a pathogenic bacterial infection. In a challenge experiment, 65% of the F2 transgenic fish survived an infection of *F. columnare,* and 60% survived an infection of *Edwardsiella tarda*, whereas 100% of the control fish were killed by both pathogens. Hsieh et al. (2010) have also overexpressed tilapia hepcidin in zebrafish reporting that transgenic fish showed significantly higher bacterial clearance after *Vibrio vulnificus* challenge but not after *Streptococcus agalactiae* challenge. Transgenic zebrafish showed increased endogenous expression of Myd88, tumor necrosis factor-alpha, and TRAM1 *in vivo*. Peng et al. (2010) have produced antimicrobial peptide epinecidin-1 transgenic zebrafish, which are able to effectively inhibit bacterial growth.

Transgenesis can also be conducted by linking green fluorescent protein (GFP) to genes or promoters of interest, making it possible to visualize processes that would otherwise be difficult to observe.

An extensive database of transgenic and mutant zebrafish lines is available at the Zfin web page (http://zfin.org/cgi-bin/webdriver?MIval=aa-ZDB_home.apg).

6.4 Chemical Genetic Screens

Zebrafish can be used in a "whole animal"-based compound discovery strategy that represents an advance if it is compared to traditional biochemical drug discovery programs. The use of larval zebrafish facilitates rapid and inexpensive *in vivo* vertebrate analysis. Phenotypic screens have been successfully employed to identify compounds as candidate drugs for many different conditions (Zon and Peterson 2005; Lieschke and Currie 2007; Bowman and Zon 2010). Whereas traditional approaches look for *in vitro* inhibitors of a particular target, this approach involves a physiological process (e.g., inflammation resolution) and looks for compounds that accelerate that process (Martin and Renshaw 2009). Phenotype-based small molecule screening in zebrafish has been described in several studies (Moon et al. 2002) and is now being applied to Alzheimer's disease (Arslanova et al. 2010), hematopoiesis (Paik et al. 2010), multiple sclerosis (Buckley et al. 2010), glucocorticoid resistance (Schoonheim et al. 2010), cancer angiogenesis (Wang et al. 2010), and cardiovascular diseases (Xu et al. 2010).

6.5 Imaging

As discussed above, one of the main advantages of the zebrafish is the ease of phenotypic analysis. The zebrafish embryo is optically transparent, making it possible to detect functional and morphological changes in internal organs without having to kill or dissect the organism. These functional and morphological changes can be further emphasized by the use of transgenic lines and reporter molecules (Zon and Peterson 2005). These characteristics of the zebrafish have made it possible to assess various aspects of the immune response through microscopic observations (Levraud et al. 2008).

Lepiller et al. (2007) have shown that labeling with DAF-FM DA is an efficient method to monitor changes in NO production in live zebrafish under both physiological and pathophysiological conditions, suggesting applications to drug screening and molecular pharmacology. Mathias et al. (2009) and Renshaw et al. (2006) have described how the zebrafish system is suitable for both live time-lapse imaging of neutrophil chemotaxis and screening of the effects of chemical compounds on the inflammatory response *in vivo*.

Singer et al. (2010) have constructed a series of plasmids to label a variety of fish and human pathogens with red fluorescent protein, making it possible to observe real-time interactions between green fluorescent protein-labeled immune cells and invading bacteria in the zebrafish.

6.6 Gnotobiotic Zebrafish

Gnotobiosis, the ability to raise animals in the absence of microorganisms is a powerful tool to study the relationships between animal hosts and their microbial residents or pathogens (Pham et al. 2008).

Rawls et al. (2004) have conducted DNA microarray comparisons of gene expression in the digestive tracts of 6 dpf germ-free zebrafish and normal zebrafish, revealing 212 genes that are regulated by the microbiota and 59 responses that are conserved in the mouse intestine, related to the stimulation of epithelial proliferation, promotion of nutrient metabolism and innate immune responses. Colonization of germ-free zebrafish with individual members of its microbiota revealed the bacterial species specificity of selected host responses.

Using a gnotobiotic zebrafish-*Pseudomonas aeruginosa* model, Rawls et al. (2007) have monitored microbial movement and localization within the intestine *in vivo* and in real time, taking advantage of the transparency of this vertebrate species. Pseudomonads are rare members of the intestinal microbiota of healthy humans but their representation is increased in certain pathologic states, notably inflammatory bowel diseases. These studies have demonstrated the utility of gnotobiotic zebrafish in defining the molecular bases of host–microbial interactions in the vertebrate digestive tract.

7 Some Examples of Application to Inflammatory Human Diseases

As Renshaw et al. (2007) have pointed out, the use of fish to investigate medical problems could result peculiar. However, we note that major advances in medical knowledge and immunology have been obtained by studying genetic pathways in invertebrate animals such as the worm *Caenorhabditis elegans* and the fly *Drosophila melanogaster*, both of which are more distant from humans than vertebrates such as zebrafish.

Inflammatory diseases are an important cause of morbidity and mortality in various medical specialties. Below, we give some examples of human diseases that have been studied using the zebrafish as a model:

Lung disease: Unresolved neutrophilic inflammation is a major contributor to the tissue damage associated with many lung inflammatory disorders (Martin and

Renshaw 2009). The resolution of inflammation depends on the termination of proinflammatory neutrophil functions by apoptosis. To date, the bases of neutrophil apoptosis have been studied in purified human peripheral blood neutrophils or in mice using gene manipulation techniques; however, these studies usually have limitations (Dzhagalov et al. 2007). The range of tools developed for labeling neutrophils in zebrafish can be valuable for this research. Although much more work is needed before zebrafish are widely utilized in respiratory research, studies are already being conducted because zebrafish offer complementary benefits to existing respiratory disease models (Renshaw et al. 2007). The use of zebrafish facilitates the application of pharmacological and genetic manipulations to ascertain their effects on neutrophils during inflammation, the ability to screen for novel antiinflammatory compounds, the generation of forward and reverse genetic screens to identify regulators of the resolution of inflammation, and the visualization of cell behavior *in vivo*.

Cardiomyopathy: Human dilated cardiomyopathy (DCM) is a myocardial disease characterized by dilatation and impaired systolic function of the ventricles. DCM is the single largest cause of heart failure and cardiac transplantation (Towbin and Bowles 2006). Accumulating evidence suggests that inflammatory and autoimmune mechanisms play a role in this idiopathic disease (Takeda 2003): Inflammatory infiltrates and proinflammatory cytokines have been observed in DCM patients (Maisch et al. 2005). Recently, Friedrichs et al. (2009) have identified a genomic region containing genes associated with cardiac function and DCM. These authors used zebrafish to complement and confirm these studies because cardiac phenotypes could be readily assessed through direct monitoring of the heart in the living animal (Driever and Fishman 1996). Functional knock-down studies have been conducted for eight genes using morpholino (MO) antisense experiments. Knockdown of three of the genes (*HBEGF*, *IK* and *SRA1*) resulted in impaired cardiac function phenotypes.

Septic shock: In mammals, microbial products, such as lipopolysaccharide (LPS), are potent inducers of inflammation that stimulate immune system cells after they are recognized (mainly by TLRs). In particular, Gram-negative enterobacterial LPS signals are transmitted through TLR4, whereas Gram-positive bacteria usually activate cells in a TLR2-dependent fashion, leading to the production of proinflammatory cytokines, proteases, eicosanoids, and reactive oxygen and nitrogen species (West and Heagy 2002). If this inflammatory response to infection is not tightly controlled, several pathological processes may develop, including endotoxin shock, which is a severe systemic inflammatory response characterized by fever, myocardial dysfunction, acute respiratory failure, hypotension, multiple organ failure, and often death (West and Heagy 2002; Power et al. 2004). It is well known in mammals that a previous exposure to LPS induces “endotoxin tolerance”, which is thought to protect the host from endotoxic or septic shock, although the mechanisms involved are not fully understood.

Zebrafish larvae (2 dpf) are able to produce an inflammatory response when exposed to LPS, although the minimum lethal LPS concentration is much higher than in mammals. *P. aeruginosa* LPS is more lethal than *E. coli* LPS and pretreat-

ment with a non-lethal LPS dose induces a hypo-responsive state that protects fish subsequently exposed to the *P. aeruginosa* LPS (Novoa et al. 2009). Furthermore, two administrations of lipoteichoic acid (a component of the surface of Gram-positive bacteria) convey complete protection against exposure to a lethal concentration of LPS, demonstrating heterotolerance, as described previously in mammals (Dobrovolskaia et al. 2003). In these studies, when a mutant fish (*Odysseus*), in which CXCR4 function is inhibited, is used or when AMD3100 (a pharmacological specific CXCR4 inhibitor) was applied, the fish did not acquire tolerance to LPS. CXCR4 is a G protein-coupled chemokine receptor; these results confirm that CXCR4 belongs to the cluster involved in LPS recognition and may be involved in controlling excessive inflammatory response (Triantafilou et al. 2008).

The use of complete organisms, such as zebrafish larvae, presents an excellent opportunity to further study this model of endotoxin shock. Indeed, zebrafish have recently been used to study the WHIM syndrome, a primary immunodeficiency disorder characterized by neutropenia and recurrent infections in which CXCR4 seems to be associated with recurrent infections (Walters et al. 2010).

Intestinal inflammatory diseases: The zebrafish has emerged as a model organism for the study of host–microbe interactions related to the digestive function (Dahm and Geisler 2006; Hama et al. 2008; Kanther and Rawls 2010) because anatomical and functional conservation has been reported between the zebrafish and mammalian intestines (Ng et al. 2005; Bates et al. 2007; Flores et al. 2008). Members of the microbiota influence intestinal epithelial cell proliferation rates independent of inflammation via direct modulation of β-catenin signaling (Cheesman et al. 2010). However, a breach of this intestinal host–microbe homeostasis contributes to the pathogenesis of inflammatory bowel disease (IBD), commonly manifested as Crohn's disease or ulcerative colitis (Kaser et al. 2010).

Brugman et al. (2009) have developed zebrafish model of enterocolitis to study the interactions between host intestinal cells and bacteria and to understand the pathogenesis of IBD. Enterocolitis was induced by intrarectal administration of the hapten oxazolone in adult wild-type and myeloperoxidase-reporter transgenic zebrafish. Fleming et al. (2010) have developed another model of IBD in zebrafish larvae, together with a method for the rapid assessment of gut morphology and an *in vivo* compound screening technique. In this case, IBD was induced by the addition of 2,4,6-trinitrobenzenesulfonic acid (TNBS) to the medium and changes in goblet cell number and tumor necrosis factor alpha (TNF-alpha) antibody staining were used to quantify disease severity.

These studies affirm that zebrafish can be a powerful model suitable for medium-throughput chemical screens in the study of gastrointestinal disease.

Other studies have been conducted to analyze the expression of genes related to these inflammatory processes in the intestine. For example, Oehlers et al. (2010a) have studied the expression gradients of antimicrobial peptide genes along the zebrafish intestine; Flores et al. (2010) have studied the zebrafish ortholog of the human *DUOX1* and *DUOX2* genes, which play an important role in gut immunity; and Oehlers et al. (2010b) have examined Cxcl8 signaling, which is associated with gut inflammation.

8 Conclusions

In summary, zebrafish can be a valuable tool to increase our knowledge of innate immune responses and the regulation of inflammation. The use of genetic and compound screens should help to identify new pathways involved in inflammation resolution and also new compounds to modify these pathways.

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Monocyte-derived Inflammatory Dendritic Cells in the Granuloma During Mycobacterial Infection

Heidi A. Schreiber and Matyas Sandor

Abstract The monocyte-derived, inflammatory dendritic cell subset plays an important role during immune responses against infections. This review will focus on the complex, changing role of this subset during mycobacterial infection. Studies demonstrate that in addition to sustaining a systemic anti-mycobacterial response, the inflammatory dendritic cell subset present in *Mycobacterium*-induced granulomas also influences local immune regulation within the granuloma over the course of infection. This review will also survey the literature on how similar and different inflammatory dendritic cell subsets during other infections.

Keywords Mycobacteria • Dendritic cells • Granuloma • Acute and chronic infection • $CD4^+$ T cells • BCG

1 Introduction

The discovery of dendritic cells (DCs) nearly four decades ago has provided immunologist with the missing link to efficiently bridge the innate and adaptive immune response (Steinman et al. 1973, 1974a, b). DCs are comprised of heterogeneous populations, with each subset uniquely qualified to support either immunity during infection and tolerance in the face of autoimmunity. Under steady-state conditions, conventional DCs make up a very small percentage of tissue and lymphoid-resident cells. In the face of infection the immune system must respond quickly. Circulating, peripheral blood monocytes provide a powerful reservoir of immune artillery, as they can efficiently give rise to DCs under infectious conditions. This monocyte-derived DC (moDCs) lineage that arises during infection shares many functional and phenotypic similarities to classical DCs, and are therefore termed 'inflammatory DCs' (*inflam*DCs). There has been a recent surge in data that irrefutably dem-

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onstrates the importance of this subset during infection by a wide array of pathogens (viruses, bacteria, fungi, protozoan, etc.). This review will discuss the origin and phenotype of moDCs, and their involvement during infection by various pathogens. The newly immerging role *inflam*DCs play during *Mycobacterium* infection will be specifically highlighted in this review.

2 Development and Phenotype

DC precursors, both classical and monocyte-derived inflammatory, originate in the bone marrow from hematopoietic stem cells. Both subsets share the same myeloid precursor that gives rise to monocyte/macrophage and DC lineages, known as the macrophage-DC precursor (MDP) (Fogg et al. 2006). However, the classical DC and monocyte developmental pathway diverges henceforth (Liu et al. 2009). Although *in vitro* culture systems can generate a large number of DCs by culturing monocytes with cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) (Inaba et al. 1992; Sallusto and Lanzavecchia 1994), and early *in vivo* data suggested the generation of a DC-like population from both murine and human monocytes (Randolph et al. 1998, 1999), recent *in vivo* data strongly supports the idea that monocytes do not give rise to classical lymphoid organ DCs during steady-state conditions. *In vivo* experiments that utilize either adoptive cell transfer models or GFP reporter mice driven by the monocyte-specific lysozyme M promoter, all demonstrate that monocytes do not have classical DC progenitor activity or are able to reconstitute classical lymphoid organ DCs during steady-state conditions (Fogg et al. 2006; Naik et al. 2006; Varol et al. 2007; Jakubzick et al. 2008; Liu et al. 2009). However, monocytes have been shown to replenish some DC populations in peripheral organs, such as the intestines, lung, and skin (Holt et al. 1994; Ginhoux et al. 2006; Varol et al. 2007). MDP exposure to fms-like tyrosine kinase 3 ligand (Flt3L) (McKenna et al. 2000; Waskow et al. 2008) or GM-CSF (Dai et al. 2002) in the bone marrow results in the divergence of conventional DC and monocyte developmental pathways, respectively. Conventional DC (cDC) development is reviewed in depth elsewhere (Liu and Nussenzweig 2010). In addition to early exposure of the growth factor GM-CSF, the monocyte lineage is further selected for at the transcriptional level. Increased expression of transcription factor PU.1 has been shown to favor monocyte lineage development (Dahl et al. 2003). Interestingly, continued expression of PU.1 further promotes monocyte differentiation into DCs by suppressing the macrophage-inducing transcription factor MafB, and vice versa (Bakri et al. 2005). Conversely, the transcription factor STAT3 sustains cDC Flt3-dependent differentiation (Laouar et al. 2003). Table 1 summarizes this and other defining characteristics between cDCs and monocyte-derived *inflam*DCs.

From the bone marrow, monocytes can be classified into two subpopulations based on high- or low-surface expression of Ly6C. Ly6C^{high} and Ly6C^{low} populations were originally and often times currently used interchangeably with Gr-1^{high} and Gr-1^{low}, respectively. However, in addition to recognizing Ly6C, the monoclo-

Table 1 Defining characteristics between cDCs and monocyte-derived *inflam*DCs. (Source: Fogg et al. 2006; Liu et al. 2009; D'Amico et al. 2003; Geissmann et al. 2010; Laouar et al. 2003; Dahl et al. 2003; Liu el al. 2007; Auffray et al. 2009; Fleming et al. 1993; Geissmann et al. 2003; Cheong et al. 2010; Kamphorst et al. 2010)

	Conventional DCs	Monocyte-derived *Inflam*DCs
BM precursor	CDP	MDP
Growth factor for development	Flt3L	M-CSF
Transcription factors	STAT3	PU.1
Precursor frequency in blood	Low (0.2%)	High (10%)
Distinguishing markers	Flt3 (CD135)	CD115, Gr-l/Ly6C, CD209a, CD14
T cell priming ability		
$CD8^+$ T cell (cross presentation)	+++	+++
$CD4^+$ T cell (MHCII)	+++	+

nal antibody Gr-1 (RB6-8C5) also recognizes Ly6G, a marker not expressed on monocytes (Fleming et al. 1993). Therefore, Ly6C expression is the more accurate description of murine monocyte subsets. The $Ly6C^{high}$ and $Ly6C^{low}$ monocyte populations in mice are believed to correspond to the human $CD14^{high}CD16-$ and $CD14^{int}CD16^+$ populations, respectively (Randolph et al. 2008). The two murine $Ly6C^{high}$ and $Ly6C^{low}$ monocyte populations can be further phenotyped by expression of different chemokine and adhesion molecules.

The conventional $Ly6C^{high}$ monocytes are also $CCR2^{pos}$, $CD62L^{pos}$, $CCR5^{neg}$, $CD11c^{neg}$ and CX_3CR1^{low}, and the $Ly6C^{low}$ population is CX_3CR1^{high}, $CCR2^{neg}$, $CD62L^{neg}$, CX_3CR1^{pos}, and $CD11c^{int}$ (Geissmann et al. 2003; Sunderkotter et al. 2004; Tacke et al. 2007). These two populations are not independently derived as the $Ly6C^{high}$ population has been shown to give rise to the $Ly6C^{low}$ population, both in bone marrow and in the periphery (Sunderkotter et al. 2004; Tacke and Randolph 2006; Arnold et al. 2007; Nahrendorf et al. 2007; Varol et al. 2007). Monocyte emigration from the bone marrow is controlled by chemokine receptors CCR2 on the $Ly6C^{high}$ population, and CX_3CR1 and CCR5 on the $Ly6C^{low}$ counterpart, but the mechanisms behind this control are not known (Combadiere et al. 2008). Once within the blood, the $Ly6C^{low}CX_3CR1^{high}$ population has been elegantly imaged crawling along vessels under steady-state conditions regardless of the direction of flow, but rapidly extravasated into tissue in the presence of infection (Auffray et al. 2007). A recent study from the Steinman group definitively demonstrated the differentiation of monocytes into DCs *in vivo* in response to microbial stimuli (Cheong et al. 2010). This study demonstrated the inflammation-induced recruitment of moDCs into the T cell areas of the affected lymph node and subsequent expression of DC-SIGN (CD209a). The moDC mobilization they observed was dependent on CCR7, TLR4, CD14, and Trif expression on the DC. In spite of the many characteristics moDCs share with cDCs, antigen presentation is not one of them. A recent, definitive report by Kamphorst et al. exhaustively compared the antigen presentation capacity of cDCs and moDCs following several mechanisms of antigen capture (Kamphorst et al. 2010). This study demonstrated that while moDCs are efficient at priming $CD8^+$ T cells through cross presentation, the cDC compartment was much

more effective at presenting antigen to MHCII-restricted $CD4^+$ T cells. While the inflamDCs may appear very similar to cDCs in terms of costimulatory molecules, MHCII expression and morphology, they may differ internally upon acquisition of antigen. A recent study by McCurley and Mellman demonstrated that the moDC subset in humans had a higher protease content, and as a result, had higher lysosomal proteolysis compared to cDCs (McCurley and Mellman 2010). This high lysosomal proteolytic activity results in quick degradation of intracellular antigen and may explain the poor ability of *inflam*DCs to present MHCII peptide.

In the face of infection the monocyte lineage provides a quick, profuse source of cells able to differentiate into the necessary populations of antigen presenting cells (APCs) necessary for quick combat. In this next section we will discuss their potential to differentiate into *inflam*DCs and the role they play during infection by bacteria, viruses, fungi and protozoa.

3 Function of Monocyte-derived Inflammatory DCs During Infection

3.1 Mycobacteria

All things considered (i.e., prevalence, new cases and deaths per year, economic burden, drug resistance, etc.) infection with *Mycobacterium* remains one of the most devastating diseases worldwide. The granuloma is the hallmark of mycobacterial infections, and is the interface for the host immune response and bacterial persistence. Functional granulomas protect an estimated two billion individuals from active disease by attenuating bacterial growth and dissemination (Ulrichs and Kaufmann 2006). One of the defining characteristics of *Mycobacterium* infection is that even in the face of an early, strong immune response, the host often fails to completely eradicate the bacteria, resulting in a long-term chronic infection. Therefore, when bearing in mind *Mycobacterium* infection it is best to view both the immunological and bacteriological events in two separate stages, acute and chronic. For example, granulomas formed during the acute stage of infection largely protect the host by limiting bacterial dissemination and inadvertent tissue damage. However, during chronic infection these well-formed granulomas provide the mycobacteria with a long-term survival niche (Volkman et al. 2004; Russell 2007). Understanding the role of DCs during *Mycobacterium* infection is still in its infancy, but the growing body of data supports the idea that they have an essential, influential role during both the acute and chronic stages. The predominating DC subset involved during both stages is monocyte-derived *inflam*DCs, largely characterized by $Ly6C^{int}CD11b^{high}CD11c^{low}$ expression (Reljic et al. 2005; Humphreys et al. 2006; Wolf et al. 2007; Schreiber et al. 2010). However, the mechanisms governing monocyte recruitment, differentiation, infection, lymphatic antigen transport, and T cell priming are not equally understood for both the acute and chronic stages. There is more information regarding the role of *inflam*DCs during the acute phase

of infection. However, recent studies are now focusing on the chronic phase. Data from these studies suggests a changing role of monocyte-derived *inflam*DCs during chronic *Mycobacterium* infection.

Recruitment of this subset to the site of acute *Mycobacterium* infection seems to occur in both a CCR2- and CCR5-dependent manner and not CX_3CR1-dependent, as demonstrated by infection of mice with mutated expression of these chemokine receptors (Hall et al. 2009). Following *Mycobacterium tuberculosis* (MTb) infection, CCR2 deficient mice were highly susceptible to high doses and had a delayed immune response when challenged with low infectious doses (Peters et al. 2001; Scott and Flynn 2002). Conversely, mice over expressing CCR2 better controlled *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) infection by faster granuloma formation, lower bacterial burden and increased transport of bacterial antigen to the draining lymph nodes by DCs (Schreiber et al. 2008). Interestingly, humans with a single nucleotide polymorphism in the MCP-1, which resulted in increased peripheral levels of the chemokine, had increased susceptibility of MTb infection, suggesting a possible desensitization to the CCR2 ligand over time (Flores-Villanueva et al. 2005; Serbina et al. 2008). In the absence of a CCR2-expressing monocyte subset, CCR5 expression may compensate and vice versa as CCR5-deficient mice also showed no significant difference in Mtb control (Badewa et al. 2005). Studies examining the immune response in the absence of both chemokine receptors have not been done. The global requirement of $CD11c^{pos}$ DCs for the initiation of T cell immunity and bacterial control following MTb infection was definitively demonstrated by transiently depleting DCs using pCD11c-diphtheria toxin receptor transgenic mice (Tian et al. 2005). Depleting the CD11c population prior to infection resulted in a delayed $CD4^+$ T cell response to MTb and impaired control of MTb replication. In back to back reports, Wolf and colleagues demonstrated that the $CD11b^{pos}CD11c^{pos}$ DC population recruited to the lung after MTb infection is readily infected and facilitates the transport of MTb antigen to the draining lymph node (Wolf et al. 2007). Eliminating a primary chemokine network utilized by this subset en route to lymph nodes using *plt* mice, which lack CCR7 ligands CCL19 and CCL21ser, they demonstrated the necessity of mycobacteria transport by $CD11b^{pos}CD11c^{pos}$ DCs (Wolf et al. 2007) and *Mycobacterium*-specific IFNγ-producing $CD4^+$ T cells(Khader et al. 2009). Early studies that demonstrated a potent T cell stimulatory activity by lymph node DCs after BCG infection did not entirely separate lymph node-resident conventional DCs from recruited monocyte-derived *inflam*DCs (Jiao et al. 2002). However, recent studies specifically addressing the activation capacity of $CD4^+$ T cells specific for *Mycobacterium* antigen 85B (P25) during early Mtb infection found *inflam*DCs to be poor T cell stimulators and elicit much less IFNγ production compared to other DC subsets (Wolf et al. 2007). As previously described for *Listeria monocytogenes* and Salmonella, evidence for the transfer of antigen from *inflam*DCs to lymph node-resident DCs also seems to be a phenomenon occurring during acute *Mycobacterium* infection as well. Eliminating the mechanism of *inflam*DC transport to the lymph nodes by using *plt* mice, resulted in 95% fewer bacilli reaching the lymph nodes, which effectively eliminated the source of antigen (Wolf et al. 2007). In addition to CCR7-expressing

migratory DCs aiding in antigen delivery to the lymph node, it was also found that CCR7-deficient mice infected via aerosol with Mtb had decreased bacterial load in the spleen during chronic infection, demonstrating the requirement for CCR7-expressing migratory DCs for bacterial dissemination during acute Mtb infection (Kahnert et al. 2007). There are numerous studies testing the ability of DCs *ex vivo*, and bone marrow and moDCs *in vitro,* to take up mycobacteria, mature, produce cytokines and prime T cells. This is reviewed in greater detail elsewhere (Schreiber et al. 2010). Collectively, these studies yield conflicting results by demonstrating the ability of DCs to both stimulate and suppress immunity *ex vivo* and *in vitro*. Which further reaffirms the idea that DCs, particularly *inflam*DCs, play a multifaceted role during mycobacterial infection.

Recent data from our group has supported this multifunctional role for *inflam*-DCs during *Mycobacterium* infection, particularly within the granuloma. Using *in vivo* imaging, a recent study by Egen and colleagues demonstrated the intense movement of T cells within the acute granuloma (Egen et al. 2008). Their data showed that newly arrived T cells efficiently scan granuloma-resident APCs. This scanning is necessary, as newly primed T cells require a second antigen encounter at the site of inflammation in order to rapidly secrete cytokines (Mohrs et al. 2005). By phenotyping monocyte-derived *inflam*DCs in both acute and chronic BCG-induced granulomas, we found that their phenotype varied greatly from the progression of acute to chronic infection. *Inflam*DCs found in acute granulomas had high expression of MHCII, CD40, CD80 and CD86, and were able to support IFNγ production from newly arrived primed *Mycobacterium*-specific P25 $CD4^+$ T cells (Schreiber et al. 2010). However, as infection progressed the *inflam*DCs within the chronic granuloma had decreased expression of MHCII and T cell costimulatory molecules, and had a significant increase in T cell inhibitory molecules PD-L1 and PD-L2. These DCs could no longer support IFNγ production from newly arrived *Mycobacterium*-specific P25 $CD4^+$ T cells. This inability was in part dependent on PD-L1/2:PD-1 signaling, as blockade of this pathway partially restored IFNγ production. Monocyte-derived *inflam*DCs, by affecting local IFNγ availability, may contribute to granuloma maturation and bacterial persistence (Fig. 1).

Enabling bacterial persistence during mycobacterial infection has partially led to the belief that chronic granulomas are ‘walled-off’ structures, inaccessible to immune surveillance. One defining feature of moDCs is their profound ability to migrate. Therefore, we asked the question of whether chronic granulomas were assessable to *inflam*DCs, and if so, were they able to then leave the granuloma with antigenic cargo en route to the draining lymph nodes? Using a kidney capsule transplant model, which consists of grafting granuloma-containing tissue underneath the kidney capsule of uninfected recipients, we were able to study $CD11c^{pos}$ cellular migration into and out of both acute and chronic *Mycobacterium*-induced granulomas using CD11c enhanced yellow fluorescent protein (CD11c-EYFP) mice with ubiquitously fluorescing DCs (Schreiber et al. 2010. *Manuscript Under Review*) (Lindquist et al. 2004). This study demonstrated that *inflam*DCs had considerable access to enter and exit chronic granulomas, even more so than acute lesions, with a turnover rate of ~60% and 30%, respectively, by one week. Determining the life

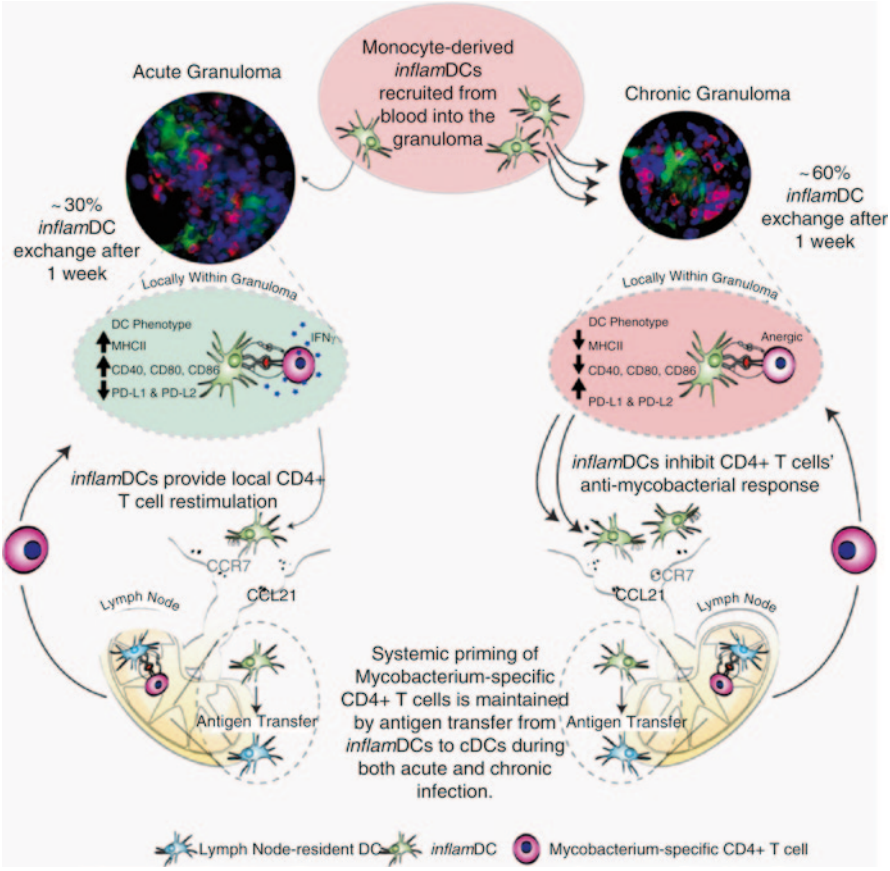

Fig. 1 Changing role of *inflam*DCs in *Mycobacterium*-induced granulomas over the course of infection

span of *inflam*DCs within the granuloma is complicated. Circulating monocytes have a relatively short half-life, as do migrating DCs; however, monocytes have also been shown to live for months after differentiation (Whitelaw 1966; Kamath et al. 2002; Liu et al. 2007; Gonzalez-Mejia and Doseff 2009). By staining acute and chronic granulomatous lesions with a marker for apoptosis and CD11c, the DCs present do not appear to be dying locally within the lesions during either acute or chronic infection (Fig. 2). It is difficult to discern whether the change in DC flux throughout the course of infection is due to a general shrinking of the lesions or potential differences in DC expansion locally due to M-CSF availability. *Inflam*-DCs leaving granulomas migrated not only to the draining lymph nodes, but also to other systemic lymphoid organs. This systemic migration supported priming of P25 $CD4^+$ T cells during both acute and chronic infection, albeit to a lesser extent in the latter. Grafting both acute and chronic granulomas into $MHCII^{-/-}$ recipients resulted in total abrogation of P25 $CD4^+$ T cell activation, demonstrating the necessity for

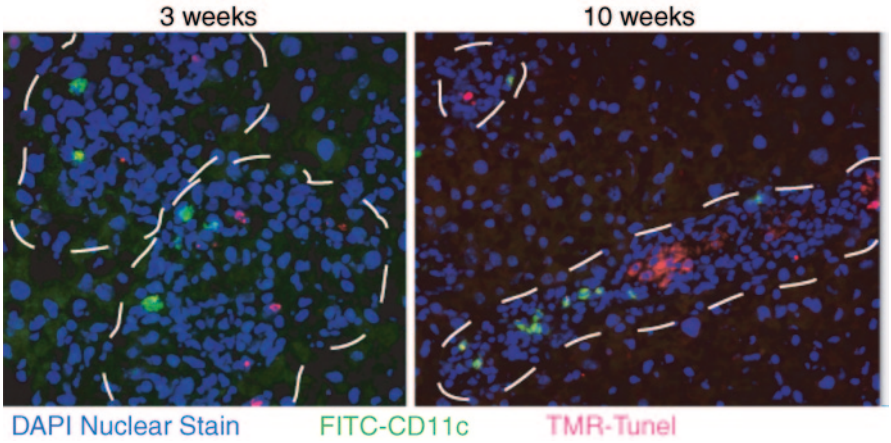

Fig. 2 Tunel staining on sections from 3- and 10-week BCG-infected mice. White dashed line indicates periphery of granuloma lesions. Staining with CD11c (green) and apoptosis indicator, Tunel (red), demonstrates that DCs are not undergoing apoptosis within the granuloma throughout chronic infection

recipient MHCII^{pos} cells. Eliminating the mechanism used by inflamDCs to migrate from the granuloma to the draining renal lymph node by transplanting granulomas from CCR7$^{-/-}$ mice, abrogated P25 CD4^{+} T cells activation during acute infection. Collectively demonstrating the role of *inflam*DCs during T cell priming as antigenic ferries (Randolph 2006).

It is evident that the changing phenotype and function of *inflam*DCs in mycobacterial granulomas plays an important role, for better or worse, in the disease course. Better understanding this cellular compartment during both acute and chronic *Mycobacterium* infection will inevitably lead to a better understanding of the disease and potential targets for therapeutic intervention.

3.2 Other Bacteria

Pioneering work on *inflam*DCs largely came from studies done by the groups of Pamer and Leenen on *Listeria monocytogenes* (Auffray et al. 2009). A Gram-positive intracellular bacterium, *L. monocytogenes* got its species name '*monocytogenes'* after the observation of increased monocytes in infected rabbits (Murray et al. 1926; Serbina et al. 2008). The early requirement for monocyte-derived Ly6C-expressing cells was further confirmed by depleting mice of this population using the RB6-8C5 monoclonal antibody within 24 h of *L. monocytogenes* infection, resulting in death within six days (Czuprynski et al. 1994). Upon infection with *L. monocytogenes*, Ly6C^{high} cells egress from the bone marrow in considerable numbers by CCR2-mediated signals largely in response to monocyte chemoattractant protein-1 (MCP-1)

(Kurihara et al. 1997; Sunderkotter et al. 2004; Serbina et al. 2006; Tsou et al. 2007). Induction of MCP-1 expression during *L. monocytogenes* infection is dependent on cytosolic recognition of the bacterium and independent of the MyD88 signaling pathway (Serbina et al. 2003). MCP-1 is also significantly induced after infection by *Toxoplasma gondii*, *Aspergillus fumigatus* and *Mycobacterium tuberculosis* (Lin et al. 1998; Blease et al. 2001; Robben et al. 2005). Mice lacking CCR2 succumb to fatal infection by *L. monocytogenes* and lack the $CD11b^{int}CD11c^{int}$ population that is morphologically similar to the splenic $CD11c^{high}$ population (i.e., pleomorphic nuclei, dendrites and numerous vacuoles) (Kurihara et al. 1997; Serbina et al. 2006). This $CD11b^{int}CD11c^{int}$ population present during *L. monocytogenes* infection also produced TNF- and iNOS (Tip), thereafter referred to as TipDCs (Serbina et al. 2003). Kang et al. recently demonstrated that the differentiation of TipDCs from the recruited $Ly6C^{high}$ monocytes was dependent on the ability of splenic $CD11c^{pos}$ cells to induce IFNγ production from NK cells (Kang et al. 2008). In addition to CCR2, Auffrey and colleagues demonstrated a role for chemokine receptor CX_3CR1 on the recruitment of $Ly6C^{pos}(GR1^{pos})CD115^{pos}$ moDC precursors to the spleen during *L. monocytogenes* infection (Auffray et al. 2009). A follow up study by this group used $Cx_3cr1^{gfp/+}$ mice to elegantly image *in vivo* monocyte recruitment during *L. monocytogenes* infection (Auffray et al. 2007). Here, the $GR1^{neg}CX_3CR1^{pos}$ monocytes observed patrolling the vasculature rapidly extravasated into inflamed tissue and initiated a macrophage-like transcriptional program by up regulating cMaf and MafB. Conversely, the $GR1^{neg}CX_3CR1^{pos}$ subset took on a DC transcriptional profile by up regulation of Pu.1 and RelB genes within 2 h post infection. Once recruited to the site of infection both differentiation of monocytes into TipDCs and production of IL-12 wash shown to be MyD88 dependent (Serbina et al. 2003; Zhan et al. 2010). CX_3CR1 is also required by moDCs recruited to the intestinal lamina propria during *Salmonella typhimurium* infection in order to extend their dendrites into the intestinal lumen and transport antigen to the lymph node (Niess et al. 2005). MoDCs are among the better migratory DC populations; however, their ability to directly prime the immune system has been called into question in infectious models across the board (Randolph et al. 2008). Using a *Salmonella typhimurium* bacterial infection model, Ravindran and colleagues demonstrated that although a recruited $Ly6C^{high}$ DC-like population was required for induction of an anti-Salmonella $CD4^+$ T cell response, the $Ly6C^{high}$ phagocytes themselves were not directly responsible for this induction (Ravindran et al. 2007). Rather, the $Ly6C^{high}$ population was necessary for transporting the antigen from the site of infection to the draining lymph nodes. Rydstrom and colleagues also demonstrated the inability of a monocyte-derived TipDC-like population to activate antigen-specific T cells following *Salmonella* infection (Rydstrom and Wick 2007).

3.3 Viruses

In addition to bacteria, studies using viral infection models have significantly broadened our understanding of *inflam*DCs. Under steady state conditions, few

monocytes are recruited to the vaginal epithelium. Although, following intravaginal infection with herpes simplex virus-2 (HSV-2), recruited GR-1^{pos} monocytes readily gave rise to vaginal epithelial DCs, including CD11c^{pos}MHCII^{pos} subsets (Iijima et al. 2007; Eidsmo et al. 2009). Similar to the granuloma kidney capsule transplant model previously discussed, Wakim and colleagues grafted HSV-infected dorsal root ganglia under the kidney capsule of recipient naïve mice, which resulted in viral reactivation (Wakim et al. 2008). They sophisticatedly demonstrated that the recruited monocyte-derived Ly6C^{high} DCs were able to restimulate memory viral-specific T cells within the graft. In addition to HSV, monocyte-derived *inflam*DCs are also readily involved in influenza infection. Upon infection with the virulent influenza A virus CD11c^{pos}CD11b^{high}Ly6C^{pos} *inflam*DCs are recruited in a CCR2-dependent manner directly into the lymph nodes, as their recruitment was not hindered in neither *plt* nor CCR7$^{-/-}$ mice (Nakano et al. 2009). When purified and stimulated *ex vivo*, these LN recruited *inflam*DCs produced IL-12(p70) and had the ability to stimulate OTII CD4^{+} T cells. A study by Aldridge et al. also found CCR2-dependent recruitment of Ly6c^{pos}CD11b^{pos} TipDCs into the lungs following influenza A infection (Aldridge et al. 2009). Interestingly, they found that these TipDCs presented antigen to cytotoxic CD8^{+} T cells in the lung and limiting the number of DCs recruited using pioglitazone, a synthetic pharmacological agent that suppressed CCL2 secretion, they could dampen inflammation-induced pathology. That indicated that too many TipDCs are not additive in protection and could even be lethal if not controlled. Viral models have also supported evidence for the dual requirement of inflamDCs migrating from the site of infection and lymph node-resident DCs in the initiation of T cell immunity. Belz and colleagues demonstrated that during both HSV and influenza infection, efficient antigen-specific T cell priming required the collaboration of both lymph node-residing DCs and DCs originating from the site of infection (Belz et al. 2004). Using an HSV skin infection model, Allan and colleagues further demonstrated that skin-originating DCs were required to shuttle antigen to the draining lymph node, where lymph node-resident CD8α^{pos} DCs were principally responsible for initiating the T cell response (Allan et al. 2006).

3.4 Fungi

The role of *inflam*DCs during fungal infection remains one of the less understood areas. *Aspergillus fumigatus* was previously shown to recruit monocytes in a CCR2-dependent mechanism in mice (Blease et al. 2000). A recent study from the Pamer group reconfirmed Ly6C-expressing monocyte recruitment using CCR2 and demonstrated that this cellular compartment was responsible for transporting *Aspergillus* antigen from the lung to the draining lymph node for CD4^{+} T cell priming (Hohl et al. 2009).A more recent study demonstrated that *A. fumigatus* infection of neutropenic hosts resulted in an even more robust recruitment of *inflam*DCs, suggestive of a novel defense mechanism to circumvent loss of neutrophils (Park et al. 2010).

Antigenic hand-off was also demonstrated in a recent study by Erlsand and colleagues, whom investigated the immune response to an attenuated vaccine strain of *Blastomyces dermatitidis* (Ersland et al. 2010). They found that monocyte-derived *inflam*DCs were quickly recruited to the subcutaneous immunization site and along with skin-migratory DCs, shuttled antigen to the lymph nodes; however, lymph node-resident DCs were responsible for priming $CD4^+$ T cells.

3.5 Protozoan

More so than other infectious models, the recruitment and differentiation of *inflam*DCs from recruited monocytes was definitively demonstrated by Leon and Ardavin using the protozoan parasite *Leishmania major* (Leon et al. 2007). Mice infected with *L. major* received an adoptive transfer of purified monocytes, traceable by the Ly5.1/5.2 congenic markers. After 72 h post transfer, two DC lineages arose from the transferred monocytes: $CD11c^{low}$Ly-$6C^{high}$$MHCII^{int}$ and $CD11c^{int}$Ly-$6C^{int\text{-}high}$MHC $II^{int\text{-}high}$ populations. The moDCs were also the predominate cellular compartment infected and the only cells presenting *L. major* peptide LACK in the context of MHCII, suggesting that they contribute to anti-parasitic $CD4^+$ T cell response. In a follow up study, this same group demonstrated that during *L. major* infection monocytes were recruited through inflamed dermal venules by binding of PSGL-1 with P/E-selectin and L-selectin with PNAd, and migration through high endothelial venules relied only the latter interaction (Leon and Ardavin 2008). A recent study also confirmed that the same monocyte-derived *inflam*DC ingesting the parasite *L. major* in mice was the same counter subset in humans (Zhan et al. 2010). Monocyte-derived *inflam*DCs have also been studied in the protozoan pathogen, *Toxoplasma gondii*. During infection, GR-$1^{pos}$$CCR2^{pos}$$CX_3CR1^{low}$ monocytes are recruited to inflamed tissue in a CCR2-dependent manner, demonstrated by lethality observed in CCR2 or MCP-1 deficient mice (Robben et al. 2005). Adoptive transfer of inflammatory monocytes into $CCR2^{-/-}$ *T. gondii* infected mice restored protection (Dunay et al. 2008).

4 Conclusions

The dynamic, crucial role *inflam*DCs play during infection is only beginning to be fully understood and appreciated. From our discussion here, *inflam*DCs represent a common thread that can be drawn between the various infectious models. This monocyte-derived lineage is rapidly recruited to the site of infection, be it liver, lung, spleen or skin, within hours. The TNF and iNOS-producing capacity of these cells in the face of various pathogens represents a common line of innate defense. One of the most significant commonalities of *inflam*DCs shared between the various pathogens is their role in shuttling antigen from the site of infection to the respective drain-

ing lymph nodes and transferring this precious cargo to lymph node-resident DCs, which relies on their professional migratory abilities. The mechanism(s) behind this antigenic transfer are currently not known. Based on the current literature, a recent review by Randolph et al. speculates that this transfer may be occurring by two potential mechanisms (Randolph et al. 2008). The first scenario involves the moDCs dying upon arrival to the lymph node and lymph node-resident DCs reprocessing the dead DC and its antigenic cargo. The second model suggests that moDCs transfer intact portions of their plasma membrane that contain MHC/peptide complexes to lymph node-resident DCs. Better understanding the mechanism behind antigenic exchange will increase our understanding of the initiation of adaptive immunity during infection and may potentially provide a target for immune intervention.

Our discussion on the role of *inflam*DCs during *Mycobacterium* infection demonstrates the plasticity this monocyte-derived linage can have during a single infection over time. Figure 1 illustrates this changing role. During acute infection *inflam*DCs facilitate an anti-*Mycobacterium* T cell response by reboosting newly recruited $CD4^+$ T cells within the granuloma to produce IFNγ, an essential cytokine to promote macrophage killing of intracellular bacteria. As infection progresses, traffic of inflamDCs into and out of chronic granulomas supports *Mycobacterium*-specific T cell priming in the lymph nodes by providing a continuous source of granuloma-residing antigen. However, once these newly primed T cells reach the granuloma, the phenotype of the local *inflam*DCs does not support IFNγ production, thereby contributing to bacterial persistence.

In closing, this review demonstrates that the monocyte-derived *inflam*DCs are often the dominant DC subset present at sites of inflammation induced by a variety of infectious agents. DCs are a necessary element of both the innate and adaptive immune response; therefore, making it important to increase our understanding of their function. At the site of infection *inflam*DCs have been shown to both induce and suppress the immune response, and can also critically effect the host-pathogen interaction. Many questions still remain regarding this DC subset. Some of them include: How does the *inflam*DC regulate components of the inflammatory response? What is their expected lifespan within an inflammatory lesion? What is the extent of their traffic to and from the inflammatory lesions? And are they able to sustain systemic immunity? In regards to *Mycobacterium*-host interactions and the anatomical structure of the granuloma, which is required for mycobacterial containment, the *inflam*DC plays a critical role in the pathogenesis. They facilitate dissemination of bacteria and bacterial antigens, which drives long-term immunity, and regulate intragranuloma processes; thereby representing an important part of the puzzle of long-term bacterial containment

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NK/DC Crosstalk in Anti-viral Response

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Abstract In recent years, it has been emphasized the role of the crosstalk between natural killer (NK) cells and monocyte-derived dendritic cells (moDCs) in the regulation of the early phases of innate immunity and of the subsequent adaptive immune responses.

NK cells and DCs coordinate their response communicating through direct cell-to-cell contact and soluble factors. NK cells appear to contribute to the quality control of immature DCs (iDCs) undergoing maturation. On the other hand, DCs may shape the magnitude of innate immune responses by modulating the NK-mediated cytolytic activity against tumors or infected cells.

Recent studies suggest that the cooperation between NK cells and DCs is also critical in several anti-viral responses. In particular, NK cells are capable of effectively counteracting viral immune evasion strategies by eliminating infected DCs, that display impaired antigen presenting functions, thus indirectly favoring the development of adaptive immune responses to viral antigens cross-presented by healthy DCs.

Keywords Natural killer cells • Dendritic cells • Viral infection • Activating receptors • Cytokines

1 Introduction

Natural killer (NK) cells and monocyte-derived dendritic cells (moDCs) are cells of innate immunity that cooperatively interact to determine the course of an innate response and its switch to adaptive immunity (Gerosa et al. 2002; Piccioli et al. 2002;

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Moretta 2002; Zitvogel 2002; Cooper et al. 2004; Walzer et al. 2005). NK cells are particularly implicated in defense against tumors and viral infections, which they provide through killing of transformed cells and the production of cytokines, such as interferon-gamma (IFN-γ) (Trinchieri 1989). MoDCs (DCs) represent the most potent antigen presenting cells (APC) and play a crucial role in the initiation of an immune response (Banchereau and Steinman 1998). Sites of NK–DC interactions may be either lymphoid organs (Ferlazzo et al. 2004a, b; Ferlazzo and Munz 2004; Martin-Fontecha et al. 2004) or non-lymphoid peripheral tissues (Moretta 2002; Della Chiesa et al. 2005). In tissues, DCs, in an immature form (iDCs), have efficient mechanisms for the detection and the uptake of pathogens. Microbial stimulation causes DCs to convert from the immature state, in which they induce abortive or tolerogenic T cell responses, to a mature state, in which they elicit a productive adaptive response (Moretta et al. 2005; Agaugué et al. 2008). In particular, upon antigen-uptake, DCs undergo maturation, by increasing their expression of MHC and co-stimulatory molecules. In addition they amplify their secretion of chemokines and proinflammatory cytokines (Banchereau et al. 2000). The soluble factors released by DCs can recruit NK cells to the infected tissue where the two cell types can communicate through both direct cell-to-cell contact and release of soluble factors, thus coordinating their response. DC-derived IL12 and NK-derived IFN-γ create a positive feedback loop that drives cell-mediated T helper1 (Th1) immunity (Moretta et al. 2005).

It has been demonstrated that activated NK cells, via the NKp30 receptor, recognize and kill iDCs while sparing mature DCs (mDCs). The cytolytic effect against iDCs is mediated by a phenotypically defined subset of NK cells characterized by the expression of CD94/NKG2A and by the lack of Killer Ig-like Receptors (KIRs) for self-HLA class I molecules (Ferlazzo et al. 2002; Della Chiesa et al. 2003). By this mechanism, termed "NK cell-mediated editing of DCs", and by the release of cytokines, that favor the process of DC maturation, NK cells appear to contribute to the quality control of DCs undergoing maturation toward mDCs. On the other hand, DCs may shape the magnitude of innate immune responses by modulating the NK-mediated cytolytic activity as well as their cytokine production.

Importantly, recent studies provide novel insights on the role played by the cross-talk between NK cells and DCs and demonstrate that NK cells can efficiently react against infected DCs, overcoming viral immune evasion strategies (Draghi et al. 2007; Magri et al. 2011).

2 NK Cell Receptors

Human NK cell function is regulated by a balance between activating and inhibitory receptors. Some inhibitory receptors recognize MHC class I molecules, which are present on virtually all autologous healthy cells, and prevent NK cell attack against these cells. Loss of MHC class I molecules from autologous cells, a frequent event that occurs during viral infection or tumor transformation, can lead to NK cell activation, as proposed by the "missing self-hypothesis" (Kärre 2008), provided that

activating receptors are engaged. The MHC-specific inhibitory NK cell receptors are represented by the KIRs (also referred to as CD158) that are able to distinguish among different HLA-A, -B and -C allotypes (Moretta et al. 1996) and the CD94/NKG2A heterodimer that directly recognizes HLA-E, a non-classical MHC molecule characterized by a limited polymorphism (Braud et al. 1998).

The activating receptors involved in the process of natural cytotoxicity have been identified more recently and they are represented by NKp46, NKp30 and NKp44 (collectively termed Natural Cytotoxicity Receptors, NCR) (Moretta et al. 2000). NKp46 and NKp30 are on most human peripheral blood NK cells, whereas, NKp44 is only on activated NK cells (Sivori et al. 1997; Pessino et al. 1998; Vitale et al. 1998; Pende et al. 1999). The cellular ligands recognized by these receptors are still elusive, with the exception of B7-H6, a recently identified ligand for NKp30 (Brandt et al. 2009). In addition, it has been demonstrated that NKp46 binds the influenza hemagglutinin (HA) protein as well as the HA-neuraminidase of parainfluenzavirus (Mandelboim et al. 2001; Arnon et al. 2004). The central role of NCR in the NK-mediated tumor cell lysis was also underscored by the strict correlation existing between their surface density and the magnitude of the NK-mediated cytotoxicity. Indeed, only donors that expressed high NCR surface density (NCR^{bright}) killed efficiently most NK-susceptible tumor cell lines, whereas donors with NCR^{dull} phenotype were associated with a low cytolytic activity of NK cells (Sivori et al. 1999).

Other activating receptors involved in target cell recognition and lysis are NKG2D and DNAM-1. NKG2D mediates NK cytolysis of tumors and, at least in mice, of cytomegalovirus (CMV)-infected cells (Diefenbach and Raulet 2001; Sutherland et al. 2001). The endogenous ligands for human NKG2D are MHC class I chain-related proteins (MIC)A and MICB and another family of class I-related molecules, the UL16-binding proteins (ULBP)1–5. Some NKG2D ligands may be up-regulated in cells infected with CMV (Dunn et al. 2003; Wu et al. 2003), but the signals regulating the expression of MIC and ULBP in the context of other viral infections have yet to be defined. DNAM-1 recognizes the poliovirus receptor (PVR, CD155) and Nectin-2 (CD112), two members of the Nectin family that are also involved in cell-to-cell adhesion and in leukocyte extravasation (Raulet 2003; Bottino et al. 2005; Reymond et al. 2004).

Another group of stimulating surface molecules may contribute to the induction of NK cell triggering. Their ability to induce NK cell activation depend on the simultaneous co-engagement of other triggering receptors, including NKp46. Based on this characteristic, these molecules have been considered as co-receptors rather than as classical "primary" receptors. Analysis at clonal level has shown that these co-receptors' function is mainly restricted to NK cells expressing a NCR^{bright} phenotype. Three such co-receptor molecules are represented by 2B4 (specific for CD48) (Brown et al. 1998; Sivori et al. 2000; Moretta et al. 2001), NTB-A (that mediates homotypic interactions) (Bottino et al. 2001; Falco et al. 2004) and NKp80 (specific for AICL1) (Vitale et al. 2001; Welte et al. 2006).

Some studies have led to the identification and molecular characterization of a number of activating forms of KIRs, characterized by a short cytoplasmic tail lacking ITIM motifs (Moretta et al. 1995; Biassoni et al. 1996), but only for some of

them (such as KIR2DS1 and KIR2DS4) the specificity for HLA class I molecules has been demonstrated (Moretta et al. 1995; Stewart et al. 2005; Chewning et al. 2007; Graef et al. 2009).

In addition to these triggering receptors that recognize cellular ligands on tumor or infected cells, most NK cells express also CD16, an activating receptor (also known as Fc-γ-RIII) with low-affinity for the Fc portion of IgG (Perussia et al. 1983).

Finally, recent reports have revealed that NK cells also express different Toll-like receptors (TLRs), independent of their activation status. These receptors, including TLR3 (that recognizes dsRNA of viral origin), TLR9 (that recognizes microbial unmethylated CpG-DNA) and TLR2 (that recognizes bacterial lipoprotein, BLP), allow NK cells to respond both to viral and bacterial products (Sivori et al. 2004; Marcenaro et al. 2008).

3 NK Cell Subsets in Peripheral Tissues and Lymphoid Organ

Different NK cell subsets exist that display differences in their cytolytic activity, cytokine production and homing capabilities. In particular, $CD56^{bright}$ $CD16^{-}$ KIR^{-} $NKG2A^{+}$ NK cells, that largely predominate in lymph nodes, have little cytolytic activity but release high amounts of cytokines; whereas $CD56^{dull}$ $CD16^{+}$ KIR^{+} and/or $NKG2A^{+}$ NK cells, that predominate in peripheral blood and inflamed tissues, display lower cytokine production, but potent cytotoxicity (Cooper et al. 2001). $CD56^{dull}$ NK cells carry homing receptors for inflamed peripheral tissues (CXCR1, CX3CR1 and ChemR23) (Parolini et al. 2007; Marcenaro et al. 2009) and perforin to rapidly mediate cytotoxicity, whereas $CD56^{bright}$ NK cells express CCR7, the homing receptor for Secondary Lymphoid Compartments (SLCs), and lack perforin but produce large amounts of IFN-γ (Mailliard et al. 2005; Moretta et al. 2008; Marcenaro et al. 2009). The developmental relationship between these two NK cell subsets remains controversial although recent reports demonstrated that $CD56^{bright}$ $KIR^{-}$and $CD56^{dull}$ KIR^{+} and/or $NKG2A^{+}$ NK cells correspond to sequential steps of differentiation and that secondary lymphoid tissues may represent sites of NK cell development and self-tolerance acquisition (Ferlazzo et al. 2004b; Freud and Caligiuri 2006; Romagnani et al. 2007).

It is important to note that, during maturation, NK cells undergo an "education" process based on the recognition of self-HLA class I molecules that allows the acquisition of a full functional competence. These data clearly suggest that this process must be confined to those NK cells that express at least one receptor for self-HLA class I although, even in normal individuals, some NK cells lacking these receptors are generated. Such cells, however, would not be dangerous since, in the absence of adequate "education", they would not acquire functional competence. KIR acquisition in lymph nodes appears to be confined to NK cells that have previously been subjected to the "education" process (Anfossi et al. 2006), upon rec-

ognition of HLA-E by NKG2A. Consequently, in lymph nodes a "switch" from a KIR^- to a KIR^+ phenotype occurs rather than a step of NK cell maturation from immature NK precursors.

4 Crosstalk Between NK Cells and Dendritic Cells

Different studies have highlighted an important immunomodulatory role of the crosstalk between NK cells and different types of DCs, in particular with moDCs (Gerosa et al. 2002; Moretta 2002; Piccioli et al. 2002; Zitvogel 2002; Cooper et al. 2004; Marcenaro et al. 2005a; Walzer et al. 2005). The interaction with DCs can modulate NK-cell functions as the result of mechanisms of cell-to-cell contact (favoring receptor–ligand interactions) and of the activity of soluble factors. This close cellular contact appears to be required for promoting a series of events, including DC-induced NK-cell proliferation, NK-mediated killing of iDCs and NK-dependent DC maturation. During this interaction, DCs, that are undergoing maturation after antigen uptake, release cytokines that will deeply influence the functional behavior of NK cells. For example, DC-derived IL12 is crucial for inducing IFN-γ release and enhancing cytotoxicity by NK cells. Thus, DCs may act as activators of NK response in the early phases of the immune response, that is, before an adaptive immune response had been evoked and T cell-derived cytokine, such as IL2, could be produced (Ferlazzo et al. 2002; Vitale et al. 2004). In turn, NK cells can influence the progression of DC maturation by releasing cytokines, including tumor-necrosis-factor-alpha (TNF-α) and IFN-γ, and by providing necrotic material from the killing of tumors and virus-infected cells (Vitale et al. 2005).

The orchestrated response of NK cells and different types of DCs is likely regulated by pattern recognition receptors (PRRs), including TLRs. In particular, TLR3 is expressed by both NK cells and DCs, whereas TLR9 is expressed by both NK cells and plasmacytoid DCs (pDCs). Thus, dsRNA of viral origin can simultaneously act on TLR3 expressed by both iDCs and NK cells, recruited by chemokine gradients to inflammatory sites. In the presence of IL12 (released by DCs after TLR engagement), NK cells respond to dsRNA by increasing their anti-tumor cytotoxicity and become capable of killing iDCs (Sivori et al. 2004). It has been proposed that killing of iDCs (NK cell-mediated "editing" of DCs undergoing maturation) may be crucial for the selection of the most appropriate DCs which, upon migration to SLCs, will be responsible for T cell priming.

Remarkably, responses to microbial CpG-DNA should involve pDCs rather than DCs. Indeed, in humans, TLR9 is expressed by NK cells and pDCs, but not by DCs. Thus, dsRNA may trigger simultaneously NK cells and iDCs via TLR3, whereas CpG-DNA may simultaneously stimulate NK cells and pDCs via TLR9. In this context, a soluble factor released by pDCs, such as IFN-α, could play an important role in supporting the activation of TLR9-responsive NK cells (Della Chiesa et al. 2006). Recently, we have described how extracellular microbial CpG-DNA can be internalized into NK cells to interact with intracellular TLR9. In particular,

we have demonstrated that NK cells certain KIRs characterized by the expression of the extracellular D0 domain (such as KIR3DL2), can bind, internalize and shuttle CpG-ODN to early endosomes (where TLR9 are located). Thus, KIRs function has been extended from receptors for HLA class I recognition to sensors of microbial products (Sivori et al. 2010a, b).

NK cells also express TLR2, a receptor expressed by other innate cells, including APC and involved in the recognition of bacterial lipoprotein. Our recent studies suggest that bacille Calmette-Guérin (BCG), by inducing simultaneous activation of NK and DCs via their "shared" TLR2, can promote efficient bidirectional NK–DC interactions (Marcenaro et al. 2008).

Other innate cells, that are either resident (such as Mastocytes) or recruited (such as Eosinophils) at the inflammatory sites, may participate to the early phases of innate immune responses by releasing opposite sets of cytokines in response to the engagement of their TLRs by one or another pathogen-derived product. By the release of either type-1 or type-2 cytokines, these cells may further modulate the ability of NK cells to induce DCs editing and maturation. Thus while, short-term exposure to APC-derived IL12 promotes the release by NK cells of high levels of both IFN-γ and TNF-α and the acquisition of cytolytic activity, exposure to IL4 results in poor cytokine production and low cytolytic activity. Accordingly, NK cells exposed to IL12 may favor the differentiation/selection of appropriate mDCs for subsequent Th1 cell priming in lymph nodes. On the contrary, NK cells exposed to IL4 would not exert DC selection, may impair efficient Th1 priming and favor either tolerogenic or Th2-type responses (Marcenaro et al. 2005b; Agaugué et al. 2008). Thus, the outcome of the cognate interaction between NK and DCs, may be conditioned by the nature of invading pathogens (Della Chiesa et al. 2005).

5 NK/DC Crosstalk in CMV Infection

Although persistent viruses can encode viral evasion genes that allow interference with the generation of the immune response, cooperation between NK cells and DCs is critical in several anti-viral responses (Lodoen and Lanier 2005; Belz et al. 2009). The central role of NK cells in anti-viral response was initially highlighted in mice that after NK-depletion became highly susceptible to murine CMV (MCMV) infection (Bancroft et al. 1981; Biron 1999). It was later determined that the viral gene m157 (a distant homologue of MHC class I proteins), recognized by the murine NK cell activating receptor Ly49H, provided a mechanism for direct recognition of MCMV infection (Arase et al. 2002; Smith et al. 2002). Moreover, it has been described that mice, which are susceptible to MCMV infection, fail to amplify the NK cell populations in late infection and therefore fail to limit viral replication. Despite the importance of NK cells in protection from viral infection, activation of NK cells coordinating the response appeared predominantly to depend critically on signals originating from the DCs (Andrews et al. 2003; Degli-Esposti and Smyth 2005; Andoniou et al. 2005; Barr et al. 2007). DCs, specifically CD8α DCs, are a

major target of MCMV infection (Andrews et al. 2003) and following infection can secrete IL18 and IL12 in addition to IL2 and type-I IFN (IFN-α and IFN-β), thus promoting NK cell IFN-γ production and enhancing NK cytotoxic function (Orange and Biron 1996; Gerosa et al. 2002; Granucci et al. 2004). In particular, IL18 appears to play a critical role in NK cell activation. In fact, depletion of IL12, either in MCMV or HSV infection, does not appear to significantly alter the capacity of NK cells to produce IFN-γ, while removal of IL18 diminished the capacity of DCs to stimulate NK cells (Andoniou et al. 2005; Barr et al. 2007).

Magri et al. (2011) have recently demonstrated that human NK cells may efficiently react against human CMV (HCMV)-infected DCs, overcoming viral immune evasion strategies. HCMV-infected DCs down-regulate the expression of HLA class I molecules and specifically activate autologous NK cell populations. NKG2D has been reported to be involved in the response to MCMV-infected DCs, and is believed to participate as well in the defense against HCMV. In this case, the evidence is essentially indirect and based on the identification of viral immune evasion molecules in HCMV (i.e., UL16, UL142 and miR–UL112) that selectively interfere with the surface expression of NKG2D ligands, similar to those employed by MCMV (m138, m145, m152, m155) (Jonjic et al. 2008a). In fact, in humans, NKG2D ligands appear virtually undetectable in infected DCs, reflecting the efficiency of immune evasion mechanisms, and explain the lack of antagonistic effects of NKG2D-specific mAb. By contrast, DNAM-1 and DNAM-1 ligands specific mAbs inhibit the NK response at 48 h post-infection. Time-course analysis revealed that at 48 h post-infection the expression of both DNAM-1 ligands on DC was minimally altered as compared to a marked down-regulation detected at 72 h in infected cells. The data indicate that the DNAM-1 receptor plays a relevant role in the NK cell response at early stages of HCMV infection, while the effects of HCMV-dependent down-regulation of DNAM-1 ligands are perceived at later stages (Magri et al. 2011).

The NK-mediated response to HCMV-infected moDCs is also dependent on NKp46, whereas NKp30 plays a dominant role in the response of IL-2-activated NK cells to immature non-infected DCs (Ferlazzo et al. 2002) (Fig. 1). NKp46 was originally reported to interact with influenza HA, contributing with NKG2D to trigger the NK cell response against influenza-infected DCs (Mandelboim et al. 2001; Arnon et al. 2004; Gazit et al. 2006; Draghi et al. 2007; Mao et al. 2009). By contrast, no HCMV molecules interacting with this NCR have been identified so far and the nature of its cellular ligands on DCs remains unknown. It is of note that the molecular basis for NKp30-mediated recognition of non-infected DCs is also uncertain, as they do not express the B7-H6 ligand (Brandt et al. 2009). Notably also the ligands for NKp46 and NKp30 are partially reduced at 72 h upon HCMV infection, thus stressing the importance of the kinetics of expression of immune evasion mechanisms. Downregulation of NCR ligands is more evident for NKp46 and has an important impact on the NK cell-mediated response (Magri et al. 2011).

It is conceivable that NKp46 may trigger NK activation simply as a result of the down-regulation of HLA class I expression (included HLA-E) in infected cells

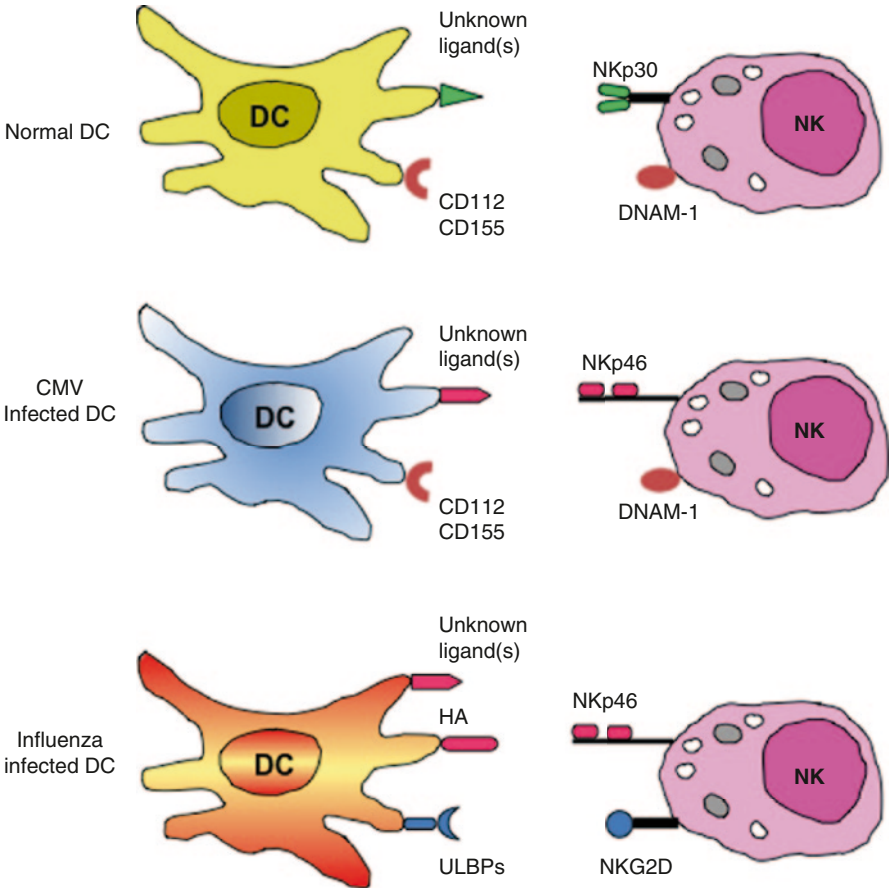

Fig. 1 NK cell activation by autologous normal or infected DCs. NK cell activation is induced by different soluble factors released by normal or infected DCs and by the engagement of different NK cell receptors recognizing cell surface ligands expressed by normal or virus-infected DCs. NKp30 and DNAM-1 receptors play a dominant role in the response of NK cells to non-infected DCs (*upper panel*). The NK-mediated response to HCMV-infected DCs is primarily dependent on NKp46 and DNAM-1 (*middle panel*). The precise molecular interactions occurring between NKp30 or NKp46 receptors and their cellular ligands expressed on normal or HCMV-infected DCs remain, at least in part, to be defined since these molecules have yet to be identified. In influenza-infected DCs, NKp46 appears to interact with influenza hemagglutinin (HA) and to contribute with NKG2D to NK cell triggering. In this case, however, the involvement of additional ligand(s) for NKp46 cannot be ruled out (*lower panel*)

resulting in the loss of inhibitory signaling, in agreement with the "missing self-hypothesis".

These results provide an insight on key receptor–ligand interactions involved in the NK cell response against HCMV-infected DCs and support that human NK cells are capable of effectively counteracting viral immune evasion strategies and responding to infected DCs, that have impaired their antigen presenting functions,

thus indirectly favoring the development of adaptive immune responses to viral antigens cross-presented by healthy DCs.

6 NK/DC Crosstalk in Influenza Infection

Previous studies have demonstrated that NK cells are also involved in host defenses against influenza virus infection. Virus-infected accessory cells, including epithelial cells, DCs, macrophages and even T cells, induce NK cell activation. The activated NK cells, in turn, kill virus-infected cells (He et al. 2004; Spies and Groh 2006; Draghi et al. 2007). The activation of NK cells is dependent on the cytokines released from infected cells as well as on the interaction between NK cell receptors and the corresponding ligands expressed on the surface of infected accessory cells. Draghi et al. (2007) analyzed the molecular mechanisms by which influenza-infected human DCs activate resting, autologous NK cells and demonstrated that NKG2D and NKp46 NK receptors play a central role in human NK–DC cooperation in the response to influenza infection (Fig. 1). These results agree with previous data reporting that mice lacking NKp46 fail to control influenza infection (Gazit et al. 2006). In particular, three events have been distinguished in the process of NK cell activation by autologous influenza-infected DCs: acquisition of the activation marker CD69, up-regulation of cytolytic activity and enhancement of IFN-γ production. These effects depend on both soluble factors secreted by infected DCs and signals arising from direct cellular contact with infected DCs. Enhanced NK cell cytolysis is primarily dependent on DC-derived type-I IFN and does not require direct contact between NK cells and DCs. Increased CD69 surface expression requires not only DC-secreted type-I IFN but also direct contact with DCs, mediated by NKp46 and NKG2D receptors on NK cells and their respective ligands (the influenza protein HA and ULBPs) on DCs (Draghi et al. 2007).

In response to the pressure of NK cells, influenza virus has developed evasion strategies to counteract their functions (Jonjic et al. 2008b; Lodoen and Lanier 2005). Different studies suggest that influenza virus directly target NK cells as part of its immunoevasion strategies. Mao et al. (2009) have demonstrated that this virus infects human NK cells and induces marked cell apoptosis. Patients with severe influenza infection were shown to have diminished NK cells in peripheral blood and an almost complete absence of pulmonary NK cells (Welliver et al. 2007; Heltzer et al. 2009). Recently, Mao et al. (2010) have demonstrated that both the intact influenza virion and the free HA protein can inhibit the cytotoxicity of fresh and IL2-activated primary human NK cells. Indeed, HA can bind and internalize into NK cells via the sialic acids. This interaction does not decrease NKp46 expression, but causes the downregulation of the ζ chain associated with NKp46 (and NKp30) through the lysosomal pathway, and this event induces the decrease of NK cell cytotoxicity mediated by NKp46 and NKp30. These findings suggest that influenza virus may develop evasion strategy to block NK cell innate immune defense and facilitate viral transmission.

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Innate-Adaptive Crosstalk: How Dendritic Cells Shape Immune Responses in the CNS

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Abstract Dendritic cells (DCs) are a heterogeneous group of professional antigen presenting cells that lie in a nexus between innate and adaptive immunity because they recognize and respond to danger signals and subsequently initiate and regulate effector T-cell responses. Initially thought to be absent from the CNS, both plasmacytoid and conventional DCs as well as DC precursors have recently been detected in several CNS compartments where they are seemingly poised for responding to injury and pathogens. Additionally, monocyte-derived DCs rapidly accumulate in the inflamed CNS where they, along with other DC subsets, may function to locally regulate effector T-cells and/or carry antigens to CNS-draining cervical lymph nodes. In this review we highlight recent research showing that (a) distinct inflammatory stimuli differentially recruit DC subsets to the CNS; (b) DC recruitment across the blood-brain barrier (BBB) is regulated by adhesion molecules, growth factors, and chemokines; and (c) DCs positively or negatively regulate immune responses in the CNS.

Keywords Dendritic cell (DC) • Inflammatory (iDC) • Plasmacytoid (pDC) • Conventional (cDC) • Central nervous system • Chemokines • T-cell responses

1 Introduction to Dendritic Cells and Their Subsets

Since the discovery of dendritic cells (DCs) (Steinman et al. 1975), an extensive body of literature has accumulated showing that these cells are the most efficient stimulators of T-lymphocytes. DCs are hematopoietic cells derived from pre-DCs that migrate from the bone marrow into circulation and eventually give rise to sev-

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eral DC subsets in lymphoid and non-lymphoid tissues (Banchereau and Steinman 1998). From studying skin (Randolph et al. 2005a, b), intestine (Milling et al. 2010; Tezuka and Ohteki 2010), granulomatous tissue (Schreiber et al. 2010), and lung (Hintzen et al. 2006), it is well accepted that in the steady state immature migratory DCs uptake antigen from the local environment, process these antigens, and present them on their surface in the context of major histocompatibility (MHC) molecules. Immature DCs express low levels of MHC and costimulatory molecules (CD80, CD83, CD86) below the threshold for T-cell activation. In the absence of inflammatory signals, immature DCs migrate through lymphatic vessels to regional lymph nodes at basal rates and present self-antigen to naïve T-cells, inducing tolerance by way of T-cell anergy, apoptosis, and regulatory T (Treg)-cell differentiation (Torres-Aguilar et al. 2010). In contrast, activated maturing DCs upregulate MHC and costimulatory molecules, change their expression of chemokine receptors to promote active migration to the T-cell areas in regional lymph nodes, and there activate naïve antigen-specific T-cells (Steinman et al. 1997; Lanzavecchia 1999; Caux et al. 2002).

Under steady state conditions two types of DCs can be distinguished: plasmacytoid (pDCs) and conventional (cDCs). Both pDCs and cDCs are derived from a committed DC progenitor (CDP) present in the bone marrow (Naik et al. 2007). PDCs are notable for their plasmoid morphology (Corcoran et al. 2003) and their ability to secrete copious amounts of interferon (IFN)-α upon activation (Cella et al. 1999; Siegal et al. 1999; Ito et al. 2004).CDPs give rise to pre-DCs that enter the blood and develop into cDCs in lymphoid and non-lymphoid tissue. Pre-DCs can enter lymphoid organs and become "lymphoid tissue resident DCs", or they can give rise to migratory antigen-collecting DCs that continuously survey most non-lymphoid tissues, pick up antigens and carry them to lymph nodes (Randolph et al. 2005a, b). Pre-DCs that enter non-lymphoid tissue give rise to all $CD103^+$ and some $CD103^-$ migratory cDCs. The fact that these cells need to function in radically different tissue environments might itself provide a reason why there are several distinct subsets of migratory DCs (Steinman and Idoyaga 2010). Even in the same lymphoid organ, different DC subsets can be detected amongst non-migratory DCs including $CD8^+$ and $CD8^-$ cDCs (Shortman and Heath 2010). Compared to their counterparts, $CD8^+$ lymphoid tissue resident cDCs and $CD103^+$ migratory cDCs have been shown to be efficient cross-presenters capable of priming cytotoxic lymphocyte responses (Liu and Nussenzweig 2010). Additionally, TNF-α and iNOS producing (TIP)-DCs also referred to as "inflammatory" (iDCs) have also been reported to accumulate in target tissues during immune responses (Serbina et al. 2003; Lowes et al. 2005; Engel et al. 2006). The origin of these DCs is still debated, but research has indicated that they may be derived from tissue infiltrating monocytes as well as blood pre-DCs (Romani et al. 1996; Randolph et al. 1998, 2002; Geissmann et al. 2003; Tacke and Randolph 2006; Shortman and Naik 2007; King et al. 2009). We will review recent work investigating recruitment of pDC, cDC, and iDC subsets to the CNS in response to different inflammatory stimuli, how these cells are

recruited, and how these DC subsets function to positively or negatively regulate CNS immunity.

2 Dendritic Cell Subsets Accumulate in the CNS During Neuroinflammation

2.1 Dendritic Cells in the Healthy CNS

The CNS is considered an immune privileged tissue due to the relative dearth of resident DCs and the presence of the blood-brain barrier (BBB), which limits immune cell immigration. While healthy CNS parenchyma is essentially devoid of MHC II^+ DCs, recent studies have demonstrated that DCs do populate several CNS compartments under steady state conditions. Based on morphology and surface expression MHC II^+ cDCs have been identified in human choroid plexus (Serot et al. 1997) as well as the meninges and choroid plexus of rats (McMenamin 1999, 2003; Chinnery et al. 2010). Both the meninges and the choroid plexus are highly vascularized, suggesting that DC accumulation in these organs might be due to increased recruitment from blood. Indeed, in wild-type mice reconstituted with bone marrow from mice expressing enhanced yellow fluorescent protein under the DC marker CD11c (CD11c-eYFP), DCs were fully repopulated in these tissues 8 weeks after reconstitution (Chinnery et al. 2010), suggesting that these CNS DCs are replenished by continuous recruitment from blood. Other studies employing CD11c-eYFP mice have revealed ovoid, bipolar, and dendriform $CD11c^+$ DCs extensively distributed in the layer II of the piriform cortex as well as periventricular tissues including the rostral migratory stream, which extends caudally from the subventricular zone to the anterior commisure of the olfactory bulb (Bulloch et al. 2008). More recently, $CD11c^+$ DC-like cells have been detected in the parenchyma surrounding the vasculature of healthy mouse brain tissue (Prodinger et al. 2010). These DC-like cells seem to be integrated into the glial wall that delimits the perivascular space from the CNS parenchyma, and thus these cells may be poised for communication with infiltrating immune cells.

2.2 Dendritic Cell Subset Accumulation in the CNS During Neuroinflammation

It has been proposed that DCs might be part of the immune environment of the CNS, as during neuroinflammation or CNS injury iDCs can be identified in the CNS (Pachter et al. 2003). A large portion of the current literature investigating DC involvement in neuroinflammation comes from experimental autoimmune en-

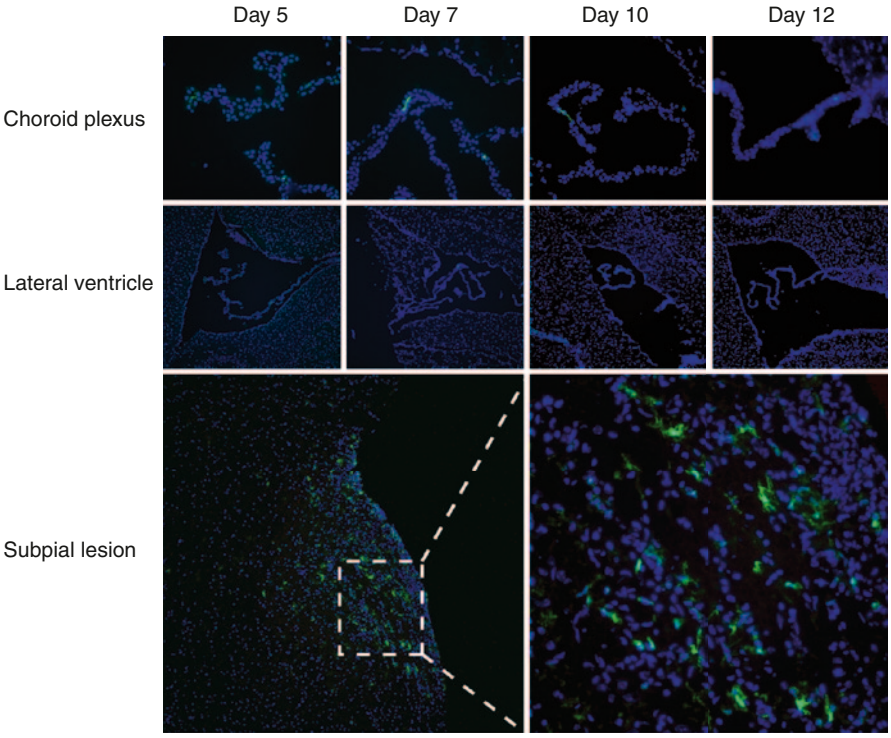

Fig. 1 CD11c-eYFP cells accumulate in the CNS following MOG immunization prior to clinical onset of EAE. CD11c-eYFP mice were immunized s.c. with MOG in complete Freund's adjuvant (CFA) and 5–12 days later their brains were isolated for analysis by fluorescent microscopy. CD11c-eYFP^{+} cells are shown in choroid plexus and lateral ventricles 5–12 days after immunization (*top*). CD11c-eYFP cells were first detected in the subpial space on day 12 after immunization (*bottom*)

cephalomyelitis (EAE)—a mouse model of MS. We have shown that DCs rapidly accumulate in the CNS of mice with EAE and that their accumulation precedes clinical onset. Upon histological survey we found CD11c-eYFP DCs accumulating in choroid plexus, periventricular tissue, and subpial spaces prior to clinical onset (Fig. 1). This corresponds well with previous reports showing CD205^{+} CD209^{+} cDCs accumulating in the meninges, choroid plexus, and subpial space of the spinal cord preclinically and in perivascular cuffs circa demyelinating lesions during acute disease (Serafini et al. 2000). While several DC subsets accumulate in the CNS of EAE mice, iDCs expressing the myeloid cell marker CD11b^{+} predominate (Matyszak and Perry 1996; Suter et al. 2000; Bailey et al. 2007). These cells have been suggested to be derived from circulating inflammatory Ly6c^{hi} monocytes that are released from the bone marrow during immune responses. Others have shown that Ly6c^{hi} monocytes are enriched in bone marrow and blood prior to their accumulation in CNS during preclinical EAE and that these CNS infiltrating monocytes dif-

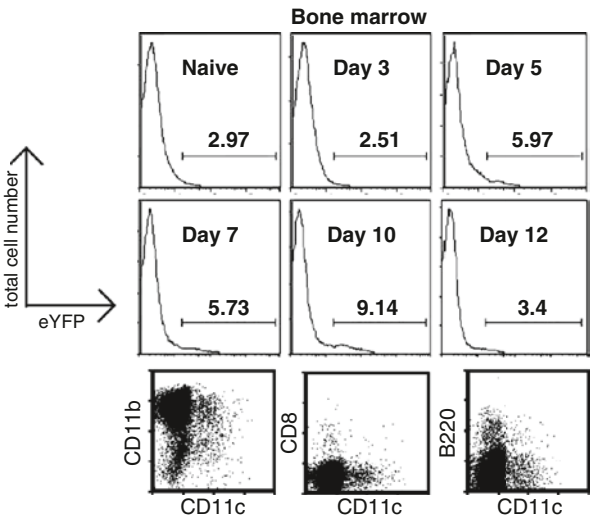

Fig. 2 CD11c$^+$ CD11b$^+$ cells transiently accumulate in bone marrow following MOG immunization. CD11c-eYFP mice were immunized s.c. with MOG in CFA and 5–12 days later their bone marrow was isolated for analysis by flow cytometry using mAb for CD11b, CD8, and B220. Histograms show fraction of total mononuclear cell population expressing eYFP. *Dot plots* show representative surface expression of DC markers on bone marrow cells

ferentiate into both macrophage and DC-like CD11c$^+$ cells (King et al. 2009). Likewise, using CD11c-eYFP mice we observed that the frequency of CD11b$^+$CD11c$^+$ cells in bone marrow is increased on days 5–10 following EAE induction (Fig. 2), suggesting that along with monocytes other DC precursors may be released from the bone marrow following EAE induction.

Mature myelin-containing CD209$^+$ cDCs (Plumb et al. 2003; Serafini et al. 2006; Cudrici et al. 2007) as well as CD123$^+$ pDCs (Lande et al. 2008) have been found in meninges, perivascular cuffs, active demyelinating lesions, and non-lesional gray matter of patients with MS. Both cDCs and pDCs are present at low levels in cerebrospinal fluid (CSF) of patients with non-inflammatory neurological disease and both DC subsets are increased in CSF of patients with neuroinflammatory conditions (Pashenkov et al. 2001). Patients with bacterial meningitis and Lyme neuroborreliosis have elevated levels of DCs in their CSF, with cDCs predominating in bacterial meningitis and pDCs predominating in neuroborreliosis (Pashenkov et al. 2002). Circulating levels of cDC and pDC precursors were reported to transiently decrease following acute stroke, and CD209$^+$ cDCs and CD123$^+$ pDCs have been detected in postmortem brain tissue from stroke patients (Yilmaz et al. 2010).

DCs have also been shown to accumulate in the CNS in mouse models of prion disease, viral encephalitis, brain ischemia, parasitic CNS infections, and CNS bacterial infections (Fischer et al. 2000; Kostulas et al. 2002; Reichmann et al. 2002; Trifilo and Lane 2004; Rosicarelli et al. 2005; Brehin et al. 2008; Gelderblom et al. 2009; Steel et al. 2009; Savarin et al. 2010). CNS infection with neurotropic mouse hepatitis virus (Trifilo and Lane 2004) or vesicular stomatitis virus (Steel et al. 2009) promotes the accumulation of predominantly CD11b$^+$ cDCs, whereas CNS infection with west nile virus promotes the selective recruitment of pDCs to CNS as

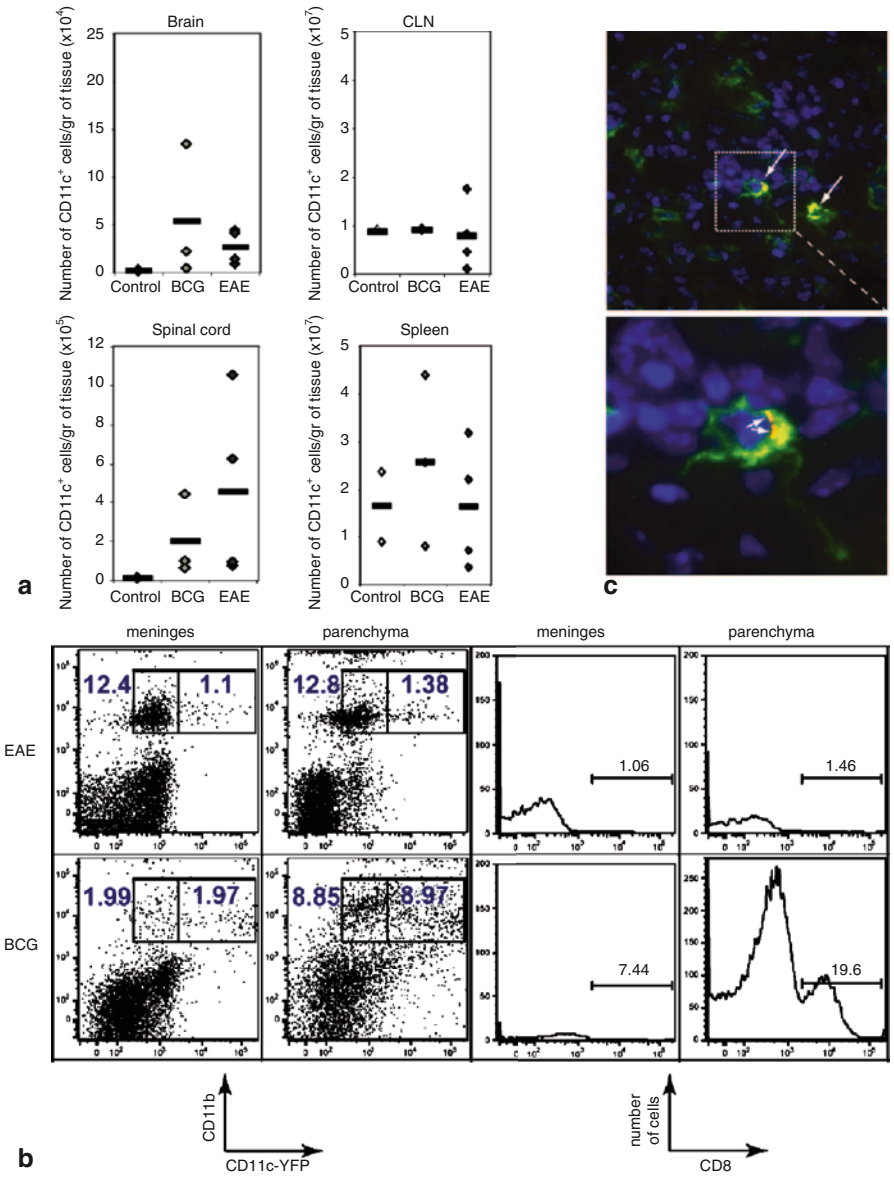

Fig. 3 EAE and intracerebral (i.c.) BCG infection lead to differential recruitment of CD11c$^+$ subsets. More CD8$^+$, CD11c^{high} cells accumulate in brain tissue following BCG infection compared to EAE. 14 days after MOG immunization or 35 days after i.c. BCG infection mice were sacrificed and perfused transcardially. Single cell suspensions were prepared from brain, spinal cord, spleen, and cervical lymph nodes and stained with anti-CD11c mAb. **a** Graphs show total number of CD11c$^+$ cells/gram tissue. CD11c$^+$ cells accumulate in the brain of mice i.c. infected with BCG and the spinal cord of mice s.c. immunized with MOG. **b** Expression of CD11b and CD11c among total mononuclear cells in meninges and brain parenchyma is displayed. Boxes and numbers show percentage of CD11c^{low}Cd11b$^+$ and CD11c^{high}Cd11b$^+$ DC subsets infiltrating the brain (*left 2*

early as 4 days after infection (Brehin et al. 2008). In models of focal brain ischemia and reperfusion injury $CD11b^+$ DCs have been reported to accumulate at the CNS as soon as 1 h after occlusion and persist for weeks, occupying the border zone of the stroke penumbra area (Kostulas et al. 2002; Reichmann et al. 2002; Gelderblom et al. 2009). Chronic CNS infection with *Toxoplasma gondii* promotes accumulation of $33D1^+CD8\text{-}F4/80^+$ myeloid DC-like cells in CNS though these cells seem to be brain-derived as *T. gondii* infection of primary brain cell culture promoted differentiation of $33D1^+$ "dendriform" cells (Fischer et al. 2000).

In order to further investigate whether different inflammatory challenges alter DC recruitment to the CNS, we compared the absolute number and phenotype of CNS-infiltrating $CD11c^+$ cells in mice following intracerebral (i.c.) mycobacterial infection or EAE induction. We demonstrated that $CD11c^+$ cells could be detected in higher numbers in the spinal cord of mice with EAE compared to brain tissues; whereas i.c. infection with Bacillus Calmette-Guérin (BCG) induced a higher level of recruitment of $CD11c^+$ cells to the brain compared to spinal cord (Fig. 3a). The $CD11c^+$ DC population can further be divided into two subtypes based on higher or lower surface expression of CD11c. $CD11c^{low}$ $CD11b^+$ DCs were a dominant subset among DCs isolated from mice during EAE. In contrast, BCG infection of the CNS resulted in increased recruitment of the $CD11c^{high}$ $CD11b^+$ subpopulation, represented by the increased $CD11c^{high}$: $CD11c^{low}$ ratio in both meninges and brain parenchyma(Fig. 3b left). Interestingly, we observed that—as in liver—CNS mycobacterial infection promoted the accumulation of DCs highly expressing the coinhibitory molecule B7H2 (Lee et al. 2009a). Additionally, we found that mycobacterial infections promoted higher CNS recruitment of the $CD8^+$ and $CD11c^{high}$ subpopulations of DCs compared to EAE (Fig. 3b right). On brain cryosections from BCG infected mice we identified dsRed BCG rods residing in $CD8^+CD11c^+$ cells (Fig. 3c), which supports data showing that $CD8^+$ DCs play an important role in mediating BCG-specific immune responses.

While $CD8^+$ cDCs are usually restricted to lymphoid tissue, it is possible that pre-DCs migrating into non-lymphoid tissues can differentiate into $CD8^+$ "lymphoid tissue resident DCs" under conditions of ectopic lymphogenesis—a feature of chronic inflammation that has been shown to be induced by mycobacterium in lung (Maglione et al. 2007). Additionally, it is suggested that the formation of tissue granulomas—a histological hallmark of mycobacterial infections—shares several features with tertiary lymphoid tissue development (Voswinkel et al. 2008; Lamprecht et al. 2009; Day et al. 2010). Our results are consistent with previous reports showing higher accumulation of cDCs during CNS mycobacterial delayed type hypersensitivity responses compared to EAE (Matyszak and Perry 1996).

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columns) and CD8 expression among total $CD11c^+$ brain-infiltrating DCs (*right 2 columns*). **c** Brain cryosection from BCG infected mouse stained with anti-CD11c (*green*), anti-CD8 (*red*), and DAPI (*blue*). On upper image, *arrows* point at double positive $CD8^+CD11c^+$DCs at 1000x total magnification. *Dashed area* is digitally magnified 4x and shown in lower image. *Arrowheads* on lower image point at dsRed BCG rods residing in a $CD8^+CD11c^+$DC

3 Mechanisms of Dendritic Cell Migration into the CNS

3.1 Regulation of DC Migration Across the BBB: Adhesion Molecules

While several studies have documented the accumulation of DCs in the CNS during neuroinflammation, less is known about DC migration into the CNS, mechanistically. Natalizumab, a monoclonal antibody against α4 integrin used to treat MS, was shown to reduce DC accumulation in the CNS (del Pilar Martin et al. 2008). In another recent study, intravital microscopy during ongoing EAE demonstrated that immature blood DCs adhere to inflamed vessel endothelium in a β-1 integrin dependent manner (Jain et al. 2010). The α4β1 complex also known as VLA4 is known to bind VCAM-1, which is upregulated on the surface of endothelial cells during neuroinflammation (Shaftel et al. 2007; Gobel et al. 2011; Li et al. 2011). Indeed, VLA-4 has been widely implicated in leukocyte adhesion to brain microvessels during neuroinflammation; however, directed migration of vessel-associated DCs into demyelinating lesions in the CNS parenchyma is restricted by the presence of the BBB. This barrier is comprised of two layers, the vessel endothelium and the glia limitans. The endothelial cells that comprise the wall of post-capillary venules express occludin and form tight junctions, which restrict transmigration of immune cells and fluid exchange (Pachter et al. 2003). Surrounding this layer is the Virchow-Robin space (perivascular space), which is continuous with the CSF-filled subarachnoid space. The perivascular space is separated from the neuropil by a second layer called the glia limitans formed by a basement membrane surrounded by tightly interlocking astroglial endfeet (Pachter et al. 2003).

3.2 Regulation of DC Migration Across the BBB: Chemokines and Growth Factors

Migration across the endothelium and glia limitans is thought to critically depend upon specific DC growth factors and chemoattractants, which are expressed in the inflamed CNS. Granulocyte-macrophage colony stimulating factor (GM-CSF) and Fms-like tyrosine kinase 3 ligand (Flt3L) are growth factors that bind the DC receptors CD115 and FLT3, respectively. GM-CSF promotes expansion of monocytes and iDCs, whereas Flt3L is considered to be specific for the expansion of DCs and committed DC precursors. I.c. injection of Flt3L promotes the recruitment of pDCs (Curtin et al. 2006), whereas injection of GM-CSF—producing cells promotes the recruitment of monocytes and iDCs (Hesske et al. 2010). GM-CSF can also transform brain-resident microglia into DC-like cells and accumulation of DC-like cells in CNS tissues during chronic *T. gondii* infections has been shown to be GM-CSF dependent (Fischer et al. 2000). Additionally it has been shown that human blood monocytes can transform into mature DC-like cells following migration across ac-

tivated human BBB endothelial cells. These newly transformed cells upregulate markers of cDCs (CD209) and pDCs (CD123) as well as DC activation markers (HLA-DR, CD80, CD86, and CD83) and induce stronger effector T-cell responses than untransformed blood monocytes. Importantly, this transformation was dependent upon BBB-derived TGF-β and GM-CSF (Ifergan et al. 2008). More recently, it was shown that Ly6c^{hi} inflammatory monocytes emigrate from bone marrow and accumulate in blood prior to their accumulation in the CNS during EAE (King et al. 2009), and that this accumulation was promoted by GM-CSF which acted to promote production of the DC chemoattractant CCL2 (Serbina and Pamer 2006; Hesske et al. 2010).

The receptor for CCL2, CCR2, has been shown to be required for emigration of inflammatory monocytes from bone marrow during bacterial infection in mice (Serbina and Pamer 2006). CCL2$^{-/-}$ mice have impaired monocyte recruitment to the perivascular space during CNS viral infection (Savarin et al. 2010) and experiments in bone marrow chimera mice have shown that glia-derived CCL2 is required for recruitment of iDCs during EAE (Dogan et al. 2008), demonstrating that CCL2 is also involved in monocyte and DC immigration into CNS tissues. Indeed, CNS-specific overexpression of CCL2 leads to spontaneous asymptomatic accumulation of perivascular monocytes in the brain with little infiltration into the CNS-parenchyma (Toft-Hansen et al. 2006). Interestingly, conditional expression of Flt3L in these mice leads to the expansion of monocytes and DCs in the CNS and the onset of ascending hind-limb paralysis within 9 days of gene induction (Furtado et al. 2006). In summary, these data suggest that Flt3L promotes pDC recruitment to the CNS whereas the GM-CSF–CCL2 axis is involved in recruitment of monocyte-derived DCs. Indeed, CCL2$^{-/-}$, CCR2$^{-/-}$, and GM-CSF$^{-/-}$ mice as well as mice treated with CCL2 neutralizing antibodies are markedly resistant to EAE (Fife et al. 2000; Izikson et al. 2000; Zaheer et al. 2007).

Importantly, CCL2 does not seem to promote cell progression across the glia limitans, which is thought to be a rate-limiting step in the induction of neuroinflammation. Pertussis toxin can be used in the induction of EAE, and pertussis toxin-induced encephalitis is associated with disruption of the glia limitans (Toft-Hansen et al. 2006). However, the mechanism by which DCs migrate across the glia limitans and into CNS parenchyma during neuroinflammation is poorly understood. In epithelial tissues, circulating DCs are recruited across the vascular endothelium by CCL2 and subsequently directed to the site of injury via a CCL20 gradient (Caux et al. 2002). It is believed that these chemokines are produced by stromal cells and resident macrophages present in the inflammatory site. Likewise, it is postulated that astrocytes and microglia produce chemoattractants that recruit DCs into the CNS parenchyma. Both cDCs and pDCs express CXCR3 and CCR6 which promote directional migration in response to the injury-associated chemokines CXCL10 and CCL20, respectively (Cravens and Lipsky 2002; Kohrgruber et al. 2004; Charles et al. 2010). CXCL10 is also known as interferon inducible protein 10 owing to the fact that it is highly upregulated by interferon signaling and accordingly highly expressed during viral infection. During CNS herpes simplex virus-1 infection, CXCL10$^{-/-}$ and CXCR3$^{-/-}$ mice had similar deficiencies in the recruit-

ment of pDCs, while cDC recruitment to the CNS was unaffected (Wuest and Carr 2008). CXCL10 is also elevated in the CSF of patients with MS and optic neuritis (Sorensen et al. 2001, 2004) and in MS lesions (Simpson et al. 2000) but has mainly been correlated with recruitment of $CXCR3^+$ T-cells in CNS autoimmunity. CCL20 mRNA has been detected in the CNS by day 13 of EAE, and protein has been detected in mouse brain tissue sections in cells with astrocytic morphology (Serafini et al. 2000). Astrocytes stimulated with IL-1β and TNF-α secrete CCL2 (CCR2 ligand); CCL3, CCL4, CCL5 (shared ligands for CCR1 and CCR5); CCL20 (CCR6 ligand) and CXCL12 (CXCR4 ligand) and promote the transmigration of immature DCs across an artificial BBB *in vitro* (Ambrosini et al. 2005). Additionally, $CCR1^+$ and $CCR5^+$ mononuclear cells have been identified in MS lesions (Trebst et al. 2001) along with their ligands (Boven et al. 2000).

We have demonstrated that CCL3 (a shared ligand for CCR1 and CCR5, also known as macrophage inflammatory protein (MIP-1α)) increases the transmigration of bone marrow-derived GFP-labeled DCs across brain microvessel endothelial cell monolayers. We have also shown that the tight junction protein occludin is reorganized when DCs migrate across brain capillary endothelial cell monolayers, and DCs produce matrix metallopreintases (MMPs) 2 and 9 when cultured with brain endothelial cells. Interestingly, in our experiments DCs showed an activated phenotype upon migration, and migration could be abrogated by treatment with an MMP inhibitor (Zozulya et al. 2007). Recently others have shown that dystroglycan—a glycoprotein that anchors astrocytic endfeet to the basement membrane of the glia limitans—is a cleavable substrate of MMP-2 and MMP-9, suggesting that MMP expression may be required for DCs to gain access to the CNS parenchyma. Indeed, it has been shown that $MMP2^{-/-}$ $MMP9^{-/-}$ double knockout mice are resistant to EAE (Agrawal et al. 2006). CCL3 is also required for upregulation of costimulatory markers on CNS-infiltrating DCs during murine viral encephalitis (Trifilo and Lane 2004) and both CCL5 and CCL3 promote secretion of matrix metalloproteinase (MMP9) by monocytes (Robinson et al. 2002) and pDCs (Hu and Ivashkiv 2006) *in vitro*.

3.3 Regulation of DC Migration across the Blood-CSF Barrier

Other studies have begun to describe an alternative route by which DCs and DC precursors could migrate into the CNS. In this route, cells cross the more permissive endothelium of the choroid plexus and meninges. These cells could then enter CSF or migrate through choroid plexus stroma into periventricular tissue. Indeed, both monocytes and DCs have been identified on electron micrographs of choroid plexus mounts from naïve mice (Serot et al. 1997, 1998; McMenamin et al. 2003). Monocytes detected within the stroma of the choroid plexus have increased proliferative capacity compared to blood monocytes and are capable of giving rise to DCs upon treatment with GM-CSF and IL-4 (Nataf et al. 2006). Interestingly, monocytes were more or less restricted to the internal stromal compartment of the choroid plexus,

whereas DCs were found on the surface of the choroid plexus epithelium. This suggests that during steady state conditions, DCs and DC precursors are recruited to the choroid plexus where they are poised for migration into the CSF. Indeed, as mentioned above DCs are elevated in CSF of patients with neuroinflammatory diseases (Pashenkov et al. 2001, 2002). CXCL10 and CXCL12 are highly upregulated in the CSF of patients with bacterial meningitis and Lyme neuroborreliosis. Moreover, DC accumulation in CSF of these patients could be partially blocked by neutralizing antibodies to CXCL12 (Pashenkov et al. 2002). In mice, DCs injected into the CSF during EAE were recruited to periventricular demyelinating lesions, meninges and the parenchyma of the cerebellum and brain stem (Hatterer et al. 2008), suggesting that DCs in the cerebroventricular system may actively contribute to neuroinflammation. Both CCL20 and CCL2 are highly expressed in the healthy choroid plexus epithelium (Mitchell et al. 2009; Reboldi et al. 2009) and the choroid plexus has been shown to be an important route of entry for T-cells and neutrophils during EAE and traumatic brain injury models, respectively (Mitchell et al. 2009; Reboldi et al. 2009; Szmydynger-Chodobska et al. 2009); however, the extent to which this migratory route is used by DCs *in vivo* during neuroinflammatory conditions is unknown.

4 Dendritic Cell Functions in the CNS

4.1 Dendritic Cells can be Positive or Negative Regulators of CNS Immunity

Monophasic EAE can be induced in C57BL/6 mice by subcutaneous immunization with myelin oligodendrocyte glycoprotein peptide ($MOG_{35–55}$). We have shown that i.c. injection of mature MOG-loaded DCs prior to induction of EAE decreases the proportion of regulatory T-cells infiltrating the brain and increased activation of CNS infiltrating T-cells. This effect was associated with acceleration of clinical disease, indicating that CNS DC accumulation might represent a limiting factor during acute EAE. In contrast, when we i.c. injected TNF-α treated "semi-mature" DCs we observed diminished production of the encephalitogenic cytokine IL-17 by CNS-infiltrating lymphocytes, which substantially delayed or prevented EAE (Zozulya et al. 2009b). Surprisingly, injection of DCs lacking the coinhibitory molecule B7H1 also ameliorated EAE clinical disease. This effect was associated with the recruitment of $CD122^+$ $CD8^+$ regulatory cytotoxic T-lymphocytes (Zozulya et al. 2009a). While these studies demonstrate that CNS DCs can be both positive and negative regulators of CNS immunity, the mechanism of their action is still poorly understood. For example, it is not known whether these DCs function principally by locally restimulating and directing T-cell differentiation or by capturing antigens and transporting them to draining lymph nodes for the priming of naïve T-cells.

4.2 *Dendritic Cells Migrate Out of the CNS to Deep Cervical Lymph Nodes*

The CNS is considered immune privileged owing to reduced immune surveillance in these tissues. As such, little is known about the mechanisms of DC migration out of the CNS during neuroinflammation. The CNS does not have typical lymphatic vessels that drain directly to regional lymph nodes. Nevertheless, mice injected intracerebroventricularly with exogenous antigen develop humoral and cell-mediated immune responses in cervical lymph nodes (Cserr et al. 1992). Indeed, in chronic relapsing EAE excising the deep cervical lymph nodes during acute disease partially ameliorates relapse (van Zwam et al. 2009). We have shown that in mice injected i.c. with OVA protein, $CD205^+$ $CD11c^+$ DCs accumulate in the CNS and uptake this antigen. Following this, $CD8^+$ OVA-specific T-cells accumulate first in the cervical lymph nodes and subsequently in the CNS, demonstrating that afferent immunity is intact in the CNS and that antigens drain or are trafficked to the cervical lymph nodes (Ling et al. 2003). Chronic relapsing EAE can be induced in SJL mice by subcutaneous immunization with peptide sequences from proteolipid protein (PLP). It is thought that relapse is the consequence of epitope spreading, whereby secondary immune responses develop against antigens released during the course of neuroinflammation. Myelin antigen-containing cells have been detected in the cervical lymph nodes of monkeys with EAE as well as patients with MS. In both cases, these cells were increased in frequency compared to healthy controls and predominantly expressed DC surface markers (de Vos et al. 2002). Recently we have shown that i.c. injected mature OVA-loaded DCs migrate from CNS tissue to the cervical lymph nodes and prime OVA-specific T-cell responses (Karman et al. 2004). This migration could be blocked by pretreatment of DCs with pertussis toxin, which suggests that this migration is not passive and is mediated by G protein-dependent motility—strongly implicating DC maturation-associated chemokine receptors.

CXCR4 and CCR7 chemokine receptors are both important in mature DC migration. CXCR4 binds CXCL12 and mediates directional migration into tissue and lymphatic vessels under steady state conditions, whereas CCR7 binds CCL19/CLL21 and directs activated mature DCs to T-cell areas in draining lymph nodes under inflammatory conditions. Both cDCs and pDCs upregulate CCR7 upon maturation; however, whereas mature cDCs express high levels of CXCR4, activated maturing pDCs downregulate CXCR4 expression and lose responsiveness to CXCL12 (Cravens and Lipsky 2002). It is thought that mature DCs migrate through interstitial fluid along arterial walls of cerebral vessels and through CSF which drains across the cribriform plate into the nasal mucosa and cervical lymph nodes (Furukawa et al. 2008; Weller et al. 2009, 2010). Both CXCL12 and CCL19 are elevated in the CSF of patients with neuroinflammatory diseases (Pashenkov et al. 2003; Krumbholz et al. 2007) and have been correlated with total CSF cell number (Pashenkov et al. 2002, 2003), suggesting these chemokines may direct DC migration out of the CNS. Indeed, DCs expressing CCR7 have been detected in the CSF of MS patients (Kivisakk et al. 2004). These data suggest that CCR7 and possibly

CXCR4 mediate DC migration from CNS to cervical lymph nodes; however, it is also possible that these chemokine receptors promote retention of DCs in the CNS. CCL19 and CCL21 have been detected perivascularly in the CNS during EAE (Alt et al. 2002) and have been implicated in the generation of tertiary lymphoid tissues in the CNS, which have been associated with disease progression in both MS and EAE (Magliozzi et al. 2004, 2007; Serafini et al. 2004).

4.3 Dendritic Cells might Locally Restimulate Effector T-cells

In T-cell stimulation assays using antigen presenting cells (APCs) isolated from the CNS of mice with relapsing EAE, DCs were superior to monocyte and macrophages in restimulating effector T-cells *ex vivo*. Additionally, only DCs were capable of priming naïve T-cells specific for a non-immunizing antigen (Miller et al. 2007), suggesting that DCs may play an important role in T-cell restimulation and epitope spreading locally in the CNS. EAE can also be induced by adoptive transfer of pre-activated myelin specific $CD4^+$ T-cells. This model allows the dissection of afferent and efferent immune components as the activated effector T-cells no longer require secondary lymph tissue to induce disease; though they still require professional MHC II^+ APCs in the target tissue in order "see" their antigen and carry out effector functions. Importantly, MHC II expression on CNS-resident APCs (such as microglia and astrocytes) has been shown to be dispensable for induction of adoptive transfer EAE, whereas MHC II expression on CNS-infiltrating APCs is required. Moreover, selective expression of MHC II only on $CD11c^+$ DCs is sufficient for local restimulation of adoptively transferred encephalitogenic T-cells and EAE disease progression (Greter et al. 2005). These data provide strong evidence that CNS-infiltrating DCs are crucial for local restimulation of encephalitogenic effector T-cells.

We have previously shown that DCs may act as platforms for cooperative interactions between infiltrating T-cells. In our model system, i.c. injection of DCs loaded with multiple antigens synergistically promoted recruitment of antigen-specific T-cell receptor (TCR) transgenic T-cells restricted to different MHC II molecules. This suggested that T-cell–T-cell cooperation occurs when antigens are presented on the same DC—a hypothesis that was corroborated in experiments where we showed i.c. injection of antigen fusion proteins promoted more antigen-specific T-cell recruitment than injection of mixtures of multiple antigens. Furthermore, following i.c. injection of two antigens into TCR transgenic mice with T-cells specific for one of the antigens, transfer of T-cells specific for the other antigen promoted recruitment of host T-cells to the CNS—an effect not observed when antigens were injected in spatially distinct compartments. This could be blocked by retrovirally inhibiting CD40L or IL-2 expression in the donor T-cells, suggesting that perhaps paracrine IL-2 signaling between closely associated T-cells as well as CD40L-induced DC activation may be important for T-cell–T-cell cooperation (Karman et al. 2006). Recently, we generated transgenic mice that express $OVA_{257–264}$-$OVA_{323–339}$-$PCC_{88–104}$ T-cell epitopes in oligodendrocytes (manuscript in preparation). We demonstrated

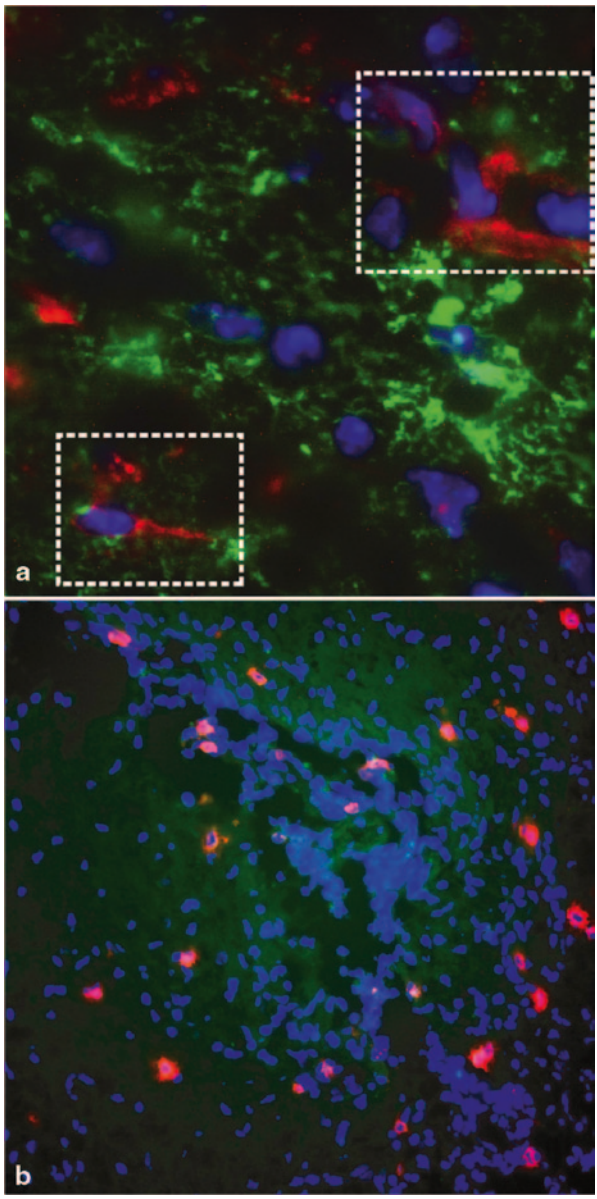

Fig. 4 Neuroinflammation leads to recruitment of DCs and effector T-cells specific for exogenously expressed CNS antigens. EAE was induced in transgenic mice expressing $OVA_{257–264}$-$OVA_{323–339}$-$PCC_{88–104}$ epitopes in oligodendrocytes and 9 days later 5×10^5 Thy1.1 $OVA_{257–264}$–specific OT-I (MHC-I restricted) and Thy1.1 $OVA_{323–339}$–specific OT-II (MHC II restricted) T-cells were adoptively transferred into the Thy1.2 transgenic hosts. Six days after transfer brains were isolated for analysis by fluorescent microscopy using anti-CD11c APC, anti-Thy1.1 PE, anti-CD8 APC, rabbit anti-CNPase, and goat anti-rabbit. **a** *Dotted lines* indicate $CD11c^+$ DCs (*red*) juxtaposed to $CNPase^+$ oligodendrocytes (*green*). **b** Adoptively transferred $Thy1.1^+$ (*red*) $CD8^+$ (*green*) OT-I T-cells are detected in brain parenchyma. *Blue staining* represents DAPI

that upon T-cell epitope upregulation in the CNS, DCs and adoptively transferred antigen-specific T-cells localize around oligodendrocytes in the inflamed brain and spinal cord of these mice (Fig. 4). These data suggest that initial neuroinflammation may induce oligodendrocyte death leading to the release of cytoplasmic antigens and recruitment of DCs, which capture and present antigen to T-cells locally or in regional lymph nodes—thus amplifying the neuroinflammatory processes.

4.4 Dendritic Cells Subsets Skew Effector T-cell Responses

According to the current view, at least four well-characterized helper T-cell lineages can be defined: Th1 cells predominantly secrete IFN-γ and are protective against intracellular pathogens; Th2 cells migrate to B cell areas within lymph nodes and promote humoral immunity by secreting IL-4, IL-5, and IL-13; Th17 promote mucosal immunity by secreting IL-17, IL-22, and IL-21 and have also been implicated in organ-specific autoimmunity; while Treg predominantly secrete IL-10 and are indispensible for maintenance of peripheral tolerance (reviewed in Weaver et al. 2007). DC-derived IL-12 promotes Th1 skewing. In its absence, Th2 differentiation is promoted by T-cell autocrine IL-4 signaling. TGF-β promotes the differentiation of Th17 and regulatory T-cells. In the presence of DC-derived IL-23/IL-6 responding T-cells differentiate into Th17 effector cells, whereas regulatory T-cell differentiation is promoted by incomplete T-cell activation due to increased expression of coinhibitory molecules relative to costimulatory molecules (reviewed in Romagnani 2006).

DC subsets differ in their propensity to skew responding effector T-cell phenotypes. For example, cDCs and pDCs can both induce Th1 or Th2 responses from responding T-cells; however, $CD8^+$ cDCs are strongly Th1 skewing. Evidence from this comes from $Id2^{-/-}$ mice, which are both selectively deficient in $CD8^+$ DCs and strongly Th2 skewed despite the fact that $Id2^{-/-}$ T-cells could be differentiated into Th1 cells *in vitro* (Kusunoki et al. 2003). As mentioned above, cDCs induce Th1 phenotype by IL-12 secretion. In contrast, pDCs promote Th1 differentiation with IFN-α (Ito et al. 2002). Additionally, pDCs are the only DC subset expressing IL-3R (CD123) and pDCs matured with IL-3 upregulate OX40L, which promotes secretion of Th2 cytokines from responding T-cells. Furthermore, TLR-matured pDCs but not cDCs upregulate ICOS-L as part of their maturation process and induce IL-10-producing Tregs (Ito et al. 2007). DC subsets also differ in their ability to promote Th17 differentiation. In mice, resistance to experimental asthma is associated with diminished Th17 responses, reduced IL-23 secretion, and lower cDC to pDC ratios in lung infiltrate. Interestingly, disease susceptibility could be restored by transferring antigen-loaded cDCs to lung tissue (Lewkowich et al. 2008). This is consistent with previous reports of deficient IL-23 translation in TLR-stimulated pDCs compared to cDCs (Waibler et al. 2007).

It has been demonstrated that $CD11b^+$ DCs isolated from mouse CNS during ongoing EAE are better activators of naïve and effector T-cells than CNS pDCs or $CD8^+$ cDCs (Miller et al. 2007). As mentioned above, we have previously shown that i.c. mycobacterial infection promoted differential recruitment of DC subsets to the CNS, with increased recruitment of $CD11c^{high}$ $CD8^+$ cDCs to the CNS during mycobacterial infection. We have also shown that this differential recruitment was associated with differences in effector T-cell responses. Specifically, mycobacterial infection promoted strongly Th1 skewed effector T-cell responses, whereas EAE was associated with both Th1 and Th17 responses. We also investigated whether pre-existing CNS mycobacterial infection could modulate T-cell responses during subsequent EAE. We ob-

served that concomitant mycobacterial infection locally suppressed Th17 responses and partially ameliorated EAE in mice (Lee et al. 2008). This is in agreement with data showing that $CD8^+$ cDCs are strongly Th1 polarizing (see above).

PDCs are a major source of type-1 IFNs *in vivo*. IFN-β is a widely used therapy for the treatment of relapsing remitting MS; however, its mechanism of action is unknown. Interestingly, IFN-β treatment has recently been shown to decrease the frequency of circulating cDCs relative to pDCs (Lopez et al. 2006). In addition, circulating pDCs isolated from MS patients have an immature phenotype and show deficient maturation in response to both IL-3 and CpG (Stasiolek et al. 2006). These data suggest that in the context of CNS autoimmunity pDCs may be tolerogenic. Indeed, IFN-β treatment has been shown to further suppress MHC II expression and increase expression of the coinhibitory molecule B7H1 on circulating pDCs in MS patients (Lande et al. 2008). Transient depletion of pDCs has been shown to exacerbate both acute EAE and relapse and is associated with increased CNS T-cell activation (Bailey-Bucktrout et al. 2008). Moreover, specifically suppressing MHC II surface expression on pDCs worsens disease (Irla et al. 2010), suggesting that pDCs are directly T-cell suppressive during EAE.

Recent research has revealed that T-cell lineages—once thought to be very rigid effector fates for differentiating CD4 T-cells—are in fact much more plastic, especially during early differentiation (Lee et al. 2009b; Afzali et al. 2010; Zhu and Paul 2010). For example, *in vitro* differentiated Th2 effector memory cells can act as IL-10 secreting suppressor T-cells *in vivo* (Xu et al. 2010), and Th17 cells can be transformed into either Th1 or Th2 effector cells given the proper stimulus ex vivo (Zhu and Paul 2010). It has also been shown that Tregs can transform into both Th1 (Wei et al. 2009) and Th17 cells *in vitro* (Xu et al. 2007; Yang et al. 2008), in mice (Lochner et al. 2008), and in humans (Voo et al. 2009). This newly discovered plasticity might imply that target tissue factors, such as local DC subset composition and cytokine milieu, may largely determine the effector phenotype of infiltrating T-cells—an implication with far-reaching consequences for immunotherapy. For example, sterile immunity is the Holy Grail for vaccine research targeting latent *M. tuberculosis* infection. Yet it was recently suggested that vaccine-induced systemic immunity may be insufficient for bacterial clearance because the chronic granuloma is replete with tolerogenic DCs that shut down cytokine secretion in infiltrating lymphocytes (Schreiber et al. 2010). Likewise—in light of evidence showing Treg transformation into inflammatory Th1 and Th17—it is important to consider that DCs present locally in the inflamed CNS may represent obstacles for tolerance-based immunotherapies targeting autoimmune or chronic inflammatory diseases of the CNS.

5 Summary

During neuroinflammation cDCs, pDCs, and iDCs accumulate in the CNS; however, it seems that different DC subsets predominate in response to different neuroinflammatory stimuli. For example, while EAE promotes the recruitment of pre-

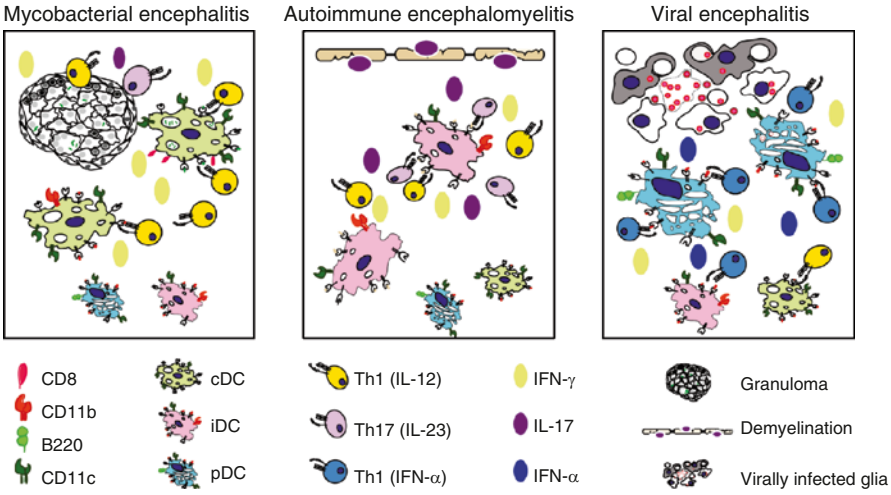

Fig. 5 Different DC subsets dominate CNS immune responses during bacterial, viral, and autoimmune neuroinflammation. CNS BCG infections promote recruitment of $CD11b^+$ and $CD8^+$ $CD11c^{hi}$ cDCs, the latter of which strongly skew T-cells toward a Th1 phenotype. In contrast, EAE predominantly favors recruitment of $CD11b^+CD11c^+$ iDCs, which secrete IL-12/IL-23, skewing T-cells toward Th1 and Th17 phenotypes. During viral encephalitis, CNS-infiltrating $B220^+$ $CD11c^+$ pDCs secrete copious amounts of IFN-α and thus skew T-cells toward Th1 phenotype

dominantly $CD11b^+$ iDCs through GM-CSF, CCL2, and other chemokines; mycobacterial encephalitis promotes granuloma formation and is associated with the accumulation of $CD8^+$ cDCs. $CD8^+$ cDCs strongly skew T-cell responses toward an IFN-γ secreting Th1 phenotype whereas iDCs secrete IL-12 and IL-23, which promote Th1 and Th17 differentiation. A minor fraction of CNS-infiltrating DCs, pDCs are thought to promote Treg development during EAE, perhaps due to deficiency in IL-23 translation or the lack of viral stimuli, which would promote pDC maturation. In contrast, during viral encephalitis pDCs can be selectively recruited to the CNS, perhaps through pDC-selective chemoattractants, such as CXCL10, Flt3L, or chemerin. These pDCs respond to viral stimuli by secreting IFN-α, which dominates the immune response and promotes IFN-γ secretion by Th1 cells (Fig. 5).

We outlined evidence indicating that CNS-DCs can restimulate effector T-cells locally and/or migrate to cervical lymph nodes where they prime naïve T-cells, both of which might shape immune responses in the CNS. Despite these advances in our understanding, many questions remain unanswered. What chemokines govern DC recruitment across the glia limitans? To what extent do DCs migrate into the inflamed CNS via the choroid plexus? How do CNS danger- and pathogen-associated molecular patterns dictate the chemokine expression patterns that govern selective DC migration? How do DCs migrate out of the CNS to cervical lymph nodes? Answers to these questions might lead to better therapies for CNS autoimmune and chronic inflammatory diseases that target subset-specific modulation of DC migration and activation status.

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Nutritional Immunity: Homology Modeling of Nramp Metal Import

Mathieu F. M. Cellier

Abstract The *N*atural *r*esistance-*a*ssociated *m*acrophage *p*roteins (Nramp1 and 2) are proton-dependent *s*olute *c*arriers of divalent metals such as Fe^{2+} and Mn^{2+} (Slc11a1 and 2). Their expression in both resting and microbicidal macrophages which metabolize iron differently, raises questions about Nramp mechanism of Me^{2+} transport and its impact in distinct phenotypic contexts. We developed a low resolution 3D model for Slc11 based on detailed phylogeny and remote homology threading using *Escherichia coli* Nramp homolog (*proton*-dependent *Mn*$^{2+}$ *t*ransporter, MntH) as experimental system. The predicted fold is consistent with determinations of transmembrane topology and activity; it indicates Slc11 carriers are part of the LeuT superfamily. Homology implies that inverted structural symmetry facilitates Slc11 H^+-driven Me^{2+} import and provides a 3D framework to test structure-activity relationships in macrophages and study functional evolution of MntH/Nramp (Slc11) carriers.

Keywords Nramp (natural resistance-associated macrophage protein) • Phagosome • Proton-dependent efflux • Divalent metals ($Fe2^+$, $Mn2^+$,…) • Membrane transport •Homology threading • Phylogeny • Structure 3D • Inverted symmetry

1 In Defense of Host Metalome

Cells and organisms generally rely on metals as pools of inorganic, re-usable small positively charged ions whose availability through metabolism can sustain the most elaborate forms of life on earth (Williams 2007). One of the most abundant and versatile metals in terms of redox cycling, Fe, is used by virtually every cell in all domains of life as a cofactor of numerous activities that catalyze oxygen evolution, signaling, energy metabolism, and cell replication. Also, Zn is of prime importance

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for transcriptional regulation and signaling while Mn serves for metabolic catalyses and oxidative reactions (Williams 2007). Divalent metal (Me^{2+}) uptake, distribution and efflux are key for human development and growth as well (Xiu 1996) thus suggesting an obvious function for the immune system: to protect the metalome from invaders.

The discovery of ovotransferrin as anti-infective agent in egg white led to the concept of nutritional immunity, which depicted host withholding growth-essential iron as an important vertebrate nonspecific defense against microbial invasion (Weinberg 1977). This microbe starving strategy applies not only to iron (Ganz 2009) but also to Mn and Zn (Kehl-Fie and Skaar 2010) and it is consistent with the important roles that metal metabolisms play in microbial virulence (Jakubovics and Valentine 2009). However, the efficiency of this defense can be limited in the long term by a pathological outcome, known as anemia of inflammation, which restricts severely iron supply for body needs (Muñoz et al. 2009; Weinberg 2009).

Macrophages exposed to microbial molecular patterns (e.g., Gram negative bacteria LPS) and innate defense stimuli (e.g., interferon-γ) polarize in response toward the so-called classically activated macrophage phenotype (M1) (Takeuchi and Akira 2010), which exemplifies host metal sequestering strategy by synthesizing and secreting a number of molecules for metal capture (e.g., Lipocalin2, H-Ferritin, S100A8-A9 heterocomplex or calprotectin) and expressing corresponding receptors (such as Megalin, Tim2) (Ganz 2009; Weinberg 2009; Kehl-Fie and Skaar 2010) among a plethora of other antimicrobial functions including generation of radicals. M1 activation primarily aims at killing intracellular parasites and tumors; it differs in many aspects from the phenotype of M2 or alternatively activated macrophages (Martinez et al. 2006), notably regarding Fe metabolism (Fig. 1). M2 macrophages provide a niche preferred by some intracellular pathogens (Gordon and Martinez 2010).

2 Metabolism of Metals Scavenged from Ingested Corpses

During steady-state conditions, resting macrophages express predominantly M0 or unpolarized phenotype and serve as recycling factories for heme/iron (and other metals) and cholesterol, which they efflux through specific transporters (FPN, ABCA1,…) into the circulation for systemic transport (via transferrin, lipoproteins) to sustain body development and renewal as well as to avoid toxic intracellular accumulation (Muñoz et al. 2009; Töröcsik et al. 2009). Thus ~90% of daily iron needs transit through phagocytes normally recycling effete cells (e.g., RBC, PMNs, thymocytes) (Muñoz et al. 2009).

Macrophage M0 phagocytic activity is immunologically silent but it maintains actively anti-inflammatory conditions, contributing to preserve self-tolerance (secretion of IL-10 vs. IL-12, IL-23, PGE2, TGFβ) (Bellora et al. 2010; Elliott and Ravichandran 2010). However, the vast repertoire of pattern recognition receptors

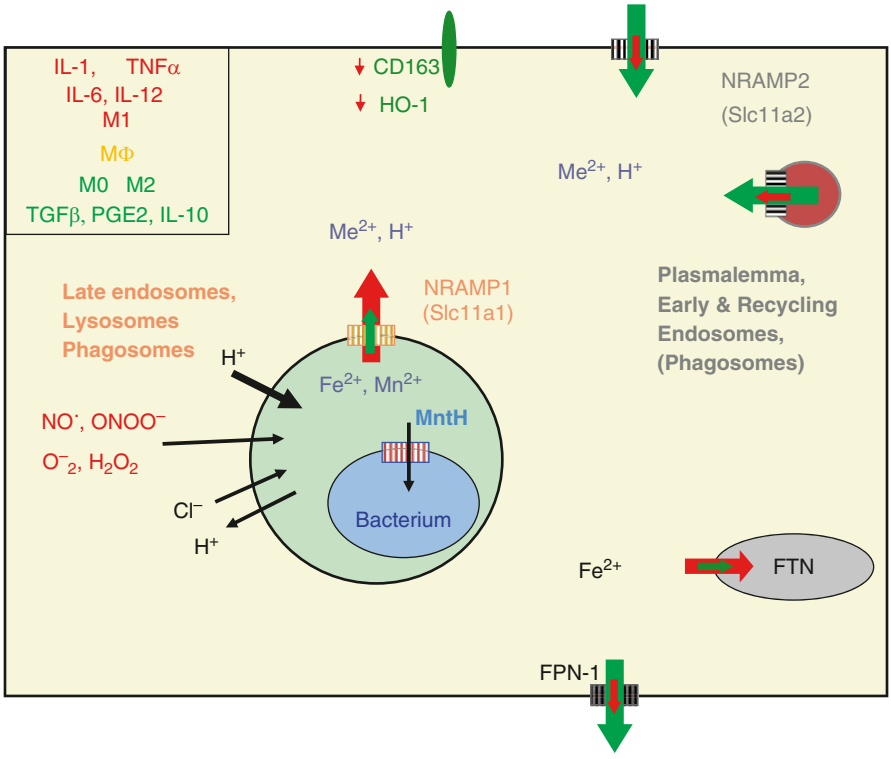

Fig. 1 Macrophage metal metabolism and immune polarization. In the resting state, recycling of apoptotic cells by body macrophages enables signaling through molecules such as phosphatidyl-l-serine, oxysterols, oxidized phopsholipids, and heme and their corresponding receptors to maintain macrophage de-activated state, M0, characterized by the secretion of mediators such as TGFβ, PGE2, IL-10, and active metal metabolism (illustrated in *green*). During infection various molecular determinants that indicate the presence of microbes and/or tissue damage are released and activate macrophages responses: M1 cells express high levels of microbicidal and potentially harmful functions and stop metabolizing metals (represented in *red*); M2 response maintains metal trafficking and favor tissue repair and resolution of inflammation (in *green*). Nramp1 activity is preponderant in M1 macrophages and Nramp1 is also expressed in M0/M2 macrophages (see text for details)

expressed by resting macrophages, dendritic and epithelial cells, together with soluble receptors in body fluids (e.g., complement components, collectins, ficolins, and pentraxins) aims at immediate detection of infection and rapid mounting of an appropriate response (Jeannin et al. 2008; Takeuchi and Akira 2010). In turn, macrophage M1 activation can halt metal trafficking (Cairo et al. 2010; Corna et al. 2010; Recalcati et al. 2010); still, virulent microbes avoid adverse consequences of detection and exploit host-derived nutrients (Brown et al. 2008; Ganz 2009; Kehl-Fie and Skaar 2010).

An intriguing regulatory aspect of macrophage phenotype spectrum relates to apoptotic cell clearance and involves nuclear receptors believed to act as molecular

sensors of phagocytic meals, such as PPAR-δ, LXR-α, β, and Rev-Erb-α, β (Elkon 2009; Fontaine et al. 2008; Schug and Li 2009). In addition to phosphatidyl-l-serine, molecular patterns derived from apoptotic cells such as polyunsaturated fatty acids, oxysterols (oxidized derivatives of cholesterol) and heme can bind to specific intracellular receptors which then instruct macrophages to increase clearance of apoptotic cells and sustain anti-inflammatory secretions (A-Gonzalez et al. 2009; Elliott and Ravichandran 2010; Marro et al. 2010; Mukundan et al. 2009). Thus, macrophages determine their phenotype in response not only to colony stimulating factors (e.g., M-CSF, GM-CSF) (Bellora et al. 2010; Cairo et al. 2010), lymphokines such as IFN-γ and IL-4 (Bensinger and Tontonoz 2008) and anatomical location *in vivo* (thymus, spleen marginal zone) (Geissmann et al. 2010) but also by sensing directly their phagocytic meal and integrating metabolism and inflammation (Bensinger and Tontonoz 2008; Jeannin et al. 2008; Vallelian et al. 2010). And steady-state clearance of apoptotic cells provides, contrary to M1 activation, potent anti-inflammatory determinants (A-Gonzalez et al. 2009; Elliott and Ravichandran 2010; Marro et al. 2010; Mukundan et al. 2009) that favor macrophage Fe metabolism (Cairo et al. 2010; Corna et al. 2010; Recalcati et al. 2010; Marro et al. 2010; Odegaard et al. 2008; Sierra-Filardi et al. 2010).

3 Divalent Metal Import by Natural Resistance-Associated Macrophage Proteins (Nramp)

Nramp1 and Nramp2 (Dmt1) are integral membrane proteins both constitutively expressed, respectively in professional phagocytes or rather ubiquitously (Cellier et al. 2007). The HUGO nomenclature introduced the acronyms Slc11a1 and Slc11a2 for Slc11 member 1 & 2 (Fredriksson et al. 2008). These very hydrophobic proteins are highly conserved in sequence and share similar predicted transmembrane topologies (Czachorowski et al. 2009). Besides differences in tissue-specific expression Nramp1 and 2 also showed distinct intracellular locations; alternative splicing yields Nramp2 isoforms with various termini expressed at the plasma membrane and in recycling and/or late endosomes (Lam-Yuk-Tseung et al. 2006). Iron-dependent regulation of Nramp2 isoforms includes mRNA stabilization by the IRE/IRP system and protein targeting for ubiquitination and degradation by Nedd4 family-interacting proteins (Howitt et al. 2009). In contrast, Nramp1 sequences contain a conserved motif that targets the proteins to intracellular late endosome–lysosome vesicles (Cellier et al. 2007; Lam-Yuk-Tseung and Gros 2006).

Lack of Nramp2 (Dmt1) is lethal, since it is required for intestinal non-heme iron absorption and for hemoglobin production in erythroid precursors, and several other mutations were linked to microcytic anemia (Gunshin et al. 2005; Blanco et al. 2009). Nramp1 deficiency can be fatal after infection by virulent pathogens that survive and proliferate in macrophages (Vidal et al. 1993). In addition, both Nramp1

and 2 promote hemoglobin iron recycling in macrophages (Soe-Lin et al. 2009) and accordingly the corresponding mRNA transcripts were detected in human and mouse M2 macrophages (Corna et al. 2010; Recalcati et al. 2010). Also, stimulation with IFNγ and LPS showed that both genes were upregulated in M1 macrophages (Cellier et al. 2007).

Nramp1 and 2 thus broadly contribute to (divalent metals and) iron trafficking either to facilitate Me^{2+} recycling in normal conditions or to deplete intracellular vacuoles and sequester Me^{2+} in response to infection (Cellier et al. 2007; Soe-Lin et al. 2009). As these activities correspond to rather opposite macrophage phenotypes and immunological outcomes (Biswas and Mantovani 2010) it is yet unclear how the environmental context may affect Nramp transport activity and whether pro- or anti-inflammatory effects may be attributed to Slc11 Me^{2+} import depending on conditions. In any case, the question of the mechanism of Me^{2+} and H^+ transmembrane import appears central to establish the physiological roles of Nramp and elucidate their antimicrobial potential.

Perhaps this is most pertinent given that numerous microbes can express Nramp homologs or several other types of transporters which may compete with host carriers during infection to acquire divalent metals (Anderson et al. 2009; Ganz 2009; Jakubovics and Valentine 2009; Kehl-Fie and Skaar 2010). An experimental challenge in molecular understanding of the mechanism of transport is to gain the necessary protein structure information despite poor solubility of integral membrane proteins. Bioinformatics can help establishing a 2D transmembrane (TM) topology, which allows probing separate predicted structural segments such as TM helices (Courville et al. 2004; Czachorowski et al. 2009) and locating potential functional residues based on protein family-specific sequence conservation pattern (Chaloupka et al. 2005; Courville et al. 2008).

4 A Phylogenetic Approach to Study Nramp Mechanism of Membrane Transport

The Nramp family was defined across all domains of life with a cut-off of 30% identity over 80% of the sequence length, which corresponds to a highly conserved hydrophobic core comprising 10 predicted transmembrane segments (TMS) (Cellier et al. 1995). Biochemical evidence from several groups showed that bacterial MntH homologues as well as yeast, plant and animal Nramp homologues performed similar Mn^{2+} uptake (Anderson et al. 2009; Cailliatte et al. 2010; Courville et al. 2006; Jensen et al. 2009; Settivari et al. 2009). Also, studying bacterial, fungal or animal Nramp homologues yielded consistent TM topology data thus indicating that sequence conservation supported functional preservation (Courville et al. 2004; Czachorowski et al. 2009). However, high number of identical residues (138 between *Homo sapiens* Nramp1 and *Escherichia coli* MntH, for instance) complicates their detailed analyses and poses the question of their relative significance.

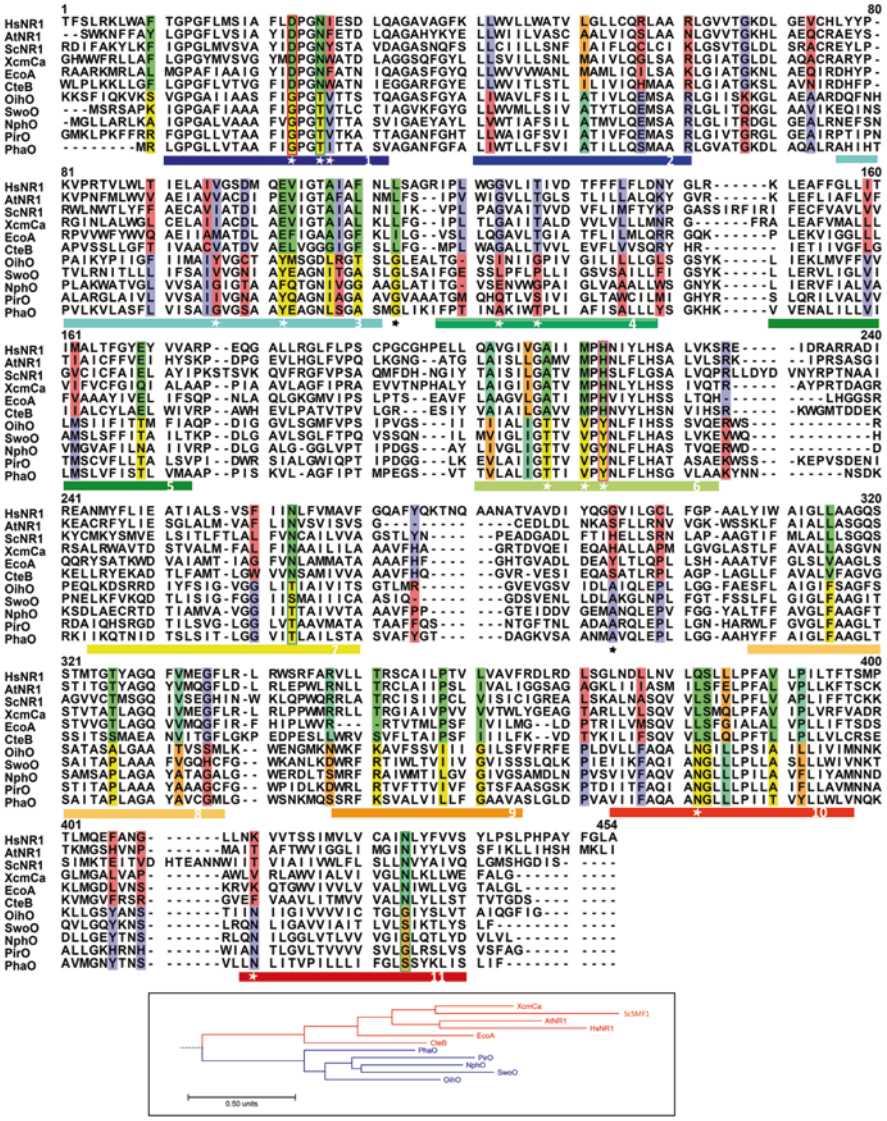

Fig. 2 A phylogenetic approach toward Nramp mechanism of transport. Evolutionary rate-shifts among the Nramp/MntH (Slc11) family and its phylogenetic outgroup (*Inset*) were predicted using representative sets of diverse sequences (CteB, *Chlorobium tepidum* MntH B, EcA, *Escherichia coli* MntH A, XcaCa, *Xanthomonas campestris* MntH Ca, ScNR1, *Saccharomyces cerevisiae* SMF1p, AtNR1, *Arabidopsis thaliana* Nramp1, HsNR1, *Homo sapiens* NRAMP1) and (OihO, *Oceanobacillus iheyensis*, SwoO, *Shewanella woodyi*, NphO, *Natronomonas pharaonis*, PirO, *Pirellula sp. 1*, PhaO, *Pseudoalteromonas haloplanktis*). Rate-shifts were calculated using an instantaneous matrix deduced from known transmembrane (vs. globular) proteins by searching the alignment for positions representing possible type I, type II or type I & II functional divergence (Chaloupka et al. 2005). Nramp family invariant sites are boxed and coloured in *red* or *magenta*,

To implement a phylogenetic approach aimed at locating functional residues we reasoned that sites varying moderately and according to a defined phylogeny may be informative and correspond to key evolutionary steps or represent functional adaptations. We thus identified a phylogenetic outgroup, i.e., a separate set of sequences somewhat related to but distinct from Nramp (20–30% identity over 80% of the sequence) and generated a full-length multiple sequence alignment (MSA). The presence of few Nramp-invariant polar residues was noted in the predicted TMS 1, 6, 7, and 11 and we posited that these residues represented potential sites of functional divergence, because they matched outgroup amino acid moieties also conserved but different (Fig. 2). To address this possibility we used *E. coli* MntH as a model for detailed *in vivo* and vitro characterization (Chaloupka et al. 2005; Courville et al. 2008).

Because this transporter was previously unknown in bacteria we subjected *E. coli* MntH to functional tests adapted from data reported for eukaryotic Nramps, and to determine the relative importance of the targeted sites we compared the activity of WT MntH and derived point mutants (several mutations/site). In whole bacteria we measured intracellular Me^{2+} accumulation by disk-assays of either growth inhibition due to cytotoxicity or restoration of growth by complementation of a metallo-dependent Ts defect (Makui et al. 2000); ICP-MS measurements of intracellular metals, and ratiometric fluorescence assay of cytoplasmic pH reporting on proton uptake in living bacteria, allowed combined measurements of metal/proton symport (Chaloupka et al. 2005). In cation uptake assays, we examined the impact of mutagenesis on the transporter substrate saturation kinetics and energy of activation for transport catalysis. *In vitro*, the well-established systems of right-side out and inside out membrane vesicles served to study both forward and reverse modes of transport and to detail MntH dependency on the electrochemical gradient of the proton. Membrane vesicles were also used to determine site-accessibility after single Cys introduction and reaction with thiol reagents of various sizes (Courville et al. 2008).

Mutating MntH potentially charged residues Asp^{34} and His^{211} within the predicted TMS1 and TMS6 (Fig. 2) yielded relatively similar results: replacement of Asp^{34} abrogated proton uptake while exchanging His^{211} prevented metal uptake at pH 7.5; TMS1 Cys^{24} was accessible to fluorescein-5-maleimide and N-ethyl-maleimide (NEM) while TMS6 Cys^{211} reacted with NEM only; also, mutating His^{211} affected the Arrhenius energy of activation for uptake of both Cd^{2+} and H^+. Regarding the three other Nramp-specific sites, MntH Asn^{37} in TMS1, Asn^{250} in TMS7 and Asn^{401} in TMS11, we noted important elevation of the Arrhenius energy of activation for mutants within the 10 TMS hydrophobic core; replacing TMS11 Asn^{401} showed mainly increased proton uptake compared to metal import. None of these three sites was accessible to thiol reagents, implying possible involvement in inter-helix interactions. To support this interpretation we sought to determine whether comparative

for solvent accessible residues that affected proton and metal transport, respectively, or in *green* for residues affecting activity less directly (Courville et al. 2008). Candidate rate-shifted sites with likelihood-ratio tests values above the significance cutoff are underscored by *stars*, *white* in predicted TMS and *black* in hydrophilic loops; TMS are *underlined* according to *rainbow colors* similar to 3D models in Fig. 3 (*Blue*, N-terminus, *red*, C-terminus)

modeling based on solved 3D crystal structures would identify a fold compatible with the experimental data.

5 Structural Origins of the Nramp Family

The amino acid transporter LeuT provided a 3D fold that fitted the results of MntH physical and chemical analyses, despite little sequence similarity: this crystal structure contains an internal symmetry with two direct repeats encoding five TMS topologically inverted, which place close to each other, in the core of the membrane, two short extended sequence motifs that interrupt the TM α-helices 1 and 6 in their middle, forming an unusual structural pattern that was found in various families of cation-driven transporters (Boudker and Verdon 2010). Cosubstrate binding to LeuT involves also two long, bent helices which correspond in comparative models to highly conserved parts of Nramp sequences (TMS 3 and 8), suggesting functional concordance for cation-driven transport among Nramp model and LeuT structural scaffold and implying possible homology (Courville et al. 2008; Czachorowski et al. 2009).

Several X-ray crystal structures of transporters were reported to be superimposable to the LeuT fold with an overall root mean square deviation (RMSD) less than 4 Å and despite the absence of clear sequence relationship. They include vSGLT a bacterial homologue of the solute:sodium symporter (SSS) family (Slc5) (Faham et al. 2008), Mhp1 from the nucleobase:cation symporter-1 (NCS1) family (Shimamura et al. 2010; Weyand et al. 2008), BetP and CaiT that belong to the betaine/carnitine/choline transporter (BCCT) family (Ressl et al. 2009; Schulze et al. 2010; Tang et al. 2010) as well as AdiC and ApcT from the amino acid-polyamine-organocation (APC) transporter family (Slc7) (Fang et al. 2009; Gao et al. 2010; Shaffer et al. 2009). Together with LeuT, a bacterial homologue of the neurotransmitter: sodium family symporters (NSS, Slc6) (Yamashita et al. 2005), these carriers share a common pseudo-symmetric hydrophobic core that is a tandem of structural units in inverted topology and comprising five TMS which are intertwined and form a novel TM fold (the LeuT fold). These structural studies have revealed that seemingly unrelated families of transporters in fact form a novel structural superfamily (Abramson and Wright 2009; Krishnamurthy et al. 2009).

Functional unification of this superfamily may seem somewhat difficult because some of the carriers are symporters driven either by Na^+ (Faham et al. 2008; Weyand et al. 2008; Yamashita et al. 2005) or H^+ (Shaffer et al. 2009) while others function as antiporters Na^+- and H^+-independent (Fang et al. 2009; Gao et al. 2010; Tang et al. 2010). However, it was predicted for the Slc6 family (Forrest et al. 2008) and observed with crystal structures of the nucleobase:cation symporter-1 Mhp1, representing outward- and inward-facing states together with an intermediate structure in which bound cosubstrates are occluded from both sides of the membrane (OF, IF and Occ, respectively) (Shimamura et al. 2010; Weyand et al. 2008) that the overall

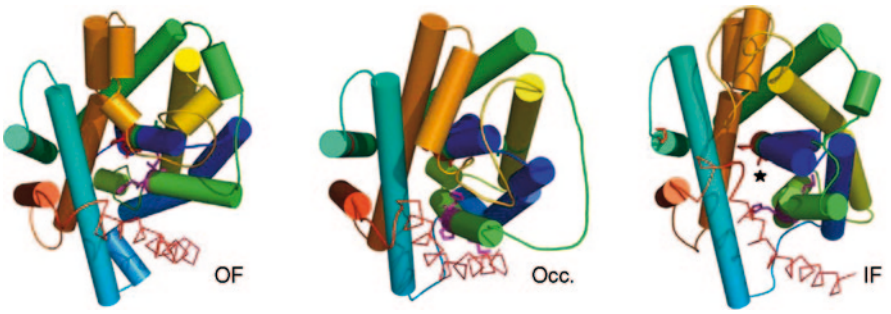

Fig. 3 Homology modeling of Nrampl potential mechanism of metal transport. Cartoon of predicted 3D structures of NRAMP1 hydrophobic core with TMS1-9 represented as cylindrical helices and TMS 10 shown as a ribbon (view from cell exterior). Consensus models were obtained using EasyModeller (Eswar et al. 2008; Kuntal et al. 2010) and crystals structure templates representing 3 possible steps in a carrier cycling mechanism common to the LeuT superfamily: Outwardly Facing (*OF*), based on Mhp1, AdiC, and LeuT templates (PDB IDs: 2JLN, 3LRB, 2A65), Occluded (*Occ*), using Mhp1, AdiC, BetP, and CaiT structures (PDB IDs: 2JLO, 3L1L, 2WIT, 3HFX) and Inwardly Facing (*IF*) following Mhp1, CaiT, vSGLT, and ApcT structures (PDB IDs: 2X79, 2WSX, 3DH4, 3GIA). Models were obtained by threading the sequence of the human NRAMP1 hydrophobic core on these templates using pairwise alignments derived from the multiple sequence alignment used in phylogenetic analyses (Fig. 5a–c). A *black star* indicates the potential transmembrane translocation pathway

transporter conformation exchange mediating cosubstrate translocation proceeds through a mechanism that is dictated by the internal structural symmetry of the fold (Forrest and Rudnick 2009; Forrest et al. 2008) (see for instance, Nramp1 models OF, Occ. and IF Fig. 3).

The LeuT fold intertwines repeats in alternative configurations and substrate binding triggers configuration substitution in each repeat. Such a process may be relatively adaptable, as suggested by the diversity of substrates transported (amino acid-polyamine-organocation, sugar, betaine-choline-carnitine, nucleobase), providing that inverted structural symmetry preserves the mechanism. From this point of view, and by analogy with another superfamily, EmrE (Korkhov and Tate 2009; Nasie et al. 2010), it is possible that carriers sharing the LeuT fold derive from common ancestors, i.e., ancestral five TMS protomers able to adopt inverted topologies and alternative configurations, which evolved with various substrates and by gene fusion (Fig. 4).

Notably, a candidate ancestral five-TMS protomer family (DedA) identified by hydropathy profiling and sequence alignment (Khafizov et al. 2010) shows low level of sequence conservation supporting the notion that protomer ability to adopt inverted topologies and swap configurations is functionally predominant (Forrest and Rudnick 2009; Khafizov et al. 2010). Though sequence identity level is inadequate for identifying new protein families that share inverted symmetry (Choi et al. 2008), demonstrating sequence homology among the LeuT superfamily may provide an assay to test families suspected to share also this fold.

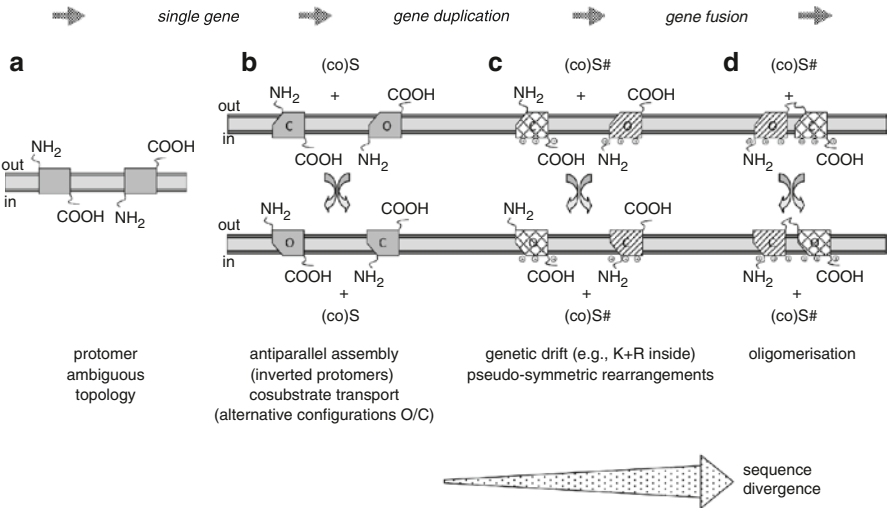

Fig. 4 Evolution of a diverse superfamily of large transporters with inverted symmetry and driven by cations. a Single gene coding for a five TMS integral membrane protein with ambiguous topology that inserts in cell membrane in two anti-parallel orientations (protomer). **b** Protomer adopting alternative configurations (open-O/closed-C) and inverted orientations could dimerise to form a full-transporter conformation presenting an external cation binding site. Specific cation binding would trigger internal rearrangement (each protomer swapping alternative O/C configurations) leading to the carrier opposite conformation, and resulting in cation delivery on the other side of the membrane. **c** Gene duplication allowed protomer divergence driven by e.g., (1) accumulation of $Lys^{+}Arg$ residues in cytoplasmic segments of protomer molecules,which fixed unambiguous topology, (2) erosion of protomers symmetry leading to pseudo-symmetric dimers that favored directional transport, and (3) novel cosubstrate specificities. **d** Fusion of genes encoding pseudo-symmetric, antiparallel protomers resulted in larger transporters which could further evolve novel transport kinetic and structural properties, including oligomerisation. Hence, genes encoding large cation-driven transport carriers with inverted symmetry evolved to a point where homologous families show unrelated sequences but share a common architecture, allowing variations in the mechanism of secondary transport (e.g., Na^{+}- or H^{+}-driven symport; Na^{+}- and H^{+}-independent antiport), which typifies the LeuT superfamily

6 Structural Homology Among the LeuT Superfamily Connects the Nramp Family

The crystals structures similar to LeuT revealed discrete transport intermediates compatible with a general type of alternating gated-pore mechanism for cation-driven transport of diverse solutes: opening of the extracellular gate exposes the substrate binding site; the carrier affinity lowers after substrate binding while both gates are closed (occluded configuration) and when the intracellular gate opens (Claxton et al. 2010; Forrest and Rudnick 2009; Krishnamurthy et al. 2009). Known cation-driven intermediates include: (1) a carrier that is “open to out” with Na^{+} bound but without substrate (Mhp1) (Weyand et al. 2008), (2) an “open to out” car-

rier with both the driving cation (two Na^+) and substrate (Leu) bound—and lightly occluded (LeuT) (Yamashita et al. 2005), (3) another occluded intermediate not open to any side (BetP) (Ressl et al. 2009), and two intermediates that are "open to in", displaying no firmly bound driving cation, and (4) with or (5) without bound substrate (vSGLT (Faham et al. 2008) and ApcT (Shaffer et al. 2009), respectively). Though these structural intermediates represent snapshots in elaborate translocation processes for which many aspects relating to discrete steps in the transport cycles remain undefined in each family, their global similarity nevertheless demonstrates a common functional architecture that identifies a novel superfamily of carriers (Abramson and Wright 2009; Krishnamurthy et al. 2009).

Some of these transporters were crystallized in different conformations, providing common grounds for a mechanism widely applicable to this superfamily (considering family-specific variations as well). The three structures of Mhp1 in conformations OF, Occ, and IF led the authors to propose a mechanism achieved by movement *en bloc* of TMS 3, 4, 8, and 9 relative to the rest of the protein (Shimamura et al. 2010; Weyand et al. 2008). Similar conformations were also reported for antiporters from the Slc7 and BCCT families: transition between "open to out" and occluded states of the arginine/agmatine exchanger AdiC involved rearrangements in TMS6, 2 and 10 (Fang et al. 2009; Gao et al. 2010), while switching toward the occluded state from fully open, inward-facing conformation showed concentric movement of TMS 1, 5, 6, and 8 of the carnitine-butyrobetaine antiporter CaiT (Schulze et al. 2010; Tang et al. 2010).

Superposition of relatively similar X-ray conformers demonstrates common cores spanning 314, 224, and 210 as residues at 3.25, 3.00, and 3.39 Å RMSD respectively, for transporter structures "open to out" (LeuT, Mhp1, AdiC) (Fang et al. 2009; Weyand et al. 2008; Yamashita et al. 2005), occluded (Mhp1, AdiC, BetP, CaiT) (Gao et al. 2010; Ressl et al. 2009; Schulze et al. 2010; Weyand et al. 2008), and "open to in" (Mhp1, vSGLT, ApcT, CaiT) (Faham et al. 2008; Gao et al. 2010; Shaffer et al. 2009; Shimamura et al. 2010). In some cases, functional divergence was linked to single residue variations: ApcT Lys^{158} is central to (Slc7) H^+-driven transport and this cation appears to fulfill a role equivalent to one of the Na^+ ligands in the (Slc6) LeuT structure (Na2) (Shaffer et al. 2009) while LeuT Glu^{290} was shown to mimic the role of a Cl^- anion key to the function of the Slc6 homolog dopamine transporter (Zhao et al. 2010). Also, CaiT Na^+-independent transport was linked to the presence of Met^{331} sulphur in place of the Na^+ ion that coordinates the substrate in the central transport site (LeuT Na1), while LeuT Na2 position in CaiT is occupied by Arg^{262} (Schulze et al. 2010). Such preservation of equivalent sites among diverse families thus supports the existence of an ancestral functional 3D framework that mediated transport based on its inverted symmetry and cosubstrate-induced conformation rearrangements.

To test remote homology in the LeuT superfamily we deduced an MSA from the superposed structures (Guda et al. 2004) of the hydrophobic cores (TMS1-10) for each conformer (OF, Occ., and IF). Alignments were edited to (1) minimize gaps in TMS, (2) reach consensus among three possible alignments for Mhp1 and 2 possible alignments for AdiC and CaiT and (3) combine into a single alignment which was

subjected to phylogenetic analyses. We used as outgroup families that typify ion carriers with 3D inverted symmetry but distinct folds (Clc chloride channels-transporters (Dutzler et al. 2002), and ammonia transporters Slc42 (Javelle et al. 2008)). The results of Maximum Likelihood, Minimal Evolution and Maximum Parsimony approaches (Schmidt and von Haeseler 2007; Tamura et al. 2007) were congruent, indicating with confidence a single node that joins the representatives of distant families such as vSGLT (Slc5), LeuT (Slc6), ApcT (Slc7), BetP and Mhp1 and their homologs (Fig. 5a–c); similar results were obtained using Slc42 sequences as outgroup (not shown). These results were confirmed by unbiased sequence clustering analyses using sets of sequences from NCBI eukaryotic homology groups sampling families of Slcs and Clcs together with 339 other sequences (Slc11 & outgroup). Clans analysis (Frickey and Lupas 2004) indicated significant relationships among the Slc families 5–7 and 38, but excluded both the Slc42 and Clc families (Fig. 5d).

To align Nramp sequence set with pre-aligned representatives of the LeuT superfamily, ClustalX-based alignment was refined using conformer-based 3D consensus Nramp models obtained using EasyModeller (Fig. 3) (Eswar et al. 2008; Kuntal et al. 2010). The MSA was edited to minimize gaps in TMS and optimize the 3D position of conserved/variable helix faces in Nramp TMS (Cellier et al. 1995). Phylogenetic analyses using this alignment demonstrate that Nramp (Slc11) carriers are homologous to the Slc5-7 and the BCCT and NCS1 families (Fig. 5a–c), a result confirmed by independent sequence clustering analyses (Fig. 5d).

7 Functional Implications for Nramp Immunological Roles

Structural homology to the LeuT superfamily provides a testable hypothesis to study structure-function relationships in MntH/Nramp (Slc11) transporters; it is supported by both TM topology analyses using functional homologs (Courville et al. 2008; Czachorowski et al. 2009) and *in vitro* biophysical analyses of Nramp2 polypeptides corresponding to the pseudo-symmetric TMS 1 & 6 (Wang et al. 2010; Xiao et al. 2010). Low-resolution 3D models of distinct conformers can be useful to interpret the results of site-directed mutagenesis studies, and the significance of genetic variants observed in the Nramp family. For instance, Nramp alignment could be tested by modeling a novel homolog, Nrat1 recently described in plants, which diverged to transport Me^{3+} instead of Me^{2+} (Xia et al. 2010). Nramp models also provide a 3D framework to study the functional significance of high level sequence conservation in this family which can be traced back to the iron-ages of immunity. Since the Nramp family likely emerged before aerobiosis (Cellier et al. 2001; Richer et al. 2003) evolution-driven analyses of MntH/Nramp structure relationships could reveal adaptations to function in oxidative environments and sustain host/microbe interactions. It will be also of interest to determine how modulating Nramp Me^{2+} and H^+ transport activity can impact macrophage phenotypes both during infection and in resting conditions.

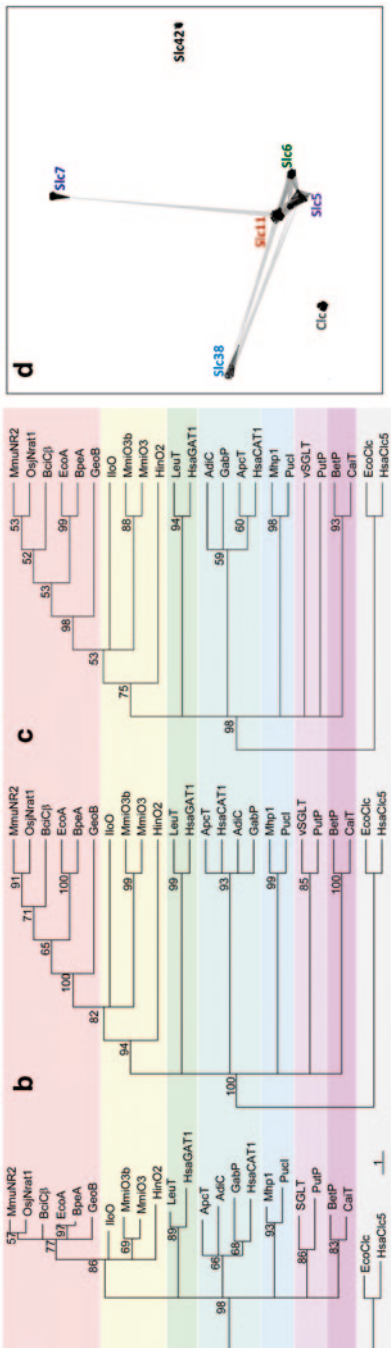

Fig. 5 Homology within the LeuT superfamily. A compound MSA was deduced by combining 3 conformer-specific MSAs derived from superposed structures (Guda et al. 2004) of carriers in conformations OF (Mhp1, PDB 2JLN: AdiC, PDB 3LRB; LeuT PDB 2A65), Occ. (Mhp1, PDB 2JLO; AdiC, PDB 3L1L; BetP, PDB 2WIT; CaiT, PDB 3HFX) or IF (Mhp1, PDB 2X79; CaiT, PDB 2WSX; vSGLT, PDB 3DH4; ApcT, PDB 3GIA), aligning 1 or 2 homologs per family (Slc5, PutP; Slc6, GAT1; Slc7, GabP, and HsaCAT1; BetP and CaiT; Mhp1, PucI) and aligning also sequences representing the Slc11 family and its outgroup (GeoB, BpeA, BciCb, OsjNrat1, MmuNR2 and IloO, HinO2, MmiO3, Mmi3b, respectively), as well as sequences encoding ion ports with inverted symmetry but unrelated (e.g., Clc channels). 310 parsimony-informative sites without missing or ambiguous information were used for calculations. Numbers indicate % occurrence per node after 3000 bootstrap re-samplings. **a** Maximum Likelihood tree (Schmidt and von Haeseler 2007; substitution matrix WAG, γ-distributed substitution rate variation among sites $\alpha = 8.15^{+/-} 0.82$); length of horizontal branches indicates relationships, based on the scale of 1 substitution/site. **b** and **c** Minimal Evolution tree using a Poisson correction for multiple substitutions per site (**b**) and Maximum Parsimony tree (**c**) were condensed by collapsing nodes with occurrence <50% (Tamura et al. 2007). **d** Slc11 relationships to the LeuT superfamily visualized using Psi-Blastall full-length pairwise sequence similarity scores calculated using the Blosum 62 matrix and NR database (Frickey and Lupas 2004), 339 Slc11/Nramp family sequences and sequence sets picked from NCBI Homologenes (LeuT superfamily: Slc7:41; Slc38:27; Slc5:108; Slc6:145; LeuT outgroup: Slc42/Amt:18; Clc:72). *Links* between *dots* indicate sequence similarity with p value better than 1e–45; *darker lines* indicate lower p values

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Index

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