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## The Role of Complement in Danger Sensing and Transmission

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### Abstract

Self–non-self discrimination has long been considered the main function of the immune system. Increasing evidence supports the view of the immune system as a network of complex danger sensors and transmitters in which self–non-self discrimination is only one facet. To meet the challenge of danger sensing, the immune system carries a large stock of germline-encoded, highly conserved molecules that can recognize microbial as well as modified host structures. Among those are the Toll-like receptors (TLR), which comprise a dozen membrane-bound pattern-recognition receptors that directly link danger recognition to danger transmission through activation of several distinct cellular signaling pathways. Here, I discuss the function and biology of a complex, evolutionary ancient system, the complement system, which has long been considered critical to host defense. In contrast to TLRs, the complement system senses danger by a panel of soluble molecules that can directly bind to specific complement receptors and/or initiate a complex cascade of proteolytic events that lead to the generation of soluble complement fragments able to bind to another, distinct set of specific complement receptors. As I will outline in this review, complement-mediated danger sensing and the complex transition of this information into distinct cellular activation profiles is critical for tissue homeostasis under steady-state conditions and in response to infection and cell injury. Furthermore, I will discuss recent findings that support a concept of intense cross-talk between the complement system and TLRs, which defines the quality and the magnitude of immune responses *in vivo*.

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### Key Words

Complement  
Immunity  
Inflammation  
Danger  
Toll-like receptor

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## Introduction

The main purpose of the immune system is to guarantee the functional integrity of the body. As such it needs to recognize potential threats that could jeopardize this integrity and to eliminate such threats. This view demands sensors that have the ability to identify all kinds of potential danger. Initially, immunologists considered everything that is non-self as a threat, in particular, pathogenic bacteria. Vice versa, this model implied that self-derived molecules are innocuous and that the immune system is designed to discriminate self from non-self (1,2). Importantly, immunity was mainly considered as cellular immunity, neglecting “unspecific” immune responses of innate immunity, including complement. As we know today, this view was certainly oversimplified as the immune system can recognize self and promote strong inflammatory responses against self-derived molecules eventually leading to autoimmunity. Clearly, antigen-presenting cells (APCs) randomly sample molecules in their surrounding, present those antigens to T cells, and activate T cells through co-stimulatory signals suggesting that cellular immunity per se cannot discriminate between self and non-self.

A loophole for this dilemma was provided by the suggestion that germline-encoded structures may exist on APCs that recognize conserved pathogen-associated molecular patterns (PAMPs) and that only this recognition licenses APCs to induce T cell activation (3). This infectious non-self (INS) model fueled new interest into innate immunity research resulting in the discovery of pattern recognition receptors (PRR) such as Toll-like receptors (TLR) that recognize conserved structures on all sorts of microorganisms. Data showing that TLR activation on dendritic cells (DCs) induces upregulation of co-stimulatory molecules and secretion of T cell-activating

cytokines supported the model non-infectious self versus infectious non-self discrimination (4). However, several obstacles remained, as the model does not provide satisfactory mechanistic concepts to explain transplant rejection, development of autoimmunity, adjuvants activity of non-bacterial origin, or spontaneous regression of occasional tumors (5). In order to reconcile these pitfalls, Matzinger proposed a “danger model” of immunity (5,6). In this model, immunity is not designed to discriminate self from non-self but to recognize danger-associated molecular patterns (DAMPs) irrespective of their nature. In other words, immunity evolved as a system to recognize tissue damage rather than foreignness. In support of this view, endogenous ligands of PRRs have been found that have the ability to license APCs for T cell activation independent of infection (7). The functional integrity of tissues is not only jeopardized by infectious or injury-related non-infectious threats but by ongoing cell death and the need to adequately clear and replace these cells. Obviously, immune responses are necessary to adequately address such physiological threats; however, the quality of such physiologic immune responses is different. Inflammatory responses that are beneficial under pathological conditions, i.e., infection, toxic, or traumatic tissue injury, are undesirable in response to physiologic threats. If so, how does the immune system discriminate between physiological and pathological threats? Furthermore, certain humoral and cellular effectors such as complement factor 3 (C3) and natural killer (NK) cells are powerful weapons of innate immunity that can induce cell killing independent of any danger motifs. Tissue cells are protected from killing through expression of self-derived inhibitory molecules and only the lack of these regulators instructs complement or NK-mediated suicide. Thus the INS or the danger model may not be considered apodictic dogmas but helpful

frameworks that allow for a better understanding of how innate and adaptive immunity responses face the challenge of tissue integrity.

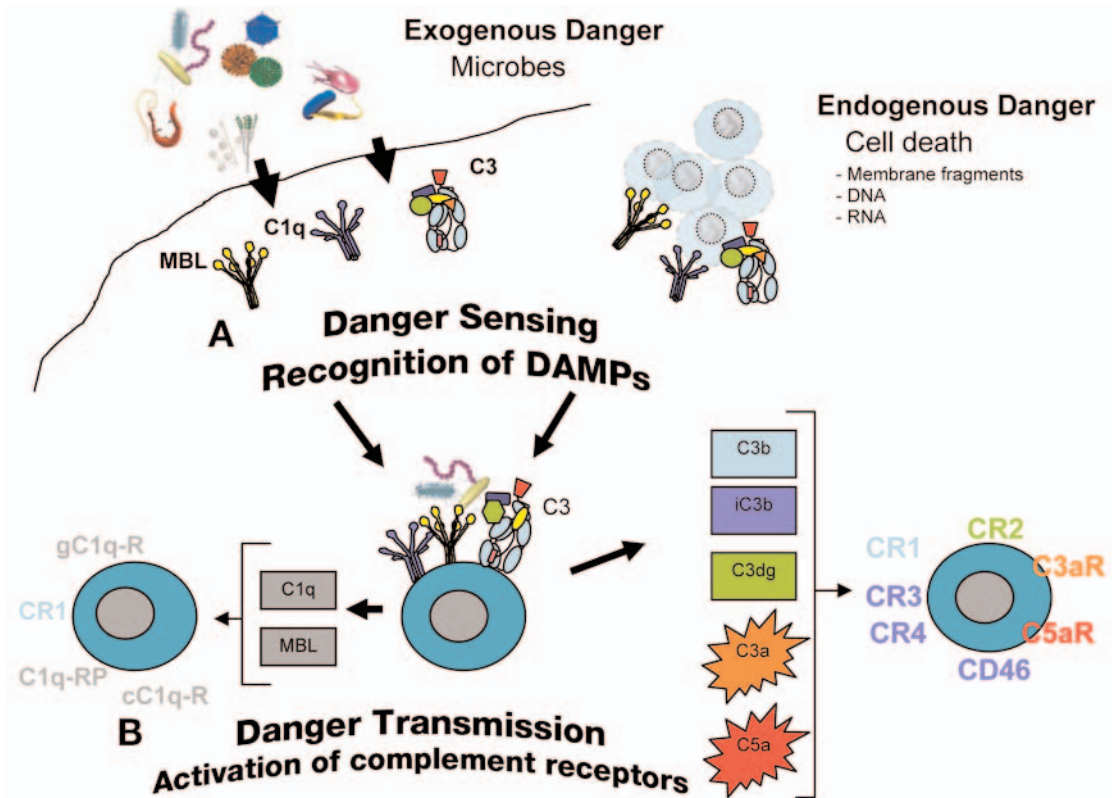
INS and danger model-driven research mainly focused on search for novel pattern-recognition receptors and ligands for such receptors. This focus neglected contributions of other arms of innate immunity such as the complement system that have the potential to recognize exogenous and endogenous threats and to shape innate as well as adaptive immune responses. Complement has been primarily regarded as a host defense system against infection that attracts leukocytes, flags microorganisms for facilitated phagocytosis, and kills bacteria through cell lysis. As I will outline below, complement is critically involved in tissue homeostasis, danger sensing, translation of danger signals into cellular innate, and adaptive immune responses as well as missing self responses.

### **Complement as a Humoral “Master Alarm System” of Innate Immunity**

The complement system is a central arm of innate immunity comprising a network of more than 30 serum and cell-surface proteins that is primarily considered to recognize and to eliminate microorganisms (8), clear immune complexes and apoptotic cells (9), and mediate inflammation (10,11). Recent findings provide evidence that complement also regulates innate as well as adaptive immune responses (12,13). In evolutionary terms, complement is a phylogenetically ancient arm of innate immunity that has evolved more than 700 million years ago. Currently available data suggest alternative and lectin pathway functions even in non-vertebrates such as *Ciona intestinales* (14). During evolution, the complement system has been build around C3 (e.g., by gene duplication in case of C4, C5) stressing the critical importance of C3 and complement in general for the survival of complex organisms.

C3 can be cleaved by different mechanisms, i.e., in response to lectin, alternative and classical pathway activation. Mannan-binding lectin (MBL), a C-type lectin, recognizes conserved carbohydrate patterns on pathogens and binds to MBL-associated serine proteases MASP-1 and MASP-2 (15). The initial step of alternative complement pathway activation is the thioester activation within C3 by polysaccharide hydroxyl groups (16), nucleating the formation of a protease complex. Finally, the classical complement pathway is activated in response to natural or induced antibodies that recognize conserved or variable molecule structures to form immune complexes that bind C1q. As I will outline below, complement activation is much more complex than that.

Considering that the immune system has evolved to prevent tissue damage (5), complement may be regarded as humoral “master alarm system,” providing (a) critical “danger sensors” that recognize harmful structure of either infectious or non-infectious origin and (b) “danger transmitters” that translate the danger signals into adequate immune responses (Fig. 1). A prerequisite for the role of complement as a critical alarm system is that the initial “sensors” of the distinct complement pathways, i.e., C1q, MBL, and C3, recognize exogenous as well as endogenous danger motifs. Indeed, in addition to the well-appreciated binding to immune complexes, C1q has been found to bind cell wall components and membrane proteins of all kinds of microorganisms, the prion infectious agent (17) as well as fragments of cellular and subcellular membranes (e.g., mitochondrial membranes) and other modified host proteins and phospholipids (18–21). Furthermore, it can bind C-reactive protein (CRP) and serum amyloid P (SAP) protein of the pentraxin family (22). MBL recognizes polysaccharide structures on bacteria, fungi, viruses, and parasites (23) as



**Fig. 1.** Danger sensing and danger transmission by the complement system. C1q and mannan binding lectin (MBL) are soluble proteins that recognize danger-associated molecular patterns (DAMPs) of microbes (exogenous danger) or of injured/apoptotic cells (endogenous danger). C1q and MBL not only recognize DAMPs but translate the danger information into specific cellular responses by interaction with specific receptors on distinct cells (danger transmission; lower left). C3 acts as an ubiquitous danger sensor, which is activated through nucleophilic attack of its thioester to covalently bind as C3b to its activator surface. Specificity is gained by the environment of the activator surface (i.e., the content of complement regulator molecules), which ultimately decides the fate of the C3b molecule. This can be (a) termination of C3 cleavage; or (b) amplified C3 cleavage to generate huge amounts of C3b and its degradation products iC3b, C3dg, and C3a. The latter outcome results in danger transmission through activation of C3 cleavage product-specific receptors (danger transmission; lower right).

well as on apoptotic host cells (24). C3 has a complex role as it functions as a sensor of the alternative pathway, serves as substrate for proteolytic cleavage by the C3 convertases formed in response to classic and lectin pathway activation, and nucleates the formation of the C3 amplification loop. The thioester in nascent C3, which allows covalent attachment to surfaces, is highly protected. Data from the recently uncovered C3 crystal structure provide

a rationale for the protection of the thioester from rapid hydrolysis or reaction with nucleophiles. C3 (as well as C4 and C5) belongs to the  $\alpha$ 2-macroglobulin family of proteins comprising a core of eight homologous macroglublin domains (MG; already found in metazoans) that evolved from a common ancestor (25). Inherent structural properties of the MG domains within the C3 molecule restrict the accessibility of the thioester (25).

However, the protection is not absolute as C3 spontaneously hydrolyses to form C3(H<sub>2</sub>O) at a very low rate (in which C3a is retained) and, in addition, reacts with nucleophils at low pace eventually leading to C3 cleavage. In fact, C3a can be found in plasma in the range of 10<sup>-8</sup>M in healthy individuals (26) supporting the view of permanent low-level C3 cleavage, something that has also been observed *in vitro* (27). Numerous examples exist for spontaneous C3b deposition on microorganisms as well as on tissues (e.g., tumors) in response to alternative pathway activation. When the cells express complement regulators such as membrane cofactor protein (CD46) or complement receptor 1 (CR1;CD35), C3b is converted into inactive products through cleavage by factor I. If the integrity of the cell is distracted, by apoptosis, injury, infection, or tumor transformation, cells can lose these regulators and become target of complement-mediated immune responses. Collectively, these data suggest that the complement system provides a sophisticated system of “alarm sensors” that are able to detect changes in the physiologic environment of tissues.

### **Complement Activation Liberates Danger Transmitters That Shape Innate and Adaptive Immune Responses**

The immune system needs to distinguish between physiologic changes in the environment, e.g., physiologic cell death, and the necessity to clear these cells without substantial inflammation and pathological changes that demand inflammation. In short, danger-sensing needs to translate in adequate danger-transmission that tailors the immune response. The complement system is well suited for this task as it releases a panel of bioactive cleavage fragments, the function of which is regulated by interaction with soluble and cell-bound proteins. Cleavage fragments

of C3 (e.g., C3a, C3b, iC3b, C3dg) have the ability to act as “danger transmitters” that instruct cells of the innate and the adaptive immune systems through interaction with specific receptors (C3aR, CR1[CD35], CR2 [CD21], CR3 [CD11b/CD18] and CR4 [CD11c/CD18]). Likewise, cleavage products of C5, i.e., C5a as well as the membrane attack complex (C5b-9; MAC), can transmit humoral inflammation and translate humoral into cellular inflammatory responses through activation of G protein-coupled receptors (GPCR). Differential expression of the distinct complement receptors on distinct cell populations as well as regulatory feedback loops that regulate their expression provide an important platform to tailor innate and adaptive immune response. In addition to the cleavage fragments of C3 and C5, C1q and MBL can function as danger transmitters, in addition to their roles as danger sensors (Fig. 1). In fact, complement-derived signals shape innate and adaptive immune responses at many levels to adequately address physiological and pathological challenges. This includes the clearance of apoptotic and necrotic cells, the removal of self-reacting B cells, the generation of the natural antibody repertoire, the maintenance of mucosal tolerance, and the allocation of powerful antimicrobial defense mechanisms.

### **The Impact of C1q/MBL Binding to Distinct C1q Receptors**

C1q and MBL bind to several receptors including cC1q-R/CR, or calreticulin (CR), which is also known as collectin receptor, gC1q-R, C1q-Rp (CD93), and CR1 (CD35), the receptor for C3b (28) (Fig. 1). On macrophages and DCs, cC1q-R/CR and gC1q-R promote the uptake of apoptotic cells and of immune complexes. Furthermore, they mediate phagocytosis and killing of all sorts of microorganisms. In addition to cC1q-R/CR

and gC1q-R, C1q-RP can enhance ingestions of C1q-targeted cells (29). The view of C1q-coating as an important physiological clearance mechanism for apoptotic cells is supported by the fact C1q-deficient mice show profound impairment in such clearance resulting in severe glomerulonephritis (30). In humans, the lack of C1 promotes a form of cutaneous lupus (31). The activation of cC1q-R/CR and gC1q-R on platelets and endothelial cells has been associated with increased cell adhesion and thrombosis suggesting a link between complement and coagulation. This view is further supported by data showing that high-molecular-weight kininogens bind to a complex composed of gC1q-R, cytokeratin1, and urokinase plasminogen activator receptor on endothelial cells that is crucial for the assembly and activation of the intrinsic coagulation/kinin-forming cascade (32). On B and T cells, cC1q-R/CR and gC1q-R regulate cell activation and proliferation. As may have been expected in terms of evolutionary pressure, pathogens have found ways to exploit many of the physiological functions of complement receptors for their survival. As an example, hepatitis C virus can bind through its core protein to gC1q-R, leading to suppression of T cell proliferation (33).

### C3 Cleavage Fragments Activate Distinct Cellular Receptors

#### *The Impact of C3b Binding to CR 1*

C3 cleavage products provide a remarkably complex pattern of signals to direct immune responses. The primary cleavage product of C3, C3b, binds to CR1, which is a single-chain, type 1 transmembrane glycoprotein (Fig. 1). In the circulation it is predominantly expressed on red blood cells. Here, it serves as an immune adherence receptor for C3b-loaded immune complexes (IC) that are shuttled to the liver and the spleen where they are transferred to and processed by tissue

macrophages through an Fc receptor-mediated process (34,35). This is an important physiological mechanism for the safe removal of IC. When defective, it results in deposition of IC in tissues and promotes severe inflammation as observed in SLE. In B cells, it is supposed to play important roles in maintaining tolerance against tissue-derived antigens together with CR2 (see below). In neutrophils and monocytes, CR1 can mediate phagocytosis (36), although other complement receptors appear more important in this respect (i.e., C1qR and CR3). Importantly, CR1 is the only co-factor protein for factor I that is able to promote degradation of C3b to C3dg, which shifts the specificity from CR1 to CR2 (discussed below). In addition to this physiologic properties, pathogens such as *Leishmania*, *Mycobacteria*, and HIV use C3b deposition on their surface to enter host cells through CR1 (37). Furthermore, several publications suggest an important role for CR1 in the pathogenesis of severe malaria which is related to rosette formation (38).

#### *The Impact of C3dg Binding to CR 2*

The terminal cleavage fragments of C3, C3dg or C3d, bind to complement receptor 2 (CR2; CD21; Fig. 1), which is primarily expressed on B cells and follicular DCs. Furthermore, CR2 expression has been found on subsets of CD4 and CD8 thymic and peripheral T cells (39,40), on activated T cells (41), basophils, mast cells, keratinocytes, and epithelial cells. On B cells, CR2 is part of the B cell receptor complex. C3d binding to CR2 results in dramatic (1000-fold) reduction of the B cell activation threshold (42). Furthermore, B cell responses are substantially impaired in mice in which the *Cr2* locus is disrupted resulting in a deficiency of both CR1 and CR2. These mice suffer from a defect in T cell-dependent antigen responses (43) and are prone to develop autoimmunity as a result of defective elimination of self-reactive B

cells (44). In addition to the regulatory effects on T cell-dependent antigens, an increasing body of data suggests a critical role for CR2 in shaping the natural antibody repertoire (reviewed in ref. (45)). Natural antibodies are mainly produced by a subset of long-lived, self-replenishing B cells, i.e., B-1 cells. As germline-encoded molecules, they serve as low-affinity pattern-recognition receptors that recognize pathogen as well as tissue-derived patterns. Importantly, *Cr2*<sup>-/-</sup> mice lack a subset of the natural antibody repertoire that is critical to recognize hypoxia-related damage on endothelial cells. On the one hand, this can be beneficial, as *Cr2*<sup>-/-</sup> mice are protected from intestinal ischemia/reperfusion injury (46,47). However, the impaired ability to recognize damaged cells may also lead to defective clearance and promote autoimmune responses. Collectively, these data demonstrate that the complement system is an integral part of the B cell compartment that shapes and regulates both the innate and the adaptive part of the B cell response (reviewed in ref. 48).

#### *The Impact of iC3b Binding to CR 3 and CR4*

Once C3b is covalently attached to a cell membrane, it can become part of the amplification loop of the alternative pathway that results in accelerated C3b deposition or it can be further degraded to iC3b, C3c, and C3dg depending on the soluble (factors B, H) and membrane-bound (CR1, CD46) molecules that serve as binding partners for C3b. The decision on the fate of C3b is primarily depending on the carbohydrate environment (49). Provided that a sialic acid-rich environment favors factor H binding to C3b, it acts as a cofactor for factor I to cleave C3b into iC3b and C3f. iC3b can bind to CR3 and CR4, both of which are  $\beta$ 2 integrins, functioning as important phagocytic receptors. CR3/4 are promiscuous receptors that bind a panel of

different endogenous and exogenous ligands such fibronectin, ICAM-1/2,  $\beta$ -glucan, or LPS (50). Importantly, CR3/4-mediated phagocytosis per se does not elicit proinflammatory signals in phagocytes nor does it provoke respiratory burst. Considering that CR3 has primarily evolved to serve physiologic functions necessary to balance tissue homeostasis, i.e., by clearing of apoptotic cells, the lack of inflammation is not surprising. However, CR3 is also considered an important receptor of innate immune defense against pathogens, promoting leukocyte adhesion and transmigration through the endothelium as well as inflammatory responses. Importantly, CR3 needs to be pre-activated to adopt a high affinity state before ligation results in an inflammatory response. Signaling pathways that induce the high affinity status include those downstream of activating immunoglobulin G receptors (Fc $\gamma$ Rs) or chemoattractant GPCR (51). Of note, oleic acid (52) or the 14-kDa myeloid-related protein (MRP-14) (53), which are released after tissue or cell injury, are also able to induce the high affinity state of CR3 suggesting that danger signals per se, but not the discrimination between infectious- or not- infectious danger signals, are critical to CR3-mediated inflammatory effector functions.

As described for other complement receptors, several pathogens exploit CR3 to invade phagocytes and to escape the immune response; examples are *Histoplasma capsulatum*, *Leishmania major*, and several *Mycobacteria*. Cell-mediated immunity is a critical defense mechanism to kill these microorganisms and/or to limit their systemic spreading. Interferon gamma (IFN- $\gamma$ ) released from Th1-polarized CD4<sup>+</sup> lymphocytes is crucial to infection control. Importantly, ligation of CR3 (by iC3b and other ligands) has been found to suppress the production of IL-12 from monocytes in response to bacterial stimulation (54).

IL-12 is a heterodimeric cytokine that plays a key role in bridging innate and adaptive immune responses as it activates and skews CD4<sup>+</sup> T cells towards Th1 lineage commitment (55). Thus, suppression of IL-12 is an important mechanism of intracellular pathogens to escape elimination by adaptive immune responses. Obviously, it is harmful for the host to shut down adaptive immunity following infection with these intracellular pathogens. In light of the physiologic functions of CR3, i.e., apoptotic cell clearance (see above), IL-12 suppression might be beneficial as it prevents unwanted activation of cell-mediated immunity.

### *The Impact of C3b Binding to CD46*

Another example of the regulatory impact of the danger transmitter C3b comes from its interaction with the regulator protein CD46, which is ubiquitously expressed on all nucleated cells. CD46 acts as cofactor for factor I and promotes the cleavage of C3b into iC3b. Its main control function is to protect tissues from complement-mediated attack by preventing deposition of C3b in response to alternative pathway activation and formation of the amplification loop. Importantly, CD46 expression is reduced in dying cells (56) suggesting that the decrease of this self-derived molecule is important in the recognition and clearing of apoptotic cells. In support of this view, the Gasque lab has recently shown that CD46 is rapidly translocated from cytoplasmic, nuclear, and membrane stores into membrane-bound apoptotic blebs, budding from the cell surface (57). They further demonstrated early exposure of nucleic acids on the cell membrane, functioning as a pattern-recognition motif for C1q, activating the classical pathway. In response to CD46 deprivation, associated with preserved CD55 and CD59 expression, preventing MAC formation, the cells become heavily loaded with

C3b, making them an excellent target for CR3-mediated phagocytosis. Collectively, these data suggest that CD46 may play a similar role as the major histocompatibility complex class I, the lack of which allows innate immunity to recognize altered self and to eliminate the altered cells through NK cell-mediated cytotoxicity.

In addition to its regulatory role in apoptotic cell elimination, CD46 impacts the development and maintenance of adaptive immune responses. Similar to the effect of iC3b binding to CR3, ligation of CD46 on monocytes by C3b dimers suppresses the production of IL-12 (58). This pathway has been demonstrated to be exploited by measles virus and may explain the immunosuppression following measles virus infection (58). In addition, CD46 serves as a receptor for several other pathogens such as *Neisseria*, Herpesvirus 6, *Streptococcus pyogenes*, and Adenoviruses (for review see ref. 59). On CD4<sup>+</sup> T cells, C3b-mediated activation of CD46 in the presence of TCR stimulation results in proliferation of a T cell population with a regulatory phenotype (60).

### **The Anaphylatoxic Peptides C3a and C5a Play Critical Roles in Danger Transmission Beyond Inflammation**

Serine proteases generated in response to activation of the three pathways of the complements system, as well as allergen and tissue-derived proteases can cleave the low-molecular-weight anaphylatoxic peptides (AT) C3a and C5a from C3 and C5, respectively. The ATs act as potent mediators of inflammatory effector functions such as phagocyte and mast cell recruitment and activation, including the release of granule enzymes, reactive oxygen species, cytokines, and chemokines as well as the upregulation of adhesion molecules (11). Most of these properties are mediated

through ligation of specific and distinct C3a and C5a receptors (see below). Consonant with the proinflammatory properties, ATs have been implicated in the pathogenesis of several infectious, autoimmune, and allergic diseases such as pneumonia (61), sepsis (62), rheumatoid arthritis (63,64), systemic lupus erythematosus (9), multiple sclerosis (65), and allergic asthma (66–68). In short the release of the AT is considered to contribute significantly to the benefit and burden of inflammation. In line with the view of complement as a crucial alarm system, several reports provide evidence for an important role of the ATs (and the membrane attack complex) in the development of the inflammatory response following ischemia/reperfusion injury (reviewed in ref. 11). Here, exposure of hypoxia-induced expression of neopeptides on endothelial cells can activate the three different pathways of the complement system to release C3a and C5a.

These well-appreciated pro-inflammatory properties of the AT are critical to shape the innate inflammatory response in response to tissue damage/destruction and/or attack by pathogens. As I will outline below, ATs exert regulatory functions far beyond their role as proinflammatory effector molecules of innate immunity. I will provide examples demonstrating that ATs regulate tissue repair, tissue remodeling, shape-adaptive immune responses at the mucosal surface, and impact TLR-induced danger responses.

### Anaphylatoxin Receptor-Dependent and -Independent Effects

The ATs unfold their functions through distinct GPCR expressed preferentially on myeloid lineage-derived leukocytes (69–72). In leukocytes, C3aR and C5aR (CD88) mediate their effects via coupling to the pertussis-sensitive and -insensitive G proteins  $G\alpha_i$  and  $G\alpha_{16}$ , respectively (73–76). In endothelial cells, the C3aR also couples to  $G\alpha_{12}$  and  $G\alpha_{13}$

(77). The downstream pathways have been intensively studied in neutrophils, monocytes, and macrophages. In neutrophils, C5aR signals through  $G\alpha_i$  and activates phosphoinositide-3-kinase  $\gamma$  (PI3K $\gamma$ ). PI3K $\gamma$  belongs to a family of lipid kinases involved in generating distinct phosphoinositides that are important second messengers for intracellular signaling. C5a activates PI3K $\gamma$  through coupling to distinct G proteins: (a)  $G\alpha_i$ , which activates the small GTPase Ras, and (b)  $G\beta\gamma$ . In turn, Ras or PI3K $\gamma$  can activate the Raf/mitogen-activated protein kinase (MAPK)/extracellular-signal regulated kinase (ERK) kinase (MEK)/ERK cascade, which links C5aR ligation to gene expression through effects on transcription factor activation and translocation to the nucleus. In monocytes and macrophages, coupling of the C5aR to  $G\alpha_i$  and  $G\alpha_{16}$  has been described. The  $G\alpha_{16}$  subunit and the  $\beta\gamma$ -subunit can activate phosphoinositide-specific phospholipase C (PLC) subtype  $\beta$ , which is a multidomain phosphodiesterase that generates the second messenger inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). These latter molecules mediate  $Ca^{2+}$  mobilization and the activation of protein kinase C (PKC). Downstream of PKC, signal transducer and activator of transcription 3 (STAT3) can be activated by C5a [through Janus kinase (Jak) phosphorylation] (77,78), suggesting a role for C5a in cell proliferation. In fact, C5a (and C3a) were found to be essential for liver regeneration (79). In further support of this view, C5aR signaling induces epidermal-growth-factor (EGF) receptor phosphorylation in endothelial cells (77). EGF is a strong mitogen for human airway smooth muscle cells via activation of the ERK MAPK pathway (80).

In addition to CD88, the orphan receptor C5L2 has been described as a second receptor for C5a (81–83). Interestingly, C5L2 binds C5adesArg with a 10-fold higher affinity than CD88 (82,83). In contrast to CD88, C5L2 is

uncoupled from G proteins. C5L2 ligation does not result in degranulation, increase in intracellular  $\text{Ca}^{2+}$ , or receptor internalization, suggesting that C5L2 signaling does not follow the classical pathways of chemoattractant receptors. The overall expression mRNA expression levels of C5L2 in murine tissues is much lower than that of CD88 (84). However, the situation can change dramatically in acute inflammation, which may impact the inflammatory response. During septic peritonitis, C5L2 surface expression markedly increased on neutrophils whereas CD88 expression decreased. In contrast, expression of both receptors increased in tissues (lung, liver, kidney, and heart). Blockade of either receptor had an opposite effect on the production of IL-6, i.e., C5aR blockade resulted in reduction whereas C5L2 blockade resulted in increase of IL-6 (85). Similarly, inflammation was enhanced in C5L2<sup>-/-</sup> mice in a model of immune complex alveolitis, as evidenced by increased cellularity and pro-inflammatory cytokines in BAL (84). Collectively, these data suggest that C5a/C5adesArg binding of C5L2 limits the pro-inflammatory properties of these ATs. The opposing roles of the CD88/C5L2 tandem provide a sophisticated feedback mechanism that allows the complement system to regulate C5a-mediated danger transmission.

Several reports in the literature suggest AT effects that are independent of AT receptor ligation. This is particularly true for C3a and its degradation product C3adesArg. The latter is devoid of binding to the C3aR; however, similar to C3a it can suppress LPS-induced cytokine production from tonsil-derived B cells and monocytes (86–88). Furthermore, C3adesArg stimulates pituitary cells to release prolactin, growth hormone, and adrenocorticotropin (89). C3adesArg has also been termed acylation stimulating protein (ASP) by one group referring to its role in triacylglycerol synthesis in human adipocytes (90). All of

the C3adesArg biology has been enigmatic as it does not fit in our mechanistic understanding of biological functions as receptor-mediated entities. Although a recent publication suggests that C5L2 maybe the missing link, C3adesArg binding to this receptor and signaling is controversial (91,92).

Data from the crystal structure of C3 may provide a loophole from this dilemma (25). The paper shows that important domains with specialized functions have been added to the core region of C3 through a series of gene-insertion events, one of which is the insertion of C3a into a loop within the MG6 domain. Thus the “original” function of C3a prior to this insertion may have been distinct from its function related to C3aR signaling found later in evolution. C3a is a highly cationic peptide containing four  $\alpha$ -helical regions, structural features that resemble those of antimicrobial peptides. Indeed, C3a and C3adesArg exert antimicrobial activity towards Gram negative and Gram positive bacteria in the low micromolar range (93). As most of the C3a generated in the circulation is rapidly converted into C3adesArg by means of serum carboxypeptidase N, the original function of C3a (direct microbial killing) is likely to be preserved in situations of massive systemic complement activation such as sepsis. Interestingly, antimicrobial peptides have been found to exert immunoregulatory functions as described for C3a (94). These functions may originate from cell activation by peptide insertion into cell membranes or through binding to promiscuous GPCRs as has been shown for cathelicidins that can activate FPRL1 or  $\beta$ -defensins, which can signal through CCR6.

### **A Critical Role for Anaphylatoxin-Receptor Signaling in Tissue Repair and Fibrosis**

In addition to expression on professional inflammatory cells, AT-receptor expression has been demonstrated on non-myeloid cells such as endothelial cells (77,95–97), epithelial cells

(96,98), smooth muscle cells (98), fibroblasts (99,100), as well as on tissue cells of kidney (101,102), lung (98,103), liver (100,103), and brain (104,105). The presence of AT receptors on tissue cells suggests AT-mediated immune functions beyond inflammation. Complement activation in response to exogenous and endogenous danger signals is often associated with cell and/or tissue injury. In addition to the safe removal of cells (discussed above), cells need to be replaced to maintain or reestablish normal tissue function. The mechanisms underlying tissue repair and remodeling are complex. Out of all organs, the liver has the greatest potential to regenerate damaged or destroyed cell mass. Liver regeneration proceeds through distinct stages, including priming of hepatocytes, cell cycle progression, proliferation, and cessation of regeneration. In models of acute toxic liver injury and partial hepatectomy, the Lambris lab has recently shown that liver regeneration is severely impaired in AT-receptor deficient mice (79,106). Mechanistically, they found direct effects of AT-receptor signaling on hepatocytes and on Kupffer cells that increased IL-6 and TNF- $\alpha$  production, which in concert, activated transcription factors (NFk-B, STAT-3) crucial for liver cell regeneration.

Chronic liver tissue injury of infectious or non-infectious origin often leads to fibrotic remodeling of the tissue architecture. Recently, a gene locus in the mouse genome was identified by quantitative trait locus analysis that confers susceptibility to liver fibrosis. Importantly, the gene locus included the C5 gene. In a model of chronic liver injury, pharmacological targeting of the C5aR had antifibrotic effects in vivo (100). Furthermore, the authors found that common polymorphisms of the human gene C5 were associated with advanced fibrosis in chronic hepatitis C virus infection. Collectively, their data suggest that C5 has a causal role in fibrogenesis across

species (100). In a second, independent study, C5 had a detrimental effect during chronic stages of bleomycin-induced pulmonary injury, also pointing toward a profibrotic role of C5 (99).

These data suggest that in case of acute cell injury, AT-receptor activation on tissue cells and on tissue macrophages is an important signal for tissue repair. In contrast, triggering of the C5aR on fibroblasts through ongoing activation of complement-derived danger sensors and subsequent release of the danger transmitter C5a results in defective repair, i.e., fibrotic remodeling of chronically injured tissues.

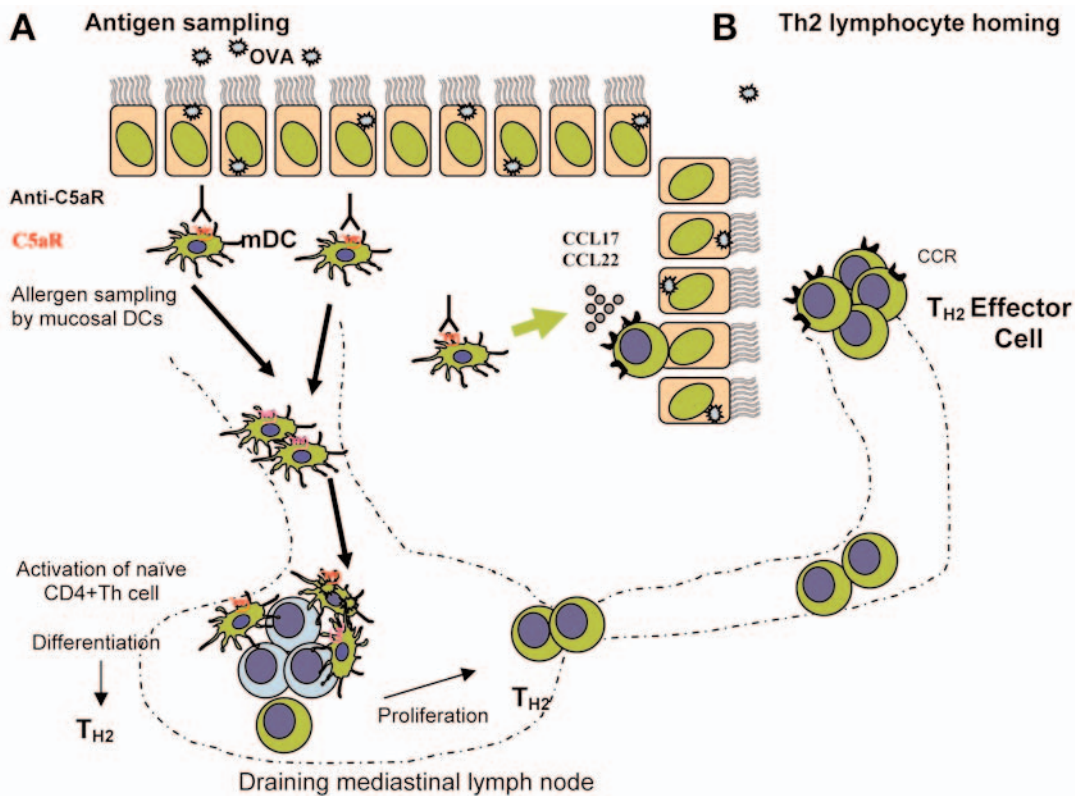
### **C5a Receptor Signaling in Pulmonary DCs Regulates Inhalation Tolerance**

As outlined above, ATs instruct innate immune responses in many ways. The presence of AT receptors on APCs suggests that they may also be involved in the development of adaptive immune responses. AT-receptor expression has been found on distinct DC populations and was shown to induce several effector functions: (a) DCs isolated from rat respiratory tract tissue, or generated in vitro from human monocytes, migrate in response to C5a (107); (b) human Langerhans cells (a type of dermal immature DC) express C5aR and migrate toward a C5a gradient (108); (c) Langerhans cell C5aR expression increases during maturation and tissue trafficking (109); (d) human dermal DCs and monocyte-derived immature DCs express C5aR and C3aR (110); (e) C5a induces calcium fluxes in such dermal DCs, whereas C3a does not; (f) expression of both AT receptors is downregulated on such dermal DC by TNF- $\alpha$  (111); and (g) both human and murine monocyte-derived immature DCs and mature DCs respond to C5a as determined by chemotaxis and Ca<sup>2+</sup> mobilization (111). Thus, the interaction between

C5a has potent functional effects on both immature DCs and mature DCs.

Mucosal DCs play important regulatory roles in the respiratory and the intestinal tract, where they control the immune response to inhaled or ingested antigens. As immature DCs, they continuously sample proteins, process these proteins, and migrate to the regional lymph nodes (Fig. 2). During this process they change their phenotype and express high levels of MHC II and a panel of co-stimulatory molecules. Upon contact with naïve T cells, they stimulate T cell proliferation. Importantly, this DC/T cell interaction does not induce immunity but tolerance (112). Under steady-state conditions, immature DCs take up harmless exogenous as well as endogenous antigens such as airborne particles or apoptotic cells. According to the INS model, endogenous antigens or harmless exogenous antigens are unable to promote full T cell activation, as the mucosal DC has not received an infectious (non-self) danger signal, which would then induce/upregulate all of the pathways (MHC II, co-stimulatory molecules and cytokines) necessary to promote adaptive immunity. As pointed out above, apoptotic cells are recognized by complement-derived danger sensors, suggesting low-level complement activation at mucosal surfaces. Indeed, trace amounts of C3a and C5a have been found in bronchoalveolar lavage samples from healthy volunteers (113,114). Furthermore, C5aR are expressed on distinct subsets of airway DCs. Together these data suggest a possible role for C5a in mucosal tolerance, in particular, in the lung. In support of this view, we recently found that ablation of C5aR signaling in mice results in the spontaneous development of a Th2-polarized immune response. Moreover, repeated pulmonary exposure to ovalbumin, resulting in inhalation tolerance, induced a strong Th2 adaptive immune response, associated with

eosinophilic and lymphocytic airway inflammation and increased airway responsiveness in the absence of C5aR signaling. Mechanistically, C5aR blockade increased the ability of pulmonary myeloid DCs to produce CCL17 and CCL22 chemokines, both of which are critical for pulmonary homing of Th2 effector cells (115) (Fig. 2). These data suggest a critical role of C5aR signaling at the DC/T cell interface that prevents from the development of maladaptive immune responses at the mucosal surface. Moreover, they suggest a dynamic model of inhalation tolerance in which C5aR signaling in pulmonary DCs is necessary to prevent their full activation and subsequent induction of adaptive immunity. Thus, adaptive immune responses not only develop when infectious (non-self) or non-infectious danger signals trigger TLRs, but also in the absence of complement-derived danger transmitters. As is evident from these data, the term danger transmitter does not adequately describe the suppressive effect of C5a on pulmonary DCs. Rather, C5a functions as an endogenous, soluble self-recognition signal, the lack of which indicates danger activating DCs and initiating T cell activation. Thus, C5a may be considered a “balance transmitter” that prevents inadequate and potentially harmful immune responses. The decision, which of the functions dominates (danger vs balance) may depend on the ratio of CD88/C5L2 expression, the cell type and/or additional signals provided by pattern recognition receptors such as TLRs (see below). Collectively, these data complement the missing-self concept in which the lack of cell bound receptors instructs the innate immune system (NK cells, complement system) to eliminate cells with altered self motifs. Here, the lack of C5a or, more precisely, that of C5aR signaling, instructs innate immunity (DCs) to activate adaptive immune responses. Such a concept suggests that “bal-



**Fig. 2.** Model of the mechanisms underlying the breakdown of inhalation tolerance in the absence of C5aR signaling. Inhibition of C5aR signaling during primary antigen sampling by pulmonary myeloid DCs results in the development of airway inflammation and Th2 adaptive immune responses in murine experimental allergic asthma (115). In the absence of C5aR signaling, allergen-sampling mDCs migrate to the draining mediastinal LN and promote Th2 lineage commitment of naïve CD4+ Th lymphocytes. Th2 effector cells circulate into the periphery, undergo CCR4 upregulation, and eventually, home back to the lung in response to CCL17 and CCL22 chemokines. Primary allergen uptake of mDCs in the absence of C5aR signaling promotes the production of CCL17/CCL22 from mDCs which is substantially enhanced during repeated allergen uptake.

ance transmitters” may have important control functions that prevent the development of autoimmune diseases, e.g., following apoptotic cell uptake by APCs.

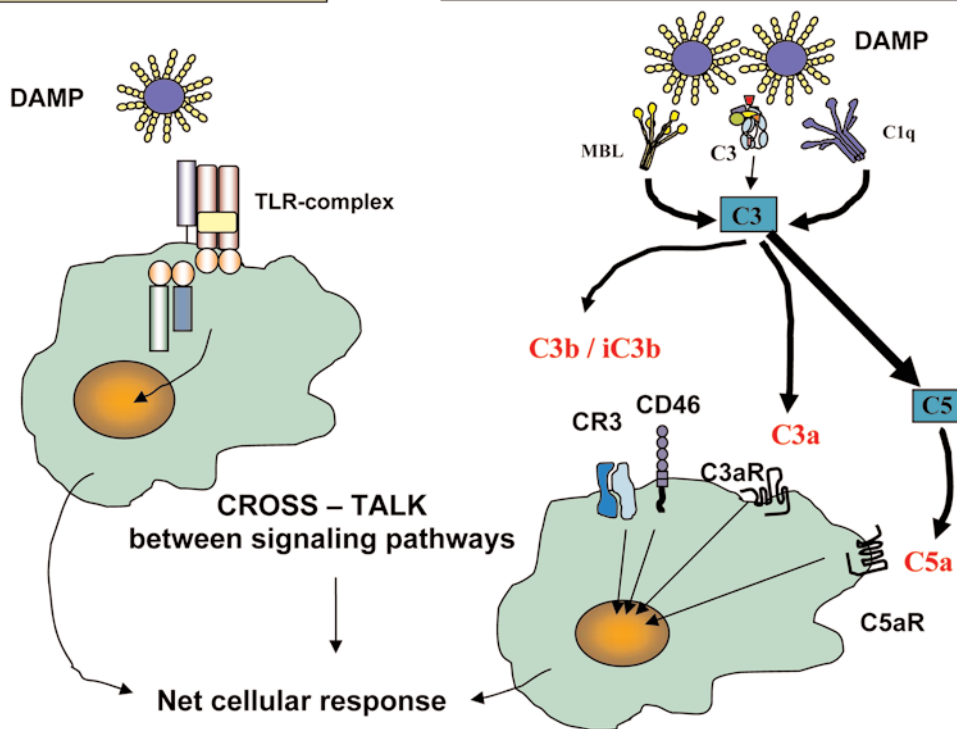
### C5a Receptor Signaling on APCs Impacts Danger Transmission Through TLRs

Many exogenous or endogenous molecular patterns, either derived from pathogens such as LPS, glycolipids, teichoic acid or from tissues such as modified LDL or necrotic cells can be recognized by complement-derived

danger sensors and TLRs suggesting that complement receptor pathways may intersect with TLR pathways (Fig. 3). First evidence of a regulatory link between the complement system and TLR-mediated immune responses was provided by reports demonstrating that activation of CR3 (54) and the complement regulator molecule CD46 (58) by C3 cleavage products promote efficient control of TLR-induced synthesis of IL-12 from human monocytes. Furthermore, the C5 cleavage fragment C5a was found to downregulate LPS or *Staphylococcus* Cowan strain 1

## TLR ACTIVATION

## COMPLEMENT ACTIVATION

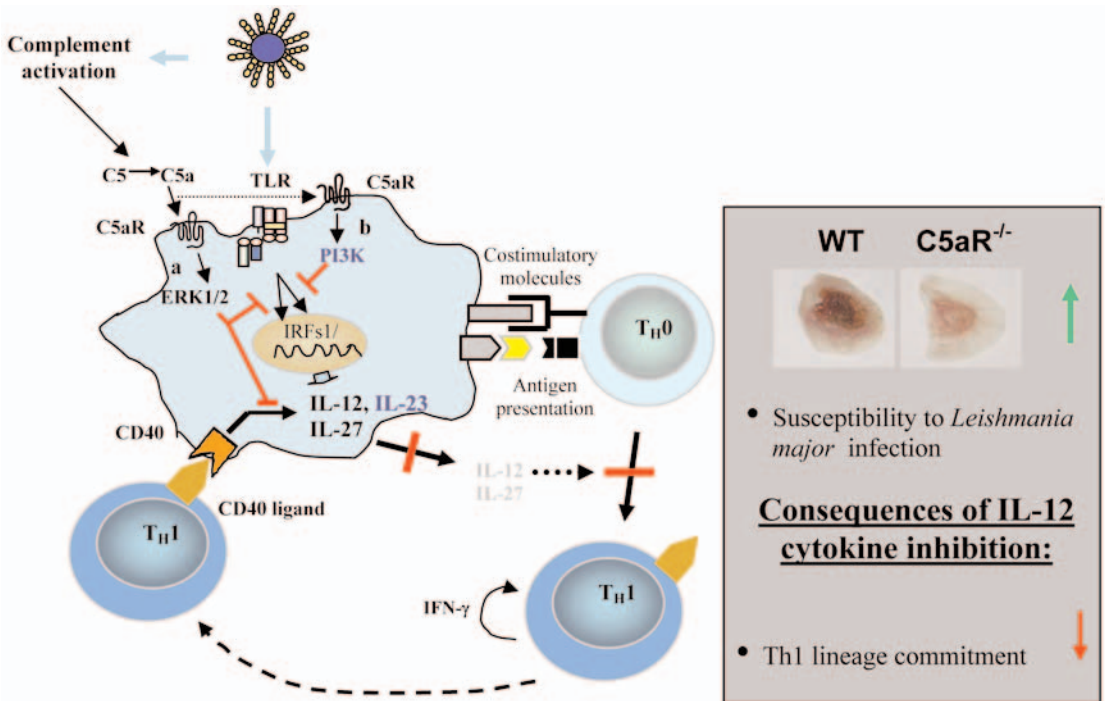


**Fig. 3.** Crosstalk between complement receptors and TLRs. DAMPs can be recognized by TLRs as well as by soluble danger sensors of the complement system such as MBL, C1q, and C3. Many cells of the myeloid lineage co-express TLRs and receptors for the cleavage products of C3, i.e., C3a, C3b/iC3b, and C5, i.e., C5a. Ligation of single TLRs or complement receptors in vitro results in activation of receptor-specific signaling pathways leading to typical cellular reaction patterns such as chemotaxis, phagocytosis, cytokine production, etc. In vivo, it is more likely that DAMPs activate several PRR (TLRs) and complement receptors in parallel, leading to different cellular activation profiles.

(SAC)-induced synthesis of IL-12 in human monocytes but not in monocyte-derived DCs (116,117). In contrast, ablation of C5 in murine macrophages led to reduced IL-12 production in response to IFN- $\gamma$  + SAC stimulation, suggesting that C5a enhances IL-12 production (118). These conflicting data suggest a complex, cell-dependent role of C5a in IL-12 regulation. The underlying molecular mechanisms and the consequences of such regulation on adaptive immunity and infection are poorly understood.

Mechanistic insights into the cross-talk between TLR4 and C5aR signaling pathways

have recently been provided in a report, demonstrating that C5a negatively impacts TLR4-induced synthesis of IL-12 family cytokines IL-12, IL-23, and IL-27 from murine macrophages through activation of signaling pathways that involve extracellular signal regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K) (119). In addition to its role in dampening TLR4-driven synthesis, C5a also suppressed CD40-induced IL-12 family cytokine production through the signaling intermediate ERK1/2 (Fig. 4). The CD40-CD154 (CD40 ligand) interaction provides an important feedback loop by which



**Fig. 4.** C5a-mediated negative regulation of TLR4 and T cell-driven synthesis of IL-12 family cytokines. DAMPs are able to activate TLR4, which directs the synthesis of IL-12 family cytokines IL-12, IL-23, IL-27, and activate the complement system to generate C5a. Binding of C5a to C5aR suppresses TLR4-induced mRNA synthesis of IL-12 family cytokine subunits IL-12/IL-23p40, IL-12p35, IL-23p19, and IL-27p28 through (a) ERK1/2- and (b) PI3K-dependent pathways. ERK1/2 phosphorylation and PI3K activation suppress the synthesis of IRF-1 and ICSBP transcription factors as an important mechanism to reduce mRNA synthesis of IL-12p35 and/or IL-12/IL-23p40. The C5a-induced inhibition of TLR4-induced IL-12 and IL-27 production negatively regulates Th1 polarization of naïve Th cells. C5a Furthermore inhibits T cell-driven synthesis of IL-12 induced by CD40/CD40 ligand interaction. This negative impact on Th1 polarization becomes critical in *Leishmania* infection, as normally susceptible BALB/c mice are almost protected from disease development in the absence of C5aR signaling.

activated CD4<sup>+</sup> T cells amplify the production of IL-12. Furthermore, IFN regulatory factor 1 (IRF-1) and IFN consensus sequence binding protein (ICSBP; IRF-8) were identified as crucial transcription factors downstream of ERK and PI3K pathways, respectively. These data indicate that C5a modulates both innate (TLR4) and adaptive (CD40) immune responses that drive the production of IL-12 family cytokines. Such broad inhibition is likely to affect the host response to infection and to modulate autoimmunity. In support of this view, a central role of C5/C5a was sug-

gested in the pathogenesis of autoimmune arthritis (64), systemic lupus (120), DTH responses (121), and allergy (118) as well as in resistance to *Listeria* (122) and to blood stage-malaria infection (123). Common to all of these models is their dependency on or exacerbation by IL-12 family cytokines. In the same report (119), a suppressive effect of C5a on Th1 polarization was found, the in vivo relevance of which was documented by the acquisition of resistance to *L. major* infection by the genetic deficiency of the C5a receptor in normally susceptible BALB/c mice (Fig. 4).

In this model, *L. major* takes advantage of the activation of one important defense mechanism of innate immunity (the complement system) to suppress cell-mediated immunity induced by another crucial arm of innate immunity, i.e., the TLR system. This immune evasion strategy may be used by other intracellular pathogens as well, such as *Mycobacteria*, *Listeria*, *Histoplasma*, or even HIV.

The crosstalk between TLR4 and the C5aR is not restricted to the release of IL-12 family cytokines. The febrile response following TLR4 ligation appears to depend on complement activation and the interaction of C5a with its receptor as pharmacological targeting of the C5aR prevents the LPS-induced fever, at least in mice. Several lines of evidence suggest that C5a stimulates Kupffer cells to release prostaglandin E<sub>2</sub>, which binds to vagal afferents in the liver transmitting the pyrogenic information to the brain (124).

### Concluding Remarks

Complement is a sophisticated system of molecules that are critical to the functional integrity of the body. Initially considered as a defense system to ward off infections, it becomes increasingly clear that the complement system is one of the most important humoral systems to sense danger, i.e., to recognize conserved patterns on pathogens and on altered/damaged self. In addition to this important role in danger recognition, the complement system has the ability to translate the danger information into an adequate cellular innate or adaptive immune response. This is accomplished by two distinct mechanisms: (a) danger sensors that have recognized altered cells or pathogens can directly activate cell-bound receptors (e.g., C1q / C1q receptor interaction) and/or (b) danger sensors initiate cleavage of complement factors C3 and C5, the fragments of which acquire the ability to bind to complement receptors and/or regulators (Fig. 1). It is

the specific interaction of the danger sensors and of the cleavage fragments with distinct cell-bound receptors/regulators that directs the immune response toward an innate or an adaptive phenotype. Furthermore, the expression pattern of the complement receptors critically impacts the shape of the immune response. Complement has the ability to discriminate between physiological and pathological danger, i.e., physiological cell death and death in response to injury. In the former case, cells are merely flagged for enhanced phagocytosis (by C3 fragments) without accompanying inflammation (through CR3), whereas in the latter case, inflammatory signals are accessorially triggered (e.g., by the release of ATs, which recruit and activate neutrophils, eosinophils, etc.). This function is of major importance for apoptotic cell clearance and tissue repair but also plays important roles in fibrotic tissue remodeling in response to chronic tissue injury. Furthermore, complement cleavage fragments may prevent the development of maladaptive immune responses at the mucosal surface. Here, complement fragment C5a does not act as a danger transmitter but as “balance transmitter” as its interaction with the C5a receptor on DCs provides a signal that prevents DCs from activating CD4<sup>+</sup> T cells. The generation of regulatory T cells in response to CD46 ligation may have a similar function, as injured cells lose CD46 expression, which may lead to decreased proliferation of Tregs and, consecutively, enhanced activation of T effector cells.

Although we are still at the beginning of our understanding of the complex interaction patterns within the complement system, recent data suggest substantial crosstalk between the signaling pathways downstream of complement receptors and other receptors of the innate immune system that function as immune sensors and/or transmitters [i.e., the TLRs, FcγRs (125,126)]. Given the importance of comple-

ment as a sensor and effector system of innate and adaptive immune responses, a complement-related view at the immune system may help to unravel some enigmas of autoimmunity, allergy, and transplantation.

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