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The Role of the Anaphylatoxins in Health and Disease

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Abstract

The anaphylatoxins (AT) C3a, C5a and C5a-desArg are generally considered pro-inflammatory polypeptides generated after proteolytic cleavage of C3 and C5 in response to complement activation. Their well appreciated effector functions include chemotaxis and activation of granulocytes, mast cells and macrophages. Recent evidence suggests that ATs are also generated locally within tissues by pathogen-, cell-, or contact system-derived proteases. This local generation of ATs is important for their pleiotropic biologic effects beyond inflammation. The ATs exert most of the biologic activities through ligation of three cognate receptors, i.e. the C3a receptor, the C5a receptor and the C5a receptor-like, C5L2. Here, we will discuss recent findings suggesting that ATs regulate cell apoptosis, lipid metabolism as well as innate and adaptive immune responses through their impact on antigen-presenting cells and T cells. As we will outline, such regulatory functions of ATs and their receptors play important roles in the pathogenesis of allergy, autoimmunity, neurodegenerative diseases, cancer and infections with intracellular pathogens.

Keywords

Complement; anaphylatoxins; inflammation; sepsis; allergy; Alzheimer disease; adaptive immunity

1. Introduction

The complement system is an ancient danger sensing system that recognizes exogenous threats such as conserved microbial motifs as well as endogenous threats including altered-self molecules (e.g. following injury or hypoxia, after virus-infection or tumor-related) and apoptotic cells (Köhl, 2006b). Danger sensing molecules that activate the complement system comprise soluble C-type lectins such as Mannan-binding-lectin (MBL), ficolins, the C-type lectin-like molecule C1q and, as recent evidence suggests, properdin (Kemper and Hourcade,

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2008). In addition to this direct recognition of conserved danger motifs, C1q binds strongly to another class of innate danger sensors, i.e. natural immunoglobulins (Ciurana *et al.*, 2004). Once bound to danger motifs, C1q, MBL, ficolins and properdin initiate activation of proteolytic cascades that result in the cleavage of the central molecule of the complement system, C3, followed by cleavage of C5. Together with other cleavage products of C3 (i.e. C3b, iC3b, C3dg, C3d and C3c), the smaller cleavage products of C3 and C5, C3a and C5a, form a set of soluble mediators that bind distinct cell surface receptors expressed on a variety of target cells. The interaction of C3a and C5a with their cognate receptors induces pleiotropic effector functions, translating the danger information from the fluid phase into defined cellular responses. Here, we will review recent findings that provide a better understanding of the mechanisms and pathways underlying the various pro-inflammatory and regulatory functions of the anaphylatoxins (AT) mediated through the three distinct AT receptors.

2. Anaphylatoxins

C3a and C5a are small polypeptides consisting of 77 and 74 amino acids, respectively. With 36% amino acid identity, they share high similarity, but only C5a harbours an N-linked carbohydrate moiety. Furthermore, C5a and C3a are both highly cationic molecules (pI 9.0), the core structure of which is based on a 4-helix bundle that is stabilized by three disulphide bonds (Nettesheim *et al.*, 1988). Interestingly, these features render C3a, but not C5a, a highly potent antimicrobial peptide (Nordahl *et al.*, 2004). Both AT comprise highly conserved C-terminal pentapeptide sequences that are required for activation of their cognate receptors. For C3a, it is LGLAR (Caporale *et al.*, 1980); for C5a, it is MQLGR (Fernandez and Hugli, 1976). Especially for C5a, cyclic C-terminal-derived peptide analogs have been generated such as AcF-(OPdChaWR) that are now in clinical trials for the treatment of several inflammatory diseases (Köhl, 2006a).

The ATs are potent inflammatory mediators targeting a broad spectrum of immune and non-immune cells. C3a and C5a regulate vasodilation, increase the permeability of small blood vessels, and induce contraction of smooth muscles (Ember *et al.*, 1998). In macrophages (Murakami *et al.*, 1993), neutrophils (Elsner *et al.*, 1994b), and eosinophils (Elsner *et al.*, 1994a) C3a and C5a can trigger oxidative burst. Basophils (Kretzschmar *et al.*, 1993) and mast cells (el Lati *et al.*, 1994) react upon AT stimulation with release of histamine. In eosinophils, C3a and C5a regulate the production of eosinophil cationic protein, their adhesion to endothelial cells as well as their migration (Takafuji *et al.*, 1996;DiScipio *et al.*, 1999). C3a further promotes serotonin release from guinea pig platelets (Fukuoka and Hugli, 1988) and modulates synthesis of IL-6 and TNF-α from B cells and monocytes (Fischer and Hugli, 1997;Fischer *et al.*, 1999). C5a is a powerful chemoattractant for macrophages (Aksamit *et al.*, 1981), neutrophils (Ehrengruber *et al.*, 1994), activated B (Ottonello *et al.*, 1999) and T cells (Nataf *et al.*, 1999a), basophils (Lett-Brown and Leonard, 1977) and mast cells, the latter of which also migrate towards a C3a gradient (Hartmann *et al.*, 1997).

In addition to their pro-inflammatory properties ATs regulate tissue regeneration (Mastellos *et al.*, 2001;Strey *et al.*, 2003) and tissue fibrosis (Hillebrandt *et al.*, 2005;Addis-Lieser *et al.*, 2005;Strey *et al.*, 2003) as well as brain development (see 4.2) (Benard *et al.*, 2004). Further, C3a has been shown to enhance SDF-1 (CXCL12)-induced homing of hematopoietic stem cells into the bone marrow and their retention in this compartment (Ratajczak *et al.*, 2004).

It is obvious that control mechanisms have evolved that regulate the activity of these powerful bioactive molecules. Indeed, AT activity in the circulation and in tissues is tightly controlled by carboxypeptidases which rapidly cleave off a C-terminal arginine residue (Bokisch and Müller-Eberhard, 1970;Matthews *et al.*, 2004). While the resulting C5a-desArg retains 1-10%

of the inflammatory activity of C5a, C3a-desArg is devoid of any pro-inflammatory activity (Sayah *et al.*, 2003).

Intriguingly, C3a-desArg, which is also called acylation stimulating protein (ASP), has been described to possess metabolic hormone activity that drives triglyceride synthesis and glucose uptake in adipose tissue in numerous studies from the Cianflone laboratory (reviewed in (Cianflone *et al.*, 2003)). Surprisingly, ASP concentrations in the range from 1-10 μM, which is 2-3 logs above the concentrations usually needed for biological functions of AT, are required for such metabolic activity. In most studies, serum-purified but not recombinant C3a-desArg has been used. C3a-analog C-terminal peptides, which bind and stimulate C3aR, did not cause any changes in lipid metabolism. These findings may point toward a different mode of binding, a different binding partner or may indicate ASP-independent effects, for example induced by co-purified serum proteins. Even more importantly, the *in vivo* relevance of ASP remains elusive as it still needs to be demonstrated that high micromolar ASP levels can be achieved in adipose tissue. Further, no specific ASP receptor has yet been described on adipocytes (for review see also (Johswich and Klos, 2007)).

2.1 Anaphylatoxin receptors

The ATs bind to a family of three receptors, so-called AT receptors, which belong to the superfamily of G-protein-coupled receptors (GPCR). The AT receptor family comprises the C3a receptor (C3aR), C5a receptor (C5aR) and C5a receptor-like 2 (C5L2). They share high sequence homology (Lee *et al.*, 2001) and are closely related to other chemotactic receptors such as the N-formyl-methionine-leucine-phenylalanine (fMLP) receptor, ChemR23 (Samson *et al.*, 1998) and the chemokine receptors CXCR1 and CXCR2. Despite their similarity, the AT receptors differ in ligand specificity, signal transduction capacity and function.

2.1.1 C3aR—The C3aR specifically binds C3a with a K_d of about 1 nM, but does not recognize its desarginated form or C5a (Crass *et al.*, 1996;Wilken *et al.*, 1999). It is a 54 kDa molecule comprising 482 amino acids with two glycosylation sites located at asparagines 9 and 195 and a sulfated tyrosine at position 174 (Crass *et al.*, 1999;Ames *et al.*, 1996). Compared to the other AT receptors, C3aR possesses a remarkable large second extracellular loop that accounts for almost a third of its size and is indispensable for ligand binding. Also, the sulfated tyrosine 174 that resides within this loop plays a pivotal role for the interaction of C3aR with C3a (Gao *et al.*, 2003). However, the receptor N-terminus seems not to be involved in C3a binding (Crass *et al.*, 1999). Upon C3a binding to the C3aR, intracellular signal transduction is promoted via heterotrimeric G-proteins. In neutrophils, C3aR signals through pertussis-toxin-sensitive G-proteins (most likely $G\alpha_i$) and mobilizes calcium fluxes from the extracellular medium but not from intracellular stores, suggesting that it does not activate phosphatidylinositol-bisphosphate-3-kinase gamma (PI3K-γ) (Norgauer *et al.*, 1993). However, C3aR may also encounter pertussis-toxin-insensitive $G_{\alpha 16}$ for signal transduction (Crass *et al.*, 1996). In endothelial cells, C3aR may couple to pertussis-toxin insensitive $G\alpha_{12}$ or $G\alpha_{13}$ (Schraufstatter *et al.*, 2002). Downstream signaling events include activation of protein kinase C by phospholipase C and, in astrocytes, the mitogen activated protein (MAP) kinases Erk1 and Erk2 (Langkabel *et al.*, 1999;Sayah *et al.*, 2003). Furthermore, in mast cells, C3a promotes cytokine expression by signaling pathways that require activation of PI3K and subsequent Akt phosphorylation, as well as MAP kinases Erk1 and Erk2 (Venkatesha *et al.*, 2005).

The C3aR is expressed on cells of myeloid origin like neutrophils, basophils, eosinophils, mast cells, monocytes/macrophages, dendritic cells (DC) and microglia (Glovsky *et al.*, 1979;Daffern *et al.*, 1995;Klos *et al.*, 1992;Zwirner *et al.*, 1998a;Zwirner *et al.*, 1998b;Gutzmer *et al.*, 2004). Additionally, non-myeloid cells express C3aR. These include astrocytes from

inflamed brain (Gasque *et al.*, 1998;Ischenko *et al.*, 1998), endothelial cells (Monsinjon *et al.*, 2003), epithelial cells, smooth muscle cells, submucosal and parenchymal vessels of the lung from patients suffering from asthma (Fregonese *et al.*, 2005) and activated but not naive human T cells (Werfel *et al.*, 2000). Other studies have confirmed the absence of C3aR on unstimulated human T cells and on naive or activated human B cells (Zwirner *et al.*, 1999;Martin *et al.*, 1997). More recently, minor C3aR expression has been described on murine $CD4^+$ T cells which was upregulated upon DC stimulation (Strainic *et al.*, 2008). Furthermore, northern blot analysis suggests expression of C3aR mRNA in tissues of lung, liver, kidney, brain, heart, muscle and testis (Hsu *et al.*, 1997).

2.1.2 C5aR—The C5aR (CD88) binds C5a with high affinity (K_d ~1 nM) and C5a-desArg with somewhat lower affinity (KD~660nM) whereas C3a and C3a-desArg are not recognized (Gerard and Gerard, 1991;Gerard *et al.*, 1989;Okinaga *et al.*, 2003). C5aR is a 42 kDa protein consisting of 350 amino acids, of which asparagine at position 5 is glycosylated. Additional posttranslational tyrosine sulfation occurs at positions 11 and 14 (Farzan *et al.*, 2001). The essential structures required for ligand binding of C5aR have been elaborated in numerous studies (Mery and Boulay, 1993;DeMartino *et al.*, 1994;Crass *et al.*, 1999;Siciliano *et al.*, 1994;Baranski *et al.*, 1999;Gerber *et al.*, 2000;Geva *et al.*, 2000;Klco *et al.*, 2005;Klco *et al.*, 2006;Matsumoto *et al.*, 2007). The bottom line is that C5a binding involves two distinct sites at the C5aR. First, the aspartate-rich acidic N-terminus of C5aR interacts with the basic core of C5a. Then, the agonistic C-terminus of C5a interacts with a binding pocket formed by hydrophobic residues from the transmembrane domains and charged residues at the base of the extracellular loops. The latter step is indispensable for receptor activation.

C5aR signal transduction depends on heterotrimeric G-proteins. It is mainly achieved by the pertussis toxin sensitive alpha units $G\alpha_{i2}$ (Skokowa *et al.*, 2005) or the pertussis toxin insensitive $G\alpha_{16}$ (Monk and Partridge, 1993;Amatruda *et al.*, 1993) which is expressed by cells of the hematopoietic lineage. A unique feature of the C5aR is precoupling to G-proteins in the absence of ligands, thereby apparently lacking a low affinity conformation found in other GPCRs (Siciliano *et al.*, 1990). When C5aR is uncoupled from its G-protein by GTPγS, its affinity is dramatically decreased. In fact, the binding affinity of C5a to the C5aR lacking G-protein coupling is too low for detection by competitive ligand binding assays (Raffetseder *et al.*, 1996). C5a binding to C5aR causes calcium fluxes from both, intracellular stores as well as from extracellular medium. After activation, β-arrestins 1 and 2 bind to C5aR, targeting it for receptor internalization via clathrin coated pits (Braun *et al.*, 2002). Binding of arrestins depends on phosphorylation of the C-terminus of the receptor by G-protein coupled receptor kinases (GRKs). In addition to their function as kinases, GRKs can also interact with other components of intracellular signaling such as Akt, MAPK/ERK kinase (MEK) and PI3K-γ (for review see (Ribas *et al.*, 2007)). It has been shown that C5aR activation leads to downstream activation of several components of different signaling pathways like PI3K-γ kinase (Perianayagam *et al.*, 2002;la Sala *et al.*, 2005), phospholipase C β2 (Jiang *et al.*, 1996), phospholipase D (Mullmann *et al.*, 1990) and Raf-1/B-Raf mediated activation of MEK-1 (Buhl *et al.*, 1994). Another intracellular molecule that binds to the C-terminus of activated C5aR is the Wiskot-Aldrich syndrome protein (WASP) (Tardif *et al.*, 2003). This interaction is significantly increased in the presence of cell division cycle 42 (cdc42), a GTP binding protein which is thought to induce a conformational change of WASP to its active state. WASP is a multifunctional protein that regulates actin dynamics and therefore might play an important role in the C5a dependent chemotaxis.

C5aR is expressed in various cell types. It is most abundantly expressed in neutrophils, eosinophils and basophils, monocytes/macrophages, mast cells and DCs (Chenoweth and Goodman, 1983;Chenoweth and Hugli, 1978;Gerard *et al.*, 1989;Werfel *et al.*, 1997;Morelli *et al.*, 1996). The expression of C5aR in cells of lymphoid origin is not generally accepted.

Several reports have shown expression in human and mouse T cells (Nataf *et al.*, 1999a;Connelly *et al.*, 2007;Strainic *et al.*, 2008;Lalli *et al.*, 2008). Further, C5a-mediated migration has been demonstrated for B and T cells (El-Naggar *et al.*, 1980). In contrast, in another study binding of C5a has been solely detected in a small subpopulation of 6% of lymphocytes (Van-Epps and Chenoweth, 1984) and anti-C5aR antibodies failed to demonstrate receptor expression in murine lymphoid cells (Soruri *et al.*, 2003). In addition to immune cells, a body of evidence has accumulated that the C5aR is expressed in a broad range of non-immune tissue cells (reviewed in (Monk *et al.*, 2007)). These include endothelial cells (Laudes *et al.*, 2002a), neurons (Farkas *et al.*, 1998), astrocytes and microglia (Gasque *et al.*, 1997) as well as cells from kidney, lung, liver, spleen, intestine, skin and heart (Fayyazi *et al.*, 2000;Wetsel, 1995). However, data regarding its expression in epithelial cells of lung, liver and intestine were negatively re-evaluated (Fayyazi *et al.*, 1999;Fayyazi *et al.*, 2000) and found to be misleading due to cross-reactivity of some anti-C5aR antibodies to desmosomal antigens (Werfel *et al.*, 1996).

2.1.3 C5a-receptor-like 2 (C5L2)—C5L2 was discovered in 2000 as putative orphan receptor (GPR77) (Ohno *et al.*, 2000). It is a 37 kDa protein consisting of 337 amino acids with Asparagine 3 as potential glycosylation site. In the conserved transmembrane regions, C5L2 shares 58% sequence identity with C5aR and 55% with C3aR (Lee *et al.*, 2001). C5L2 is expressed in various tissues of myeloid and non-myeloid origin and transcripts were detected in brain, placenta, ovary, testis, spleen and colon (Gavrilyuk *et al.*, 2005;Lee *et al.*, 2001). Surface expression of C5L2 was detected in lung, liver, heart, kidney (Gao *et al.*, 2005), in adipose tissue and in skin fibroblasts (Kalant *et al.*, 2005), in neutrophils (Huber-Lang *et al.*, 2005) and in immature, but not in mature DCs (Ohno *et al.*, 2000). In combination with a C5aR-antagonist, binding of C5a could be demonstrated on differentiated myeloblastic HL-60 and U937 and epithelial HeLa-cells (Johswich *et al.*, 2006). C5L2 and C5aR seem to be frequently co-expressed in most cells or tissues (Okinaga *et al.*, 2003;Gao *et al.*, 2005).

C5a binds with high affinity (K_d: 2.5 nM) to C5L2. Suggesting a slightly different function, C5a-desArg binds with a 20-30 fold higher affinity to C5L2 than to C5aR (Cain and Monk, 2002). Moreover, binding kinetics of the two receptors differ for the same ligand: the on-rate of C5aR is about 100 fold faster than that of C5L2. It is important to consider that only a small percentage of the split products of C5 and C3 (close to the nucleus of complement activation) are available as C5a and C3a in the circulation as they are rapidly degraded to their desArg products. C5L2 appears to bind C5a and C5a-desArg by different mechanisms. Unlike C5aR, C5L2 uses critical residues in its N-terminal domain for binding only to C5a-desArg (Scola *et al.*, 2007).

In addition to C5a and C5a-desArg binding, C5L2 has been considered a binding partner for C3a, C3a-desArg, C4a and C4a-desArg (Cain and Monk, 2002;Kalant *et al.*, 2003;Okinaga *et al.*, 2003). Re-evaluation of such data revealed “unspecific” binding mainly to plastic surfaces due to the highly cationic nature of the ligands C3a and C3a-desArg; a binding which mimics in the absence of any cells saturable, specific, high-affinity binding to the C3aR (Johswich *et al.*, 2006). Similar problems had already been reported before with rat mast cells (Kajita and Hugli, 1991). Indeed, using assay conditions that take the cationic nature of the ligands into account, no binding to the recombinant human C5L2, at least not in the low or medium nanomolar range, has been observed (Johswich *et al.*, 2006). This does not exclude a biological role for C3a-desArg (ASP) in lipid and glucose metabolism in fat cells and fibroblasts (Kalant *et al.*, 2005). Yet, it makes it highly unlikely that C5L2 is the suggested binding partner for C3a-desArg or C3a.

C5L2 is an enigmatic receptor as a couple of reports suggest opposing functions. On the one hand, it has been described as a non-signaling scavenger receptor for C5a and C5a-desArg. In

contrast, at least two reports indicate that C5L2 alone, or in conjunction with other receptors, serves as a signaling receptor. In support of a role as a decoy receptor, no mobilization of intracellular Ca^{2+} occurs in C5L2 transfected cells after AT administration (Cain and Monk, 2002;Okinaga *et al.*, 2003). Moreover, no calcium fluxes have been observed in neutrophils from $C5aR^{-/-}$ mice after stimulation with C5a (Hopken *et al.*, 1996) or in endogenously, C5L2 expressing cell lines (Johswich *et al.*, 2006). In heptahelical receptors, a highly conserved DRY motif in the third transmembrane domain is important for its interaction with the corresponding G-proteins. The DRY motif is DRF in C5aR and DRC in C3a, but DLC in C5L2. The central arginine residue plays a key role in coordinating the transmembrane domains 3 and 6 and mutagenesis of this residue in C5aR leads to loss of function (Kolakowski, Jr. *et al.*, 1995). When DLC in C5L2 is restored to DRC, C5L2 couples weakly to intracellular Ca^{2+} fluxes in HEK293 cells co-expressing $G\alpha_{16}$ (Okinaga *et al.*, 2003). Rat basophilic leukaemia cells (RBL. 2H3)-cells are frequently used to study functional intracellular coupling and signaling of AT receptors. Intriguingly, no Ca^{2+} fluxes occurred in these cells using a C5L2-mutant where the DRY-motif and two additional regions typically involved in G-protein coupling were replaced by the corresponding C5aR-sequences (Scola *et al.*, 2009). Taken together, these findings strongly suggest that C5L2 on its own is uncoupled from G-proteins.

In functional studies, C5L2 failed to promote biologic activities. Preloading of the tyrosine kinase coupled IgE-receptors (FcεRI) with DNP-specific IgE resulted in a small increase in β-hexosaminidase release upon AT pretreatment which was absent in C5L2 transfected RBL cells (Cain and Monk, 2002). Further, C5a binding to C5L2 in bone marrow cells derived from $C5aR^{-/-}$ mice failed to promote any changes in mRNA expression (Okinaga *et al.*, 2003). Thus, C5L2 has been considered to belong to the group of decoy receptors negatively regulating primary inflammatory chemokines (reviewed in (Locati *et al.*, 2005)). Decoy receptors such as US28, CCX CKR, DARC and D6 not only lack the DRY motif and functional coupling, but also constantly recycle giving rise to a high proportion of intracellular receptors combined with highly efficient ligand internalization and degradation.

Rapid ligand-induced receptor internalization is a central feature of functional G-protein coupled receptors. C5aR and C3aR are no exception (Bock *et al.*, 1997;Settmacher *et al.*, 1999). Expressed in RBL.2H3-cells, C5aR rapidly internalizes upon C5a stimulation whereas C5L2 does not (Scola *et al.*, 2009). This view is supported by two independent studies which found no internalization of C5L2 upon stimulation with ATs (Cain and Monk, 2002;Okinaga *et al.*, 2003). On the other hand, C5L2 but not C5aR constitutively recycles in transfected RBL cells leading to major intracellular expression. For example, in transfected CHO cells C5L2 is more efficient than C5aR at internalizing and retaining C5a and C5a-desArg. Additionally, in human neutrophils and HeLa-cells, C5L2 is responsible for internalization and degradation of C5a and C5a-desArg (Scola *et al.*, 2009).

The phosphorylation of C-terminal serine or threonine residues by GRKs, protein kinase A or protein kinase C, followed by their association with arrestins to the receptor is a prerequisite for receptor internalization. C5L2-transfected mouse L1.2 cells elicited only a very basal level of phosphorylation upon C5a stimulation despite a serine/threonine rich C-terminus (Okinaga *et al.*, 2003). In contrast, using transfected HEK293 cells, C5L2 was found to be robustly phosphorylated after stimulation with very high concentrations of C3a-desArg (2×10^{-5}M). Further, a cotransfected β-arrestin-GFP fusion protein translocated from the plasma membrane to endocytic vesicles after stimulation with C5a, C3a or C3a-desArg, thereby suggesting functionality of C5L2 (Kalant *et al.*, 2005). However, this result has recently been questioned, as no translocation of β-arrestin has been found after C5a-stimulation of transfected RBL.2H3-cells (Scola *et al.*, 2009).

Taken together, there is accumulating evidence that C5L2 is a decoy receptor in primary cells and transfected cell-lines suggesting that it acts as a functional antagonist of the C5aR *in vitro* and *in vivo*. Thus biological effects mediated by C5aR can be expected to be stronger in the absence and weaker in the presence of C5L2. Indeed, neutrophils and macrophages from $C5L2^{-/-}$ mice produce more TNF-α and IL-6 in response to combined stimulation with C5a and LPS than their wildtype littermates. Further, C5a and LPS stimulation drive more IL-6 production from rat neutrophils when C5L2 is blocked (Gao *et al.*, 2005). *In vivo*, $C5L2^{-/-}$ mice suffer from augmented inflammatory responses (IL-6 and TNF-α) and higher numbers of infiltrating neutrophils when compared to their wildtype littermates in a model of pulmonary immune complex injury (Gerard *et al.*, 2005). The anti-inflammatory role of C5L2 is further supported by a study in which LPS-injected $C5L2^{-/-}$ mice showed higher IL-1β levels and decreased survival rates (Chen *et al.*, 2007) and a report in which $C5L2^{-/-}$ mice displayed higher serum concentrations of IL-6 as compared to wildtype and $C5aR^{-/-}$ mice in a model of septic peritonitis (Rittirsch *et al.*, 2008).

However, under the same conditions, a strong reduction of other inflammatory mediators, such as IL-1β, MIP-1α and MIP-2 was observed in $C5L2^{-/-}$ mice compared to wildtype mice. Indeed, the concentrations of these mediators were comparable to those of $C5aR^{-/-}$ mice. Furthermore, $C5L2^{-/-}$ mice, like $C5aR^{-/-}$ mice, or animals in which each of the receptors was blocked by antibodies, showed a higher survival rate in mid-grade sepsis (Rittirsch *et al.*, 2008). Contrasting the *in vitro* findings that the C5a/LPS-driven IL-6 production of mouse neutrophils is increased when C5L2 is blocked by antibodies (see above), Chen et al. found decreased IL-6 release from $C5L2^{-/-}$ neutrophils (Chen *et al.*, 2007). Moreover, C5a+LPS-induced Mac-1 surface expression on neutrophils was also diminished. Likewise, whereas C5a or C5a+LPS led to strong ERK1/2- and AKT-phosphorylation in neutrophils from wildtype mice, there was only a weak effect in the absence of C5L2. Additionally, in $C5L2^{-/-}$ macrophages, there was an impaired induction of co-stimulatory molecules (CD40, CD86). Even effects which are mediated by C3a such as ERK1/2- and AKT-phosphorylation or F-actin formation on neutrophils were impaired in $C5L2^{-/-}$ mice (Chen *et al.*, 2007). In addition to these *in vitro* findings, inflammatory responses were reduced *in vivo* in models of thioglycollate induced peritonitis, thioglycollate induced migration into dorsal air pouches, or OVA induced airway hyperresponsiveness. Thus, the studies of Rittirsch et al. and of Chen et al. point to a more complex role of C5L2 in inflammation with C5L2 acting not only as a decoy receptor but also as positive modulator of C5a or even C3a. Although C3a-desArg (ASP) does not bind directly to C5L2 (Johswich *et al.*, 2006), over-expression of C5L2 or its down-regulation by antisense oligonucleotides influenced effects of C3a-desArg (ASP) (Kalant *et al.*, 2005), suggesting that C5L2 can modulate signaling pathways of other receptors. As GPCRs, including C5aR, tend to homo- or hetero-oligomerize (Klco *et al.*, 2003;Rabiet *et al.*, 2008) it is tempting to speculate that such oligomerization may be one mechanism underlying the positive modulatory effect of C5L2 on C5a or C3a effector functions (Rabiet *et al.*, 2008).

3. Anaphylatoxins in acute and chronic inflammation

ATs have been shown to promote inflammatory responses during the effector phase of allergic, infectious and autoimmune diseases (reviewed in (Guo and Ward, 2005;Köhl, 2001)). In the following paragraphs, we will focus on recent findings related to the role of C5a in sepsis and the impact of AT receptor signaling on the development of adaptive immune responses in allergy, transplantation, tumor biology and infection.

3.1 Anaphylatoxins in sepsis

The sepsis syndrome is still the leading cause of death in intensive care units in the Western world. It is well accepted that invading microorganisms induce the release of a large number of humoral and cellular proinflammatory mediators causing a systemic inflammatory response

syndrome which defines the patients' outcome. Most microbes activate the complement system through their interaction with C1q, MBL/ficolins, or properdin leading to local and/or systemic complement activation with subsequent generation of C3a and C5a. Indeed, high plasma or serum concentrations of C3a-desArg and C5a-desArg (Bengtson and Heideman, 1988;Weinberg *et al.*, 1984;Hack *et al.*, 1989;Stove *et al.*, 1996;Selberg *et al.*, 2000) have been observed in septic patients and in experimental models of sepsis (Ward, 2008a). Importantly, C3a-desArg plasma levels have been associated with fatal outcome (Hack *et al.*, 1989;Selberg *et al.*, 2000) suggesting a causal role of the ATs in sepsis pathogenesis. The Ward laboratory has contributed tremendously to our understanding of the role of the ATs, and in particular C5a, in experimental sepsis. Most of the work has recently been reviewed (Ward, 2008b;Ward, 2004; Ward, 2008a) and will be summarized only very briefly. Based on the initial observation that interruption of the C5a/C5aR interaction in a model of septic peritonitis significantly improved survival (Czermak *et al.*, 1999), several mechanisms have been worked out underlying the detrimental effects of C5a in sepsis. C5a paralyzes immune functions of neutrophils (Huber-Lang *et al.*, 2001), promotes septic cardiomyopathy (Niederbichler *et al.*, 2006), drives apoptosis of thymocytes (Guo *et al.*, 2000) and adrenal medullary cells (Flierl *et al.*, 2008) and contributes to consumptive coagulopathy (Laudes *et al.*, 2002b). Importantly, C5aR is markedly upregulated in several organs including lung, liver, kidney, heart as well as in the thymus (Huber-Lang *et al.*, 2002b). Initially, the “dark side of C5a” has been exclusively attributed to the activation of the C5aR. However, assessing the septic peritonitis model in more detail, it turned out that in addition to C5aR, C5L2 is involved in C5a-mediated sepsis. In “mid-grade” sepsis with mortality rates of 60-70% absence of either C5aR or C5L2 improves survival. In “high-grade” sepsis (mortality rate 100%) only the combined inhibition of C5aR and C5L2 is protective, suggesting that C5aR and C5L2 synergistically promote septic inflammation (Rittirsch *et al.*, 2008). Importantly, C5L2 but not C5aR was found to regulate the production of the high mobility group box 1 protein (HMBG1) from phagocytes, the ablation of which protects from organ damage and failure in experimental sepsis (Wang *et al.*, 1999). Further, LPS-induced HMBG-1 production from $C5L2^{-/-}$ macrophages was significantly reduced as compared with WT controls suggesting that C5L2 regulates LPS-driven Toll-like receptor (TLR) 4 activation. Indeed, cross-talk between C5a and TLR signaling has been shown before, although related to C5aR signaling (Hawlisch *et al.*, 2005;Zhang *et al.*, 2007), indicating complex interactions between the complement system and other danger sensing systems (Köhl, 2006b) (see also 3.2.5). In support of this view, MBL and TLR2/6 signaling cooperate in *S. aureus* infection to specify and amplify host defense (Ip *et al.*, 2008).

3.2 Anaphylatoxins regulate adaptive immune responses

It is now well appreciated that complement contributes to the regulation of adaptive immune responses (for review see (Carroll, 2004; Köhl, 2006b; Kemper and Atkinson, 2007)). Initially, complement and particularly C3-derived cleavage products have been considered to regulate B cell immunity (Fearon and Locksley, 1996). In a series of elegant studies, it was demonstrated that CD21 (CR2) and CD35 (CR1) are critical for the elimination of self-reactive B cells, selection and and/or maintenance of B1 cells, and the amplitude of humoral responses to thymus-dependent and thymus-independent antigens (summarized in (Carroll, 2004)). During the past 10 years, we have learned that in addition to CD21 and CD35, signaling through other complement receptors including the AT receptors regulates adaptive immune responses. In contrast to CD21 and CD35, AT-mediated regulation focuses on T cell-dependent immune responses promoting inflammation in allergy, autoimmunity, transplantation, tumors and infection with intracellular microorganisms. In the following paragraphs, we will summarize recent findings in these areas.

3.2.1 Anaphylatoxins in allergic asthma—The worldwide prevalence and severity of allergic asthma in industrialized countries have increased dramatically in recent decades reaching epidemic proportions. In the U.S. alone, 15 million people suffer from this chronic inflammatory disease of the lung. The mechanisms underlying the development of pulmonary allergy in individuals from industrialized countries remain elusive. One important observation is that asthmatics suffer from a dysregulated adaptive immune response which accounts for most of the pathophysiological characteristics found in asthmatics including airflow obstruction, airway hyperresponsiveness (AHR) and airway inflammation. Specifically, environmental factors drive a T helper cell type 2 (Th2)-biased immune response associated with strong production of cytokines such as IL-4, IL-5, and IL-13 which orchestrate the pulmonary inflammatory response, involving the recruitment and the activation of eosinophils, the production of allergen specific IgE, induction of AHR and mucus hypersecretion (Wills-Karp, 2004). One of the current challenges is to understand the mechanisms that regulate tolerance towards environmental allergens at the mucosal surface, preventing the development of the maladaptive, Th2-biased immune response in healthy individuals.

ATs are generated in the lungs of both healthy individuals and asthmatics. Under physiologic or steady state conditions, ATs are locally generated in the pulmonary tissue at low levels, while in the inflamed asthmatic environment, high concentrations of ATs are produced (Krug *et al.*, 2001). C3a and C5a play distinct roles in the pathogenesis and the pathology, depending on the conditions under which they are generated and the cell types that become activated. Under steady state conditions, C5aR signaling at the DC/T cell interface controls the development of the maladaptive immune response towards innocuous aeroallergens (Karp *et al.*, 2000;Köhl *et al.*, 2006;Drouin *et al.*, 2006;McKinley *et al.*, 2006). In an inflamed environment that promotes strong complement activation, C3a (Bautsch *et al.*, 2000;Humbles *et al.*, 2000;Drouin *et al.*, 2002;Drouin *et al.*, 2001;Baelder *et al.*, 2005) as well as C5a (Abe *et al.*, 2001;Baelder *et al.*, 2005;Köhl *et al.*, 2006;Peng *et al.*, 2005) act mainly on infiltrating cells such as eosinophils, mast cells and basophils and promote a proinflammatory scenario. Interestingly, an exclusive role for C3a has recently been shown in the development of anaphylactic shock in peanut allergy (Khodoun *et al.*, 2009).

The mechanisms underlying the protective role of C5a during allergen sensitization include control of the pulmonary accumulation of immunogenic myeloid DCs, increased release of the Th2 effector cell-homing chemokines CCL17 and CCL22 and the regulation of mDC susceptibility toward the suppressor activity of naturally occurring regulatory T cells (Köhl *et al.*, 2006;Lewkowich *et al.*, 2005). Further insights into the regulatory role of C5 on distinct pulmonary DC population result from a recent study comparing the mechanisms underlying asthma-susceptible C5-deficient A/J mice and resistant C3H mice. A/J mice favor allergen uptake by mDCs leading to upregulation of co-stimulatory molecules and production of a Th2 and Th17-promoting cytokine profile. In contrast, in C3H mice, allergens are preferentially taken up by tolerogenic plasmacytoid DCs (Lewkowich *et al.*, 2008). These data suggest that C5 and possibly C5a regulate maladaptive immunity in asthma through an impact on pDC. In support of this view, C5aR blockade during allergen sensitization decreases expression of the co-stimulatory molecules B7-H1 (pD-L1) and B7-DC (pD-L2) on pDCs but not on mDCs. Importantly, B7-H1 and B7-DC regulate Th2 cytokine production from $CD4^+$ T effector cells (Zhang *et al.*, 2009). Together, these data suggest that C5/C5a sets the threshold for the development of maladaptive immunity by defining the pathways of allergen uptake (mDC vs. pDC) and the potential of pDCs to suppress mDC-induced activation of T effector cells. In future studies, it will be important to delineate the molecular mechanism by which C5a regulates mDC and/or pDC function. At this point, the role of C5L2 in this scenario is unclear. C5L2-deficiency results in a decreased allergic phenotype similar to that seen in C3aR-deficient mice (Chen *et al.*, 2007).

3.2.2 AT and transplantation—First evidence for an important contribution of local complement production to allograft survival came from the Sacks lab showing that the lack of C3 in donor kidneys is associated with long-term graft survival in experimental transplantation (Pratt *et al.*, 2002). The association of C3 polymorphisms with late graft failure confirmed the importance of this finding (Brown *et al.*, 2006) although these data were negatively re-evaluated in a larger cohort (Varagunam *et al.*, 2009). Searching for mechanisms contributing to this C3-dependent effect, the same lab found that APCs are the source of C3 and that macrophages or DCs lacking C3 have an impaired ability to stimulate alloreactive T cells and to drive Th1-biased adaptive immune responses (Zhou *et al.*, 2006;Peng *et al.*, 2006). In a follow up study, Peng et al. were able to assign the contribution of C3 to the interaction of C3a with the C3aR on DCs. They found that DCs not only synthesize C3 but also harbor the machinery to proteolytically cleave C3 into C3a leading to autocrine C3aR signaling. Taking advantage of C3aR-deficient DCs, they showed that such cells suffer from reduced MHC-II, co-stimulatory molecule expression and IL-12 production, which was associated with impaired allospecific T cell stimulation (Peng *et al.*, 2008). More recently, C3aR signaling in DCs was shown to decrease cAMP levels as one mechanism by which C3a regulates DC activation and T cell responses (Li *et al.*, 2008). In addition to C3a, C5a might be important in allograft rejection. Inhibition of the C5aR in murine model of life-supporting renal allotransplantation substantially improved graft survival from 11d to 12 weeks. In addition to reduced kidney inflammation and apoptosis, the authors found attenuated priming of alloreactive T cells (Gueler *et al.*, 2008). In support of these findings, pharmacological targeting of C5aRs during organ preservation was found to improve kidney graft survival (Lewis *et al.*, 2008). Thus, C3aR and C5aR signaling appear to contribute significantly to the innate and adaptive inflammatory response following solid organ transplantation. Given that several companies have potent C5aR antagonist in their pipeline; more work is required in this area to define the role of AT receptors in allotransplantation in particular with regard to the regulation of $CD4^+$ and $CD8^+$ T cell responses and long-term graft survival.

3.2.3 Role of AT receptor signaling in T cell activation—A couple of recent joint publications from the labs of Medof and Heeger suggest that the AT not only act on APCs but also on T cells. In an allogeneic setting or following antigen stimulation, they found that upon APC-T cell interaction, T cells as well as APCs start producing complement factors of the alternative pathway such as factors B and D as well as C3 (Heeger *et al.*, 2005). Further, they demonstrated downregulation of the membrane-bound complement regulator CD55 (decay accelerating factor; DAF). Importantly, DAF-deficient APCs or T cells increased T cell proliferation and Th1 cytokine production *in vitro* and *in vivo*. In follow up studies, they were able to link the decreased expression of CD55 on APCs to increased production of C5a which they found to be critical for differentiation into Th1 effector cells (Lalli *et al.*, 2007) confirming the importance of C5a signaling for Th cell differentiation (Hawlisch *et al.*, 2005;Köhl *et al.*, 2006). Extending their studies, they showed that cognate APC-T cell interaction promotes C3 and C5 production from APCs and T cells and subsequent generation of C3a and C5a. Further, they not only described C3aR and C5aR expression on DCs and naïve, unstimulated $CD4^+$ T cells but also found upregulation of both AT receptors upon antigen challenge (Strainic *et al.*, 2008). Mechanistically, AT generation and AT receptor upregulation was linked to costimulation by B7-CD28 and CD40-CD40L molecules. Importantly, the authors were able to define autocrine and paracrine feedback loops between AT production, AT receptor and costimulatory molecule expression suggesting that the ATs and their receptors on APCs and T cells contribute to the network of costimulation required for optimal T cell activation and differentiation. Further, they showed that naïve T cells already produce low amounts of C3a and C5a under steady state conditions leading to autocrine activation of AT receptors, which seems to promote sustained viability of naïve T cells. The latter finding was recently extended to effector T cells. Here they demonstrated that C5aR signaling on effector T cells controls

extrinsic and intrinsic pathways of apoptosis by regulating T cell expression of the apoptosis regulator proteins Fas and Bcl-2 (Lalli *et al.*, 2008). These data suggest a critical role for complement in the maintenance of naïve T cells and the expansion of effector T cells. Whether this regulatory effect on T cell differentiation, expansion and apoptosis applies to all subpopulations of $CD4^+$ T cell as well as to $CD8^+$ T cells remains to be determined in future studies.

3.2.4 C5a and cancer—Another example of C5a-mediated regulation of adaptive immunity has recently been uncovered in tumor immunology. The fate of tumors depends on the balance between innate and adaptive immune responses and the potential of the malignant cells to evade immune surveillance. Of particular importance is the interplay between myeloid-derived suppressor cells (MDSC) and $CD8^+$ cytotoxic T cells. MDSC are a heterogeneous population of regular myeloid cells trapped in intermediate stages of differentiation toward mononuclear cells such as monocytes, macrophages or DCs or polymorphonular granulocytes (Marigo *et al.*, 2008). Markiewski et al. found that MDSC express C5aR and that C5a attracts MDSCs to TC-1 tumor cells *in vivo*. Further, activation of the C5aR results in increased production of reactive oxygen and nitrogen species from MDSC, which are known mediators of MDSC that suppress $CD8^+$ T cell functions. Importantly, in a model of cervical cancer, genetic or pharmacological C5aR targeting resulted in decreased numbers of MDSCs within the tumor, associated with high numbers of $CD8^+$ effector T cells and decreased tumor growth (Markiewski *et al.*, 2008). These data underscore the view of C5a as an important immunoregulatory molecule of adaptive immunity. Of note, C5a may not only be generated in the tumor environment through tumor sensing by C1q-mediated mechanisms. In fact, C5a can be generated from C5 by cell-derived proteases (Huber-Lang *et al.*, 2002a), serine proteases of the clotting and the fibrinolysis system (Huber-Lang *et al.*, 2006;Markiewski and Lambris, 2007) and several pathogens (see 3.2.5) which fits well with the observation that inflammation and infection can promote and enhance tumor growth. In line with this view, C5a cooperates with pathogen-induced TLR activation to drive IL-6 and IL-1β production from mononuclear cells, which are pro-inflammatory mediators that also increase the accumulation and activation of MDSCs in tumors (Ostrand-Rosenberg, 2008).

3.2.5 Cross talk of AT receptors with TLRs and its impact on adaptive immune responses—The complement system and TLRs are ancient danger sensing systems that have co-evolved over hundreds of millions of years. As might have been expected, cross talk between complement proteins and TLRs have been described that suggest rather complex mechanisms of pathogen sensing. Soluble complement proteins such as C1q, MBL/ficolins and properdin (Kemper and Hourcade, 2008) recognize conserved microbial motifs resulting in the activation of the classical, lectin or the alternative pathway of complement. Similarly, conserved microbial patterns are recognized by cellular pattern recognition receptors (PRR) including TLRs. Direct cooperation between the soluble complement-derived sensing molecule MBL and membrane bound TLR2 has been found in *S. aureus* infection which specifies and amplifies the host response. Importantly, this cooperation takes place in the special environment of the phagosome, suggesting that soluble complement proteins are multifunctional molecules that promote their antimicrobial defense functions not only in the fluid phase but in defined cell compartments (Ip *et al.*, 2008). Another example is the cooperation between TLR2 and CR3 in periodontopathic bacterial infection. *Porphyromonas gingivalis* exploits the host danger sensing system to shut down adaptive immunity allowing the bacterium to enter host cells and survive within phagosomes. *P. gingivalis* fimbriae are recognized by TLR2 which then stimulates inside-out signaling to promote clustering of CR3, providing a safe and efficient entry pathway for the pathogen. This CR3 activation further leads to suppression of IL-12 production which would otherwise result from TLR2 activation of the phagocyte (Hajishengallis *et al.*, 2008). Interestingly, *P. gingivalis* can degrade C5 to release a C5a-like

fragment which is biologically active (Wingrove *et al.*, 1992). As C5a can also promote clustering of CR3 (Jones *et al.*, 1998), this may serve as an additional mechanisms of how the microbe exploits host defense. Further, C5a has been shown to cross talk with TLR2, TLR4 and TLR9. In peritoneal macrophages, C5a (and to a lesser extent also C3a) downregulates TLR4-induced production of IL-12 family cytokines including IL-12, IL-27 and IL-23 by PI3K-γ and ERK1/2-dependent pathways (Hawlisch *et al.*, 2005). Importantly, this suppressive effect of C5a also applies to CD40-CD40L-induced amplification of IL-12 production in macrophages. The *in vivo* importance of this effect was demonstrated in a model of cutaneous leishmaniasis. BALB/c mice, which are sensitive to *L. major* infection, become resistant when C5aR-signaling is ablated in C5aR-deficient mice. This protection is associated with increased proliferation of $CD4^+$ and $CD8^+$ T cells and high IFN-γ production. Of note, *L. major* uses a similar entry mechanism as *P. gingivalis*, i.e. through CR3 and shuts down IL-12 production upon macrophage infection suggesting that this pathway is an Achilles heel that serves intracellular survival of several pathogens (Liese *et al.*, 2008). In fact activation of CR3 by several ligands has been shown to shut down IL-12 (Marth and Kelsall, 1997). The negative regulatory impact of C5a and C3a on TLR-driven IL-12 production has been confirmed *in vivo* injecting TLR ligands into mice (Zhang *et al.*, 2007). The bottom line of this study is that the ATs downregulate IL-12 production but act synergistically with TLRs 2, 4 and 9 in promoting TNF-α, IL-1β, IL-6 and IL-10 production from blood cells.

On the other hand, C5aR blockade has been shown to suppress *S. aureus*-induced production of IL-12 from human monocytes and to enhance the production of IL-10 (Karp *et al.*, 2000) suggesting a synergistic role for C5aR signaling in microbial-derived IL-12 production. Similarly, mice lacking C3aR and C5aR die rapidly following *T. gondii* infection which is associated with a markedly reduced ability to mount protective Th1 immunity (Strainic *et al.*, 2008). Taken together, these data suggest that the impact of C5aR and C3aR signaling on microbial-induced production of IL-12 and Th1 protective immunity is complex. The emerging model from the available data is that C5aR and C3aR signaling in macrophages and monocytes suppresses TLR-induced IL-12 production. In DCs and T cells, C3aR and C5aR signaling may act synergistically with TLR signaling or activation of other PRR such as NOD-like receptors in case of *S. aureus* infection. More studies are needed that delineate the fascinating cross talk between the distinct AT receptors and PRRs in APCs and T cells in infection in particular with regard to intracellular pathogens.

3.3 Cross-talk between C5aR and immunoglobulin G receptor (FcγR) in autoimmune diseases

Autoimmune diseases are chronic disabling disorders in which maladaptive, self-directed immune responses drive severe inflammation. More than 80 autoimmune diseases have been identified, including systemic lupus erythematosus (SLE), multiple sclerosis, type I diabetes and rheumatoid arthritis (RA). Immune complexes (ICs) are integral to the pathogenesis of several autoimmune diseases, including SLE and RA. They activate the classical and the alternative pathway of the complement system and thus interact with both receptors for Fc of immunoglobulin G (FcγR) and a variety of complement receptors (Köhl, 2001). When clearance mechanisms are overwhelmed, ICs can become an important cause of tissue damage. In such settings, ICs promote proinflammatory processes characterized by activation of myeloid cells at sites of IC deposition.

In mice, four different FcγRs have been described, the activating FcγRs I, III and IV and the inhibitory FcγRIIB (Nimmerjahn and Ravetch, 2006). IC binding to activating FcγRs promotes inflammation through an immunoreceptor tyrosine-based activation motif (ITAM) whereas ligation of the inhibitory FcγRIIB blocks the inflammatory response upon co-ligation with activating FcγRs through an immunoreceptor tyrosine-based inhibitory motif (ITIM). This

coupling of activating and inhibitory signals from cellular receptors which recognize similar ligands has emerged as a general principle during evolution in which the overall response is determined by the relative contribution of each signaling pathway (Ravetch and Lanier, 2000). In line with this view, FcγRIIB deficient mice suffer from augmented inflammation in experimental models of IC-disease, systemic anaphylaxis, and show enhanced IgG-mediated clearance of pathogens and tumor cells (Takai, 2002).

Several studies of experimental IC-disease have shown that both activating FcγRs (Sylvestre and Ravetch, 1994), as well as the C5aR (CD88) (Heller *et al.*, 1999a;Heller *et al.*, 1999b;Baumann *et al.*, 2000;Baumann *et al.*, 2001), drive effector responses in IC-mediated inflammation and that cross-regulation exists between the two receptor signaling pathways (Shushakova *et al.*, 2002;Godau *et al.*, 2004). The emerging paradigm is that activating FcγRs provide IC-mediated cellular effector responses, while C5a sets the threshold for FcγR activation by upregulation of activating FcγRs and downregulation of FcγRIIB. C5aR signaling through $G_{\alpha i2}$-dependent mechanisms (Skokowa *et al.*, 2005) that activate PI3K-γ and δ (Konrad *et al.*, 2008) are critical for the regulatory impact of C5a on FcγR expression. Indeed, contribution of both FcγRs and C5aR has been shown in models of IC-alveolitis and peritonitis (Baumann *et al.*, 2000;Heller *et al.*, 1999a), rheumatoid arthritis (Ji *et al.*, 2002), autoimmune vitiligo (Trcka *et al.*, 2002), and autoimmune hemolytic anemia (AIHA) (Kumar *et al.*, 2006). Further, a positive amplification loop has been described between FcγR signaling and the complement system in a model of AIHA in which activating FcγRs promote the production of C5 from tissue macrophages for its subsequent cleavage into C5a (Kumar *et al.*, 2006).

Taken together, these data suggest that the interplay between FcγRs and C5aR contributes significantly to the effector phase of IC diseases, i.e. the enhancement of FcγR-mediated clearance of ICs by macrophages and the pro-inflammatory scenario through the recruitment and activation of neutrophils. Given that FcγRs, in particular FcγRIIB, contributes to the development of autoimmunity (Takai, 2002), it will be important to delineate in future studies whether the regulatory link between C5aR and FcγRIIB will also affect afferent immunity and the development of autoimmune diseases such as SLE.

4. Anaphylatoxins and their receptors in CNS

4.1 Evidence for a role for C5a/C5aR in Neurodegenerative disease

Degenerative diseases are becoming a larger issue in the modern age in which lifespan has been prolonged. One example of age related neurodegenerative disorders is Alzheimer's Disease (AD) with over 4.5 million individuals in the US alone afflicted with the disease, most above the age of 65 (Hebert *et al.*, 2003). Symptoms of the disease include: loss of memory, decreased reasoning and communication ability and loss of independence. The characteristic neuropathological changes include synaptic and neuronal loss, extracellular amyloid beta (Aβ) plaques and neurofibrillary tangles of hyperphosphorylated tau mainly within the hippocampus and cortex of the brain (Terry *et al.*, 1999;Trojanowski and Lee, 2005;Aizenstein *et al.*, 2008). The observation that individuals who take non-steroidal anti-inflammatory drugs (NSAIDs) show decreased susceptibility to AD onset (Pasinetti, 2002), as well as a plethora of *in vitro* data (Bales *et al.*, 2000;Shaftel *et al.*, 2008;Meda *et al.*, 2001;Veerhuis *et al.*, 2003), have led many to speculate that inflammation may play a substantial role in AD pathogenesis. In contrast to diffuse plaques seen in brain tissue from non-demented individuals, the Aβ peptide in plaques in AD brain is in a β-sheet structure conformation (can be stained with a dye thioflavine) and has been termed fibrillar in contrast to diffuse. Components of the complement system have been observed almost exclusively in association with these fibrillar Aβ plaques (Afagh *et al.*, 1996;Loeffler *et al.*, 2008). Some positive complement immunostaining has been shown in thioflavine-negative, cognitively normal brains, but to a far lesser extent (Lue *et al.*, 2001). *In vitro* studies have shown that these β-sheet rich Aβ

assemblies activate both the classical and alternative complement pathways (Rogers *et al.*, 1992;Jiang *et al.*, 1994;Bradt *et al.*, 1998;Watson *et al.*, 1997), thus generating complement activation products, including C3b, C3a and C5a. Prolonged complement activation triggered by fibrillar Aβ plaques or activation in the absence of proper regulation may contribute to many of the manifestations present in the disease (Cotman *et al.*, 1996) due to C5a-recruited and -activated glia that promote inflammatory events (Akiyama *et al.*, 2000;Grammas and Ovase, 2001;Yao *et al.*, 1990;O'Barr and Cooper, 2000). Furthermore, studies have suggested that C5aR activation may be involved in neuronal apoptosis (Farkas *et al.*, 2003) and decreased cell viability (Humayun *et al.*, 2009). Similar scenarios may apply to Parkinson disease (McGeer and McGeer, 2004), Huntington's disease (Singhrao *et al.*, 1999) and age relate macular degeneration (AMD) (Hageman *et al.*, 2005). For example, complement components, glia and complement AT receptor mRNAs are strongly expressed in caudate in Huntington's disease. Complement components are found in drusen in AMD, and over 70% of the risk of AMD can be attributed to polymorphisms predominantly in the endogenous complement pathway regulator, Factor H, with some contributions from polymorphisms in C2, C3, and Factor B (Maller *et al.*, 2007;Jakobsdottir *et al.*, 2008;Spencer *et al.*, 2008;Spencer *et al.*, 2007). Complement activation is strongly implicated in cerebral ischemia and hemorrhagic stroke, although the precise contributions and mechanisms of complement-dependent neuronal degeneration are yet to be unequivocally defined. The evidence for, and consequences of, C3a and C5a generated as a result of this injury has recently been thoroughly reviewed by Arumugan and colleagues and thus will not be discussed here further (Arumugam *et al.*, 2009).

The role of the complement system in neurodegenerative disease progression appears to be a complex one, with evidence demonstrating both complement-dependent detrimental effects and protective effects. Indeed, C3a and C5a are generated during many/most pathological events in the CNS (Bonifati and Kishore, 2007). A vital tool in exploring the possible mechanisms and overall contribution of complement initiated pathogenesis has been the use of animal models of disease. Murine models are particularly important as mice genetically deficient in specific complement components such as C1q, C3, C5, and Factor B are available for use in dissecting the role of complement in general and/or the function of the individual component. In addition, novel approaches such as “ectopic” expression of C3a or C5a may also prove useful in validating the direct role of these mediators in disease (Boos *et al.*, 2004). Since it has been demonstrated that receptors for C5a are expressed in the brain (Gasque *et al.*, 1997;Gasque *et al.*, 1998;Singhrao *et al.*, 1999;O'Barr *et al.*, 2001;Nataf *et al.*, 1999b) and that CNS cells do respond to C5a (Sayah *et al.*, 2003), the complement activation product C5a has been the focus of several recent CNS studies. For example, a lack of C5/C5a rendered mice resistant to cerebral malaria (Patel *et al.*, 2008), and significantly reduced neutrophil recruitment and CNS damage in a traumatic brain injury model (Sewell *et al.*, 2004), supporting the hypothesis that C5a can exacerbate inflammation in the CNS, with involvement of either mononuclear phagocytes or infiltrating neutrophils. Interestingly, C3a but not C5a, had a dominant effect in accelerating experimental autoimmune encephalomyelitis (EAE) (Boos *et al.*, 2004).

Mouse models expressing human mutated forms of the amyloid precursor protein (APP) have been shown to develop age-related accumulation of Aβ in the brain, and have been reported to mimic certain other features associated with human age-related AD pathology, including the association of complement components, such as C1q, C3 and C4 (Fonseca *et al.*, 2004;Zhou *et al.*, 2005;Zhou *et al.*, 2008), as well as reactive/inflammatory astrocytes and microglia, with the fibrillar Aβ plaques. Treatment of one AD mouse model (APP_{23}) with a low molecular weight heparin, enoxaparin, which is an inhibitor of Aβ mediated classical complement activation reduced Aβ load and astrocyte activation (Bergamaschini *et al.*, 2004), thus suggesting complement inhibition may result in reduced AD associated pathology. Further and more direct evidence for complement involvement in AD pathogenesis was demonstrated by

crossing an AD mouse model (Tg2576) to a C1q knock out to generate an AD mouse lacking the ability to activate the classical complement pathway. These C1q-deficient mice had significant reductions in glial activation as well as increased neuronal integrity in contrast to C1q-sufficient Tg2576 mice (Fonseca *et al.*, 2004). Interestingly, however, the genetic deficiency of C5 has been shown to be one of a limited number of genetic differences that are associated with decreased amyloid deposition in DBA/2J mice vs. C57Bl6 mice transgenic for the human APP gene (Ryman *et al.*, 2008). As is the case for peripheral monocytes, C5a has been shown to be chemotactic for microglia and astrocytes (Miller and Stella, 2008;Yao *et al.*, 1990), and thus in AD may provide a significant signal for glial recruitment to the plaque vicinity. In addition, C5a can synergize with other ligands to induce proinflammatory cytokine and/or chemokine production (O'Barr and Cooper, 2000), and thus can contribute to a secondary/synergistic inflammatory reaction to the plaques, particularly since fibrillar amyloid may bind TLR2 and TLR4 (Jana *et al.*, 2008;Lotz *et al.*, 2005) and TLR and C5aR have been shown to synergize in other tissues (Zhang *et al.*, 2007;Hawlisch and Köhl, 2006). This exacerbated inflammatory nidus likely contributes to neurotoxicity and the subsequent cognitive loss symptomatic of AD.

Since many of the detrimental effects of complement can result from the influx and activation of inflammatory myeloid-derived cells (neutrophils, macrophages, microglia) or synergistic signaling with other receptors, such as P2Y6 (Flaherty *et al.*, 2008) or TLRs (Zhang *et al.*, 2007;Hawlisch *et al.*, 2005), developing inhibitors of C5a activation of myeloid cells and/or receptor antagonists of C5a has been targeted as major mechanism for inhibiting acute C5a-induced inflammatory disorders as well as chronic debilitating disorders (Konteatis *et al.*, 1994). The C5a receptor antagonist PMX-205, a derivative of PMX-53 (Köhl, 2006a), which is an orally active CD88-specific receptor antagonist, has been shown to reduce disease activity in several animal models, including models of CNS disease such as brain trauma (Sewell *et al.*, 2004), Amyotrophic Lateral Sclerosis (ALS) (Woodruff *et al.*, 2008) and Huntington-like neurodegeneration (Woodruff *et al.*, 2006). Mouse models of AD, treated with PMX-205, have also shown significant decreases in both fibrillar Aβ and inflammatory glia in cortex and hippocampus (Fonseca, Ager, et al., submitted). These reductions in pathology were correlated with improvements in cognitive performance. These observations provide experimental evidence for a role for C5aR/CD88 in neurodegenerative disease progression. In addition, the results suggest that blockade of a receptor for complement-mediated inflammation reduces neuroinflammation and behavioral deficits, and may serve as a potent therapeutic target for those afflicted with AD and other neurodegenerative diseases that are accelerated by complement mediated inflammation.

4.2 Neurogenesis and Development

Interestingly, seemingly contradictory results were reported in other studies of the influence of the complement component C5 on inflammation. C5a, when given with kainic acid intraventricularly or 24 hours prior to glutamate treatment in neuronal mouse cultures, was shown to be neuroprotective against glutamate mediated caspase-3 activation (Osaka *et al.*, 1999). It was subsequently hypothesized that the C5a mediated protection may be dependent on the modulation of Ca^{2+} and MAP-kinase activity (Mukherjee and Pasinetti, 2000;Mukherjee and Pasinetti, 2001). In other systems, C5a, as well as C3a, provided direct neuroprotection (Van Beek *et al.*, 2001;Mukherjee and Pasinetti, 2001;O'Barr and Cooper, 2000); however, these were cell lines and/or neurons perhaps at different stages of maturation which may align with the studies of Fontaine and colleagues in newborn rat brain. These researchers demonstrate that in the developing cerebellar cortex brain, C5aR stimulation triggered increased BrdU incorporation by granule neurons and a C3aR agonist promoted migration of cells to their proper location (Jauneau *et al.*, 2006;Benard *et al.*, 2004;Benard *et al.*, 2008). However, since defects in cerebellum have not been reported in C3, C3aR, C5 or C5aR deficient animals,

further studies will be necessary to determine if these systems are redundant, residual or involved in facilitating survival and development during infection. The underlying basis for the differences in outcome due to C5a/C3a engagement of their receptors are likely different differentiation states of the cells and/or the cell signaling resulting from mixed cell interaction. Another example of the complexity of these responses is the report that C5a (but not C3a) drives up expression of microglial (but not astrocyte) glutamate receptor (GLT-1) which should provide increased glutamate uptake and thus protect neurons in the environment against glutamate toxicity (Humayun *et al.*, 2009). Since the two C5aR (CD88 and C5L2) may "cooperate" with other receptors (see 3.2.5 and 3.3), it is possible that a diverse, but precise set of responses to a changing environment could be orchestrated depending on the repertoire of interacting receptors available in the sensing cell. Clearly a "systems" approach to these responses will facilitate the clarification of these pathways and identification of targets for therapeutic interventions.

The ATs have been shown to be important for both hepatocyte proliferation and regeneration (Reca *et al.*, 2003;Daveau *et al.*, 2004;Mastellos *et al.*, 2001;Markiewski *et al.*, 2004). Recent reports in addition to those mentioned above involving development of rat cerebellum, have implicated C3a (and possibly C5a) in neural stem cell regeneration and directed migration of stem cells. After demonstrating that both clonally derived rat hippocampal neural stem cells and murine neural progenitor cells expressed receptors for C3a and C5a, mice lacking C3, C3aR, or had C3aR activity inhibited with an antagonist, showed reductions in migrating neuroblasts and newly formed neurons in areas of basal adult neurogenesis (Rahpeymai *et al.*, 2006). A similar reduction in neuroblast migration and number of newly formed neurons was observed after ischemic injury in the deficient or C3aR-inhibited mice. Although their study did not provide a mechanism behind the C3a receptor activation that would lead to the creation of new neurons nor a source for the generation of C3a or C5a (their results did not rule out a contribution of C5a), their results do provide evidence that complement components can play a role in neurogenesis. Interestingly, it has been reported that C3a induces or synergizes with IL-1β to enhance NGF expression (Heese *et al.*, 1998;Jauneau *et al.*, 2006) in human microglial cell cultures, which implicates the possible involvement/cross talk between these two systems. Taken together, it will be important to try to preserve and/or enhance the potential protective/reparative effects of specific ATs in the CNS when designing therapeutic targets.

5. Conclusions

Since the discovery of the ATs almost 40 years ago and their respective receptors 10-15 years ago, our view of the ATs as mere pro-inflammatory mediators generated in response to complement activation has changed. Today we consider ATs as immunoregulatory molecules with pleiotropic biologic functions, the development of which can be complement system-dependent or independent through serine proteases of different origins. Except the ancient antimicrobial activity and the metabolic activity of C3a/C3adesArg, ATs exert their biologic activities through ligation of their cognate receptors, belonging to the most abundant receptor family in vertebrates, i.e. the GPCRs. Depending on the local environment and the circumstances under which ATs are generated, they can contribute to cell and tissue homeostasis, the benefit or burden of inflammation as well as to tissue regeneration or fibrosis. Based on the strong pro-inflammatory properties, C5a and the C5aR in particular have been considered attractive pharmacological targets. Several companies have C5aR antagonists in their pipelines to attenuate inflammation in allergic asthma, ischemia/reperfusion injury, rheumatoid arthritis or age-related macular degeneration. While C5aR targeting appears an attractive approach in acute inflammation, long term approaches should consider direct or indirect effects of AT receptor signaling on adaptive immune responses, cell apoptosis and tissue regeneration/fibrosis that may impact on such treatments.

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