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Review

Anaphylatoxins coordinate innate and adaptive immune responses in allergic asthma

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ABSTRACT

Allergic asthma is a chronic disease of the airways in which maladaptive Th2 and Th17 immune responses drive airway hyperresponsiveness (AHR), eosinophilic and neutrophilic airway inflammation and mucus overproduction. Airway epithelial and pulmonary vascular endothelial cells in concert with different resident and monocyte-derived dendritic cells (DC) play critical roles in allergen sensing and consecutive activation of TH cells and their differentiation toward TH2 and TH17 effector or regulatory T cells (Treg). Further, myeloid-derived regulatory cells (MDRC) act on TH cells and either suppress or enhance their activation. The complement-derived anaphylatoxins (AT) C3a and C5a are generated during initial antigen encounter and regulate the development of maladaptive immunity at allergen sensitization. Here, we will review the complex role of ATs in activation and modulation of different DC populations, MDRCs and CD4⁺ TH cells. We will also discuss the potential impact of ATs on the regulation of the pulmonary stromal compartment as an important means to regulate DC functions.

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1. Introduction

Asthma is a complex inflammatory disease of the lung characterized by intense infiltration of the bronchial mucosa by mast cells, lymphocytes and eosinophils, goblet cell hyperplasia, together with elevation of serum IgE. The cellular findings are associated with the two main pathologic changes in lung physiology, airway hyperresponsiveness (AHR) and airway obstruction. The role of the anaphylatoxins (AT) C3a and C5a as pro-inflammatory effector molecules of the complement system is well appreciated. As such, they have long been considered as drivers of bronchoconstriction and airway inflammation during the effector phase of allergic asthma [1]. More than a decade has passed since the first description that C5 can protect from the development of allergic asthma in a mouse model [2]. In the meantime, a body of evidence has accumulated showing that the ATs and their corresponding AT receptors regulate the activation of different DC populations which can have a strong impact on the development of maladaptive TH2 and TH17 immune responses in allergic asthma. Further, the pulmonary epithelium is no longer considered as a mere barrier but an active sensor for allergens, which, together with endothelial cells,

produces cytokines and chemokines that drive DC maturation and transmigration of innate immune cells into the lung. Similar to other innate immune cells, pulmonary epithelial and endothelial cells express complement components and express AT receptors, suggesting that the ATs regulate innate immune cell cross-talk already during initial allergen encounter. In addition to innate immune cells, production of C3 and C5 has also been demonstrated in CD4⁺ TH cells [3]. Also, activation of AT receptors on CD4⁺ TH cells is critical for their survival and differentiation into effector [3–6] or Treg cells [7,8]. The available data suggest that the ATs tip the scales at the cross-talk between APCs and CD4⁺ TH cells, thereby regulating the differentiation of TH cells into TH2 and TH17 effector and Treg cells at allergen sensitization. In addition to a direct regulatory impact at the APC/T cell interface, recent data point toward an additional regulatory role of the ATs at the level of myeloid-derived regulatory cells (MDRC), a heterogeneous cell population that can either suppress or enhance pulmonary allergy [9,10].

2. The anaphylatoxins and their receptors

2.1. Generation of anaphylatoxins in the airways

The ATs C3a and C5a are generated by several mechanisms. First, they originate from their respective mother molecules when the soluble complement-sensing molecules C1q, mannan-binding lectin, the ficolins or properdin sense exogenous or endogenous danger [11,12]. This sensing initiates the assembly of different C3 and C5 convertases that cleave C3 into C3a and C3b and C5 into

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C5a and C5b. Indeed, low-level pulmonary complement activation already occurs under steady state conditions [13] but is markedly enhanced under asthmatic conditions [13–15]. Secondly, activation of the clotting cascade can lead to the generation of the ATs as several coagulation factors can cleave C3 and C5 [16–18]. In addition to these fluid phase activation pathways, ATs can also be generated in lung tissue by cellular mechanisms. Activated alveolar macrophages can cleave C5 into C5a by a serine protease [19]. Interestingly, such protease activity is not present in the supernatants of activated macrophages, pointing toward protease activity at the cells surface. Further, the serine protease granzyme B, which is expressed in neutrophils and lymphocytes [20] and the aspartic protease cathepsin D [21] can cleave C5 to generate C5a. Cathepsin D is primarily found in azurophilic lysosomes of neutrophils but can be released in the cytosol. These data suggest that cellular pathways exist promoting C5 cleavage and generation of C5a already within cells of the innate and the adaptive immune system. ATs can also originate directly from proteolytic cleavage by allergens. Extracts of several allergens including house dust mite (HDM), *Aspergillus fumigatus* and perennial ryegrass induce *in vitro* production of ATs in serum in a dose- and time-dependent manner [22]. Also, proteases from *Dermatophagoides* species (in particular *Der p3* and *Der f3*) can cleave C3 and C5 into their active fragments *in vitro* [23]. Finally, microorganisms in the upper airways can secrete proteases that exert C3 and/or C5 cleavage activity. *Porphyromonas gingivalis* is an anaerobic bacterium residing in the oral cavity. It promotes periodontitis by a mechanism that includes digestion of C3 and C5 by the cysteine protease gingipain resulting in C3a and C5a-like fragments [24,25].

2.2. Anaphylatoxin receptors

The ATs bind to a family of three AT receptors belonging to the superfamily of G-protein-coupled receptors (GPCR). The AT receptor family is composed of the C3a receptor (C3aR) [26,27], the C5a receptor (C5aR) [28,29] and C5a receptor-like 2 (C5L2) [30,31]. They share high-sequence homology and are closely related to other chemotactic receptors such as the N-formyl-methionine-leucine-phenylalanine (fMLP) receptor, ChemR23 and the chemokine receptors CXCR1 and CXCR2. Despite their similarity, the AT receptors differ in ligand specificity, signal transduction capacity and function.

The C3aR specifically binds C3a but does not recognize its degradation product C3adesArg or C5a. C5a and C5adesArg bind to two distinct receptors, i.e. C5aR and C5L2. C5L2 binds C5a with the same high affinity as C5aR but has a 20-fold higher affinity for the C5a metabolite, C5a-desArg, than C5aR [9]. Binding of C5a to C5aR activates cells through the recruitment of G protein subtypes, resulting in increased intracellular calcium concentration, Mitogen-activated protein kinase (MAPK), phosphoinositide-3-kinase gamma (PI3K γ) and Protein kinase (PKB/Akt) activation among other pathways [32]. In contrast, C5L2 is uncoupled from G-proteins due to the lack of critical intra-cellular amino acid motifs such as DRY and NPXXY. This lack of signaling together with its constitutive recycling and the internalization of its ligands suggests that C5L2 is a decoy receptor regulating the bioavailability of C5a/C5adesArg, thereby limiting C5aR-dependent cell activation. In support of this view, immune complex-mediated alveolitis is significantly enhanced in C5L2-deficient mice [33]. Mechanistically, C5L2 negatively modulates C5aR signaling through the β -arrestin pathway in neutrophils [34]. However, several reports provide evidence that C5L2 can also mediate pro-inflammatory properties in systemic inflammatory disorders (reviewed in [32]). Chen et al. have demonstrated that C5L2-deficient mice are protected from AHR in an ovalbumin (OVA) model of allergic asthma [35]. In a more recent study, it has been demonstrated that the absence of C5L2 is

associated with decreased TH2 cytokine production, eosinophilic airway inflammation, serum IgE, and mucus production in OVA and HDM models of allergic asthma [36].

3. The role of airway dendritic cells in allergic asthma

3.1. Development and phenotypes of airway dendritic cells

Airway DCs are a heterogeneous population within the innate immune system that sense exogenous and endogenous danger at the mucosal surface [37]. Airway DCs comprise conventional DCs (cDCs) and plasmacytoid DCs (pDCs), both of which derive from common DC progenitors that express the hematopoietic cytokine receptor Fms tyrosine-like kinase (Flt3) [38]. Among the cDCs, two distinct populations have been identified expressing either the CD103 or CD11b integrins [39]. Functionally, CD11b+CD103–cDCs but not CD11b–CD103+ appear to drive the development of maladaptive TH2 immunity in HDM-induced experimental allergic asthma [40] although opposing data have been published [41]. In support of a division of labor between CD103+ and CD11b+ cDCs, the latter cDC subset has been demonstrated to exclusively cross-present antigens to CD8+ T cells, whereas CD11b+ cDCs are specialized to present innocuous antigens to CD4+ T cells [42–44]. Pulmonary pDCs exert regulatory functions. They suppress cDC-mediated activation of CD4+ T cells [45] and induce Treg cells [46]. In addition to common DC progenitor-derived cDCs and pDCs, circulating monocytes can leave the bloodstream by a CCR2-dependent mechanism and differentiate into both CD11b+ and CD103+ DCs that reside in the lung under steady state conditions [47,48]. Upon exposure to HDM allergen, lung epithelial cells produce CCL2 and CCL7 chemokines [49], which can mobilize more CCR2+ monocytes from the bone marrow, resulting in an increased frequency of CD11b+ cDC in the lung [50]. Monocyte-derived DCs are an important source of chemokines attracting eosinophils and T cells into the airways [40].

3.2. Anaphylatoxins regulate conventional and plasmacytoid DCs in allergic asthma

C5aR expression has been demonstrated on pulmonary CD103+ [51], CD11b+ cDCs and pDCs [45]. Several studies have shown that mice lacking C5 [2,52], C5aR or in which C5aR signaling has been ablated during allergen sensitization [45,53], suffer from robust TH2 and TH17 immune responses that are associated with an enhanced allergic phenotype, suggesting that activation of the C5a/C5aR axis during primary allergen encounter protects from the development of maladaptive immune responses. The enhanced allergic phenotype is associated with an increased frequency of pulmonary CD11b+ cDCs and a decreased frequency of pDCs [45]. The same study showed an increased production of the TH2-cell selective CCL17 and CCL22 chemokines in cDCs from C5aR-targeted mice. Resident CD103+ DCs are the main producers of these two chemokines [54] although monocyte-derived DCs may also produce such chemokines and become a rich source during allergen challenge at high allergen encounter [40]. At this point, it is unclear whether the protective effect of C5a results from a direct regulatory effect on CD11b+ and/or CD103+ pulmonary cDCs during primary allergen sensitization and/or consecutive allergen challenges. Alternatively, C5a may suppress pulmonary DC functions by regulation of allergen-driven epithelial and/or endothelial cell activation (see Sections 4.1 and 4.2). In addition to the modulation of the distinct cDC subsets, pDC functions are altered by pulmonary C5aR blockade. After HDM exposure, pDCs express less of the co-stimulatory molecules PD-L1 and PD-L2, both of which regulate TH2 cytokine production from CD4+ T cells [55]. Thus, the

available data suggest a broad impact of C5a on the different pulmonary DC populations. However, no data have been published that show a direct impact of C5aR signaling on either pulmonary CD11b⁺, CD103⁺, monocyte-derived DCs or pDCs *in vivo* or *ex vivo* in models of allergic asthma.

Studies with naïve pulmonary DCs are difficult to perform due to their low numbers. As a surrogate, several groups have adoptively transferred antigen-pulsed BMDCs into the airways. Indeed, such GM-CSF-induced BMDCs promote TH2 and Th17 responses associated with AHR, strong airway inflammation and mucus production [56–58]. However, GM-CSF-induced BMDCs are phenotypically and functionally different from tissue-resident CD11c⁺ and CD103⁺ lung cDCs, as they resemble monocyte-derived inflammatory DCs [59,60]. To assess the direct impact of C5aR signaling in cDCs on TH cell activation in allergic asthma, Schmutte et al. have adoptively transferred OVA-pulsed BMDCs from C5aR^{−/−} mice into the airways of wt mice that were OVA-challenged 10 days later. In sharp contrast to previous findings, C5aR^{−/−} BMDCs failed to drive a strong asthmatic phenotype, which was associated with marginal TH2 cytokine production [61]. Surprisingly, C5aR^{−/−} BMDCs promoted TH2 cytokine production from OVA-transgenic CD4⁺ T cells with the same potency as wt DCs *in vitro*. However, C5aR^{−/−} BMDCs accelerated activated CD4⁺ T cell death *in vitro*, which was associated with an impaired ability to induce stable TH2 effector cell responses *in vivo*. Further, the frequency of MDRCs was significantly higher in C5aR^{−/−} than in wt BM cultures (Fig. 1 and Section 5.2). Several explanations may account for the opposing finding that C5aR^{−/−} mice show an increased allergic phenotype whereas C5aR^{−/−} BMDCs fail to induce pulmonary allergy: (1) The regulatory impact of C5a on BMDCs and lung resident and/or monocyte-derived CD103⁺ or CD11b⁺ DC may be different; (2) C5a may not directly act on pulmonary DCs but on other innate immune cells that regulate DC function such as epithelial, endothelial cells (see Section 4) or MDRCs (see Section 5); (3) C5a may mainly act on pDCs. Whatever reason may account for this unexpected result, it suggests that it might be too simplistic to consider BMDCs as appropriate surrogates to mimic TH cell activation by airway dendritic cells in allergic asthma, at least with regard to the role of complement.

To this end, several mechanistic studies have been performed *in vitro* with spleen-derived (sDC) or bone marrow-derived DCs (BMDC) to assess the impact of C5a on TH cell activation. One of these studies showed that C5aR-deficient sDCs produce less IL-12 and IL-6 but more TGF- β and IL-23 than wt sDCs when stimulated with OVA in the presence of the TLR2 ligand Pam3Cys, resulting in *de novo* generation of Treg cells and the differentiation of TH17 cells [62]. In line with these findings, Lajoie et al. recently reported that C5aR-deficient BMDCs produce more IL-23 than wt DCs following *in vitro* stimulation with HDM, which was associated with strong TH17 immune responses in a HDM-model of pulmonary allergy [53]. In contrast, OVA-pulsed BMDCs from C5aR-deficient mice produce less IL-1 β , IL-6 and IL-23 resulting in a decreased TH17 response when such BMDCs were adoptively transferred into wt mice [61]. In line with these data, Strainic et al. found reduced IL-23p19 mRNA levels in OVA_{323–330} peptide-stimulated BMDC/OT-II T cell co-cultures, when the C5aR and the C3aR were pharmacologically targeted [3]. Together, these findings suggest a complex regulatory role for the C5a-C5aR axis in TH17 differentiation that depends on the nature of the antigen (HDM vs. OVA) and the subset of DCs that is activated (BMDCs vs. spleen-DCs). The importance of the APC cell type is further stressed by data obtained with mouse peritoneal macrophages or human monocytes. In such settings, C5a drives TH17 cell differentiation by a strictly IL-6-dependent mechanism [63–65].

In contrast to C5 and C5aR-deficient mice, C3 [66] and C3aR-deficient mice [14,67] and guinea pigs [68] show a reduced allergic

phenotype. C3aR (and C5aR) expression has been demonstrated on human monocyte-derived DCs and pDCs [69,70]. In addition to human DCs, C3 and C3aR expression has also been shown in mouse BMDCs [3,71,72]. C3 and C3aR-deficient BMDCs have an impaired ability to promote TH cell proliferation and differentiation into IFN- γ -producing TH1 cells similar to what has been described for C5 or C5aR-deficient DCs [3,62,73]. Mechanistically, C3a suppress BMDC cAMP-levels, which is associated with enhanced capacity of antigen uptake, T cell proliferation and TH1 lineage commitment [74]. In line with these data, C3a treatment of sDCs suppresses the IL-4 production from OVA-transgenic T cells by an IL-12-dependent mechanism. These data suggest that C3aR signaling in BMDCs is an important driver of IL-12 production, which is critical for TH1 differentiation. Moreover, data from Lajoie et al. suggest that C3a stimulates the production of IL-23 and IL-6 and suppresses IL-10 production from BMDCs, thereby promoting TH17 differentiation in HDM-mediated experimental allergic asthma [53]. These findings are in line with high C3a plasma concentration in asthmatics with acute exacerbation [75]. However, in an *Aspergillus*/OVA model of allergic asthma, C3aR-deficient mice suffer from an increased frequency of IL-17A-producing TH cells [51]. This effect was not due to an impact of C3a on DC activation but on other cells of the myeloid compartment that need to be determined. The apparently contradictory findings of the two studies may result from different pathways of allergen sensing and distinct roles of C3a in such pathways. In fact, in HDM-mediated asthma, mucosal DCs are not directly activated by the allergen, but by epithelial-derived cytokines such as thymic stromal lymphopoietin (TSLP), IL-25, IL-33 and GM-CSF, all of which are released upon TLR4 activation [49]. The mechanisms underlying *Aspergillus* sensing are ill-defined. Studies with primary epithelial and pulmonary DC populations will help to elucidate the role of C3a in TH17 differentiation.

Further insights into the role of the different pulmonary DC subsets and AT-mediated regulation of allergic asthma come from studies of viral infections. It is well appreciated that allergic asthma can exacerbate following viral infections. It has been shown in an experimental influenza virus models that C3-deficient mice suffer from an impaired ability to recruit virus-specific CD4⁺ and CD8⁺ T cells into the lung-draining lymph nodes [76]. A more recent study provides evidence that it is the CD103⁺ DC subset that produces high levels of C3 and C5 during influenza infection and that C3a and C5a mediate the trafficking of CD103⁺ and CD11b⁺ pulmonary DCs as a critical means to activate CD4⁺ and CD8⁺ virus-specific T cells and clear the virus [77]. Although it is not known whether CD103⁺ airway DC are also the main source of C3 and C5 in allergic asthma, this study indicates that C3a and C5a may regulate the dominant allergen sensing by the CD11b⁺ cDC population or the regulatory pDCs by a paracrine mechanism that involves CD103⁺ cDCs.

4. The pulmonary stromal compartment in allergic asthma

4.1. Expression and function of anaphylatoxins and their receptors in airway epithelial cells

Although the lung epithelium is at the forefront of allergen encounter, its roles in allergen uptake and immunoregulation have long been underrated. This view has substantially changed during recent years. Epithelial cells are now considered an important modulator of airway mucosal structure and function regulating innate and adaptive immune response [78,79]. In HDM-mediated asthma, TLR4 activation in epithelial cells is critical to initiate the adaptive immune response through maturation of cDCs by production of pro-allergic cytokines [49]. Human alveolar epithelial cell lines and primary epithelial cells control cDC functions through

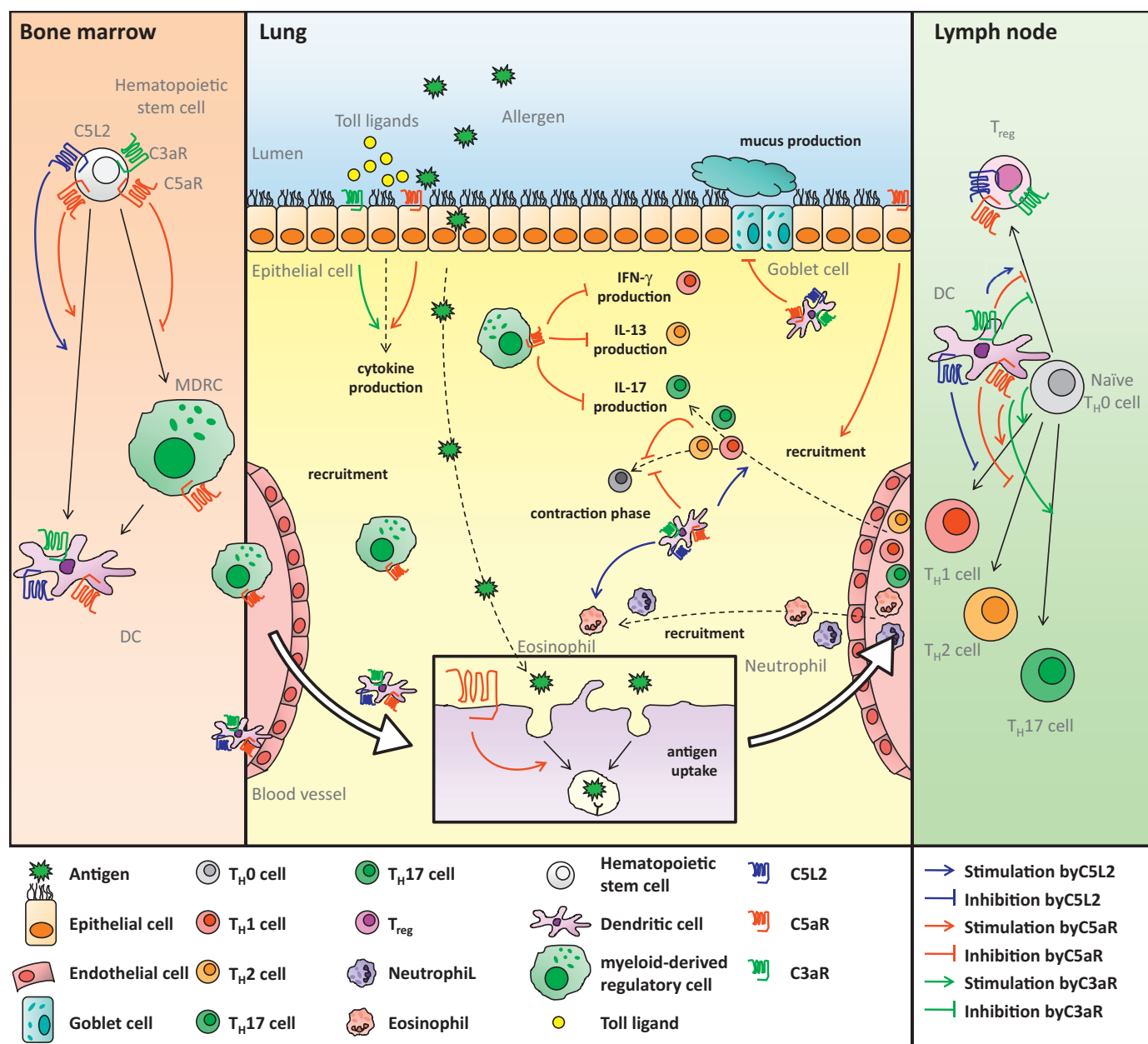


Fig. 1. Regulation of innate and adaptive immune responses by ATs and their cognate receptors. The ATs regulate the development of allergic asthma at several levels: (a) during bone marrow cell differentiation of cDCs and MDRCs (left panel); (b) in the draining lymph nodes at the level of TH cell differentiation (right panel); and (c) within the lung tissue regarding the complex interactions between epithelial cells, airway DCs, MDRCs, TH cells and pro-inflammatory effector cells (middle panel). (a) In the bone marrow, C5aR signaling suppresses the development of monocytic MDRCs. C5aR and C5L2 signaling also affect the differentiation of BMDCs in response to GM-CSF treatment. (b) In the draining lymph nodes, ATs can regulate the differentiation of naïve TH cells toward TH1, TH2 and/or TH17 effector cells as well as toward Treg cells by autocrine (in T cells) and paracrine (in cDCs) induction of complement factors, up-regulation of AT receptors and activation of such receptors. (c) Within the lung, the AT/AT receptors axis regulates initial allergen sensing at the epithelial/cDC interface, the homing of T effector cells, their regulation by MDRCs and the recruitment and activation of innate effector cells such as neutrophils, eosinophils, basophils or mast cells. Further ATs stimulate goblet cells to produce mucus.

Interferon Type I and IL-6 signaling pathways [80] and through the secretion of GM-CSF at steady state conditions and upon viral infection [81]. HDM-activated airway epithelial cells secrete several chemokines including CCL2 and CCL20 [82–84] that are involved in the recruitment of monocytes and cDCs. Taken together, the epithelium serves as a crucial sentinel system of innate immunity, attracting and activating monocytes and DCs that cross-talk with CD4⁺ T cells to define their lineage commitment. Further, they can activate type 2 innate lymphoid cells to pave the first wave of TH2 cytokine production in allergic asthma and drive smooth muscle contraction, mucus production and eosinophilic airway inflammation [85].

The involvement of the ATs in airway inflammation has already been demonstrated 30 years ago. C5a was found to recruit proinflammatory cells into the lung [86] and trigger pulmonary inflammation in a hyperoxic model [87]. Expression of various complement components of the classical and alternative pathways, in particular C3 and C5, were identified in primary rat type II epithelial cells [88,89] and the human type II pneumocyte cell line A549 [90]. C3 is produced by the epithelium upon activation by organic dust, particulate matter or smoke [91–93]. C3a induces the expression of Muc5A and promotes the recruitment of inflammatory cells by epithelial cells [94]. C5a triggers the release of IL-8 by bronchial epithelial cells upon stimulation by cigarette smoke [95]

and adhesion molecules [96]. In addition, C5a is cleaved from C5 by a serine protease-dependent mechanism and secreted by transformed human bronchial epithelial cells [97]. The mouse CLARA cell line C22 and primary mouse alveolar epithelial cells also express C3 and C5 (Laumonnier, unpublished data).

In addition to complement factors, AT receptor expression has been demonstrated in airway epithelial cells. Originally thought to be limited to leukocyte cells, C5aR expression was soon described in non-myeloid cells, in particular in lung bronchial and alveolar epithelial cells [98,99]. Further, the expression of C5aR and C3aR in mouse bronchial epithelium has been demonstrated [92]. Activation of bronchial epithelial cells by cigarette smoke markedly increased the expression of C5aR [100]. In contrast, C3aR expression was down-regulated during respiratory syncytial virus infection in a C5-dependent manner [101].

The functional relevance of C5aR signaling in epithelial cells has been shown in a model of IgG-induced lung injury. C5aR targeting in epithelial cells improved the inflammatory response [102]. Upon stimulation with C5a alone or synergistically with TLR4, rat epithelial cells secrete large amounts of TNF- α and CCL2 and to a lower extent IL-1 β [19] (Fig. 1), supporting the idea that C5a can cooperate with TLR signaling in epithelial cells similar to what has been observed in APCs.

Despite clear evidence for the expression of AT receptors in the epithelial layer of the airways, a definite role for AT receptor signaling in epithelial cell function during allergen encounter remains to be determined. Moreover, the impact of allergen exposure on the production of complement factors, the generation of the ATs and the expression of their receptors during the different phases of allergic asthma is poorly understood.

4.2. Endothelial cells in allergic asthma and their regulation by anaphylatoxins

The airway epithelium and the endothelium form a cellular compartment that is integral to appropriate steady state function of the lung. The vascular endothelium plays critical roles in the transmigration of innate immune cells such as neutrophils or monocytes from the blood vessels into the airways. Similar to epithelial cells, microvascular endothelial cells produce CXC and CC chemokines such as CXCL2 and CCL2. The expression of C3aR and C5aR has been demonstrated in HUVEC cells and human microvascular endothelial cells [103]. C5aR is also found in the endothelium of the lung [104,105] and can assist the emigration of leukocytes from the pulmonary vasculature by up-regulation of P-selectin and modulating their adhesiveness to the endothelium [106–108]. Further, C5a synergizes with LPS, IFN- γ and IL-6 to drive the production of CXCL2 and CCL2 from mouse microvascular endothelial cells in a sequential manner. Importantly, LPS, IFN- γ and IL-6 alone promote some production of the chemokines and up-regulate C5aR expression. Subsequent exposure to C5a significantly and selectively enhanced CXCL2 and CCL2 production, suggesting an important role of C5a in neutrophil and monocyte trafficking [105]. Further, neovascularization of the airways has been described in allergic asthma even before the development of maladaptive immune responses [109]. Endothelium lining vessels together with BM-derived proangiogenic progenitor cells produce IL-25 and TSLP in a model of experimental allergic asthma. Inhibition of angiogenesis blunted IL-25 and TSLP production, which was associated with a markedly reduced allergic phenotype [110]. These data suggest that the endothelial compartment significantly contributes to allergen-driven TH2 polarization in allergic asthma. C3a and C5a can promote neovascularization by VEGF- [111] or bFGF-dependent mechanisms [97]. On the other hand, C5a may also inhibit angiogenesis [112], most likely by a macrophage-dependent mechanism [113]. Clearly, the ATs have the ability to regulate the

development of TH2 and TH17 immune responses at the endothelial level. However, at this point no data are available demonstrating C3a and/or C5a-mediated regulation of pulmonary endothelial cells in allergic asthma.

5. The role of myeloid-derived regulatory cells (MDRC) in allergic asthma

5.1. Development and functions of MDRCs

In addition to the differentiation of inflammatory DCs, GM-CSF treatment of BM cultures promotes the development of MDRCs [9,114]. These cells comprise a heterogeneous group of myeloid progenitor cells and immature myeloid cells that is characterized by the surface expression of CD11c, CD11b and Gr-1 in mice or CD11b, CD33, CD34 and the lack of HLA-DR expression in humans, respectively (reviewed in [115]). Although most of the MDRCs exert immunosuppressive properties, a recent study identified a macrophage-like MDRC population with pro-inflammatory functions in a model of experimental asthma [10].

MDRCs can be subdivided into three subpopulations that differ in surface marker expression, morphology and regulatory effector functions. Monocytic MDRCs in mice are characterized by the expression of CD49d, Ly6C and F4/80 [115,116] and their monocytic cell shape with a round nucleus and an intermediate sized cytoplasm [10]. In humans, the phenotype of monocytic MDRCs is Lin-HLA-DR-CD33+CD14+ [115]. Compared to monocytic MDRCs, the more prevalent granulocytic MDRCs show a reciprocal expression of CD49d and F4/80 as well as a strong expression of Ly6G and low levels of Ly6C. Morphologically, they are characterized by their polymorphonuclear structure. Human granulocytic MDRCs express CD15 and CD33 but only low levels of HLA-DR. In mice, a third population of MDRCs has been described which can be distinguished by its macrophage-like morphology with a round nucleus and a large, strongly granulated cytoplasm. In terms of surface markers, this subpopulation expresses Ly6G but not Ly6C [10].

Monocytic and granulocytic MDRCs suppress proliferation and cytokine production by effector T cells, B cells and NK cells in several disease models such as cancer [116,117], autoimmune and viral encephalitis [118] or inflammatory bowel disease [119], among others. Moreover, infections with several microorganisms including bacteria [120], fungi [121], parasites [122] and helminths [123] have been demonstrated to increase the number of MDRCs in lymphoid organs and to stabilize the infection. The mechanisms underlying their suppressive properties include the metabolism of L-Arginine by Arginase 1 or nitric oxide synthase 2 (iNOS), the generation of reactive oxygen species or the production of cytokines. Monocytic MDRCs act mainly through the up-regulation of iNOS and Arginase 1, resulting in the consumption of L-Arginine, which inhibits T cell proliferation due to a loss of the CD3 ξ chain [124] or by blocking the cell-cycle progression [125]. Furthermore, Arginase 1 has been implicated in the induction of Treg cells [126] although controversial data exist [127]. On the other hand, iNOS generates an increasing concentration of nitric oxide that either inhibits T cell function by blocking the downstream pathway of the IL-2 receptor [128] and MHC-II expression [129] or by the induction of T cell apoptosis [130]. In contrast, the suppressive mechanisms of granulocytic MDRCs are based on the generation of reactive oxygen species by NADPH oxidase (Nox2) [127]. Such reactive oxygen species react with nitric oxide and result in the formation of peroxynitrite, which is able to nitrate the T cell receptor of CD8+ T cells [131]. This effect leads to an altered T cell receptor/MHC-peptide recognition and T cell tolerance [131].

In contrast to monocytic and granulocytic MDRCs, macrophage-like MDRCs, which were discovered in a murine model of

experimental allergic asthma, exert immunostimulatory functions. These functions are based on the superoxide production by NADPH oxidase. However, in contrast to reactive oxygen species produced by granulocytic MDRCs, the superoxide rather leads to an increased proliferation of T cells [10] which is in line with other studies [132]. Further, macrophage-like MDRCs produce VEGF and MMP-9.

5.2. Anaphylatoxins and their receptors in MDRC regulation

The role of ATs and their receptors in the regulation of MDRC biology is poorly understood. The involvement of C5a in MDRC function was first described in a model of cervical carcinoma [133]. In this model, MDRCs protect the tumor tissue from immune attack by down-regulation of anti-tumoral CD8⁺ T cell responses in a C5a/C5aR-dependent manner. In this model, locally generated C5a activates the C5aR on MDRCs. Interestingly, C5aR activation in granulocytic and monocytic MDRCs results in different outcomes. In monocytic MDRCs, C5a enhances the production of reactive oxygen and nitrogen species, leading to an enhanced tumor suppressive activity but has no impact on the migration of monocytic MDRCs into the tumor. In contrast, C5a is a strong chemoattractant for granulocytic MDRCs and drives their migration toward the tumor. This observation is in line with the general chemotactic effect of C5a on granulocytes and the up-regulation of adhesion molecules such as CD11b on MDRCs in response to C5a stimulation. Indeed, tumor growth is substantially decreased in C5aR-deficient mice or in wt mice in which the C5aR has been pharmacologically targeted. In another tumor model, an increased frequency of MDRCs in the spleen has been reported in response to a C5a-producing lymphoma, which was associated with a decreased frequency of CD4⁺ and CD8⁺ T cells in the tumor, tumor-draining lymph nodes and the spleen [134].

Only one study has demonstrated a regulatory effect C5a/C5aR signaling in MDRCs in experimental allergic asthma [61]. In GM-CSF cultures of BM cells from C5aR-deficient mice, the frequency of monocytic MDRCs was 2.5-fold higher than in cultures from wt mice (Fig. 1). Functionally, C5aR^{-/-} MDRCs expressed Arginase 1 and iNOS, the latter of which was significantly higher than in MDRCs from wt mice. To assess potential functional differences between wt and C5aR^{-/-} MDRCs *in vivo* both populations were adoptively transferred into wt mice together with antigen-pulsed wt BMDCs. Following adoptive transfer of wt cDCs in combination with wt MDRCs, the asthmatic phenotype was markedly decreased as compared with adoptive transfer of wt cDCs alone. Importantly, C5aR expression of MDRCs was critical for some of the suppressive properties of MDRCs including the reduction of airway inflammation and the TH2 cytokines IL-4 and IL-5. MDRCs from wt mice reduced the asthmatic phenotype in terms of airway resistance, TH1, TH2- and TH17 cytokine production and pulmonary infiltration with inflammatory cells. In contrast, C5aR-deficient MDRCs failed to improve lung function, which was associated with high IL-13 production. Further, IFN- γ and IL-17A production was not affected (Fig. 1). This study demonstrates that activation of the C5a/C5aR axis controls the differentiation of monocytic MDRCs from BM precursors by GM-CSF. Further, it shows that C5aR signaling during MDRC development not only regulates the frequency of MDRCs but also their function as evidenced by the increased iNOS levels *in vitro* in the absence of C5aR signaling, which were associated with a selective failure to suppress IL-13 production from TH2 effector cells resulting in failure to suppress AHR development (Fig. 1). No studies have been published determining the impact of C3a on MDRC differentiation, function or migration. However a recent study described a positive regulatory effect of C3 on the differentiation of MDRCs in DC cultures, which was related to its degradation product iC3b [135].

Altogether, the published data provide evidence that the C5a/C5aR axis regulates MDRC-differentiation and several MDRC functions that regulate innate and adaptive immune responses. However, the exact mechanisms underlying the C5a/C5aR-mediated regulation of MDRC differentiation and function are currently unknown. Further, it remains open whether the regulatory functions of C5a will also apply to the generation and function of pulmonary MDRCs.

6. CD4⁺ T helper cells in allergic asthma

It is now well appreciated that allergic asthma develops in response to the development of maladaptive TH2 and TH17 immune response in genetically susceptible individuals. The TH2 response is characterized by the production of IL-4, IL-5, IL-9 and IL-13, which promote antibody class switching to IgG1 and IgE. Also, TH2 cytokines mediate AHR and mobilize and attract effector cells such as eosinophils, basophils and mast cells to the airways, eventually driving goblet cell hyperplasia and mucus production. The major cytokines expressed by TH17 cells are IL-17A, IL-17F and IL-22, which not only recruit and activate neutrophils but stimulate different pulmonary cells to produce cytokines, chemokines and metalloproteinases (reviewed in [136]). Several recent publications provide evidence that CD4⁺ TH cells produce complement factors and express complement receptors upon activation leading to the generation of AT and activation of their cognate receptors in CD4⁺ Th cells [3,7,8].

6.1. The importance of the anaphylatoxins and their receptors for naïve and effector T cell development and survival

Cognate interaction between APCs and T cells leads to the local generation of alternative-pathway (AP) complement proteins C3, factors B and D and the down-regulation of the complement regulator CD55, promoting the dissociation of C3 and C5 convertases. As DAF-deficiency is associated with an increased frequency of effector T cells, these data suggested a role of alternative pathway activation and surface complement regulators as modulators of T cell immunity [137,138]. These findings were corroborated in another study showing that APC/T cell interaction also up-regulates C5, C3aR, and C5aR in OVA-transgenic T cells, mainly by CD28/CD80 and CD154/CD40 interactions [3]. Further, differentiation of OT-II cells into IFN- γ -differentiating TH1 cells was inhibited, when C3aR and C5aR were pharmacologically targeted or when naïve CD4⁺ T cells from AT receptor-deficient mice were stimulated with anti-CD3 and CD28. TH1 differentiation was also reduced *in vivo* in C3aR/C5aR-double-deficient mice in models of infection or autoimmunity [3]. C3a and C5a generated upon T cell activation activate PI3K γ formation and Akt phosphorylation, thereby up-regulating anti-apoptotic Bcl2 and down-regulating proapoptotic Fas expression [5]. Surprisingly, no evidence for C5aR receptor expression in naïve or effector T cells has been found in a C5aR/GFP reporter gene knockin mouse strain [139].

At this point it is unclear, whether complement factors and AT receptors are also up-regulated in T cells, when airway DCs activate naïve TH cells to differentiate into TH2 and TH17 cells following allergen sensing. Regulation of T cell apoptosis by C5a in allergic asthma has only been shown by modulation of DC functions. In an adoptive transfer model, C5aR^{-/-} BMDCs promote accelerated T cell death by decreasing the expression of Bcl2 and increasing the expression of BimEL/BimL in TH2 effector cells [61].

6.2. The anaphylatoxins regulate Treg cell differentiation and function

Regulatory Foxp3⁺ T cells are a heterogeneous population of T cells that arise either from the thymus as natural Treg cells (nTreg) or in the tissue during an immune response as adaptive or induced Treg cells (iTreg) [140]. There is evidence that allergic patients suffer from a decreased frequency of both Treg populations and that such Treg cells further exert functional defects resulting in decreased potency to block proliferation and cytokine production from effector T cells [141]. In experimental asthma, transfer of antigen-specific Tregs blocked the development and suppressed established AHR and airway inflammation [142]. In another study, depletion of nTregs increased AHR and airway inflammation in C5-sufficient C3H/HeJ mice but not in C5-deficient A/J mice, suggesting a role for C5 in Treg-mediated regulation of the allergic phenotype [143]. Interestingly, this effect was associated with increased numbers of highly active cDCs, suggesting that the suppressive effect of nTregs on cDCs [144] is also C5-dependent.

Initially described as a DC-dependent mechanism [62], more recent studies have highlighted an important role for the AT/AT receptor axis on T cells for the development and function of nTregs [8] and iTregs [7]. The frequency of Foxp3⁺ iTreg cells was dramatically increased in cultures of CD3 and CD28-stimulated C3aR and C5aR-deficient T cells as compared with cultures derived from C3aR^{−/−} or C5aR^{−/−} single KO or wt animals. These findings suggest that the lack of AT receptor signaling in CD4⁺ T cells paves the way for Treg cell lineage commitment. Functionally, activated naïve T cells from C5aR/C3aR double KO mice had a strong suppressive capacity, were anergic and unable to produce IL-2 upon re-stimulation. Further, the absence of AT receptor-mediated signals during T cell activation resulted in endogenous TGF-β1 production from naïve CD4⁺ T cells, fueling a positive feed back loop and enhancing their suppressor functions. Surprisingly, C5L2 contributes to Treg development by scavenging local C5a, which ensures sustained absence of C5aR engagement [7]. Similar to the impact of AT/AT receptor activation on iTregs, signaling through the AT receptors in nTreg cells inhibited their suppressive functions. Activation of AKT in response to AT receptor ligation drove the phosphorylation of the transcription factor Foxo1, resulting in decreased Foxp3 expression associated with reduced nTreg function [8]. Given that allergic asthma results, at least in part, from the imbalance between TH2/TH17 effector T cell and different Treg populations, it is likely that the strong impact of the AT/AT receptor axis on the fate decision of naïve T cells will also regulate the balance of effector T cell and Treg cells in allergic asthma.

7. Therapeutic implications

C3a and C5a can act on several cell populations that either primarily reside in the lung such as epithelial cells, endothelial cells, macrophage and DCs or traffic into the lung in response to chemokines and cytokines such as monocyte-derived DCs, neutrophils, eosinophils, basophils, MDRCs and CD4⁺ T cells. The activation and regulation of this complex cellular network by the ATs has to be taken into account, when it comes to targeting of the ATs or their corresponding receptors in allergic asthma. Unfortunately, our knowledge is scarce regarding the spatial and timely resolution of cellular activation by the AT/AT receptor axes during the different phases of the allergic response. Clinical studies have demonstrated that ATs are generated under steady state conditions in the airways and that allergen provocation markedly increases the production of the ATs in allergic individuals [13–15]. However, we do not know the source, *i.e.* the dominant cell type producing the complement factors or the type of activation, *i.e.* pathway (classical,

alternative, lectin) or cellular activation leading to AT generation. Further, not much is known about the expression of the AT receptors during allergen sensitization or the allergic effector phase. Such knowledge is critical for therapeutic considerations, as it lays the ground for the selection of potential targets and the timing of the therapeutic approach.

The available data suggest that C3a and C5a exert opposing roles during allergen sensitization, *i.e.* C3a drives, whereas C5a suppresses TH2 and TH17 development (see above). In contrast, selective targeting of C3 [63,93], C5 [145], the C3aR [14,67,68] or the C5aR [45,146,147] during the allergic effector phase decreased the allergic phenotype in different models of experimental allergic asthma. These data suggest that blocking of C3 and/or C5 cleavage, direct targeting of the ATs or their corresponding AT receptors might be beneficial in established asthma. Unfortunately, the oral C5aR antagonist NGD-1000 developed by Neurogen did not improve the asthmatic phenotype in a Phase IIa asthma trial back in 2004, indicating that the results from the animal studies cannot be simply transferred to the human situation. A more recent study suggests that blocking of C5 cleavage by eculizumab may attenuate some allergic responses, although the effect was minor [148].

Taken together, the published data suggest that we need a better timely and spatial resolution of the mechanisms underlying the regulation of the allergic phenotype by C3a and C5a in experimental models of allergic asthma. Once such knowledge will be available, we can begin to assess whether the pathways, by which the ATs regulate the asthmatic response in mice, will also be of relevance for the development of the allergic phenotype in humans. Using such knowledge may ultimately help to target the complement system for the benefit of allergic patients.

8. Concluding remarks

The emerging paradigm resulting from the last 15 years of complement-related asthma research is that C3a and C5a regulate the allergic sensitization and effector phases at several different levels. The expression of the ATs and their receptors in airway epithelial and endothelial cells as well as airway DCs suggest a regulatory impact at the epithelial/endothelial/DC interface that controls DC recruitment and activation. Further, accumulating evidence exists that the AT/AT receptor axis is controlling the lineage commitment of CD4⁺ T cells at the DC/T cell and MDRC/T cell interfaces. To fully understand the role of the ATs in allergic asthma development and maintenance, it will be important in future research to delineate the individual contribution of C3a, C5a, and their corresponding receptors at the level of epithelial/endothelial/DC, DC/T and MDRC/T cell cross-talk. Such understanding keeps the promise of a better asthma therapy.

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