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Abstract

Allergic diseases such as asthma result from inappropriate immunologic responses to common environmental allergens in genetically susceptible individuals. Following allergen exposure, interaction of dendritic cells (DC) with $CD4^+$ T cells leads to the production of Th2 cytokines, which induce B cells to synthesize IgE molecules (sensitization phase). These IgE molecules bind to their high affinity receptors (FcεRI) on the surface of mast cells and basophils and their subsequent cross-linking by allergen results in the release of preformed and newly synthesized mediators, which cause bronchoconstriction, lung inflammation and airway hyperresponsiveness (AHR) in asthma (effector phase). The complement components C3a and C5a levels are increased in the lungs of patients with asthma and are likely generated via the actions of both allergen and mast cell proteases. In vivo studies with rodents have shown that while C3a facilitates allergen sensitization in some models C5a inhibits this response. Despite this difference, both anaphylatoxins promote lung inflammation and AHR in vivo indicating that cells other than DC and T cells likely mediate the functional effects of C3a and C5a in asthma. This review focuses on the contribution of C3a and C5a in the pathogenesis of asthma with a particular emphasis on mast cells and basophils. It discusses the mechanisms by which anaphylatoxins activate mast cells and basophils and the associated signaling pathways via which their receptors are regulated by priming and desensitization.

Keywords

Anaphylatoxin; Asthma; Basophil; C3a; C5a; Complement; Desensitization; G protein; Mast cell; Priming; Signal transduction

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Short review

Regulation of human mast cell and basophil function by anaphylatoxins C3a and C5a

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ABSTRACT

Allergic diseases such as asthma result from inappropriate immunologic responses to common environmental allergens in genetically susceptible individuals. Following allergen exposure, interaction of dendritic cells (DC) with $CD4^+$ T cells leads to the production of Th2 cytokines, which induce B cells to synthesize IgE molecules (sensitization phase). These IgE molecules bind to their high affinity receptors (FcεRI) on the surface of mast cells and basophils and their subsequent cross-linking by allergen results in the release of preformed and newly synthesized mediators, which cause bronchoconstriction, lung inflammation and airway hyperresponsiveness (AHR) in asthma (effector phase). The complement components C3a and C5a levels are increased in the lungs of patients with asthma and are likely generated via the actions of both allergen and mast cell proteases. *In vivo* studies with rodents have shown that while C3a facilitates allergen sensitization in some models C5a inhibits this response. Despite this difference, both anaphylatoxins promote lung inflammation and AHR *in vivo* indicating that cells other than DC and T cells likely mediate the functional effects of C3a and C5a in asthma. This review focuses on the contribution of C3a and C5a in the pathogenesis of asthma with a particular emphasis on mast cells and basophils. It discusses the mechanisms by which anaphylatoxins activate mast cells and basophils and the associated signaling pathways via which their receptors are regulated by priming and desensitization.

1. Role of mast cells in asthma

Allergic diseases such as rhinitis and asthma are the most prevalent respiratory diseases in industrialized societies affecting ~20% and ~7% of the US population, respectively [1,2]. These diseases are caused by an overzealous immune response to allergens in which immunoglobulin E (IgE) and mast cells play critical roles. It is therefore not surprising that tremendous efforts have been directed towards developing therapy based on the modulation of IgE and its receptor, FcεRI. A recent exciting development in mast cell research has been the approval by the U.S. Food and Drug Administration of a humanized monoclonal antibody omalizumab for the treatment of allergic diseases. Omalizumab binds free IgE molecules and the resulting complexes are removed from the circulation. Over time, IgE comes off its receptors on mast cells and they lose their ability to respond to allergen [3,4]. Omalizumab is difficult to manufacture, is expensive, effective on a subset of allergic patients and may not be sufficient alone to prevent hyperresponsiveness [5]. Another approach has been to target the intracellular signaling pathway via which IgE–FcεRI activates mast cells. Given that Syk kinase plays a central role in FcεRI signaling, a number of Syk inhibitors have been developed [6]. One compound, R112, was the first Syk inhibitor to enter clinical studies [7]. These findings suggest that other pathways that also activate mast cells could be targeted for the development of asthma therapeutics.

As discussed in this review, the complement components C3a and C5a are involved in the pathogenesis of asthma and their effects have variously been proposed to involve dendritic cells, T cells, airway epithelial cells and smooth muscle cells [8–16]. Although a number of excellent reviews have recently been published on the roles of C3a and C5a in asthma [17–21], the possible involvement of mast cells and basophils have not been discussed in detail. It is noteworthy that murine bone marrow-derived mast cells (BMMC) and rat basophilic leukemia RBL-2H3 cells, which have been extensively used to study FcεRI signaling in mast cells, do not express G protein coupled receptors (GPCRs) for C3a and C5a [22–24]. The purpose of this brief review article is to summarize what is known about the activation and regulation of human mast cells and basophils by C3a and C5a. This review is particularly timely as basophils, which express C3a and C5a receptors, have recently been shown to have previously unrecognized role in the development and maintenance of allergic diseases [25–27]. Thus, understanding the molecular mechanism by which anaphylatoxins activate mast cells and basophils and delineating the signaling pathway via which

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their functions are regulated may provide a novel therapeutic target for asthma and other allergic diseases.

2. Roles of complement component C3a in the pathogenesis of allergic asthma

The complement system forms an important part of innate immunity against bacteria and other pathogens. As a system of 'pattern recognition molecules', foreign surface antigens and immune complexes initiate a proteolytic pathway leading to the formation of a lytic membrane attack complex. The anaphylatoxins C3a and C5a are generated as byproducts of complement activation, and they interact with cell surface GPCRs in target cells to mediate a variety of inflammatory responses [28–30]. Recent studies have shown that C3a and C5a levels are elevated in bronchoalveolar lavage (BAL) fluid after segmental allergen challenge in asthmatic but not healthy subjects [9,31,32]. Furthermore, plasma C3a and C5a levels are elevated in acute exacerbations of asthma [31] and C3a receptor is unregulated in subjects who died with asthma compared with subjects who died from other causes [33]. Additionally, single nucleotide polymorphism in C3 or C3a receptor (C3aR) gene increases susceptibility to asthma [34]. In animal models, complement activation modulates both AHR and airway inflammation [35,36]. Furthermore, deletion of C3aR gene or administration of C3aR inhibitors attenuates both AHR and lung inflammation [9,37–40]. Collectively, these findings demonstrate an important role of C3aR in the pathogenesis of asthma.

The mechanism by which C3a regulates AHR and inflammation in asthma is unknown and has been the subject of considerable debate. C3aR is expressed in both antigen-presenting cells (APCs) and activated T cells indicating that C3a may promote asthma by inducing Th2 cytokine production and IgE synthesis [41–44]. Indeed, Drouin et al. [37] reported that in models of *Aspergillus fumigatus* and ovalbumin-induced pulmonary allergy, C3aR-deficiency in mice on C57BL/6 background results in significant decrease in Th2 cytokine production and IgE synthesis. More recently, Zhang et al. [13] showed that in house dust mite (HDM)-induced allergic asthma $C3aR^{-}/^{-}$ mice produce less Th2 cytokine when compared to wild-type mice. These findings are in contrast with previous reports, which showed that C3aR-deficiency in guinea pigs and mice on the BALB/c background are not protected from serum IgE secretion, Th2 cytokine secretion [9,39]. These differences might reflect differences in species and strains of animals, nature of allergen and methods of sensitization used. Despite this, C3aR-deficiency protects animals from allergen-induced AHR and lung inflammation. Furthermore, administration of complement inhibitor in mice after sensitization but before challenge prevented the development of AHR and blocked lung inflammation [36]. Additionally, a small molecule antagonist of C3a receptor, when administered after sensitization but before challenge also significantly inhibited airway inflammation [38]. These findings suggest that although C3a has variable effect on allergen sensitization, its effect on AHR and lung inflammation in animal models of allergic asthma is likely mediated via the activation of C3aR in effector cells such as mast cells and basophils [12,21,36,38].

3. Dual roles of C5a in the pathogenesis of allergic asthma

As described above, development of allergic asthma in animal models can be modulated either at the level of allergen sensitization or the effector phase. Administration of C5aR monoclonal antibody after sensitization but before allergen challenge leads to substantial improvement of AHR and reduction in airway inflammation [38]. These findings are consistent with the idea that C5a also contributes the pathogenesis of allergic asthma via the modification of the effector phase. However, this contention was challenged by Karp et al. [45], who showed that C5-deficient mice are more susceptible to experimental asthma when compared with C5-sufficient mice indicating that C5a may instead play a protective role in the pathogenesis of asthma. Kohl et al. [15] recently utilized three experimental approaches to resolve this paradox. These included (a) administration of anti-C5a receptor (C5aR) monoclonal antibody to the lung, (b) expression of a lung-inducible mutant form of C5a (C5aRA $A8^{\Delta 71-73}$) that acts as a C5aR antagonist and (c) C5aR-deficient mice. They found that blocking or deleting C5aR prior to initial allergen sensitization in murine model of allergic asthma either induces or causes a marked enhancement of Th2-polarized immune responses, airway inflammation, and AHR. These effects result from an increase in the number of myeloid dendritic cells and in the production of Th2-selective chemokines. However, when C5aR was blocked during airway allergen challenge in already Th2-sensitized mice, AHR and lung inflammation were attenuated. Based on these findings, it has been proposed that C5a plays a dual role in allergic asthma; protection from the development of maladaptive Th2 immune responses during allergen sensitization at the level of myeloid dendritic and the production of Th2 cytokines but enhancement of airway inflammation and AHR in an established inflammatory environment [15]. This suggests that, as for C3a, the effect of C5a on asthma likely involves the activation of effector cells such as mast cells and basophils.

3.1. Activation of human mast cells by C3a and C5a

Mast cells are important effector cells that orchestrate the development of AHR and inflammation via their close interaction with airway smooth muscle (ASM), T cells and leukocytes [46–50]. In lungs of asthmatic individuals, mast cells are found in different compartments including bronchoalveolar space beneath the basement membrane, adjacent to blood vessels and scattered throughout the ASM bundles [51,52]. The ability of allergen to cross-link FcεRI on mast cells to induce mediator release is well documented [53–55]. In addition to FcεRI, mast cells express C3a and C5a receptors [56,57,49,21,58], which have been implicated in the pathogenesis of asthma.

Two subtypes of human mast cells were initially recognized based on the composition of their secretory granules. Thus, mast cell granules that contain both tryptase and chymase are designated MC_{TC} whereas those that contain only tryptase are known as MC_T [59]. Interestingly, MC_T cells predominate in the alveolar wall and the epithelium of the lung whereas MC_{TC} cells favor bronchial smooth muscle and glandular regions [60]. Furthermore, MC_T cell number in the respiratory epithelium increases during pollen season [61,62] and markedly elevated levels of MC_{TC} cells are found in bronchial smooth muscle cells of patients with asthma [63]. These findings suggest that different mast cell types may play distinct roles in the pathogenesis of asthma.

Studies performed in the 1980s indicated that while C3a and C5a induce mediator release in human skin mast cells, lung mast cells are unresponsive to these anaphylatoxins [56,64,65]. One possible reason for the discrepancy might reflect the fact that while MC_{TC} cells are the predominant cell type present in the skin they are the minority cell type found in the lung [66,67]. Indeed, Oskeritzian et al. [60] recently showed that MC_T cells in the lung do not express C5aR whereas M_{TC} cells do and that this is correlated with substantial C5a-induced degranulation in MC_{TC} cells. It is noteworthy that RBL-2H3 cells and BMMC, which are thought to be counterparts of human MC_T mast cells do not express C3aR or C5aR and are unresponsive to anaphylatoxins for mediator release [23,24,68,69].

Although the effects of C3a on human lung MC_{TC} cells are unknown, C3aR are expressed in a human mast cell line, HMC-1 cells [70–72], highly differentiated $CD34^{+}$-derived primary human

mast cells and a newly characterized MC_{TC} type human mast cell line, LAD2 [57,58]. Furthermore, C3a is one of the most potent mast cell chemoattractant known [72,73] and it causes sustained Ca^{2+} mobilization, degranulation as well as chemokine production in primary human mast cells and LAD2 cells [58,71]. In addition, cell–cell contact between airway smooth muscle (ASM) cells and MC_{TC} cells enhance C3a-induced mast cell mediator release [49]. Given that MC_{TC} cells favor bronchial smooth muscle and glandular locations, it supports the idea that effects of C3a and C5a in the effector phase of asthma are mediated, at least in part, by mast cells.

3.2. Novel pathway for the generation of C3a and C5a requiring mast cells

The best known mechanism for the generation of C3a and C5a is the classic IgG/antigen immune-complex pathway but this pathway does not appear to play a major role in the pathogenesis of asthma. The lectin and alternative pathways may participate in the production of these anaphylatoxins but several proteolytic enzymes outside of these complement pathways also can generate anaphylatoxin-like activity, including thrombin, kallikrein, and house dust mite protease [74–78]. Since C3a and C5a are generated in the lung of asthmatic but not in normal individuals, this raises the possibility that mast cells could participate in the generation of these anaphylatoxins. Indeed, Fukuoka et al. [79] recently showed that β-tryptase, the major protease of human mast cells, can directly generate bioactive C3a and C5a *in vitro*. Furthermore, activation of human MC_{TC} cells via the cross-linking of FcεRI results in the release of tryptase at sufficient concentrations to generate C3a and C5a from C3 and C5, respectively. Mast cell-derived mediators such as histamine can increase vascular permeability and the resulting exudation may serve to recruit C3 and C5 at the site of mast cell activation. The extravascular C3 and C5 are likely to be targets for mast cell-derived tryptase resulting in the generation of C3a and C5a. Thus, initial IgE-mediated release of histamine and tryptase may serve to amplify the allergic reaction through the generation of C3a and C5a, and additional mediator release via the subsequent activation of their GPCRs (see Fig. 1).

3.3. GPCR-dependent pathway for the activation of mast cells by C3a and C5a

C3aR and C5aR belong to a family of seven transmembrane domain GPCRs that couple to the Gαi family of heterotrimeric G proteins. Under resting conditions, G proteins exist as heterotrimeric complexes consisting αβγ complex with GDP bound to the α subunit (Gα). Receptor activation leads to a conformational change in Gα, resulting in an exchange of GTP for GDP. This interaction causes the dissociation of the βγ subunit (Gβγ) from the heterotrimeric complex. Gβγ, of which there are many subtypes, plays essential roles in mediating diverse functions of GPCRs. C3a and C5a induce chemotaxis of human mast cell line HMC-1, human cord blood-derived mast cells (CBMC) and cutaneous mast cells *in vitro* and these responses are inhibited by receptor-specific antibodies and pertussis toxin, inhibitor of Gαi family of G proteins [72,73]. Rat basophilic leukemia RBL-2H3 cells have been used extensively study to the molecular details of FcεRI signaling in mast cells. This cell line does not endogenously express receptors for C3a or C5a and does not respond to the anaphylatoxins for degranulation. However, RBL-2H3 cells ectopically expressing C3aR or C5aR are responsive to the anaphylatoxins for signaling and mediator release [22,23,80]. These findings suggest that the effects of C3a and C5a in mast cells are mediated via the activation of their respective GPCRs.

In addition to chemotaxis and degranulation, C3a and C5a also induce chemokine gene expression in mast cells [70,71,81]. The ability of C3a and C5a to induce early degranulation and delayed chemokine production release involves the activation of distinct signaling pathways including phospholipase Cβ (PLCβ)-mediated Ca^{2+} mobilization and protein kinase C (PKC) as well as phosphoinositide 3 kinase (PI3K) and extracellular signal regulated kinase (ERK) activation. C5a induces degranulation in mast cells via signaling pathways that require PLCβ but not PI3K or ERK [49,56,81,82].

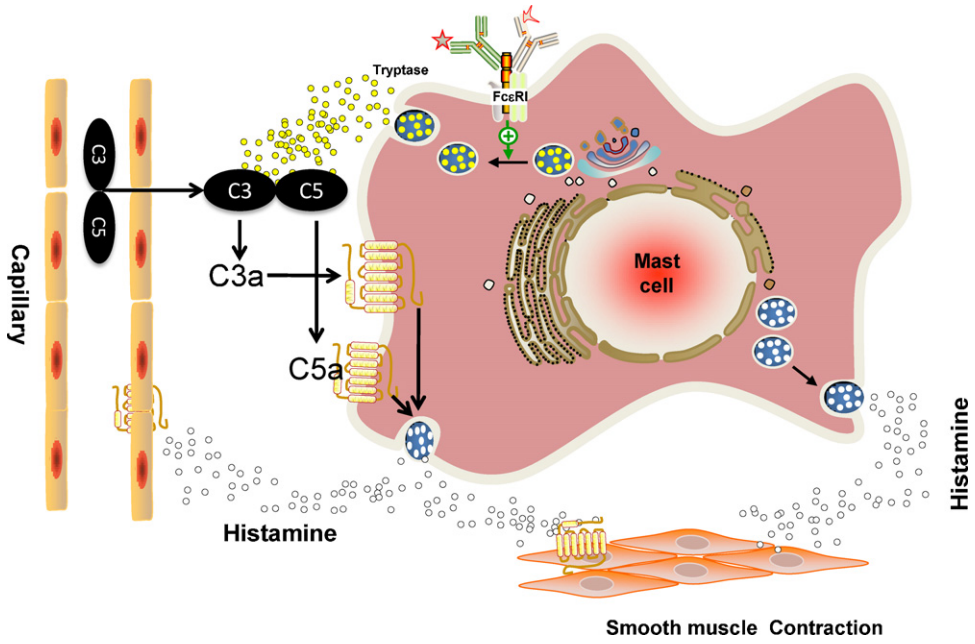

Fig. 1. Model for the role of FcεRI and mast cells on C3a and C5a generation and amplification of mediator release. Mast cell numbers are increased in the lung of allergic individuals, which are likely to be activated via FcεRI. The release of mast-derived mediators such as histamine causes increase in vascular permeability and the resulting exudation likely contains C3 and C5. Tryptase released from activated mast cells acts on C3 and C5 to locally generate C3a and C5a. These anaphylatoxins activate MC_{TC} mast cells present in bronchial smooth muscle to further exacerbate symptoms associated with asthma. See text and Fukuoka et al. [79] for further details.

By contrast, C3a promotes cytokine gene expression in mast cells via signaling pathways that require PLCβ, PI3K as well as ERK [70,81,71].

3.4. GPCR-independent pathway for the activation of mast cells by C3a

Basic peptides such as compound 48/80, substance P and mastoparan have been known for many decades to cause degranulation of rat peritoneal mast cells and human skin mast cells [56,83,84]. High concentrations of these peptides (micromolar range) are required for mast cell degranulation and their effects are blocked by neuraminidase, which hydrolyzes sialic acid residues on the cell surface, decreasing its negative charge. These peptides also activate purified Gαi proteins and treatment of mast cells with benzalkonium chloride, an inhibitor of Gαi, blocks degranulation. Based on these findings, it has been proposed that basic peptides utilize negatively charged residue on the surface of mast cells to induce degranulation by directly activating G proteins. It is noteworthy that C3a is a basic protein and is one of the few plasma proteins that can be generated at micromolar levels [85]. Furthermore, C3a (1–30 μM) causes degranulation of rat peritoneal mast cells and this response is inhibited by neuraminidase, pertussis toxin and benzalkonium chloride [85,86]. These findings suggest that C3a activates mast cell by two pathways; one at low concentration via the activation of cell surface GPCR and the other at high concentration involving the direct activation of G proteins.

4. Role of basophils in allergic diseases

Tissue mast cells and blood basophils share several features including surface expression of FcεRI, the presence of basophilic granules in the cytoplasm and the release of shared important chemical mediators. While the availability of genetically mast cell-deficient mice have provided a valuable tool to study the role of mast cells in allergic diseases, no mutant mice have been reported that selectively lack basophils. This, together with the fact that basophils represent a minor component of circulating blood leukocytes (<1%) and their similarities with mast cells, they have been neglected as minor and possibly redundant "circulating mast cells". However, the recent development of two monoclonal antibodies that selectively deplete murine basophils have been instrumental in identifying novel roles for basophils in promoting allergen-induced Th2 cell differentiation, enhancing humoral memory immune responses [26,87,88], mediating IgG-mediated systemic anaphylaxis and IgE-mediated chronic allergic inflammation [89–91]. In most of these situations, complements are likely activated generating both C3a and C5a. These anaphylatoxins, particularly C5a, have the capability to release histamine, leukotriene C_4 (LTC_4) and the Th2 cytokines IL-4 and IL-13 comparable in magnitude to those induced via FcεRI cross-linking [92–95]. In the sections below, I discuss the roles of C3a and C5a in mediator release in human basophils and the signal transduction pathways involved in their activation and regulation.

4.1. C3a and C5a-induced histamine and LTC_4 release in human basophils; priming by IL-3

Whether or not leukocytes express C3aR has been the subject of considerable debate. Zwirner et al. [11] recently utilized monoclonal antibodies against two different epitopes on the third extracellular domain of the human C3aR to show that human basophils express ~8100 receptors/cells. However, C3a, at concentrations that activate RBL-2H3 cells stably expressing human C3aR [68,96], do not induce mediator release in human basophils [12].

Table 1
Regulation of C3a and C5a receptor function in human basophils by IL-3.

	Histamine release	LTC_4	IL-4	IL-13
C3a	–	–	–	–
IL-3	–	–	–	–
IL-3 + C3a	+	+	–	–
C5a	++	–	–	–
IL-3 + C5a	++++	++++	+++	++++

Human basophils express ~8100 C3aR and ~13,500 C5aR on their surface. (–) indicates no or little mediator release. (+) to (++++) low to very high mediator release. See text for detail.

The reason for this difference is unknown but could reflect differences in the expression levels of C3aR in human basophils and transfected RBL-2H3 cells.

Hematopoietic growth factors such as interleukin-5 (IL-5), granulocyte/macrophage colony stimulating factor (GM-CSF) and in particular interleukin-3 (IL-3) profoundly modify the effector function of mature human basophils. IL-3 is generated in large amounts from T cells and antigen/IgE-activated human basophils [97]. Interestingly, preincubation of human basophils with concentrations of IL-3 that are ~100-fold lower than those required for colony formation render them responsive to extremely low concentration of C3a (1 nM) for histamine release and the generation of LTC_4 [12]. The release of these mediators in IL-3-primed cells is very rapid, being complete within 0.5–2 min. It has been proposed that IL-3 may induce priming of C3aR-mediated response via the induction of a high affinity state of the C3aR. However, this contention has yet to be verified experimentally. Using $C3aR^{-}/^{-}$ mice and a monoclonal antibody to selectively deplete basophils in mice *in vivo*, it has been shown that C3aR expressed in basophils greatly contribute to peanut extract-induced anaphylaxis [98]. It is, however, unknown whether C3a induces mediator release in mouse basophils via the activation of C3aR and if this effect requires priming by IL-3 and other cytokines.

Human basophils express approximately twice as many C5aR on their surface as C3aR [11]. Unlike C3a, C5a causes rapid histamine release in the absence of IL-3 [12,99] (Table 1). Neither C3a nor C5a causes LTC_4 production in human basophils [12,99]. However, basophils preincubated with low concentrations of IL-3 profoundly synergize with C3a and C5a to induce large quantities of LTC_4 [100]. The magnitude of IL-3/C5a-induced LTC_4 synthesis is much larger than that induced by IL-3 and C3a combination [8,12,94]. The effect of IL-3 on priming has been investigated in some detail for C5a, which occurs in two phases. The first phase occurs very rapidly after exposure to IL-3, starting at 1 min, with optimal effects at 5–15 min but reduced at 2 h [8,93,99]. The second phase is observed after 18–24 h of IL-3 treatment, and the magnitude of the C5a response is often greater than that observed after acute IL-3 pretreatment [101]. It is noteworthy that basophils are known to participate in the chronic phase of allergic diseases and lipid-derived mediators accumulate at these sites at amounts exceeding those found during immediate reaction [94]. It is likely that both C5a and IL-3 are generated continuously and concomitantly at the site of inflammation to induce LTC_4 generation [94]. It is noteworthy that leukotriene antagonists have been used in the treatment of chronic allergic inflammation such as asthma [102]. It is therefore possible that C5a contributes to allergic diseases via the production of LTC_4 in cytokine-primed basophils.

4.2. C5a, but not C3a, induces IL-4 and IL-13 in IL-3-primed human basophils

IL-4 and IL-13 are key immunoregulatory cytokines, which induce and amplify Th2-type immune responses and promote IgE

synthesis [103,104]. Receptors for IL-4 and IL-13 are also expressed on airway smooth muscle cells and IL-13 causes smooth muscle contraction, promotes mucous secretion and lung remodeling [105–107]. Not surprisingly, these cytokines have been targeted for asthma therapeutics [108,109].

Basophils are a prominent source of IL-4 and IL-13, which are rapidly produced upon FcεRI cross-linking [95,97,110]. It is noteworthy that neither C3a nor C5a induce IL-4 or IL-13 in human basophils. However, in the presence of IL-3 co-stimulation, C5a but not C3a, induces large quantities of these cytokines [92,100,110,111] (Table 1). The magnitude and duration of the IL-4/IL-13 induction in response to IL-3/C5a are often greater than that induced via IgE cross-linking. The priming effect of IL-3 on C5a-induced Th2 cytokine generation does not depend on the sequence of their addition but requires their sustained presence. Furthermore, monoclonal C5aR antibody, and pertussis toxin block IL-3/C5a-induced Th2 cytokine production [92]. In addition, the effect of C5a on this response can be mimicked by C5a-derived peptides that are known to activate C5aR [92]. These findings, in total, suggest that basophil-specific modulation of C5aR or its signaling pathways may modulate both the Th2 response and the effector phase of asthma.

4.3. Signaling pathways for C3a and C5a-induced mediator release in human basophils and priming by IL-3

The abilities of C3a (+IL-3) and C5a to induce histamine and LTC_4 release in human basophils are likely mediated via the activation of their respective cell surface GPCRs [11]. Although the mechanism by which IL-3 primes C3a-induced LTC_4 generation remains unknown, the signaling pathway via which IL-3 mediates both early and late phases of C5aR priming for the LTC_4 generation has been studied in some detail. Elegant work by Miura et al. [112,113] supports the view that synchronized regulation of cytosolic phospholipase A_2 ($cPLA_2$) activity is required for the generation of arachidonic acid, which acts as a substrate for LTC_4 synthesis. For optimal action, $cPLA_2$ requires Ca^{2+} for phospholipid binding and its phosphorylation by extracellular signal regulated kinase (ERK). It is interesting to note that in the absence of IL-3, C5a causes a transient increase cytosolic Ca^{2+} that lasts for about 30–45 s and induces $cPLA_2$ phosphorylation that is not apparent until after the Ca^{2+} response returns to basal level [93]. Thus, Ca^{2+}-mediated translocation of $cPLA_2$ may be dissociated from the membrane before phosphorylation and activation of the enzyme can occur. However, short-term pretreatment of human basophils with IL-3 causes rapid $cPLA_2$ phosphorylation but does not alter the characteristics of C5a-induced Ca^{2+} response. Thus, it has been proposed that the ability of IL-3 to allow C5a to promote LTC_4 release results from the preconditioning of $cPLA_2$ due to its phosphorylation. Under this condition, brief transient Ca^{2+} mobilization that occurs following C5a stimulation overlaps with the pre-existing phosphorylated $cPLA_2$ to allow its full enzymatic activity.

The ability of IL-3 to prime C5a-induced LTC_4 generation after 18 h preincubation also involves synchronization of $cPLA_2$ phosphorylation and Ca^{2+} mobilization but by different mechanisms [8]. In this situation, exposure of basophils to IL-3 has no effect on the delayed $cPLA_2$ activation by C5a but it converts a transient C5a-induced Ca^{2+} response to a sustained one, thus facilitating overlap of two synergistic signals; $cPLA_2$ phosphorylation and Ca^{2+} mobilization, which are required for optimal $cPLA_2$ activity and LTC_4 generation. An important factor that distinguishes between early and late priming by IL-3 involves new protein synthesis. Thus, while treatment of basophils with cycloheximide inhibits both the sustained phase of the Ca^{2+} response to C5a and late priming effect of IL-3, it has no effect on early priming [8]. It is therefore possible that chronic exposure of basophils to IL-3 increases the expression of C5aR to induce priming. However, this explanation is unlikely as IL-3 has a global effect in modulating LTC_4 generation in response to basophil stimulation by other receptor-mediated pathways [93,100,114,115].

5. Role of receptor phosphorylation on C3aR and C5aR desensitization in mast cells and basophils

Most, if not all GPCRs undergo desensitization that dampens cellular responses in the presence of continued stimulation. Importantly, desensitization regulates mediator release and thus prevents tissue damage [116]. This process involves agonist-induced receptor phosphorylation and β-arrestin recruitment [117]. The carboxyl terminus of GPCRs expressed in mast cells and basophils display low sequence conservation except for a large number of clustered phosphorylation sites [118]. C3aR possesses ten potential phosphorylation sites in two distinct clusters. C3a causes rapid phosphorylation of its receptors in RBL-2H3 cells stably expressing human C3aR or HMC-1 cells natively expressing the receptor [68,119]. Phosphopeptide mapping analysis showed that C3a causes phosphorylation of the receptor at both serine and threonine residues. Replacing all ten serine and threonine residues with alanine leads to more robust G protein activation and greater degranulation when compared to wild-type receptors [120,22,121–124]. These findings are consistent with the notion that, as in many other cell types, receptor phosphorylation desensitizes C3aR function in mast cells.

In addition to mast cells and basophils, C5aR is expressed in human neutrophils. Boulay and co-workers [125–127] have utilized neutrophil-like HL-60 cells and transfected COS-7 cells to show that although C5aR possesses six serine and five threonine residues at its carboxyl terminus, C5a causes phosphorylation of the receptor only at the serine residues with a maximal stoichiometry of 6 mol of PO_4/mol of receptor at Ser^{314}, Ser^{317}, Ser^{327}, Ser^{332}, Ser^{334}, and Ser^{338}. Using a mutagenesis approach they have shown that C5aR undergoes sequential phosphorylation with Ser^{334} as the major initial site followed by residues at positions Ser^{332} and Ser^{338} playing significant roles. Christophe et al. [126], demonstrated that phosphorylation of either of two serine pairs, namely Ser^{332} and Ser^{334} or Ser^{334} and Ser^{338}, is critical for the phosphorylation of C5aR and its subsequent desensitization. Replacement of Ser residues at these sites with Ala and their transfection in undifferentiated HL-60 cells results in a more sustained calcium mobilization, enhanced ERK phosphorylation and greater superoxide generation when compared to cells expressing wild-type receptors [126]. C5a also causes phosphorylation of its receptor in HMC-1 cells natively expressing C5aR and RBL-2H3 cells stably expressing the human receptor [23,119]. Pollok-Kopp et al. [128] recently showed that when compared to RBL-2H3 cells expressing wild-type receptor, C5a causes enhanced Ca^{2+} mobilization in RBL-2H3 cells expressing a mutant C5aR in which all six serine residues were replaced with alanine. This enhanced Ca^{2+} mobilization was partially reversed in cells expressing C5aR that had intact residues at positions Ser^{327}, Ser^{334} and Ser^{338}. This indicates that C5aR phosphorylation at multiple sites regulates signaling in mast cells and presumably in basophils.

5.1. Role of GRKs on C3aR and C5aR phosphorylation and desensitization

GPCRs are phosphorylated by a family of protein kinases, collectively known as GRKs (G protein coupled receptor kinases). Of the seven known GRKs, four (GRK2, GRK3, GRK5 and GRK6) are expressed in peripheral blood leukocytes, myeloid cell lines [129–133] and human mast cells (Hariharan, S, Guo, Q and Ali; unpublished data). All GRKs (60–80 kDa) possess a similar struc-

tural organization consisting of an amino terminal domain (185 amino acids), a catalytic domain (270 amino acids) and a carboxyl terminal domain (105–230 amino acids). There are, however, important differences in the mechanism via which GRK2/GRK3 *vs.* GRK5/GRK6 are localized to the proximity of the receptor to induce receptor phosphorylation [134]. GRK2 and GRK3 are found primarily in the cytoplasm and undergo translocation to the plasma membrane upon G protein activation, via their interaction with Gβγ subunit and membrane phospholipids. By contrast, GRK5 and GRK6 do not associate with Gβγ but interact with phospholipids or require lipid modification for their association with receptors. Overexpression of GRK2, GRK3, GRK5 and GRK6 with C3aR in COS cells enhanced agonist-induced receptor phosphorylation [119]. However, only GRK2 and GRK3 caused significant inhibition C3a-induced G protein activation. Furthermore, introduction of monoclonal antibodies to GRK2 and GRK3 inhibited agonist-induced C3aR phosphorylation but antibodies to GRK5 or GRK6 had no effect. These findings suggest that recruitment of GRK2 or GRK3 following C3aR activation leads to receptor phosphorylation and desensitization. The role of C3aR phosphorylation by GRK5 and GRK6 has yet to be determined.

Langkabel et al. [119] showed that as for C3aR, overexpression of GRK2, GRK3, GRK5 or GRK6 augmented C5a-induced phosphorylation of its receptors in transfected COS-7 cells. By contrast, Milcent et al. [135] demonstrated that overexpression of GRK2 or GRK6 has no effect on agonist-induced C5aR phosphorylation. Despite this difference, GRK6$^{-/-}$ mice have elevated serum IL-6 in an *in vivo* K/BxN model of inflammatory arthritis and enhanced granulocyte migration towards C5a *in vitro* [136]. These findings suggest that GRK6 may desensitize inflammatory responses by regulating granulocyte trafficking and reducing cytokine generation in response to C5a *in vivo*. It remains to be determined which GRK regulates C5aR phosphorylation in mast cells and basophils to modulate allergic asthma.

5.2. Role of β-arrestin on the regulation of C3aR and C5aR signaling in mast cells and basophils

One of the most intensely studied proteins that interact with phosphorylated GPCRs is β-arrestin. Two isoforms of β-arrestins (β-arrestin 1 and 2) are expressed in many cell types including mast cells [137,138]. In transfected RBL-2H3 cells, β-arrestin associates with wild-type but not phosphorylation-deficient C3aR [68]. Furthermore, overexpression of β-arrestin with C3aR enhances receptor internalization [20]. These findings are consistent with the notion that agonist-induced receptor phosphorylation leads to β-arrestin recruitment which promotes desensitization and internalization. However, the specific phosphorylation site in the carboxyl terminus of C3aR that interacts with β-arrestin and the GRK which mediates these responses remains unknown. Boulay and co-workers showed that agonist-induced C5a phosphorylation in neutrophil-like HL-60 cells, caused β-arrestin recruitment resulting in desensitization and internalization [125,126].

In addition to its role in receptor desensitization, β-arrestin acts as an adapter molecule to regulate diverse cellular function independent of desensitization [139–141]. For example, β-arrestins directly interact with several Src family kinases, ubiquitin ligases, protein phosphatases, microtubules, etc., and serve as scaffolds facilitating signaling in two MAP kinase cascades, leading to the activation of ERK1/2 and JNK3 [142–144]. Although the role of β-arrestin on the activation of downstream signaling pathways has not been studied in detail in mast cells and basophils, it does not appear to be required for C3a and C5a-mediated ERK phosphorylation. For example phosphorylation-deficient C3aR and C5aR, which do not associate with β-arrestin support greater ERK phosphorylation [22,126]. This suggests that unlike many GPCRs, β-arrestin plays inhibitory rather than stimulatory role in C3a and C5a-induced ERK phosphorylation in mast cells and basophils (Fig. 3).

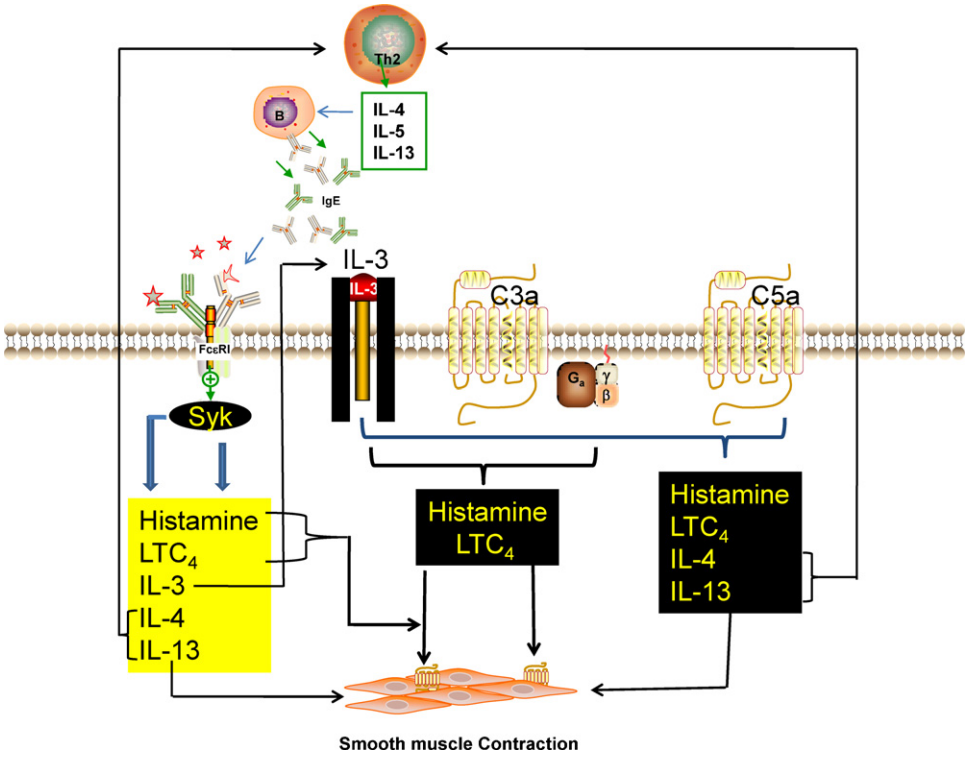

Fig. 2. Possible role of basophil-derived IL-3 on the priming of C3a and C5a-induced mediator release. IL-3 generated via the activation of FcεRI in basophils interacts with its cell surface receptors on basophils to prime both C3a-induced histamine release and LTC_4 generation. IL-3 also enhances C5a-induced histamine release, LTC_4 generation (early phase and later phase) as well as Th2 cytokines IL-4 and IL-13. These basophil-derived mediators are likely to have profound influence on allergen sensitization, bronchial smooth contraction and delayed inflammation.

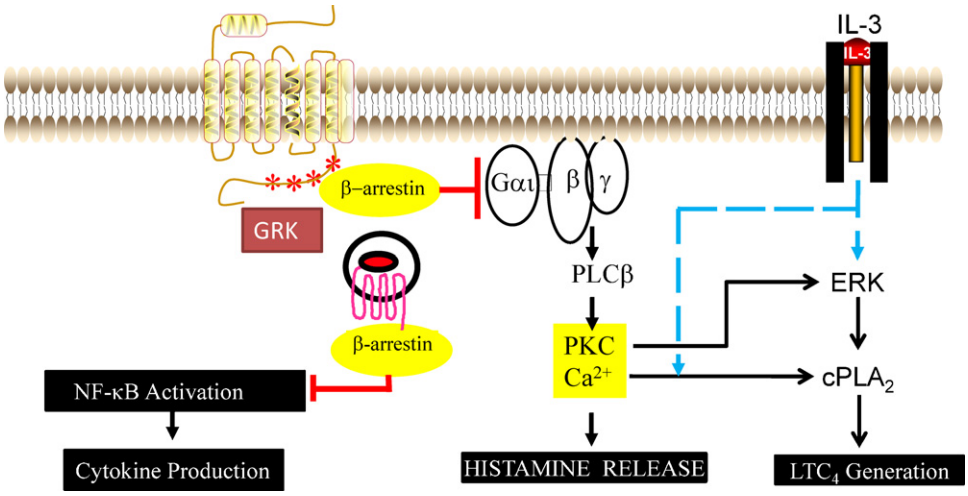

Fig. 3. Model for the role of GRKs and β-arrestin on the regulation of C3a and C5a receptors. C3a and C5a bind to their GPCRs on mast cells and basophils to activate PLCβ, $cPLA_2$ and NF-κB to induce release of different mediators. IL-3 enhances C3a and C5a-induced mediator release in human basophils. Effects of IL-3 on enhanced C5a-induced LTC_4 generation involve ERK phosphorylation (early) and greater C5a-induced Ca^{2+} mobilization (late) (blue dashed lines). Receptor phosphorylation by GRKs serves to recruit β-arrestin and this complex interacts with G protein to desensitize degranulation and LTC_4 generation. Internalized β-arrestin-associated receptor inhibits NF-κB activation to block delayed cytokine gene expression (red lines). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

NF-κB is a transcription factor that regulates the expression of a variety genes leading to the formation of chemokine and cytokines. In resting cells, most of the NF-κB is bound to a potent inhibitor IκB, thus retaining this complex in the cytoplasm [145]. Upon cell activation IκB is phosphorylated by IκB kinase (IKK) leading to its proteosomal degradation. NF-κB, once dissociated from IκB, rapidly translocates to the nucleus where it binds to specific promoters of the target genes. Although several IκB isoforms are known, Gao et al. [146] made the surprising observation that β-arrestin 2 directly interacts with IκBα to inhibit GPCR-mediated NF-κB activity. Witherow et al. [147], showed that although both β-arrestin 1 and β-arrestin 2 associate with IκBα as well as upstream kinases such as IKKα, IKKβ and NIK, only β-arrestin 1 inhibits NF-κB activity and cytokine production. Our recent studies with platelet activating factor (PAF) showed that β-arrestin inhibits NF-κB activity and chemokine induction in mast cells [20,148]. Furthermore, overexpression of β-arrestin enhances agonist-induced C3aR internalization and blocks chemokine CCL2 generation [20]. Also, similar to the situation with PAFR [148], expression of C3aR in mouse embryonic fibroblasts deficient in both β-arrestin-1 and β-arrestin-2, inhibits agonist-induced C3aR internalization but enhances NF-κB activity (Ali, unpublished data). These findings, in total suggest that unlike the situation with many GPCRs, β-arrestin plays a critical role in inhibition of C3a and C5a-induced degranulation, ERK phosphorylation, NF-κB activation, LTC_4 generation and cytokine synthesis (Fig. 3).

6. Summary and conclusions

This review discusses the role of anaphylatoxins in the pathogenesis of allergic asthma. Studies with $C3aR^{-}/^{-}$, $C5a^{-}/^{-}$ mice as well as receptor-specific antibodies and inhibitors have shown that although C3a and C5a have opposing effects on allergen sensitization, they promote two important features of asthma, AHR and lung inflammation. These findings indicate that effects of C3a and C5a in allergic asthma involve the activation of effector cells. Given that mast cells are important effector cells in asthma and that basophils play a critical role in chronic allergy, this review has focused mainly on the activation of these cells by C3a and C5a and the regulation of their receptors by priming and desensitization.

Emerging evidence suggests that tryptase released from FcεRI-activated mast cells generate C3a and C5a from C3 and C5, respectively, and that these anaphylatoxins act on MC_{TC} mast cells found in bronchial smooth muscles to induce mediator release causing smooth muscle contraction (Fig. 1). GPCRs for C3a and C5a are expressed on the surface of human basophils but there are important differences in the magnitude and diversity of mediators induced by these anaphylatoxins and their synergy with IL-3 (Table 1 and Fig. 2). It appears that, as for other GPCRs, agonist-induced receptor phosphorylation plays a critical role in the desensitization of C3aR and C5aR. In many cell types, β-arrestin acts as an adapter molecule to activate ERK and other intracellular signaling pathways. However, studies with transfected cell lines indicate that β-arrestin is not only involved in the desensitization of C3a and C5a-induced degranulation, it inhibits both ERK phosphorylation and NF-κB activation (Fig. 3). This suggests that β-arrestin could be targeted in mast cells and basophils for the modulation of allergic diseases. It must be pointed out that although most of mediator release and signaling studies discussed in this review were performed with primary human mast cells and basophils, phosphorylation and desensitization studies utilized cell lines such as RBL-2H3 cells, neutrophil-like HL-60 cells and COS-7 cells. Future studies are therefore required to confirm these findings in primary mast cells and basophils and ultimately in animal models to evaluate the potential for the development of novel asthma therapeutics by targeting anaphylatoxin signaling in these FcεRI-bearing immune cells.

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