# Characterizing the role of IgG antibodies in anaphylaxis

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*For my family*

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‘Dans les champs de l’observation le hasard ne favorise que les esprits préparés’

‘In the fields of observation, chance favors only the minds that are prepared.’

Louis Pasteur, 1854

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**Abstract**

Anaphylaxis is the most extreme manifestation of an allergic reaction and is characterized by an acute, systemic and potentially fatal response upon contact with an allergen. The current paradigm states that, in human, anaphylaxis is triggered by allergen-specific IgE antibodies (Abs). However, several reports in mice indicate that mouse IgG Abs can also trigger anaphylaxis, but conflicting results have been obtained regarding the identity of the effector cell(s) mediating this reaction. The main goal of my thesis was to better understand the role of IgG in anaphylaxis, and the pathways through which these Abs can mediate allergic shock. We first evaluated the contribution the IgE and IgG Fc receptors, effector cells and mediators in an adjuvant-free mouse model of active systemic anaphylaxis. We observed a modest contribution of the 'classical' pathway mediated by IgE, FcεRI, mast cells and histamine in this model. However, anaphylaxis was largely mediated by an 'alternative' pathway driven by IgG, FcγRIII, macrophages and platelet-activating factor (PAF). We then examined whether human IgG can also trigger anaphylaxis. Omalizumab, a humanized IgG1 anti-IgE mAb, has been reported to induce adverse events, including anaphylaxis, in some patients. We found that Omalizumab forms immune complexes (ICs) with IgE, which can engage FcγRs and activate neutrophils. We further discovered that such ICs induce both skin inflammation and systemic anaphylaxis when injected into $hFc\gamma R^{KI}$ mice (which express all human FcγRs in place of the mouse proteins). We then developed an Fc-engineered mutant version of Omalizumab which cannot bind FcγRs, and demonstrate that this antibody is equally potent as Omalizumab at blocking IgE-mediated allergic reactions, but does not induce FcγR-mediated anaphylaxis. Finally, I describe ongoing work in a new humanized model of peanut anaphylaxis, in which $hFc\gamma R^{KI}$ mice are passively sensitized with IgG purified from peanut allergic subjects. Our preliminary data indicate that IgG from allergic subjects can induce anaphylaxis in this model. Surprisingly, we observed that anaphylaxis is increased in $Fc\gamma R^{null}$ mice, which do not express any FcγR. We are now investigating the mechanism(s) of this reaction, and in particular the implication of the complement pathway, and the potential role of the inhibitory FcγRIIB.

**Résumé**

L'anaphylaxie est la manifestation la plus extrême d'une réaction allergique et se caractérise par une réponse aiguë, systémique et potentiellement fatale au contact d'un allergène. Le paradigme actuel stipule que, chez l'homme, l'anaphylaxie est déclenchée par des anticorps de type IgE spécifiques de l'allergène. Cependant, il a été montré que dans des modèles murins d'anaphylaxis, les anticorps de type IgG peuvent également déclencher un choc allergique, mais des résultats contradictoires ont été obtenus en ce qui concerne l'identité de la ou des cellules effectrices responsable(s) de cette réaction. Le but principal de ma thèse a été de mieux comprendre le rôle des IgG dans l'anaphylaxie et les voies par lesquelles ces anticorps peuvent déclencher un choc allergique. Nous avons tout d'abord développé un modèle murin actif d'anaphylaxie dans lequel les souris sont sensibilisées avec de l'ovalbumine sans utilisant d'adjuvant artificiel. Dans ce modèle, nous avons démontré que le choc allergique dépend en partie de la voie « classique » IgE, FcεRI, mastocyte et histamine, mais surtout d'une voie « alternative » induite par les IgG, leur récepteur FcγRIII, les macrophages et le facteur d'activation des plaquettes (PAF). Nous avons ensuite examiné si les IgG humaines peuvent également déclencher une anaphylaxie. L'anticorps thérapeutique anti-IgE « Omalizumab » (qui est une IgG1 humanisée recombinante) peut induire des effets secondaires, notamment des chocs anaphylactiques, chez certains patients. Nous avons constaté que l'Omalizumab forme des complexes immuns (IC) avec les IgE, et que ces IC peuvent lier les FcγR et activer les neutrophiles. Nous avons démontré que de tels IC peuvent induire à la fois une inflammation dans la peau (au niveau du site d'injection) et une anaphylaxie systémique lorsqu'ils sont injectés à des souris hFcγR$^{KI}$ (qui expriment tous les FcγRs humains à la place des protéines de souris). Nous avons ensuite développé une version mutante de l'Omalizumab avec une mutation ponctuelle dans la portion Fc (N297A) qui empêche l'anticorps de se lier aux FcγRs. Nous avons démontré que cet anticorps muté est aussi efficace que l'Omalizumab pour bloquer les réactions allergiques médiées par les IgE, mais n'induit pas d'inflammation cutanée et d'anaphylaxie via les FcγRs. Enfin, je décris des travaux en cours sur un nouveau modèle humanisé d'anaphylaxie aux arachides, dans lequel des souris hFcγR$^{KI}$ sont sensibilisées passivement avec des IgG purifiées provenant de sujets allergiques aux arachides. Nos données préliminaires indiquent que les IgG de sujets allergiques peuvent induire une anaphylaxie dans ce modèle. De manière surprenante, nous avons observé une augmentation de l'anaphylaxie lorsque ces anticorps de patients sont transférés dans des souris FcγR$^{null}$, qui n'expriment pas de FcγR. Nous étudions actuellement le ou les mécanismes de cette réaction, et en particulier l'implication de la voie du complément et le rôle potentiel du récepteur inhibiteur FcγRIIB.

## 1. Introduction

The immune system is a highly developed network of specialized cells and tissues able to protect the host from pathogen invasion. As a first line of defense, physical and chemical barriers, such as antimicrobial proteins present at the mucosal barriers, are responsible for protecting the host against pathogen invasion. If this barrier has trespassed, components of the innate immune system take place. Soluble factors may have antimicrobial functions, flag pathogen for elimination or facilitate the action of effector cells. Leukocytes recognize and phagocyte bacteria and viruses, and fight against parasites by producing an array of antimicrobial agents. Innate immune system components can be found, at least to some extent, from plants to higher vertebrates, highlighting the importance of this primordial system in the survival of almost all living organisms.

The development of the adaptive immune system arose with the evolution of jawed vertebrates. To be more successful against predatory attacks, the most flexible and efficient way was to diversify the defense mechanisms. This is successfully achieved through the expression of immunoglobulins (Igs), B cell receptors (BCRs) and T cell receptors (TCRs) and the generation of somatic gene rearrangements of these molecules. The infinity of possible combinations allows the receptors of the acquired immune response to respond to virtually any antigen one might encounter. Recognition of antigens by these receptors and subsequent receptor activation can then elicit an appropriate response to eliminate the threat. Furthermore, one of the most important consequences of the initiation of an adaptive immune response is the establishment of immunological memory, which allows subsequent rapid immune response in the case of reencounter with the same pathogen/antigen.

Innate and adaptive immunity work in constant collaboration to maintain the organism's homeostatic state. The natural course of the immunological responses consists of the recognition of the pathogen, development of protective immune responses that leads to the elimination of the pathogen and successfully reestablishment of homeostasis. Inappropriate or excessive immune activation, i.e. dysregulation of such immune responses can break tissue homeostasis and cause pathology. In some cases, apparently innocuous agents may even trigger an unbalanced immune response, which is the case in allergic diseases. Harmless for most of people, these innocuous agents (allergens) are found in dust mites, pets, pollen, insects, ticks, moulds, foods and some medicines. Importantly, allergies can affect up to 20% of the population and are increasing worldwide with unprecedent complexity and severity [1].

The most common manifestations of allergies are observed in the skin and the mucous membranes, representing the frontier between the individual and the environment. Allergies include several clinical conditions such as asthma, urticaria, atopic dermatitis, allergic rhinitis as well as a big spectrum of food- or drug-hypersensitive reaction, among others [2]. In the case of a sensitize subject, reactions can be elicited rapidly (minutes to hours) after the contact with the allergen and include mostly local-specific reactions. The immediate allergic reaction can be followed by a more sustained inflammation known as the late-phase reaction. Depending on the dose and the route of entry, symptoms may range from irritating sniffles of hay fever when pollen is inhaled or nausea/diarrhea when offending food is ingested. Yet, in some cases, symptoms may expand to a life-threatening circulatory collapse: systemic shock (or anaphylaxis).

Anaphylaxis is classically described to rely on the so-called 'classical' pathway, in which allergen-specific IgE antibodies are bound to the high-affinity receptor expressed on mast cells and basophils; upon allergen exposure, such cells are able to release mediators responsible for the systemic shock. The main focus of this thesis will be to question the paradigm of the sole contribution of the classical pathway in anaphylaxis by using innovative mouse approaches. In the first instance, the focus will be in an adjuvant-free mouse model of anaphylaxis. With a better understanding of the mechanisms in murine anaphylaxis, this work examines the clinical implications of the findings in two well-defined clinical situations: anaphylaxis occurring upon treatment with high dose of a therapeutic antibody, and peanut-induced anaphylaxis.

## 1.1 The innate Immune System: myeloid cells

Myeloid cells derive from hematopoietic stem cells (HSCs) in the bone marrow. HSCs are characterized by their potential to self-renew and their multipotency, *i.e*. their capacity to give rise to all blood cells. Maturation of these progenitors is a process known as hematopoiesis, and which is tightly regulated by transcriptional factors at distinct differentiation branches. A common myeloid progenitor gives rise to the myeloid compartment, as well as erythrocytes and platelets [3] (**Figure 1**). Some cells, like mast cells and macrophages, leave the bone marrow as immature precursors, and will niche in the peripheral tissues to fully achieve maturation. Others, like granulocytes (neutrophils, basophils and eosinophils), exit the bone marrow as fully mature cells and are able to rapidly respond to environmental stimulation.

Unlike the adaptive immune system, myeloid cells rely on a limited number of receptors to recognize pathogens. Pathogen-associated molecular patterns (PAMPs) are small motifs conserved within microbes. PAMPs are recognized via pattern recognition receptors (PRRs) expressed by myeloid cells. PRRs can be expressed on the cell surface or intracellularly. PRRs are also able to sense damage-associated molecular patterns (DAMPs). DAMPs are generated following tissue injury and help initiate the immune response.

This introduction will highlight some important aspects from the development of myeloid cells to their homing in tissues, both in homeostatic and inflammatory conditions. It will focus in particular on some myeloid cell populations – mast cells, basophils, neutrophils and monocyte/macrophages – which have all been implicated in a specific pathological condition: the allergic shock.

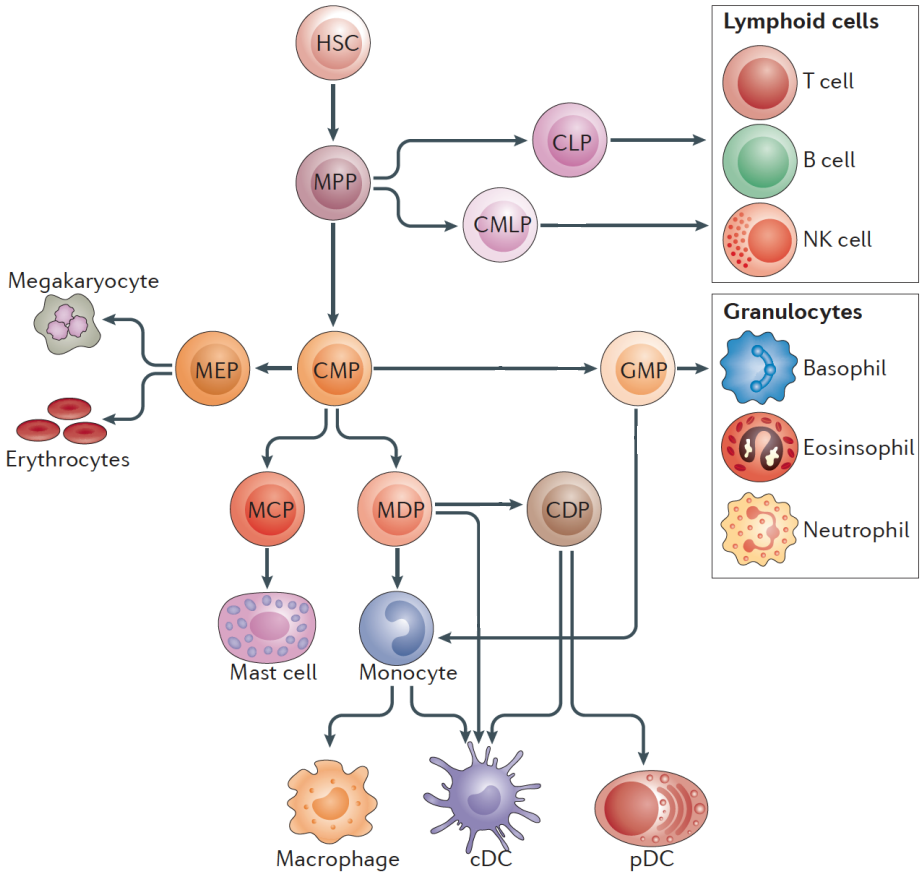



**Figure 1. Myeloid and lymphoid cell differentiation under normal physiological conditions.** Myeloid cells originate from haematopoietic stem cells (HSCs) and multipotent progenitor cells (MPPs). The figure illustrates the network (rather than hierarchical) of progenitor cells that gives rise to the various haematopoietic cell lineages. cDC, conventional DC; CDP, common DC progenitor; CLP, common lymphoid progenitor; CMLP, common myelolymphoid progenitor; CMP, common myeloid progenitor; DC, dendritic cell; GMP, granulocyte and macrophage progenitor; MCP, mast cell progenitor; MDP, macrophage and DC progenitor; MEP, megakaryocyte and erythroid progenitor; NK, natural killer; pDC, plasmacytoid DC. Adapted from [4]

### 1.1.1 Mast cells

Mast cell (MCs) are present in virtually all vascularized tissues but are most abundant in tissues exposed to the external environment such as the skin, the airways, and the intestinal tract. This strategic location in close proximity to blood, lymphatic vessels and nerves allows MCs to rapidly respond to harmful pathogens or other stimuli. Along with dendritic cells (DCs), MCs are the first cells of the immune system to interact with the external environment, being among the primary inducer and amplifier of both innate and adaptive immune response. After specific recognition, the response to various stimuli is done through the release of an array of biologically active mediators such as histamine, proteases, and other enzymes, cytokines, chemokines, growth factors, arachidonic acid metabolites, and reactive oxygen and nitrogen species [5].

MCs originate from hematopoietic stem cells in the bone marrow (**Figure 1**) that migrate through blood as immature progenitors and enter peripheral tissues where they can fully mature [6, 7]. The maturation of MCs depends on multiple molecules. Among these, stem cell factor (SCF), the ligand of the KIT receptor (CD117), acts as the main survival and developmental factor. Many other growth factors, cytokines, and chemokines account for MC phenotype and tissue distribution, including interleukin-3 (IL-3), IL-4, IL-6, IL-9 and IL-10 [8].

MC constitutively express high levels of KIT and the high-affinity receptor for IgE: FcεRI; the expression level of FcεRI on MC's surface is positively correlated with the concentration of IgE, independently of its antigen specificity in both mouse models [9] and clinical setting in humans [10]. While FcεRI is expressed in all human and mouse MCs, the expression of IgG Fcγ receptors (FcγR) on MCs is highly dependent on the species and environmental conditions. Murine MCs express the activatory IgG receptor FcγRIII and the inhibitory FcγRIIB [11]. Skin-derived human MCs constitutively express FcγRIIA, but not FcγRIIB [12], whereas expression of FcγRI can be induced by IFN-γ and the crosslink of the receptor leads to MC activation [13]. Human MCs cultured from cord-blood express FcγRIIB, but not FcγRIIA [14]. More recently, Burton and colleagues confirmed that human skin MCs lack expression of FcγRIIB, while demonstrated its presence in MC of the gastrointestinal tract [15].

The most characteristic morphological feature of MCs is their high content of secretory granules occupying a big proportion of the cytoplasm of the mature cells. Those granules are filled with a panel of pre-formed compounds, such as histamine, proteases (such as tryptases, chymases, and carboxypeptidase A3), cytokines (such as tumor necrosis factor - TNF) and growth factors (such as vascular endothelial growth factor - VEGF). MCs also produce newly formed-lipid mediators, including prostaglandins ($PGD_2$) and leukotrienes ($LTB_4$, $LTC_4$, $LTD_4$ and $LTE_4$) (**Figure 2**) [16]. Based on the location, histochemical staining and content of proteases, two major subtypes of MCs have been described in rodents: mucosal MCs and connective tissue-type MCs. Mucosal MCs preferentially express mouse MC protease (MCPT)-1 and -2. Connective tissue-type MCs express MCPT-4, -5, -6, -7 and carboxypeptidase A [5, 17]. Heterogeneity is also observed in humans MCs. Human MCs are classified by their content of serine proteases: Tryptase-only MCs ($MC_T$) or tryptase-chymase MCs ($MC_{TC}$) [18].

Given MCs' association with blood and lymphatic vessels, epithelial surfaces, and smooth muscles, it is not surprising that MCs play roles in several physiological and pathological processes. Their mediators may impact flow, permeability, secretion, and contraction in many sites, as well as during all phases of the inflammatory process and during wound healing [19, 20]. Upon contact with the threat, MCs promote the influx of inflammatory cells (neutrophils at the first instance, followed by monocytes) to the injury site by secreting histamine [21], TNF-α and other mediators capable of activating endothelial cells [22, 23].

MCs are well known for their fundamental protective role in host defense against certain parasites [24, 25]. Studies employing MC-deficient mice or MC-protease deficient mice suggest that MC play a pivotal role to resistance to infections, including *Strongyloides venezuelensis*, *S. ratti* and *Heligmosomoides polygyrus*, for example. Increased MC number in the gastrointestinal tract is common feature of infection with these nematodes; MC derived proteases and other mediators can be toxic to the parasites, as well as stimulate gut contraction (leading to worm expulsion) or enhancing mucosal permeability, all contributing to anti-parasitic immunity [25]. MCs express a great variety of receptors, including complement receptors, cytokine and chemokine receptors, toll-like receptors (TLRs) and pattern recognition receptors (PRRs), enabling them to respond to a wide range of cellular, viral and bacterial triggers [26, 27]. As an example, their importance has been shown in MC-deficient mice, which are more susceptible to septic shock and suffer increased mortality upon infection [28, 29]. MC can also be activated without direct contact with pathogens as they

respond also to danger signals (DAMPs) released by surrounding cells [26]. When engaged by multiple PRRs, activated MCs are able to immediately release pre-formed mediators such as histamine, proteoglycans, serotonin, chymases, tryptases, lipid mediators (prostaglandins, leukotrienes and platelet-activating factor) and pre-stored cytokines such as TNF-α. MCs can also secrete newly synthesized mediators including cytokines, such as IL-1, IL-3, IL-4, IL-9, IL-13, chemokines and angiogenic factors [30]. Another recently described protective function of MCs is to enhance innate resistance to venoms by releasing proteases (i.e. the carboxypeptidase A3 and the chymase MCPT4 in mice). In mice, these proteases are able to degrade toxins from several species of snakes and honeybee [31, 32]

However, MCs are better known for their detrimental role in allergic disorders. MCs are involved in the pathology of several hypersensitivity reactions that, not surprisingly, manifests in the physical barriers of the body, such as the airways, the skin and the gastrointestinal tract. In an allergic subject, whose tissue MCs have antigen-specific IgE bound to the high-affinity receptors FcεRI, re-exposure to the same antigen induces crosslinking of cell-surface bound IgE, resulting in aggregation of the receptor and activation of a complex signaling cascade that ultimately leads to the secretion of pre-formed biologically active products [22]. For example, histamine, proteases, proteoglycans, and lipid mediators are released within minutes of antigen exposure and are responsible for the early phase of the allergic reaction and depending on the localization of the stimulus, will trigger specific symptoms. If localized to the gastrointestinal tract for example, this response is characterized by increased vascular permeability, contraction of smooth muscles and altered ion transport leading in diarrhea, an important feature of food allergy [5]. Finally, some individuals also develop a late phase response directed not only by continued MC mediators release but also by activation of newly recruited and tissue-resident cells, typically beginning a few hours after antigen exposure. These late phase reactions are thought to cause the recurrent and chronic symptoms of allergic individuals [33].

As described above, MCs are very versatile sentinels of the immune system which can play both protective and detrimental roles, and whose phenotype and functions are tightly regulated by the local microenvironment.

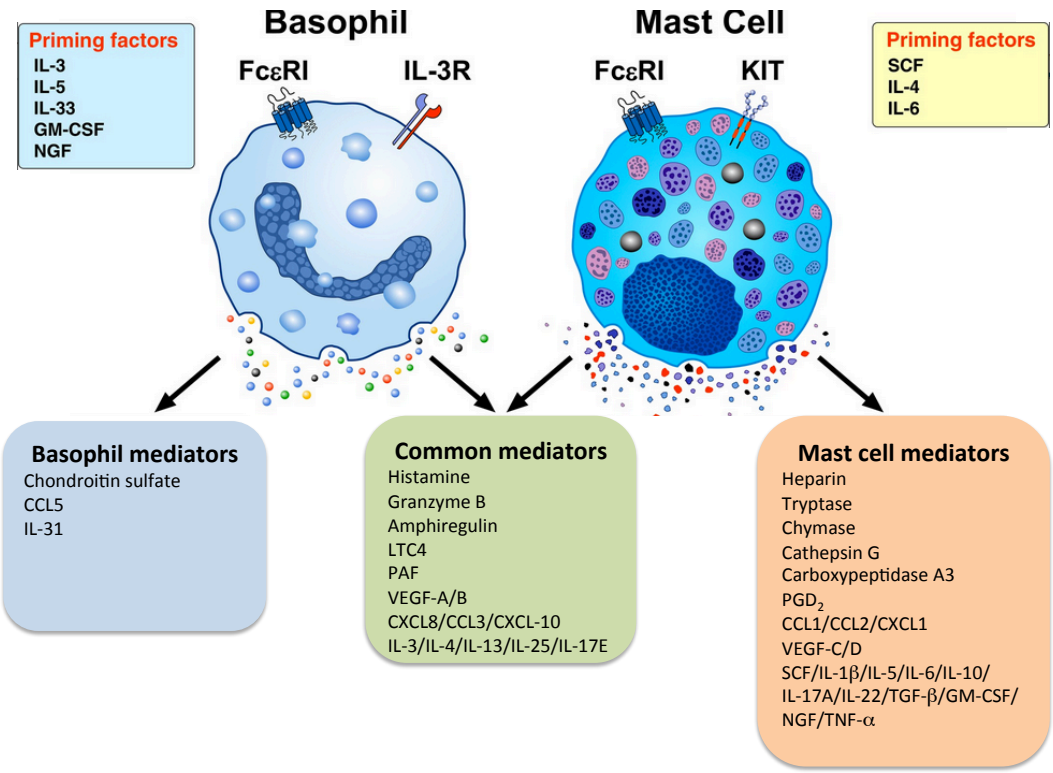



**Figure 2. Pro-inflammatory and immunomodulatory mediators of human basophils and mast cells**. Basophils contain histamine in secretory granules complexed with chondroitin sulfate. Secretory granules contain tryptase at levels of less than 1% of mast cells. Immunologic activation of basophils leads to the selective release of histamine and the production of IL-4, IL-31, CCL5/RANTES. A wide spectrum of mediators is release by both basophils and mast cells (ie, granzyme B, amphiregulin, LTC4, PAF, IL-3, VEGF-A, VEGF-B, IL-25/IL-17E, CXCL8/IL-8, CCL3/MIP-1α, and CXCL10/IP-10). Secretory granules of human mast cells can selectively contain several preformed mediators (ie, heparin, tryptase, chymase, cathepsin G, carboxypeptidase A3). In addition, activated mast cells can produce PGD2, chemokines (CCL1/I-309, CCL2/MCP-1, CXCL-1/GRO-α), angiogenic factors (VEGF-C and VEGF-D), and a constellation of cytokines (SCF, TNF-α, IL-1β, IL-5, IL-6, IL-9, IL-10, IL-11, IL-16, IL-17A, IL-18, 1L-22, TGF-β, NGF, FGF-2, and GM-CSF). Several factors (ie, IL-3, IL-5, IL-33, GM-CSF, and NGF) can prime human basophils, whereas fewer factors (ie, SCF, IL-4, and IL-6) prime human mast cells. CCL:chemokine C-C motif ligand; CXCL: C-X-C motif ligand; LTC: leukotriene; VEGF: Vascular Endothelial Growth Factor; PGD: prostaglandin; NGF: nerve growth factor. Adapted from [16]

### 1.1.2 Basophils

Basophils are the rarest granulocytes, representing less than 1% of peripheral blood leukocytes in both human and mice [34]. Based on their unique microscopic appearance containing basophilic granules in the cytoplasm, basophils were first identified by Paul Ehrlich in 1879 [35]. Because their relative lack of abundance in the bloodstream and phenotypical similarity to MCs, basophils were long considered as a redundant granulocyte population. However, the advent of mouse technology allowed the experimental manipulation of basophils *in vivo* and highlighted their critical contribution to anti-helminthic immunity, allergic inflammation and the pathogenesis of several disorders in models of human diseases [36, 37].

Basophils develop in the bone marrow from a common precursor and enter the bloodstream as mature cells with a relatively short lifespan of 1-2 days [38]. Functionally closely related to MCs, basophils also express the high-affinity IgE receptor (FcεRI), which is used as a surface marker to distinguish these cells from other cell types in the blood; basophils also express high levels of IL-3Rα, CD200R, and CD49b. IL-3 is an important growth and survival factor for basophils. Moreover, murine basophils express the IgG receptors FcγRIIB and FcγRIII, and human basophils express the IgG receptors FcγRIIA and FcγRIIB [11].

Cross-linking of IgE bound to their high-affinity receptor FcεRI is definitely the most studied basophil activation mechanism. IgE cross-linking induces basophils degranulation and the rapid release of several cytokines (including IL-4 and Il-13), histamine, leukotrienes (LTC4, LTD4, and LTE4), proteases (such as MCPT8 and MCPT11) and platelet-activating factor (PAF) [39](**Figure 2**). Another antibody-mediated activation of basophils was demonstrated in mice, in which IgG-antigen complexes were able to promote systemic anaphylaxis in wild-type mice, but not in mice in which basophils had been depleted using anti-CD200R3 antibodies (clone Ba103) [40]. However, one should note that these results are still controversial, as depending on the exact anaphylaxis model used, and the approach used to deplete basophils, basophils were found to either contribute [41-45] or play no significant role [46-49] in anaphylaxis. C3a and C5a can also activate human basophils through their receptors (C3aR and C5aR) [50]. Additionally, basophils can also be activated by cytokines, such as IL-3 and thymic stromal lymphopoietin (TSLP) [51]. Some proteases originated from allergens can also stimulate basophils directly. As an example, the protease Der p 1 from house dust mite promotes the release of IL-4, IL-5, and IL-13 in human basophils Phillips, 2003 #40}. Basophil-derived IL-4 has been shown to play a role in recruiting several inflammatory cells, including macrophages, innate lymphoid cells, and fibroblasts [52], and to upregulate the expression of VCAM-1 in vascular endothelium, a step required for eosinophil recruitment [53].

As regarding the biological importance of basophils, many studies have revealed non-redundant-functions in various immune responses such as in a model of IgE-mediated chronic allergic inflammation and in host defense against parasites (reviewed in [54]). However, the role of basophils in allergic diseases in humans still remains unclear.

Nevertheless, one should mention the important role of human basophils in the clinical diagnostics of allergy through the use of the so-called “Basophil Activation Test” (BAT). This test uses flow cytometry to detect the upregulation of CD63 or CD203c (which are hallmarks

of degranulation in these cells) on the surface of basophils upon antigen stimulation. In the BAT, basophil degranulation is used as an indirect readout of the presence of allergen-specific IgE. Therefore, BAT can reflect the patient's sensitization towards an antigen and it is used, along with other tests (such as skin prick tests), for the diagnostic of allergic diseases [55].

### 1.1.3 Neutrophils

Neutrophils are the most abundant circulating leukocytes in humans, accounting for fifty to seventy percent, while represented between ten to twenty-five percent in naïve mice (housed in a regular animal facility) [56]. Neutrophils represent the first line of host defense against infectious pathogens including bacteria, virus, and protozoa; neutrophils are the most important arm of the innate immune system and highly conserved throughout the evolutionary tree. This is best exemplified by a wide range of primitive organisms, such as insects that lack adaptive immune cells, relying on the function of their innate phagocytes [57]. Neutrophils are generated at a rate of $10^{11}$ cells per day in healthy individuals, a number that can increase up to ten-fold during infection [58]. Produced in the bone marrow by a process named granulopoiesis, mature neutrophils differentiate from hematopoietic stem cells (HSCs) in response to several cytokines, notably granulocyte-colony stimulating factor (G-CSF) (**Figure 1**). In order to become a fully mature PolyMorphoNuclear granulocyte (PMN), precursors are governed by a large transcriptional network that leads their differentiation through several stages: myeloblast, promyelocyte, metamyelocyte, band cell and finally PMN (**Figure 3A**). The upregulation of the transcription factor *Gfi-1* (growth factor independent -1) is critically required for neutrophil differentiation; and its additional role is to repress the monocyte-promoting transcription factor *Egr2* (early growth response 2) and *Csf1* (colony stimulating factor 1), both responsible for monocyte development [59]. The importance of the Gfi-1 in neutrophil development has been demonstrated by mutations causing neutropenia both in humans [60] and mice [61]. Relatively short-lived, early reports showed that human neutrophils survive for 8-12 hours in the circulation and up to 2 days in tissues [62], whereas more modern approaches suggest that under homeostatic conditions, neutrophils may have a circulatory life span of up to 5 days [63]. By contrast, the lifespan of neutrophils can be extended in inflammatory conditions, due to delayed apoptosis [64].

In the circulation, mature neutrophils measure between 7 and 10 μm and are filled with granules and secretory vesicles. Granule biogenesis is intrinsically linked with neutrophil development, so granules formed at different stages during maturation carry a distinctive

cargo of matrix and membrane proteins, which are critical for the antimicrobial and proinflammatory characteristics of the cell. Granules are classified as three distinct subsets: primary granules containing myeloperoxidase (MPO), alternatively called azurophil; second granules containing lactoferrin and finally tertiary or gelatinase granules filled with metalloproteinases [65](**Figure 3A**). Neutrophils are able to rapidly release secretory vesicles, which upon stimulation mobilize and bring integrins to the surface (for example, CD18 and β2 integrins), necessary for the cell to adhere to the vasculature. The release of proteases from gelatinase granules and reactive oxidative intermediates that cause local destruction of the extracellular matrix allows migration of neutrophils into tissues [66].

After their release from the bone marrow, neutrophils patrol the circulation and reach sites of tissue inflammation in response to chemoattractive signals originated from tissue-resident immune cells such as macrophages and MCs. The recruitment cascade and the mechanism underlying neutrophil extravasation from the bloodstream into the tissues have been well elucidated. This event takes place largely at postcapillary venules and is determined by the activation of endothelial cells by TNF-α, IL-1β and IL-17 generated during infection or inflammation. The classical adhesion cascade consists of *a)* initial attachment of the neutrophil to the endothelium (tethering), *b)* rolling of the neutrophil along the endothelium (rolling), *c)* firm arrest of the neutrophil with cell spread (adhesion), *d)* crawling of the neutrophil along the endothelium (crawling) and *e)* transmigration into the tissue (transmigration). The migration through the endothelial barrier may be via paracellular (between cells) or paracellular (through the cell) [58] (**Figure 3B**).

Circulating neutrophils are quiescent cells. After their entry to the inflammatory tissue, neutrophils can become fully activated in response to pro-inflammatory stimuli, which is characterized by the release of granules and acquisition of phagocytic capabilities. Generally, neutrophils need to respond to several stimuli to become fully activated. However, exposure to a sole stimulus (TNF, chemokines, growth factors, lipopolysaccharide) can prime neutrophils, thereby allowing enhanced activation in response other stimuli [67].

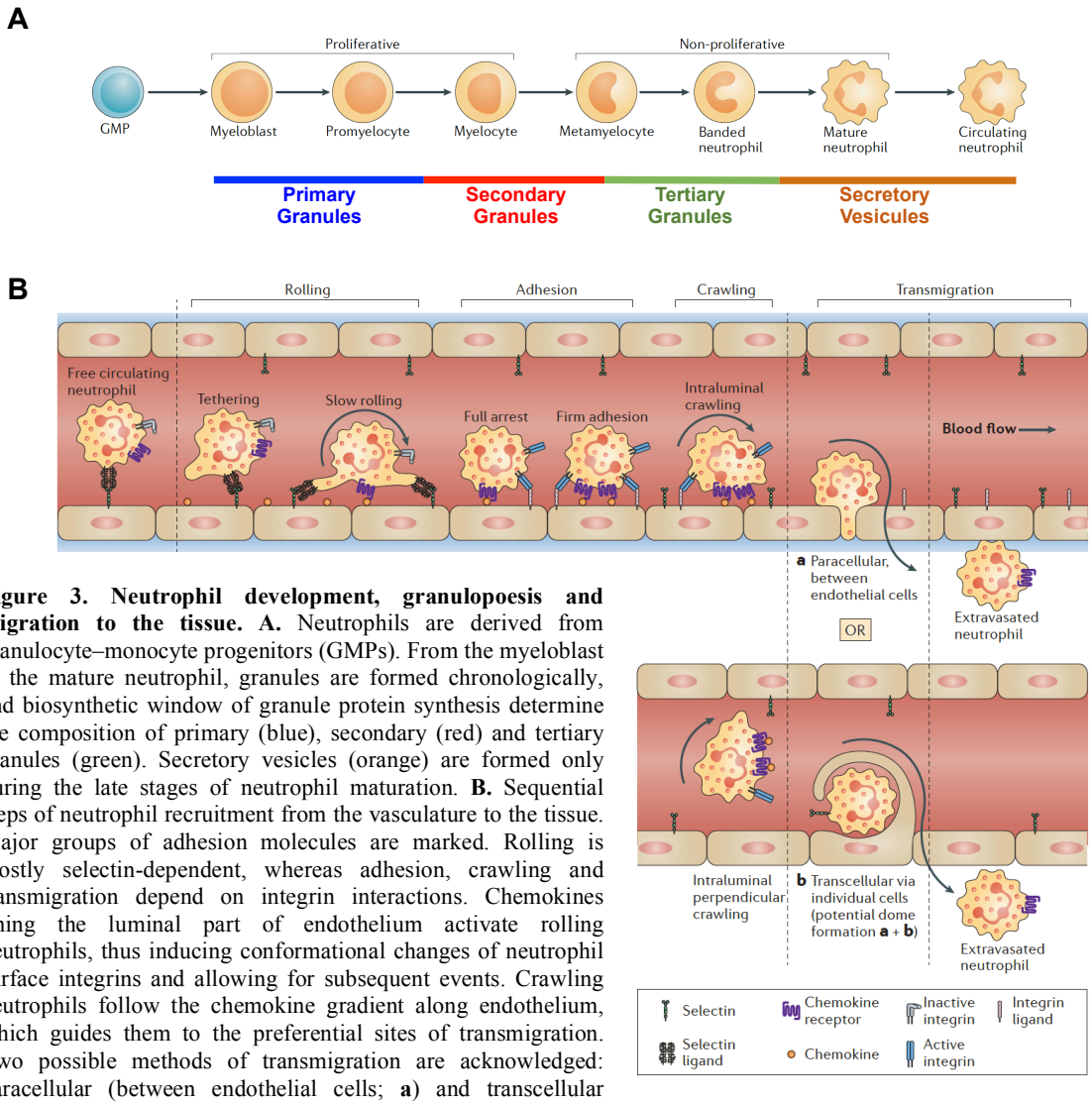



**Figure 3. Neutrophil development, granulopoesis and migration to the tissue. A.** Neutrophils are derived from granulocyte–monocyte progenitors (GMPs). From the myeloblast to the mature neutrophil, granules are formed chronologically, and biosynthetic window of granule protein synthesis determine the composition of primary (blue), secondary (red) and tertiary granules (green). Secretory vesicles (orange) are formed only during the late stages of neutrophil maturation. **B.** Sequential steps of neutrophil recruitment from the vasculature to the tissue. Major groups of adhesion molecules are marked. Rolling is mostly selectin-dependent, whereas adhesion, crawling and transmigration depend on integrin interactions. Chemokines lining the luminal part of endothelium activate rolling neutrophils, thus inducing conformational changes of neutrophil surface integrins and allowing for subsequent events. Crawling neutrophils follow the chemokine gradient along endothelium, which guides them to the preferential sites of transmigration. Two possible methods of transmigration are acknowledged: paracellular (between endothelial cells; **a**) and transcellular (through endothelial cells; **b**). Adapted from [68]

Neutrophils express several pattern recognition receptors (PRRs) and therefore can respond to different PAMPs and DAMPs. The major endocytic PRRs expressed by neutrophils are c-type lectin receptors, such as Dectin-1. Neutrophils also express toll-like receptors which are able to recognize lipids, carbohydrates, peptides, DNA and single or double-strain RNA. Furthermore, neutrophils express several receptors that recognize host proteins opsonizing microorganisms (such as antibodies and C3 complement activation product C3b). PMN are indispensable in the context of infection, yet their detrimental role in

the case of sterile inflammation cannot be neglected; neutrophils can contribute to tissue damage and are described to participate in immunopathology of many diseases, including autoimmunity, trauma, ischemic injuries, and allergy [69]. Indeed, in mice, the constitutive expression of the FcγRIII and FcγRIV allow neutrophils to be activated by immune complexes, as described during anaphylactic reactions [42]. Likewise, human neutrophils also express low-affinity IgG receptors: FcγRIIA and FcγRIIIB. FcγRIIA expressed on neutrophils seems to be sufficient to induce anaphylaxis in a FcγRIIA transgenic mouse model [70] and in a humanized mouse model where human low-affinity FcγRs were replaced by the mouse counterparts [71].

Functionally, after their activation by several stimuli, neutrophils can eliminate pathogens by phagocytosis, degranulation, generation of reactive oxygen species (ROS), cytokine production and ultimately by NETosis (secretion of neutrophil extracellular traps [NETs]). Pathogen recognition via direct PRRs or ligation of opsonized agents by Fc receptors/complement results in phagocytosis, with ingested material uptake by phagosomes and fusion with neutrophil granules resulting in the killing of the microorganism. The dramatic increase in oxygen consumption associated with the ROS generated by the activation of the NADPH (Nicotinamide Adenine Dinucleotide Phosphate Hydrogen) complex is an important mechanism of pathogen killing. Oxidative burst enables NADPH complex to reduce oxygen to superoxide anion, which then can dismutate into hydrogen peroxide or react with NO to form peroxynitrite. Both ROS and reactive nitrogen species have the capacity to alter and damage pathogens. Neutrophil granules can also fuse with the plasma membrane, causing the release of their content to the extracellular space. This degranulation is responsible for pathogen killing, but it can also contribute to local tissue damage. Finally, PMNs also extend antimicrobial activity beyond their lifespan by the formation of the neutrophils extracellular traps (NETs). NETosis occurs when large stands of decondensed DNA are extruded from the cell decorated with granule-derived antimicrobial peptides and enzymes, such as elastase and MPO and NETs ensnare microorganisms [58].

### 1.1.4 Monocytes & Macrophages

Monocytes and macrophages belong to the so-called ‘mononuclear phagocytic system', which also includes DCs [72]. A key function of these cell populations is to act as antigen-presenting cells (APCs). In addition, monocytes and macrophages also play important

effector functions during inflammation and infection, where they act both as phagocytes and immunomodulatory cells, participating in the initiation, maintenance and resolution of inflammation. Monocytes and macrophages derive from a common precursor named CMP (for "common-myeloid progenitor") present in the bone marrow and shared with granulocytes and dendritic cells (DCs) [72] (**Figure 1**).

Monocytes constitute 5-10 % of peripheral blood leukocytes in humans. Their development is highly dependent on the transcription factors *Egr2* (early growth response 2) and *Csf1* (colony stimulating factor 1) [59], and on the growth factor M-CSF (macrophage colony-stimulating factor). Monocytes exit the bone marrow and enter the blood circulation where they exist as two major subsets: 'patrolling' monocytes (promoting endothelial integrity by removing damaged cells and debris from the vasculature) or 'classical' monocytes that can be rapidly recruited into tissues, following tissue alterations in response to injury (infection and/or inflammation). In humans, these monocytes subsets are typically distinguished by their surface expression of $CD14^{hi}CD16^{-}$ (classical monocytes) and $CD14^{low}CD16^{+}$ (patrolling monocytes); the analogous population in mice are $Ly6C^{hi}CD43^{-}$ (expressed on the migrating population and not on those remaining on the circulation) and $Ly6C^{low}CD43^{hi}$ [73].

Once recruited to tissues, monocytes can differentiate into macrophages and DCs. Indeed, macrophages were long thought to only originate from the monocyte lineage. However, new studies using fate-mapping data suggest that interstitial macrophages actually represent a hybrid population originating both from monocytes and from embryonically derived self-renewing cells [74, 75]. The mechanism responsible for the maintenance of this self-renewing population in adult tissues still remains unknown [75]. Most tissues of the body harbor resident macrophages, most of them named in specialized tissues, including Kupffer cells (liver), alveolar macrophages (lung), Langerhans cell (skin), microglia (nervous system), and others. The functions of different tissue macrophages are highly dependent on both the transcriptional control of gene expression and tissue specific factors [76]. For example, Kupffer cells in the liver are responsible for clearance of aged erythrocytes as well as clearance of microorganisms and cell debris from the blood [77]. On the other hand, bone marrow macrophages are reported to clean nuclei expelled from erythroid precursors [78]. Macrophages possess critical roles in homeostatic clearance of apoptotic cells in various tissues, and a dysregulation of this process contribute to autoimmunity and chronic inflammatory disorders [77].

Tissue resident macrophages express a wide range of receptors for recognition of PAMPs and DAMPs, such toll like receptors (TLR), NOD-like receptors, lectins and scavengers' receptors, among others [79]. Following microbial challenge, activated macrophages (alongside with other tissue resident cells, such mast cell and DC) can then produce pro-inflammatory cytokines (such as TNF-α) and chemoattractants (such as CXCL1, CXCL2, and MCP-1 [monocyte chemoattractant protein-1]). The resultant effect is the influx of inflammatory leukocytes, notably neutrophils, but also monocytes at a source of inflammatory macrophages. The importance of resident macrophages in initiating the inflammatory response was demonstrated by studies in which resident macrophages were depleted, mainly by the use of clodronate liposomes. This depletion has several consequences, such as reduced production of chemokines, cytokines and lipid mediators and thus altered inflammatory cell recruitment [80, 81].

According with the type of activation, macrophages were long delineated as two distinct populations: classically ('M1') or alternative ('M2') activated macrophages. This classification considers the stimulus resulting in prototypical cellular phenotype [76]. Classical activation ('M1') of macrophages is elicited by stimuli such as LPS through TLR4 and is associated with macrophage secretion of IL-6, TNF-α, IL-1β production and nitric oxide, resulting in effective pathogen killing [82]. Alternative activation ('M2') of macrophages is elicited by an alternative spectrum of alternative signatures such as IL-4, IL-13, IL-10 and tumor growth factor beta (TGF-β) [82]. These 'M2' have high phagocytic capacity and can mitigate inflammatory response promoting wound healing; by the production of anti-inflammatory cytokines such IL-10 and upon the uptake of aging and apoptotic cells (notably neutrophils), macrophages help to limit the inflammation and potential tissue injury [83, 84]. It is noteworthy that the polarization nomenclature 'M1/M2' has been questioned recently with the considerable amount of evidence that macrophages do not form stable subsets *in vivo* [82]. The status of activation may change depending on the stimuli and the environment, creating far more complex and mixed phenotypes that 'M1/M2' accounts for [76, 82].

### 1.1.5 Platelets

Platelets are small anucleated cell fragments characterized by a discoid shape. Platelets are derived from megakaryocytes, which are large cells present in the bone marrow [85]. Thrombopoietin (TPO) is the dominant hormone controlling megakaryocyte development, but

many other cytokines and hormones, including IL-3, IL-6 and IL-11, also participate to this process [86]. Megakaryocytes are localized next to sinusoidal walls and the fragmentation of the cytoplasm into individual platelets results from a shear force of the circulating blood [87]. Sized from 1 to 3 μm, platelets are indispensable for a process such as homeostasis, wound healing, angiogenesis, and inflammation. Platelet secretion is essential in the normal response to vascular damage, as platelet activation and aggregation at the injury site promotes coagulation by forming a hemostatic plug [88]. The cytoplasmic contend of platelets is composed mainly of microtubules, granules and mitochondrias [89]. The membrane is composed of several receptors, such as integrins, selectins, protease activated-receptors (PAR), PAF receptors, histamine receptors and Fc receptors (see below for details) [90]. Upon activation, platelets change from the normal disc shape to a compact sphere and release secretory granules. Dense granules contain small molecules (e.g. serotonin, ADP, polyphastases); alpha granules contain numerous proteins, including platelet-derived growth factor, fibrinogen, Willebrand factor, fibronectin, P-selectin (or CD62P), coagulation factors and adhesion proteins [89]. The process of coagulation starts with the release of alpha granules; adhesion proteins (including CD62P) are expressed on platelet surface after activation along with the release of the coagulation factors and co-factors [91]. Platelets release also numerous chemokines, cytokines and lipid mediators, such as PAF and histamine [92, 93].

In addition to their important role in coagulation, platelets can interact directly with microorganisms through their expression of TLRs (both in humans and mice) [94]. TLR engagement was demonstrated to modulate LPS-induced thrombocytopenia and induce the production of TNF-α *in vivo* [95]. Platelets may also have a detrimental role in certain pathologies, such as arteriosclerosis and sepsis [89]. For example, in a murine model of sepsis, in which an organism has disseminated systemic reaction driven by an infection, activated platelets (increased expression of P-selectine) interact with neutrophils favoring their recruitment, activation, degranulation and NET formation [96]. Furthermore, human platelets (but not mouse) were reported to express antibody receptors, such as the IgG receptor FcγRIIA, which might give them potential functions during adaptive immune responses [11]. This was recently demonstrated in our laboratory in the case of anaphylaxis, where platelets were found to participate to IgG-mediated anaphylaxis through the direct engagement of FcγRIIA [97]. Indeed, the depletion of platelets attenuated anaphylaxis in mice expressing the human FcγRIIA; and serotonin released by activated platelets contributed to

anaphylaxis severity in mice [97]. In addition, early reports may suggest that platelets express the IgE receptors FcεRI and FcεRII (CD23), however these data are still controversial [98].

### 1.1.6 Eosinophils

Eosinophils are fully differentiated cells derived from granulocyte and macrophage progenitor (GMP) in the bonne marrow (**Figure 1**). IL-5 plays a central regulators of eosinophil proliferation, whereas eotaxin is responsible for eosinophil tissue accumulation. Granule-containing leukocytes, human eosinophils are rich in four cationic proteins: major basic protein 1 (MBP1), eosinophil cationic protein, eosinophil-derived neurotoxin and eosinophil peroxidase (EPX). Mouse eosinophils contain only two orthologs of the granule containing proteins: MBP1 and EPX. These basic proteins are toxic to microbes and particularly parasites. Eosinophils can also produce a variety of cytokines upon stimulation including IL-4, IL-6, TNF-α. Their effector functions also include lipid-derived mediators such as LTC4, PGE2, thromboxane and PAF. Unlike basophils and mast cells, human eosinophils express low levels of FcεRI, but exhibit expression of IgG receptor FcγRIIA [99].

## 1.2 The complement system and innate immunity

When a pathogen is able to break the epithelial barrier and cross the first antimicrobial peptides of the host (*e.g.* defensins and cathelicidins), soluble factors from the innate immunity are primordial to initiate the immunological response. These soluble factors are known as the complement system. Complement is comprised of more than 30 circulating and membrane-bound proteins that are distributed within the blood and interstitial fluids [100]. These proteins were first described by Jules Bordet in 1980 as a thermolabile component of plasma with the capacity to 'complement' antibodies in their ability to opsonize and kill bacteria [100]. Jules Bordet was awarded the Nobel Prize in Physiology and Medicine in 1919 because of his work on the role of antibodies and the complement system [100].

The majority of complement circulating proteins are synthesized by hepatocytes [101], however complement biosynthesis may occur in many other cell types, including adipocytes, endothelial cells, fibroblasts, and a range of myeloid and lymphoid cells [102]. In homeostatic state, complement proteins circulate as inactive forms. In the case of pathogen encounter, either directly or opsonized by antibodies, the complement system is activated and start a chemical cascade. The ultimate goal is to help pathogen elimination, by either direct lysis that results from the insertion of the membrane attack complex, or by targeting the pathogen with opsonins that are covalently bound onto the cell surface and in turn are recognized by receptors on effector cells. Critically, there is also an increased inflammatory response by soluble factors produced during complement activation [103].

Complement activation is described to occur through the classical pathway, alternative pathway and the lectin pathway. The first to be described was the classical pathway and involves antibody-mediated activation via the C1 complexes (comprised of C1q, C1s and C1r); the alternative pathway involves direct activation of C3 through surface binding; and the lectin pathway is triggered by carbohydrates on cell surface [103]. The pathway by which the complement cascade will be initiated is fully dependent on the type of recognition molecule, but all three typically described pathways will ultimately converge in a common pathway [100]. Critically, all of three aforementioned focus on cleaving the central molecule of the cascade C3 [104]. The cleavage of inactive C3 by C3 convertase leads to the formation of two functional fragments: C3a, a pro-inflammatory mediator and C3b, an opsonin responsible for tagging any surface in close proximity to the site of its generation. A new enzymatic complex if formed when C3b binds to C3 convertase: C5 convertase, which then cleaves C5 to bioactive fragments C5a and C5b. C3a and C5a are also known as

anaphylatoxins, due to their capacity of acting as inflammatory mediators [104]. These anaphylatoxins are highly cationic peptides with a very low molecular weight, possessing C-terminal argynil residues that are critical for their full pharmacological function [105].

Anaphylatoxins exert their biological activity through binding to specific receptors (C3aR and C5aR) expressed in several immune cells (mast cells, macrophages, neutrophils, B cells and other cells) and non-immune cells (e.g. endothelial cells, smooth muscle) [103]. C3a and C5a elicits broad responses: it enhances vascular permeability, induces contraction of smooth muscle, and acts as a chemotactic for a wide variety of leukocytes (neutrophils, eosinophils, basophils, macrophages and monocytes) [105]. To enhance inflammation, anaphylatoxins were demonstrated to induce oxidative burst on human neutrophils [106, 107]. Human mast cells respond to C3a and C5a with the release of histamine [108].

## 1.3 Antibodies and their receptors: conferring immune cells with adaptive specificity

Antibodies represent an important arm of the adaptive immunity for being implicated in host defense against pathogens. However, they can also have a detrimental role in some pathologies, as for example in autoimmune diseases and in allergy. The first evidence that molecules present in the plasma were responsible for immunity against diphtheria and tetanus came in the late 1800s. Shortly after, through the demonstration that passive transfer of immune serum could confer protection against diphtheria to a naïve recipient, von Behring and Kitasato showed for the first time the great importance of antibodies.

The different immunoglobulin classes (in humans and mice: IgM, IgD, IgG, IgE and IgA), their receptors and their pattern of expression in different cells and tissues will dictate the outcome of the encounter of a pathogen, or the development of inflammation, disease progression, as well as the kinetic of return to the homeostatic status.

This thesis focuses particularly on the roles of IgE and IgG and their receptors in allergic shock (anaphylaxis). The following section will thus be focus on these two antibody classes, and dedicated to depicting how IgE and IgG antibodies are generated, their different subclasses (in the case of IgG), and Fc receptors. Major differences between mouse and human IgE, IgG and Fc receptors will be discussed, which will be informative on how one can use mouse models to understand human IgE- and IgG-mediated anaphylaxis.

### 1.3.1 IgE antibodies and their receptors

Among the five isotypes of antibody described in humans, IgE antibodies are found at the lowest concentration in the circulation (50–200 ng/ml IgE in healthy individuals *vs.* 1~10 mg/ml for other immunoglobulin isotypes) [109]. It is important to note that IgE levels can increase dramatically in individuals with allergic diseases [22, 110]. Shortest of all immunoglobulin isotypes, the serum IgE half-life ranges from ~5-12 hours in mice and ~2 days in humans [109, 111]. IgE exists in two forms: a membrane form expressed by B cells that have undergone class switch to IgE and serves as a B cell receptor (BCR) involved in antigen uptake and presentation; and a secreted form produced by plasma B cells, and which can exert its biological functions by binding two main receptors: FcεRI and FcεRII (CD23).

IgE antibodies are composed of two identical heavy chains (each comprising a variable VH domain and four constant Cε domains) and two identical light chains (composed

of a variable VL domain and a constant CL domain) with a total molecular weight of 190 kDa [112, 113]. Similar to other antibody classes, the Fab region (fragment-antigen binding) of IgE is responsible for antigen recognition and binding, while the effector function of IgE is determined by the carboxy-terminal Fc portion (fragment crystallizable region) [112, 113]. IgE possess four domains in the heavy chain with Cε2 being a major stability enhancer of the interaction between IgE and its high-affinity receptor FcεRI [114]. The FcεRI binding site is located in the Cε3 domain and in the Cε2-Cε3 linker region [115] and the binding site to the low-affinity IgE receptor CD23 is also primarily located within the Cε3 domain, with contributions from the Cε4 domain. Several disulfide bridges control the structure and activity of IgE, which is also regulated by glycosylation at different sites. In particular, disruption of the glycosylation site found in the Cε3 domain at asparagine 394 (N394) in human, and N384 in mouse, abrogates the binding of IgE to FcεRI, highlighting the importance of glycosylation modifications in IgE biology [116].

FcεRI is the high-affinity receptor for IgE (Kd of ~$10^{-9}$ to $10^{-10}$ M). It is constitutively expressed at high levels on both human and rodent mast cells and basophils as a tetramer formed of one α subunit, one β subunit, and a dimer of disulfide-linked γ subunits [117] (**Figure 4A**). The α subunit (FcεRIα) belongs to the immunoglobulin superfamily with an extracellular portion containing the IgE binding sites, a transmembrane domain and a short cytoplasmic domain which is thought to have no signaling function [118]. FcεRIβ has a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM), which acts as a signal amplifier (please refer to *Section 1.2.2.2* for more detailed ITAM signaling). The FcεRIγ homodimer also contains two ITAM domains, which are responsible for signal transduction [119, 120]. In humans, but not mice, FcεRI is also constitutively expressed as a αγ2 at the surface of dendritic cells [121], monocytes [119, 122], neutrophils [123], eosinophils [119, 124], and platelets [125]. FcεRI plays a key role in mediating the biological functions of IgE, whereas the biological functions of the αγ2 trimer of FcεRI are less well understood. It has been suggested that the αγ2 FcεRI trimer expressed on human dendritic cells (DCs) and monocytes contributes to serum IgE clearance [126], and also (in the case of DCs) to allergen uptake and presentation to naive T cells [121].

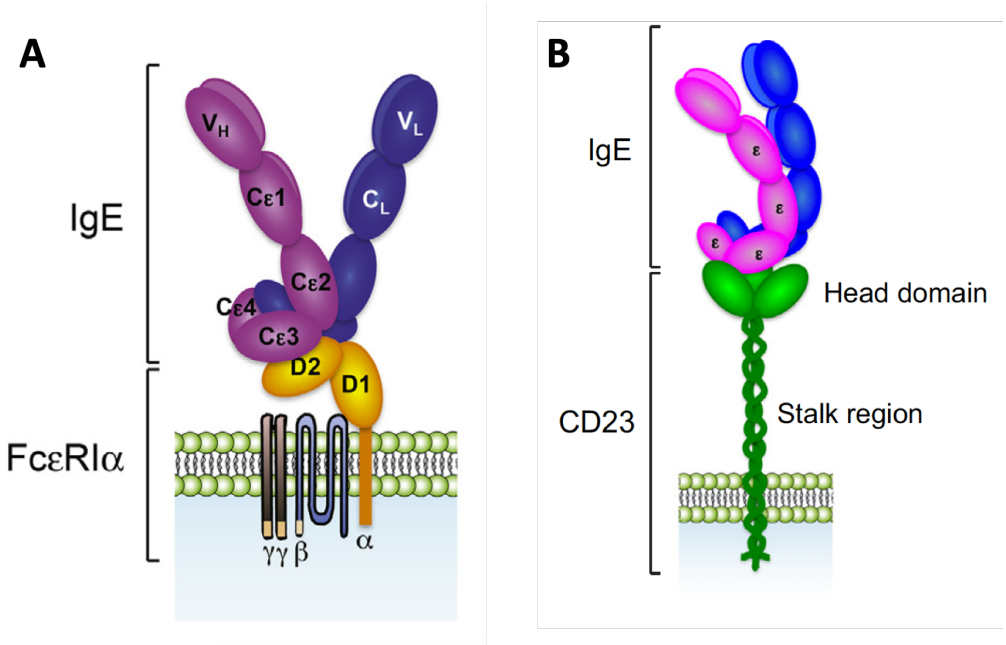



**Figure 4. Structure of FcεRI and CD23 and its interaction with IgE. A**. FcεRI is expressed on mast cells and basophils as a tetramer formed with one α subunit, one β subunit and a dimer of disulfide-linked γ subunits. IgE binds the receptor via surface loops in Cε3, with contributions from the Cε2–Cε3 linker region. The two Cε3 domains of IgE bind distinct sites on FcεRIα, one site found in the D2 domain, and a second site formed by a cluster of four surface-exposed tryptophan residues in the D1-D2 interface. **B**. CD23 self-associates as a trimer, and is composed of an IgE-binding 'head domain' (which belongs to the C-type lectin superfamily) linked to the membrane by an extracellular coiled-coil stalk region, and a small cytoplasmic N-terminal domain. The IgE binding site of CD23 is located in the C-terminal head domain, with some additional contributions from the stalk region (not shown). Two CD23 molecules bind to each IgE heavy chain, primarily to the Cε3 domains but with a contribution from Cε4. Adapted from [127]

CD23, also known as FcεRII, is the low-affinity receptor for IgE (Kd = $10^{-5}$ M) [128]. CD23 self-associates as a trimer and is composed of an IgE-binding 'head domain' (which belongs to the C-type lectin superfamily) linked to the membrane by an extracellular coiled-coil stalk region and a small cytoplasmic N-terminal domain (**Figure 4B**). CD23 exists in a membrane form (mCD23), as well as in soluble forms of various sizes (sCD23) which are released by proteolytic cleavage at several sites in the stalk region [129]. CD23 is expressed on the surface of B cells, neutrophils, eosinophils, follicular DCs and intestinal epithelial cells and it is thought to contribute as a regulator of IgE production. Several publications show increased levels of IgE in mice deficient for CD23 [130-132], whereas transgenic mice overexpressing CD23 have markedly reduced levels of circulating IgE after immunization [133]. The mechanism by which CD23 regulates serum IgE levels still remains unclear. In B cells, CD23 has also been implicated in IgE-dependent antigen uptake and presentation to T cells; and in intestinal epithelial cells (IEC), CD23 seems to be important for transepithelial transport of IgE and IgE/antigen complexes into the intestinal lumen, which could play an

important role in food allergy, since it could explain how IgE and allergens are delivered to mast cells located in the lamina propria [112, 134].

Production of antigen-specific IgE requires that such antigen is uptake and processed by antigen presenting cells (DCs, B cells or other APC) and, in the presence of the cytokines IL-4 and IL-13, presented to cognate naïve T cells that will then acquire the so-called T helper 2 cell (Th2) phenotype. Th2 cells can induce B cells to undergo class-switch recombination (CSR), a genomic modification that replaces the expressed constant region (for example cμ or cγ) of the heavy chain with a downstream region cε, thereby determining B cell production of IgE [22].

Several observations have helped to increase our understanding of the physiological role IgE. IgE and its receptors are believed to have evolved as defense mechanisms against infections, notably against parasitic infections; host response to intestinal helminthic infection is typically characterized by Th2 immunity and levels of antigen-specific and non-specific IgE correlate with host resistance. The actual contribution of non-specific *vs.* specific IgE antibodies in parasite clearance and host defense is still not fully understood, and has been recently reviewed [25]. Furthermore, evidence using mouse models also demonstrated the potential contribution of IgE in host defense against venoms; in one example, bee venom was able to induce robust venom-specific Th2 cells and IgE; this acquired immune response was associated with increased resistance of mice against subsequent challenge with a high dose of the same venom [135].

Despite their importance in host defense against helminths and venoms, IgE antibodies are mostly studied in the context of allergic reactions, which not surprisingly, was how they were first discovered and described. In allergic individuals, mast cells and basophils have specific-IgE bound to FcεRI on their surface. Upon exposure to the allergen, binding of the allergen to IgE on the surface of mast cells and basophils induces FcεRI crosslink and activation, leading to the rapid release of pre-formed granule-stored mediators such as histamine (please refer to *Section 1.1.2 Mast cells* for more details). These mediators can act locally or systemically leading to the clinical features of immediate hypersensitivities, such as bronchoconstriction, urticaria, and diarrhea.

### 1.3.2 IgG antibodies and IgG-receptors

#### 1.3.2.1 IgG Subclasses

Immunoglobulin G (IgG) consists of two identical heavy chains (50kDa each) and two identical light chains (25kDa each) linked by an inter-chain disulfide bridge. Heavy chains and light chains assembled to form the functional domains: two Fab regions and one Fc portion. The light chain associates to the variable region and CH1 domain of the heavy chain to form the Fab arm responsible for antigen recognition and binding. IgG can have either activating or inhibitory effector functions, and such functions relies on binding of the CH2-CH3 Fc portion to Fcγ receptor (FcγRs) expressed on the surface of various effector cells (please refer to *Section 1.3.2.2 IgG antibody Fc receptors* for more details) or binding to complement (reviewed in [136] (please refer to *Section 1.2 The complement system and innate immunity* for more details).

A highly conserved N-linked glycosylation site at influences the quaternary structure of the Fc portion, and the interactions of the glycan with the protein backbone stabilize the Fc portion [137]. The N-glycan contains one core structure, which is invariable and contains four N-acetylglucosamine (GlcNAc) and three mannose (Man) moieties. Additional monosaccharides can appear, such as fucose, galactose and N-acetylneuraminic acid, leading to multiple compositions of the N-glycan moiety attached to IgG-Fc portion [136]. Oligosaccharides moieties determine the 'open' conformation of IgG Cγ2 domain and the conformation of the Cγ2 domain is directly linked to the capacity of an IgG to interact with FcγRs [138]. This was confirmed by the progressive removal of the sugar residues leading to an approach in the Cγ2 domain ('closed conformation') [138]. In addition, the glycan has been suggested to impact antibody conformation via specific glycan-protein and glycan-glycan interaction [136]. The close proximity of glycan and FcγR might directly contribute to a glycan-protein interaction [139]; or Fc-glycans might also interact with glycan conserved in FcγRIIIA and FcγRIIIB, for example [140].

Mice and humans share the remarkable feature of possessing four IgG subclasses [141-143]. Even if this encourages the belief of shared functions between IgG subclasses of these two species, this feature might be more a coincidence than a fact [144]. The different IgG subclasses have distinctive patterns of production during an immune response, and different affinities for the various FcγRs and for the complement component C1q. These

subclasses can thus trigger different effector functions based on their complement and receptor binding specificities. The proportion of different IgG subclasses vary as a result of the cytokine environment, and the response development depends on the migration and interactions of various cell populations within the organized lymphoid tissue.

Mouse IgG subclasses are IgG1, IgG2a (or IgG2c depending on the mouse strain), IgG2b and IgG3. The total IgG concentration in serum is around 6 mg/mL. Among the four IgGs, IgG3 is believed to elicit the first response to an antigen, even in the absence of T-cell helper cells; IgG3 is able to bind complement with high affinity [145], with still debatable ability to bind the high-affinity FcγRI [146, 147]. IgG2a and IgG2b subclasses have similar functions *in vivo*, being considered part of the T-cell dependent response, but were also described to be generated in the absence of any T-dependent stimulus [148]. Interestingly, IgG2a and IgG2b bind to all FcγRs. This very strong FcγR-mediated activity allows these two IgGs subclasses to drive pathogen clearance. IgG1 has distinct properties from the other murine subclasses, not being able to activate complement and engaging only FcγRIIB and FcγRIII [11] (please refer to *Section 1.1.2.3 Mouse FcγR expression* for more details).

As mentioned above, humans also possess four distinct IgG subclasses. Total IgG levels in human serum is around 10 mg/mL. The most abundant subclass is IgG1, followed by IgG2, IgG3, and IgG4 is the least abundant subclass. Although sharing 90% homology, each subclass has a unique profile with respect to complement activation, activation of effector cells, immune complexes formation, half-life, and placental transport. Classically, IgG1 and IgG3 responses are elicited against soluble proteins and membrane proteins and are very effective against viral infections; IgG2 are against encapsulated bacteria, more precisely to T-independent polysaccharide antigens. Repeated antigen exposure, as in allergic desensitization, elicits IgG4 antibodies. In the case of the long-term exposure in a non-infectious setting, IgG4 may become more predominant than other IgG subclasses, as in the case of beekeepers and allergic individuals that underwent immunotherapy [149] (the relatively terminal position of the Cγ4 cassette may be the explanation for this). In addition to isotypic variation, the polymorphic epitopes of immunoglobulins can differ between individuals and ethnic groups, accounting for allelic variations found among IgG subclasses [150]. All the human IgGs are able to cross the placental barrier through their ability to bind to the neonatal Fc receptor FcRn, and all IgG subclasses bind to complement to different extents, except IgG4.

It is important to notice that immune responses are not only polyclonal but also involves several immunoglobulin isotypes. No antigen contact generates only one type of antibody response, but some isotype can be favored depending on the antigen concentration, natural or artificial adjuvants and route of immunization.

#### 1.3.2.2 IgG antibody Fc receptors[1]

The Fc receptors of mice and human are expressed in myeloid cells, NK cells, as well as B cells, and platelets (in human only), and can interact differently with antibodies of each different IgG subclass (reviewed [11]). In mice, cells express three classical activating FcγRs: mFcγRI, mFcγRIII, mFcγRIV, together with the inhibitory mFcγRIIB; as well as the recycling receptor mFcRn. On the other hand, humans express six classical receptors for IgGs: the activating receptors hFcγRI (CD64), hFcγRIIA (CD32A), hFcγRIIC (CD32C), hFcγRIIIA (CD16A) and FcγRIIIB (CD16B), the inhibitory receptor hFcγRIIB (CD32B), and the recycling receptor hFcRn. In the intracellular compartment, both in humans and mice also express the tripartite motif-containing protein 21 (TRIM21), a cytosolic protein able to bind IgG with nanomolar affinity, and which play a critical role in viral degradation through the proteasome [151]. Additionally, humans express Fc-receptor like proteins, FcRL4 and FcRL5, on B cells, and these receptors are associated with inhibition of B cell activation via recruitment of SHP-1 (SH2-domain-containing inositol polyphosphate 5-phosphatase) [152].

FcγRs belong to the immunoglobulin receptor superfamily and their extracellular domain consists of two or three IgG-like domains, structure highly conserved in mice and humans [153]. All FcγRs, except hFcγRIIIB, endow one α chain responsible for the antibody binding, a transmembrane region and a cytoplasmic tail attached to an accessory γ subunit [11]. FcγRIIIB is a glycosyl-phosphatylinisol (GPI)-linked receptor lacking cytoplasmic tail whose signaling is less elucidated, although known to be able to induce effector functions via integrins [154].

Functionally, there are two classes of FcγRs: the activatory FcγRs and the inhibitory receptor FcγRIIB. The key to generating an effective immune response is the balanced expression of activating and inhibitory molecules within the same cell. Activating receptors require an immunoreceptor tyrosine-based activation motif (ITAM) for their signaling and

[1] Note for easy prose, this part will refer mostly to mouse proteins as *m* and human proteins as *h* (e.g. the distinction between mFcγRI and hFcγRI).

consequent effector functions. For hFcγRI, hFcγRIIIA and all murine activating receptors, ITAM is present in the non-covalently associated FcR γ-chain, whereas in hFcγRIIA and hFcγRIIC this motif is present in its own cytoplasmic domain and does not associate to the γ-chain [155]. Receptor clustering is responsible for the initial signal, which leads to the phosphorylation of tyrosines residues in the ITAM-motif by associated Src family kinases (Lyn and/or Fyn). The stable recruitment of Syk tyrosine-kinase is responsible for downstream signaling, such as calcium mobilization and Protein kinase C activation that drives Fc-dependent cell activation [156]. The inhibitor receptor FcγRIIB, both in mice and human, depends on an immunoreceptor tyrosine-based inhibitor motif (ITIM) for signaling. In this case, Scr kinases recruit SHP1 or SHP2, counterbalancing the activating pathway, upon co-engagement with an activating receptor. It is important to notice that in certain cases of low-affinity interactions of FcγRIIA and FcγRIIIA, ITAMs signal emanating from these receptors can be solely inhibitory, a novel mechanism referred as inhibitory ITAM signaling (ITAMi) [157].

Structurally, the neonatal IgG receptor (FcRn) is similar to MHC class I molecules, co-expressed with β2-microglobulin and the α chain [158]. FcRn is responsible for the long-half-life of IgG *in vivo* and the efficient transport of IgG from mother to young across the placenta. Besides recycling IgG, FcRn has also been found to bind and recycle albumin [159]. FcRn protects IgG from degradation by binding its ligand in the acidic environment if endocytic vacuoles, where the pH is ~6.5. This binding triggers FcRn translocation of IgG back to the cell surface, where IgG is released at a neutral pH [159].

Additionally, FcγRs are characterized as high-affinity (grossly corresponding to a $K_A$ higher than $10^7$ $M^{-1}$) and low-affinity (generally corresponding to a $K_A$ lower than $10^7$ $M^{-1}$) [160]. High-affinity receptors are defined by their property to bind and retain free/monomeric immunoglobulins, and it is thus expected that the binding sites of those receptors are constitutively saturated by IgG's *in vivo*. Both mFcγRI and mFcγRIV in mice and hFcγRI are considered as high-affinity receptors. On the other hand, low-affinity FcγRs are only able to bind multimeric immunoglobulins (present in immune complexes, aggregated or opsonized).

1.3.2.3 Mouse FcγRs expression

Most mFcγRs do not appear to be specific for one class or subclass of immunoglobulins nor in their pattern of expression on the cell surface. mIgG1 is able to bind only to the activating mFcγRIII, as well to the inhibitory mFcγRIIB; mIgG2a and mIgG2b bind to all the activating and the inhibitory receptors; IgG3 may not bind any mFcγRs, even if it was reported to interact with a very low-affinity with mFcγRI [147] – data that has not been reproduced and reported since (**Figure 5**).

mFcγRI is expressed on the surface of dendritic cells (DCs), Ly6C$^{hi/lo}$ monocytes, bone marrow monocytes and tissue macrophages (spleen, kidney and alveolar) [161, 162]; the inhibitory mFcγIIB is expressed on all cells of the myeloid compartment, and is highly expressed on B cells (and the sole FcγR expressed in these cells); mFcγRIII is highly expressed by all myeloid cells and in a lower extent on NK cells; finally mFcγRIV is expressed on Ly6C$^{lo}$ monocytes, macrophages and neutrophils, being absent on other cells [163]. Interestingly, it is important to keep in mind that mFcγRIIB, mFcγRIII and FcγRIV are also able to bind, with lower affinity, mouse IgE [163, 164].

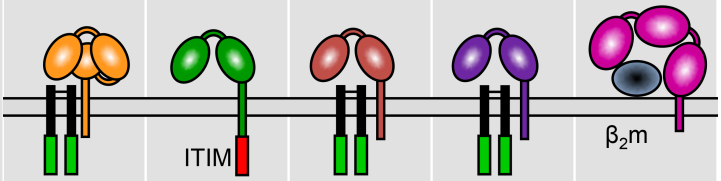
| Name | FcγRI | FcγRIIB | FcγRIII | FcγRIV | FcRn |
|---|---|---|---|---|---|
| ***Gene*** | *Fcgr1* | *Fcgr2b* | *Fcgr3* | *Fcgr4* | *Fcgrt* |
| |  | | | | |
| **IgM** | - | - | - | - | - |
| **IgG1** | - | 3x10$^6$ | 3x10$^5$ | - | **8x10$^6$** |
| **IgG2a** | **3x10$^7$** | 4x10$^5$ | 7x10$^5$ | **3x10$^7$** | **+** |
| **IgG2b** | 1x10$^5$ | 2x10$^6$ | 6x10$^5$ | **2x10$^7$** | **+** |
| **IgG3** | **+*** | - | - | - | **+** |
| **IgE** | - | 2x10$^4$ | 2x10$^4$ | 3x10$^5$ | - |
| **IgA** | - | - | - | - | - |
| **Major role** | Activation | Inhibition | Activation: ITAMi-inhibition | Activation | IgG recycling; transport |

**Figure 5. Mouse Fcγ Receptors and mouse FcRn**. Schematic representation of mouse FcγRs in respect to the cell membrane (grey bar). ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibition motif. Binding affinities for the various immunoglobulins subclasses are given as $K_A$ ($M^{-1}$); high-affinity interactions with monomeric Ig are indicated in bold; + binding but no affinity values available; - no binding; *under debate [146-147]. Adapted from [11]

### 1.3.2.4 Human FcγRs expression

For humans, the binding of human IgG to hFcγRs is much less straightforward than what is described in mice. Globally, IgG1 and IgG3 can bind to all hFcγRs; IgG2 binds hFcγRIIA and hFcγRIIIA. Finally, IgG4 is able to bind hFcγRI, hFcγRIIA, hFcγRIIB, hFcγRIIC and hFcγRIIIA. Along with different patterns of expression on immune cell populations, the multiplicity of human FcγRs is increased by a series of genetic polymorphisms. Distinct genes encode six human receptors for IgG, three of which have two or three polymorphic variants (reviewed in [165]). A polymorphism resulting in the presence of histidine or arginine at the position 131 in the second Ig-domain of hFcγRIIA is equally distributed in the overall population. The $H_{131}$ allele has moderate affinity for IgG2, whereas the $R_{131}$ has very low affinity for the same immunoglobulin. Two alleles of the gene-encoding hFcγRIIIA generate two variants differing at the position 158 ($V_{158}$ and $F_{158}$). hFcγRIIIB possesses also two alleles that generate 2 variants differing at 4 positions (NA1 and NA2), a

point mutation $A_{78}D$ of the NA2 allele generates another hFcγRIIIB variant named SH [160]. Polymorphisms can also be observed in hFcγRIIB and hFcγRIIC but will not be described herein.

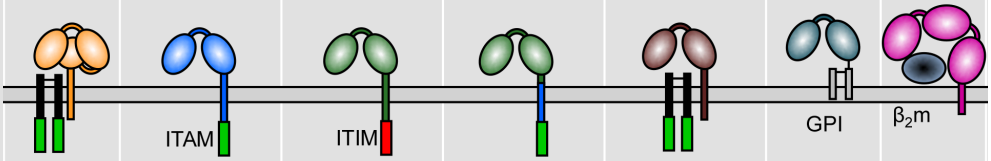
| Name | FcγRI | FcγRIIA | | FcγRIIB | | FcγRIIC | | FcγRIIIA | | FcγRIIIB | FcRn |
|---|---|---|---|---|---|---|---|---|---|---|---|
| CD | CD64 | CD32A | | CD32B | | CD32C | | CD16A | | CD16B | - |
| *Gene* | *FCGR1A* | *FCGR2A* | | *FCGR2B* | | *FCGR2C* | | *FCGR3A* | | *FCGR3B* | *FCGRT* |
| |  | | | | | | | | | | |
| Alleles | / | $H_{131}$ | $R_{131}$ | $I_{232}$ | $T_{232}$ | $Q_{13}$ | $stop_{13}$ | $V_{158}$ | $F_{158}$ | NA1, NA2, SH | / |
| IgM | - | - | - | - | - | - | - | - | - | - | - |
| IgG1 | **$6x10^7$** | $5x10^6$ | $3x10^6$ | $1x10^5$ | ND | $1x10^5$ | - | $2x10^5$ | $1x10^5$ | $2x10^5$ | **$8x10^7$** |
| IgG2 | - | $4x10^5$ | $1x10^5$ | $2x10^4$ | ND | $2x10^4$ | - | $7x10^4$ | $3x10^4$ | - | **$5x10^7$** |
| IgG3 | **$6x10^7$** | $9x10^5$ | $9x10^5$ | $2x10^5$ | ND | $2x10^5$ | - | **$1x10^7$** | $8x10^6$ | $1x10^6$ | **$3x10^7$** |
| IgG4 | **$3x10^7$** | $2x10^5$ | $2x10^5$ | $2x10^5$ | ND | $2x10^5$ | - | $2x10^5$ | $2x10^5$ | - | **$2x10^7$** |
| IgE | - | - | | - | | - | | - | | - | - |
| IgA | - | - | | - | | - | | - | | - | - |
| Major role | Activation | Activation; ITAMi-inhibition | | Inhibition | | Activation | | Activation; ITAMi-inhibition | | Decoy; Activation | IgG recycling; transport. Ag pres. |

**Figure 6. Human Fcγ Receptors and human FcRn.** Schematic representation of human FcγRs in respect to the cell membrane (grey bar). ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibition motif. Alleles are identified by the amino acid variant in the protein (e.g. $H_{131}$), or by the name of the allelic variant (NA1, NA2, SH). Binding affinities for the various immunoglobulins subclasses are given as $K_A$ ($M^{-1}$); high-affinity interactions with monomeric Ig are indicated in bold; - no binding; (adapted from [11])

hFcγRI is restricted to DCs and monocytes/macrophages, and can be induced on neutrophils and mast cells [13] under inflammatory conditions. hFcγRIIA is expressed on all myeloid cells, but not on lymphocytes. It's the only FcγR expressed on the surface of platelets (exclusively in humans). hFcγRIIB is the sole receptor present on B cells and is also expressed on basophils, tissue macrophages, and DCs; it is also expressed at low levels on circulating monocytes and circulating neutrophils [166]. More recently, hFcγRIIB was identified on mast cells of the gastrointestinal tract [15]. hFcγRIIC is present on the surface of NK cells, monocytes, and neutrophils in around 20% of individuals that carry the polymorphism Q13 or ORF; in the remaining population, a SNP on the position 13 generates

a stop codon (stop13) in which *FCGR2C* represents a pseudogene [167]. hFcγRIIIA is expressed by NK cells and monocyte/macrophages; and FcγRIIIB is densely expressed by neutrophils, and can also be found at a lower extent on basophils from rare patients [168]. Finally, FcRn is expressed by epithelial, endothelial cells, monocyte/macrophages, dendritic cells, neutrophils [169] and syncytiotrophoblast (responsible for IgG transport across the placenta) [158].

## 1.4 Anaphylaxis: from local to systemic reactions

This thesis work focuses on the role of IgG and IgE antibodies in the context of systemic allergic shock, also known as anaphylaxis.

### 1.4.1 Definition and Epidemiology

Anaphylaxis is the most extreme manifestation of an allergic reaction and is characterized by an acute, systemic and potentially fatal response upon contact with an allergen. Few minutes after allergen exposure, a multisystemic process is triggered affecting mostly the skin, pulmonary tract, cardiovascular and gastrointestinal systems [170] (**Box 1**). Fatalities can occur via respiratory tract obstruction and/or rapid onset of hypotension, together with cardiac failure [1]. Even if most manifestations are comprised of only an acute episode, some patients might experience a biphasic allergic reaction, with anaphylaxis symptoms appearing hours after the early phase of the reaction [171].

Current treatments, for allergic anaphylaxis, rely primarily on the allergen avoidance (if the allergen is known) or the use of medication to reverse the physiological effects caused by the release of mediators. The first line of treatment is epinephrine (adrenaline) administration. This drug acts on α1-adrenergic receptors to induce vasoconstriction, and on β1 and β2-adrenergic receptors to induce, respectively, positive inotropic and chronotropic effects in the heart and bronchodilatation. When the patient is no longer at risk of death, as a second line of treatment, anti-histamines (receptor H1 and H2 antagonist) could be indicated to reduce cutaneous and upper respiratory manifestations. Glucocorticoids can also be included to prevent the biphasic episodes, although they have no effect on the initial symptoms [172].

The clinical diagnosis of anaphylaxis is primarily based on the levels of circulating histamine and mast cell tryptase. Other tests are performed later to detect any biological signs of immunologic-dependent activation, such as: skin prick test with the suspected allergen and measurement of allergen specific-IgE in the serum. In some cases, a basophil degranulation test called BAT (for 'Basophil Activation Assay') can be performed *ex vivo*, by incubating whole blood, PBMCs or purified basophils with the allergen, and assessing potential basophil degranulation by flow cytometry [55] (please refer to *Section 1.1.2 Basophils* for more details).

Relying only on the parameters aforementioned to confirm the clinical anaphylaxis can be insufficient. Mainly because symptoms may occur in the absence of IgE-immune activation. In a particular case of anaphylactic reaction due to anesthesia, for example, the proportion of anaphylaxis occurring in the absence of detectable allergen-specific IgE levels is 40% to 50% [173]. Thus, a lot of effort has been put to elucidate and emphasize the potential contribution of other immunological pathways in the induction of anaphylaxis. This has led to a new definition of anaphylaxis by the World Allergy Organization (WAO), with the term 'allergic anaphylaxis' defined as reactions mediated by IgE, IgG or immune complexes, and 'non-allergic anaphylaxis' for all other non-immunologic causes [1]. This thesis will focus on antibody-mediated anaphylaxis only.

**Box 1. Clinical Criteria for Diagnosis of Anaphylaxis**

Anaphylaxis is highly likely if any one of the following three conditions is satisfied.

1. Acute onset of illness with:
   Mucocutaneous involvement (pruritus, flushing, urticaria, angioedema) and one of the following:
   A. Respiratory complications (wheezing, stridor, hypoxemia/cyanosis)
   B. Hypotension or end-organ damage (encephalopathy, kidney injury, etc.)
2. Two or more of the following occurring rapidly after exposure to a known or likely allergen:
   - Mucocutaneous involvement (pruritus, flushing, urticaria, angioedema)
   - Respiratory complications (wheezing, stridor, hypoxemia/cyanosis)
   - Hypotension or evidence of end-organ hypoperfusion (encephalopathy, kidney injury, etc.)
   - Persistent gastrointestinal symptoms (pain, nausea, vomiting)
3. Reduced Blood Pressure soon after exposure to a known allergen (minutes to hours).

(adapted from [174])

Accurate characterization of anaphylaxis epidemiology can be complicated, due to unreported (symptoms which do not lead to hospitalization) and under-diagnosed cases (variability in symptoms, signs and time course). However, a systematic review of epidemiological studies demonstrated that the incidence for all-cause anaphylaxis range from 1.5 to 7.9 per 100,000 persons in Europe per year [175]; moreover, anaphylaxis probably affects 0.3% of the population at some point during their lifetime [175].

### 1.4.2 Drivers of anaphylaxis

The causative agents of anaphylaxis are mostly introduced via injection or ingestion, and less commonly via the airways. The etiology of the disease varies with age and geography. The most common causative agent in children is food and in adults, drugs. Foods that usually trigger anaphylaxis are milk, egg, wheat, soy, fish, shellfish and nuts. Medications associated with anaphylaxis include beta-lactams, cephalosporin, quinolones and sulphonamides [174]. Anesthetic drugs such as curare have also been implicated in perioperative anaphylaxis and monoclonal antibodies have been described as important triggers of anaphylaxis (e.g.: targeting CD20, HER-2 or IgE) [176]. *Hymenoptera* venom appears as a third important trigger of anaphylactic reactions [174].

Even if the trigger may vary, the concomitant factors and co-factors that can amplify anaphylaxis are similar worldwide [172]. These include presence of certain diseases (asthma, mastocytosis and cardiovascular diseases), along with alcohol, emotional stress, premenstrual status and the co-administration of non-steroidal anti-inflammatory drugs [177].

### 1.4.3 Discovery of anaphylaxis

Paul Portier and Charles Richet reported for the first time, in 1902, the phenomenon of anaphylaxis, starting the field of allergy. They described the clinical shock syndrome developed in dogs after a second encounter to a first innocuous dose of toxins from a jellyfish-like extract. The two physiologists named this new phenomenon anaphylaxis, to express the antithesis to the already known term prophylaxis (protection) [178]. Another interesting observation was that a ‘toxin substance’, present in the serum of dogs undergoing anaphylaxis, was also able to induce the same symptoms in dogs that had never encountered the toxin; describing then, the first demonstration of passive anaphylaxis. Close to a decade later, Charles Richet was awarded the Nobel Prize in Medicine or Physiology for this discovery [178].

In the same period, Maurice Arthus described local anaphylaxis (Arthus reaction), characterized by local hemorrhagic and necrotic responses to repeated injections of protein antigens. Finally, Clemens von Pirquet and Bela Schick described unusual local and systemic reactions in patients receiving antitoxin horse serum for both diphtheria and tetanus.

Those were the first evidence that the immune system, supposed to protect the host against pathogens and toxins, could also mediate extreme adverse reactions. This concept

was, at the time, not well accepted by many scientists. In this regard, Von Pirquet wrote '*The conception that antibodies, which should protect against the disease, are also responsible for the disease, sounds at first absurd*' [179]. Such reactions were finally given the name "allergy", from the Greek words *allos* and *ergon*, which means "altered reaction".

### 1.4.4 Models to study anaphylaxis *in vivo*

Since anaphylaxis is a complex and systemic reaction, it cannot, with current technology, be reproduced *in vitro*. Therefore, several animal models – mainly mouse models – have been developed to study the mechanisms of this reaction. Including both "Passive Systemic Anaphylaxis (PSA)" and "Active Systemic Anaphylaxis (ASA)" models, all derived from the initial two models used by Richet and Portier (detailed in *1.4.3*).

ASA can be induced by means of active immunization (or sensitization) with an antigen (or allergen), usually together with an adjuvant. This immunization induces a specific and polyclonal antibody response. A few weeks later, administration of the same antigen is able to induce the anaphylactic reaction.

In PSA models, mice are sensitized passively by transfer of antigen-specific antibodies, followed by challenge with the antigen (usually 16-24h later) to trigger anaphylaxis. Those antigen-specific antibodies can be in monomeric form, immune complexed or even in the aggregated format. In the case of monomeric antibodies, a challenge (exposure to the allergen) phase must take place to trigger the reaction.

### 1.4.5 The paradigm of anaphylaxis: IgE *vs* IgG

The elusive 'reagenic' antibodies responsible for human atopic allergies were described 60 years after Portier and Richet (please refer to *Section 1.4.3 Discovery of anaphylaxis* for more details), in 1966. The Ishizakas' group, in Japan, described a new immunoglobulin, different from the known immunoglobulin classes, which they called γE antibody [180]. Also, the group of Johansson and Bennich in Sweden isolated another new immunoglobulin class, which they called IgND [181]. It was soon realized that γE and IgND belong to the same and unique antibody class, receiving the official name IgE in 1968. IgE was then described as the key to hypersensitivity reactions, notably anaphylaxis. Progressively, anaphylaxis was described as dependent on IgE antibodies and FcεRI expressed on the surface of mast cells and basophils (classical pathway). Yet, mouse models

allowed the identification of alternative pathways of anaphylaxis relying on IgG antibodies and FcγR expressed on myeloid cells, particularly neutrophils and monocytes/macrophages.

This next section will be dedicated to explore the state-of-art of both the classical and alternative pathways of anaphylaxis.

### 1.4.6 The classical pathway of IgE-dependent mast cell and basophil activation: Evidence from humans to mouse models

Anaphylaxis is classically described to rely on monomeric IgE antibodies bound to the FcεRI expressed on mast cells and basophils. In humans, but not in mice, other myeloid cells and platelets can also express FcεRI. In allergic individuals, sensitization elicits the production of allergen-specific IgE. Upon allergen re-exposure, the recognition of a bivalent or multivalent antigen by FcεRI-bound IgE induces cross-linking of FcεRI, which triggers cell activation and an immediate and sustained release of diverse vasoactive mediators, including histamine and tryptase. This cross-link also induces the synthesis of many inflammatory mediators, as mentioned previously (please refer to *Section 1.1.1 and 1.1.2* for more details).

IgE-mediated hypersensitivity could be passively transferred in humans, in which purified IgE antibodies were able to transfer skin sensitivity from a sensitized human subject to non-sensitized individuals upon subsequent injection of the relevant allergen [180, 181]. The key role of IgE in anaphylaxis was also demonstrated in mouse models. Passive transfer of antigen-specific IgE into wild-type mice prior challenge with the cognate antigen can recapitulate the systemic signs of shock: hypotension, loss of mobility and severe hypothermia [182]. Also, passive skin sensitization with IgE and subsequent challenge with the relevant antigen induces immediate local oedema and skin inflammation at the site of IgE sensitization [183].

Highlighting the importance of IgE and mast cell-mediated reactions, IgE-mediated passive anaphylaxis was abrogated in mice deficient for the high-affinity IgE receptor FcεRI [183, 184], as well as in several strains of mast cell deficient mice [44, 183, 185-188]. Therefore, IgE-dependent mast cell activation can indeed produce many of the pathological changes that are associated with anaphylaxis.

#### 1.4.6.1 Role of mast cells, basophils and their mediators

There is also additional compelling evidence that mast cells and mast cell granule contents play a key role during acute anaphylaxis. The release of granule contents, such as histamine and tryptase, is used as part of the diagnosis of anaphylaxis. Pharmacological inhibition of histamine receptors (mainly H1R) abrogates anaphylaxis in mouse models, whereas the exogenous intravenous administration of histamine can induce anaphylactic shock-like syndrome [189]. In humans, the intravenous administration of histamine can reproduce many symptoms of anaphylaxis. After the intravenous exposure, volunteers developed cutaneous, pulmonary and cardiac manifestations, the clinical signs of anaphylactic reaction [190]. Nevertheless, it is important to mention that histamine is not a mast-cell specific product because it can also be released by other myeloid cells, including basophils and neutrophils [171].

Tryptase, on the other hand, is much more stable and is considered a product derived from mast-cells [191]. Low levels of tryptase are continuously released in the serum and account for baseline tryptase levels, while the transient release of tryptase upon mast cell activation accounts for the rise of serum tryptase levels. Indeed, elevated levels of the mast cell-specific protease tryptase have been detected during anaphylactic reactions in humans [192-195]. Additional evidence for the role of mast cells comes from observations of increased incidence of anaphylaxis in patients with mastocytosis, a disease characterized by clonal expansion and accumulation of mast cells in several organs [196].

The role of basophils in the pathogenesis of anaphylaxis is much less well elucidated. Recently, Korosec and colleagues demonstrated a marked reduction in the absolute number of basophils in the circulation during an anaphylactic reaction to *Hymenoptera venom* in humans [197]. These results suggest a potential activation of basophils during an anaphylactic reaction in humans, however, they do not demonstrate a significant contribution of basophils in the pathophysiology of human anaphylaxis. The role of murine basophils in IgE-mediated anaphylaxis is also still a matter of debate. In some reports, mouse basophils were ablated using a depleting antibody and this depletion did not show any significant effect on IgE passive cutaneous anaphylaxis or IgE-mediated systemic anaphylaxis [40, 198]. On the other hand, as indirect evidence of basophils importance in *vivo,* passive cutaneous anaphylaxis was shown to be reduced in mice deficient for the mast cell protease-11 (that is preferentially expressed by basophils rather than mast cells), as compared to wild-type mice. This study demonstrates a reduction of swelling, vascular permeability, and leukocyte infiltration in the

local IgE-mediated reaction, suggesting a potential role of basophils in the onset of allergic inflammation [199]. Thus, the role of basophils in anaphylaxis is unsettled both in humans and mice. In humans, it is difficult to assess the contribution of basophils alone (independently of mast cell activation), mainly because both cells most likely have concomitant activation in the context of anaphylaxis. It is also difficult to distinguish the contribution of basophil mediator release in anaphylaxis, as tryptase quantities in basophils account for less than 1% of those in tissue mast cells [200].

#### 1.4.6.2 Role of Interleukin 4 and IL-4Rα in IgE-mediated anaphylaxis

IL-4 play a key role in allergic sensitization and IgE production. The potential implication of IL-4 in the effector phase of IgE-mediated anaphylaxis has also been explored using mouse models of IgE-mediated reactions. IL-4 has been shown to increase sensitivity to vasoactive mediators during IgE-mediated anaphylaxis, largely through exacerbation of vascular leak [201]. Indeed, a fluid leak has been shown to be a side effect of IL-4 therapy in human cancer patients [202].

More recently, Yamani and colleagues showed evidence that vascular endothelial IL-4Rα is necessary for the development of IgE-dependent anaphylaxis and this signaling regulates the severity of anaphylactic reactions [203]. Moreover, IL-4 seems also responsible for increased susceptibility to food-induced anaphylaxis by inducing mast cell expansion in the intestine of mice in an active peanut anaphylaxis model that entirely depends on mast cells and IgE [204]. Taking advantage of animals expressing Il4*rαF*709 (an activating variant of the IL-4 receptor α-chain), it was demonstrated that IL-4 produced by type 2 innate lymphoid cells (ILC2) is able to break T cell tolerance and promote food allergy [205].

#### 1.4.6.3 Clinical demonstration of the role of IgE in anaphylaxis using the anti-IgE monoclonal antibody Omalizumab.

Finally, one of the most important arguments sustaining the implication of IgE in allergy and anaphylaxis comes from examples of patients treated with the anti-IgE (Omalizumab) monoclonal antibody. Omalizumab is able to recognize the Cε3 region and impair IgE to bind to FcεRI and CD23. In some studies, the treatment with Omalizumab was efficient in reducing recurrent anaphylaxis and skin symptoms in patients with systemic

mastocytosis [206, 207]. Several reports also demonstrate that the use of Omalizumab as an additional treatment during food or venom immunotherapy can decrease the severity of the allergic reactions, including anaphylaxis [208-210].

Altogether, the results of clinical observations and experiments performed using mouse models of anaphylaxis demonstrate the role of IgE and mast cells in mediating systemic anaphylaxis. However, IgE levels alone cannot explain completely a subject's susceptibility to developing anaphylaxis. First, allergen-specific IgE can be detected in the plasma of many individuals who do not develop anaphylaxis when exposed to that allergen [211]. Secondly, some patients may experience near-fatal anaphylaxis without detectable IgE-specific antibodies or any sign of IgE-dependent reaction [212]. Hence, this suggests that some other mechanism may play along with IgE in the orchestra of systemic anaphylaxis development.

### 1.4.7 The alternative IgG-mediated pathway of anaphylaxis: Evidence from mouse models to humans

As described above, IgE and mast cells can play a key role in the development of anaphylaxis in both humans and mice. However, some experimental evidence also demonstrates the potential role of IgG antibodies in anaphylaxis. In this case, IgG recognized the antigen, binds to the activator FcγR expressed on the surface of myeloid cells, leading to the release of mediators, such as histamine and platelet-activating factor (PAF). The release of the mediators causes then physiological manifestations similar to those observed in the IgE-mediated anaphylaxis (described previously). Three of the four mouse IgG subclasses in the mouse, IgG1, IgG2a, and IgG2b, have been reported to enable the induction of systemic anaphylaxis [42, 43, 46].

#### 1.4.7.1 IgG-dependent anaphylaxis in mouse models

IgG-induced passive systemic anaphylaxis (PSA) can be elicited in three different ways in mice: *a)* systemic injection of antigen-specific IgG followed by intravenous challenge with the corresponding antigen; *b)* injection of pre-formed IgG-immune complexes, consisting of antibody bound to a soluble antigen; or *c)* injection of *in vitro* aggregated IgGs (to mimic immune complexes). Passive sensitization with mIgG1 specific antibodies conferred mice the ability to develop systemic anaphylaxis upon allergen exposure [44, 213].

By using either knock-out mice for the different low-affinity FcγRs or by selectively blocking these receptors with blocking antibodies, different studies have highlighted the importance of the low-affinity IgG receptors, with a notable role for mFcγRIII [46, 182]. Moreover, in mIgG1 and IgG2b-induced PSA, mFcγRIIB seems to be important in the control of the anaphylaxis, since in FcγRIIB knock-out mice showed an enhanced reaction. In the same recent study developed by our group, IgG-induced PSA depends on neutrophils, monocytes/macrophages, and/or basophils, depending on the IgG subclass [46].

Evidence for IgE-independent, IgG-dependent anaphylaxis was provided by studies in which mice were actively immunized (either in the presence or absence of adjuvants) and then challenged with the same model antigen. In these active systemic anaphylaxes (ASA) models, mice develop a polyclonal IgE and IgG response before the challenge. However, ASA can still develop in the absence of IgE [214], FcεRI [44] and even mast cells or basophils [40, 44]. In one study using active immunization process, anaphylaxis was suppressed if mice were treated with an antibody able to block FcγRII/III activation, suggesting a role for these receptors in the development of anaphylaxis [43]. Regarding the cells involved in ASA, some studies have identified an important role for monocytes/macrophages in inducing severe shock [182], whereas our lab has identified that neutrophils are necessary and sufficient for the induction of ASA [42].

#### 1.4.7.2 Platelet-activating factor

Platelet-activating factor (PAF) is a potent phospholipid-derived biological mediator and is able to act through the PAF receptor. PAF has been shown as the dominant mediator responsible for mouse models for IgG-dependent anaphylaxis [42, 182] and is positively correlated with the reaction severity[215]. PAF has also been proposed as a mediator in human anaphylaxis [92]. In fact, the serum PAF levels directly correlate while PAF acetylhydrolase activity (the enzyme that breaks down PAF) was inversed correlated with anaphylaxis severity [216]. Human neutrophils, monocytes/macrophages are able to secrete PAF in response to FcγR crosslink, however basophils, mast cells and platelets have also been described to produce and respond to PAF following antibody-dependent activation [92]. Hence, it can be argued that most of the data available nowadays bring some insights into the possible contribution of PAF to human anaphylaxis, but not direct evidence that PAF is sufficient to induce anaphylaxis in human subjects.

#### 1.4.7.3 Evidence of human IgG-mediated anaphylaxis

Even with several pieces of evidence in mice and indirect data of mediator release during anaphylaxis, it remains under debate whether IgG can contribute to anaphylaxis in humans. Importantly, in the mouse models of anaphylaxis described above, the challenge involves high amounts of allergen administrated by the intravenous route to trigger the reaction. Results of studies that compared the doses of antigen required to induce IgG-mediated anaphylaxis in mice suggest that the high doses are necessary to trigger the reaction [217]. Accordingly, the most likely case in which IgG-dependent anaphylaxis may be evoked in humans is via intravenous exposure to injectable drugs. Indeed, our group had recently reported the detection of antigen-specific IgG in the serum of patients with suspected anaphylaxis to a neuromuscular blocking agent (NMBA) during general anesthesia [218]. In this study, neutrophils were activated early after anaphylaxis onset, and plasma purified anti-NMBA IgG antibodies could trigger antigen-specific neutrophil activation *ex vivo*. Interestingly, antigen-specific IgE was undetectable in 72% of patients with anaphylaxis [218].

Additional evidence in favor of IgG-mediated anaphylaxis comes from several clinical observations in which patients develop anaphylaxis after exposure to a biological agent and had specific-IgG but no detectable specific-IgE antibody, including to monoclonal therapeutic antibodies (which will be discussed in more details in the next paragraph) [176, 219], aprotinin [220], dextran [221], transfusion of von Willebrand factor [222] or even serum transfer (reviewed in [223]). Anaphylaxis was observed following treatment with intravenous immunoglobulin therapy (IVIG), possibly due to the generation of IgG against IgA in patients with common variable immunodeficiency (CVID) [224]. Finally, in the presence of immune complexes, the gain-of-function on the receptor FcγRIIA was also associated with increased risk of anaphylaxis in CVID patients [225].

Monoclonal antibodies (mAbs) are now established as targeted therapies for neoplastic, inflammatory, transplant and auto-immune diseases. The first use of this drug class dates back to the 1970s, but the use of mAbs became widespread in the past decade [176]. Along with the increase of use, there was a rise in reported drug hypersensitivity reactions, limiting the prescription of the mAb or even preventing the use as first-line therapies. Subjects treated with a variety of chimeric mouse-human, humanized or fully human mAb may experience acute reactions following mAb infusion, ranging from local

inflammation at the injection site to systemic anaphylaxis [226]. The clinical manifestation can range from local skin reactions, nausea, chills, and fever to acute anaphylaxis, which could be life-threatening in rare cases [226]. The first exposure to mAb can lead to severe symptoms, as it can be observed with rituximab (anti-CD20) [227] and omalizumab (anti-IgE) [228], or after multiple exposures. As most of these therapeutic antibodies are human or humanized IgG mAbs injected at high dose, anaphylaxis to such mAb might be due, at least in some cases, to passive IgG (mAb)-mediated anaphylaxis in humans. However, this still needs to be demonstrated.

Acute reactions subsequent to the infusion of mAbs correlates with clinical risk factors, such as a history of atopy [229]; as an example, omalizumab is not recommended for patients with a history of anaphylaxis [230]. However, the mechanism by which mAb induce infusion reactions is in most cases poorly characterized [231]. The immunologic mechanism associated with mAb administration can be broadly classified as IgE-mediated and non-IgE mediated reactions. Several studies demonstrated the presence of anti-mAb IgE in the serum and positive skin test after the re-exposure to the mAb [232, 233], suggesting that anti-mAb IgE could be responsible for mAb-induced adverse reactions and anaphylaxis. Another study showed that the majority of patients experiencing severe reactions to cetuximab (epidermal growth factor receptor – EGRF) had detectable levels of IgE against the galactose-α-1,3-galactose oligosaccharide, a sugar moiety present in the Fab portion of the mAb [234]. Conversely, there have been conflicting reports about the nature of the hypersensitivity reaction to mAb infusion, and whether IgE is truly implicated. The presence of anti-drug specific immunoglobulin requires previous exposure to the drug, which is not the case when the reaction is observed during the first infusion [226], as is the case for omalizumab [230].

Omalizumab (Xolair®) is a recombinant humanized IgG1 mAb directed against IgE. It is administered subcutaneously and it is used in the treatment of steroid-resistant asthma and chronic idiopathic urticaria [228]. Anaphylaxis to omalizumab considered a rare event and is estimated to occur in 0.1 to 0.2% of patients [235]. Regardless of the cause, a history of anaphylaxis was recently shown to increase the risk of developing anaphylaxis upon omalizumab administration. In this same study with 30 patients, 70% of the anaphylaxis events occurred within 1 hour of omalizumab administration and only one occurred after 2 hours [230]. Delayed reactions (> 24h of the injection) have also been reported in a patient with asthma [236]. The mechanism of anaphylaxis in patients receiving omalizumab remains still unknown. No IgE- or IgG-specific against omalizumab was detected and none of the patients had a positive skin reaction to omalizumab or the excipient in a study including 21

patients [230]. In other studies, serum tryptase was normal in all patients with anaphylaxis to omalizumab [235]. Moreover, the sole report attributing anaphylaxis to the excipient polysorbate of omalizumab included two cases only [237].

Hence, anaphylaxis induced by monoclonal antibodies can be mediated by mAb-IgE, but the mechanism by which mAb induce first-infusion or later reactions still remains largely unknown and might represent pure cases of IgG (mAb)-mediated passive anaphylaxis in human.

### 1.4.8 The complement pathway: another potential trigger of anaphylaxis

Activation of the complement cascade occurs in response to many stimuli and leads to the generation of potent inflammatory mediators known as anaphylatoxins. Several lines of evidence suggest that these small molecules (named C3a and C5a) might be involved in the development of anaphylaxis, both in mice and humans.

In mice, passive cutaneous anaphylaxis (PCA) studies have demonstrated that the injection of exogenous C3a and C5a stimulates mast cell degranulation (and consequent skin oedema) through the activation of C3a and C5a receptors, respectively [238]; this activation is also important to enhance IgE-mediated PCA [238]. Furthermore, a role of complement in human anaphylaxis have been suggested by a correlation between the increased levels of anaphylatoxins and severity of anaphylaxis [192]. In this study, however, the most important parameter correlated with severity of anaphylaxis were increased levels of tryptase and histamine [192].

In the case of peanut anaphylaxis, it has been suggested that peanut could directly trigger activation of the complement through both classical and lectin pathways in mice [239, 240]. C3a produced upon peanut injection could induce anaphylaxis through activation of macrophages and basophils, and in a lesser extent mast cells; this reaction was mostly PAF-dependent. In this study, mice were pre-treated with a long-lasting formulation of IL-4 and β-adrenergic antagonist, a cocktail known to increase responsiveness of the mice to PAF, and thereby to enhance susceptibility to anaphylaxis [239]. Interestingly, in a passive model of IgE-mediated anaphylaxis, the administration of peanut extract had an additive effect in the development of hypothermia, suggesting a likely synergistic role of complement and antibody-mediated activation in triggering systemic shock. These results suggest that activation of the complement system by peanut, along with IgE-dependent mast cell activation, might be one explanation for the severity of peanut-induced anaphylaxis in certain subjects [223].

In humans, there is also clinical evidence of anaphylaxis induced by agents that can directly activate complement. Liposomal drug infusions and monoclonal antibodies are listed among the biologicals capable of activating complement in humans [241]. For example, the side-effects of Rituximab have been associated with the activation of classical complement pathway in a few relapsed non-Hodgkin's lymphoma patients [242]. One should keep in mind, however, that the aforementioned studies have limitations, including limited sample number or the lack of specific inhibitors of complement activation or anaphylatoxin receptors

during an anaphylactic shock, and that we still have no direct evidence that complement could be the sole actor in the development of human anaphylaxis. It is possible that the complement system plays a role in amplifying anaphylaxis in synergy with other major pathways, such as engagement of antibody Fc receptors.

### 1.4.9 Humanized models: on our way to understand human anaphylaxis

Several humanized mouse models have been developed to better understand the role of human IgE and IgG, Fc receptors and cells implicated in human anaphylaxis. It is well established that both IgE and IgG-dependent anaphylaxis can occur in mice. It is, however, important to understand how these findings can translate to the human system. To address this question, several studies have employed transgenic mice expressing human proteins or immunodeficient mice engrafted with human hematopoietic stem cells.

First attempts came with transgenic mice expressing human FcεRI subunits instead of the mouse protein (FcεRI$^{Tg}$ mice). The expression of the FcεRI in transgenic mice seems to recapitulate the overall expression observed in humans [119, 243, 244]. FcεRI$^{Tg}$ mice are able to develop passive cutaneous anaphylaxis when sensitized with human IgE and challenged with the cognate antigen [244]. Systemic anaphylaxis was also induced in response to intravenous sensitization with human IgE followed by systemic antigen challenge [243]. Moreover, FcεRI$^{Tg}$ mice can also exhibit cutaneous anaphylaxis when they are sensitized intradermally with serum from patients with cat or peanut allergy and then intravenously challenged with the respective allergen [245, 246].

Transgenic mice expressing hFcγRIIA (on a background deficient for endogenous mouse FcγRs) are able to develop anaphylaxis when injected intravenously with human IgGs followed by challenge with respective antigen [70]. FcγRIIA$^{Tg}$ expression was sufficient to trigger IgG-mediated PSA and blockade of this receptor *in vivo* abolished anaphylaxis. PSA mediated by hFcγRIIA was dependent on the presence of neutrophils and monocytes/macrophages [70]. On the other hand, hFcγRIIA expressed by mast cells were responsible for passive cutaneous anaphylaxis [70]. Moreover, the transfer of platelets expressing the FcγRIIA$^{Tg}$ into FcγR$^{null}$ mice (resistant to anaphylaxis) was sufficient to restore sensitivity to anaphylaxis after passive transfer of IgG immune complexes [97]. These findings in FcγR transgenic mice support the importance of the human receptor FcγRIIA, expressed by monocytes/macrophages, neutrophils and platelets, in mediating anaphylaxis *in*

*vivo*. Importantly, transgenic mice expressing all the human FcγRs ($hFc\gamma RI^{Tg}IIA^{Tg}IIB^{Tg}IIIA^{Tg}IIIB^{Tg}$ mice) in a $Fc\gamma R^{null}$ background was susceptible to anaphylaxis induced by human IgG complexes [247].

Recently, our lab has generated in collaboration with Regeneron Pharmaceuticals a novel mouse model in which the human low-affinity IgG receptor locus has been knock-in into the equivalent mouse locus. Mice were knock-in with both activating (FcγRIIA, IIIA, and IIIB) and inhibitory (FcγRIIB) human receptors [71]. These mice recapitulate the low-affinity receptor expression found in human subjects and are susceptible to PSA induced by IgG immune complexes. IgG-mediated anaphylaxis was predominantly dependent on FcγRIIA and neutrophils, with a minor contribution of basophils. Finally, both histamine and PAF partially contributed to the reaction [71]. More recently, a full version of the knock-in mice (called $hFc\gamma R^{KI}$ mice) was developed by our group. Along with the low-affinity FcγRs, mice now express also the high-affinity FcγRI [97]. Corroborating with the previously mentioned results, $hFc\gamma R^{KI}$ mice develop PSA induced by IgG immune complexes. This reaction still critically dependent on hFcγRIIA, as revealed in experiments using an anti-FcγRIIA blocking antibody [97].

Several groups also generated humanized models of anaphylaxis using different mouse strains on a highly immunodeficient background engrafted with human stem cells [248-250].

Bryce and colleagues used NOD-SCID γ (NSG) mice expressing human stem cell factor, IL-3 and GM-CSF transgenes (NSG-SGM3 mice) engrafted with human fetal thymus, liver and hematopoietic stem cells (termed the BLT model). They observed engraftment of a large number of human mast cells in the peripheral lymphoid tissues, mucosal tissues, and peritoneal cavity. The NSG-SGM3-BLT mice developed an antigen-specific IgE-mediated passive cutaneous anaphylaxis response upon IgE sensitization followed intradermal or intravenous challenge [248]. The authors speculate that this reaction is fully mast-cell dependent due to the presence of high-number of engrafted mast cells, but other cells were not evaluated.

The other two humanized mouse models of anaphylaxis used peanut as an allergen. Burton *et al.* engrafted hematopoietic stem cells in NSG mice carrying a stem cell factor transgene, and observed robust engraftment with human cells, including mast cells in the intestinal mucosa [249]. Mice were fed by gavage with peanut and mounted a specific IgE-antibody response. Following peanut challenge, mice developed an IgE-mast cell-dependent

systemic anaphylaxis with increased plasma tryptase levels [249]. They concluded that markedly elevated tryptase levels is likely to be from a human mast cell-mediated reaction. Finally, Pagovich *et al*. also used NSG mice in which the immune system was reconstituted using mononuclear cells from patients allergic to peanut. These mice produced peanut-specific IgE and IgG in response to sensitization with peanut, and anaphylaxis was observed after challenge with peanut by gavage [250]. In both models, anaphylaxis could be blocked in mice treated with the anti-human antibody Omalizumab [249, 250].

Altogether, these results in humanized models of anaphylaxis suggest that both human IgE and IgG have the potential to induce anaphylaxis throught activation of their human Fc receptors.

## 2. Summary and objectives

This thesis work aims at better understanding the pathways of systemic anaphylaxis, and in particular the role of IgG antibodies. Our main goal was to answer the following questions:

- What are the respective contributions of IgE *vs.* IgG in mouse models of active systemic anaphylaxis (ASA)?
- Through which pathways do IgE and IgG antibodies mediate their functions in ASA?
- Could human IgG also contribute to anaphylaxis? Through which mechanism(s)?

To do so, we divided our work in two main parts:

First, we compared the implication of the classical (IgE-mediated) and alternative (IgG-mediated) pathways in a novel adjuvant-free mouse model of active systemic anaphylaxis to the allergen ovalbumin (OVA). Using this model, we found that both the classical and alternative pathways are involved in full development of anaphylaxis, with a major contribution from the alternative pathway. IgE antibodies induced anaphylaxis through FcεRI-dependent activation of mast cells and subsequent release of histamine. IgG antibodies mediated their effects through FcγRIII and activation of monocytes/macrophages, leading to the release of platelet-activating factor (PAF).

Secondly, to assess the clinical relevance of our findings obtained with mouse antibodies, we studied whether human IgG can also contribute to anaphylaxis. Because of the life-threatening nature of anaphylaxis, it is difficult to perform clinical investigations of human anaphylaxis. We therefore decided to study the role of human IgG in anaphylaxis using a unique humanized mouse model (named hFcγR$^{KI}$ mice) developed in our laboratory, and which expresses all human FcγRs in place of the mouse FcγRs, and therefore can respond to all human IgG subclasses (IgG1-4). Using this mouse model, we investigated the potential contribution of human IgG in two well defined cases of anaphylaxis: (1) anaphylaxis-induced by the monoclonal therapeutic anti-IgE antibody Omalizumab, and (2) peanut-induced anaphylaxis.

(1) Omalizumab-induced anaphylaxis:

Omalizumab is a humanized anti-IgE IgG1 antibody approved for the treatment of severe asthma and chronic spontaneous urticaria. However, use of Omalizumab is associated with reported side effects, ranging from local skin inflammation at the injection site to systemic anaphylaxis. To date, the mechanisms through which Omalizumab induces adverse reactions are still unknown. We hypothesized that the side effects of Omalizumab could be due, at least in part, to activation of Fcγ Receptors (FcγR) by Omalizumab/IgE immune complexes (ICs). To address this, we found that Omalizumab forms ICs with IgE that can engage IgG Fcγ receptors (FcγRs) and activate neutrophils *ex vivo*. We further discovered that such ICs induce both skin inflammation and systemic anaphylaxis when injected into FcγR-humanized mice (expressing hFcγRI, IIA, IIB, IIIA & IIIB in place of all mouse FcγRs). We then developed an Fc-engineered mutant version of Omalizumab that cannot bind FcγRs, and demonstrate that this antibody is equally potent as Omalizumab at blocking IgE-mediated allergic reactions, but does not induce FcγR-dependent adverse reactions. In conclusion, we envision that IC-mediated engagement of FcγRs could be a more general mechanism of therapeutic monoclonal antibodies-mediated adverse reactions.

(2) Peanut-induced anaphylaxis:

Peanut (PN) allergy is a particularly severe type of allergy since it tends to persist throughout life, and is more likely to induce severe anaphylaxis. In collaboration with clinicians and researchers from Stanford University, we showed that plasma from PN allergic patients contains high levels of PN-specific IgG from all subclasses, as compared to plasma from healthy donors; and levels of PN-specific IgG correlated with levels of PN-specific IgE in these allergic subjects. We then developed a humanized model of PN anaphylaxis, in which $hFc\gamma R^{KI}$ mice were treated with a long-lasting formulation of IL-4 (a protocol known to increase susceptibility to anaphylaxis [251]), before adoptive transfer of IgG purified from PN allergic subjects, followed by challenge with PN extract. Our preliminary data indicates evidence of anaphylaxis in $hFc\gamma R^{KI}$ mice sensitized with IgG from allergic subjects. Surprisingly, we found that $Fc\gamma R^{null}$ mice (which do not express any FcγR) sensitized with IgG from PN allergic patients develop strong anaphylaxis with high mortality rate in this model. Our ongoing work now addresses the mechanisms of these reactions, in particular the implication of the complement pathway, and the potential role of the inhibitory human IgG receptor FcγRIIB.

# 3. Chapter 3 - Pathways of immediate hypothermia and leukocyte infiltration in an adjuvant-free mouse model of anaphylaxis

This first part aimed at exploring the *in vivo* contributions of various antibody receptors, effector cells and mediators in a new adjuvant-free mouse model of active systemic anaphylaxis (ASA). Anaphylaxis is generally thought to rely on allergen-specific IgE, mast cells (and perhaps blood basophils) and the release of histamine. However, the understanding of key pathways that can induce anaphylaxis in humans remains largely limited. Therefore, many mouse models of anaphylaxis have been developed to assess the roles of effector cells and mediators. Notably, most of these models employ non-physiological adjuvants during the sensitization phase. Surprisingly, anaphylaxis in many of these mouse models can be induced with little or no contribution of mast cells and IgE.

We hypothesized that the use of adjuvants might have masked the contribution of mast cells and IgE by enhancing IgG-mediated "alternative" pathways. We thus decided to develop an adjuvant-free mouse model of anaphylaxis, in which we evaluated both immediate hypothermia and late-phase inflammation. We then studied the contribution of various antibody receptors, effector cells and mediators in this model of anaphylaxis. To do so, we used both genetically engineered mice lacking various cells or receptors, as well as pharmacological approaches.

ASA was induced by 6 consecutives intraperitoneal (i.p.) sensitizations with ovalbumin (OVA) once a week, and an i.p. challenge with a high dose of OVA two weeks later. We found that, in this adjuvant-free model, the manifestations of anaphylaxis and late-phase reaction were partially but significantly reduced in mice lacking the high affinity IgE receptor FcεRI or deficient for mast cells, confirming the key roles of mast cells and IgE in these settings. However, both immediate anaphylaxis and late-phase peritoneal inflammation were reduced to a higher extent in mice deficient for the IgG receptor FcγRIII or after depletion of monocytes/macrophages (using pretreatment with clodronate liposomes), indicating that additional mechanisms of anaphylaxis contributed to a significant extent to the pathology in this model. Conversely, neutrophils and basophils had no significant role in this model. Finally, by using a pharmacologically approach, we demonstrated that immediate anaphylaxis and the late-phase inflammation in this model were dependent on two key mediators: histamine and platelet activating factor (PAF), with mast cells likely representing the main source of histamine and monocytes/macrophages likely representing the main source of PAF.

## 3.1 PAPER I

### Pathways of immediate hypothermia and leukocyte infiltration in an adjuvant-free mouse model of anaphylaxis

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# Pathways of immediate hypothermia and leukocyte infiltration in an adjuvant-free mouse model of anaphylaxis

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**Background: Conflicting results have been obtained regarding the roles of Fc receptors and effector cells in models of active systemic anaphylaxis (ASA). In part, this might reflect the choice of adjuvant used during sensitization because various adjuvants might differentially influence the production of particular antibody isotypes.**

**Objective: We developed an "adjuvant-free" mouse model of ASA and assessed the contributions of components of the "classical" and "alternative" pathways in this model.**

**Methods: Mice were sensitized intraperitoneally with ovalbumin at weekly intervals for 6 weeks and challenged intraperitoneally with ovalbumin 2 weeks later.**

**Results: Wild-type animals had immediate hypothermia and late-phase intraperitoneal inflammation in this model. These features were reduced in mice lacking the IgE receptor FcεRI, the IgG receptor FcγRIII or the common γ-chain FcRγ. FcγRIV blockade resulted in a partial reduction of inflammation without any effect on hypothermia. Depletion of monocytes/macrophages with clodronate liposomes significantly reduced the hypothermia response. By contrast, depletion of neutrophils or basophils had no significant effects in this ASA model. Both the hypothermia and inflammation were dependent on platelet-activating factor and histamine and were reduced in 2 types of mast cell (MC)–deficient mice. Finally, engraftment of MC-deficient mice with bone marrow–derived cultured MCs significantly exacerbated the hypothermia response and restored inflammation to levels similar to those observed in wild-type mice.**

**Conclusion: Components of the classical and alternative pathways contribute to anaphylaxis in this adjuvant-free model, with key roles for MCs and monocytes/macrophages. (J Allergy Clin Immunol 2017;139:584-96.)**

***Key words:*** *Rodents, mouse model, mast cells/basophils, monocytes/macrophages, neutrophils, antibodies, Fc receptors, allergy, inflammation, anaphylaxis*

Anaphylaxis is an acute, life-threatening systemic allergic reaction with a lifetime prevalence of 0.05% to 2.0% in developed countries.[1-3] In human subjects it is largely accepted that anaphylaxis can be triggered by histamine and other mediators released in response to antigen cross-linking of IgE bound to its high-affinity receptor, FcεRI, on mast cells (MCs).[1-3] However, IgG might also contribute to anaphylaxis in some patients, especially those treated with infused drugs, such as therapeutic mAbs.[4,5]

Several mouse models of anaphylaxis have been developed and used to assess the contributions of IgE and IgG antibodies and the

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*Abbreviations used*
ASA: Active systemic anaphylaxis
BMCMC: Bone marrow–derived cultured mast cell
Cpa3: Carboxypeptidase A3
DT: Diphtheria toxin
DTR: Diphtheria toxin receptor
MC: Mast cell
Mcpt: Mast cell protease
OVA: Ovalbumin
PAF: Platelet-activating factor
PAF-AH: Platelet-activating factor acetylhydrolase
PCMC: Peritoneal cell–derived mast cell
PI: Propidium iodide
PSA: Passive systemic anaphylaxis
WT: Wild-type

roles of various effector cells and mediators. The analysis of passive local anaphylaxis or passive systemic anaphylaxis (PSA) models has allowed identification of 2 major pathways of anaphylaxis in mice: a "classical" pathway consisting of IgE, FcεRI, MCs, and histamine[6-8] and an "alternative" pathway consisting of IgG, FcγRIII and/or FcγRIV,[5,9-11] platelet-activating factor (PAF),[12,13] and, depending on the exact model used, macrophages,[5,14] basophils,[5,13] and/or neutrophils.[5,11,15]

Active systemic anaphylaxis (ASA) models, which are arguably more reflective of the clinical situation, have generated more conflicting results. Some ASA models critically depend on IgE, FcεRI, and MCs,[16-19] whereas some others can develop, at least with respect to the features analyzed, in $IgE^{-/-}$, $Fc\varepsilon RI^{-/-}$, and/or MC-deficient mice.[9,13,20] Depending on the mouse strain and ASA model used, basophils have been shown to either contribute to the systemic response[13,19] or play little to no significant role.[17,21] Similarly, depletion of monocytes/macrophages or antibody-mediated neutrophil depletion reduces anaphylaxis in some but not all ASA models.[11,17,19,21]

We hypothesize that such conflicting results in ASA models might reflect, at least in part, the choice of adjuvant used during sensitization because various adjuvants might differentially influence the production of individual antibody isotypes. Moreover, in human subjects with anaphylactic reactivity, sensitization to antigen generally occurs in the absence of an artificial adjuvant. Therefore we developed an "adjuvant-free" mouse model of ASA and assessed the contributions of components of the classical and alternative pathways of anaphylaxis in this model.

## METHODS

### Mice

C57BL/6J (wild-type [WT]) mice were purchased from Jackson Laboratories (Bar Harbor, Me) or Charles River (Lyon, France). $Fc\gamma RIII^{-/-}$ mice (B6.129P2-*Fcgr3*$^{tm1Sjv}$/J mice backcrossed to C57BL/6 for 12 generations) were purchased from Jackson Laboratories. $Fc R\gamma^{-/-}$ mice (B6.129P2 *Fcer1g*$^{tm1Rav}$ mice backcrossed to C57BL/6 for 12 generations) were from Taconic (New York, NY). C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice were originally provided by Peter Besmer (Memorial Sloan-Kettering Cancer Center, New York, NY); we then backcrossed these mice to C57BL/6J mice for more than 11 generations.[22] $Fc\varepsilon RI^{-/-}$ mice[7] (FcεRI α chain–deficient mice backcrossed to C57BL/6 for more than 8 generations and kindly provided by Jean-Pierre Kinet, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Mass), mast cell protease $(Mcpt8)^{DTR}$ mice (backcrossed to C57BL/6 for at least 10 generations),[23] and carboxypeptidase A3 *(Cpa3)-Cre; Mcl-1*$^{fl/fl}$ mice (backcrossed to C57BL/6 for at least 9 generations)[24] were bred and maintained at the Stanford University Research Animal Facility. Age-matched male mice were used in all experiments. Except as otherwise noted, we used littermate controls in all experiments. All animal care and experimentation was conducted in compliance with the guidelines of the National Institutes of Health and specific approval of the Institutional Animal Care and Use Committee of Stanford University and the Animal Ethics Committee CETEA (Institut Pasteur, Paris, France) registered under #C2EA-89.

### Ovalbumin-induced adjuvant-free model of active anaphylaxis

Six- to 8-week-old mice were sensitized intraperitoneally with 10 μg of endotoxin-free ovalbumin (OVA; Endograde OVA; BioVendor, Asheville, NC; <0.01 EU endotoxin per injection) in 100 μL of PBS once a week for 6 weeks. Two weeks after the last intraperitoneal sensitization, mice were challenged intraperitoneally with 500 μg of OVA. Rectal measurements of body temperature were performed immediately before (time 0) and at different time points for up to 2 hours after challenge. Mice were killed at various time points after challenge (as indicated) for assessment of inflammatory cell numbers in the peritoneal cavity and histology.

### Other methods

See the Methods section in this article's Online Repository at www.jacionline.org for the methods for flow cytometry; peritoneal lavage; differential cell counts; depletion of basophils, monocytes/macrophages, and neutrophils; blockade of FcγRIV; histologic analysis; measurement of serum OVA-specific $IgG_1$ and $IgG_{2c}$ antibodies; IgE-mediated PSA; ASA with adjuvant; treatment with an $H_1$-antihistamine or a PAF receptor antagonist; quantification of histamine and platelet-activating factor acetylhydrolase (PAF-AH); and generation and adoptive transfer of bone marrow–derived cultured mast cells (BMCMCs).

### Statistical analyses

Results represent means ± SEMs or means + SEMs, with values for individual mice represented for quantifications of histamine, PAF-AH, and leukocytes. We used an unpaired Student *t* test (body temperature) or an unpaired Mann-Whitney *U* test (all other data) to assess the significance of differences between 2 sets of data. *P* values of less than .05 are considered statistically significant.

## RESULTS

### Development of an adjuvant-free model of ASA in C57BL/6 mice

We developed an adjuvant-free mouse model of ASA consisting of intraperitoneal sensitization with endotoxin-free OVA once a week for 6 weeks and intraperitoneal challenge with OVA 2 weeks after the last sensitization (Fig 1, *A*). OVA-sensitized C57BL/6 mice had hypothermia after OVA challenge that was maximal at 30 minutes and decreased thereafter (Fig 1, *B*). Sensitized and challenged mice also had a late-phase inflammatory response with increased numbers of total cells, eosinophils, macrophages, and lymphocytes in the peritoneal cavity, with the highest numbers of leukocytes on day 3 after challenge (Fig 1, *C-H*). Consistent with the increase in eosinophil numbers, we detected significant amounts of IL-5 in the plasma of 3 of 5 OVA-sensitized mice 18 (but not 72) hours after challenge, whereas levels of IL-4, IL-6, IL-10, TNF-α, and IFN-γ were all less than the detection limit of standard ELISAs at both time points (data not shown).

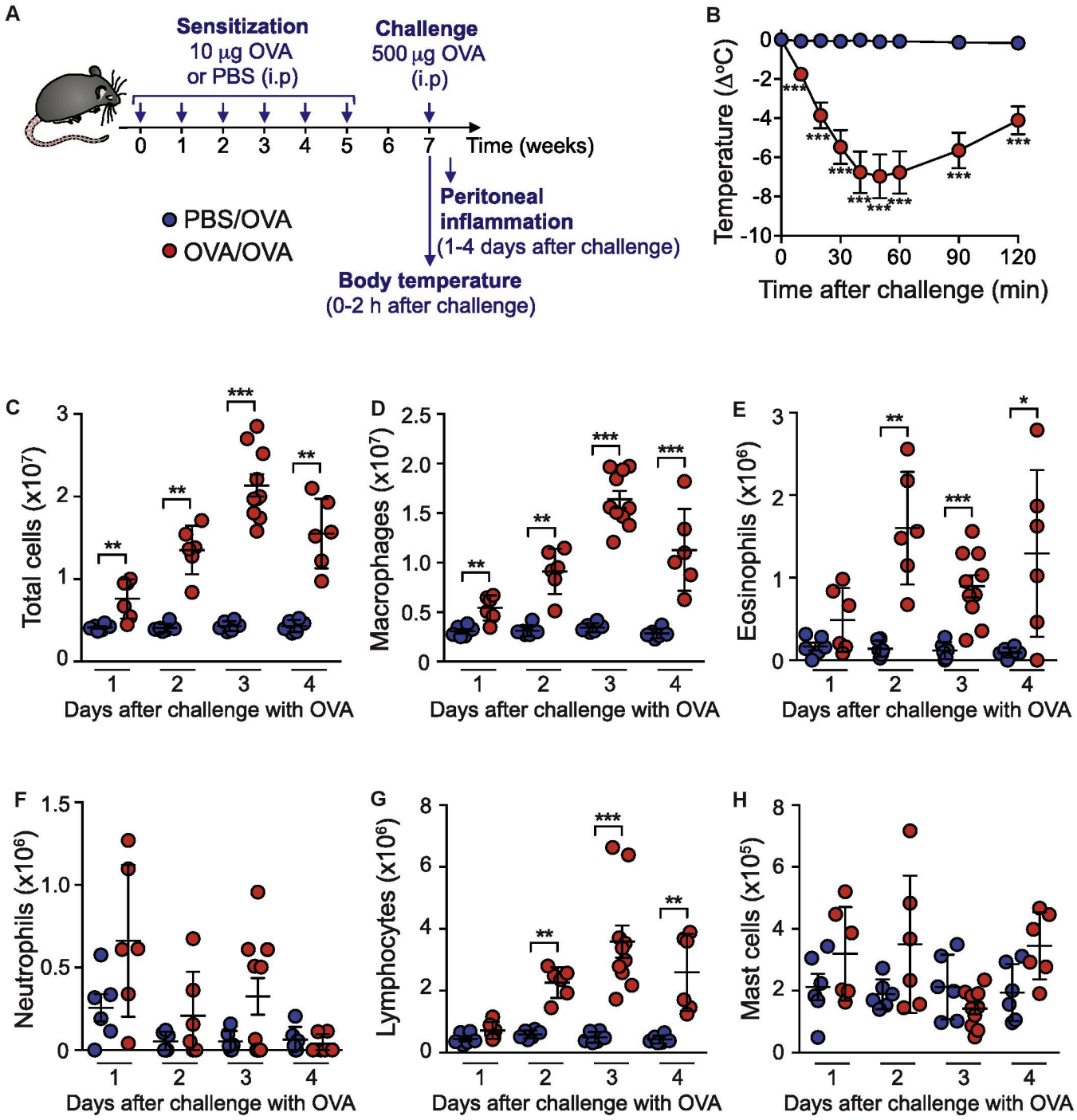


**FIG 1.** Development of hypothermia and intraperitoneal *(i.p.)* inflammation in mice sensitized and challenged with OVA in the absence of adjuvant. **A,** Experimental outline. **B,** Changes in body temperature after challenge with OVA in OVA-sensitized *(OVA/OVA)* or PBS-treated *(PBS/OVA)* mice. **C-H,** Numbers of leukocytes in peritoneal lavage fluid at the indicated time points. Data are pooled from 2 or 3 independent experiments (n = 6-10 per group). $*P < .05$, $**P < .01$, or $***P < .001$.

### OVA-induced hypothermia and inflammation depend on the high-affinity IgE receptor FcεRI, the IgG receptor FcγRIII, and their common activating subunit FcRγ

OVA sensitization induced a significant increase in OVA-specific $IgG_1$ and $IgG_{2c}$ antibody levels in C57BL/6 mice (see Fig E1, *A* and *B*, in this article's Online Repository at www.jacionline.org). We did not detect significant levels of OVA-specific IgE in sera by using a standard ELISA (data not shown). However, we observed degranulation of peritoneal cell–derived mast cells (PCMCs) incubated *in vitro* with sera from OVA-sensitized mice, followed by stimulation with OVA

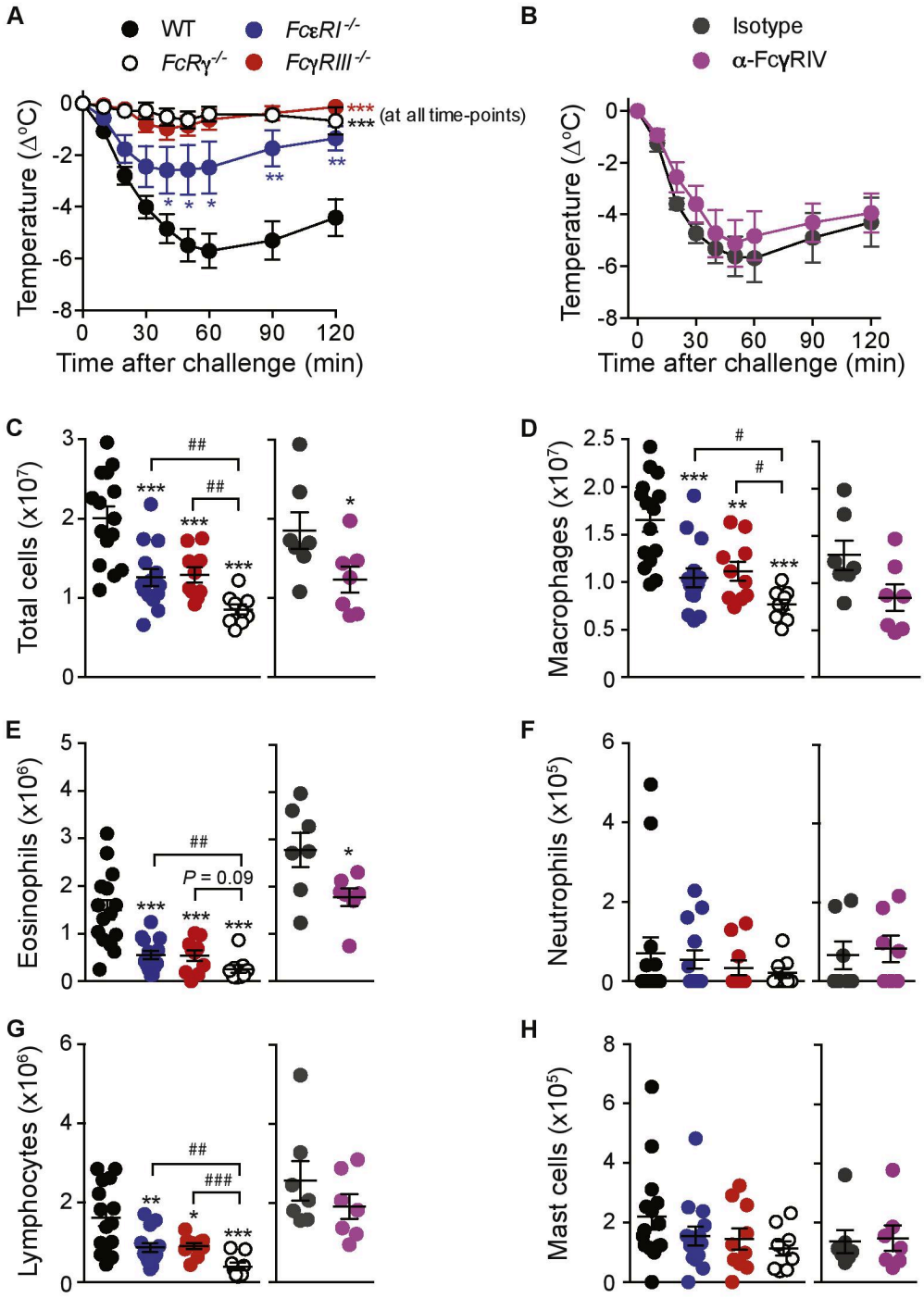


**FIG 2.** Roles of Fc receptors in OVA-induced ASA. **A** and **B,** OVA-induced hypothermia in OVA-sensitized WT (C57BL/6J), *FcεRI*$^{-/-}$, *FcγRIII*$^{-/-}$, and *FcRγ*$^{-/-}$ mice (Fig 2, *A*) or WT mice treated with an anti-FcγRIV antibody or an isotype control (Fig 2, *B*). **C-H,** Numbers of leukocytes in peritoneal lavage fluid 3 days after challenge. Data are pooled from 2 (*Isotype* and *α-FcγRIV*, n = 7 per group) or 3 (all other groups, n = 9-15 per group) independent experiments. **P* < .05, ***P* < .01, or ****P* < .001. #*P* < .05, ##*P* < .01, or ###*P* < .001.

(see Fig E1, *C* and *D*). Such degranulation was not observed with PCMCs incubated with sera from PBS-treated mice or with sera from OVA-sensitized mice, which had been preincubated with anti-IgE antibodies (see Fig E1, *C* and *D*), demonstrating the presence of functional OVA-specific IgE in the sera of sensitized mice.

We then assessed responses of C57BL/6 mice lacking the IgG receptor FcγRIII, the high-affinity IgE receptor FcεRI, or their common activating subunit FcRγ. OVA-induced hypothermia was abolished in *FcRγ*$^{-/-}$ and *FcγRIII*$^{-/-}$ mice and partially reduced in *FcεRI*$^{-/-}$ mice compared with that seen in WT mice (Fig 2, *A*). Antibody-mediated blockade of FcγRIV had no effect

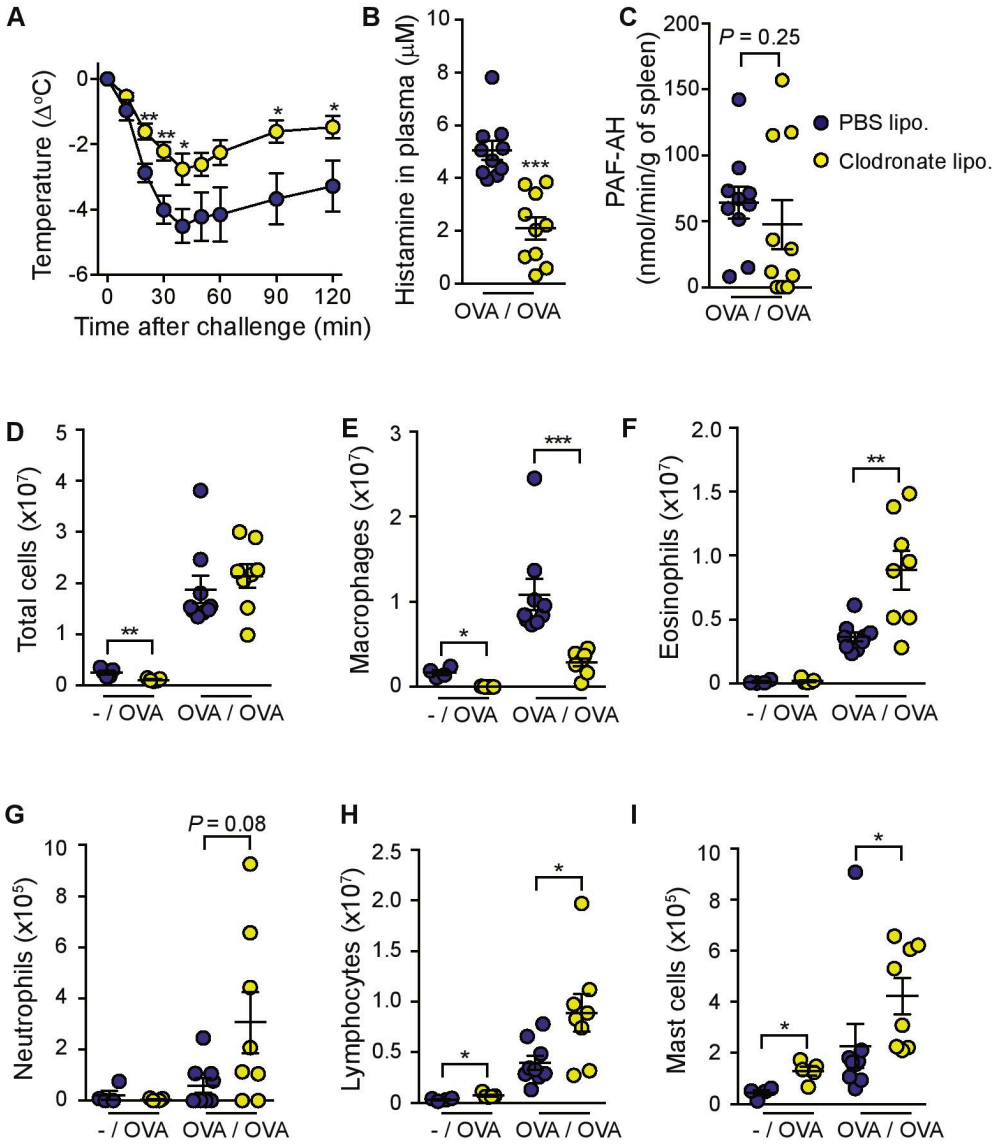


**FIG 3.** Assessment of the effect of monocyte/macrophage depletion on OVA-induced ASA. Mice were treated with clodronate liposomes *(Clodronate lipo.)* or PBS liposomes *(PBS lipo.)* 24 hours before challenge. **A,** OVA-induced hypothermia. **B,** Levels of histamine in the plasma 20 minutes after challenge. **C,** PAF-AH activity in the spleen 20 minutes after challenge. **D-I,** Numbers of leukocytes in peritoneal lavage fluid 3 days after challenge in nonsensitized mice *(−/OVA)* or OVA-sensitized mice *(OVA/OVA)*. Data are pooled from 3 independent experiments for all OVA-sensitized groups (total n = 8-9 per group) and from 1 experiment for nonsensitized control mice (n = 4-5 per group). *$P < .05$, **$P < .01$, or ***$P < .001$.

on immediate hypothermia (Fig 2, *B*). WT, *FcεRI*$^{-/-}$, and *FcγRIII*$^{-/-}$ mice had similar levels of intraperitoneal leukocytes at baseline (except for a small increase in macrophages and lymphocytes in *FcγRIII*$^{-/-}$ mice, see Fig E2 in this article's Online Repository at www.jacionline.org). However, we observed decreased numbers of total cells, macrophages, eosinophils, and lymphocytes in the peritoneal cavities of *FcεRI*$^{-/-}$ and *FcγRIII*$^{-/-}$ mice compared with WT mice 3 days after challenge (Fig 2, *C-G*). These numbers were further decreased in mice lacking FcRγ (Fig 2, *C-G*). Antibody-mediated blockade of FcγRIV induced only a moderate decrease of peritoneal leukocyte numbers at day 3, with reduced numbers of total cells and eosinophils compared with those seen in mice treated with an isotype control antibody (Fig 2, *C-G*).

## Monocyte/macrophage depletion with clodronate liposomes decreases immediate hypothermia and enhances late-phase intraperitoneal inflammation

We injected OVA-sensitized mice with clodronate liposomes (or PBS liposomes as a control) 24 hours before challenge with OVA to deplete monocytes/macrophages and assess their potential contribution to anaphylaxis through the alternative pathway. Clodronate liposome–treated mice exhibited depletion of circulating CD11b$^{high}$ monocytes at the time of OVA challenge, with no effect on blood Ly6G$^{+}$ neutrophils (see Fig E3, *A*, *C*, and *D*, in this article's Online Repository at www.jacionline.org). OVA challenge led to a decrease in CD11b expression in blood monocytes in PBS liposome–treated mice at day 3 (see Fig E3, *A*). Consistent with a previous report,[25] we found similar

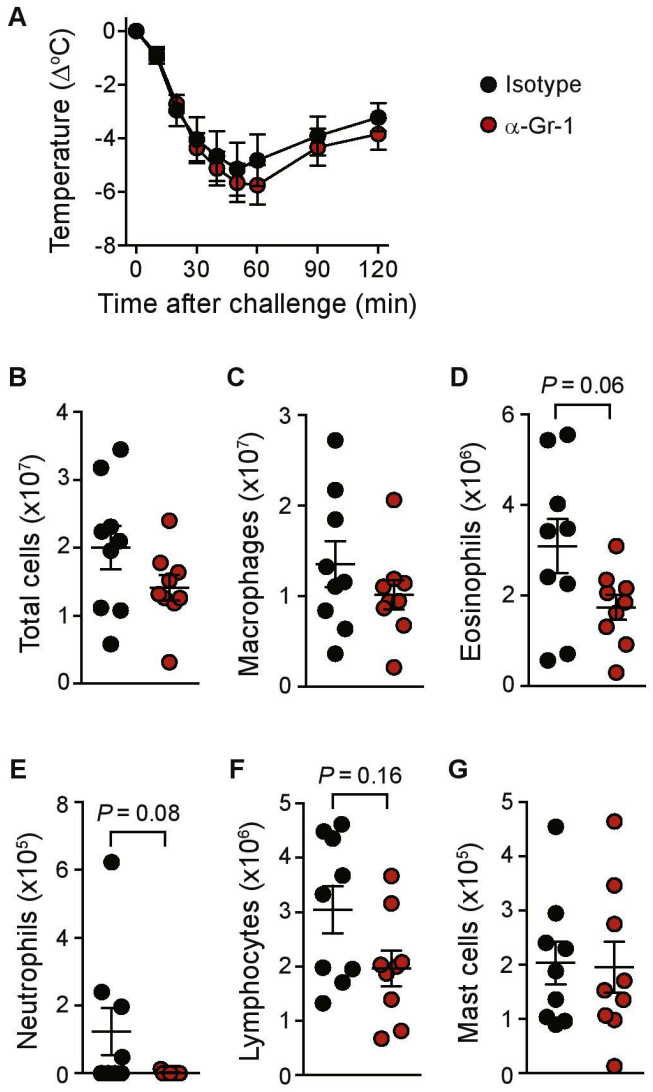


**FIG 4.** Assessment of antibody-mediated depletion of neutrophils on OVA-induced ASA. OVA-sensitized mice were treated with an anti–Gr-1 neutrophil-depleting antibody *(α-Gr-1)* or an isotype control *(Isotype)* 40 hours before and 24 hours after challenge with OVA. **A,** OVA-induced hypothermia. **B-G,** Numbers of leukocytes in peritoneal lavage fluid 3 days after challenge. Data are pooled from 3 independent experiments (total n = 9 per group).

percentages of blood monocytes in both PBS liposome– and clodronate liposome–treated mice at day 3 (ie, 4 days after the injection of liposomes; see Fig E3, *A*, *D*, and *E*). However, F4/80$^+$ macrophages were depleted in the spleens of clodronate liposome–treated mice at this time point, with no effect on neutrophils (see Fig E3, *B*, *F*, and *G*). Confirming the efficiency of depletion, we also found that levels of peritoneal macrophages were reduced by 73% in clodronate liposome–treated mice compared with those in control mice at day 3 (Fig 3, *E*).

In line with previous reports,[17,26] treatment with clodronate liposomes reduced features of anaphylaxis in an ASA model using sensitization with OVA together with the adjuvant alum and *Bordetella pertussis* toxin (see Fig E4, *B* and *C*, in this article's Online Repository at www.jacionline.org). Treatment with clodronate liposomes also significantly diminished the hypothermia response in OVA-sensitized and challenged mice in the adjuvant-free ASA model (Fig 3, *A*). Clodronate liposome–treated mice had lower levels of histamine in plasma 20 minutes after challenge compared with those in PBS liposome–treated mice (Fig 3, *B*). Because such a reduction in histamine levels could reflect a toxic effect of the liposomes on MCs, we also assessed responses of mice treated with clodronate or PBS liposomes in an MC-dependent IgE-mediated PSA model. Hypothermia was slightly but not significantly reduced (except at 10 minutes after challenge) in clodronate liposome–treated mice, which suggests that the clodronate liposomes have only modest effects on MC activity in this model (see Fig E3, *H*). The activity of PAF-AH (an enzyme that degrades PAF) has been shown to inversely correlate with the severity of anaphylaxis in human subjects.[27] However, we found similar PAF-AH activity in the spleens of mice treated with clodronate or PBS liposomes 20 minutes after challenge (Fig 3, *C*). By contrast, clodronate liposome–treated mice displayed significantly higher levels of eosinophils, lymphocytes, and MCs than PBS liposome–treated mice in the peritoneal cavity 3 days after challenge (Fig 3, *F-I*). Levels of blood neutrophils were also higher in such clodronate liposome–treated, macrophage-depleted mice compared with those in control mice at day 3 (see Fig E3, *A* and *C*). However, we also found a small but significant increase in lymphocyte and MC numbers 3 days after OVA challenge in naive mice treated with clodronate liposomes, which could reflect some proinflammatory effects of the clodronate liposomes (Fig 3, *D-I*).

## Neutrophils are not required for OVA-induced hypothermia and inflammation

We injected OVA-sensitized mice with an anti–Gr-1 antibody (or an isotype control antibody) 40 hours before and 24 hours after challenge with OVA to deplete neutrophils. Such treatment led to complete ablation of circulating neutrophils at the time of challenge (see Fig E5, *A* and *C*, in this article's Online Repository at www.jacionline.org), and neutrophils remained absent in the blood and spleens of anti–Gr-1–treated mice 3 days after challenge (see Fig E5, *A-C* and *F*). By contrast, treatment with anti–Gr-1 antibodies did not deplete circulating monocytes or spleen macrophages (and the percentages of these cells were even slightly higher than those in mice treated with the isotype control antibody; see Fig E5, *A*, *B*, *D*, *E*, and *G*).

In agreement with findings obtained in IgG-mediated PSA or ASA models using adjuvants for the sensitization,[5,11] we found that anti–Gr-1–treated mice had markedly reduced features of anaphylaxis compared with isotype control–treated mice in an ASA model using sensitization with OVA together with the adjuvants alum and *B pertussis* toxin (see Fig E4, *D* and *E*). By contrast, isotype control–treated mice and anti–Gr-1–treated mice had similar levels of immediate hypothermia after OVA challenge (Fig 4, *A*). Both groups also had similar levels of leukocytes in the peritoneal cavity 3 days after challenge. Although eosinophil and lymphocyte numbers were slightly decreased in the anti–Gr-1–treated group compared with those in the isotype control–treated mice, those differences did not reach statistical significance (Fig 4, *B-G*).

## *Cpa3-Cre*$^+$*; Mcl-1*$^{fl/fl}$ mice have less OVA-induced hypothermia and inflammation

We next assessed responses of MC-deficient, basophil-depleted *Cpa3-Cre*$^+$*; Mcl-1*$^{fl/fl}$ mice[24] in this adjuvant-free ASA model.

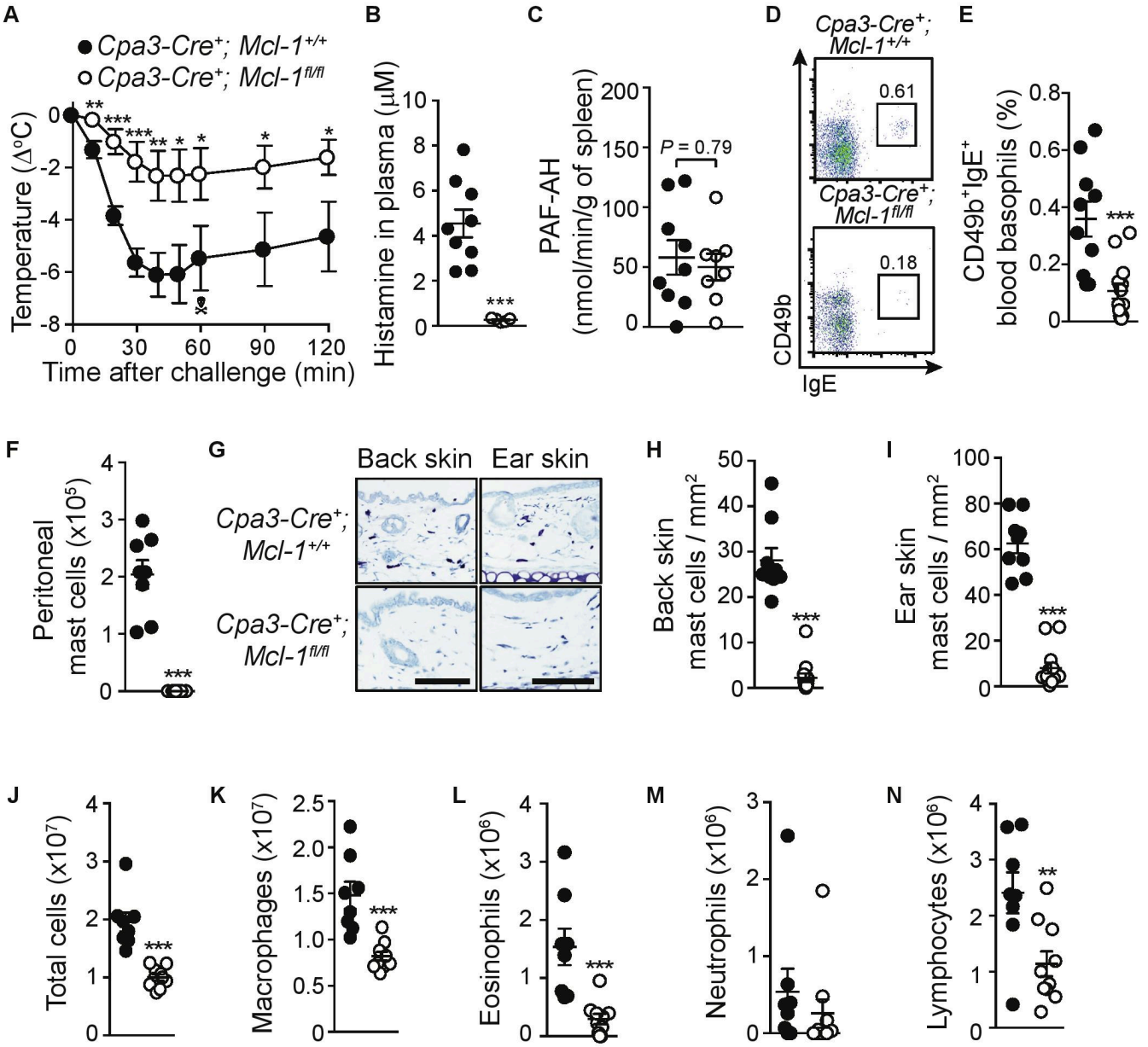


**FIG 5.** Assessment of OVA-induced ASA in genetically MC-deficient and basophil-depleted *Cpa3-Cre; Mcl-1$^{fl/fl}$* mice. **A,** OVA-induced hypothermia in OVA-sensitized *Cpa3-Cre$^{+}$; Mcl-1$^{+/+}$* and *Cpa3-Cre$^{+}$; Mcl-1$^{fl/fl}$* mice. **B,** Levels of histamine in the plasma 20 minutes after challenge. **C,** PAF-AH activity in the spleen 20 minutes after challenge. **D** and **E,** Representative fluorescence-activated cell sorting profile (Fig 5, *D*) and percentage (Fig 5, *E*) of blood basophils (CD49b$^{+}$; IgE$^{+}$) 24 hours before challenge. **F,** Numbers of MCs in peritoneal lavage fluid 3 days after challenge. **G-I,** Toluidine blue staining for MCs (Fig 5, *G*) and MC numbers (Fig 5, *H* and *I*) in sections of back skin and ear pinna. **J-N,** Numbers of leukocytes in the peritoneal lavage fluid 3 days after challenge. Data are pooled from 3 independent experiments (total n = 9-14 per group). **P* < .05, ***P* < .01, or ****P* < .001. *Crossbones symbol* indicates the death of 1 mouse. *Scale bars* = 100 μm.

*Cpa3-Cre$^{+}$; Mcl-1$^{fl/fl}$* mice remained highly deficient in MCs and had reduced numbers of basophils, even after repeated sensitizations and challenge with OVA (Fig 5, *D-I*). These mice had similar levels of OVA-specific $IgG_1$ and $IgG_{2c}$ antibodies as *Cpa3-Cre$^{+}$; Mcl-1$^{+/+}$* littermate control mice (see Fig E6, *A* and *B*, in this article's Online Repository at www.jacionline.org) and displayed similar amounts of antigen-specific IgE activity in the serum (see Fig E6, *C* and *D*), suggesting that MCs and basophils do not contribute substantially to the sensitization phase in this model. However, sensitized *Cpa3-Cre$^{+}$; Mcl-1$^{fl/fl}$* mice had significantly reduced levels of hypothermia after OVA challenge, suggesting an important role for MCs, basophils, or both in this feature of the model (Fig 5, *A*). *Cpa3-Cre$^{+}$; Mcl-1$^{fl/fl}$* mice had markedly reduced levels of histamine in the plasma but similar PAF-AH activity in the spleen 20 minutes after challenge (Fig 5, *B* and *C*).

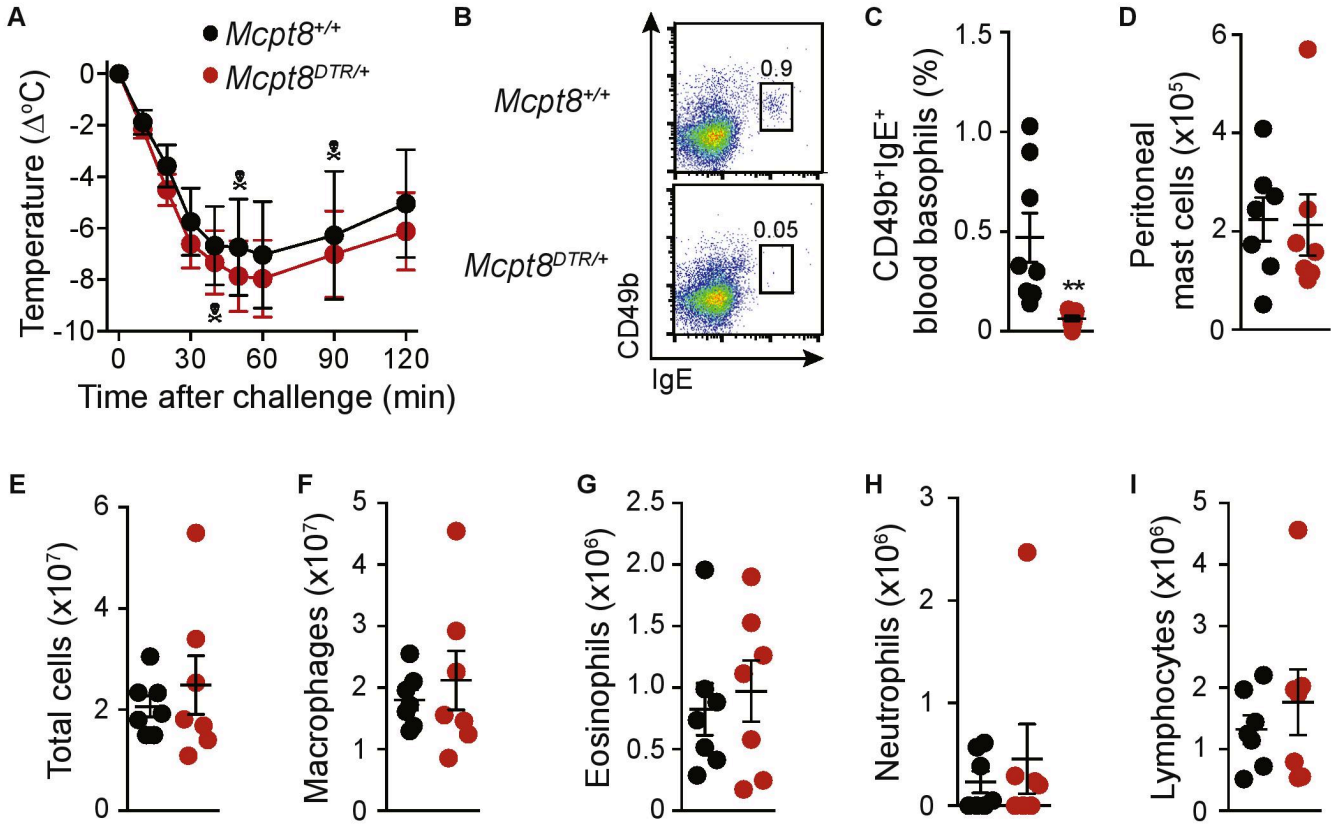


**FIG 6.** Assessment of the effect of DT-mediated basophil depletion on OVA-induced ASA in *Mcpt8*$^{DTR}$ mice. OVA-sensitized *Mcpt8*$^{+/+}$ and *Mcpt8*$^{DTR/+}$ mice were treated with DT 48 hours before and 24 hours after challenge with OVA. **A,** OVA-induced hypothermia. **B** and **C,** Representative fluorescence-activated cell sorting profile (Fig 6, *B*) and percentage (Fig 6, *C*) of blood basophils (CD49b$^+$; IgE$^+$) 2 hours before challenge. **D-I,** Numbers of leukocytes in peritoneal lavage fluid 3 days after challenge. Data are pooled from 2 independent experiments (total n = 7-9 per group). $^{**}P < .01$. Each *crossbones symbol* indicates the death of 1 mouse.

*Cpa3-Cre*$^+$*; Mcl-1*$^{fl/fl}$ mice also had diminished intraperitoneal inflammation, with decreased numbers of total leukocytes, macrophages, eosinophils, and lymphocytes compared with *Cpa3-Cre*$^+$*; Mcl-1*$^{+/+}$ mice, indicating that MCs, basophils, or both can also contribute importantly to the late-phase leukocyte numbers in this model (Fig 5, *J-N*).

## Basophils are not required for OVA-induced hypothermia and inflammation

We assessed the potential role of basophils using *Mcpt8*$^{DTR}$ mice, which express the diphtheria toxin receptor (DTR) in basophils only and in which basophils can be selectively ablated by injection of diphtheria toxin (DT).[23] We first confirmed that treatment with DT induces ablation of basophils, but not MCs, in OVA-sensitized *Mcpt8*$^{DTR}$ mice (Fig 6, *B-D*). We ruled out a significant role for basophils in this model by showing that DT-mediated depletion of basophils in OVA-sensitized *Mcpt8*$^{DTR}$ mice does not affect OVA-induced hypothermia (Fig 6, *A*) or late-phase intraperitoneal leukocyte numbers (Fig 6, *E-I*).

## MCs exacerbate OVA-induced hypothermia and inflammation

We used *Kit* mutant MC-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice to assess further the contributions of MCs in this "adjuvant-free" ASA model. Because *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice have many KIT-related phenotypic abnormalities in addition to their MC deficiency,[28-33] we also included a group of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice that had been engrafted with BMCMCs (BMCMCs → *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice) both intraperitoneally and intravenously 12 weeks before the first sensitization with OVA.

We found no significant difference in the severity of anaphylaxis between *Kit*$^{+/+}$ and MC-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice in an ASA model using sensitization with OVA together with the adjuvants alum and *B pertussis* toxin (see Fig E4, *F* and *G*), confirming previous findings obtained with MC-deficient *Kit*$^{W/W\text{-}v}$ mice.[9] By contrast, MC-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice had significantly less hypothermia after challenge with OVA compared with *Kit*$^{+/+}$ mice (Fig 7, *A*). BMCMCs → *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice had significantly more hypothermia than did *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice after challenge with OVA, although levels of hypothermia in these mice did not reach those in *Kit*$^{+/+}$ mice (Fig 7, *A*). The intermediate body temperature response in MC-engrafted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice compared with WT or MC-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice is consistent with our previously reported data in a peanut-induced ASA model.[19] We think it very likely that the technical limitations of such systemic MC engraftment experiments contributed to the intermediate temperature response seen in MC-engrafted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. Specifically, compared with the corresponding

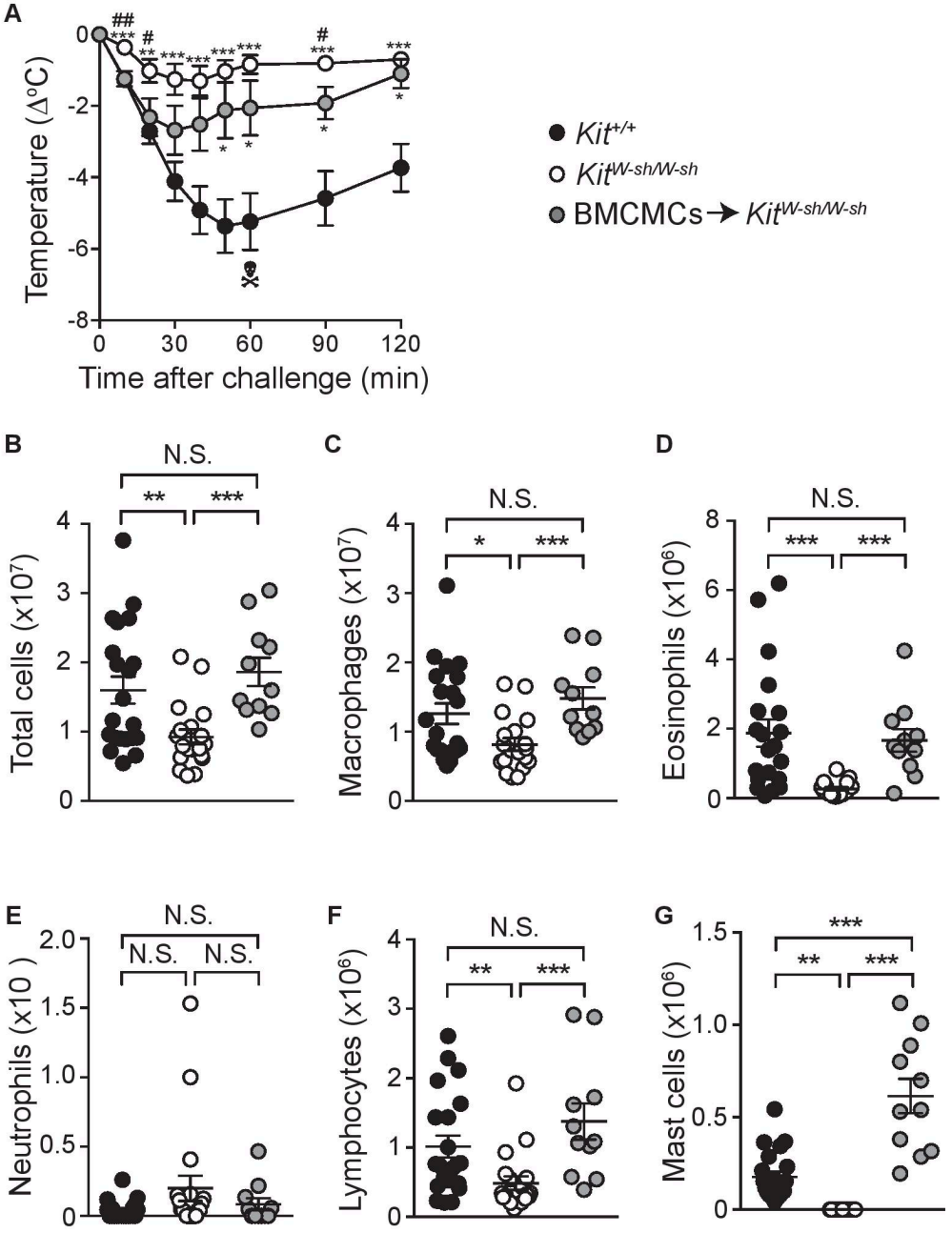


**FIG 7.** Responses of WT, MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$, and $Kit^{W\text{-}sh/W\text{-}sh}$ mice engrafted with BMCMCs in OVA-induced ASA. **A,** OVA-induced hypothermia in $Kit^{+/+}$, MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$, and $Kit^{W\text{-}sh/W\text{-}sh}$ mice engrafted with WT BMCMCs. **B-G,** Numbers of leukocytes in peritoneal lavage fluid 3 days after challenge. Data are pooled from 3 to 5 independent experiments (total n = 11-25 per group). $*P < .05$, $**P < .01$, or $***P < .001$ versus the $Kit^{+/+}$ group (Fig 7, *A*) or indicated group (Fig 7, *B-G*). $\#P < .05$ or $\#\#P < .01$ versus BMCMCs→$Kit^{W\text{-}sh/W\text{-}sh}$ group (Fig 7, *A*). *Crossbones symbol* indicates the death of 1 mouse.

WT mice, MC-engrafted $Kit^{W\text{-}sh/W\text{-}sh}$ mice had similar levels of MCs in the peritoneal cavity and mesenteric windows and even higher numbers of MCs in the spleen. However, these mice had no detectable MCs in the skin, thus eliminating the numerically large population of skin MCs as a potential source of mediators in such mice (Fig 7 and see Fig E7 in this article's Online Repository at www.jacionline.org).

Finally, MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice had significantly diminished numbers of leukocytes during late-phase intraperitoneal inflammation compared with $Kit^{+/+}$ mice (Fig 7, *B-F*). Confirming the role of MCs in this feature of our ASA model, engraftment of $Kit^{W\text{-}sh/W\text{-}sh}$ mice with BMCMCs restored levels of total peritoneal leukocytes, neutrophils, eosinophils, macrophages, and lymphocytes quantified 3 days after challenge to those observed in $Kit^{+/+}$ mice (Fig 7, *B-F*). Altogether, these results show that MCs can amplify both the immediate hypothermia and the late-phase inflammatory reaction in $Kit^{W\text{-}sh/W\text{-}sh}$ mice in this adjuvant-free ASA model.

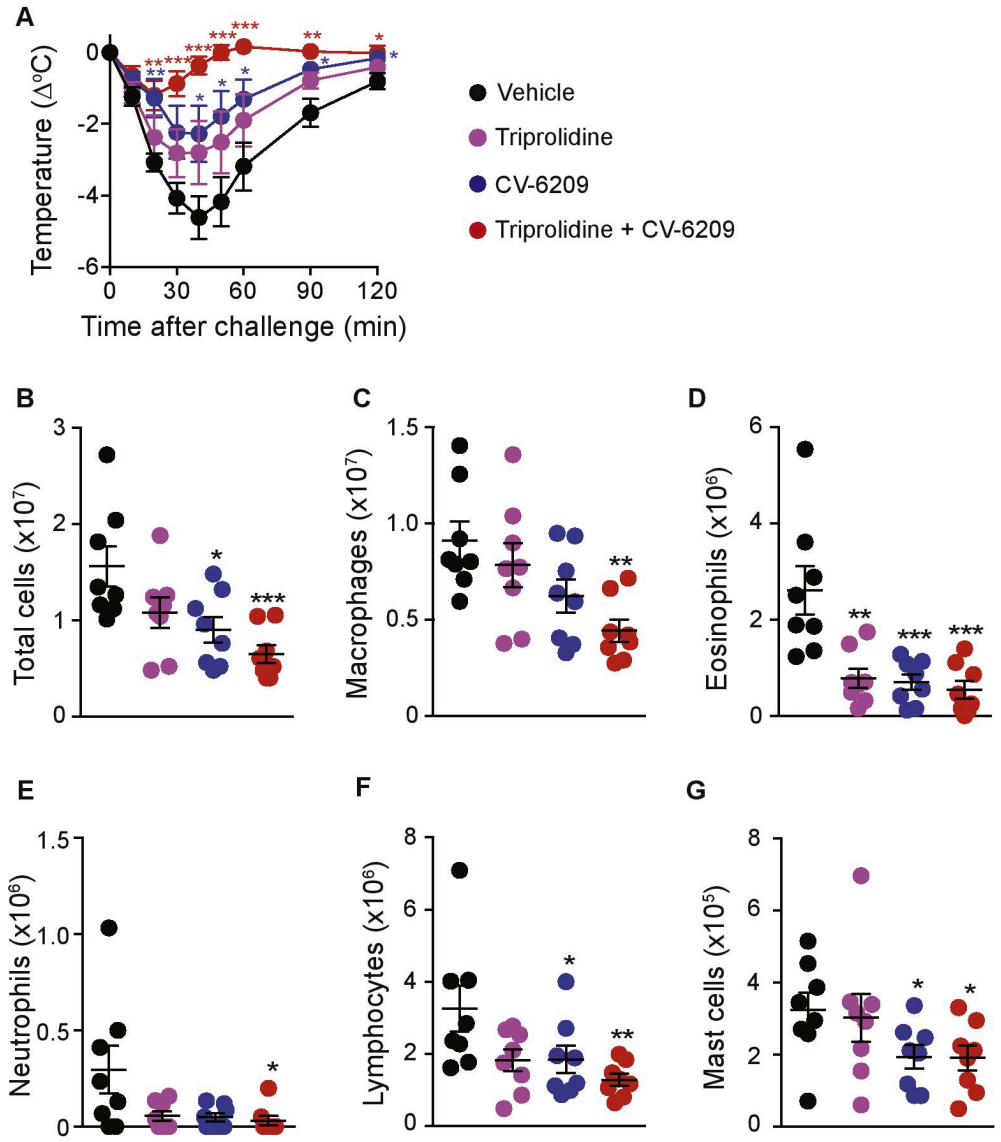


**FIG 8.** Roles of histamine and PAF in OVA-induced ASA. OVA-sensitized mice were treated with the $H_1$-antihistamine triprolidine or with the PAF receptor antagonist CV-6209 alone or in combination 30 minutes before and 1 day after challenge with OVA. Control mice were injected with vehicle (saline) only. **A,** OVA-induced hypothermia. **B-G,** Numbers of leukocytes in peritoneal lavage fluid 3 days after OVA challenge. Data are pooled from 2 independent experiments (total n = 8 per group). $*P < .05$, $**P < .01$, or $***P < .001$ versus the vehicle group.

## Histamine and PAF contribute to OVA-induced hypothermia and inflammation

Pretreatment of mice with the PAF receptor antagonist CV-6209 significantly decreased both the immediate hypothermia and numbers of leukocytes in the late-phase inflammatory response (Fig 8). Pretreatment with the $H_1$-antihistamine triprolidine slightly reduced immediate hypothermia and the numbers of leukocytes in the late-phase response (Fig 8), although differences did not reach statistical significance, except for eosinophil numbers (Fig 8, *D*). However, combined treatment with the antihistamine and PAF receptor antagonist almost entirely blocked both the hypothermia and increased numbers of leukocytes observed during inflammatory responses (Fig 8). By contrast, treatment with triprolidine markedly reduced both the hypothermia and numbers of intraperitoneal leukocytes in mice pretreated with clodronate liposomes, whereas CV-6209 had no significant effects on these features (see Fig E8 in this article's Online Repository at www.jacionline.org). Collectively, these data demonstrate the involvement of both histamine and PAF in this ASA model in WT mice and suggest that monocytes/macrophages represent the main source of PAF in this model.

## DISCUSSION

Most ASA models use adjuvants during the sensitization phase, and such methods typically prime the animals to exhibit allergic reactions that require little or no contribution from MCs and IgE.

We hypothesized that use of adjuvants might boost the production of certain IgG isotypes, favoring activation of alternative pathways of anaphylaxis, which might mask contributions from the classical IgE- and MC-dependent pathway. Therefore we designed a new adjuvant-free model of ASA and assessed the potential roles of various components of the classical and alternative pathways in that model.

Our sensitization protocol resulted in production of OVA-specific $IgG_1$ and $IgG_{2c}$ antibodies (see Figs E1 and E4), 2 isotypes that can induce anaphylaxis through FcγRIII, FcγRIV, or both.[5,10,11] Indeed, we found that *FcγRIII*$^{-/-}$ mice had markedly reduced hypothermia and reduced numbers of peritoneal leukocytes compared with WT mice in this model (Fig 2). We used an anti-FcγRIV blocking antibody to assess the contribution of this receptor and found no role for FcγRIV in the hypothermia response and a relatively minor contribution to numbers of intraperitoneal leukocytes (Fig 2). Altogether, our results confirm the important involvement of FcγRIII in allergic shock[10,11,17,26,34] and reveal that this receptor also has an important role in the development of antigen-induced allergic inflammation.

Although we did not detect significant levels of OVA-specific IgE in the sera of most mice by using a classical ELISA (data not shown), we demonstrated the presence of functionally active specific IgE in sera from OVA-sensitized mice using an *ex vivo* MC activation test (see Figs E1 and E6). These levels of antigen-specific IgE were sufficient to contribute to anaphylaxis and allergic inflammation because mice lacking the high-affinity IgE receptor FcεRI displayed significantly diminished hypothermia and numbers of intraperitoneal leukocytes compared with WT mice (Fig 2).

These results are in agreement with previous findings showing less hypothermia in *FcεRI*$^{-/-}$ or *IgE*$^{-/-}$ mice in models of peanut-induced ASA.[16,17,35] However, our results are in sharp contrast with reports showing no evidence for involvement of IgE or FcεRI in models of OVA-induced ASA that use alum as an adjuvant during the sensitization phase.[9,10,20] In line with the strong contributions of both FcγRIII and FcεRI in our model, we found that *FcRγ*$^{-/-}$ mice, which lack the common activating subunit of Fcε and Fcγ receptors,[36] were completely protected from hypothermia and developed even lower numbers of intraperitoneal leukocytes than *FcεRI*$^{-/-}$ or *FcγRIII*$^{-/-}$ mice (Fig 2).

In mice FcγRIII is expressed on all myeloid cells,[37] whereas FcεRI is mainly expressed on MCs and basophils.[38] Depending on the model used, MCs, basophils, monocytes/macrophages, and/or neutrophils have been reported to contribute to ASA.[11,13,14,16-19,21,26,35] Several studies using passive or active models of anaphylaxis have reported an important role for monocytes/macrophages in the immediate hypothermia response after antigen challenge.[5,14,17,21,26,39-41] We confirmed these findings in an ASA model by using the adjuvants alum and *B pertussis* toxin for sensitization (see Fig E4). Our depletion experiments also demonstrated that monocytes/macrophages contributed to the hypothermia response in our adjuvant-free ASA model (Fig 2). By contrast, little is known about the function of monocytes/macrophages in the late-phase allergic inflammation after anaphylaxis. We found that mice treated with clodronate liposomes to deplete monocytes/macrophages exhibited significantly increased numbers of intraperitoneal leukocytes (Fig 2). This raises the possibility that monocytes/macrophages can contribute to the resolution phase in this ASA model. However, care should be taken in the interpretation of these findings because such increased leukocyte numbers might also reflect, at least in part, proinflammatory effects of the clodronate liposomes. Indeed, we also found slightly increased numbers of intraperitoneal MCs and lymphocytes in nonsensitized mice treated with clodronate liposomes.

The importance of neutrophils and basophils in anaphylaxis is debated.[15] Some reports indicate that antibody-mediated depletion of neutrophils can reduce $IgG_2$-mediated PSA[5,11] and ASA[11] in mice, whereas others found no role for neutrophils in the hypothermia reaction after antigen challenge in IgG-mediated PSA[13,41] or ASA[19,21] models. In the present study we used anti–Gr-1 antibodies to deplete neutrophils. We found that such treatment markedly reduced anaphylaxis in an ASA model by using the adjuvants alum and *B pertussis* toxin for sensitization (see Fig E4). By contrast, we found no significant contribution for neutrophils in either the immediate hypothermia reaction or the late-phase accumulation of intraperitoneal leukocytes in the adjuvant-free ASA model (Fig 4). Similarly, some reports indicate a contribution of basophils to IgG-mediated PSA[5,13,34] or ASA,[11,17,19] whereas others found no significant role for basophils in anaphylaxis models.[21,41,42] Here we used conditional basophil-deficient *Mcpt8*$^{DTR}$ mice[23] and found no roles for basophils in either the immediate hypothermia reaction or the late-phase allergic inflammation in our adjuvant-free ASA model (Fig 6).

It is largely agreed that MCs play important roles in food allergy and anaphylaxis.[14,43-45] Although most of the literature on the roles of MCs in experimental anaphylaxis is based on data obtained by using *Kit* mutant genetically MC-deficient mice, several new models have been developed in which the MC deficiency is not dependent on mutations affecting c-*kit* structure or expression.[24,30-32,46-49] Although discordant findings have been reported in some disease models in newer versus older MC-deficient strains,[30,46,47,50,51] the importance of MCs to both IgE-mediated PSA[24,47,52] and peanut-induced ASA[19] has been confirmed by using multiple MC-deficient mouse strains. In agreement with these findings, we demonstrate here that both *Kit*$^{W\text{-}sh/W\text{-}sh}$ and *Kit*-independent *Cpa3-Cre; Mcl-1*$^{fl/fl}$ MC-deficient mice had reduced immediate hypothermia reactions and diminished numbers of late-phase intraperitoneal leukocytes in our adjuvant-free ASA model (Figs 5 and 7). We obtained additional evidence for an important contribution of MCs in this model by showing that engraftment of genetically MC-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice with bone marrow–derived cultured MCs partially restored the immediate hypothermia and completely restored the late-phase allergic inflammation induced by antigen challenge (Fig 7).

These results stand in marked contrast with previous reports[9,13] and our own data (see Fig E4) showing that anaphylaxis can fully develop after challenge with OVA in MC-deficient mice sensitized with OVA together with the adjuvant alum. One potential explanation for such results is that MCs are particularly potent at promoting allergic reactions at low levels of antibodies and/or that increased levels of antibodies (promoted by the use of adjuvants during the sensitization phase) lead to a greater contribution of alternative pathways that mask or render redundant the role of MCs. Indeed, previous reports show that the contribution of MCs to some ASA models is greater when using low doses of adjuvant, antigen, or both for sensitization

and low doses of antigen for challenge.[17,19] Our results are also in line with previous reports demonstrating that genetically MC-deficient mice exhibit significantly diminished OVA-induced allergic airway inflammation when sensitized without adjuvant[53-56] but can fully develop airway inflammation when alum is used as an adjuvant during the sensitization phase.[53,57]

Finally, consistent with previous reports, we found that both histamine and PAF contributed to the immediate hypothermia reaction in WT mice.[14,20,31,58-60] These 2 mediators also contributed to the late-phase inflammatory reaction, with a more pronounced effect of PAF (Fig 8). By contrast, treatment with a PAF receptor antagonist had no effect in clodronate liposome–treated mice, suggesting that monocytes/macrophages represent the major source of PAF in this adjuvant-free ASA model. As expected, we found that plasma histamine levels were markedly reduced in MC- and basophil-deficient *Cpa3-Cre*$^{+}$*; Mcl-1*$^{fl/fl}$ mice. More surprisingly, however, we also found reduced plasma histamine levels in clodronate liposome–treated mice (although the reduction was not as substantial as that found in *Cpa3-Cre*$^{+}$*; Mcl-1*$^{fl/fl}$ mice), suggesting that monocytes/macrophages might also directly or indirectly contribute to histamine release in this model.

In conclusion, we demonstrate here that FcεRI- and FcγRIII-dependent signaling, histamine, and PAF are required for the full development of hypothermia and intraperitoneal leukocyte accumulation in an adjuvant-free model of ASA. In this model both MCs and monocytes/macrophages are critically involved in the immediate hypothermia reaction. In addition, MCs are also required for the full development of intraperitoneal inflammation, as assessed based on numbers of intraperitoneal leukocytes. Thus our data strongly support the hypothesis that MCs and monocytes/macrophages are the main effector cells of anaphylaxis in this setting.

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**Key messages**

- **The IgE receptor FcεRI, the IgG receptor FcγRIII, histamine, and PAF contribute to hypothermia and leukocyte numbers in an adjuvant-free ASA model.**
- **MCs are required for full development of hypothermia and leukocyte numbers in this ASA model, with no significant role for basophils or neutrophils.**
- **Monocytes/macrophages contribute to hypothermia in this ASA model.**

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# METHODS

## Flow cytometry

We used flow cytometry to identify blood basophils ($CD49b^+IgE^+$), neutrophils ($Ly6G^+CD11b^+$) and monocytes ($Ly6G^-CD11b^+$), as well as spleen neutrophils ($Ly6G^+F4/80^-$) and macrophages ($Ly6G^-F4/80^+$). Briefly, red blood cells were lysed by means of treatment with pH 7.3 ACK lysis buffer (0.15 mol/L $NH_4Cl$, 1 mmol/L $KHCO_3$, and 0.1 mmol/L EDTA [pH 8.0]) 2 times for 5 minutes each. Cells were blocked with unconjugated anti–CD16/CD32 antibodies (BioXcell, Beverly, Mass) on ice for 5 minutes and then stained with combinations of anti-CD49b (DX5), anti-IgE (R35-72), anti-Ly6G (1A8), anti-CD11b (M1/70), and anti-F4/80 (BM8; all from eBioscience, San Diego, Calif, or BioLegend, San Diego, Calif) antibodies on ice for 30 minutes. Data were acquired with LSRII and Accuri C6 flow cytometers (all from BD Biosciences, San Jose, Calif). Data were analyzed with FlowJo 9.5.3. software (TreeStar, Ashland, Ore). Dead cells (identified by means of staining with propidium iodide [PI; Gibco, Carlsabd, Calif] or LIVE/DEAD Aqua amine [Invitrogen, Carlsbad, Calif]) were not included in the analysis.

## Peritoneal lavage and differential cell counts

To harvest peritoneal cells, 2 mL of ice-cold PBS was injected into the peritoneal cavity 1 to 4 days (3 days for routine experiments) after OVA challenge, and the abdomen was massaged gently for 20 seconds. Fluid containing peritoneal cells was aspirated, and the cells were resuspended in ice-cold PBS. Total cell numbers were measured with a hemocytometer chamber, and cells were cytocentrifuged onto glass slides and stained with May-Grünwald-Giemsa. Differential cell counts on at least 300 cells were obtained by using standard morphologic criteria.

## Depletion of basophils, monocytes/macrophages, or neutrophils and blockade of FcγRIV, PAF, and histamine

For depletion of basophils, $Mcpt8^{DTR/+}$ basophil-deficient mice and $Mcpt8^{+/+}$ basophil-sufficient littermates received intraperitoneal injections of 500 ng of DT (Sigma-Aldrich) 2 days before and 1 day after challenge with OVA. The efficiency of depletion was assessed by means of flow cytometric analysis of blood basophils ($CD49b^+IgE^+$) in blood samples collected by means of retro-orbital bleeding 1 hour before OVA challenge.

For neutrophil depletion, mice were injected intraperitoneally with 150 μg of an anti–Gr-1 antibody (RB6-8C5; BioXcell) 40 hours before and 24 hours after challenge with OVA.[E1] Control mice were injected with a rat $IgG_{2b}$ isotype control antibody (LTF-2; BioXcell). The efficiency of neutrophil depletion was assessed by means of flow cytometric analysis of neutrophils in blood samples ($Ly6G^+$; $CD11b^+$) collected by using retro-orbital bleeding 1 hour before and 3 days after OVA challenge, as well as in spleen samples ($Ly6G^+$; $F4/80^-$) collected 3 days after challenge.

For depletion of monocytes/macrophages, mice were injected with clodronate liposomes (150 μg administered intraperitoneally plus 150 μg administered intravenously; ClodronateLiposomes.com) 24 hours before challenge with OVA.[E1] Control mice were injected in the same manner with the same amount of PBS liposomes. The efficiency of monocyte/macrophage depletion was assessed by means of flow cytometric analysis of monocytes in blood samples ($Ly6G^-$; $CD11b^+$) collected by using retro-orbital bleeding 1 hour before and 3 days after OVA challenge, as well as in spleen samples ($Ly6G^-$; $F4/80^+$) collected 3 days after challenge.

For blockade of FcγRIV, mice were injected intravenously with 200 μg of an anti-FcγRIV (9E9) antibody 30 minutes before and 2 days after OVA challenge.[E1] The hybridoma cell line producing anti-FcγRIV antibodies (9E9) was kindly provided by J. V. Ravetch (Rockefeller University, New York, NY); control mice were injected with polyclonal Armenian hamster isotype control IgG antibodies (BioXcell).

The potential contributions of histamine and PAF in this model were analyzed based on specific inhibition of the actions of these mediators, according to previously described methods,[E2] with slight modifications: the $H_1$ receptor–specific antihistamine triprolidine was solubilized at 1 mg/mL in sterile saline, and 200 μL was injected intraperitoneally into mice 30 minutes before and 1 day after OVA challenge. The PAF receptor antagonist CV-6209 (Santa Cruz Biotechnology, Dallas, Tex) was diluted at 330 μg/mL in saline, and 200 μL was injected intravenously 30 minutes before and 1 day after OVA challenge.

## Evaluation of MCs in mesenteric windows

Evaluation of MCs in mesenteric windows was performed, as previously described.[E3,E4] Briefly, 4 to 5 mesenteric windows from approximately the same locations in each mouse were arranged onto slides and fixed for 1 hour in Carnoy solution (3:2:1 vol/vol/vol of ethanol, chloroform, and acetic acid). The preparations were stained with Csaba stain, which contains both safranin (red, identifying "mature" MCs) and Alcian blue (blue, identifying "less mature" MCs), which bind to MC cytoplasmic granules.[E3,E4]

## Histologic analysis

Ear skin (ear pinnae), back skin, and spleen specimens were fixed in 10% formalin and embedded in paraffin, and 4-μm sections were stained with 0.1% Toluidine blue for histologic examination and enumeration of MCs. Images were captured with an Olympus BX60 microscope using a Retiga-2000R QImaging camera run by using Image-Pro Plus Version 6.3 software (Media Cybernetics, Rockville, Md).

## Measurement of OVA-specific $IgG_1$ and $IgG_{2c}$ antibodies

Peripheral blood was collected by mean of retro-orbital bleeding 24 hours before OVA challenge. Serum OVA-specific $IgG_1$ and $IgG_{2c}$ antibody levels were measured by means of ELISA. Briefly, 96-well EIA/RIA plates (Costar, Cambridge, Mass) were coated with 2 μg/well of OVA in 50 nmol/L carbonate-bicarbonate buffer (pH 9.6; Sigma-Aldrich, St Louis, Mo) overnight at 4°C. Coated plates were blocked with 1% BSA for 2 hours at room temperature. Plates were washed 3 times, and diluted serum samples were incubated overnight at 4°C. We diluted samples 1:100,000 for $IgG_1$ and 1:50 for $IgG_{2c}$. Plates were then washed 3 times and incubated with biotinylated anti-mouse $IgG_1$ (A85-1; BD PharMingen) or biotinylated anti-mouse $IgG_{2a/c}$ (R19-15; BD PharMingen) for 1 hour at room temperature. Plates were washed 5 times and incubated with streptavidin–horseradish peroxidase (BD PharMingen) for 30 minutes at room temperature. Plates were washed 3 times and incubated with TMB substrate (Sigma), and the reaction was stopped by addition of 2 mol/L $H_2SO_4$. Absorbance was measured at 450 nm. Anti-OVA $IgG_1$ (4B4E6; Chondrex) and $IgG_{2a}$ (M12E4D5; Chondrex, Redmond, Wash) were used as standard antibodies. C57BL/6 does not express $IgG_{2a}$ but does expresses $IgG_{2c}$[E5] (the R19-15 detection antibody we used in our ELISA recognizes both $IgG_{2a}$ and $IgG_{2c}$[E6]), and therefore we expressed levels of OVA-specific $IgG_{2c}$ as arbitrary units.

## Generation and adoptive transfer of BMCMCs

Female mouse femoral bone marrow cells were cultured in Dulbecco modified Eagle medium supplemented with 10% FCS, penicillin, and streptomycin and 20% WEHI-3 cell–conditioned medium (as a source of IL-3) to generate BMCMCs. Cells were cultured for 6 weeks, with medium changed twice a week until more than 95% were $Fc\varepsilon RI\alpha^+KIT^+$ (assessed by using flow cytometry. data not shown). BMCMCs were transferred by means of intraperitoneal plus intravenous injections ($2 \times 10^6$ cells administered intraperitoneally plus $10^7$ cells administered intravenously) into male $Kit^{W\text{-}sh/W\text{-}sh}$ mice at 4 to 6 weeks of age. Sensitization with OVA was initiated 12 weeks after BMCMC transfer.

## Generation of PCMCs

To generate PCMCs,[E7] peritoneal cells from C57BL/6J mice were maintained *in vitro* for 4 weeks in medium containing stem cell factor (10 ng/mL; R&D Systems, Minneapolis, Minn) until MCs represented greater

than 95% of the total nonadherent cells, as indicated by May-Grünwald-Giemsa staining.

## *In vitro* MC degranulation assay

We assessed the ability of serum samples (collected 24 hours before the challenge) to sensitize PCMCs for activation in response to OVA *in vitro* as an *ex vivo* method to quantify the presence and functional activity of OVA-specific IgE.[E8] PCMCs were incubated overnight with sera from PBS-treated or OVA-sensitized mice diluted 1:10 in the PCMC complete culture medium (described above). Cells were washed with PBS and stimulated with 10 ng/mL OVA for 30 minutes at 37°C. PCMCs were washed and incubated with avidin–fluorescein isothiocyanate (a probe that specifically binds to heparin contained in MC granules) and PI for 10 minutes on ice. The extent of MC degranulation was assessed in live ($PI^-$) cells by measuring the membrane-bound fraction of granules with flow cytometry, as described previously.[E9] In some experiments serum samples were incubated with a rat $IgG_1$ anti-IgE antibody (R35-92; 50 μg/mL; BD PharMingen) or a rat $IgG_1$ isotype control antibody (R3-34; 50 μg/mL; BD PharMingen) for 30 minutes at 37°C before incubation with BMCMCs.

## OVA-induced model of active anaphylaxis using alum and *B pertussis* for sensitization

Mice were sensitized intraperitoneally with 100 μg of endotoxin-free OVA (Endograde OVA; BioVendor; <0.01 EU of endotoxin per injection), 300 ng of *B pertussis* toxin (Sigma), and 1 mg of Aluminum Hydroxide Gel Adjuvant (alum; InvivoGen, San Diego, Calif) in 200 μL of PBS. Three weeks after sensitization, mice were challenged intraperitoneally with 500 μg of OVA. Rectal measurements of body temperature were performed immediately before (time 0) and at different time points for up to 2 hours after challenge.

## IgE-mediated PSA

Purified mouse IgE anti-TNP antibodies (clone C38-2; BD PharMingen) were administered intraperitoneally at a dose of 20 μg in 200 μL of PBS, followed by an intraperitoneal challenge with 500 μg of $TNP_{(21\text{-}31)}$-BSA (Santa Cruz Biotechnology) in PBS 24 hours later. Rectal measurements of body temperature were performed immediately before (time 0) and at different time points for up to 2 hours after challenge.

## Measurement of histamine levels and PAF-AH activity

Histamine levels were measured in plasma collected 20 minutes after challenge with OVA by using a commercially available ELISA kit (Histamine EIA; Beckman Coulter, Fullerton, Calif). For measurement of PAF-AH activity, spleens were collected 20 minutes after OVA challenge, weighed, and dissociated into single cells in 400 μL of cold buffer (0.1 mol/L Tris-HCl, pH 7.2). Cell suspensions were sonicated in ice for 10 seconds and centrifuged (20,000*g* for 10 minutes at 4°C), and supernatant was collected for the assay. PAF-AH activity was measured in the supernatant by using a commercially available assay, according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, Mich). Results were expressed as enzymatic activity per milligram of spleen.

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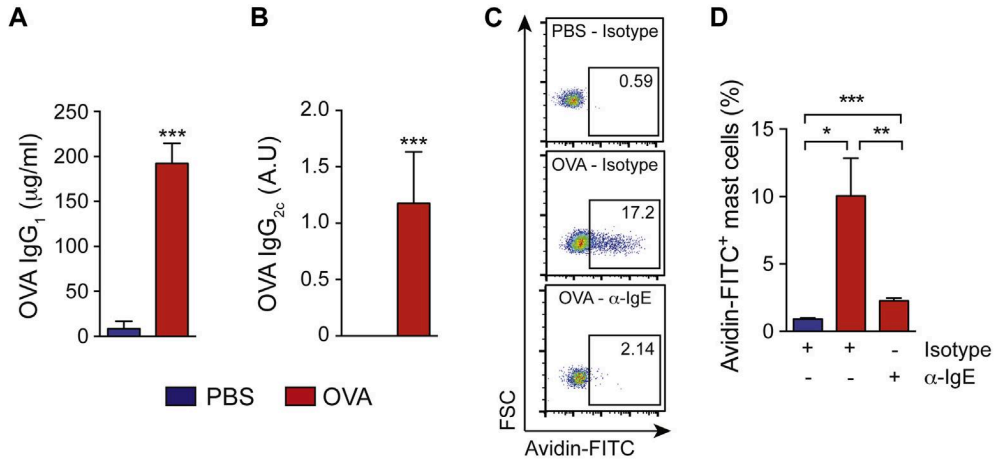


**FIG E1.** Concentrations of OVA-specific $IgG_1$ and $IgG_{2c}$ and functional activity of OVA-specific IgE in sera from PBS-treated or OVA-sensitized C57BL/6 mice. **A** and **B,** Concentrations of OVA-specific $IgG_1$ (Fig E1, *A*) and OVA-specific $IgG_{2c}$ (Fig E1, *B*) in serum samples collected 24 hours before challenge in PBS-treated or OVA-sensitized C57BL/6 mice. *A.U,* Arbitrary units. **C** and **D,** Sera from PBS-treated or OVA-sensitized mice was incubated for 30 minutes with an anti-IgE antibody (to block soluble IgE) or an isotype control antibody, as indicated. Samples were then incubated overnight with PCMCs. PCMCs were washed and stimulated for 30 minutes with OVA. MC degranulation was monitored by means of fluorescence-activated cell sorting analysis with avidin–fluorescein isothiocyanate *(Avidin-FITC)* to stain membrane-bound exocytosed granules. Representative fluorescence-activated cell sorting profiles (Fig E1, *C*) and percentages of avidin-$FITC^+$ degranulated cells (Fig E1, *D*) are shown. *FSC,* Forward scatter. Data are pooled from serum samples collected from 2 (PBS group, total n = 5) or 3 (OVA group, total n = 10) independent experiments. $*P < .05$, $**P < .01$, and $***P < .001$ versus the corresponding control group or the indicated group.

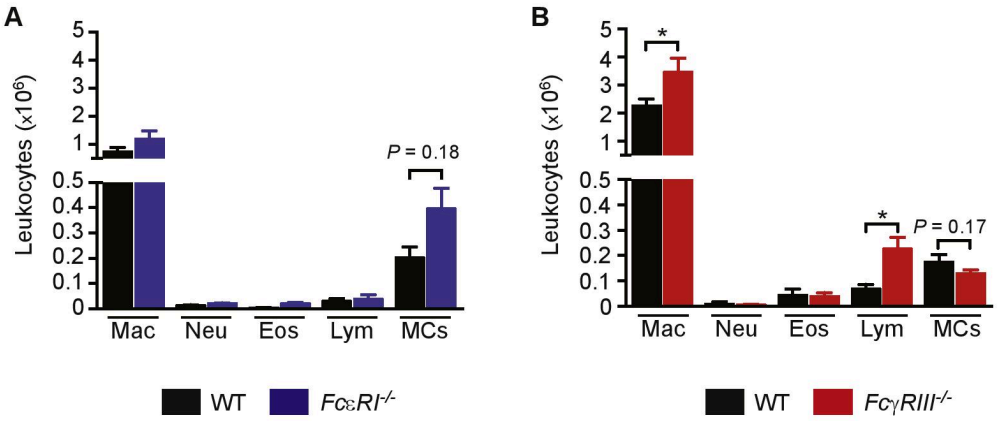


**FIG E2.** Baseline numbers of intraperitoneal leukocytes in WT, FcεRI$^{-/-}$, and FcγRIII$^{-/-}$ mice. Numbers of leukocytes in the peritoneal lavage fluid of naive WT and FcεRI$^{-/-}$ mice **(A)** or naive WT and FcγRIII$^{-/-}$ mice (**B**; n = 6 per group) are shown. *$P < .05$. *Eos*, Eosinophils; *Lym*, lymphocytes; *Mac*, macrophages; *Neu*, neutrophils.

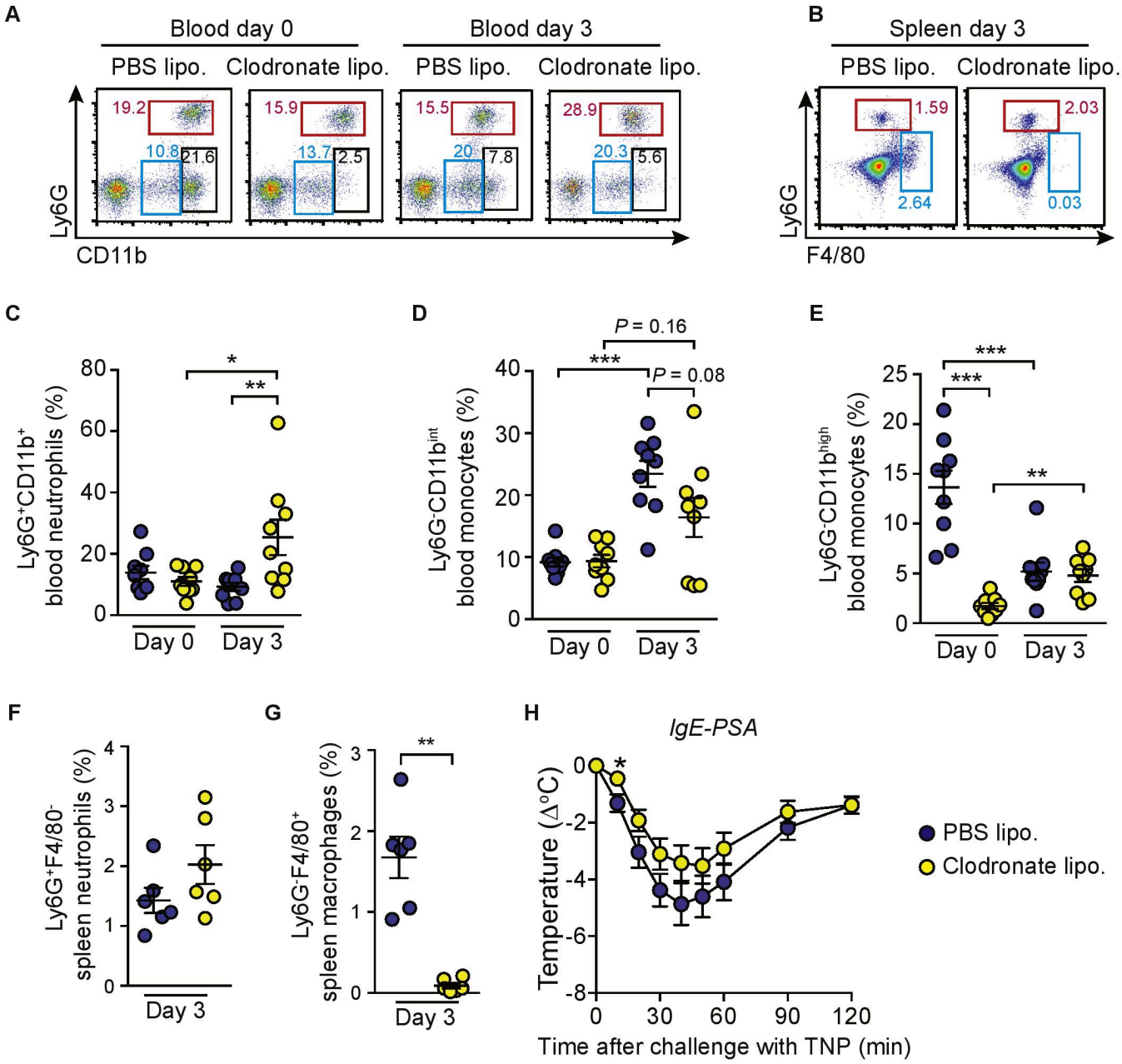


**FIG E3.** Effect of treatment with clodronate liposomes on macrophages, monocytes, neutrophils, and MCs in C57BL/6 mice. OVA-sensitized mice were treated with clodronate liposomes (150 μg administered intraperitoneally plus 150 μg administered intravenously) or the same amount of PBS liposomes (as a control) 24 hours before challenge with OVA. **A,** Representative fluorescence-activated cell sorting profile showing Ly6G$^-$CD11b$^{high}$ monocytes *(black rectangles)*, Ly6G$^-$CD11b$^{int}$ monocytes *(blue rectangles)*, and Ly6G$^+$CD11b$^+$ neutrophils *(red rectangles)* 1 hour before *(Blood day 0)* and 3 days after *(Blood day 3)* challenge with OVA. **B,** Representative fluorescence-activated cell sorting profile showing Ly6G$^-$F4/80$^+$ macrophages *(blue rectangles)* and Ly6G$^+$F4/80$^-$ neutrophils *(red rectangles)* 3 days after challenge with OVA. Numbers in Fig E3, *A* and *B*, indicate percentages of each cell population. **C-G,** Percentage of blood neutrophils (Fig E3, *C*), CD11b$^{int}$ blood monocytes (Fig E3, *D*), CD11b$^{high}$ blood monocytes (Fig E3, *E*), spleen neutrophils (Fig E3, *F*), and spleen macrophages (Fig E3, *G*) at the indicated time points. **H,** IgE-mediated PSA in mice treated with clodronate liposomes *(Clodronate lipo.)* of PBS liposomes *(PBS lipo.)*. Fluorescence-activated cell sorting profiles in Fig E3, *A* and *B*, are representative of 3 and 2 independent experiments, respectively. Data are pooled from 2 (total n = 6-8 per group; Fig E3, *C-E* and *H*) or 3 (total n = 9 per group; Fig E3, *F* and *G*) independent experiments. *$P < .05$, **$P < .01$, or ***$P < .001$ versus the indicated group.

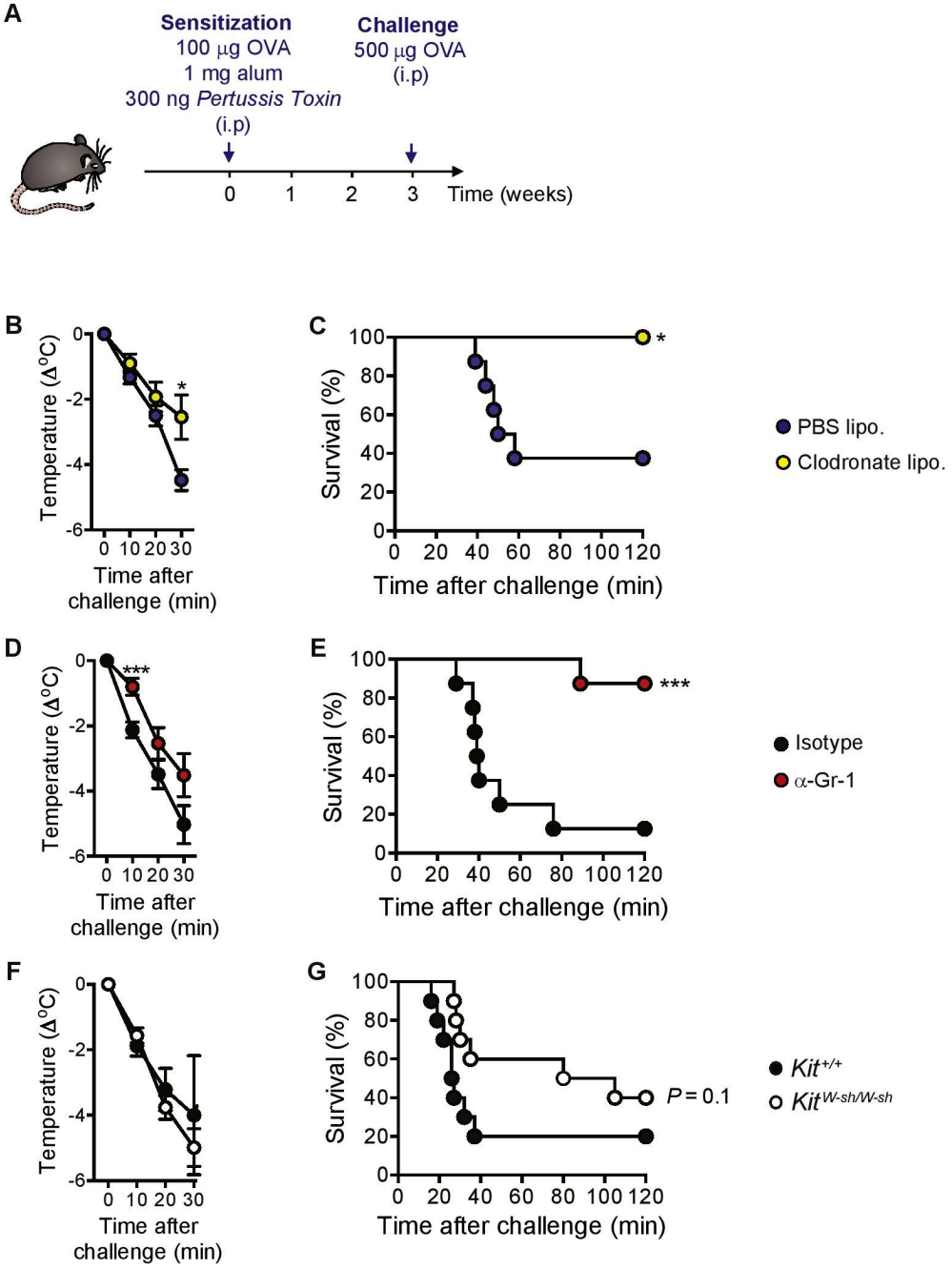


**FIG E4.** ASA in monocyte/macrophage-depleted mice, neutrophil-depleted mice, and MC-deficient mice sensitized with OVA together with alum and *B pertussis* toxin. **A,** Experimental outline. For monocyte/macrophage depletion, OVA-sensitized mice were treated with clodronate liposomes *(Clodronate lipo.)* or PBS liposomes (*PBS lipo.*; as a control) 24 hours before challenge with OVA. For neutrophil depletion, OVA-sensitized mice were treated with an anti–Gr-1 antibody or an isotype control 40 hours before challenge with OVA. *i.p.*, Intraperitoneal. **B-G,** OVA-induced hypothermia (Fig E4, *B*, *D*, and *F*) and survival (Fig E4, *C*, *E*, and *G*) in the indicated groups of mice. Data are pooled from 2 independent experiments (n = 8-10 per group). $*P < .05$ or $***P < .001$ by using an unpaired Student *t* test (Fig E4, *B*, *D*, and *F*) or a Mantel-Cox log-rank test (Fig E4, *C*, *E*, and *G*).

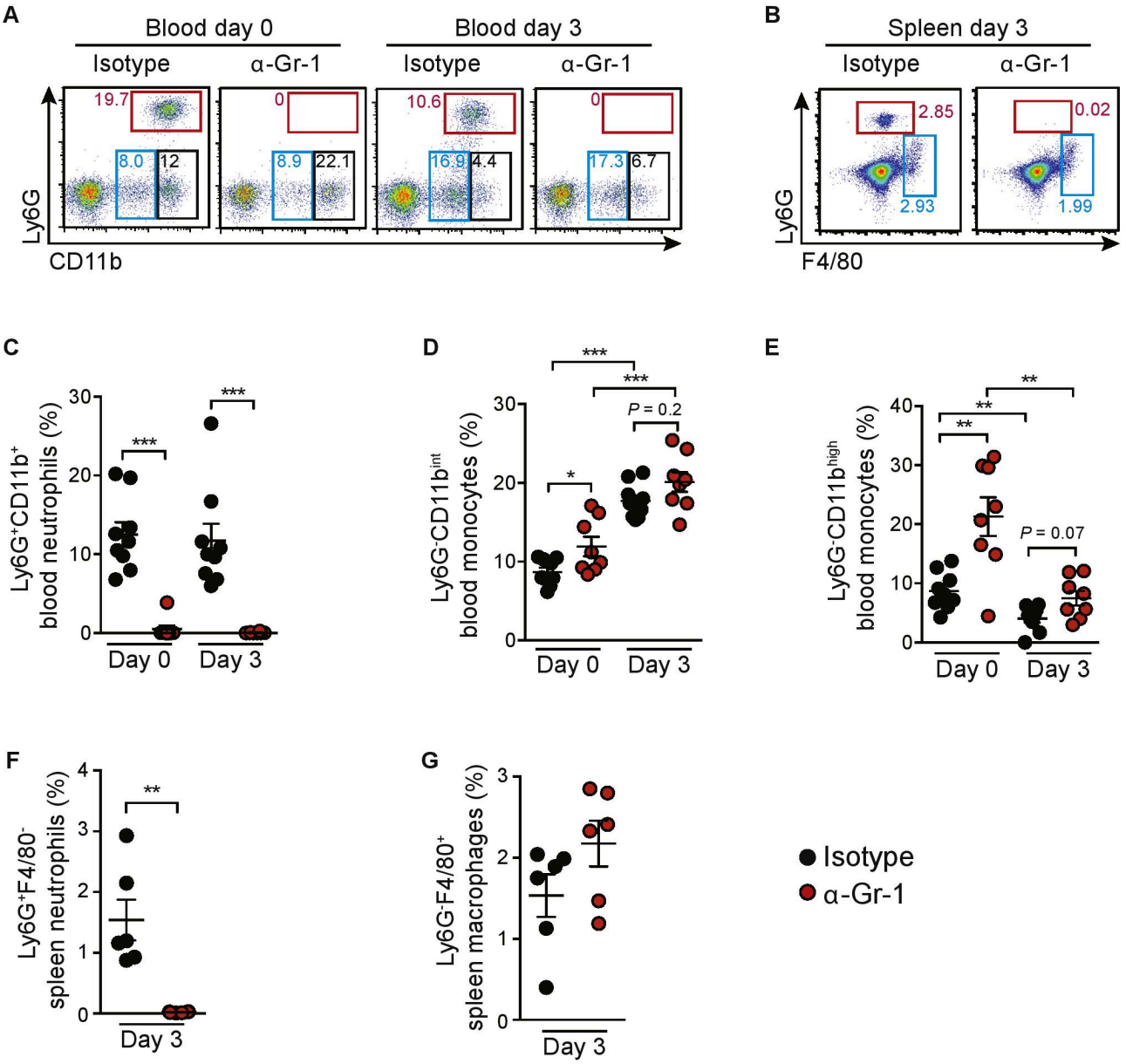


**FIG E5.** Effect of treatment with an anti–Gr-1 antibody on macrophages, monocytes, and neutrophils in C57BL/6 mice. OVA-sensitized mice were treated with an anti–Gr-1 neutrophil-depleting antibody (150 μg) or the same amount of an isotype control antibody 40 hours before and 24 hours after challenge with OVA. **A,** Representative fluorescence-activated cell sorting profile showing $Ly6G^{-}CD11b^{high}$ monocytes *(black rectangles)*, $Ly6G^{-}CD11b^{int}$ monocytes *(blue rectangles)*, and $Ly6G^{+}CD11b^{+}$ neutrophils *(red rectangles)* 1 hour before *(Blood day 0)* and 3 days after *(Blood day 3)* challenge with OVA. **B,** Representative fluorescence-activated cell sorting profile showing $Ly6G^{-}F4/80^{+}$ macrophages *(blue rectangles)* and $Ly6G^{+}F4/80^{-}$ neutrophils *(red rectangles)* 3 days after challenge with OVA. Numbers in Fog E5, *A* and *B*, indicate percentages of each cell population. **C-G,** Percentage of blood neutrophils (Fig E5, *C*), $CD11b^{int}$ blood monocytes (Fig E5, *D*), $CD11b^{high}$ blood monocytes (Fig E5, *E*), spleen neutrophils (Fig E5, *F*), and spleen macrophages (Fig E5, *G*) at the indicated time point. Fluorescence-activated cell sorting profiles in Fig E5, *A* and *B*, are representative of 3 and 2 independent experiments, respectively. Data are pooled from 2 (total n = 6 per group; Fig E5, *C-E*) or 3 (total n = 8-9 per group; Fig E5, *F* and *G*) independent experiments. **P* < .05, ***P* < .01, or ****P* < .001 versus indicated group.

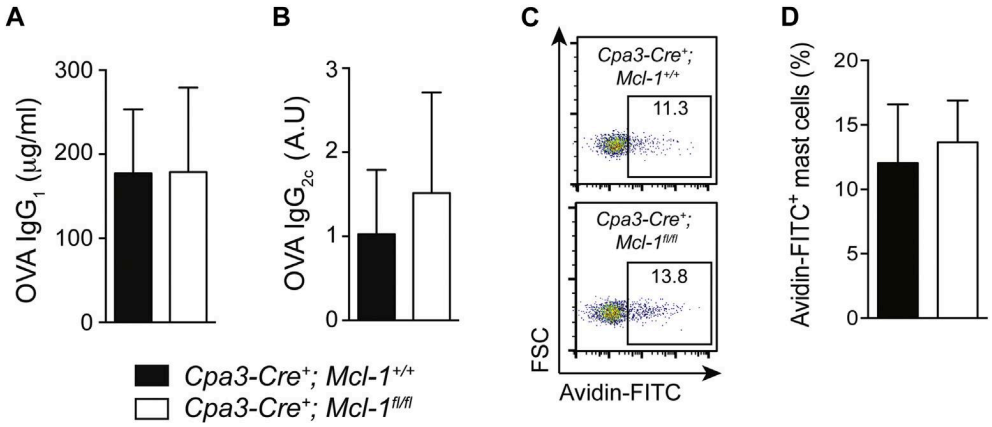


**FIG E6.** Concentrations of OVA-specific $IgG_1$ and $IgG_{2c}$ and functional activity of OVA-specific IgE in sera from OVA-sensitized MC- and basophil-deficient *Cpa3-Cre*$^+$*; Mcl-1*$^{fl/fl}$ mice. **A** and **B,** Concentrations of OVA-specific $IgG_1$ (Fig E6, *A*) and OVA-specific $IgG_{2c}$ (Fig E6, *B*) in serum samples collected 24 hours before challenge in OVA-sensitized mice. *A.U,* Arbitrary units. **C** and **D,** PCMCs were incubated overnight with sera from OVA-sensitized *Cpa3-Cre*$^+$*; Mcl-1*$^{fl/fl}$ mice or littermate control *(Cpa3-Cre*$^+$*; Mcl-1*$^{+/+}$*)* mice. PCMCs were washed and stimulated for 30 minutes with OVA. MC degranulation was monitored by means of fluorescence-activated cell sorting analysis with avidin–fluorescein isothiocyanate *(Avidin-FITC)* to stain membrane-bound exocytosed granules. Representative fluorescence-activated cell sorting profiles (Fig E6, *C*) and percentage of avidin-FITC$^+$ degranulated cells (Fig E6, *D*) are shown. Data are pooled from serum samples collected from 3 independent experiments (total n = 9-14 per group). Differences between groups are not significant ($P > .05$).

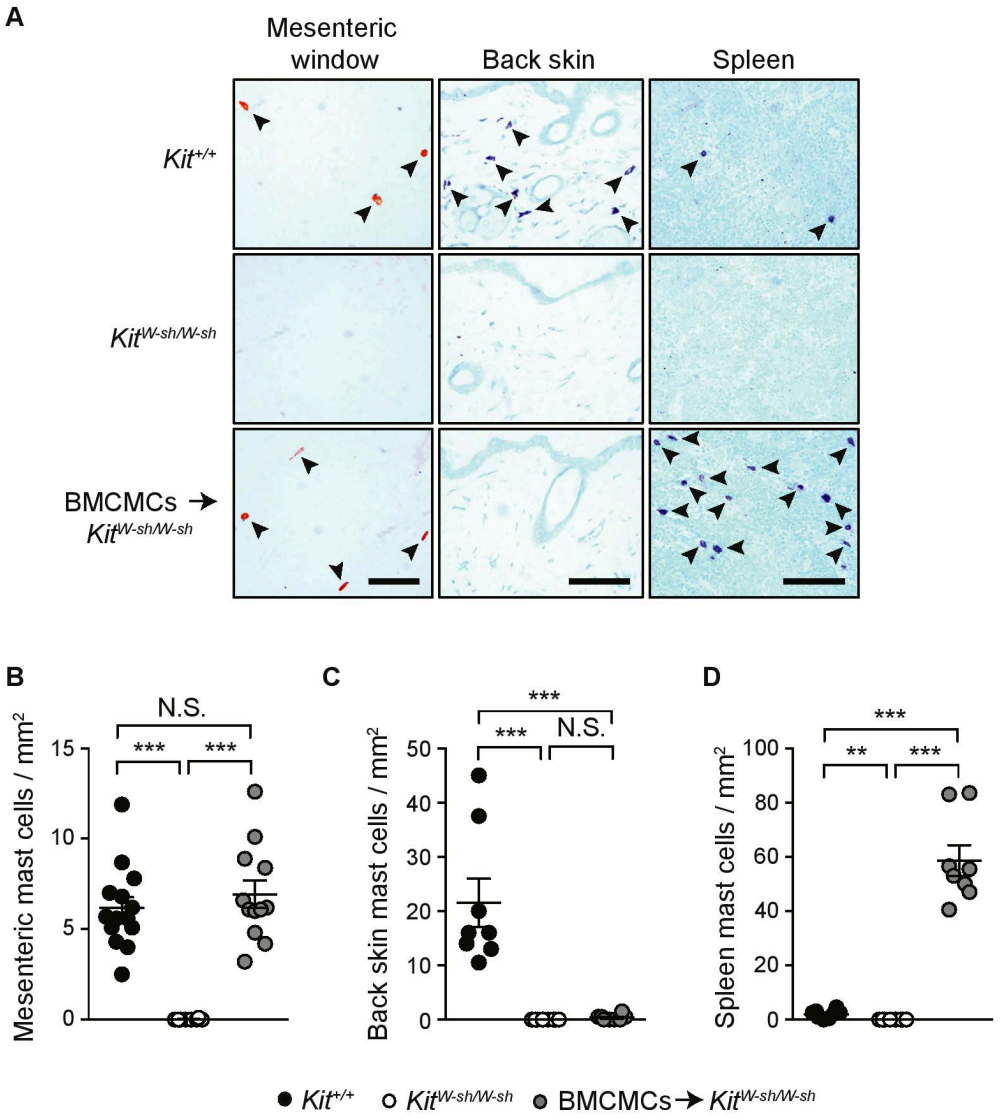


**FIG E7.** Distribution and numbers of MCs in the skin, spleen, and mesenteric windows of $Kit^{+/+}$ WT mice, MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice, and $Kit^{W\text{-}sh/W\text{-}sh}$ mice engrafted with WT BMCMCs. Tissue samples were collected 3 days after challenge with OVA in OVA-sensitized mice. Representative pictures **(A)** and quantification of MCs in the mesentery (mesenteric window; **B**), back skin **(C)**, and spleen **(D)** are shown. MCs in the mesentery (Csaba stain), back skin, and spleen (Toluidine blue stain) are indicated by *arrowheads* in photomicrographs. Pictures in Fig E7, *A*, are representative of at least 3 independent experiments. Data in Fig E7, *B-D*, are pooled from at least 3 independent experiments (total n = 8-14 per group). $^{**}P < .01$ or $^{***}P < .001$ versus the indicated group. *N.S.*, Not significant ($P > .05$).

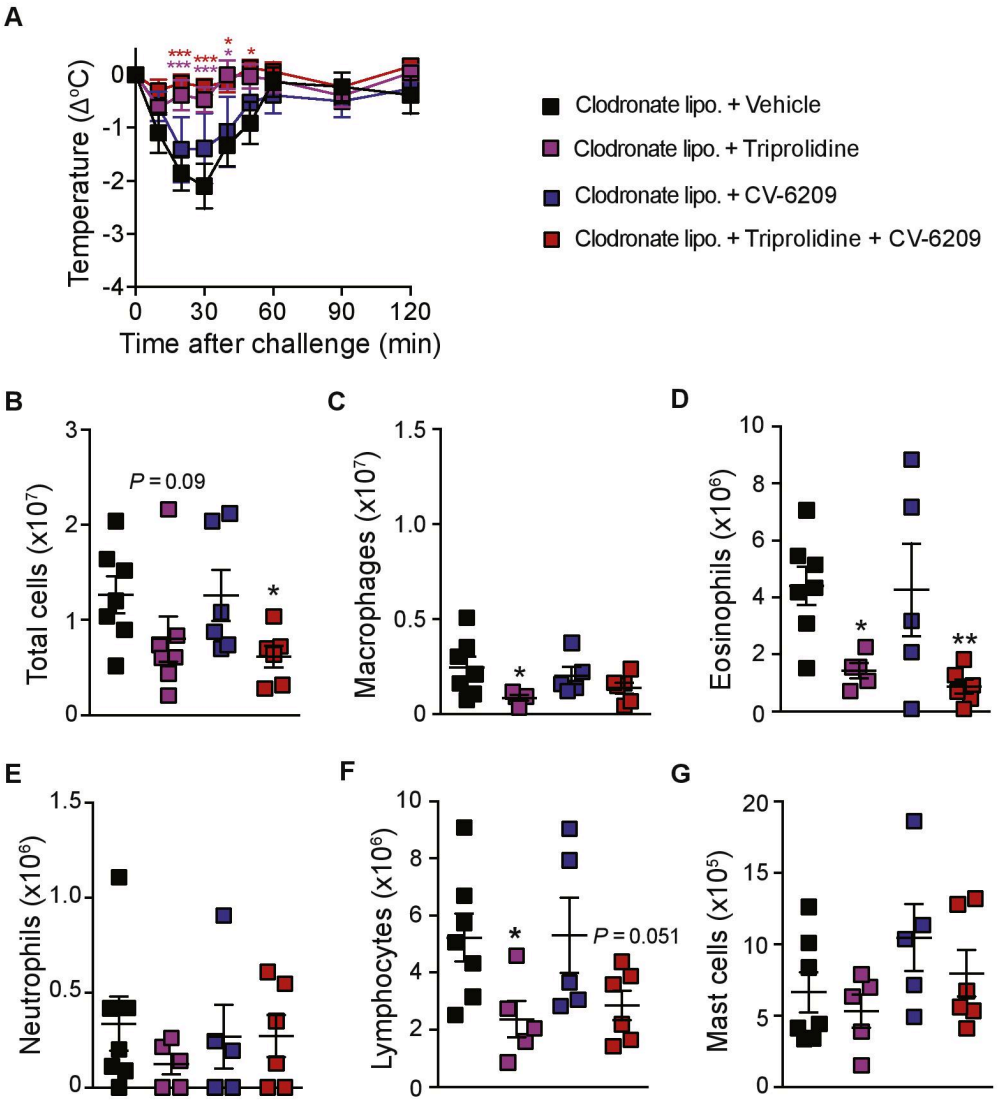


**FIG E8.** Roles of histamine and PAF in OVA-induced ASA in monocyte/macrophage-depleted mice. OVA-sensitized mice were treated with clodronate liposomes *(Clodronate lipo.)* 24 hours before challenge with OVA. Mice were then treated with the $H_1$-antihistamine triprolidine or with the PAF receptor antagonist CV-6209 alone or in combination (or vehicle [saline] as a control) 30 minutes before and 1 day after challenge with OVA. **A,** OVA-induced hypothermia. **B-G,** Numbers of leukocytes in the peritoneal lavage fluid 3 days after OVA challenge. Data are pooled from 2 independent experiments (total n = 6-8 per group). $^*P < .05$, $^{**}P < .01$, or $^{***}P < .001$ versus the vehicle group.

# 4. Chapter 4 - The anti-IgE antibody Omalizumab can induce adverse reactions through engagement of Fc gamma receptors

Our data obtained in mice indicate that mouse IgG contribute to systemic anaphylaxis when sensitization is performed in the absence of adjuvant (paper I), as it may be in humans. To assess the clinical relevance of our findings, we then studied if and how human IgG could also trigger anaphylaxis. Most therapeutic antibodies are human or humanized IgG. Some of these mAbs, including the anti-IgE mAb Omalizumab, have been reported to induce anaphylaxis in some patients. We thus decided to evaluate whether Omalizumab-induced anaphylaxis could rely on engagement of human FcγRs, and thus represent an example of the 'alternative' pathway of anaphylaxis.

We first tested whether immune complexes (ICs) formed by the omalizumab and human IgE could bind to Fcγ receptors. Our *in vitro* results clearly revealed that these ICs can bind all activating hFcγRs (but not the inhibitory hFcγRIIB), and have the potential to activate human neutrophils *ex vivo*. We then demonstrated that Omalizumab:IgE ICs can induce both skin inflammation (at the injection site) and systemic anaphylaxis in $hFc\gamma R^{KI}$ mice which express all hFcγRs in place of the mouse receptors. We further demonstrated that these effects were mediated by hFcγRs, since they were markedly reduced in $Fc\gamma R^{null}$ mice (which lack all FcγRs).

Because IgG binds their FcγRs through the Fc portion of the Abs, we decided to generate an Fc-engineered variant of Omalizumab which is mutated in a glycosylation site (N297) necessary for binding to FcγRs. This single mutation was able to significantly reduce both local and systemic adverse reactions induced by Omalizumab, without perturbing its ability to block IgE. Therefore, we propose that this Fc-engineered mAb could be a potential candidate as an alternative to Omalizumab in patients with high levels of IgE and/or a history of anaphylaxis or other adverse reactions to the drug. The potential industrial applicability of our findings is protected by a patent application (PCT/EP2019/059414 – April 12$^{th}$ 2019).

## 4.1 PAPER II

### The anti-IgE antibody Omalizumab can induce adverse reactions through engagement of Fc gamma receptors

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# The anti-IgE antibody Omalizumab can induce adverse reactions through engagement of Fc gamma receptors


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## Running title

Xolair induces FcγR-dependent inflammation

## Abstract

Omalizumab is an anti-IgE monoclonal antibody (mAb) approved for the treatment of severe asthma and chronic spontaneous urticaria (CSU). Use of Omalizumab is associated with reported side effects, ranging from local skin inflammation at the injection site to systemic anaphylaxis. To date, the mechanisms through which Omalizumab induces adverse reactions are still unknown. Here, we demonstrate that immune complexes formed between Omalizumab and IgE can induce both skin inflammation and anaphylaxis through engagement of IgG receptors (FcγRs) in FcγR-humanized mice. We further developed an Fc-engineered mutant version of Omalizumab, and demonstrate that this mAb is equally potent as Omalizumab at blocking IgE-mediated allergic reactions, but does not induce FcγR-dependent adverse reactions.

## Introduction

IgE antibodies are key mediators of allergic diseases (1-3). Upon exposure to an allergen in allergic patients, such allergen is recognized by IgE bound to their high-affinity receptor FcεRI expressed mainly on the surface of mast cells and basophils, which promotes the immediate activation of these cells, and the release of both preformed and newly synthesized inflammatory mediators such as histamine, responsible for allergic symptoms (3).

Omalizumab (Xolair®) is a recombinant humanized IgG1 mAb directed against IgE (4). Omalizumab binds to the Cε3 domain of free IgE, and thereby impairs binding of IgE to both FcεRI and the low-affinity IgE receptor CD23 (FcεRII) (5-7). Omalizumab does not recognize IgE already bound to FcεRI or CD23, and therefore cannot induce cell activation by crosslinking of IgE receptors (5, 7). Omalizumab is approved for the treatment of severe asthma (8) and chronic spontaneous urticaria (9). It also shows promises for the treatment of other allergic diseases, including food allergy (10).

Treatment with Omalizumab is associated with adverse reactions, ranging from local skin inflammation at the injection site to severe systemic allergic shock (*i.e.* 'anaphylaxis') in ~0.1-0.2% of patients (11-13). The mechanism by which this drug induces side effects is still unknown. Notably, Omalizumab does not induce the formation of anti-drug antibodies, and most cases of anaphylaxis occur within the first three injections of the drug (11-13).

79

80 We hypothesized that the formation of immune complexes (ICs) between
81 Omalizumab and IgE could be responsible for some of the adverse reactions observed
82 with this therapeutic mAb. Using mice humanized for all IgG receptors (FcγRs), we
83 demonstrate here that Omalizumab/IgE ICs can induce both skin inflammation at the site
84 of injection of the drug, as well as systemic anaphylaxis through engagement of FcγRs.
85 Finally, we developed an Fc-engineered version of Omalizumab which blocks IgE-
86 mediated allergic reactions without inducing FcγR-dependent adverse reactions.

87

## Results and Discussion

We first co-incubated Omalizumab and human IgE (termed IgE herein) *in vitro* to form ICs, and assessed the molecular mass of these ICs by size exclusion chromatography coupled to static light scattering (SEC-SLS). As reported previously both *in vitro* (14) and *in vivo* in cynomolgus monkeys (15), these ICs were of limited size, mainly consisting of trimeric structures (**Supplemental Figure 1**). It was initially suggested that such small ICs have a low potential to engage FcγRs (15). However, we found that Omalizumab/IgE ICs potently bind all activating human FcγRs (FcγRI, IIA, IIIA and IIIB), but not the inhibitory FcγRIIB that has the lowest affinity for human IgG1 among FcγRs (16) (**Figure 1A**). As expected, we also observed that Omalizumab binds human complement component C1q in a dose-dependent manner (**Figure 1B**).

As neutrophils were reported to contribute to IgG-mediated inflammation and anaphylaxis (17), we next evaluated whether Omalizumab/IgE ICs can activate neutrophils through engagement of FcγRs. We purified human neutrophils from healthy donors and incubated these cells with Omalizumab/IgE ICs. We found that such ICs induce marked upregulation of CD66b and downregulation of CD62L on the surface of neutrophils, which are considered hallmarks of neutrophil activation (18, 19) (**Figure 1, C and D**). Omalizumab/IgE ICs also induced downregulation of FcγRII (CD32) (**Figure 1E**). As human neutrophils express FcγRIIA and not FcγRIIB (20), and Omalizumab/IgE ICs do not bind FcγRIIB (**Figure 1A**), our results indicate that the ICs induce active engagement of FcγRIIA in human neutrophils.

To further confirm the role of FcγRs in neutrophil activation, we performed similar experiments with neutrophils purified from hFcγR$^{KI}$ mice (in which all mouse FcγR have been replaced with human FcγRs) (20, 21) or FcγR$^{null}$ mice (which are deficient for all FcγR) as a control (**Figure 1F**). Omalizumab/IgE ICs induced a downregulation of CD62L in neutrophils purified from hFcγR$^{KI}$ mice, but not, as expected, in neutrophils purified from FcγR$^{null}$ mice (**Figure 1F**). Altogether, these results demonstrate that Omalizumab/IgE can activate neutrophils through engagement of human FcγRs.

The most frequent side effect observed with Omalizumab is skin inflammation at the site of subcutaneous injection of the drug (13). We hypothesized that such local inflammation could be a consequence of active FcγRs engagement. To assess this *in vivo*, we injected Omalizumab/IgE ICs subcutaneously into hairless (to avoid shaving-induced skin inflammation) nude hFcγR$^{KI}$ mice and nude FcγR$^{null}$ mice, and assessed skin inflammation 2 h after injection by bioluminescence imaging of myeloperoxidase (MPO) activity after luminol administration (22, 23). We observed a strong MPO activity at the site of Omalizumab/IgE ICs injection in hFcγR$^{KI}$ mice (**Figure 2, A and B**). By contrast, MPO activity was markedly reduced at the site of injection of IgE alone or Omalizumab alone, and at the site of injection of ICs in FcγR$^{null}$ mice. Thus, our results indicate that Omalizumab/IgE ICs can induce local skin inflammation through engagement of human FcγRs.

The most dramatic side effect reported for Omalizumab is systemic anaphylaxis (12, 13). We thus assessed whether Omalizumab/IgE ICs can induce anaphylaxis in hFcγR$^{KI}$ mice. Intravenous (i.v.) injection of Omalizumab/IgE ICs induced significant hypothermia (the main readout of anaphylaxis in mice (24)) in hFcγR$^{KI}$ mice (**Figure 2C**). Importantly, hypothermia was not observed upon injection of ICs in hFcγR$^{null}$ mice, or injection of IgE or Omalizumab alone in hFcγR$^{KI}$ mice (**Supplemental Figure 2**), demonstrating that Omalizumab/IgE ICs induce systemic anaphylaxis through engagement of human FcγRs.

Since these results were obtained with injection of preformed ICs, we next assessed whether *in vivo* formation of Omalizumab/IgE ICs could trigger anaphylaxis in hFcγR$^{KI}$ mice. However, injection of IgE followed by injection of Omalizumab in naïve hFcγR$^{KI}$ mice did not induce signs of anaphylaxis (**Figure 2D**). Anaphylaxis to Omalizumab remains a rare event, occurring in 0.1-0.2% of Omalizumab-treated patients. Interestingly, three clinical studies have shown that the occurrence of anaphylaxis to Omalizumab is significantly increased in patients with prior history of anaphylaxis unrelated to Omalizumab (11-13). It is thus possible that a subpopulation of highly atopic patients is more prone to develop anaphylaxis to Omalizumab. To mimic this, we pre-treated hFcγR$^{KI}$ mice with IL-4C, a long-lasting formulation of IL-4 (25). Strikingly, IL-4C-treated mice developed marked anaphylaxis upon sequential injection of IgE and Omalizumab (**Figure 2D**). Altogether, our data in FcγR-humanized mice are consistent with prior clinical observations (11-13) which suggest that the risk of anaphylaxis to Omalizumab could be markedly increased in highly atopic subjects.

157

158 Based on these results, we decided to clone and produce an Fc-engineered form of
159 Omalizumab (using available Omalizumab $V_H$ and $V_L$ sequences (4)) lacking the N-
160 linked glycan attached to asparagine 297 on the human IgG1 Fc portion ($N_{297}A$ mutation)
161 to reduce binding to FcγRs and complement (26, 27). We refer to this mAb as 'NA anti-
162 IgE'. As a control, we also produced a non-mutated version of this mAb, which we call
163 'WT anti-IgE'. Both the WT and NA anti-IgE mainly formed trimers when incubated
164 with IgE *in vitro* (**Supplemental Figure 3, A-D**), which is consistent with the data we
165 obtained when using commercial Omalizumab (**Supplemental Figure 1**). As expected,
166 ICs made of IgE and the WT anti-IgE could bind all human activating FcγRs, while
167 binding to FcγRs was markedly reduced with ICs made of IgE and the Fc-engineered NA
168 anti-IgE (**Figure 3A**). Indeed, IgE/NA anti-IgE ICs could only bind to FcγRI, which is
169 consistent with a previous report showing that the $N_{297}A$ mutation does not abrogate
170 binding of ICs to this high affinity FcγR (28). In addition, WT anti-IgE could bind human
171 C1q (**Supplemental Figure 3E**), as observed with commercial Omalizumab (**Figure 1B**),
172 but we detected no binding to C1q with the NA anti-IgE (**Supplemental Figure 3E**).
173 Finally, we observed activation of human neutrophils with ICs made of IgE and the WT
174 anti-IgE, but markedly reduced activation with ICs made of IgE and the Fc-engineered
175 NA anti-IgE mAb (**Figure 3, B-D**). Altogether, these results show that the Fc-engineered
176 version of Omalizumab we produced has markedly reduced binding to FcγRs.
177 complement, and does not activate neutrophils.

178

FcRn/β2m heterodimers extend the half-life of IgG by reducing lysosomal degradation in endothelial cells (29). To assess the half-life of our anti-IgE mAbs *in vivo*, we generated hFcγR$^{KI}$hFcRn$^{KI}$hβ2m$^{KI}$ mice which recapitulate binding of human IgG to all human FcγRs and to the human FcRn complex (FcRn-β2m) that recycles and protects IgG from proteolytic degradation (**Supplemental Figure 4**). We injected WT or Fc-engineered NA anti-IgE into hFcγR$^{KI}$hFcRn$^{KI}$hβ2m$^{KI}$ mice, and observed similar levels of mAbs in sera collected at different time-points after injection, demonstrating that the $N_{297}$A mutation does not affect the half-life of the anti-IgE mAb *in vivo* (**Figure 3E**).

We also verified that the $N_{297}$A mutation does not affect the ability of the anti-IgE mAb to block IgE and IgE-mediated reactions. Both the WT and NA anti-IgE recognized IgE with the same affinity (**Supplemental Figure 5A**), and were equally potent at blocking binding of IgE to human mast cells *in vitro* (**Supplemental Figure 5B**). Moreover, we showed that pre-treatment of hFcεRI$^{Tg}$ mice (which express the human IgE high-affinity receptor hFcεRI, and therefore respond to human IgE (30)) with either Omalizumab or the NA anti-IgE can block IgE-mediated passive systemic anaphylaxis (**Figure 4A**). Altogether, our results demonstrate that the Fc-engineered NA anti-IgE mAb is equally potent as Omalizumab at blocking IgE and IgE-mediated allergic reactions.

We then compared skin inflammation induced by ICs made of IgE and Omalizumab or IgE and the NA anti-IgE in hFcγR$^{KI}$ mice. Injection of Omalizumab/IgE ICs induced marked MPO activity in the skin (**Figure 4, B and C**). By contrast, skin

MPO activity was reduced to levels observed with injection of IgE alone in hFcγR$^{KI}$ mice injected with ICs made of IgE and the NA anti-IgE (**Figure 4, B and C**). Finally, we compared the ability of ICs made of IgE and Omalizumab or IgE and the Fc-engineered NA anti-IgE to induce systemic anaphylaxis in hFcγR$^{KI}$ mice. As expected, we observed anaphylaxis in mice injected i.v. with Omalizumab/IgE ICs (**Figure 4D**). By great contrast, no sign of anaphylaxis was observed in mice injected with ICs made of IgE and the NA anti-IgE mAb (**Figure 4D**).

In summary, our findings demonstrate that Omalizumab forms ICs with IgE which can activate neutrophils, and induce local skin inflammation and systemic anaphylaxis through human FcγRs in FcR-humanized mice. Such findings could explain some of the side effects which have been described in patients treated with Omalizumab (12, 13). The Fc-engineered anti-IgE mAb we developed is equally potent as Omalizumab at blocking IgE-mediated allergic reactions but does not induce FcγR-mediated inflammation. It could thus potentially be used in patients with very high levels of IgE, and/or in patients with a history of anaphylaxis or other adverse reactions to Omalizumab. Finally, we envision that IC-mediated engagement of FcγRs could be a more general mechanism of therapeutic monoclonal antibodies-mediated adverse reactions.

## Materials and Methods

***Antibodies***. For anti-NP human IgE mAbs (termed anti-NP IgE herein), WT or Fc-engineered human IgG1 anti-human IgE mAbs (termed anti-IgE mAbs herein) please refer to Supplemental Information.

***Mice.*** hFcγR$^{KI}$ and FcγR$^{null}$ mice were generated by Regeneron Pharmaceuticals Inc. to express hFcγRI, hFcγRIIa$_{H131}$, hFcγRIIb$_{I232}$, hFcγRIIc$_{stop13}$, hFcγRIIIa$_{V158}$ and hFcγRIIIb$_{NA2}$ polymorphic variants, or no FcγR, respectively, as described previously (21). hFcγR$^{KI}$hFcRn$^{KI}$hβ2m$^{KI}$ mice were generated by intercrossing of *hFcγR$^{KI}$* mice with both hFcRn$^{KI}$ (VG1481) and hβ2m$^{KI}$ (VG5153) designed and generated by Regeneron Pharmaceuticals Inc. on a mixed 62.5% C57BL/6N and 37.5% 129S6/SvEv genetic background. hFcεRI$^{Tg}$ mice (30) were described previously. Nude-hFcγR$^{KI}$ and nude-hFcR$^{null}$ mice were obtained by intercrossing NMRI-Foxn1$^{nu/nu}$ (nude) (Janvier labs) mice with hFcγR$^{KI}$ and FcγR$^{null}$ mice, respectively. All mice were bred at Institut Pasteur and demonstrated normal development and breeding patterns. We used age-matched mice for all experiments. All animal care and experimentation were conducted in compliance with the guidelines and specific approval of the Animal Ethics committee CETEA (Institut Pasteur, Paris, France) registered under #2013-0103, and by the French Ministry of Research under agreement #00513.02.

**In vitro *formation of Omalizumab/IgE immune complexes (ICs).*** All antibodies were centrifuged at 13.000 *g* for 15 min to remove any possible aggregate in stock solution.

247 ICs were formed by incubating anti-NP IgE or FITC-labelled anti-NP IgE with
248 Omalizumab or Fc-engineered anti-IgE mAbs at a 1:2 ratio for 1 h at 37°C under
249 agitation (250 rpm).
250
251 ***Binding of ICs to FcγRs expressed on CHO cells.*** We use a collection of Chinese
252 Hamster Ovarian (CHO) transfectants expressing FLAG-tagged human FcγRs (16) to
253 assess binding of preformed ICs to various FcγRs. Briefly, preformed ICs made of FITC-
254 labelled IgE and Omalizumab or Fc-engineered anti-IgE mAbs were incubated with
255 $5x10^4$ CHO transfectants for 1 h on ice. Cells were washed with MACS buffer
256 (PBS/0.5% BSA/ 2mM EDTA). Cell-bound ICs were detected using MACSQuant flow
257 cytometer (Miltenyi Biotec), and data were analyzed using Flowjo Software (Tree Star).
258 CHO transfectants incubated with FITC-labelled IgE alone were used as a negative
259 control. Expression of human FcγR on the surface of each CHO transfectant was
260 confirmed by flow cytometry using antibodies against FcγRI (10.1), FcγRIIA/IIB
261 (AT.10) and FcγRIIIA/IIIB (MEM-154), all from BD Biosciences.
262
263 ***IC-mediated activation of neutrophils.*** EDTA-collected blood from healthy donors was
264 obtained from the blood bank ("Etablissement Français du Sang" EFS). Human
265 neutrophils were purified with MACSxpress Neutrophil Isolation Kit (Miltenyi)
266 according to the manufacturer's instructions, and neutrophils purity was assessed by flow
267 cytometry (human neutrophils were defined as $CD45^+CD15^+CD66^+$ cells). Purified
268 human neutrophils were kept in RPMI medium containing 10% FCS, 10 ng/ml clinical
269 grade G-CSF (Miltenyi) and 50 ng/ml recombinant human IFN-γ (Miltenyi) at $5x10^5$

cells/ml. Mouse neutrophils were purified from the tibia and femur of $hFc\gamma R^{KI}$ and $Fc\gamma R^{null}$ mice by negative selection using the EasySep Mouse Neutrophil Enrichment kit (STEMCELL Technologies; >90% $Ly6G^+$ $CD11b^+$ on average) according to the manufacturer's instructions, and neutrophils purity was assessed by flow cytometry (mouse neutrophils were defined as $CD45^+CD11b^+Ly6G^+$ cells). Purified mouse neutrophils were primed in RPMI medium containing 10% FCS, 10 ng/ml mouse M-CSF (Miltenyi) and 50 ng/ml mouse IFN-γ (Miltenyi) at $5x10^5$ cells/ml for 16 h before activation with ICs. Activation of human or mouse neutrophils by ICs was performed as previously described (31). Briefly, immobilized ICs were formed by coating 96-well plates (Costar) with IgE (50 μg/ml) in 50 mM carbonate/bicarbonate buffer (pH 9.6) for 16 h, followed by blocking with 10% Ultra Low IgG FBS (Invitrogen) in PBS for 2 h and an incubation with Omalizumab or Fc-engineered anti-IgE mAbs at 100 μg/ml for 1 h in PBST (PBS 0.05% Tween20). Plates were washed 3 times with PBS, and purified neutrophils were incubated at $5x10^4$ cells/well for 1 h at 37°C. Human neutrophils were stained with fluorescently-labeled anti-CD45, anti-CD15, anti-CD66 and anti-CD62L antibodies (all from Miltenyi) for 30 min at 4°C. Mouse neutrophils were stained with fluorescently-labeled anti-CD45, anti-CD11b, anti-Ly6G and anti-CD62L antibodies (all from BD Pharmigen) for 30 min at 4°C. Activation of mouse or human neutrophils was assessed by measuring changes in expression of CD62L. Data were acquired using a MACSQuant flow cytometer (Miltenyi), and analyzed with Flowjo Software (Tree Star). Dead cells (identified by staining with propidium iodide; Gibco) were not included in the analysis.

***ICs-induced skin inflammation and myeloperoxidase (MPO) activity.*** Pre-formed ICs were diluted in saline and injected subcutaneously (s.c.) in the back skin of nude-hFcγR$^{KI}$ or nude-hFcR$^{null}$ mice at a final concentration of 100 μg IgE and 200 μg anti-IgE IgG1 mAbs in 50 μL. We injected saline, IgE or anti-IgE alone (diluted in the same conditions) as a control. Bioluminescent imaging of MPO activity was used as readout of skin inflammation 2 h after ICs injection as described previously (22, 23). Briefly, mice were injected intraperitoneally (i.p.) with luminol (Sigma; 200 mg/kg in 100 μL). 10 min later, bioluminescence resulting from MPO-mediated oxidation of luminol (30) was imaged using an IVIS Spectrum (PerkinElmer) with 5 min acquisition time using an open filter and medium binning. Total photon flux (photons/seconds) was calculated using Living-Image-v4.5 software (Xenogen Product from PerkinElmer).

***ICs-mediated passive systemic anaphylaxis (PSA).*** Pre-formed ICs were diluted in saline and injected i.v. in hFcγR$^{KI}$ or FcγR$^{null}$ mice at a final concentration of 250 μg IgE and 500 μg anti-IgE in 100μL to induce anaphylaxis. We injected saline, IgE or anti-IgE alone (diluted in the same conditions) as a control. Rectal temperature measurements were performed using a digital thermometer (YSI) immediately before (time 0) and at different time points for up to 1 h after injection of ICs.

***Omalizumab-induced anaphylaxis in IL-4C-treated mice.*** hFcγR$^{KI}$ mice were treated i.p. at days -7, -4 and -1 with saline or IL-4C. IL-4C is a long-lasting formulation of IL-4 and consists of 10 μg IL-4 (Miltenyi Biotech) mixed with 50 μg of a neutralizing rat IgG1 anti-mouse IL-4 (clone 11B11, BioXCell). These complexes slowly dissociate *in vivo*,

releasing free active IL-4 with a $t_{1/2}$ of ~1 day (25). Mice were then injected i.v. with 250 µg of anti-NP IgE, and 30 minutes later with 500 µg of Omalizumab i.v. Rectal temperature measurements were performed using a digital thermometer (YSI) immediately before (time -30 min) and at different time points for up to 1 h after injection of Omalizumab.

***Human IgE-mediated passive systemic anaphylaxis (PSA).*** hFcεRI$^{Tg}$ mice were injected intravenously (i.v.) with 700 µg anti-IgE IgG1 (Omalizumab or Fc-engineered anti-IgE mAbs) in 100 µL saline, or saline only as a control. 30 min later, mice were sensitized i.v. with 10 µg anti-NP IgE. 16 h later, mice were challenged i.v. with 500 µg NP-BSA (ratio: >20 NP molecules per BSA molecule) (Santa Cruz). Rectal temperature measurements were performed using a digital thermometer (YSI) immediately before (time 0) and at different time points for up to 1 h after challenge with NP.

***Statistical analyses.*** Data are presented as mean ± SEM. Temperature loss during PSA was compared by using 2-way repeated-measures ANOVA. Experiments with human and mouse neutrophils were analysed using one-way analysis of variance (ANOVA) with Tukey's post test. Bioluminescent detection of MPO activity *in vivo* was analysed using a Mann-Whitney test. Statistical analyses were performed with Prism Software (GraphPad Software, La Jolla, Calif). $P$ values < 0.05 are considered statistically significant.

Further methods are detailed in Supplemental Information

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## Competing interests

B.B, P.B and L.L.R are inventors on a patent related to this work. L.E.M and A.M. are employees of Regeneron Pharmaceuticals, Inc. hold stock in the company, and are inventors on patents and patent applications related to the mice used for this work. The authors declare no additional competing financial interests.

## Author contributions

Experimental design, B.B, P.B and L.L.R; Investigation, B.B, P.H, O.G, J.S, O.R.G, B.I, D.S, S.B and L.R; Formal analysis, B.B and L.R; Writing (original draft), B.B and L.R; Writing (review and editing), all authors.

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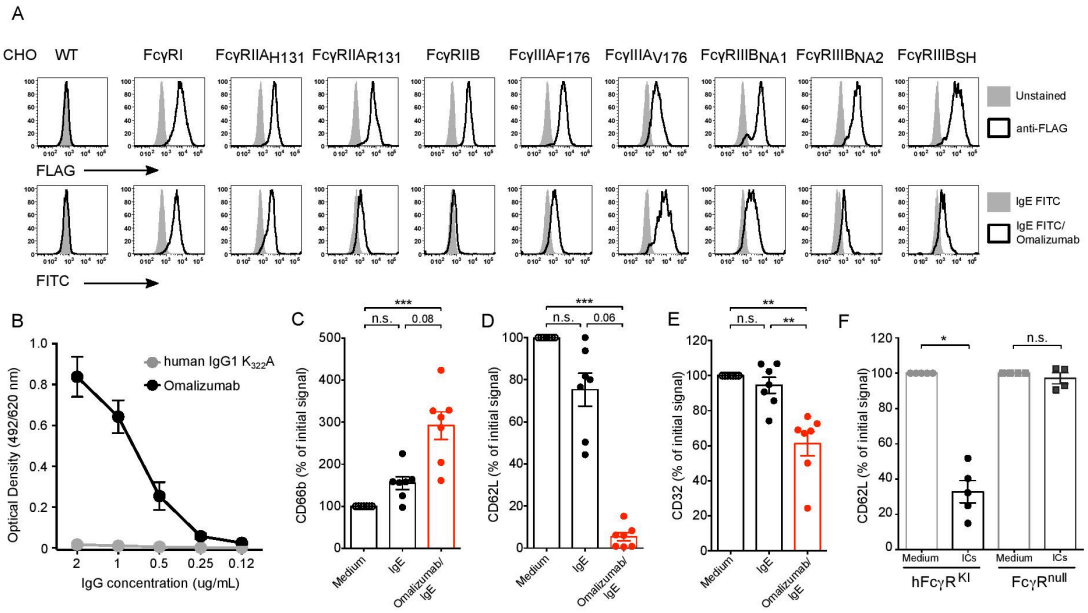


**Figure 1. Omalizumab/IgE immune complexes (ICs) bind FcγRs and activate neutrophils.** (**A**) Binding of pre-formed IgE/Omalizumab ICs to FcγRs using a bank of CHO cells stably transfected with each one of the human FcγRs (16). Upper histograms show binding of an anti-FLAG mAb (thin line), as a control for human FcγR expression, or unstained samples (solid gray). Lower histograms show binding of pre-formed IgE FITC/Omalizumab ICs (thin line) or IgE FITC alone (solid grey). Data are representative of three independent experiments. (**B**) Binding of Omalizumab to human C1q assessed by ELISA. An irrelevant human IgG1 mutated in its Fc portion at position 322 ($K_{322}A$) to preclude binding to C1q was used as negative control. Results in **B** show means ± SD from data pooled from two independent experiments (total of *n=4* replicates). (**C-E**) Expression of CD66b (**C**), CD62L (**D**) and CD32 (**E**) on purified $CD45^+CD15^+$ human neutrophils after 1 h incubation with Omalizumab/IgE immobilized ICs, IgE or medium alone. Results in **C-E** show values from neutrophils from individual donors normalized against cells stimulated with medium alone; bars indicate means ± SEM pooled from three (total *n*=7/group) independent experiments. (**F**) CD62L expression on $CD11b^+Ly6G^+$ neutrophils purified form $hFc\gamma R^{KI}$ or $Fc\gamma R^{null}$ mice after 1 h incubation with Omalizumab/IgE ICs or medium. Results in **F** show values from individual mice with bars indicating means ± SEM pooled from two ($Fc\gamma R^{null}$; total *n*=4/group) or three ($hFc\gamma R^{KI}$; total *n*=5/group) independent experiments. *, $P < 0.05$; *, $P < 0.01$; ***, $P < 0.001$ by one-way analysis of variance (ANOVA) with Kruskal-Wallis post-test. n.s., not significant ($P > 0.05$).

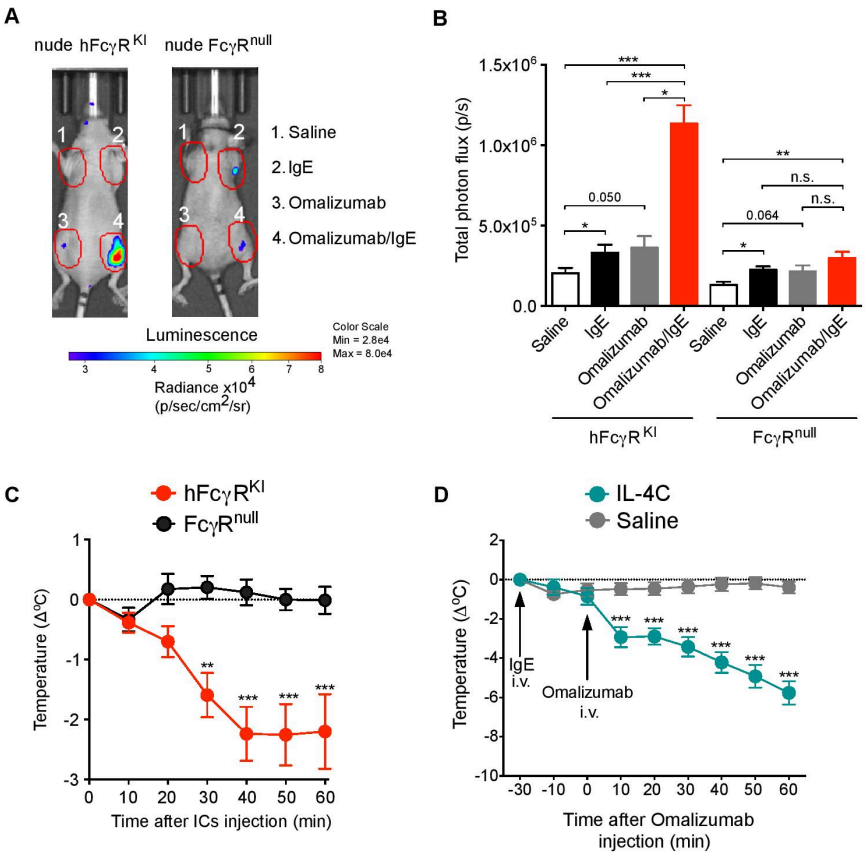


**Figure 2. Omalizumab/IgE ICs induce skin inflammation and anaphylaxis through engagement of FcγRs in FcγR-humanized mice.** Representative bioluminescent images (**A**) and quantification (**B**) of MPO activity 2 h after subcutaneous injection of pre-formed IgE/Omalizumab ICs in nude hFcγR$^{KI}$ mice or nude FcγR$^{null}$ mice. Regions of interest (ROI) outlined in red in **A** surround sites of injection; upper left limb: saline; upper right limb: IgE; lower left limb: Omalizumab; lower right limb: IgE/Omalizumab ICs. Data in **B** are means + SEM pooled from two independent experiments (total $n$=8-9/group). (**C**) Changes in body temperature (Δ°C [mean ± SEM]) after intravenous injection of pre-formed IgE/Omalizumab ICs into hFcγR$^{KI}$ mice ($n$=13) or FcγR$^{null}$ mice ($n$=9). Data in **C** are pooled from three independent experiments. (**D**) Changes in body temperature (Δ°C [mean ± SEM]) after intravenous (i.v.) injection of IgE followed by i.v. injection of Omalizumab into mice pre-treated with a long-lasting formulation of IL-4 (IL-4C) ($n$=12) or PBS ($n$=14) at days -7, -3 and -1. Results in **D** are pooled of two independent experiments. *, $P < 0.05$; *, $P < 0.01$; ***, $P < 0.001$ by Mann-Whitney $U$ test (**B**) or 2-way repeated-measures (ANOVA) (in **C** and **D**).

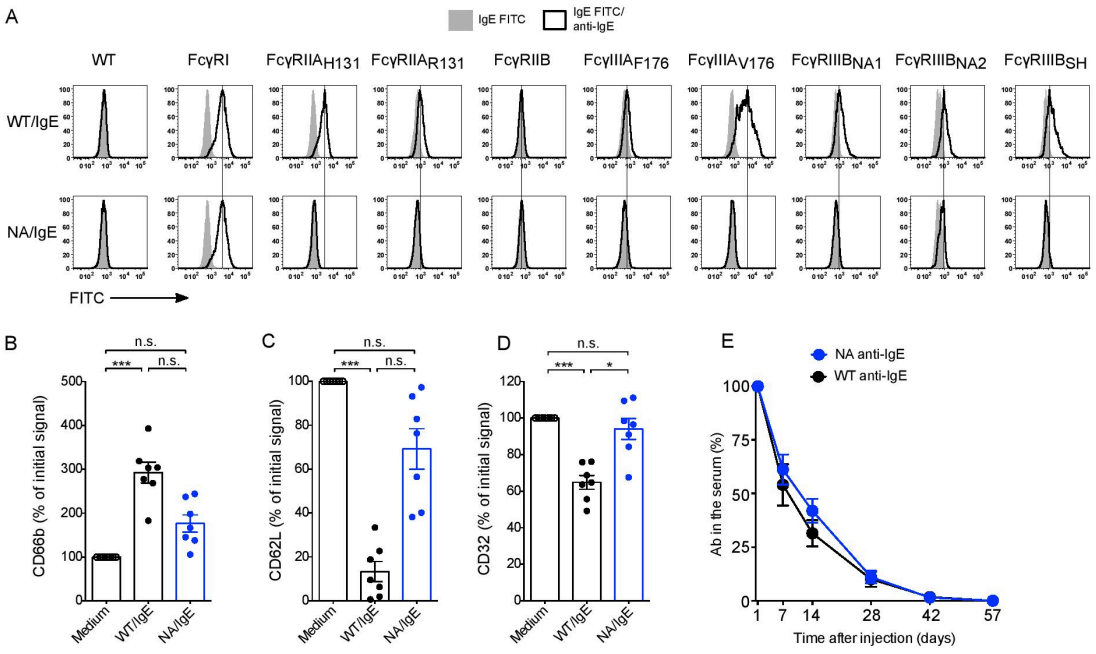


**Figure 3. Fc-engineered anti-IgE antibodies display markedly reduced FcγR-binding and neutrophil activation.** (**A**) Binding of preformed immune complexes (ICs) made of FITC-IgE and WT anti-IgE (upper panel) or Fc-engineered N297A ('NA') anti-IgE (lower panel). WT and NA anti-IgE mAbs were generated using Omalizumab $V_H$ and $V_L$ sequences. (**B-C**) Expression of CD66b (**B**), CD62L (**C**) and CD32 (**D**) on purified $CD45^+CD15^+$ human neutrophils after 1 h incubation with IgE/WT anti-IgE or IgE/NA anti-IgE ICs or medium alone. Results in **B-D** show values from neutrophils from individual donors normalized against cells stimulated with medium alone. Bars indicate means ± SEM pooled from three independent experiments (total *n*=7/group). (**E**) 100 µg of WT or NA anti-IgE was injected intraperitoneally (i.p.) into $hFc\gamma R^{KI}hFcRn^{KI}h\beta2m^{KI}$ mice, and serum was collected at different time-points. Levels of anti-IgE mAbs were measured by ELISA. Data are indicated as means ± SEM pooled from two (*n*=13/group) independent experiments.

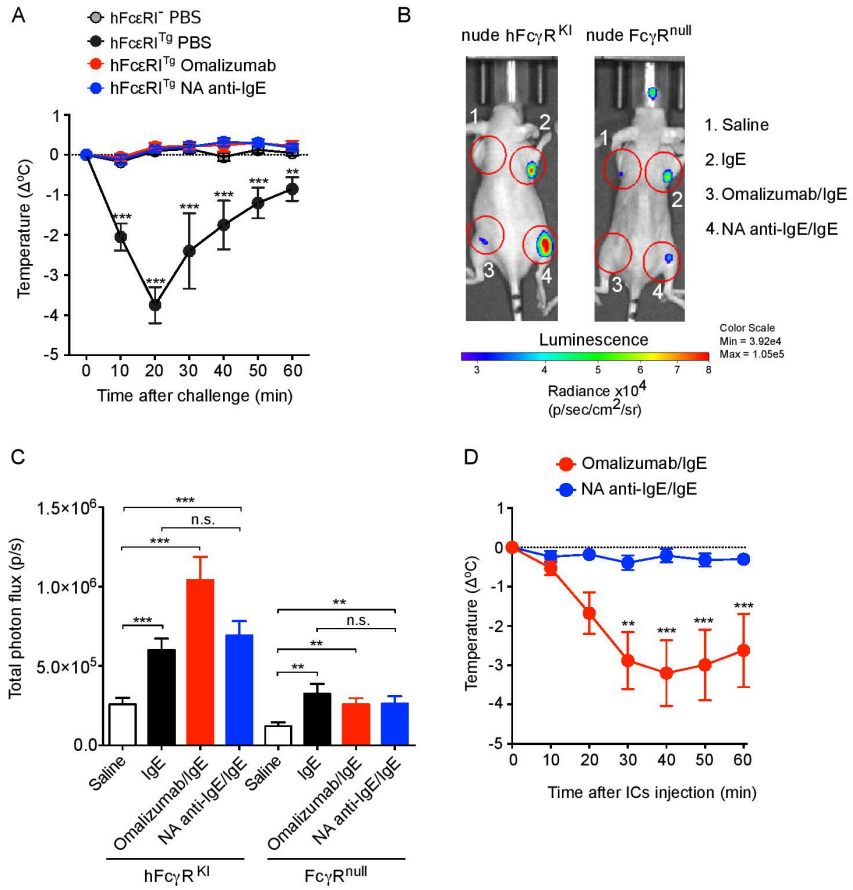


**Figure 4. Fc-engineered anti-IgE antibodies block IgE-mediated anaphylaxis but do not induce FcγR-dependent inflammation.** (**A**) Changes in body temperature (Δ°C [mean ± SEM]) after intravenous (i.v.) challenge with 500 µg of nitrophenyl-coupled BSA (NP-BSA) in hFcεRI$^{Tg}$ mice pre-treated i.v. with 700 µg Omalizumab, NA anti-IgE or PBS 30 min before sensitization with anti-NP IgE (10 µg). Data in **A** are pooled from two independent experiments (total $n$=4-6/group). hFcεRI$^{-}$ mice were used as a control. (**B** and **C**) Representative bioluminescent images (**B**) and quantification (**C**) of MPO activity 2 h after subcutaneous injection of IgE/Omalizumab or IgE/NA anti-IgE ICs in nude hFcγR$^{KI}$ or nude FcγR$^{null}$ mice. Regions of interest (ROI) outlined in red surround the site of injection; upper left limb: saline; upper right limb: IgE; lower left limb: IgE/NA anti-IgE ICs; lower right limb: IgE/Omalizumab ICs. Bars in **C** indicate means + SEM pooled from five (nude hFcγR$^{KI}$, total $n$=20) or four (nude FcγR$^{null}$, total $n$=11) independent experiments. (**D**) Changes in body temperature (Δ°C [mean ± SEM]) after i.v. injection of IgE/Omalizumab ($n$=10) or IgE/NA anti-IgE ($n$=11) into hFcγR$^{KI}$ mice. Data in **D** are pooled from two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by Mann-Whitney $U$ test (**C**) or 2-way repeated-measures (ANOVA) (**A** and **D**).

# Title: The anti-IgE antibody Omalizumab can induce adverse reactions through engagement of Fc gamma receptors


## Authors

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## Running title

Xolair induces FcγR-dependent inflammation

## Supplemental methods

***Cloning and production of WT and Fc-engineered anti-IgE mAbs***. Publicly available Omalizumab $V_H$ and $V_L$ sequences (https://www.drugbank.ca/drugs/DB00043) were reverse transcribed into DNA and codon optimized for expression in human cells using IMGT-V-Quest software. $V_H$ and $V_L$ DNA fragments were synthesized by Eurofins. The Omalizumab $V_H$ sequence was cloned into a human pUC19-Igγ1-expressing vector (a kind gift from Hugo Mouquet, Institut Pasteur, Paris) using SalI and AgeI restriction sites, and Omalizumab $V_L$ sequence was cloned into human Igκ-expressing vector using AgeI and BsiWI restriction sites, as previously described (1). For Fc-engineered mAbs, point mutations in the Igγ1-expressing vector were introduced at position 297 (N297A, thereafter named 'NA' mutant) using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies), according to the manufacturer's instructions. All vectors were sequenced before being used for antibody production. Antibodies were produced by transient co-transfection of WT or Fc-engineered $V_H$ and $V_L$ expression plasmids into exponentially growing Freestyle™ HEK 293-F that were cultured in serum-free Freestyle™ 293 Expression Medium (Life Technologies) in suspension at 37°C in a humidified 8% $CO_2$ incubator on a shaker platform rotating at 110 rpm. Twenty-four hours before transfection, cells were harvested by centrifugation at 300 *g* for 5 min and resuspended in Freestyle™ 293 expression medium at a density of 1 x $10^6$ cells/ml, and cultured overnight in the same conditions as mentioned above. For the production of mAbs, 40 µg of $V_H$ and $V_L$ expressing plasmids were diluted in 80 µl of FectoPRO reagent (PolyPlus) at a final DNA concentration of 0.8 µg/ml, incubated for 10 minutes at

RT before addition to the cells. Twenty-four hours post-transfection, cells were diluted 1:1 with Freestyle™ 293 expression medium. Cells were cultured for 6 days after transfection, supernatants were harvested, centrifuged at 4200 rpm for 30 min and filtered (0.2 μm). Antibodies were purified by affinity chromatography using an AKTA pure FPLC instrument (GE Healthcare) and HiTrap Protein G Column (GE Healthcare). After purification, mAbs were desalted with HiTrap Desalting Column (GE Healthcare).

***Production of human IgE antibodies***. JW8/5/13 (ECACC 87080706) cells were obtained from Sigma-Aldrich. This cell line produces a chimeric human IgE antibody directed against the hapten 4-hydroxy-3-nitrophenacetyl (NP), and composed of the human Fc ε chain and mouse anti-NP variable chain (we refer to this antibody as 'human IgE' in the manuscript). JW8/5/13 cells were cultured in complete Dulbecco-modified Eagle medium (DMEM, Gibco) containing 2 mM glutamine and 10% Foetal Bovine Serum (FBS) at $9x10^5$ cells/ml. After 15 days, supernatants were harvested, centrifuged at 4200 rpm for 30 min and filtered (0.2 μm). We purified IgE antibodies by affinity chromatography. Briefly, CNBr-activated Sepharose 4 Fast Flow Beads (GE Healthcare) were coupled with WT anti-IgE using a ratio of 2.5 mg of protein for each gram of beads. Beads were weighted, washed with 15 volumes of cold 1mM HCl and centrifuged for 5 min at 2500 rpm. WT anti-IgE were resuspended in coupling solution (0.1 M $NaHCO_3$ pH 8.3 containing 0.5M NaCl) and mixed with beads overnight at 4°C under agitation. Beads were washed with coupling buffer and non-reacted groups were blocked with 0.1 M Tris-HCl buffer pH 8.0. WT anti-IgE-coupled beads were then washed using alternate low (0.1 M acetate buffer pH 3) and high (0.1 M Tris-HCl pH 8) pH solutions and stored

in Borate buffer (100 mM Borate, 150 mM NaCl pH 8.0) at 4°C until use. For purification of IgE, WT anti-IgE-coupled sepharose beads were packed in XK 16/20 Column (GE Healthcare) and affinity chromatography was performed using an AKTA pure FPLC instrument (GE Healthcare). After purification, IgE antibodies were desalted with HiTrap Desalting Column (GE Healthcare), and stored at 4°C until use. For some experiments, purified IgE antibodies were conjugated with FITC using the Pierce Antibody labeling kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

***Molecular mass measurements.*** Antibodies and complexes were analyzed by size exclusion chromatography (SEC) coupled with on-line static light scattering (SLS) system. Prior to equilibration of buffer and injection of protein samples, solutions were passed through 0.2 µm filters. Samples were separated on a Superose 6 Increase 10/300 GL column (GE Healthcare) in Dulbecco's phosphate-buffered saline at 18°C. Samples were run at 0.7 mg/mL through the gel filtration column with a constant flow at 0.3 ml/min controlled by a GPCmax module. Column was coupled to a triple detector array (TDA) model 302 (Malvern Panalytical, UK) with a static light scattering cell (7 and 90°), a deflection refractometer, a photometer and a differential viscometer. Calibration was done on bovine serum albumin (Sigma) with an injection of 200 µL at 2 mg/mL. Data were recorded and processed using the Omnisec software (Malvern Panalytical, UK).

***Binding of Omalizumab or Fc-engineered anti-IgE mAbs to human C1q.*** To measure binding of Omalizumab or Fc-engineered anti-IgE mAbs to human C1q, 96-well plates (Costar) were coated with increasing concentrations of each mAb (12.5 to 200 ng/well) in 50 nM carbonate-bicarbonate buffer (pH 9.6) at 4°C for 16 h. Plates were washed 3 times with PBS containing 0.05% Tween 20 (PBST), and blocked for 2 h at room temperature in PBST containing 0.1% gelatine and 3% BSA. Plates were washed 3 times before addition of native human C1q (Abd Serotec) at 1 ng/µL. After 16 h, plates were washed with PBST and incubated 1 h with 50 µL of PBS containing 1 µg/mL anti-human C1q HRP (Abd Serotec). Plates were washed 3 times with PBST before addition of 100 µL/well OPD peroxidase (Sigma). The reaction was stopped by addition of 50 µL 2M $H_2SO_4$ and absorbance was recorded at 492 nm and corrected at 620 nm.

***Binding of Omalizumab or Fc-engineered anti-IgE mAbs to human IgE.*** To measure binding of Omalizumab or Fc-engineered anti-IgE mAbs to human IgE, 96-well plates (Costar) were coated with each mAb (0.5 µg/well) in 50 nM carbonate-bicarbonate buffer (pH 9.6) at 4°C for 16 h. Plates were washed 3 times with PBST, and blocked for 2 h at room temperature in PBST 1% BSA. Plates were washed 3 times before addition of increasing doses of IgE (1.6 to 5000 ng/well). After 3 hours, plates were washed with PBST and incubated with 1:10.000 of anti-human IgE (Bethyl) for 1 h. Plates were washed 3 times with PBST before addition of 100 µL/well OPD peroxidase (Sigma). The reaction was stopped by addition of 50 µL 2M $H_2SO_4$ and absorbance was recorded at 492 nm and corrected at 620 nm.

***Detection of mouse and human FcRn and β2m* transcripts**: Total RNA was extracted from human peripheral blood mononuclear cells or murine splenocytes using NucleoSpin RNA plus kit (Macherey-Nagel) according to the manufacturer's instructions. cDNA were generated at 50°C for 60 minutes using random primers and SuperScript III Reverse Transcriptase (Invitrogen). The primer pairs for FcRn gene (human: 5'-CTCTCCCTCCTGTACCACCTT-3'; 5'-ATAGCAGGAAGGTGAGCTCCT-3' ; mouse : 5'-AGCTCAAGTTCCGATTCCTG-3'; 5'- GATCTGGCTGATGAATCTAGGTC-3') and for β2-microglobulin gene (human : 5'-GGCTATCCAGCGTACTCCAAA-3' ; 5'-CGGCAGGCATACTCATCTTTT-3' ; mouse : 5'- CCGGAGAATGGGAAGC -3 ; 5'-GTAGACGGTCTTGGGC -3') were used for amplification with GoTaq G2 polymerase (Promega). Amplification was performed by 35 cycles PCR each consisting of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min. At the end of the 35 cycles, samples were run for an additional 10 min at 72°C and analyzed by 1.5% agarose gel electrophoresis. The expected size of the PCR products: Human FcRn: 450bp; Mouse FcRn: 240 bp; Human β2m: 240 bp; Mouse β2m: 270 bp.

***Recirculation of Fc-engineered anti-IgE antibodies* in vivo.** hFcγR$^{KI}$hFcRn$^{KI}$hβ2m$^{KI}$ mice were injected i.p. with 100 μg of WT or NA anti-IgE mAbs in 100 μL 0.9% NaCl solution. Serum was then collected every 7 or 14 days starting from day 1 post-injection and stored at -20°C until use. Serum levels of anti-IgE mAbs were quantified by ELISA. Briefly, 96-well plates (Costar) were coated with F(ab')2 Goat Anti-human IgG (5μg/mL; Jackson ImmunoResearch) in 50 nM carbonate-bicarbonate buffer (pH 9.6) at 4°C for 16 h. Plates were washed 3 times with PBST, and blocked for 2 h at room

160 temperature in PBST containing 1% BSA. Plates were washed 3 times before addition of
161 serial dilutions of serum (1/100 to 1/3000). After 3 hours, plates were washed with PBST
162 and incubated with goat anti-human kappa HRP (1:4.000; Southern Biotech) for 1 h.
163 Plates were washed 3 times with PBST before addition of 100 µL/well OPD peroxidase
164 (Sigma). The reaction was stopped by addition of 50 µL 2M $H_2SO_4$ and absorbance was
165 recorded at 492 nm and corrected at 620 nm.
166
167 ***Generation of peripheral blood derived-cultured human mast cells (hMCs).*** hMCs were
168 generated as described previously (2). Briefly, peripheral blood mononuclear cells were
169 separated using Ficoll-Paque PLUS (GE Healthcare) and $CD34^+$ cells were isolated with
170 a human CD34 positive selection kit (StemCell Technologies). Cells were seeded at
171 $1x10^6$ cells/mL in StemSpan medium (StemCell Technologies) supplemented with
172 Ciprofloxacin (10 µg/ml; Sigma-Aldrich), recombinant human IL-6 (50 ng/ml;
173 Peprotech), human IL-3 (50 ng/ml; Peprotech) and SCF (100 ng/mL; Miltenyi). Every
174 three to four days, cultures were doubled in volume with fresh supplemented medium for
175 30 days. Cells were then progressively transferred to Iscove's-modified Dulbeccos
176 medium (IMDM; Gibco) supplemented with 50 µM 2-mercaptoethanol (Life
177 Technologies), 0.5% BSA, 1% Insulin-Transferrin-Selenium (Life Technologies),
178 Ciprofloxacin (10 ug/ml), human IL-6 (50ng/mL) and human SCF (100 ng/mL). hMCs
179 were supplemented with fresh medium every week. All data presented were generated
180 with cells after 10 weeks of culture, and co-expression of FcεRI and CD117 was verified
181 by flow cytometry.

## Supplemental references

1. Tiller T, Meffre E, Yurasov S, Tsuiji M, Nussenzweig MC, and Wardemann H. Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *J Immunol Methods.* 2008;329(1-2):112-24.
2. Gaudenzio N, Sibilano R, Marichal T, Starkl P, Reber LL, Cenac N, et al. Different activation signals induce distinct mast cell degranulation strategies. *J Clin Invest.* 2016;126(10):3981-98.

## Supplemental figures and legends

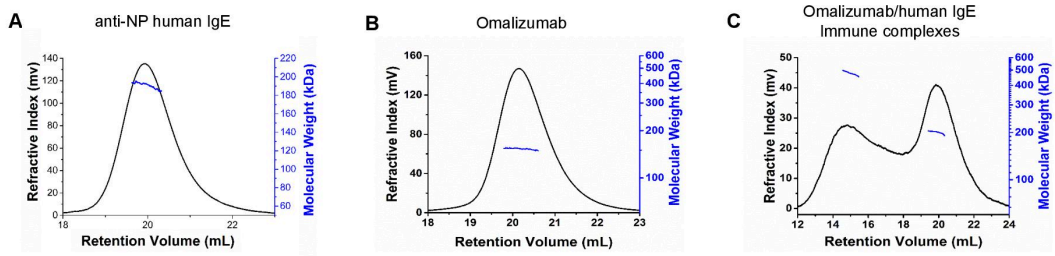


**Figure S1. Size exclusion chromatography coupled to static light scattering (SEC-SLS) profiles for human IgE and Omalizumab alone, and for the complex in solution**. Anti-NP human IgE (**A**), Omalizumab (**B**) and human IgE/Omalizumab immune complexes (ICs) (**C**) were run through a gel filtration column with a constant flow at 0.3 mL/min. ICs were formed by incubating human IgE and Omalizumab at a 1:2 mass ratio for 1 h at room temperature.

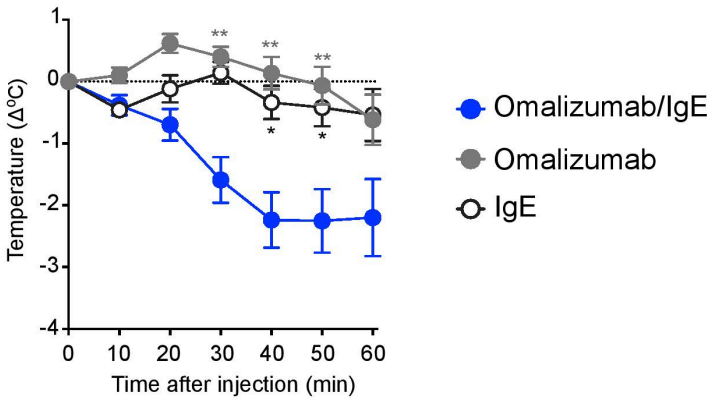


**Figure S2. Injection of Omalizumab/IgE ICs triggers passive systemic anaphylaxis in hFcγR$^{KI}$ mice, but not injection of IgE or Omalizumab alone.** Changes in body temperature (Δ°C [mean ± SEM]) after intravenous injection of pre-formed Omalizumab/IgE ICs ($n$=13) or anti-NP human IgE ($n$=5) or Omalizumab ($n$=6) alone into hFcγR$^{KI}$ mice. Data are pooled from two (IgE and Omalizumab alone) or three (Omalizumab/IgE ICs) independent experiments. *, $P < 0.05$; **, $P < 0.01$ *vs.* Omalizumab/IgE group at the same time-point; by 2-way repeated-measures (ANOVA).

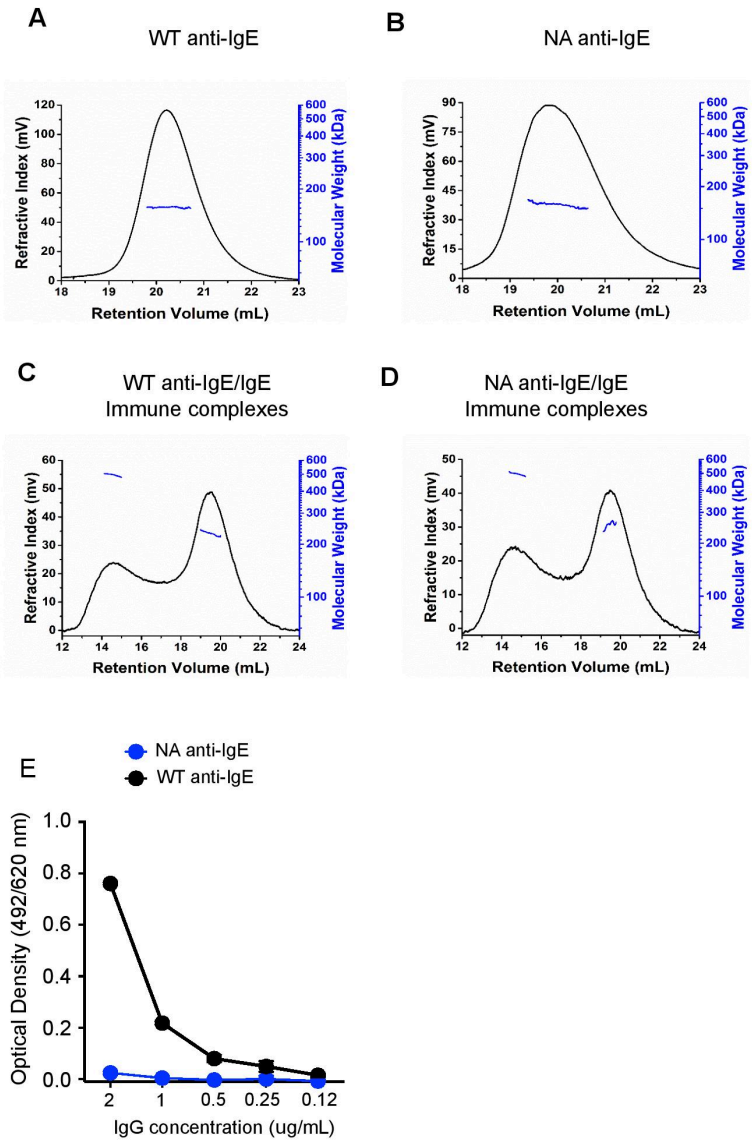


**Figure S3. Size exclusion chromatography coupled to static light scattering (SEC-SLS) profiles for WT and NA anti-IgE alone, and in complex with human IgE, and binding of WT or NA anti-IgE to human complement component C1q. (A-D)** WT anti-IgE or NA anti-IgE alone (**A** and **B**) or in complex with anti-NP IgE (**C** and **D**) were run through a gel filtration column with a constant flow at 0.3 mL/min. ICs were formed by incubating IgE and anti-IgE mAbs at a 1:2 mass ratio for 1 h at room temperature. (**E**) Binding of WT and NA anti-IgE to human C1q assessed by ELISA. Results in **E** show means ± SD from one out of 2 independent experiments (total of *n=4* replicates).

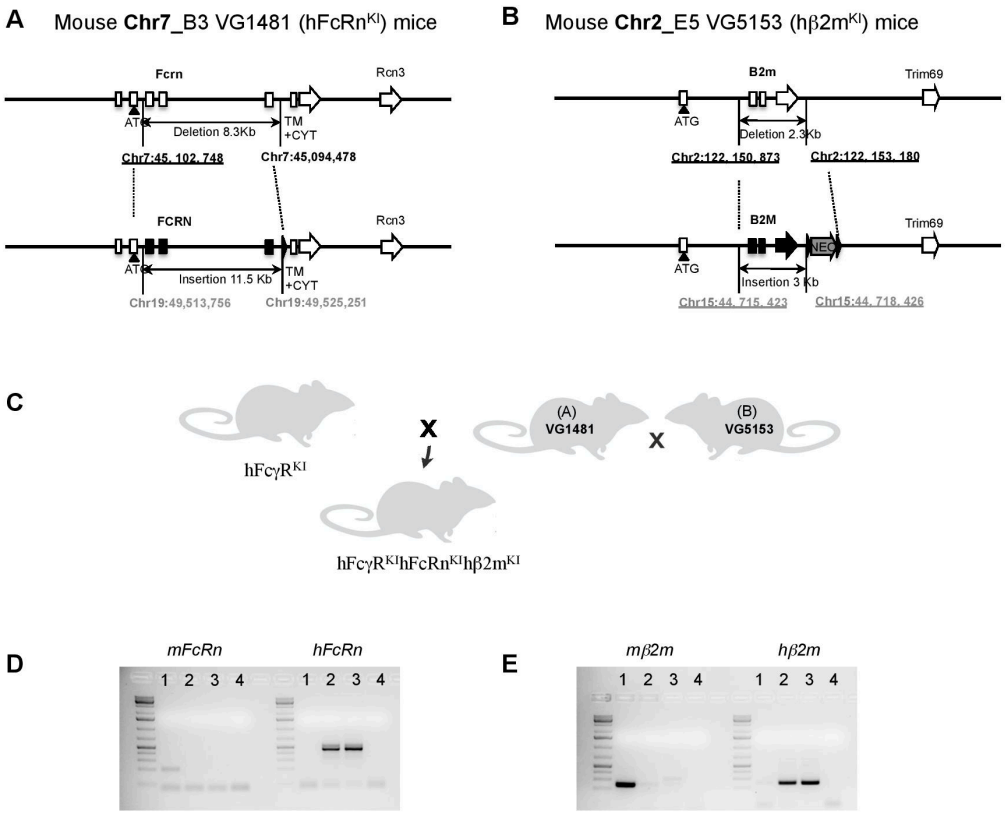


**Figure S4. Generation of hFcRn$^{KI}$ and hβ2m$^{KI}$ mice.** (**A** and **B**) Humanization of the (**A**) mouse Fcrn gene and (**B**) b2m gene. Representations are not drawn to scale. Coordinates are based on mouse (GRCm38.p4) and human (GRCh38.p7) genomic assemblies: mouse genes are in empty rectangles, genomic coordinates are in black; human genes are in solid rectangles, genomic coordinates are in grey; black triangles represent Loxp site. (**C**) Breeding scheme to obtain hFcγR$^{KI}$hFcRn$^{KI}$hβ2m$^{KI}$ mice. (**D** and **E**) RT-PCR analysis of mFcRn and hFcRn (**D**) and mβ2m and hβ2m (**E**). 1: splenocytes from hFcγR$^{KI}$ mice; 2: splenocytes from hFcγR$^{KI}$hFcRn$^{KI}$hβ2m$^{KI}$ mice; 3: Human PBMC; 4: negative control.

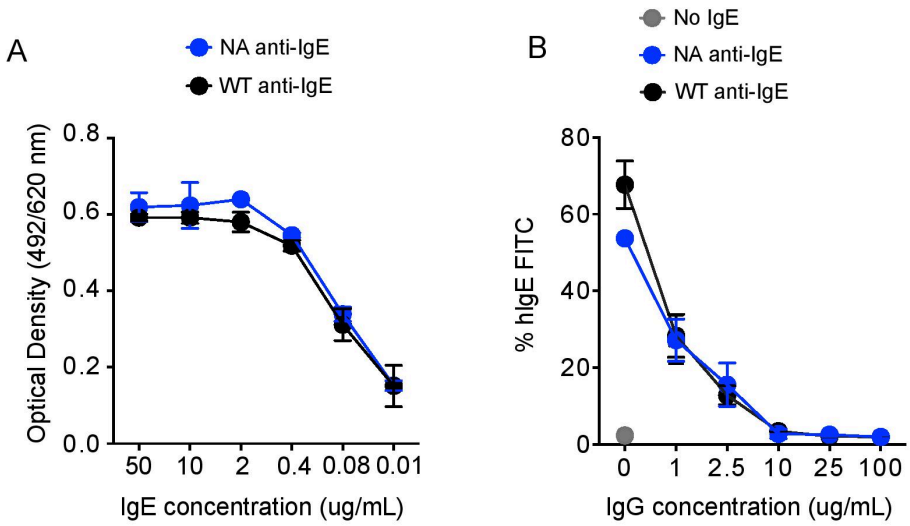


**Figure S5. Characterization of recombinant WT anti-IgE and NA anti-IgE mAbs.** (**A**) Binding of WT and NA anti-IgE to IgE assessed by ELISA. Results show means ± SD from data pooled from two independent experiments. (**B**) Human mast cells (hMCs) expressing the high-affinity IgE receptor FcεRI were preincubated with WT or NA anti-IgE at the indicated concentration. FITC-labeled IgE were added immediately after and hMCs were incubated for 16 hours before levels of FITC-IgE bound to hMCs was assessed by flow cytometry. Data show means ± SD from one representative of three independent experiments.

## 4.2 Development of a second Fc-engineered variant of Omalizumab

In paper II, we have demonstrated that Omalizumab can induce skin inflammation and systemic shock through the engagement of hFcγRs. In addition to the mutant version of Omalizumab harboring a point mutation at position N297, we also developed an Fc-engineered anti-IgE harboring mutations at positions L234 and L235 (which were substituted by alanines, we therefore call this mutant 'LALA' anti-IgE), as the LALA mutation is also known to have markedly reduce binding to hFcγRs.

We first verified that the $L_{234}AL_{235}A$ mutation does not affect the ability of the anti-IgE to recognize IgE. As shown in Figure 7A, the LALA anti-IgE recognizes IgE with the same affinity as the WT anti-IgE. In addition, we detected no binding to human C1q with the LALA anti-IgE (Figure 7B), as observed with the NA anti-IgE (Paper II – Supplemental Figure 3E). When incubated *in vitro* with IgE, the LALA-anti-IgE mainly formed trimers (data now shown), which is consistent with the data we obtained using the WT, the NA mutant and the commercial version Omalizumab (Paper II - Supplemental Figure 1 and 3). Moreover, immune complexes (ICs) made of IgE and the LALA anti-IgE showed markedly reduced binding to FcγRs (Figure 7C). Nevertheless, the anti-IgE LALA displayed residual bind to FcγRI, $FcγRIIIA_{V176}$, and, to a lesser extent, $FcγRIIIB_{NA2}$ (Figure 7C).

Finally, we observed activation of human neutrophils with ICs made of IgE and the WT anti-IgE, and a reduced but still significant activation with ICs of IgE and the LALA anti-IgE (Figure 7D-F). ICs of IgE and LALA anti-IgE induced upregulation of CD66b and a down regulation of CD62L, both considered hallmarks of neutrophil activation (Figure 7D-E). However, no significant downregulation of CD32 (FcγRIIA) was observed. To further confirm the role of FcγRs in neutrophil activation, we performed similar experiments with neutrophils purified from $hFcγR^{KI}$ mice or $FcγR^{null}$ mice as a control (Figure 7G). As expected, IgE/WT anti-IgE ICs induced a downregulation of CD62L in neutrophils purified from $hFcγR^{KI}$ mice, but not in neutrophils purified from $FcγR^{null}$ mice (Figure 7G). IgE/LALA anti-IgE ICs had reduced capacity of activate neutrophils purified from $hFcγR^{KI}$ mice.

All the *in vitro* tests allowed us to characterize both the NA and LALA mutant anti-IgE as potential candidates for the validation of *in vivo* tests. We decided to perform *in vivo* using only the NA anti-IgE Fc-engineered antibody for basically three main reasons: *a)* the

NA anti-IgE had a slightly better performance when considering neutrophil activation by ICs; *b)* to limit the number of animals used for these *in vivo* experiments; and *c)* antibodies harboring the $N_{297}A$ mutation are being investigated in different clinical stages, thus increasing the possibility of clinical applicability of our findings [252].

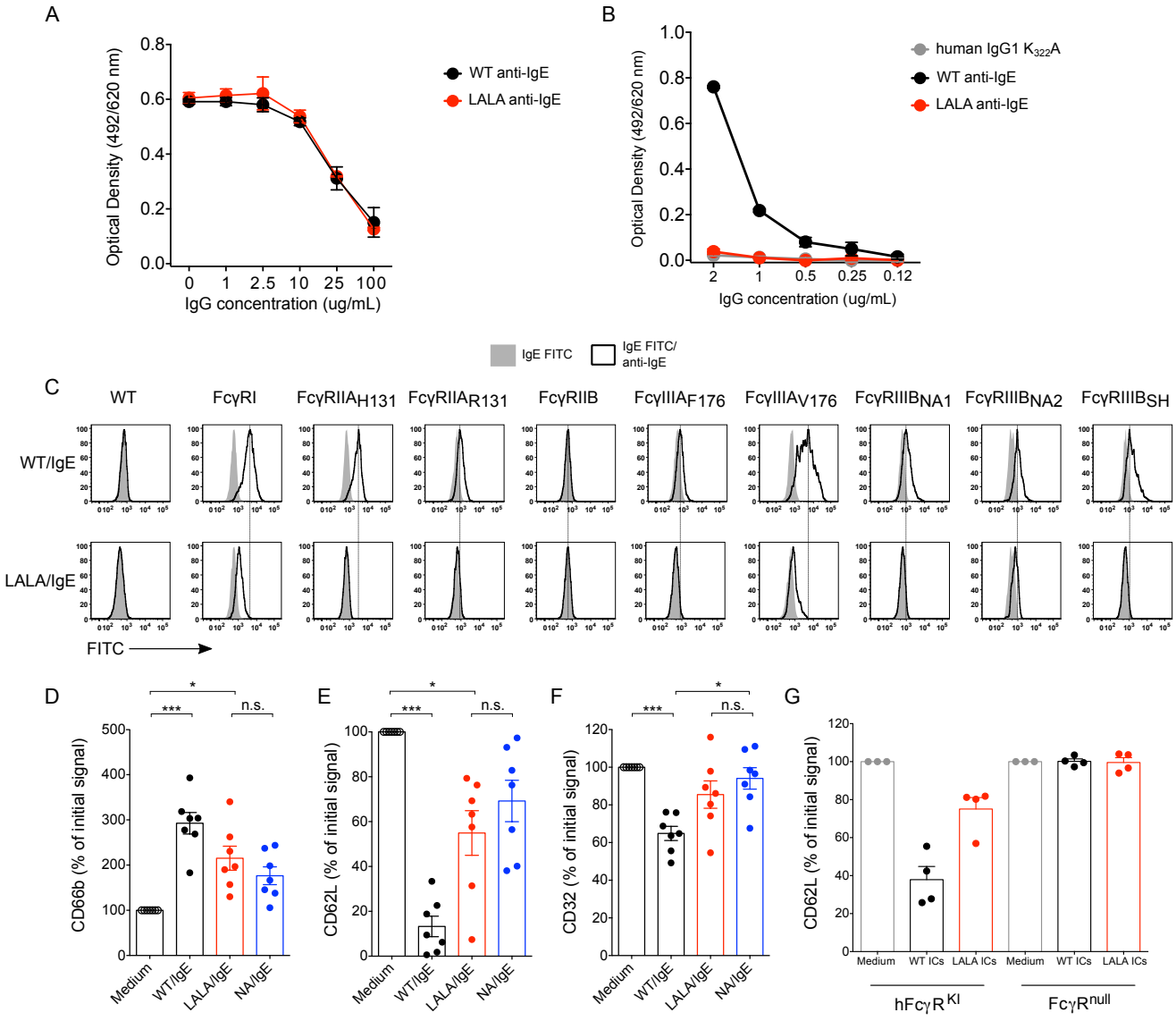


Figure 7. **Characterization of NA anti-IgE produced monoclonal antibodies.** (**A**) Binding of WT and NA anti-IgE to human IgE assessed by ELISA. Results show means ± SD from data pooled from two independent experiments. (**B**) Binding of WT and LALA anti-IgE to human C1q assessed by ELISA. An irrelevant human IgG1 mutated in its Fc portion at position 322 ($K_{322}A$) to preclude binding to C1q was used as negative control. Results in **B** show means ± SD from data pooled from two independent experiments (total of n=4 replicates). (**C**) Binding of preformed immune complexes (ICs) made of FITC-IgE and WT anti-IgE (upper panel) or Fc-engineered $L_{235}AL_{236}A$ ('LALA') anti-IgE (lower panel). WT and LALA anti-IgE mAbs were generated using Omalizumab $V_H$ and $V_L$ sequences. (**D-F**) Expression of CD66b (**D**), CD62L (**E**) and CD32 (**F**) on purified $CD45^+CD15^+$ human neutrophils after 1 h incubation with IgE/WT anti-IgE or IgE/LALA anti-IgE ICs or medium alone. Results in **D-F** show values from neutrophils from individual donors normalized against cells stimulated with medium alone. Bars indicate means ± SEM pooled from three independent experiments (total n=7/group). (**G**) CD62L expression on $CD11b^+Ly6G^+$ neutrophils purified form humanized $hFc\gamma R^{KI}$ or $Fc\gamma R^{null}$ mice after 1 h incubation with IgE/WT or IgE/LALA anti-IgE ICs or medium. Results in **G** show values from individual mice with bars indicating means ± SEM pooled from two ($hFc\gamma R^{null}$; total n=4/group) or three ($hFc\gamma R^{KI}$; total n=4/group) independent experiments. *, $P < 0.05$; *, $P < 0.01$; ***, $P < 0.001$ by one-way analysis of variance (ANOVA) with Kruskal-Wallis post-test. n.s., not significant ($P > 0.05$).

# 5. Chapter 5 - Study of the role of human IgG antibodies and human Fc gamma receptors in peanut anaphylaxis

The results we showed so far in this thesis identified two major points on IgG induced-anaphylaxis: *a)* a key role for mouse IgGs in experimental anaphylaxis in mice and, *b)* IgG-mediated anaphylaxis induced by high-doses of therapeutic antibodies in a humanized mouse model.

These two models bring important contributions to understand the physio-pathological mechanism of anaphylaxis. However, it still remains under debate whether endogenous IgG can contribute to anaphylaxis in humans. Because of a collaboration obtained with Dr. Kari Nadeau and Stephen J. Galli at Stanford, we were able to address this question taking advantage of plasma coming from a cohort of highly allergic patients. We obtained plasma samples from 107 peanut-allergic patients recruited under the clinical trial 'The Peanut Oral Immunotherapy Study: Safety, Efficacy and Discovery (POISED)' https://clinicaltrials.gov/ct2/show/NCT02103270.

All experiments showed in this chapter are part of an ongoing work. Our preliminary results demonstrate that highly peanut allergic patients have great levels of peanut-specific (PN-spe) IgG in the plasma compared to blood donors. Moreover, clinical data allowed us to correlate our findings to available levels of total and peanut-specific IgE; surprisingly, PN-spe IgG1, IgG2, IgG3 and IgG4 positively correlate to PN-spe IgE levels. Finally, we propose a new clinical-relevant model of systemic anaphylaxis by passively transferring human IgG to a humanized mouse harboring all human FcγR or lacking all endogenous mouse FcγRs.

**Clinical Trials Registration**

https://clinicaltrials.gov; National Clinical Trial Identifier: NCT02103270 29

## INTRODUCTORY PARAGRAPH

Epidemiologic studies indicate that the prevalence of food allergies, especially to peanuts, has increased worldwide in the last few decades [253]. Foods are by far the most causative agent of anaphylaxis in infants, children teens and young adults [172]. Peanut (PN) allergy is a particularly important public health problem, since it tends to persist throughout life and causes anaphylaxis and death more frequently than other food allergies [254]. It is estimated that more than 50% of patients with peanut or tree-nut allergy will experience at least one severe allergic reaction [255].

Clinical diagnoses of food allergies can be complex. Normally, it includes skin prick tests with the suspected allergens and measurements of allergen-specific IgE. However, these tests are not always definitive because of their lack of specificity and high number of false-positives results [254]. The gold standard test to confirm or exclude food allergy is the double-blind, placebo-controlled food challenge (DBPCFC). Subjects receive escalating doses of the potential allergen to see whether this results in any sign of allergic response (and if so, the food challenge is stopped at that specific allergen dose). Yet, DBPCFC still carries the risk of a systemic reaction. Hence, the development of additional clinically relevant tools for the diagnostic of food allergy could be a great value.

The current paradigm states that peanut allergy is most likely trigged by allergen-induced crosslinking of PN-specific IgE antibodies, bound to their high affinity receptor FcεRI on the surface of mast cells and basophils, leading to the release of histamine and other preformed mediators. However, evidence derived from mouse models indicates that some IgG isotypes could also mediate allergic reactions. To date, the role of human IgG in PN allergy remains largely unknown.

Here, we assessed whether level of PN-specific IgG in PN allergic subjects correlates with the clinical severity of allergy, and would have any value as part of the diagnosis of PN allergy. We then studied whether human IgG from PN allergic subjects and human FcγRs could play a role in PN anaphylaxis. To do so, we developed a clinically-relevant passive model of PN anaphylaxis, in which mice humanized for all their FcγRs ($hFc\gamma R^{KI}$ mice) are treated with a long-lasting formulation of IL-4 (named IL-4C, and consisting of murine IL-4 in complex with an anti-IL-4 mAb) to increase their susceptibility to develop anaphylaxis

[201, 251], and are then sensitized with total IgG purified from the plasma of PN allergic patients, followed by challenge with PN extract to induce anaphylaxis. As controls, mice lacking all endogenous mouse FcγRs (FcγR$^{null}$ mice) were used.

We found that levels of PN-specific IgG from all subclasses (IgG1-4) correlated with levels of PN-specific IgE, suggesting that quantification of IgG levels could have a value as part of the diagnostic of PN allergy. We then demonstrated that hFcγR$^{KI}$ mice primed with IL4C and sensitized with IgG from PN allergic patients, but not with IgG from healthy donors, developed anaphylaxis upon challenge with PN in this model. This strongly suggests that PN-specific IgG have the potential to trigger anaphylaxis. Surprisingly, FcγR$^{null}$ mice sensitized with IgG from PN-allergic subjects and challenged with PN had a higher mortality rate than hFcγR$^{KI}$ mice, suggesting that the presence of hFcγRs can have a protective role in this PN-induced anaphylaxis model. We are now further investigating the mechanism of this observation, and notably the potential protective role of the inhibitory FcγRIIB receptor, and the role of the complement pathway in this PN anaphylaxis model.

## RESULTS AND DISCUSSION

### *Levels of PN-specific IgG antibodies in plasma samples from PN allergic subjects*

We obtained plasma from PN allergic subjects included in a large phase 2, randomized clinical study of PN oral immunotherapies (OIT) (National Clinical Trial Identifier: NCT02103270 29). The study recruited 120 PN allergic patients based on several clinical parameters, including medical history and allergy tests. Data obtained by ImmunoCAP® (quantitative measurement of total or allergen-specific IgE in human plasma) showed high levels of total and PN-specific IgE (**Supplementary figure 1A and 1B**) in these highly allergic patients. Not surprisingly, levels of total IgE positively correlated with levels of PN-specific IgE (**Supplementary figure 1C**).

We then assessed the presence of PN-specific IgGs by ELISA in these plasma samples. As controls, we used plasma from healthy donors (with unknown allergic status) obtained from the French blood bank (‘*Etablissement Français du Sang’* – EFS). We detected significant levels of PN-specific IgG from all subclasses (IgG1-4) in the plasma of PN allergic subjects (*n*=107) but not in plasma samples from healthy donors (*n*=25) (**Figure 1 A-D**). Interestingly, levels of PN-specific IgG1, IgG2, IgG3 and IgG4 correlated with levels of PN-specific IgE in PN allergic subjects (**Figure 1 E-H**). These results suggest that quantification of PN-specific IgGs could be used as an additional test in the diagnosis of PN allergy. However, it is important to note that in some subjects, levels of PN-specific IgG were undetectable by ELISA, while ImmunoCAP could still detect specific IgE. This was the case for 15 out of 107 samples for IgG1, 8 out of 107 samples for IgG2, 27 out of 107 for IgG3 and 3 out of 107 for IgG4 (**Figure 1**).

### *Passive Systemic Anaphylaxis mediated by IgG from PN-allergic patients (preliminary results)*

To examine the potential role of human IgG in the development of PN-induced anaphylaxis *in vivo*, we decided to first purify IgGs from the plasma of PN allergic subjects. Due to the limited volume of plasma we obtained from each patient, we proposed to generate three representative pools of plasma from our cohort in order to proceed to the purification. Hence, to obtain homogenous groups, we have chosen to stratify our patients using the gold

standard DBPCFC [254]. All highly PN-allergic patients were included in the clinical study after undergoing through a DBPCFC. The standardized DBPCFC were performed according to validated guidelines. The protocol consisted of 7 escalating doses (5, 20, 50, 100, 100, 100, and 125 mg) of the food protein (either PN or placebo – oat) in flour form, concealed in either applesauce or pudding, ingested by the participant every 15 minutes, as tolerated. Physical examination and vital signs were recorded. To evaluate for sensitivity to PN protein quantitatively, the cumulative tolerated dose (CTD; the amount of PN ingested before treating the participant for objective symptoms) was also quantified [256]. Our strategy was to distribute subjects according to their CTD, separating groups of responders belonging to all cumulative doses (0, 5, 25, 75, 175, 275 and 375 mg of PN). From those groups, we homogenously created three pools (Pool #1-3) containing responders from every group, not being biased by antibody levels or any other clinical parameter (**Supplementary Figure 2A**). Using HiTrap Protein G columns, we could selectively extract and purify all subclasses of IgGs from plasma samples of the three pools and of a pool of plasma from 30 healthy donors. We could detect the presence of PN-specific IgGs in the purified fraction of the IgGs from allergic subjects, but not from healthy donors (**Supplementary Fig. 2B-E**).

We then passively transferred 3 mg of purified total IgGs from the plasma of either controls or PN allergic patients (from Pool #1) into hFcγR$^{KI}$ or FcγR$^{null}$ (which do not express any mouse or human FcγR) mice. Mice were then challenged i.v. with PN extract and central body temperature was followed over 60 minutes (**Figure 2A**). None of the groups were susceptible to the development of anaphylaxis upon PN challenge in these conditions (**Figure 2A**).

Anaphylaxis remains a rare event in the overall population, probably affecting 0.3% of the population at some point during their lifetime [175]. However, history of atopy, and in particular allergy to PN or nuts, can account for increased risks of more severe reactions and anaphylaxis development [257]. Moreover, among children with food allergies, approximately 30% of food allergic individuals have allergies to multiple foods [254]. It is possible that a subpopulation of highly atopic patients is more prone to multiple sensitizations and also is more susceptible to develop anaphylaxis. To reproduce this susceptibility to anaphylaxis *in vivo*, we exposed hFcγR$^{KI}$ or FcγR$^{null}$ mice to IL-4C, a long-lasting formulation of IL-4 [201, 251].

hFcγR$^{KI}$ or FcγR$^{null}$ mice were pre-treated with IL-4C before passive sensitization with purified IgGs from the plasma of either controls or PN allergic patients. 16 hours after sensitization, mice were challenged i.v. with PN extract and measurements of body temperature was performed immediately before and up to 30 minutes after challenge (**Figure 2B**). Upon PN challenge, hFcγR$^{KI}$ sensitized with IgGs from PN allergic patients displayed a rapid and sustained loss of core body temperature, indicative of anaphylaxis. Surprisingly, FcγR$^{null}$ mice that received IgGs from PN allergic patients also developed hypothermia. Moreover, hFcγR$^{KI}$ mice receiving IgGs from a pool of plasma from healthy donors had a loss of body temperature up to 20 minutes after challenge, but recovered at 30 minutes (**Figure 2B**).

Importantly, although most mice recovered from anaphylaxis in hFcγR$^{KI}$ mice, 1 out of 7 mice died in the group injected with IgG from healthy donors, and 1 out of 15 in the group injected with IgG from PN allergic subjects. These data are highly consistent with two previous reports showing that in mice pre-treated with IL-4C, PN by itself can induce signs of anaphylaxis [201, 251]. The proposed mechanism for such effect was a direct activation of both classical and lectin complement pathways by PN extracts [239, 240]. The authors showed that C3a produced upon PN injection can induce anaphylaxis through activation of macrophages and basophils, and to a lesser extent mast cells, and that this reaction was mostly dependent on platelet-activating factor (PAF) [239].

Interestingly, we observed a significantly higher mortality in FcγR$^{null}$ mice that received IgGs from PN allergic patients (7 out of 13 mice) as compared to hFcγR$^{KI}$ mice receiving either IgG from PN allergic patients or healthy donors (**Figure 2C**). Although we observed variations in the levels of hypothermia and mortality with the different pools of IgG, higher mortality in FcγR$^{null}$ mice was observed with each IgG pool (data not shown). These preliminary results could suggest a potential protective role for hFcγRs in this PN anaphylaxis model. Further investigations are required in order to understand the mechanism explaining these results. It is possible that the constitutive lack of FcγRs in FcγR$^{null}$ mice favor development of the complement pathway, and that thus FcγR$^{null}$ mice develop stronger complement-dependent anaphylaxis upon PN challenge. At this time, we have not assessed responses of FcγR$^{null}$ mice to PN extract alone (that is in the absence of transferred human

IgG). If PN-specific IgG is required for the increased mortality rate in FcγR$^{null}$ mice in this PN anaphylaxis model, another potential hypothesis would be that in the absence of FcγRs, PN-IgG immune complexes are not trapped by FcγRs and are more "available" to activate the complement pathway. Finally, PN-IgG immune complexes could directly engage the inhibitory receptor FcγRIIB, thereby limiting anaphylaxis in our model. However, while we consistently observe FcγRIIB expression on B cells in hFcγR$^{KI}$ mice, we did not observe FcγRIIB on mast cells or basophils in naïve hFcγR$^{KI}$ mice [71]. It is however possible that such expression of FcγRIIB on mast cells or basophils could be induced by the pre-treatment with IL-4C, and this will be assessed in follow-up experiments.

Since our data and that of previous investigators [239, 240] suggest an involvement of the complement pathway in PN anaphylaxis, it will be interesting to assess whether activation of C1q by PN-IgG immune complexes participate to anaphylaxis in our model. This will be performed by comparing responses of FcγR$^{null}$ mice and C1q$^{-/-}$FcγR$^{null}$ mice (available in our laboratory). Finally, since we are using human IgG to trigger anaphylaxis, it will be important to assess whether these antibodies can also activate human C1q. This will now be possible as we obtained humanized hC1q$^{KI}$ mice (as a collaboration with Regeneron Pharmaceuticals) and are now developing hC1q$^{KI}$hFcγR$^{KI}$ mice.

In conclusion, although this project requires further investigations, we have already shown that PN allergic subject have high levels of PN-specific IgG from all subclasses. Such IgG can trigger anaphylaxis when transferred into hFcγR$^{KI}$ mice pre-treated with IL-4C. This suggests that, at least in highly atopic subjects, IgG might participate to the severity of anaphylaxis. Interestingly, our preliminary data also suggest that IgG might mediate PN anaphylaxis through activation of the complement pathway. Activation of the complement pathway by PN has been reported by other groups and was not observed with other major food allergens [239]. It is tempting to speculate that this unique feature might participate to the fact that PN can induce particularly strong cases of anaphylaxis in humans.

## METHODS

### Plasma specimens

Plasma from healthy blood donors (unknown allergy status) was obtained from the French blood bank "*Etablissement Français du Sang"* (EFS) and plasma from 107 PN-allergic patients was obtained as part of their participation in a reviewed board-approved phase 2 clinical trial (https://clinicaltrials.gov; National Clinical Trial Identifier: NCT02103270 29). Patients between the ages of 7 to 55 years with a convincing history of PN allergy were recruited to undergo standardized double-blind placebo-controlled food challenges (DBPCFCs) as part of screening for clinical trial enrollment. The protocol consisted of 7 escalating doses (5, 20, 50, 100, 100, 100, 125 mg) of the food protein in flour form, concealed in either applesauce or pudding, ingested by the participant every 15 minutes. Vital signs and pertinent physical examinations were repeated every 15 minutes. If a positive reaction was obtained, the oral food challenge was immediately discontinued and appropriate treatments were administered. PN allergy was defined as having a clinical reaction to PN during a DBPCFC to PN (the reaction occurring at ≤500 mg total of PN protein) and a positive skin prick test result to PN (>5 mm). To evaluate for sensitivity to PN protein quantitatively, the cumulative tolerated dose (CTD; the amount of PN ingested prior to treating the participant for objective symptoms) was also quantified. A detailed medical history, physical examination, spirometry, skin prick test, PN-specific IgE and total IgE testing, and basophils activation test were completed at screening to confirm PN allergy. All plasma samples were collected and kept at -80 $^{o}$C until further analysis.

### PN-specific IgG measurements by ELISA

Microtiter plates (96 wells, Nunc Maxisorb, Costar) were coated with 20 μg of peanut extract (100 μl/ well) in 50 nM carbonate-bicarbonate buffer (pH 9.6) at 4°C for 16 h. Plates were washed 3 times with PBST (PBS 0.05 % Tween 20), and blocked for 2 h at room temperature in PBST 1% FBS Ultra Low IgG (Invivogen). Plates were washed 3 times before addition of plasma samples. Each plasma sample was exposed to a range of dilutions in PBST 2% BSA (Bovine Serum Albumin) starting at 1:100, then a 3-fold dilution for a total of seven dilution steps. Samples were incubated for 3 hours at room temperature. Plates were washed with PBST and incubated with 1:4000 of HRP anti-human IgG1, IgG2, IgG3 or IgG4 (all from Southern Biotechnology) for 1 h. Plates were washed 3 times with PBST before addition of

100 µL/well OPD peroxidase (Sigma). Reaction was stopped by addition of 50 µL 2M $H_2SO_4$ 2 N and absorbance was recorded at 492 nm and corrected at 620 nm.

**Mice**

hFcγR$^{KI}$ and FcγR$^{null}$ mice were generated by Regeneron Pharmaceuticals Inc. to express hFcγRI, hFcγRIIa$_{H131}$, hFcγRIIb$_{I232}$, hFcγRIIc$_{stop13}$, hFcγRIIIa$_{V158}$ and hFcγRIIIb$_{NA2}$ polymorphic variants, or no FcγR, respectively, as described previously [97]. All mice were bred at Institut Pasteur and demonstrated normal development and breeding patterns. We used age-matched mice for all experiments. All animal care and experimentation were conducted in compliance with the guidelines and specific approval of the Animal Ethics committee CETEA (Institut Pasteur, Paris, France) registered under #2015-0024, and by the French Ministry of Research under agreement #00513.02.

**IgG-mediated passive systemic anaphylaxis**

hFcγR$^{KI}$ and FcγR$^{null}$ mice were passively sensitized intravenously (i.v.) with 3 mg of human IgG from PN allergic patients or EFS donors. 16 hours later, mice were challenged i.v. with 1 mg of PN extract. Rectal temperature measurements were performed using a digital thermometer (YSI) immediately before and at different time points for up to 1 h after injection of peanut extract.

**Passive systemic anaphylaxis in IL-4C-treated mice**

hFcγR$^{KI}$ and FcγR$^{null}$ mice were treated intraperitoneally at days -7, -4 and -1 with saline or IL-4C. IL-4C is a long-lasting formulation of IL-4 and consists of 10 µg murine IL-4 (Miltenyi Biotech) mixed with 50 µg of a neutralizing rat IgG1 anti-mouse IL-4 (clone 11B11, BioXCell). These complexes slowly dissociate *in vivo*, releasing free active IL-4 with a $t_{1/2}$ of ~1 day [201, 251]. At day -1, mice were passively sensitized i.v. with 3 mg of human IgG from PN allergic patients or healthy donors. 16 hours later, mice were then challenged i.v. with 1 mg of PN extract. Rectal temperature measurements were performed using a digital thermometer (YSI) immediately before and at different time points for up to 1 h after injection of peanut extract.

**Statistical analyses**

Data are presented as mean ± SEM for hypothermia and survival. ELISAs are represented as individual values with line indicating median and were analysed using one-way analysis of variance (ANOVA). Correlation analyses between PN-specific IgG and PN-specific IgE were performed using two-tailed non-parametric Spearman's tests. Temperature loss during PSA was compared by using 2-way repeated-measures ANOVA. Survival differences between groups were assessed for statistical significance using Mantel–Cox. Statistical analyses were performed with Prism Software (GraphPad Software, La Jolla, Calif). $P$ values $< 0.05$ are considered statistically significant.

**FIGURES**

## Figure 1.

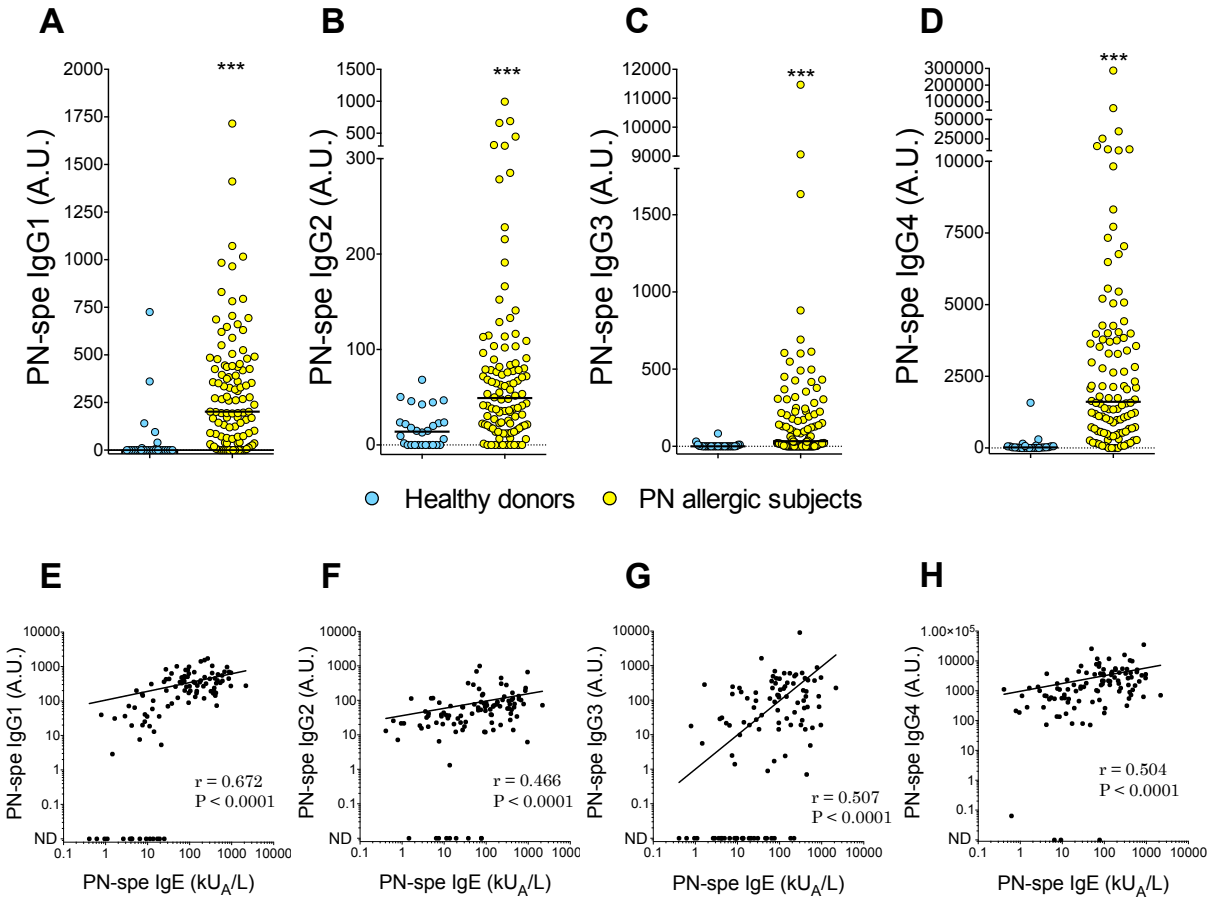


**Figure 1. PN specific IgG antibodies are elevated in PN-allergic patients and correlate with specific IgE levels. (A-D)** Measurements of PN-specific IgG from all subclasses in plasma samples from patients presenting with PN allergy (*n=107*) vs. healthy donors (with unknown allergy status) (*n=27*). One-way ANOVA test: *** P<0.0001. Results in A-D are represented as individual values and line indicating median; A.U.: arbitrary unit. **(E-H)** Plotting of individual data of PN-specific IgE (measured by standard ImmunoCAP, and indicated as kUA/L) and PN-specific IgG (in A.U.). Pearson's *R* correlation coefficients and *P* values (two-tailed) were calculated using all samples and values are indicated below the correlation curve. ND: not detected.

## Figure 2.

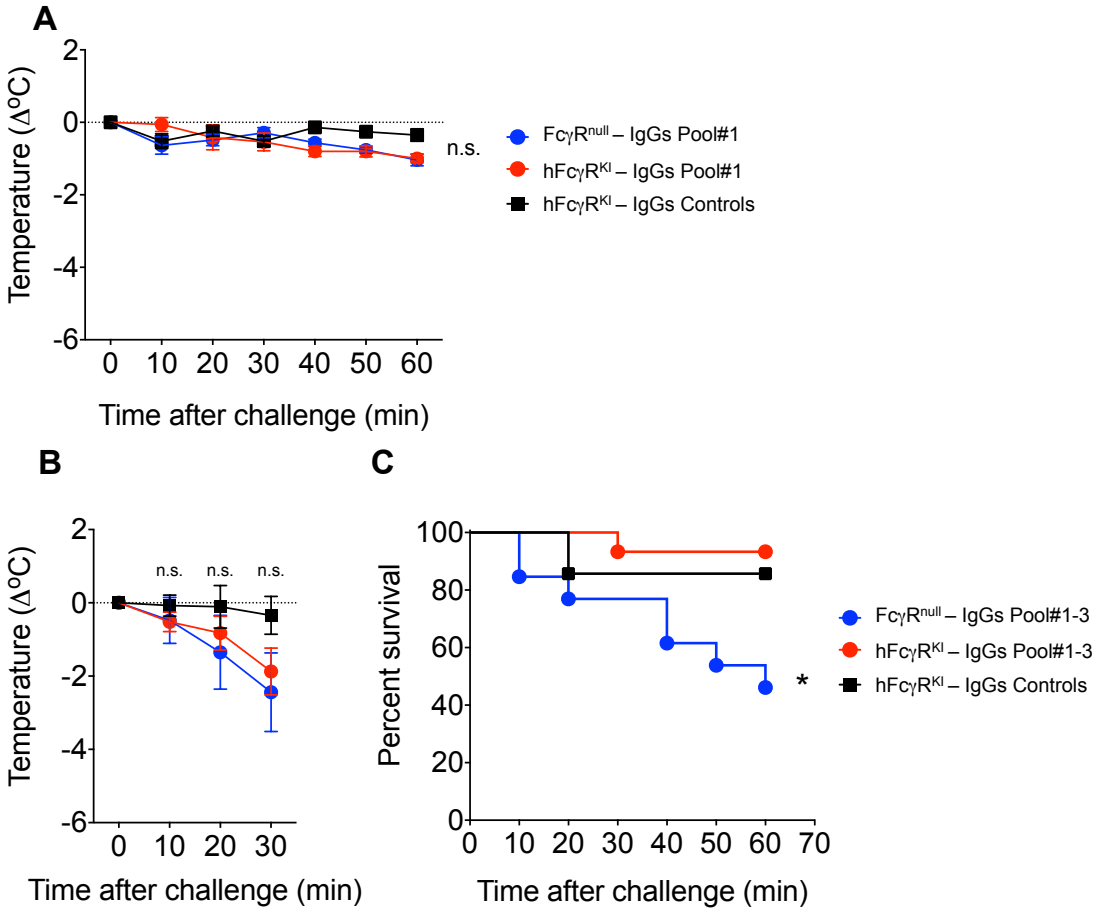


**Figure 2. Hypothermia and mortality in hFcγR$^{KI}$ and FcγR$^{null}$ mice sensitized with purified IgGs from plasma samples from PN allergic subjects or healthy donors. (A)** Changes in body temperature ($\Delta^{\circ}$ C [mean ± SEM]) after PN challenge in hFcγR$^{KI}$ mice sensitized with 3 mg of total IgGs purified from healthy donors (with unknown allergy status) (square), or in hFcγR$^{KI}$ mice (*n*=5/group; red circle) or FcγR$^{null}$ mice (*n*=4; blue circle) sensitized with 3 mg of total IgG from PN allergic subjects (Pool #1). **(B-C)** All mice were pre-treated with a long-lasting formulation of IL-4 (IL-4C) or saline at days -7, -3 and -1 before PN challenge. **(B)** Changes in body temperature ($\Delta^{\circ}$ C [mean ± SEM]) and **(C)** survival after intravenous (i.v.) injection of a pool of IgGs purified from PN allergic patients (*n=13-15*/group) or IgG purified from a pool of plasma from healthy donors (*n=7*) followed by PN challenge i.v. into hFcγR$^{KI}$ mice or FcγR$^{null}$ mice. Data in **(B-C)** are pooled from three independent experiments, each of which was performed with a different pool of IgG from PN allergic subjects (pools #1, 2 and 3). *, $P < 0.05$ by using Mantel-Cox log-rank test **(C)**. n.s. not statistically significant.

**SUPPLEMENTARY FIGURES**

## Supplementary Figure 1.

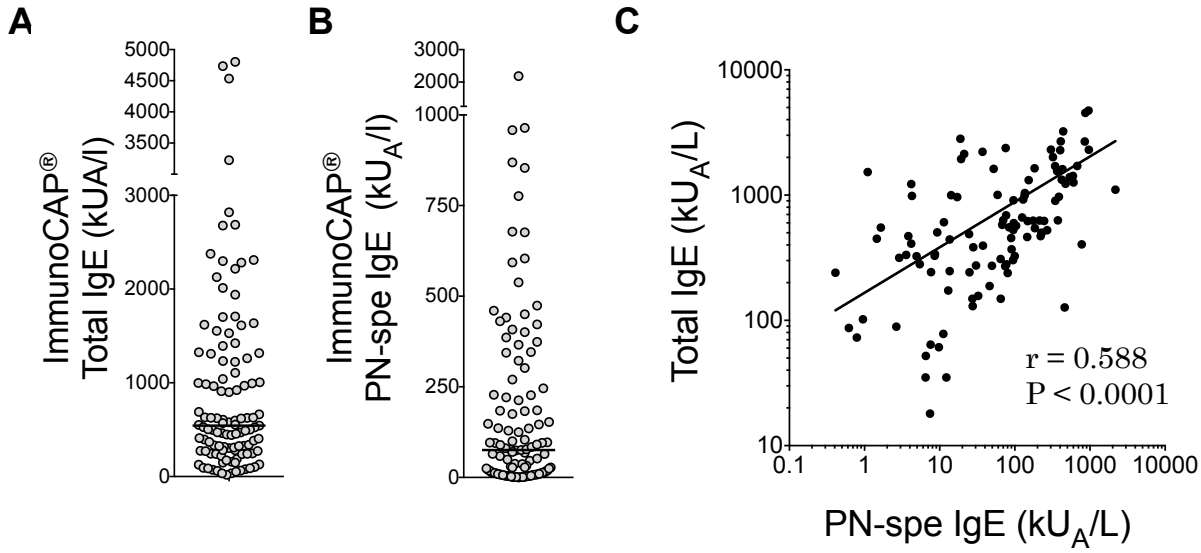


**Figure S1. Levels of total and PN-specific IgE antibodies in PN allergic subjects.** (**A-B**) Measurements of total IgE and IgE against PN extract in patients presenting with PN allergy (*n=107*) detected by ImmunoCAP. Results are represented as individual data; values are in kUA/L. (**C**) Plotting of individual data of total IgE and PN-specific IgE (in concentration). Correlation plot includes 107 peanut allergic subjects. Spearman-correlation with P indicated below the correlation curve.

# Supplementary Figure 2.

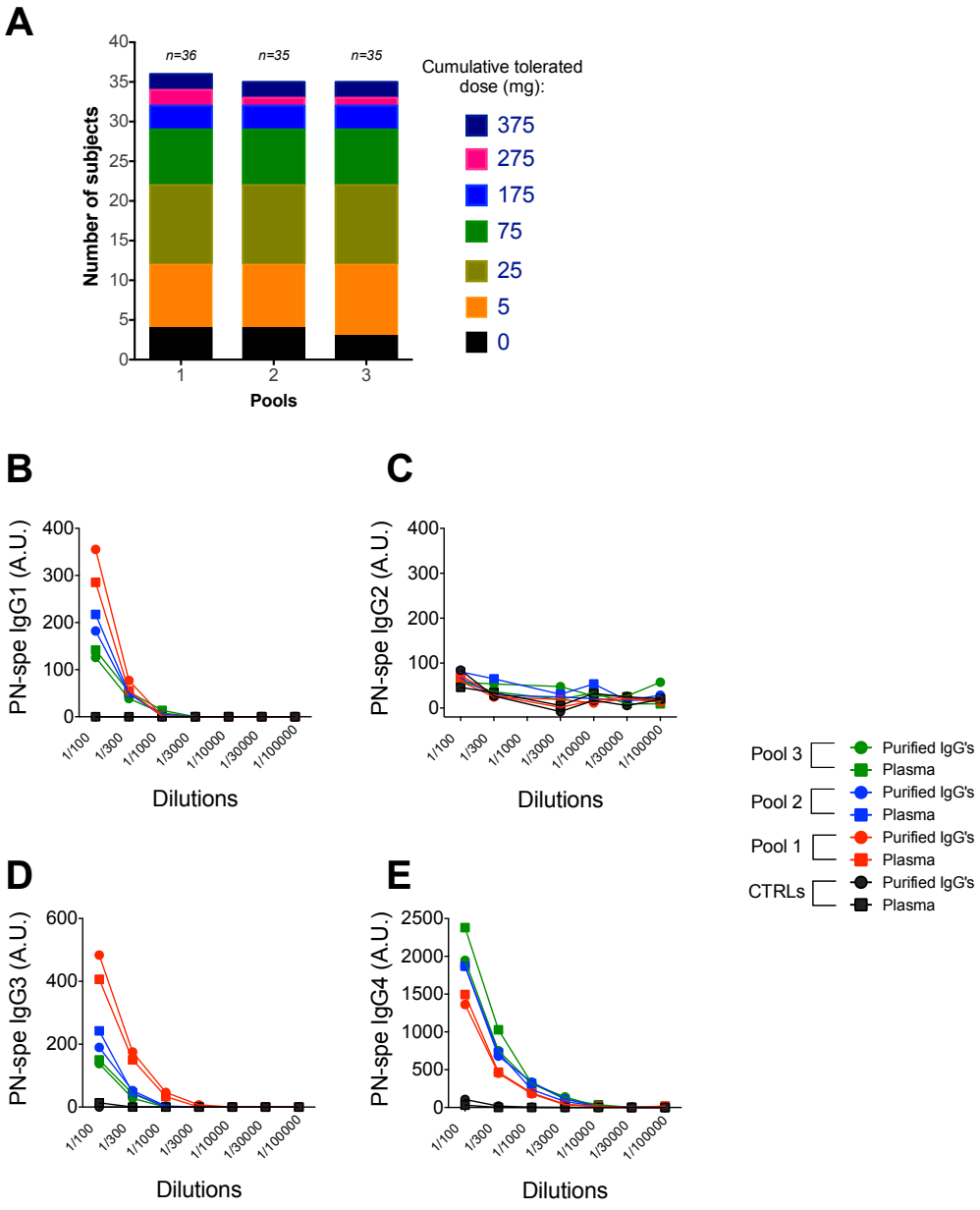


**Figure S2. Distribution and characterization of Pools 1 to 3 containing purified IgGs from PN allergic subjects.** (**A**) Plasma samples from PN allergic subjects were distributed in 3 different pools of *n*=35 or 36 plasma samples with homogeneous distribution of the patient's cumulative tolerated dose (CTD) obtained during the double-blind placebo-control oral food challenge (DBPCFC). Colours represent the different CTD values obtained during the DBPCFC (**B-E**). Measurements of IgG1-4 against PN extract in plasma or purified IgGs from pool 1, pool 2, pool 3 and in a pool of healthy donors. A.U.: arbitrary units.

# 6. Discussion

This thesis examines the contribution of IgG antibodies to systemic shock in three distinct models: an adjuvant-free mouse model of anaphylaxis; anaphylaxis induced by the therapeutic monoclonal antibody omalizumab; and peanut-induced anaphylaxis.

This discussion chapter is composed of five parts: I) sensitization and elicitation phases in mouse model of anaphylaxis; II) the detrimental role of the mAb Omalizumab in promoting FcγR-mediated adverse reactions; III) The potential benefits and limitations of the new Fc-engineered anti-IgE antibody we generated; IV) Passive anaphylaxis induced by transfer of IgG from peanut allergic subjects into FcγR humanized mice ; V) limitations of mouse models to study anaphylaxis; and finally, VI) general discussion.

## 6.1 PART I: Sensitization and challenge in the adjuvant-free mouse model of anaphylaxis

### 6.1.1 Are adjuvants reflective of the real-life sensitization process?

The word adjuvant is derived from the Latin *adjuvare*, meaning 'to help' or 'to aid'. Probably, adjuvants are best known for their use in vaccines. In these cases, adjuvants are added to vaccines formulations to enhance the immunogenicity of antigens in order to induce protection. Aluminum salts (alum) are the most widely used adjuvants in human vaccines and the first report of the adjuvant activity of aluminum compounds dates back to 1926 [258]. Glenny and colleagues reported a very strong antibody production by guinea pigs injected with toxoid precipitated with aluminum potassium sulfate when compared to animals receiving the toxoid alone [258]. Since then some other classes of adjuvants are also used in the clinics, as for example squalene-based oil-in-water emulsions, liposome or alum-adsorbed TLR4 agonists [259]. This discussion will mainly be focused on the adjuvant alum as it was our choice of adjuvant (with *B. pertussis* toxin) as a comparative to our adjuvant-free model of ovalbumin (OVA)-induced anaphylaxis.

Classically, adjuvants have been identified for their capacity to enhance the adaptive immune response to co-administered antigen [260]. In mice, alum injection in the intraperitoneal cavity induces a strong innate immune response with an influx of neutrophils, eosinophils, NK cells, monocytes, and DCs [261]. Tissue-resident macrophages and mast cells are among the first cells that sense damage caused by barrier disruption (tissue and endothelial damages) and rapidly signal to the recruitment of other innate immune cells, like

neutrophils and eosinophils [260, 261]. The link between innate and adaptive immunity is mostly done by DCs; conditional depletion of DCs and monocyte-derived inflammatory DCs during immunization in the presence of alum has been shown to abolish T-cell activation and reduce the production of antigen-specific antibodies [262]. DCs undergo an important maturation process guided signals coming from both the pathogen itself or indirectly through the recognition of DAMPs, which are released upon tissue damage [263]. This dual activation mechanism is importantly impacted by the administration of adjuvants.

Besides triggering an innate immune response, it has been proposed that aluminum-containing adjuvants ensure a long-lasting immune response through the formation of a depot [264], favoring a strong antibody response. Moreover, it has long been known that alum exerts some level of cellular stress or damage is able to produce numerous self-derived molecules associated with tissue damage such as uric acid, DNA RNA and ATP that act as endogenous danger signals (or DAMPs) [262, 263]. Indeed, the danger hypothesis was proposed by Polly Matzinger, who suggested that apart from self/non-self-discrimination against infections, danger signals from damaged cells can trigger activation of the immune system [265].

In the field of immunology, the use of adjuvant also extends to the immunization protocols, mainly for animal model studies, in order to elicit a strong immunological response. This strategic boost of the immune system leads to the production of specific antibodies towards an antigen of interest, allowing further investigation of immunological mechanisms behind both sensitization or effector phases of the response. Not surprisingly, adjuvants are therefore used in the sensitization phase in most protocols of induction of active systemic anaphylaxis (ASA).

Many different protocols for mouse models of ASA have been reported in the literature. The main differences in these models are the experimental antigens (usually, TNP-OVA, OVA, BSA and more recently, some more relevant allergen such as peanut or house dust mite - HDM) and the route of sensitization and challenge. The nature of the antigen and the route of immunization are definitely playing a role in the differences observed in ASA models; however, it is important to note that the choice of adjuvant is also crucial for the outcome of the response. Critically, certain adjuvants may favor the production of individual antibody isotypes, therefore influencing the pathway(s) leading to anaphylaxis in ASA models.

In most ASA using adjuvants in the sensitization phase, anaphylaxis can develop even in the absence of mast cells (MCs), IgE or FceRI [41, 45, 266, 267]. We hypothesized that the

use of adjuvant might have pushed IgG responses, and thereby masked the contribution of IgE and MCs. This was previously observed in mouse models of asthma, where sensitization of mice with OVA and alum followed by intranasal challenges with OVA induced full development of airway hyperresponsiveness (AHR) and eosinophilia in MC-deficient mice [268, 269]. By contrast, mice sensitized with endotoxin-free OVA in the absence of adjuvant developed AHR and airway eosinophilia which were fully dependent on MCs [268, 269]. We thus decided to evaluate whether ASA can be induced without any adjuvant in the sensitization phase, and whether such protocol would lead to anaphylaxis mediated by MCs and/or IgE.

As a whole response, we have clearly demonstrated that WT mice can be sensitized towards OVA in the absence of any artificial adjuvants during the sensitization phase. Our results demonstrated that OVA-sensitization is able to elicit both IgE- and IgG-specific antibodies when compared to PBS-immunized mice after several exposures to the allergen. Moreover, upon challenge with OVA, OVA-sensitized mice are able to display a rapid and sustained loss of core body temperature. Regarding IgG subclasses, we could detect both IgG1 and IgG2c specific antibodies in the plasma of sensitized mice [270]. Different protocols of ASA immunization described in the literature using adjuvants could detect important levels of IgG1 after exposure to allergens such as BSA [42] or peanut [41, 45, 49], however, we have less clear data when considering IgG2 levels, which are either not detected or not shown [41, 45, 49].

Considering the IgE levels, in most of the studies in which this parameter was measured it was possible to detect allergen-specific IgE in animals immunized with adjuvant [41, 45, 49]. By using standards methods of detection such as ELISA, we could not detect the presence of specific IgE in the sera of OVA-sensitized mice. This might be due to limitation in the sensitivity of our IgE ELISA. However, not being able to detect specific IgE in the serum does not necessarily mean lack of this immunoglobulin after adjuvant-free sensitization process. We were able to demonstrate the presence of OVA-specific IgE by means of *in vitro* peritoneal mast cell (PCMCs) degranulation assay in which we incubated MCs with serum of sensitized mice followed by stimulation with OVA. Using this indirect approach, we clearly showed that in our adjuvant-free model, mice develop levels of specific IgE in the blood that are able to promote MCs degranulation upon exposure to the allergen. The contribution of IgE was most definitely demonstrated by showing that mice deficient for FcεRI developed reduced hypothermia in our adjuvant-free ASA model. However, IgG-mediated responses

played a dominant role in our adjuvant-free ASA model, as mice lacking the IgG receptor FcγRIII where almost completely protected from anaphylaxis.

Another important point is that our adjuvant-free sensitization model may be more reflective of how a subject is sensitized to an allergen in 'real-life' conditions. Normally, an individual gets sensitized to an antigen in the presence of naturally-occurring adjuvants (the particle itself or contaminants and which are able to activate DCs). It is speculated that additional danger signals, such as LPS content in the preparation may be required to facilitate the sensitization process. Indeed, the presence of LPS in OVA preparations enabled intranasal sensitization, whether the absence of LPS resulted in development of tolerance in a mouse model of asthma [271]. However, in a case of subcutaneous sensitization, regardless of the presence or absence of LPS, a similar experimental asthma phenotype was generated [272]. We used endotoxin free OVA (<0.01 EU endotoxin per injection) for sensitization of mice in the adjuvant-free protocol, but it is impossible to be completely sure of the absence of traces of LPS or other toll-like receptors stimulants in the formulation, which could be extrapolated as an amount also seen in naturally occurring situations of sensitization. Nevertheless, the 'danger signal' necessary to provide the starting-point for an immune response can also possibly come from tissue damage at the injection site, due to the injection itself or the presence of a non-self-protein, such as OVA.

In conclusion, the adjuvant-free mouse model may be an attempt to explore the mechanisms of systemic anaphylaxis in a system more reflective of the clinical situation.

### 6.1.2 The importance of the challenge route for the outcome of the response

The previous part of the discussion was mainly focused on the sensitization phase and whether the presence or absence of adjuvants could make a difference in the outcome of antibody production and subsequent systemic anaphylaxis response. This section will be focused on the effector phase of the anaphylactic response, mostly considering the effector cells in our adjuvant-free model. I will try to depict the differences observed in our model compared to several ASA models described in the literature. Just as a note, all models cited below (including some of our experiments) used adjuvant in the sensitization phase.

Conflicting results have been obtained in ASA models regarding the involvement of several potential effector cells. Some ASA models are dependent on the IgE-FcεRI-MC axis [41, 45, 266, 267], whereas some others can develop, at least with respect to the features

analyzed, in IgE-, FcεRI-, and/or MC-deficient mice [40, 44, 214]. Depending on the mouse strain and ASA model used, basophils have been shown to either contribute to the systemic response [40, 45] or play little to no significant role [41, 49]. Similarly, depletion of monocytes/macrophages abrogate ASA in some models [41, 49], while antibody-mediated neutrophil depletion reduces anaphylaxis in other models [42]. The results obtained with our adjuvant-free ASA model strongly support the role of MCs and monocytes/macrophages in the development of immediate hypothermia, whereas neutrophils and basophils seem to not be necessary to the reaction.

One should consider the influence of the route of challenge in each of these reports, and the cell population(s) that would first respond, i.e. release mediators upon activation by antigen-antibody immune complexes. Regardless the demonstration of antigen-specific antibodies before challenge, either mast cells or FcεRI/IgE had a minor or no role in the development of systemic reaction when the intravenous (i.v.) route was chosen for antigen administration [40, 41, 44, 213, 214]. Conversely, MCs were necessary for the immediate hypothermia when challenge was done either intraperitoneally (i.p.) or by gavage [41, 45, 49, 266, 267]. This observation goes in line with our adjuvant-free model, in which mice were challenged by the intraperitoneal route and MCs had a partial contribution to the development of anaphylaxis (assessed by using MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice, and engraftment of these mice with bone marrow-derived cultured MCs [BMCMCs]) [270]. MCs are abundant in the peritoneal cavity and along the lamina propria, suggesting their major role in sensing and triggering the first signals for the development of immediate hypothermia when an antigen enters through intraperitoneal injection or gavage (antigen must cross the epithelial barrier). Interestingly, in a model of peanut-induced anaphylaxis, the i.v. exposure to the allergen-induced a more severe shock when compared to i.p. exposure, and MCs seems to amplify the response in both cases [41]. Because MC deficiency did not fully prevent shock, it was suggested that other cells contribute to the process.

Tissue macrophages highly populate the peritoneal cavity, and seem to either be necessary or act as an amplifier of the immediate hypothermia. When mice are sensitized in the presence of adjuvant, our observations demonstrated monocytes/macrophages as important cells contributing to the post-challenge mortality, since mice are completely protected from death when monocytes/macrophages are depleted before challenge [270]. In the adjuvant-free model, monocytes/macrophages are important enhancers of the immediate shock, because their depletion significantly (but not completely) reduced shock. Monocytes/macrophages also seem to be crucial for anaphylaxis when the antigen is

administered by gavage [49]. Monocytes constitute 5-10 % of blood leukocytes in the bloodstream, so allowing their response after the intravenous contact with the antigen. Indeed, some evidence from both active and passive models of anaphylaxis have reported an important role for monocytes/macrophages in the immediate hypothermia response after i.v. antigen challenge [46, 47, 182].

The route of challenge may also favor the contribution of neutrophils and basophils to systemic anaphylaxis. Our lab showed that neutrophils are sufficient to trigger anaphylaxis in some ASA models [42], whereas basophils contributed to a lesser extent. The same results were obtained by Khodoun and colleagues [43]. Interestingly, both reports used the intravenous route to challenge mice, suggesting that the first cells to get activated are the ones present in the bloodstream. Neutrophils seemed not necessary in the case of our adjuvant-free ASA model in which challenge with OVA was performed i.p. neither in a model in which gavage was done to elicit the systemic reaction [45, 127]. Moreover, basophils were also dispensable to the systemic reaction in our adjuvant-free i.p. challenged mouse model, but also in another report that used gavage as the route of challenge [49, 270].

Yet, it is also possible that neutrophils and/or basophils might be activated to promote anaphylaxis only in the presence of high titers of antibodies. This is suggested in our results, since we observed reduced anaphylaxis in mice pre-treated with neutrophil-depleting antibodies only in the ASA model using adjuvant (in which high titers of IgG antibodies where likely induced), but not in the adjuvant-free ASA model (in which IgG antibodies where induced at a lower level) [270]. In line with this, our group has recently shown that at high doses, both IgG2a and IgG2b are able to trigger passive anaphylaxis, which is partially blocked when mice are pre-treated with neutrophil-depleting antibodies [46].

Previous reports had implicated both histamine and PAF as the main mediators of ASA in mouse models [42, 92] and in human [92]. In line with these observations, antagonists of the histamine receptor H1R and of the PAF receptor (PAFR) partially blocked anaphylaxis in our adjuvant-free model when used separately, and almost completely abrogated anaphylaxis when used in combination. We further demonstrated that MCs where likely the main source of histamine in this model, as levels of histamine where markedly reduced after challenge in MC-deficient mice as compared to WT mice. Many cell types, including platelets, neutrophils, monocytes/macrophages and MCs, can produce PAF [216]. However, we suggest that monocytes/macrophages where the main source of PAF in our adjuvant-free ASA model, since pre-treatment with the H1R antagonist completely blocked the residual

anaphylaxis which developed in mice depleted from monocytes/macrophages, and the PAFR antagonist had no more effect in this group of mice [270].

Altogether, the data we obtain in our adjuvant-free mouse model confirm the importance of the classical IgE-FcεRI-MC-histamine pathway of anaphylaxis, but also clearly showed that, even in the virtual absence of adjuvant, an alternative pathway mediated by IgG-FcγRIII-monocytes/macrophages-PAF play an essential role in anaphylaxis. With these results in hand, we decided to focus the rest of this PhD project to study the human relevance of our findings, i.e. whether human IgG could also trigger anaphylaxis, and if so, through which pathway(s).

## 6.2 PART II: Omalizumab-induced adverse reactions

### 6.2.1 Size of immune complexes formed between Omalizumab and human IgE

Chapter 4 described that anaphylaxis can occur in hFcγR$^{KI}$ mice upon injection of Omalizumab:IgE immune complexes (ICs). We have chosen to use a ratio of one IgE molecule for 2 molecules of the anti-IgE (1:2) and we detected the formation of mainly trimeric structures. Previous reports have also shown that ICs formed were limited in size [273, 274]. Liu and colleagues demonstrated that complex formed *in vitro* when one of the interacting components is in large molar excess appear to have a trimeric structure [274]. The size of immune complexes was also assessed *in vivo* using cynomolgus monkeys. These monkeys had high quantities of endogenous IgE and received omalizumab intravenously. Eluted IgE:Omalizumab immune complexes were consistent with the *in vitro* observations, corresponding to heterodimers of 1:2 IgE:Omalizumab [273]. Altogether, we can conclude that omalizumab did not form big complexes and we can extrapolate that the ratio we chose corresponds to the probable size of immune complexes formed in humans.

Yet, the injection of IgE followed by Omalizumab was also able to induce anaphylaxis, demonstrating that immune complexes can be formed *in vivo*, mimicking how humans respond to the reception of the drug. This model can be considered clinically-relevant because it uses a drug currently implicated in the treatment of highly allergic patients in a context where myeloid cells recapitulate the human FcγR expression. However, one possible improvement could be to achieve similar results with the subcutaneous administration (exactly how humans are exposed to the drug) of omalizumab in mice expressing endogenous human IgE and IgE receptors along with human FcγRs. These mice have already been developed in our laboratory, and could be a great asset to further explore our findings.

### 6.2.2 Which FcγR could be responsible for IgE/Omalizumab ICs mediated anaphylaxis?

We demonstrated that Omalizumab:IgE ICs can mediate both skin inflammation at the injection site and systemic anaphylaxis in hFcγR$^{KI}$ mice, and that these reactions require one or several hFcγR, since they are markedly reduced in FcγR$^{null}$ mice. These results bring the following biological question: which hFcγR(s) could be the major trigger of anaphylaxis induced by Omalizumab?

We have demonstrated that immune complexes made of Omalizumab and IgE are able to bind to all activating FcγRs *in vitro*. Therefore, in theory these complexes could bind to any cell expressing activating FcγR *in vivo*. So, to simplify our understanding of the contribution of FcγR in the pathology of anaphylaxis induced by omalizumab, each of the activating FcγR will be explored individually.

IgG-FcγRI

As mentioned previously, IgE and Omalizumab immune complexes (ICs) are able to bind to the high affinity hFcγRI receptor *in vitro*. In $hFc\gamma R^{KI}$ mice, hFcγRI is restricted to dendritic cells and macrophages, and also expressed at low levels in monocytes [97]. hFcγRI is virtually occupied by monomeric IgG, however the fast dissociation of IgG from this receptor could favor the capture of small immune complexes and thus activate cells expressing this receptor [11, 275]. In the case of Omalizumab/IgE ICs however, it is very unlikely that these ICs could activate this receptor *in vivo*. The most important evidence comes from the Fc-engineered anti-IgE $N_{297}A$. We observed that this Fc-engineered mAb can still bind hFcγRI when complexed with IgE (at levels almost equivalent to WT Omalizumab), but is not able to trigger systemic anaphylaxis in $hFc\gamma R^{KI}$ mice. Such conclusion is also comforted by recent work from our lab using heat-aggregated IVIG (intravenous IgG is a pool of serum IgG of a thousand donors and contains a majority of IgG1) induced PSA [276]; hFcγRI does not contribute to PSA induced by heat-aggregated IVIG in $hFc\gamma R^{KI}$ mice because pre-treatment of mice with a blocking antibody (clone 197) against this receptor had no effect on anaphylaxis. Altogether, these results suggest that hFcγRI is not sufficient to trigger systemic anaphylaxis in the $hFc\gamma R^{KI}$ mice.

IgG-FcγRIIA

FcγRIIA is a low-affinity IgG receptor and is widely expressed in all myeloid cells and on the surface of platelets [97]. Due to its broad expression throughout the myeloid lineage, FcγRIIA could be a potential candidate to initiate/participate in the systemic anaphylaxis induced by Omalizumab. Critically, mFcγRIII is evidenced as one of the most important receptors in murine anaphylaxis and it is also expressed in all mouse myeloid compartment.

We can thus hypothesize that hFcγRIIA would have the same importance when considering anaphylaxis in humans. Indeed, previous data from our lab demonstrated that the expression of a transgenic hFcγRIIA in a mouse model was sufficient to induce IgG-dependent anaphylaxis; anaphylaxis was mediated by neutrophils and monocytes/macrophages in this model [70]. This also goes in line with recent work from our lab in which heat-aggregated IVIG intravenous administration induced anaphylaxis in hFcγRIIA$^{Tg}$, confirming the importance of this receptor to systemic shock [97]. Interestingly, this model was fully dependent on hFcγRIIA expressed on platelets, since platelet depletion attenuated anaphylaxis [97]. In a similar manner, by using blocking antibodies, hFcγRIIA was evidenced as the most important receptor in severe systemic anaphylaxis induced by heat-aggregated IVIG in humanized mice expressing low-affinity human FcγRs (FcγRIIA, FcγRIIB, FcγRIIIA, and FcγRIIIB) in place of endogenous mouse FcγRIIB, FcγRIII, and FcγRIV [71].

The use of human neutrophil activation assay *ex vivo* can also sustain our hypothesis that hFcγRIIA may be important to the development of systemic anaphylaxis. Upon stimulation with IgE and Omalizumab pre-formed ICs, human neutrophils were activated, as demonstrated by the shed of the surface marker CD62L. CD62L is the L-selectin protein expressed at the surface of non-activated neutrophils, and is shed once the cell get activated. Interestingly, we also detected a downregulation of CD32 (FcγRII) on the surface of neutrophils after incubation with Omalizumab:IgE ICs. Even though we used an anti-hFcγRII clone unable to distinguish between hFcγRIIA and hFcγRIIB, neutrophils are expected to express only hFcγRIIA on the cell surface [165], and our in vitro data using transfected CHO cells show that Omalizumab:IgE ICs do not bind FcγRIIB. Therefore, our results suggest that there was an engagement of FcγRIIA upon stimulation of neutrophils with Omalizumab:IgE ICs. The engagement of hFcγRIIA leads to rapid uptake of ICs by neutrophils; in the specific case of this experimental setting, neutrophil phagocytic receptor is engaged by ICs deposited in a large surface (the bottom of the plate). Hence, it is likely that neutrophils cannot completely engulf plate-bound ICs, generating so-called frustrated phagocytosis, that is able to induce cell activation [58]. Neutrophils purified from hFcγR$^{KI}$ mice were also activated by Omalizumab:IgE ICs, which lead to reduced expression of hFcγRIIA when using the same experimental conditions mentioned above. By contrast, neutrophils from FcγR$^{null}$ mice were

completely resistant to activation, confirming that engagement of hFcγR(s) on the surface of neutrophils is necessary for their activation.

We therefore decided to study the anaphylaxis response in mice expressing exclusively hFcγRIIA (hFcγRIIA$^{Tg}$ FcγR$^{null}$ mice). We passively injected ICs of IgE and omalizumab i.v. in hFcγRIIA$^{Tg}$ FcγR$^{null}$ versus hFcγR$^{KI}$ mice and followed body temperature for 60 minutes. The results obtained are represented in the **Figure 8** (below).

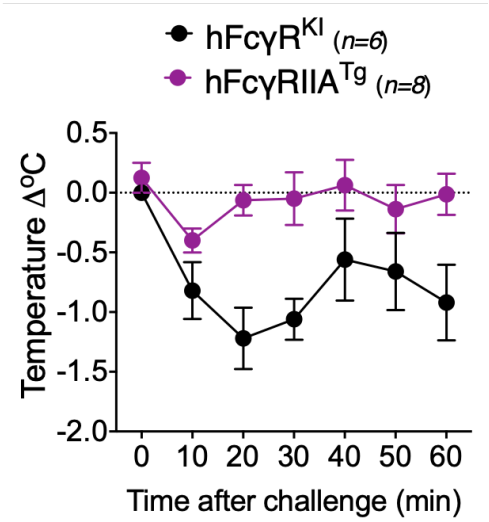


**Figure 8.Passive Systemic Anaphylaxis after injection of Omalizumab/IgE ICs**. Changes in body temperature (Δ°C [mean ± SEM]) after intravenous injection of pre-formed IgE/Omalizumab ICs into hFcγR$^{KI}$ mice (*n=6*) or hFcγRIIA$^{Tg}$ FcγR$^{null}$mice (*n=8*). Data are pooled from two independent experiments.

These preliminary results showed in Figure 8 suggest that hFcγRIIA alone is not sufficient to induce anaphylaxis by ICs made of IgE and Omalizumab. hFcγRIIA$^{Tg}$ mice express the hFcγRIIA$_{R131}$ variant, while hFcγR$^{KI}$ mice express the hFcγRIIA$_{H131}$ variant. Since these variants can have different affinity for Omalizumab:IgE ICs, we also performed one pilot experiment demonstrating that hFcγR$^{KI}$ mice were still susceptible to IC-mediated anaphylaxis even after hFcγRIIA$_{H131}$ blockade using a blocking antibody (data not shown). Altogether, these observations strongly suggest that expression of hFcγRIIA alone is not sufficient to trigger anaphylaxis mediated by immune complexes of IgE and Omalizumab. These are quite surprising results, because most of the PSA models reported in our laboratory relied on hFcγRIIA [70, 71, 97].

IgG-FcγRIIIA/B

As mentioned in Chapter 1, hFcγRIIIA is expressed by NK cells and monocyte/ macrophages, whereas FcγRIIIB is densely expressed by neutrophils, and can also be found at

a lower extent on basophils [168]. We have demonstrated *in vitro* that IgE and Omalizumab ICs are able to bind both hFcγRIIIA/B in transfected CHO cells expressing all hFcγRIIIA/B variants (Paper II – figure 1A). Therefore, we can hypothesize that these receptors could contribute to the activation of myeloid cells and to the development of anaphylaxis *in vivo* in hFcγR$^{KI}$ mice. It is noteworthy that in hFcγR$^{KI}$ mice, hFcγRIII is expressed at high levels on neutrophils, and at variable levels on monocytes/macrophages and NK cells, as seen in human subjects [71]. However, we still do not know the respective expression of the two receptors in hFcγR$^{KI}$ mice, since the anti-hFγRIII antibody clone used for characterization of hFcγRIIIA/B expression by flow cytometry did not allow distinction between CD16A (hFcγRIIIA) and CD16B (hFcγRIIIB) [71, 276].

hFcγRIIIB is highly expressed on human neutrophils with an estimated density of 200,000 to 300,000 molecules per cell. This hFcγR has been described as responsible for capturing soluble immune complexes in transgenic mice expressing hFcγRIIIB, but lacking the common γ chain (important for the expression of endogenous activating FcγRs) [277]. Linked to the membrane by a GPI anchor, hFcγRIIIB is unique among FcγRs and the downstream signaling diverge from what is seen for others FcγRs. A possible mechanism through which signal is transduced across the membrane upon engagement of hFcγRIIIB is through the interaction with other transmembrane receptors [278]. Several reports provide evidence that CR3 (CD11b/CD18) is a possible transmembrane partner of hFcγRIIIB [279], and that CD11b is in physical proximity to hFcγRIIIB on the plasma membrane of resting neutrophils [280]. hFcγRIIIB may also associate with hFcγRIIA for intracellular signaling. For instance, the cross-link of these two receptors has been implicated in synergistic increase of intracellular $Ca^{2+}$ and phagocytosis [281]. Importantly, a previous report showed that the cooperative engagement of hFcγRIIIB with hFcγRIIA promoted human neutrophil activation (with maximum $Ca^{2+}$ influx) in response to heat-aggregated IgGs [282].

In order to understand the potential contribution of hFcγRIIIA/B in our model, we will take a step back in our *in vitro* results. Unfortunately, when analyzing human neutrophil activation *ex vivo* using ICs made of IgE and Omalizumab, we did not assess the changes in expression of hFcγRIII. However, data from neutrophils of hFcγR$^{KI}$ mice demonstrated a reduction in hFcγRIII expression after incubation of IgE and anti-IgE WT ICs *in vitro* (**Figure 9**). This likely means that the receptor is downregulated via its attempt to uptake plate-bound

immune complexes (for frustrated phagocytosis, see above). In line with previous results, we observed reduced downregulation of hFcγRIII when using the Fc-engineered anti-IgE NA antibody. As expected, neutrophils purified from FcγR$^{null}$ mice did not show any downregulation of hFcγRIII. Altogether, these results suggest that hFcγRIIIA/B can be engaged by ICs of IgE and anti-IgE *in vitro*. We yet have to assess whether hFcγRIII contributes to anaphylaxis induced by Omalizumab *in vivo* in our humanized model.

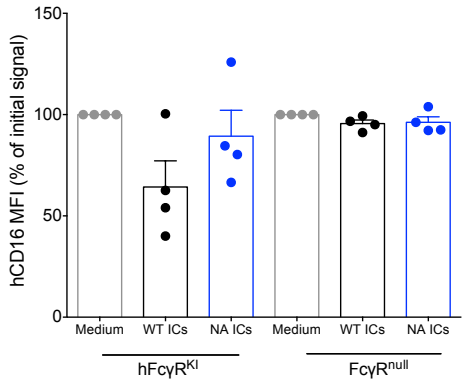


**Figure 9. Surface expression of human CD16 on neutrophils purified from hFcγRKI or FcγRnull mice**. hCD16 expression on CD11b$^{+}$Ly6G$^{+}$ neutrophils purified from hFcγR$^{KI}$ or FcγR$^{null}$ mice after 1 h incubation with IgE/WT anti-IgE or IgE/NA anti-IgE ICs or medium alone. Results show values from individual mice with bars indicating means ± SEM normalized against cells stimulated with medium alone. Data are pooled from at least two independent experiments (total n=4/group).

Previous results from our laboratory showed that expression of hFcγRIII on the surface of neutrophils is markedly reduced after IVIG-induced PSA in hFcγR$^{KI}$ mice [71], which suggest a downregulation via uptake of high levels of large immune complexes, or cleavage from the surface as the neutrophils get activated. However, the selectively blockade of hFcγRIIA was sufficient to completely block anaphylaxis in this setting (see above). Even if we have not formally analyzed the downregulation of hFcγRIII after PSA induced by ICs made of IgE and Omalizumab in hFcγR$^{KI}$ mice, it is tempting to hypothesize that this receptor could also be engaged in the presence of high-levels of pre-formed immune complexes in the bloodstream. Indeed, we have recently demonstrated that treatment of hFcγR$^{KI}$ mice with a new cocktail of blocking antibodies against all activating FcγRs (hFcγRI, hFcγRIIA and hFcγRIII) was able to protect mice from the development of hypothermia induced by ICs of IgE/Omalizumab (Richard Le-Goff *et al*. unpublished data). If we assume that anaphylaxis was not mediated by either hFcγRI or hFcγRIIA (see above), hFcγRIII would be the sole remaining receptor able to trigger the anaphylactic reaction in these experimental conditions. Additional evidence was found in the requirement of hFcγRIII to induce IgG-mediated anaphylaxis in a mouse model expressing human low-affinity FcγRs[283] in place of mFcγRIII and mFcγRIV (but retaining mFcγRIIb in the locus), or expressing hFcγRIIIb in place of mFcγRIV (retaining mFcγRIII and mFcγRIIb in the locus). In these mice, the authors observed anaphylaxis induced by the

injection of human IgG1 against an endogenous mouse protein; surprisingly, neutrophil hFcγRIIIB was sufficient and necessary to induce systemic shock [283]. It is important to note that the authors used mice expressing not only hFcγR but also mouse FcγRs, hence the possible contribution of other mouse FcγRs (as for example the potential activating effect of mFcγRIII or the potential inhibitory effect of FcγRIIB). Nevertheless, these results suggest that ICs of IgE/Omalizumab could potentially activate hFcγRIII on neutrophils to trigger anaphylaxis in $hFc\gamma R^{KI}$ mice.

Human neutrophils express much higher levels of hFcγRIIIB on the surface than hFcγRIIA, however the affinity of hFcγRIIIB ($2x10^5$ $M^{-1}$) for IgG1 is much lower than that of hFcγRIIA (3 to $5x10^6$ $M^{-1}$ depending on the allelic variant) [11]. One would expect that small immune complexes, such as the one observed in IgE and Omalizumab (Paper II – Supplementary Figure 1C), are not likely to cross-link two hFcγRIIA molecules, and induce activation of the cell (in opposition to what happens when in presence of large ICs – such as the ones formed by IVIG). Instead, ICs could be captured by hFcγRIIIB and once stabilized on the cell surface, would be able to reach and have a cooperative engagement with hFcγRIIA, leading to cell activation. A second hypothesis could be that once ICs are in contact with hFcγRIIIB, this receptor is downregulated from the cell surface, allowing additional ICs to bind remaining and accessible hFcγRIIA.

In conclusion, further investigation is needed to understand the contribution of each activating hFcγR in anaphylaxis induced by IgE/Omalizumab ICs. The availability in our laboratory of blocking antibodies for each hFcγR will now enable us to assess this in the near future.

## 6.3 PART III: Potential benefits and limitations of the Fc-engineered anti-IgE antibody we generated

An IgG molecule contains a conserved N-linked glycan attached at asparagine 297 (Asn297) in each Cγ2 heavy chain domain. The heterogeneity in oligosaccharides results in multiple glycoforms that can influence the biological activity of the antibody. The reason is because glycans play essential role for the activation of downstream effector functions, yet such glycosylation has not been reported to affect antigen binding [284]. Indeed, we have shown that the aglycosylated version ($N_{297}A$) of the anti-IgE antibody Omalizumab still retains the property of trapping free IgE antibodies and therefore impairing the binding of IgE to FcεRI.

The composition of the glycan at Asn297 has been shown to influence the quaternary structure of the Fc portion [285]. Oligosaccharides moieties determine the 'open' conformation of IgG Cγ2 domain and the conformation of the Cγ2 domain is directly linked to the capacity of an IgG to interact with FcγRs [138]. This was confirmed by the progressive removal of the sugar residues leading to an approach in the Cγ2 domain ('closed conformation') [138]. In addition, the glycan has been suggested to impact antibody conformation via specific glycan-protein and glycan-glycan interactions [136]. The close proximity of glycan and FcγR might directly contribute to a glycan-protein interaction [139]; or Fc-glycans might also interact with glycan conserved in FcγRIIIA and FcγRIIIB, for example [140].

Independently of the mechanism, the sugar in the Fc portion allows IgG to interact with the low affinity FcγRs [286]. However, the complete removal of the Fc glycan of an IgG does not impair binding to FcγRI [287, 288]. Our results are consistent with these previous observations, because immune complexes made of IgE and the anti-IgE $N_{297}A$ mutant have markedly reduced binding to the low affinity FcγRs, whereas they still bind to the high affinity receptor FcγRI in an *in vitro* binding assay (Paper II - Figure 3A). These results emphasize that the high baseline affinity for IgG1 cannot be fully overcome by the exchange of the asparagine residue for an alanine at position 297; thus, it remains available to mediate IgG-dependent effector functions. We can then question the relevance of this observation in the case of the anti-IgE NA and the possible *in vivo* effects if administered to a patient. When considering blood cells, the high-affinity FcγRI is speculated to be saturated by high-levels of serum monomeric IgG1 (and IgG3) [286]. One cannot exclude however that the presence of

immune complexes may be able to activate FcγRI, as demonstrated in *in vitro* studies [289] and several mouse models system *in vivo* [275, 290, 291]. The rapid dissociation of IgG from this receptor could favor the capture of small immune complexes or sparsely bound antigens [11, 275]. Moreover, experimental evidence suggests that under steady state conditions, hFcγRI is exchanging bound monomeric IgG, thus favoring the binding of immune complexes in certain conditions [11]. In humans, FcγRI is restricted to dendritic cells and monocytes/macrophages, but not other myeloid cells [59]. hFcγR$^{KI}$ mice recapitulate hFcγRI patterns of human immune cells, except that monocytes express the receptor at lower level as compared to human (Gillis, CM, unpublished observations), which may reflect different maturation of activation status according to the microbiological environment (SPF mice). In our experimental conditions, we have shown that immune complexes of IgE and anti-IgE NA are not able to induce systemic anaphylaxis; however, these pre-formed immune complexes may still induce a low/mild inflammation at the site of the injection when administered subcutaneously (Paper II - Figure 4C). The lack of endogenous human IgG to compete with the high-affinity receptor (even though we know that mouse IgG binds to human receptors to some extent) may have favored local inflammation induced by ICs of the NA anti-IgE.

The sequestration of free IgE has been extensively described as the major mechanism of action of Omalizumab when administered to patients [292]. Omalizumab suppresses allergen-mediated degranulation of mast cells and basophils without inducing cell activation by the cross-link of the IgE receptor, mainly because omalizumab does not bind IgE already bound to FcεRI or CD23 [292, 293]. Moreover, the therapeutic activity of Omalizumab did not seem to involve the suppression of IgE+ B cells or plasma cells [294], although a report has shown this effect when humanized mice received high doses of Omalizumab [295]. In one attempt to create an improved version of Omalizumab, Chu and colleagues have generated an Fc-engineered anti-IgE antibody with increased affinity for hFcγRIIB, XmAb7195 [295]. The authors demonstrated that XmAb7195 could block free IgE and inhibit IgE production in B cells through co-engagement of membrane IgE and hFcγRIIB, thus reducing the formation of IgE-secreting plasma cells [295]. In a first-in-human phase 1a trial in healthy volunteers (NCT02148744), XmAb7195 decreased IgE levels below the limit of detection in 90% of subjects that had detectable IgE levels at baseline. Transient thrombocytopenia was observed at a dose of 3 mg/kg, but no other major adverse events were reported [296]. Importantly, in a small study conducted in atopic subjects, thrombocytopenia was observed in all subjects

receiving ≥ 2 mg/kg of XmAb7195, meanwhile one atopic subject (with a history of seasonal allergies) experienced severe bronchospasm after drug infusion, possibly suggesting an anaphylactic reaction [296]. Interestingly, these two-point mutations in the IgG1 Fc portion of the mAb ($S_{267}E$ and $L_{328}F$) increase the binding affinity to inhibitory IgG receptor FcγRIIB by 400 times compared to Omalizumab, but it has been reported to also enhanced binding affinity to hFcγRIIA [297], the sole FcγR on human platelets. These observations suggest therefore that the mechanism by which this anti-IgE antibody induce side effects might be through the engagement of FcγRs, in particular hFcγRIIA. The data obtained in this study corroborate with our in vivo study using Omalizumab, thus providing further evidence that FcγRs can mediate side effects induced by anti-IgE therapeutic mAbs.

Critically, the study of Chu and colleagues has revealed a potential caveat involving the use of Fc-engineered anti-IgE antibodies with reduced FcγR binding. They have generated an anti-IgE antibody with two-point mutations in the Fc portion ($G_{236}R$ and $L_{328}R$) called XENP7196, that showed reduced binding to all FcγRs [295]. *In vitro* experiments showed that this antibody had reduced capacity to block IgE production by B cells as compared to Omalizumab. These results suggest that the engagement of Fc receptors may play a critical role also in the control of IgE production [295], possibly through the binding to the inhibitory hFcγRIIB. Thus, the assumption that the anti-IgE $N_{297}A$ could be used as a potential candidate to treat allergic diseases needs to be further investigated, and it would be informative to explore the possible effects of this mAb on B cells *in vivo*.

## 6.4 PART IV: Passive anaphylaxis induced by transfer of IgG from peanut allergic subjects into FcγR humanized mice

The role that endogenous IgG may play in the development of anaphylaxis in humans is still remain under debate. The ongoing work we present in this thesis brought some interesting insights on the role of human IgGs in peanut induced anaphylaxis using a humanized mouse model. Firstly, we demonstrated that highly peanut allergic patients have high levels of peanut-specific (PN-spe) IgG in the plasma compared to blood donors. Moreover, clinical data allowed us to correlate our findings to available levels of total and peanut-specific IgE; we detected PN-spe IgG1, IgG2, IgG3 and IgG4, and these levels positively correlate with PN-spe IgE levels. These results suggest that quantification of PN-specific IgGs could be used as an additional test in the diagnosis of PN allergy, mainly because all of the samples analyzed had at least one subclass of PN-spe IgG detectable in the plasma. However, it is important to note that in some subjects, levels of one or more PN-specific IgG subclass were undetectable by ELISA, while ImmunoCAP could still detect specific IgE. It could be informative to correlate the data we obtained with further clinical parameters used for the peanut allergy diagnosis, such as the levels of the major allergens Ara h1 and Ara h2 specific IgE. Those data were already shared by our collaborators at Stanford and require analysis.

We then described that anaphylaxis could be induced by sensitization with human IgGs purified from highly peanut-allergic patients followed by challenge with peanut extract in mice expressing all human FcγRs. Interestingly, the doses of purified IgG (3mg/mouse) we used in our PSA model can be considered closer to the physiological situation than most passive IgG transfer models in which high amounts of specific IgG are administered before challenge. Indeed, human have on average around 10 mg/ml of total IgG in the blood, the dose we transferred thus resulted in a circulating dose of IgG that is 5-10 times lower than in human. It is noteworthy that this quantity represented the technical limit of volume injection in mice, yet it was still sufficient to induce an anaphylactic response in this murine model. Therefore, it is reasonable to question the need of high amounts of IgG in order to study anaphylaxis in mice. Additionally, this model reflects the diversity of IgGs present in the circulation (opposing to use of only specific antibodies), and therefore, it can simulate a possible occupation of FcγR by IgGs *in vivo*. In the case of specific monoclonal antibody

passive models, one subclass of IgG is responsible for the binding of FcγR without the competition of endogenous human IgG. We have shown that even in the circumstance of polyclonal human IgGs, specific IgGs are able to induce a sustained response, representing a more representative situation of how an anaphylactic response may happen in the human context.

Although we did not formally assess the potential contribution of each IgG subclass in our model of peanut-anaphylaxis, we can conclude that the assembly of IgGs are responsible, at least in part, for the shock induction. The passive transfer of a certain human subclass of specific IgG into humanized mice could bring valuable insight to the individual contribution of each subclass to the anaphylactic shock, as already previously shown in our lab for mouse IgGs [46]. We have recently produced different subclasses of PN-specific IgGs (using VH and VL sequences from [298]) that could be used to passively sensitize our humanized mice. One particular subclass could stand out as protective in this model of peanut-induced anaphylaxis, as indeed IgG4 that has been reported to have protective roles in humans [299]. Importantly, we picture that the presence of a polyclonal antibody pool, considering also the subclass diversity in IgGs is an important advantage of this newly developed humanized PN induced anaphylaxis.

Moreover, our model considers also the competition among IgG subclasses to bind the antigen. One could expect that different IgG subclasses bind the antigen to form immune complexes and these complexes are responsible for the engagement of FcγR and the development of anaphylaxis. PN-IgG immune complexes could directly engage not only the activating receptors leading to release of mediators, but also the inhibitory receptor FcγRIIB, thereby limiting anaphylaxis in our model. Whereas we consistently observe FcγRIIB expression on B cells in hFcγR$^{KI}$ mice, we did not observe FcγRIIB on mast cells or basophils in naïve hFcγR$^{KI}$ mice [71]. It is however possible that such expression of FcγRIIB on mast cells or basophils could be induced by the pre-treatment with IL-4C (to increase their susceptibility to develop anaphylaxis [201, 251]), and this will be assessed in follow-up experiments.

Surprisingly, the passive sensitization with purified IgGs from the plasma of PN allergic patients followed by PN challenge induced hypothermia into hFcγR$^{KI}$ or FcγR$^{null}$ mice pre-treated with IL-4C. These data are highly consistent with two previous reports showing that in mice pre-treated with IL-4C, PN by itself can induce signs of anaphylaxis [201, 251]. The proposed mechanism for such effect was a direct activation of both classical and lectin

complement pathways by PN extracts [239, 240]. The authors showed that C3a produced upon PN injection can induce anaphylaxis through activation of macrophages and basophils, and to a lesser extent mast cells, and that this reaction was mostly dependent on platelet-activating factor (PAF) [239]. Moreover, this intrinsic capacity of complement activation by the allergen itself does not seem to be an exclusive characteristic of PN allergen, as it has been reported also for wasp venoms [300]. The authors found that levels of C3a increased after an induced wasp-sting anaphylaxis in patients with previous anaphylactic reactions to wasp-sting; C3a levels also correlated positively with the severity of the reaction [300].

In line with this, we have observed that mice lacking all FcγRs had a significantly higher mortality when receiving PN after administration of IgGs from PN allergic patients as compared to hFcγR$^{KI}$ mice receiving either IgG from PN allergic patients or healthy donors. These preliminary results could suggest a potential protective role for hFcγRs in this PN anaphylaxis model. Further investigations are required in order to understand the mechanism explaining these results. If PN-specific IgG is required for the increased mortality rate in FcγR$^{null}$ mice in this PN anaphylaxis model, one potential hypothesis would be that in the absence of FcγRs, PN-IgG immune complexes are not trapped by FcγRs and are more "available" to activate the complement pathway. Hence, it could be informative to assess whether activation of C1q by PN-IgG immune complexes participate to anaphylaxis in our model. This will be performed by comparing responses of FcγR$^{null}$ mice and C1q$^{-/-}$FcγR$^{null}$ mice (recently available in our laboratory). Finally, since we are using human IgG to trigger anaphylaxis, it will be important to assess whether these antibodies can also activate human C1q. This will now be possible as we obtained humanized hC1q$^{KI}$ mice (as a collaboration with Regeneron Pharmaceuticals) and are now developing hC1q$^{KI}$hFcγR$^{KI}$ mice.

It is however possible that the constitutive lack of FcγRs in FcγR$^{null}$ mice favor development of the complement pathway, and that thus FcγR$^{null}$ mice develop stronger complement-dependent anaphylaxis upon PN challenge. To date, we have not assessed responses of FcγR$^{null}$ mice to PN extract alone (that is in the absence of transferred human IgG). Activation of the complement pathway by PN has been reported by other groups and was not observed with other major food allergens [239]. It is tempting to speculate that this unique feature among food allergens might participate to the fact that PN can induce particularly strong cases of anaphylaxis in humans.

To date, there are no approved treatments for food allergy, except food avoidance. However, several potential new treatments are under investigation, including oral immunotherapy (OIT) [301]. OIT consists in the administration of slowly increasing doses of the food allergen over the course of several months; and several IgG isotypes are produced during OIT course [299]. It has been hypothesized that some of these IgG could mediate at least in part the protective effects of OIT, however, the immunological mechanisms underlying the effects of OIT remain to be fully understood. Interestingly, a systematic review of 12 studies including more than 1000 patients highlighted that current OIT treatments resulted in roughly three times increase in risk and severity of anaphylaxis as compared to food avoidance or placebo [302]. Considering our preliminary results in the humanized mouse model, we can speculate that these patients have higher risk of developing anaphylaxis possibly due to the presence of high levels of IgG that would favor the shock. However, further investigation is needed to support this hypothesis.

## 6.5 PART V: limitations of the study of anaphylaxis in mouse models

In human, anaphylactic shock is a severe and rapid systemic reaction that can potentially lead to death. Upon the first signs of reaction, patients receive immediate treatment to restrain the symptoms and avoid undesirable outcomes. In some cases, blood sampling is used for diagnostics, and is highly valuable to research purposes; however, these samples are limited and do not allow thorough investigations about the mechanisms triggering the disease in the first place. Mouse models allow a better appreciation of some parameters of systemic anaphylaxis which cannot be studied in humans. Nevertheless, these mouse models also possess several limitations, and conclusions obtained using these models should be carefully interpreted.

### 6.5.1 Readouts of anaphylaxis in mouse models

The clinical manifestations of an allergic shock in humans can be very different from those observed in mice. Classically, humans experience mucocutaneous symptoms (pruritus, flushing, urticaria, angioedema) with respiratory complications (wheezing, stridor, hypoxemia/cyanosis) and/or cardiovascular signs (hypotension, tachycardia, cardiac failure) that might lead to a fatality [174] (**Box 1 – page 35**). In the specific case of perioperative shock, values of arterial hypotension, hypocapnia, and hypoxemia positively correlated with severity of anaphylaxis [303]. Mice may experience similar symptoms, such as the drop of blood pressure, respiratory signs (conductance and compliance [213]) and sometimes death; in addition, an important loss of core body temperature and reduced activity in the cage seem to be the exclusivity of animal models. Indeed, Charles Richet described the development of severe hypothermia and ataxia in his first anaphylaxis experiments in dogs [304]. During an anaphylactic shock, hypothermia is certainly not a clinical manifestation observed in humans, however, represents a non-invasive and easy method to assess the development of anaphylaxis in mice. Clinical score based on mice behavior can also be a useful tool to assess hypersensitivity reaction in mice, as the loss of mobility correlates with the severity of shock [41, 49]. However, the clinical score is very subjective, as it depends on the experimenter judgment; thus, most of the time, the clinical score is an additional parameter to the decrease of core body temperature. Assessment of blood pressure and respiratory alterations could be an interesting option, but it would require anesthetized mice; though, previous experiments in the lab have shown that anesthesia might aggravate the course of the anaphylactic reaction.

Finally, mortality can also be an important readout in certain models of anaphylaxis in mice. As shown in Chapter 3 [270], sustained loss of body temperature may lead to death in mice sensitized in the presence of adjuvant, whereas it was not the main feature observed in the absence of adjuvant. Death was also observed in mice sensitized with human IgGs followed by intravenous challenge with peanut (Chapter 5).

*Genetically modified mouse models & tools to understand anaphylaxis*

Towards more refined models to study anaphylaxis *in vivo*, mouse models try to integrate current clinical findings of disease characteristics, pathways, and specific phenotypes. In this way, mouse models integrate and/or combine knock-out genes, the insertion of the transgene or knock-in genes. The selective blockage of a specific receptor or antibody-mediated cell depletion is also a handful in the comprehension of anaphylaxis mechanisms. Whilst the conclusions are innovative, these findings come at a particularly sensitive to the limitations of the model chosen.

*Knockout and mutant mouse models*

The contribution of mouse FcRs was extensively studied by using FcR-deficient mice. To study the role of Fc receptors, one of the most used knock-out mice is the FcRγ$^{-/-}$ mice that genetically lack the expression of the ITAM-bearing subunit γ. FcRγ$^{-/-}$ mice lack the surface expression of mouse FcεRI and all the activating FcγRs. However, the phenotypes observed in FcRγ$^{-/-}$ may be partially or completely attributed to molecules not related to FcRs since the γ chain is also expressed in non-Fc-related proteins, such as the NK cytotoxicity receptor [305], or the TCR complex that also use the γ chain for intracellular signaling, among others. Therefore, the lack of γ chain may imply in different disfunctions due to the lack of several receptors, guiding of misleading conclusions and possible overestimation of FcRs contribution to certain pathologies. The generation of FcγR$^{null}$ mice (knock-out for all mouse FcγRs) finally allowed the investigators to address the role of FcγR-deficiency without the biases introduced by the deficiency in the γ subunit [97, 247]. Importantly, FcγR$^{null}$ mice (that still express the FcRγ subunit) were used as our controls to the hFcγR$^{KI}$ mice.

Single FcR deficiency can be useful to better understand the role of a sole receptor in anaphylaxis. However, the single FcR deficiency may also affect the expression of other

FcRs; as of example, mFcγRIII$^{-/-}$ mice exhibit increased expression of mFcγRIIB inhibitory on the surface of circulating myeloid cells [46]. Hence, the contribution of other activator receptors (mFcγRI or mFcγRIV) might be masked by the overexpression of mFcγRIIB. Another example is that mFcγRIIB$^{-/-}$ and mFcγRIII$^{-/-}$ mice exhibit increased expression of mFcγRIV compared to WT mice [11]. Therefore, we can anticipate that the role attributed to mFcγRIII in our active model may be overestimated [270]; however, we could partially answer this question by using a mFcγRIV blocking antibody and discarding its contribution to our model [270]. These models do not address possible collaborative effects between different FcRs, neither compensatory mechanism may influence in the outcome of the response. Hence, the conclusions obtained from the use of these knock-out models may require caution.

Likewise, specific cell deficiency is also used to study their contribution to a variety of anaphylaxis models. In Paper I we assessed the role of mast cells in the adjuvant-free model using different MC-deficient models [270]. More coherent than relying on only one model, conclusions were drawn using different strategies to avoid misleading assumptions due to the limitations of the chosen model. For example, C57BL/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice is widely used to analyze functions of MC *in vivo*; these mice are deficient for KIT, the receptor of SCF and are commonly referred as *kit mutant mice*. Kit mutant mice are profoundly deficient in MC, but also lack melanocytes and have several other phenotypic abnormalities (KIT is expressed in several other cellular types, e.g. subpopulation of neurons, keratinocytes, tubular epithelial cells in the kidney) [306]. So, one cannot attribute the results to a sole or even partial contribution of MC when KIT mutation may direct or indirectly affect other cell lineages. The adoptive transfer of genetically-compatible *in vitro* derived mast cells can selectively replace some MC cell populations and help in better understanding the role of MC *in vivo*; we have shown that the adoptive transfer of MC contributes to partially restore susceptibility to anaphylaxis in the adjuvant-free mouse model [270].

Because of potential caveats inherent in interpreting findings based on work employing only kit mutant mice, we also sought to validate our results in other MC-deficient mice which lack abnormalities related to KIT structure and expression. The Cpa3-Cre; Mcl-1$^{fl/fl}$ mice have a transgenic expression of Cre-recombinase under the control of a Cpa3 promoter and are crossed them with mice that the gene coding for the anti-apoptotic factor *myeloid cells leukemia sequence 1* (Mcl-1) was floxed [306]. Along with MC-deficiency, these mice also exhibit substantial reduction on basophils numbers in the bone marrow, spleen and blood. Therefore, care should be taken with conclusions withdraw using only these

mutant mice, because the lack of full ablation of a certain population may mislead the contribution of these cells in a certain model. In order to rule out the contribution of basophils in the adjuvant-free mouse model, we have chosen to use a selectively ablation of a DTR model (more details below).

*Mouse FcR transgenic models*

Transgenic mice have been extensively used to study the mechanisms behind several Fc receptor mediated diseases where one or more FcR is implicated. However, two major caveats may be inherently important to evaluate. *a)* the introduction of foreign DNA generates a transgene able to randomly integrate as one or more copies into the mouse genome, even in the presence of its own human promoter and *b)* this insertion may not always recapitulate the expression seen in humans. For example, in the hFcγRI$^{Tg}$ mouse, neutrophils expressed constitutively this human receptor, whereas it is "only" inducible in human neutrophils [289]. Conversely, the expression of hFcγRIIA seems to correspond to that observed in humans in the hFcγRIIA$^{Tg}$ mice [307]; also, hFcεRIα$^{Tg}$ mice (bred on a mouse FcεRI-deficient background) express a 'humanized' FcεRI receptor with a similar cellular distribution as that found in humans [119, 243]. Finally, the heterogeneity of transgene expression between individuals of the same genotype and instability between generations due to progressive loss of receptor expression might represent inherent flaws of the transgenic approach.

*Knock-in models*

The contribution of human FcγRs *in vivo* was mostly evaluated using the novel knock-in mouse model - hFcγR$^{KI}$ mice [71, 97]. hFcγR$^{KI}$ mice were generated by a locus swap of the entire low-affinity hFcγR locus into the corresponding mouse locus, followed by the targeted insertion of the high affinity receptor gene FCGR1A in place of the endogenous Fcgr1 gene. These mice circumvent the inherent flaws of using transgenic approaches (see above) to generate human FcγR expression in the mouse model. Overall, hFcγRs seem to be uniformly expressed across different individuals without the exaggerated expression tendencies of transgenic mice [276]. Yet, these mice have limited to no expression of both hFcγRIIA and hFcγRIIB on basophils, and also hFcγRIII on eosinophils, which renders hFcγR$^{KI}$ mice still imperfect to study all hFcγR-expressing myeloid cells.

One fundamental assumption of models that employ exogenous human receptor expression in mice (both for knock-in and transgenic) is that the signaling capacity of the human receptor is able to integrate with the mouse intracellular system. There is compelling evidence that human receptor transgenic expression is functional on mouse cells for hFcγRI [289, 291], hFcγRIIA [70, 97, 307], hFcγRIIIA [308] and hFcγRIIIB [277]. The use of knock-in technology to express FcγR ($hFc\gamma R^{KI}$ mice) is somehow still crawling, and our data, along with ongoing projects in the lab, comfort assumptions of appropriate downstream signaling, notably for anaphylaxis, thrombocytopenia, arthritis and others. The careful design of blocking antibodies to target particular receptor is also under development in our lab and will allow us to assess the relative contribution of each hFcγR to physiology and disease. The future application of such blocking antibodies will thereby demonstrate the functionality of hFcγR construct in the $hFc\gamma R^{KI}$ mice.

*The use of blocking antibodies*

The selectively *in vivo* blockade of Fc receptors has been used as an alternative to knock-out approaches to study the role of Fc receptors and cells in anaphylaxis. Nevertheless, *in vivo* blockade of Fc receptors brings some important limitations. The use of blocking antibodies to target a particular receptor may induce off-target effects, including either signaling directly through FcγR or binding to non-target receptors through the Fc region.

In the case of mouse FcRs, only mFcγRIII [42, 163] and mFcγRIV [309] can be efficiently blocked *in vivo*. Even in the lack of blocking antibodies for other receptors, results obtained blocking antibodies for both mFcγRIII and mFcγRIV have revealed that these receptors are together responsible for anaphylaxis in mice [42, 163, 309]. Moreover, there are no blocking antibodies for all the human FcγRs. hFcγRIIA can be selectively blocked with a monoclonal mouse IgG2b anti-human agonistic antibody (clone IV.3); hFcγRIIB can also be blocked by a blocking antagonist antibody (clone 2B6). Blocking antibodies for hFcγRI and hFcγRIII are been currently generated in the lab. Improved antibody engineered to specifically target particular FcγR will be extremely valuable in the study of the $hFc\gamma R^{KI}$ mice, and further translation of these findings to human studies.

*Selective ablation of cells*

Antibody-mediated depletion strategies to target neutrophils have been extensively used to study the absence of these cells in the development of anaphylaxis *in vivo*. We have used the most commonly anti-Gr-1 (clone RB6-8C5) antibody to have a highly effective elimination of neutrophils [270]. This depleting antibody has also the potential to recognize Ly6C expressed on monocytes and monocyte-derived cells. We have further demonstrated that this monoclonal antibody does not affect the circulating monocytes or spleen macrophages (Paper I – Fig E5), however care should be taken when evaluating the presumably no effect on other cell functions, which is difficult to evaluate. For instance, the elimination of neutrophils from the system after administration of a large quantity of antibodies entails phagocytic clearance forcibly by FcγR-expressing cells. One could speculate that this FcγR dependent capture of antibody-bound cells could modulate subsequent macrophage function, thereby keeping these cells 'occupied' and limiting their capacity to respond to further stimulation of circulating immune complexes [310]. A possible alternative to study the role of neutrophils in the development of anaphylaxis is the use of genetic approaches to render mice constitutively neutropenic. Several neutropenic models have been developed in the recent years and are already been tested in our laboratory, such as Gfi-1$^{-/-}$ deficient mice (knock-out of Gfi-1 transcriptional repressor involved in polarization of hematopoietic precursors) [59] or PMN$^{DTR}$ (conditional diphtheria toxin receptor – DTR – expression on neutrophils) [311].

Diphtheria toxin (DT)-mediated conditional ablation of cells has been used in a great number of mouse models, as recently reviewed [312]. We have used mice expressing the DTR under the control of the mast cell protease 8 (Mcpt8) promoter to selectively deplete murine basophils. Otherwise resistant to the bacterial exotoxin, the transgenic expression of DTR on basophils allow the transient depletion of these cells in the bone marrow and the periphery upon administration of DT [313]. The cytotoxicity of DT is the result of the entry of heterodimeric DT via receptor into cell resulting in fatal protein synthesis inhibition, and subsequent cell death by apoptosis [312]. One important possible side effect of wide cell ablation is the inherent risk of triggering systemic inflammation. As an example, neutrophilia and monocytosis can be observed in several DTR mouse strains [314], but not all models [311].

Monocyte/macrophage targeting by toxic liposomes is also one strategy to deplete these cell populations and assess their role in triggering anaphylaxis *in vivo*. We have

demonstrated that monocyte/macrophage depletion with clodronate liposomes decrease immediate loss of body temperature and enhance late-phase intraperitoneal inflammation. There was no evident alteration in other cell populations (notably neutrophils) in the blood, suggesting the specific target of this treatment. However, we cannot exclude the possible effects of the use of toxic liposomes in all promoting pro-inflammatory signals. Indeed, we found a slightly increased numbers of intraperitoneal mast cells and lymphocytes in non-sensitized mice treated with clodronate liposomes, but not with PBS liposomes. Most critically, one cannot exclude either the possible effect of cell depletion or functional ablation approaches can have off-target effects in other phagocytic populations, like mast cells. Not to mention the potential immunomodulatory effect of disseminated cell death to the outcome of the response, depending on the dose and route of administration.

### 6.5.2 Pre-treatment of mice with IL-4 to increase susceptibility to anaphylaxis

Allergies are detrimental immune responses to specific environmental antigens. IL-4 (and IL-13) are crucially involved in the development of allergic responses. IL-4 has been reported to be critically required to mount full Th2 responses, which then provide help to B cells into isotype switch and orchestrate inflammation within allergen exposure. IL-4 signals through use the common γ-chain related to the IL-4 receptor α chain (IL-4Rα). The α-chain is also shared with the IL-13R to signal both IL-4 and IL-13 [315].

The importance of IL-4 in human allergic diseases is supported by numerous reports. In asthmatic patients, the levels of this cytokine are elevated in the bronchoalveolar lavage of allergic subjects when compared to those of control subjects [315]. Also, it has been reported that a single nucleotide polymorphism on IL-4α, such as IL-4Rα I75Vwith increased signaling, is associated with increased risk of atopy [316]. Lastly, clinical trials using a monoclonal antibody against the α subunit of IL-4R (dupilumab) show improvement in asthma symptoms by blocking both IL-4 and IL-13 in asthmatic patients [317].

Evidence from mouse models has also helped to understand the role of IL-4 in allergic diseases models. IL-4 is required for the sensitization of mice in a peanut-induced anaphylaxis model as showed in IL-4 deficient mice [318]. In line with this, the constitutively activating mutation in the ITIM domain of the IL-4Rα at the amino acid 709 (IL4RF709 mice) has been extensively used to explore the roles of IL-4 *in vivo*. Although a human equivalent of this mutation does not exist, this mimics a single nucleotide polymorphism mentioned above.

Allergy phenotypes in IL4RF709 results not only in increased inflammation in the lungs after allergen exposure in one model, but also promote the development of allergic sensitization to ingested protein and induction of anaphylaxis after enteral challenge [319].

In active immunization allergy models, the constitutive activation of the IL-4 receptors seems to circumvent the tolerance pathways and induce the allergic phenotype. This conclusion can be drawn due to IL4RF709 mice increased responsiveness to sensitization even in the absence of adjuvants [319]. The lack of allergen-specific regulatory T cells may be one explanation; notably, IL-4 increased signaling leads to mast cells expansion in the intestine of mice [204, 205]. In passive models, IL-4 has also been used to induce an atopic phenotype in mice. The administration of IL-4 previous to challenge have demonstrated increased susceptibility to loss of core body temperature in mice [201]. In this particular case, mice received a long-lasting formulation of IL-4 complexes with a rat IgG1 anti-mouse IL-4, herein called IL-4C. Previous reports showed that this complex is formed by one molecule of monoclonal neutralizing antibody binding two molecules of IL-4 [320]. IL-4C protects IL-4 from degradation and slowly dissociates *in vivo*, realizing biologically active IL-4. As a result, IL-4 half-life *in vivo* increases from a few minutes to roughly 24 hours [320].

We have taken advantage of this IL-4C model in both models of PSA induced by the administration of Omalizumab (Chapter 4) and peanut-specific IgG-anaphylaxis (Chapter 5). To mimic the situation when the patients have already high levels of IgE and then receive the treatment with Omalizumab, we injected IgE followed by injection of Omalizumab (Paper II – Fig 2D) in naïve hFcγR$^{KI}$. Mice did not develop signs of anaphylaxis. In chapter 5, the passive sensitization of hFcγR$^{KI}$ with IgGs from highly allergic patients followed by challenge with the cognate antigen did not induce signs of anaphylaxis (Chapter 5 - Figure 2A). It is noteworthy that in both cases of Omalizumab and peanut induced anaphylaxis, the extreme reaction in humans remains a rare event. Thus, we hypothesized that patients undergoing anaphylaxis might have an atopic phenotype, as shown for increased risks of occurrence of anaphylaxis in patients with prior severe shock episodes. In this respect, the administration of IL-4C prior to passive sensitization and challenge was suited.

We observed that the treatment with IL-4C was able to yield an allergic phenotype to mice, making them more susceptible to the anaphylactic shock. The mechanism behind this increased susceptibility still remains unknown, however we can bring some hypothesis that might be relevant to this model. IL-4 receptor is widely expressed in different cells and tissues of humans and mice. We have shown that IL-4 is able to enhance anaphylaxis potently and others have shown their rapidly effect [201], suggesting possibly that IL-4 is able to act

directly on an organ or cell type intimately involved in the effector phase of anaphylaxis. Interestingly, observation that IL-4 is able to exacerbate anaphylaxis in Rag2/$\gamma_c$-double deficient mice exclude B cells, T cells, mast cells, eosinophils, NK cells and, ILCs [201, 321] as targets of IL-4. However, we cannot exclude the effect of IL-4 in other myeloid cells than these one previously mentioned that would positively impact the development of systemic shock. Neutrophils and basophils also express the IL-4R on the cell surface. In human, neutrophils from allergic patients or stimulated *in vitro* with IL-4 showed impaired migration capacity, suggesting an activated state [322]; the same was seen with mouse neutrophils [323]. Hence, neutrophils might be good candidates as cells being directly affect by IL-4 exogenous administration and more prone to respond in case of immune complexes formation.

Additional data suggest that the most probable effect of IL-4 is the increased sensitivity to mediators (histamine, PAF, serotonin and cysteinyl leukotrienes) [201]. IL-4 does not seem to increase mediator production, but rather mediate increase anaphylaxis by a synergistic effect in vascular permeability by the presence of the cytokine and the mediator [324]. More recently, it has been suggested that the presence of IL-4R in the endothelium would cause increased vascular permeability, hence allowing mediators to act in a diminished threshold, therefore causing the system to react [203]. These results were shown using an IgE-mediated histamine dependent anaphylaxis, however it does not exclude the potential of this mechanism to participate in an IgG-mediated reaction.

Altogether, our results using IL-4C to mimic the atopic phenotype could explain a subject's susceptibility to an IgG-mediated anaphylactic shock. IL-4 (and possibly IL-13) is important in the sensitization phase; the use of inhibitors of IL-4 may ameliorate allergy by not only blocking the sensitization, but also have an effect on the effector phase of the allergic response.

### 6.5.3 Concluding remarks of part V

In conclusion, human data is required to reveal the mechanisms behind anaphylactic shocks in humans, however the special urgent and potentially fatal nature of the anaphylactic reaction leaves little or no room for trials. Hence, it is undeniable the essential role of studies in animal models. Mouse models have extensively contributed to our understanding of the pathophysiology of anaphylaxis. Although no mouse model of anaphylaxis encompasses all features of human disease, careful comparison of the clinical manifestations of human anaphylaxis reveal that current models may recapitulate some characteristics of the pathology. However, careful considerations should be given to the type of model employed (e.g. active or passive model) and timing of intervention so as the best model refer to the clinical situation. Furthermore, it is important to continuously refine mouse models in light of new clinical information so that rather than replicating 'anaphylaxis', mouse models might reflect specific phenotypes of the shock (as we did for Paper II, for example).

## 6.6 Part VI: General discussion

### 6.6.1 The role of IgE vs IgG in anaphylaxis

The first conclusions of this thesis bring insights to the mutual contribution of both classical (IgE-dependent) and alternative (IgG-dependent) pathways in a mouse model of anaphylaxis. Secondly, we evidenced the potential contribution of specific IgGs in two well-defined clinical situations: monoclonal antibody-induced anaphylaxis and peanut-induced anaphylaxis. Highlighting the importance of the alternative pathway in the clinical situation has certainly contributed to the understanding of the anaphylaxis pathogenesis in humans. Yet, it is important to mention that we used the mouse system to study the role of FcγRs. I consider this as one important limitation of this thesis work and many others in this field.

Evidence from human studies and extrapolations of mouse models surely allow the conclusion that both classical and alternative pathways contribute to anaphylaxis. Indeed, a recent multicentric study including 86 patients with suspected anaphylaxis to neuromuscular blocking agents (NMBA) suggests that the co-existence of specific IgE and IgG antibodies may aggravate NMBA-induced anaphylaxis in humans. It is however challenging to dissociate one mechanism [218]. One pathway or the other may play initial or pivotal roles in the pathogenesis of systemic shock, possibly depending on the route of exposure, quantity of allergen, co-factors, the subject's individual health status (atopic or not) etc. The activation of myeloid cells in the bloodstream alone may be enough to trigger mediator release. Yet, it is more likely that the full and complete activation of those cells occur in particular sites, or requires adhesion and potentially extravasation. Indeed, most evidence of IgG-mediated anaphylaxis in humans have in common the systemic administration of high amount of allergen, condition that seems to be likely to trigger anaphylaxis in mice (refer to part I) (**Figure 10**). Our results demonstrated that high levels of immune complexes in the bloodstream is able to induce anaphylaxis in humanized mice. Surprisingly however, with respect of the preliminary aspect of the chapter 5 findings, IgGs seem also to play a rather protective role in the development of peanut anaphylaxis in humanized mice in the presence of high amounts of peanut extract.

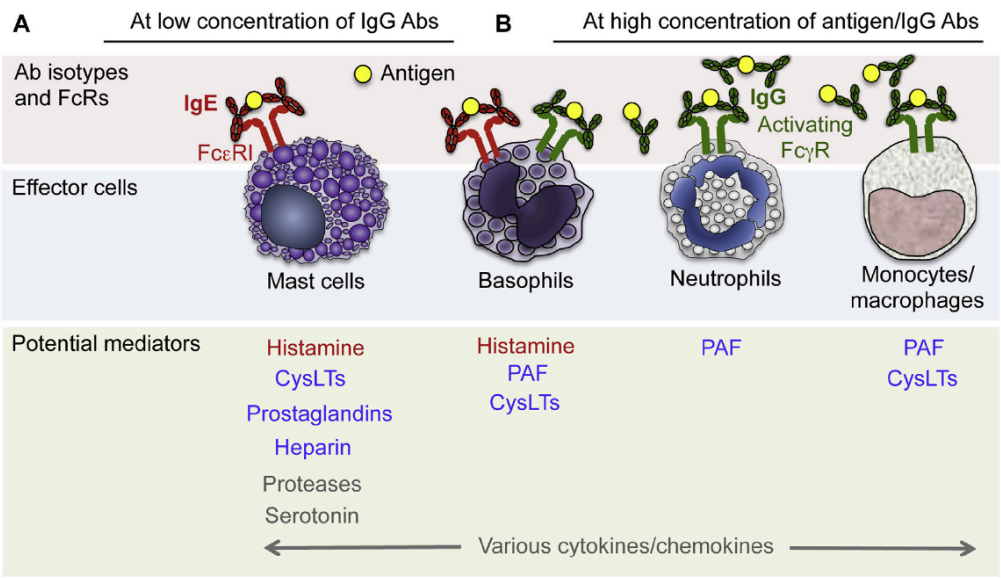


**Figure 10. Pathways in antibody-mediated anaphylaxis.** A. Antigen-specific IgE antibodies and FcεRI-bearing effector cells (eg, mast cells and basophils) play a dominant role in anaphylaxis induced (sometimes by very small amounts of bi- or multi-valent antigen) when concentrations of IgG antibodies are low. B Mouse models of anaphylaxis suggest that IgG antibodies and FcγR-bearing effector cells (eg, basophils, macrophages, neutrophils, and mast cells) can be important effectors of anaphylaxis induced by large amounts of antigen (immune complexes are formed in the presence of high concentrations of IgG antibodies). Some examples of anaphylaxis likely involve both pathways (A and B). Red indicates strong evidence for the importance of these mediators in human anaphylaxis induced by antigen. Blue indicates that these elements can participate in models of anaphylaxis in mice, but their importance in human anaphylaxis is not yet clear. Grey indicates elements with the potential to influence anaphylaxis, but their importance in human or mouse anaphylaxis is not yet clear (e.g., human mast cells are thought to make little or no serotonin). From [171]

Nevertheless, it seems reasonable to propose that if evolution engineered redundancy in mechanisms to sustain critical biological process, the same mechanistic redundancy also applies to pathological processes, including anaphylaxis. Indeed, we can speculate that there may be small number of biological responses in which the extent of a biological response would render undetectable the importance of another redundant mechanism. This might be the case as in patients with mastocytosis in which mast cells are thought to be responsible cell population for the increased susceptibility to anaphylaxis.

Accordingly, in many types of complex and systemic response as in anaphylaxis, receptors, cells and mediators may overlap among themselves. We expect then that the choice of experimental model, including the intensity of the stimulus used to elicit the response, may be critical into drawing appropriate conclusions. In this respect, mouse models are very important to increase our knowledge, being truthful with their limitations.

## 7. Final Considerations and Perspectives

This work thesis may be summarized in four major outcomes:

- In the absence of adjuvant, active systemic anaphylaxis is highly dependent on the IgE receptor FcεRI and the IgG receptor FcγRIII with key roles for mast cells and monocytes/macrophages which release histamine and PAF, respectively.
- Omalizumab, the anti-IgE monoclonal antibody (mAb) induced skin inflammation and anaphylaxis through engagement of FcγRs in FcγR-humanized mice.
- We described a new Fc-engineered version of Omalizumab, with equal capacity to block IgE-mediated reactions, but which does not induce FcγR-dependent adverse reactions.
- By way of ongoing work, we investigate if IgG from peanut allergic subjects could play a dual role in amplifying complement-mediated anaphylaxis, while also limiting allergic shock by FcγR-dependent mechanisms.

Our findings add to the knowledge of the cellular and molecular mechanisms of anaphylaxis. Yet, major questions remain open. For example, it remains largely unknown when and how an anaphylactic response is initiated and propagated; and most importantly how cellular activation drives systemic inflammatory symptoms.

The understanding of the IgG-dependent anaphylaxis pathway might have further implications than the sole description of the mechanism. More broadly, the description of IgG-mediated adverse events might also be important for diagnosis and therapeutic approaches. The screening of allergen-specific IgG could be a great asset, in particular to allergens in which there is no detectable allergen-specific IgE, or even for injectable drugs, which are more prone to induce such adverse reactions. The diagnosis of apparently ‘idiopathic’ reactions could be achieved by the detection of IgGs in patients. On the therapeutic side, particularly when considering monoclonal therapeutic antibodies, it could be informative to assess the potential of FcγR-mediated activation *in vivo*, and therefore the use of more accurate Fc-engineered techniques to reduce adverse effects of such drugs.

Conversely to the detrimental role of IgG in anaphylaxis, a lot still remains to be done to better understand the potential protective role of these immunoglobulins in the context of systemic anaphylaxis. Allergen-specific immunotherapy leads to marked increases in

allergen-specific IgG, especially IgG4. Whether the protective effects of allergen-specific immunotherapies are mediated, at least in part, by FcγR-engagement still remains to be determined. Moreover, the understanding of the protective vs. anaphylactogenic potential of each subclass of IgG still remains to be assessed.

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# 9. Annex

## 9.1 Review – Approaches to target IgE antibodies in allergic diseases

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# Approaches to target IgE antibodies in allergic diseases

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ABSTRACT

IgE is the antibody isotype found at the lowest concentration in the circulation. However IgE can undeniably play an important role in mediating allergic reactions; best exemplified by the clinical benefits of anti-IgE monoclonal antibody (omalizumab) therapy for some allergic diseases. This review will describe our current understanding of the interactions between IgE and its main receptors FcεRI and CD23 (FcεRII). We will review the known and potential functions of IgE in health and disease: in particular, its detrimental roles in allergic diseases and chronic spontaneous urticaria, and its protective functions in host defense against parasites and venoms. Finally, we will present an overview of the drugs that are in clinical development or have therapeutic potential for IgE-mediated allergic diseases.



**Contents**



*Abbreviations:* AD, atopic dermatitis; Ag, antigen; ADAM10, a disintegrin and metalloprotease 10; ADCC, antibody-dependent cell-mediated cytotoxicity; AECs, airway epithelial cells; ASST, autologous serum skin test; Cε, constant epsilon domain of IgE; $C_L$, constant region of an antibody's light chains; CSU, chronic spontaneous urticaria; DARPins, designed ankyrin repeat proteins; DC, dendritic cell; Fab, fragment antigen-binding region; Fc, fragment crystallizable region of an antibody; HRF, histamine releasing factor; IECs, intestinal epithelial cells; Ig, immunoglobulin; IL, interleukin; ITAM, immunoreceptor tyrosine-based activation motif; mIgE, membrane-bound IgE; PCA, passive cutaneous anaphylaxis; PLA2, phospholipase A2; PSA, passive systemic anaphylaxis; Tg, transgenic; $T_H2$, T cell helper type 2; TPO, Thyroperoxidase; $V_H$, variable region of an antibody's heavy chains; $V_L$, variable region of an antibody's light chains; WT, wild type.

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## 1. Introduction

Immunoglobulin E (IgE) was discovered about 50 years ago. In 1966, the Ishizakas' group in Japan described an immunoglobulin different from the known immunoglobulin classes, that could induce allergic reactions in the skin, and which they called γE antibody (Ishizaka & Ishizaka, 1967). During the same period, the group of Johansson and Bennich in Sweden isolated a new immunoglobulin class, which they called IgND (Johansson & Bennich, 1967). It soon turned out that γE and IgND belong to the same and unique antibody class, and the official name IgE was given in 1968 (Bennich et al., 1968). The story behind this discovery has been the subject of many reviews, including two recent reviews by the discoverers themselves (Ishizaka & Ishizaka, 2016; Johansson, 2016). IgE is the isotype found at the lowest concentration in the circulation (50–200 ng/ml IgE in healthy individuals *vs.* ~10 mg/ml for IgG) (Dullaers et al., 2012). However, IgE levels can increase dramatically in individuals with allergic diseases (Galli & Tsai, 2012; Platts-Mills, Schuyler, Erwin, Commins, & Woodfolk, 2016). Indeed, the importance of IgE in allergy was demonstrated at the time of its discovery, when the investigators identified that purified IgE was capable of transferring skin reactivity from sensitized human subjects to naive hosts (Ishizaka & Ishizaka, 2016; Johansson, 2016). This discovery has had great importance for both the diagnosis and treatment of allergic disorders: quantification of allergen-specific IgE is one of the main diagnostic criteria for allergies (Hamilton, MacGlashan Jr., & Saini, 2010), and the anti-IgE therapeutic antibody omalizumab is now approved for the treatment of moderate to severe persistent allergic asthma, and shows great potential for the treatment of other allergic diseases (Humbert et al., 2014; Kawakami & Blank, 2016; Pelaia et al., 2015). Omalizumab has also been approved for the treatment of chronic spontaneous urticaria (CSU), demonstrating that the pathologic functions of IgE extend beyond allergy (Chang et al., 2015; Maurer et al., 2013; Zhao et al., 2016).

IgE antibodies exist in two forms: a membrane-bound form (mIgE) expressed by B cells that have undergone class switching to IgE, and a secreted form produced by plasma B cells. mIgE serves as a B cell receptor involved in antigen uptake and presentation. The structure and functions of mIgE, as well as the regulation of IgE synthesis, have been extensively reviewed elsewhere (Geha, Jabara, & Brodeur, 2003; Gould & Sutton, 2008; Wu & Zarrin, 2014). This review will focus mainly on the effector functions of secreted IgE (hereafter referred to as 'IgE').

IgE exerts its biological functions by binding to two main receptors: FcεRI and CD23 (FcεRII). The high affinity IgE receptor, FcεRI, is expressed on the surface of blood basophils and tissue resident mast cells; and on other cell types in humans (but not in mice), including neutrophils, eosinophils, platelets, monocytes and dendritic cells (Kraft & Kinet, 2007). The low affinity receptor CD23 is expressed mainly by B cells (Sutton & Davies, 2015), but also by several other cell populations including neutrophils, eosinophils, follicular DCs and intestinal epithelial cells (IECs) (Acharya et al., 2010). CD23 on B cells serves mainly as a negative regulator of IgE synthesis (Acharya et al., 2010). Crosslinking of FcεRI-bound IgE can initiate allergic reactions by inducing the activation of mast cells and basophils, the immediate release of preformed granule-stored mediators such as histamine and proteases, and the *de novo* production of lipid mediators (*e.g.* prostaglandins, leukotrienes), cytokines and chemokines (Galli et al., 2005; Voehringer, 2013; Wernersson & Pejler, 2014).

In this review, we will describe our current understanding of the interactions between IgE and its receptors FcεRI and CD23. We will review the known and potential functions of IgE antibodies in health and disease, in particular their detrimental roles in allergic diseases and chronic spontaneous urticaria, as well as their protective functions in host defense against parasites and venoms. Finally, we will present an overview of the drugs that are in clinical development or have therapeutic potential for IgE-mediated allergic diseases.

## 2. IgE structure

IgE antibodies are composed of two identical heavy chains (each comprising a variable $V_H$ domain and four constant Cε domains) and two identical light chains (composed of a variable $V_L$ domain and a constant $C_L$ domain) with a total molecular weight of 190 kDa (Gould & Sutton, 2008; Wu & Zarrin, 2014) (Fig. 1). Similar to other antibody classes, the Fab region of IgE is responsible for antigen recognition and binding, while the effector function of IgE is determined by the carboxy-terminal Fc portion (Gould & Sutton, 2008, Wu & Zarrin, 2014). IgE shares a similar overall structure with IgG, with the exception of an additional domain in the heavy chain (Cε2). As detailed in Section 3.1.3, this additional Cε2 domain corresponds to the location of the flexible hinge region found in IgG, and plays a major role in enhancing the stability of the interaction between IgE and its high affinity receptor FcεRI (McDonnell et al., 2001). The FcεRI binding site is located in the Cε3 domain and in the Cε2-Cε3 linker region (Garman, Wurzburg, Tarchevskaya, Kinet, & Jardetzky, 2000) (described in more detail in Section 3.1.3). The binding site to the low affinity IgE receptor CD23 is also primarily located within the Cε3 domain, with contributions from the Cε4 domain (described in more detail in Section 3.2.3) (Fig. 1). The crystal structure of the human Cε3-Cε4 domains revealed that, by rotating relatively to Cε4, Cε3 can adopt either 'open' or 'closed' conformations. This conformational flexibility regulates the binding of IgE to both FcεRI and CD23 (Garman et al., 2000; Wurzburg, Garman, & Jardetzky, 2000). These features are discussed in more detail in Sections 3.1.3 & 3.2.3. Several intra- and inter-domain disulphide bridges control the structure and activity of IgE, which is also regulated by glycosylation at various sites (Fig. 1). In particular, disruption of the glycosylation site found in the Cε3 domain at asparagine-394 (N394) in humans, and N384 in mouse, abrogates the binding of IgE to FcεRI, highlighting the importance of glycosylation modifications in IgE biology (Shade et al., 2015).

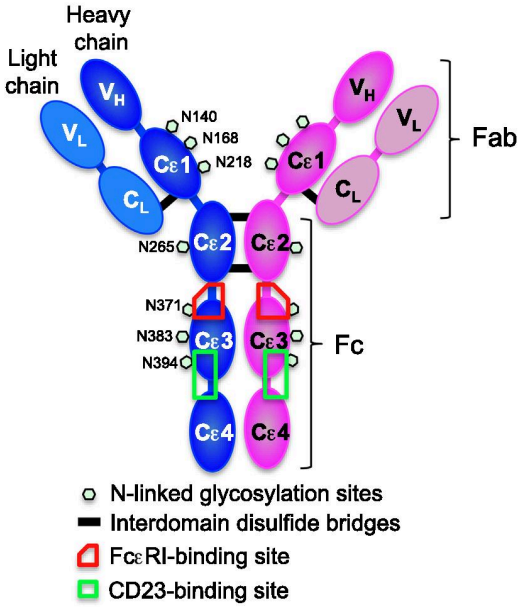


**Fig. 1. IgE structure.** IgE antibodies consist of two identical heavy chains (composed of a variable $V_H$ domain and four constant Cε domains) and two identical light chains (composed of a variable $V_L$ domain and a constant $C_L$ domain). 'Fab': region responsible for antigen recognition and binding. 'Fc': portion responsible for IgE effector functions. The positions of interdomain disulfide bridges, N-linked glycosylation sites (in human IgE), FcεRI- and CD23-binding sites are indicated.

## 3. IgE receptors

### 3.1. The high affinity IgE receptor FcεRI

#### 3.1.1. FcεRI structure and expression

FcεRI is the high affinity receptor for IgE ($K_d$ of ~$10^{-9}$ to $10^{-10}$ M). It is constitutively expressed at high levels on both human and rodent mast cells and basophils as a tetramer formed of one α subunit, one β subunit, and a dimer of disulfide-linked γ subunits (Blank et al., 1989). The α subunit (FcεRIα) belongs to the immunoglobulin (Ig) superfamily, with an extracellular portion composed of two Ig-like domains (D1 and D2), containing the IgE binding sites, a transmembrane domain and a short cytoplasmic domain which is thought to have no signaling function (Kraft & Kinet, 2007) (Fig. 2). Human FcεRIα is glycosylated at seven sites, and these glycosylations appear to be required for proper interactions with the folding machinery in the endoplasmic reticulum, rather than for binding to IgE (Letourneur, Sechi, Willette-Brown, Robertson, & Kinet, 1995; Sutton & Davies, 2015). FcεRIβ has a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM), which acts as signal amplifier. The FcεRIγ homodimer also contains two ITAM domains, which are responsible for signal transduction (Dombrowicz et al., 1998; Lin, Cicala, Scharenberg, & Kinet, 1996).

In humans, but not in rodents, FcεRI is also constitutively expressed as a αγ2 trimer at the surface of monocytes (Maurer et al., 1994; Takenaka, Tanaka, Anan, Yoshida, & Ra, 1995), dendritic cells (DCs) (Maurer et al., 1996), Langerhans cells (Bieber et al., 1992), neutrophils (Gounni et al., 2001), eosinophils (Gounni et al., 1994) and platelets (Hasegawa et al., 1999; Joseph et al., 1997). It was reported that expression of the αγ2 trimer is increased in peripheral blood monocytes from atopic patients, as compared to healthy controls (Maurer et al., 1994).

A circulating soluble form of FcεRI (sFcεRI) of about 40 kDa, and which contains an intact IgE binding site, has been described in human serum (Dehlink et al., 2011). However, the cell types that release or shed this protein in humans, and the physiological role of sFcεRI, remain to be identified (reviewed in (Platzer, Ruiter, van der Mee, & Fiebiger, 2011).

#### 3.1.2. FcεRI functions

FcεRI plays a key role in mediating the biological functions of IgE *in vivo*, which is best exemplified by the fact that FcεRI-deficient mice are fully resistant to IgE-mediated passive cutaneous anaphylaxis (PCA) and passive systemic anaphylaxis (PSA) (Dombrowicz, Flamand, Brigman, Koller, & Kinet, 1993). These findings are most likely attributable to the αβγ2 FcεRI tetramer expressed on the surface of mast cells, since mast cell-deficient mice are also resistant to IgE-mediated PCA and PSA (Feyerabend et al., 2011; Lilla et al., 2011; Miyajima et al., 1997). Studies using transgenic mice expressing the human FcεRIα chain under the control of its own promoter have also given significant insight into the functions of human FcεRI (Dombrowicz et al., 1996; Dombrowicz et al., 1998; Greer et al., 2014). *hFcεRIα*$^{Tg}$ mice (bred on a mouse FcεRI-deficient background) express a 'humanized' FcεRI receptor with a similar cellular distribution as that found in humans (Dombrowicz et al., 1996; Dombrowicz et al., 1998; Greer et al., 2014; Mancardi et al., 2008). *hFcεRIα*$^{Tg}$ mice can develop PSA reactions upon sensitization with antigen-specific human or mouse IgE and challenge with the same antigen (Dombrowicz et al., 1996; Dombrowicz et al., 1998). Notably, mouse IgE is able to bind both human and mouse FcεRI, while human IgE does not bind the mouse receptor (Conrad, Wingard, & Ishizaka, 1983). PCA reactions can even be induced in *hFcεRIα*$^{Tg}$ mice by intradermal transfer of plasma from allergic patients followed by challenge with the relevant allergen (Liu et al., 2013; Zhu et al., 2005). The αβγ2 tetramer on mast cells is also probably the main trigger of IgE-mediated systemic and cutaneous anaphylaxis in *hFcεRIα*$^{Tg}$ mice, although, to the best of our knowledge, this has not yet been unequivocally demonstrated.

The biological functions of the αγ2 trimer of FcεRI are less well understood. Greer and collaborators recently used *hFcεRIα*$^{Tg}$ mice to demonstrate that internalization of human FcεRI by conventional DCs and monocytes (which express the αγ2 trimer) contributes to serum IgE clearance (Greer et al., 2014). They injected human IgE into *hFcεRIα*$^{Tg}$ mice and control mice (deficient for both human and mouse FcεRI), and found that serum IgE clearance was markedly accelerated in the transgenic animals. They subsequently demonstrated that human IgE was rapidly endocytosed by conventional DCs and monocytes, and that this endocytosis was associated with the rapid clearance of circulating IgE observed in *hFcεRIα*$^{Tg}$ mice (Greer et al., 2014). While these findings appear convincing, it remains to be determined the extent to which trapping of circulating IgE by human FcεRI expressed on mast cells also contributes to its clearance. It was recently reported that perivascular mouse mast cells can 'sample' circulating IgE directly in the blood by extending cell processes across the vessel wall (Cheng, Hartmann, Roers, Krummel, & Locksley, 2013). However, the role of FcεRI in serum IgE clearance seems to be a specific feature of the human receptor, and not the mouse receptor, as mice deficient in FcεRI clear serum IgE to the same extent as WT mice (Cheng, Wang, & Locksley, 2010).

It has also been suggested that human peripheral blood DCs use the αγ2 FcεRI trimer for allergen uptake and presentation to naive T cells (Maurer et al., 1996). Using transgenic mice expressing human FcεRIα under the dependency of the CD11c promoter, in an attempt to restrict expression to DCs, these authors found that hFcεRI-expressing DCs can efficiently prime naive T cells for $T_H2$ differentiation, and amplify antigen-specific $T_H2$ responses *in vivo* (Sallmann et al., 2011).

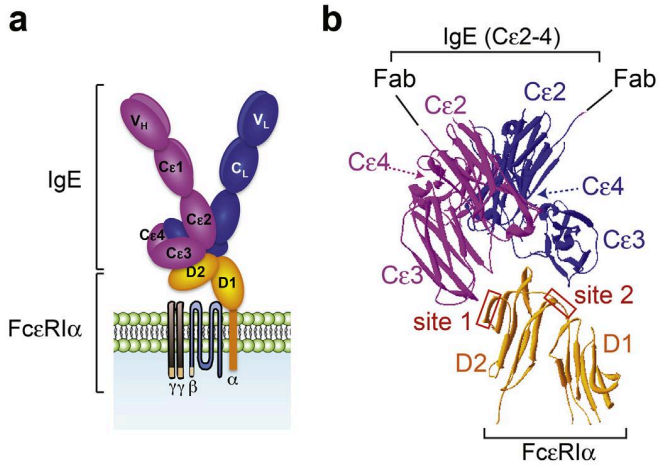


**Fig. 2. Structure of FcεRI and its interaction with IgE. a.** FcεRI is expressed on mast cells and basophils as a tetramer formed with one α subunit, one β subunit and a dimer of disulfide-linked γ subunits. IgE binds the receptor via surface loops in Cε3, with contributions from the Cε2–Cε3 linker region. **b.** The two Cε3 domains of IgE bind distinct sites on FcεRIα, one site found in the D2 domain (site 1), and a second site formed by a cluster of four surface-exposed tryptophan residues in the D1-D2 interface (site 2) (Protein Data Bank ID: 2Y7Q).

#### 3.1.3. Binding of IgE to FcεRI

Mutagenesis studies have helped define the FcεRI binding epitope on IgE. Schwarzbaum and colleagues generated a mutant form of mouse IgE with a deletion of 45 amino acids in the carboxy end of Cε3: this mutant IgE was unable to bind FcεRI (Schwarzbaum et al., 1989). Nissim and collaborators produced several chimeric IgE containing the Cε2, Cε3 and Cε4 domains of human IgE (hereafter named Cε2-4), in which various domains were replaced by their murine counterparts. This work confirmed that the FcεRI binding site mapped to the Cε3 domain of IgE (Nissim, Jouvin, & Eshhar, 1991). In 2000, Garman et al. determined the crystal structure of the IgE Cε3–4 dimer bound to the extracellular part of FcεRIα (Garman et al., 2000). Analysis of this crystal structure confirmed that each of the two chains of the IgE Cε3–4 dimer could

bind the receptor using surface loops in Cε3, and revealed contributions of the Cε2–Cε3 linker region (Garman et al., 2000).

Analysis of the crystal structures of the extracellular portion of human FcεRIα alone (Garman, Kinet, & Jardetzky, 1998) or in complex with a dimeric Cε3–4 fragment (Garman et al., 2000) have also provided invaluable insight into how IgE interacts with FcεRI. The extracellular part of FcεRIα is formed of two immunoglobulin domains of about 85 amino acids each (D1 and D2), with a heavily bent D1–D2 interface forming an overall structure of an inverted V shape (Garman et al., 1998; Garman, Kinet, & Jardetzky, 1999) (Fig. 2). The two Cε3 domains of IgE bind distinct sites on FcεRIα, one site found in the D2 domain, and a second site formed by a cluster of four surface-exposed tryptophans in the D1–D2 interface (Garman et al., 2000). The presence of these two binding sites explains the 1:1 stoichiometry of the IgE-FcεRIα complex, which is essential to ensure that receptor crosslinking and activation occurs only upon multivalent antigen binding to IgE (Garman et al., 2000).

A unique feature of the FcεRI receptor, as compared to other Fc receptors, is the distinctly slow dissociation rate of the IgE-FcεRIα complex ($k_{off} \approx 10^{-5}\ s^{-1}$). This translates into a half-life of about two weeks for IgE bound to FcεRI (compared to only hours for IgG complexes bound to Fcγ receptors), and ensures that tissue mast cells and basophils remain saturated with IgE (Geha, Helm, & Gould, 1985; McDonnell et al., 2001). McDonnell and collaborators showed that full human IgE molecules and dimeric IgE fragments comprising the Cε2, Cε3 and Cε4 domains (Cε2–4) have identical kinetics of dissociation with FcεRIα, while Cε3–4 displays a markedly enhanced dissociation kinetic (~20-fold), indicating that Cε2 plays a major role in enhancing the stability of the IgE-FcεRIα complex (McDonnell et al., 2001). More recently, Holdom et al. published the crystal structure of human Cε2–4 bound to the extracellular domain of FcεRIα, and confirmed that the Cε2 domain contributes to the slow dissociation rate of IgE-FcεRIα complexes through conformational changes rather than direct interactions with the receptor (Holdom et al., 2011).

Analysis of the crystal structures of free *vs*. receptor-bound IgE Fc domains have revealed that the Cε3 domains of IgE undergo a large conformational rearrangement upon binding to FcεRI (Holdom et al., 2011; Wan et al., 2002; Wurzburg et al., 2000; Wurzburg & Jardetzky, 2009). The free IgE Fc portion was observed in a 'closed' conformation in which the FcεRIα binding site in Cε3 is masked (Wan et al., 2002; Wurzburg et al., 2000; Wurzburg & Jardetzky, 2009). This masking is achieved as the Cε2 domains in the free Fc fragment are folded back asymmetrically onto the Cε3 and Cε4 domains, locking the Cε3 domains in a 'closed' conformation (Wan et al., 2002) (Fig. 3). The authors suggest that free 'bent' IgE may first engage FcεRI through only one Cε3 domain, followed by an important conformational change involving Cε2, whereby Cε3 would adopt an 'open' conformation, leading to engagement of the second Cε3.

### 3.2. The low affinity IgE receptor CD23 (FcεRII)

#### 3.2.1. CD23 structure and expression

CD23, also known as FcεRII, is the low affinity receptor for IgE ($K_d = 10^{-5}$ M) (Wurzburg, Tarchevskaya, & Jardetzky, 2006). The structure of CD23 and its interaction with IgE have been reviewed in detail (Sutton & Davies, 2015). CD23 self-associates as trimer, and is composed of an IgE-binding 'head domain' (which belongs to the C-type lectin superfamily) linked to the membrane by an extracellular coiled-coil stalk region, and a small cytoplasmic N-terminal domain (Fig. 4). CD23 exists in a membrane-bound form of 45 kDa (mCD23), as well as in soluble forms of various sizes (sCD23) which are released by proteolytic cleavage at several sites in the stalk region (Sutton & Davies, 2015). ADAM10 ('a disintegrin and metalloprotease 10') is considered to be the main endogenous protease responsible for cleavage and generation of sCD23 (Lemieux et al., 2007; Weskamp et al., 2006). The exogenous house dust mite cysteine protease *Der p I* is also able to cleave mCD23 at two sites (Schulz et al., 1997). mCD23 (hereafter referred to as CD23) is expressed by B cells (Sutton & Davies, 2015), and several other cell populations including neutrophils (Yamaoka et al., 1996), eosinophils (Capron et al., 1992), follicular DCs (Johnson, Hardie, Ling, & Maclennan, 1986) and IECs (Yang, Berin, Yu, Conrad, & Perdue, 2000; Yu et al., 2003). Human CD23 exists as two isoforms (CD23a and CD23b), which differ in the first seven (CD23a) or six (CD23b) amino-acid residues of the cytoplasmic N-terminal part (Sutton & Davies, 2015; Yokota et al., 1992).

#### 3.2.2. CD23 functions

CD23 is expressed on the surface of B cells, where it serves as a negative regulator of IgE synthesis. Several publications show increased levels of IgE in mice deficient for CD23 (Haczku et al., 2000; Lewis et al., 2004; Riffo-Vasquez et al., 2000; Stief et al., 1994; Yu, Kosco-Vilbois, Richards, Kohler, & Lamers, 1994). Conversely, transgenic mice overexpressing CD23 in B (and T) cells have markedly reduced levels of circulating IgE after immunization (Payet, Woodward, & Conrad, 1999). The regulation of IgE production seems to require the oligomerization of CD23, since serum IgE levels are also increased in mice treated with an antibody that binds to the stalk region of CD23 and thus blocks receptor oligomerization (Ford et al., 2006; Kilmon et al., 2001). It is possible that CD23 on B cells plays an additional role(s) in regulating serum IgE levels, independently of its effects on IgE production. This was suggested by a study showing that exogenous IgE injected into mice deficient for B cells or treated with an anti-CD23 antibody can be detected in the blood one hour later at levels two-fold higher than in the corresponding control mice (Cheng et al., 2010). The mechanism through which CD23 regulates serum IgE levels is still unclear, and appears to be independent on B cells, since the administered IgE had similar rates of clearance in B cell-deficient and -sufficient mice (Cheng et al., 2010).

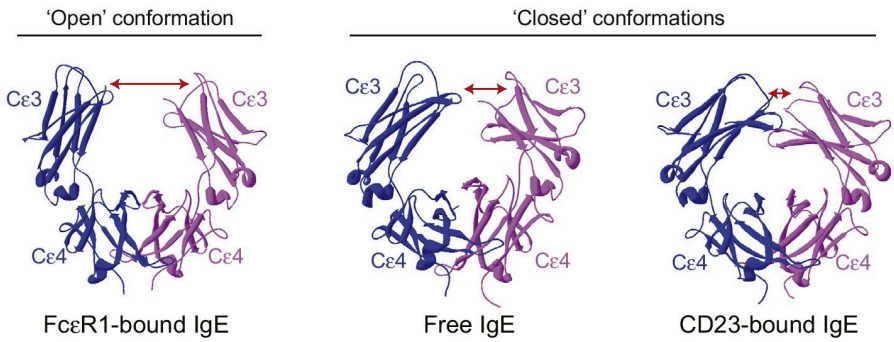


**Fig. 3. Conformational changes in IgE Fc portion upon binding to FcεRI or CD23.** The Cε3 domains of free IgE are found in a 'closed' conformation in which the FcεRIα binding site in Cε3 is masked (middle; Protein Data Bank [PDB] ID: 2WQR). Cε3 adopts an 'opened' conformation upon binding to FcεRI, which is incompatible with CD23 binding (left; PDB ID: 1F6A-2). By contrast, Cε3 adopts a 'closed' conformation upon binding to CD23, which is incompatible with FcεRI binding (right; PDB ID: 4GKO).

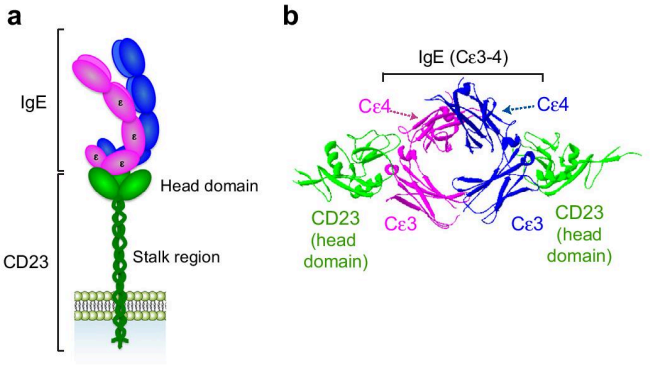


**Fig. 4. Structure of CD23 and its interaction with IgE. a.** CD23 self-associates as a trimer, and is composed of an IgE-binding 'head domain' (which belongs to the C-type lectin superfamily) linked to the membrane by an extracellular coiled-coil stalk region, and a small cytoplasmic N-terminal domain. **b.** The IgE binding site of CD23 is located in the C-terminal head domain (in green), with some additional contributions from the stalk region (not shown). Two CD23 molecules bind to each IgE heavy chain, primarily to the Cε3 domains but with a contribution from Cε4 (Protein Data Bank ID: 4GKO).

In B cells, CD23 has also been implicated in IgE-dependent antigen uptake and presentation to T cells. *In vitro* experiments showed that mouse and human B cells incubated with antigen-specific IgE were up to 100-fold more efficient than untreated B cells at presenting low concentrations of the respective antigen, and this phenomenon was markedly reduced by a CD23 blocking antibody (Kehry & Yamashita, 1989; Pirron, Schlunck, Prinz, & Rieber, 1990).

CD23 is expressed on IECs, and such expression is enhanced upon antigen sensitization in rodents (Yang et al., 2000; Yu et al., 2001), or exposure to the $T_H2$ cytokine IL-4 in humans (Tu et al., 2005). Studies using CD23 blocking antibodies or mice deficient for CD23 have demonstrated that CD23 in IECs is involved in the transepithelial transport of IgE and IgE/antigen complexes into the intestinal lumen (Tu et al., 2005; Yang et al., 2000; Yu et al., 2001). This phenomenon is potentially important for food allergy, since it could explain how IgE and allergens are delivered to mast cells located in the lamina propria beneath the epithelial lining of the gut (Gould & Sutton, 2008; Tu et al., 2005). Similarly, CD23 is expressed on human airway epithelial cells (AECs), where it is also subject to upregulation by IL-4, and *ex vivo* experiments suggest that CD23 in AECs is involved in transepithelial transport of IgE and IgE/antigen immune complexes (Palaniyandi, Tomei, Li, Conrad, & Zhu, 2011). A more recent study using CD23-deficient mice confirmed that CD23 expressed by AECs is involved in IgE and IgE/antigen transport, and showed that expression of CD23 in lung structural cells is important for the development of allergic airway inflammation (Palaniyandi et al., 2015).

The soluble form of CD23 (sCD23) can also regulate IgE synthesis. sCD23 exists in several isoforms of different sizes. All isoforms can interact with IgE, but the shorter sCD23 remains monomeric while the longer isoforms associate in trimers (reviewed in detail in (Platzer et al., 2011)). sCD23 isomers can have divergent effects on B cells. Trimeric sCD23 can upregulate IgE synthesis through the co-ligation of CD21 and membrane IgE on B cells (Aubry, Pochon, Graber, Jansen, & Bonnefoy, 1992; Cooper et al., 2012; Hibbert et al., 2005; McCloskey et al., 2007), whereas monomeric sCD23 inhibits IgE synthesis in human B cells (McCloskey et al., 2007).

### 3.2.3. Binding of IgE to CD23

Early mutagenesis studies mapped the IgE binding site of CD23 to discontinuous epitopes between residues 160-287 in the C-terminal head domain (Bettler, Maier, Ruegg, & Hofstetter, 1989; Bettler, Texido, Raggini, Ruegg, & Hofstetter, 1992). These mutagenesis studies also suggested that binding of IgE requires six out of eight extracellular cysteine residues of CD23, which are likely involved in the formation of intramolecular disulfide bridges (Bettler et al., 1992). The head domain of CD23 is involved in IgE binding, since its proteolytic cleavage by the house dust mite protease *Der p I* abrogates binding (Schulz et al., 1997). Nevertheless, one mutagenesis study suggested that the stalk region of CD23 is also involved in IgE binding (Chen et al., 2002); a finding that was recently confirmed, indicating that the IgE-CD23 interaction is more complex than previously anticipated (Selb et al., 2017). Interestingly, the latter study also demonstrated that mutation of the N-glycosylation site of CD23 (N63) alone is sufficient to enhance binding of IgE (Selb et al., 2017).

Vercelli et al. first demonstrated, using a bank of peptides spanning the IgE Cε2-4 domains, that CD23 recognizes a motif in the Cε3 domain of IgE (Vercelli et al., 1989). This was confirmed in a study using chimeric IgE molecules in which the human Cε3 domain was replaced by mouse Cε3: these chimeric molecules bound to mouse CD23 and concomitantly lost their ability to bind the human receptor (Nissim, Schwarzbaum, Siraganian, & Eshhar, 1993). Thereafter, the CD23 binding site on IgE was more precisely mapped to the A-B loop of the Cε3 domain (residues 341–356), with a key role for lysine 352 (Sayers, Housden, Spivey, & Helm, 2004). More recently, the crystal structure of the soluble head domain of CD23 bound to a Cε3–4 IgE dimer was resolved by Dhaliwal et al. (2012). These authors found that one CD23 molecule binds to each IgE heavy chain, principally via the Cε3 domains but with a contribution from Cε4 (Dhaliwal et al., 2012) (Fig. 4). Although the binding sites for FcεRI and CD23 are at opposite ends of the Cε3 domain, binding of the two receptors to IgE is mutually exclusive. Indeed, binding of IgE to CD23 induces conformational changes in Cε3, leading to a highly 'closed' conformation incompatible with FcεRI binding (Borthakur et al., 2012; Dhaliwal et al., 2012). Similarly, the 'opened' conformation adopted by Cε3 upon binding to FcεRI is incompatible with CD23 binding (Borthakur et al., 2012, Dhaliwal et al., 2012) (Fig. 3). Finally, the crystal structure of CD23 bound to a complete IgE Fc fragment was reported, revealing that the IgE Cε2 domain also contributes to CD23 binding, in addition to the known contributions of the Cε3 and Cε4 domains (Dhaliwal et al., 2017).

### 3.3. Other IgE or FcεRI binding molecules

Mast cells and basophils can be activated by the cytokine-like protein histamine-releasing factor (HRF) (reviewed in (Kawakami, Kashiwakura, & Kawakami, 2014)). It was shown that HRF could bind to a subset of IgE antibodies via their Fab regions, thereby inducing antigen-independent cross-linking of FcεRI-bound IgE molecules, and that this process could amplify inflammation in mouse models of cutaneous anaphylaxis or allergic airway inflammation (Kashiwakura et al., 2012). Similarly, the protein Galectin-3 (formerly known as ε binding molecule), which is released by several cell types, can bind to both IgE and FcεRI and induce mast cell and basophil activation via antigen-independent crosslinking of FcεRI (Frigeri, Zuberi, & Liu, 1993; Zuberi, Frigeri, & Liu, 1994). Galectin-3 is also directly produced by mast cells (it is found in the cytoplasm and nucleus of mast cells (Craig et al., 1995)), and it was shown that mast cells derived from the bone marrow of galectin-3 deficient mice displayed reduced activation by IgE and antigen *in vitro* as compared to WT mast cells (Chen et al., 2006).

Takizawa and collaborators reported that IgE immune complexes can bind to the mouse IgG receptors FcγRIIB and FcγRIII expressed on mast cells and macrophages, with an affinity similar to that of IgG immune complexes (Takizawa, Adamczewski, & Kinet, 1992). They further demonstrated that such binding to FcγRs can induce mast cell activation independently of FcεRI (Takizawa et al., 1992). IgE immune complexes were also found to bind and activate mouse FcγRIV, expressed on monocytes, macrophages and neutrophils (Hirano et al., 2007; Mancardi et al., 2008). Confirming that FcγRIV can act as a low-affinity receptor for mouse IgE, treatment of mice with an anti-FcγRIV antibody inhibited late phase reactions in a model of IgE-mediated passive cutaneous allergic inflammation (Hirano et al., 2007). In addition,

experiments performed in mice deficient for FcεRI, CD23 and all FcγRs except FcγRIV suggested that the *in vivo* engagement of FcγRIV by IgE immune complexes can synergize with mediators released by IgE-activated mast cells to induce lung inflammation (Mancardi et al., 2008).

## 4. Roles of IgE in health and disease

### 4.1. Pathologic roles of IgE

#### 4.1.1. Immediate hypersensitivity reactions

IgE antibodies are probably best known for their critical role in acute allergic reactions. In allergic individuals, mast cells and basophils have antigen-specific IgE bound to FcεRI expressed on the cell surface (Galli & Tsai, 2012). Antigen-mediated IgE/FcεRI crosslinking initiates a complex signaling cascade (Reber & Frossard, 2014; Sibilano, Frossi, & Pucillo, 2014), leading to the eventual activation of these effector cells and the immediate and rapid release of preformed granule-stored mediators (Wernersson & Pejler, 2014) (*e.g.*, histamine, serotonin, proteoglycans, proteases and cytokines) and *de novo* production and release of an impressive range of lipid mediators (*e.g.*, prostaglandins, leukotrienes), cytokines and chemokines (Galli et al., 2005; Voehringer, 2013). These mediators can act locally or systemically, leading to the clinical features of immediate hypersensitivity, such as bronchoconstriction, urticaria, diarrhea (when acting locally in the airways, the skin and the gut, respectively) (Fig. 5).

#### 4.1.2. Anaphylaxis

Anaphylaxis is the most extreme manifestation of an allergic reaction. In humans, anaphylaxis can be attributed to an IgE- and mast cell-dependent immediate hypersensitivity reaction in individuals previously sensitized to that allergen (Burton & Oettgen, 2011; Galli & Tsai, 2012; Lieberman et al., 2006). Indeed, quantification of specific IgE levels is used to identify potential triggers of anaphylaxis in patients with a personal history of anaphylaxis (Hamilton et al., 2010). IgE-dependent anaphylactic reactions can also be recapitulated in mice, in which a local or systemic injection of antigen one day after passive injection of antigen-specific IgE induces features of anaphylaxis (Dombrowicz et al., 1993; Oka, Kalesnikoff, Starkl, Tsai, & Galli, 2012; Wershil, Mekori, Murakami, & Galli, 1987).

IgE-mediated anaphylaxis is abrogated in mice lacking the high affinity IgE receptor FcεRI (Dombrowicz et al., 1993), as well as in mast cell-deficient mice (Feyerabend et al., 2011; Lilla et al., 2011; Oka et al., 2012), highlighting the importance of IgE-mediated mast cell activation in this reaction. Mast cells likely also play a key role in human anaphylaxis. Indeed, elevated levels of the mast cell specific protease tryptase have been detected during anaphylactic reactions in humans (Schwartz, Metcalfe et al. 1987, Schwartz, 2006, Brown et al., 2013). Moreover, an increased incidence of anaphylaxis was reported in patients with mastocytosis, a disease characterized by increased numbers of mast cells (Schuch & Brockow, 2017). By contrast, the role of basophils in anaphylaxis is more debated. So-called "Basophil activation tests" are used to confirm allergen sensitization in human patients. In these tests, which are performed on blood samples *ex vivo*, IgE-mediated activation of basophils is monitored by measuring up-regulation of surface markers such as CD63 and CD203c (Giavina-Bianchi, Galvao, Picard, Caiado, & Castells, 2017; Kim et al., 2016; Santos et al., 2015). Recently, Korosec and colleagues also reported an increase of CD63 expression on circulating basophils, as well as a marked reduction in the absolute number of circulating basophils, during anaphylactic reactions to *Hymenoptera venom* in humans (Korosec et al., 2017). While these data suggest that basophils are activated in human anaphylaxis, they do not however demonstrate a significant contribution to anaphylaxis pathophysiology. Even in mice, the role of basophils in IgE-mediated anaphylaxis remains contentious. Different reports indicate that depletion of basophils does not reduce IgE-mediated local or systemic passive anaphylaxis (Sawaguchi et al., 2012; Wada et al., 2010). Mukai and colleagues reported that intravenous injection of antigen-specific IgE in mice, followed one day later by subcutaneous challenge with the antigen, can induce a triphasic response (Mukai et al., 2005). The 'immediate' and 'late-phase' (6 to 10 h after challenge) responses were dependent on mast cells. However, the third-phase, beginning one to two days after challenge, was independent of mast cells and was abrogated upon depletion of

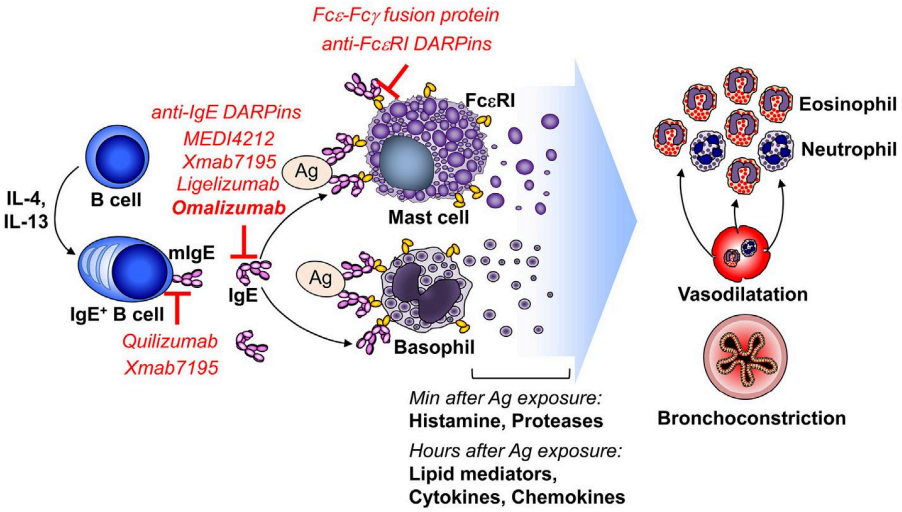


**Fig. 5. Key role of IgE in allergic reactions.** Stimulation with the $T_H2$ cytokines IL-4 and IL-13 induces class-switching of B cells into IgE-producing cells. IgE binds to its high-affinity receptor FcεRI on the surface of tissue mast cells and blood basophils. Upon exposure to an allergen, in allergic patients, allergen recognition by allergen-specific IgE on the surface of mast cells and basophils induces crosslinking of FcεRI, leading to degranulation and the immediate release of histamine, proteases and other preformed mediators, as well as *de novo* synthesis of lipid mediators (prostaglandins, leukotrienes, …), cytokines and chemokines. These mediators can act locally or systemically, leading to the clinical features of immediate hypersensitivity, such as bronchoconstriction, urticaria, diarrhea (when acting locally in the airways, the skin and the gut, respectively) and vasodilatation. These mediators are also responsible for late-phase allergic responses, entailing the recruitment of leukocytes, mainly eosinophils and neutrophils. Several drugs have been developed to counteract the effects of IgE. These drugs either target IgE production, block free IgE or compete with IgE for binding to FcεRI. The only FDA-approved anti-IgE drug is Omalizumab, a humanized anti-IgE mAb that blocks free IgE, and which is approved for the treatment of moderate to severe persistent allergic asthma, and chronic spontaneous urticaria (CSU). Ag: antigen.

basophils (Mukai et al., 2005; Obata et al., 2007; Sawaguchi et al., 2012). This third-phase delayed response was also absent in mice lacking FcRγ (a signaling subunit shared by FcεRI and activating IgG Fcγ receptors), and was restored upon engraftment of these mice with basophils purified from WT mice but not from *FcRγ*$^{-/-}$ mice (Mukai et al., 2005). Since this passive model relies on specific IgE antibodies, and not on IgG, these results strongly suggest that direct activation of basophils through FcεRI is responsible for the delayed allergic skin inflammation observed this model. Using a similar model of IgE-mediated chronic allergic inflammation, Cheng et al. also reported markedly reduced eosinophilic dermatitis in basophil-deficient mice as compared to control mice three days after cutaneous challenge with the relevant antigen (Cheng et al., 2015).

The presence of allergen-specific IgE alone does not explain an individual's susceptibility to allergy and anaphylaxis. Allergen-specific IgE can be detected in subjects who do not develop clinical symptoms when exposed to the corresponding allergen (Sicherer & Sampson, 2010). Conversely, some patients can experience near fatal anaphylaxis despite having low or undetectable levels of circulating allergen-specific IgE (Simons et al., 2007), which suggests (but does not prove) the existence of IgE-independent pathways of anaphylaxis in humans (recently reviewed in (Finkelman, Khodoun, & Strait, 2016) and (Reber, Hernandez, & Galli, 2017)). More definitive evidence for IgE-independent pathways of anaphylaxis has been obtained using mouse models of active systemic anaphylaxis (ASA), in which mice are sensitized with an antigen (to produce antigen-specific antibodies) and re-exposed later on to the same antigen to induce anaphylaxis (Finkelman et al., 2016; Munoz-Cano, Picado, Valero, & Bartra, 2016). Mice deficient for IgE or for FcεRI can still partially (Arias et al., 2011; Balbino et al., 2017; Sun et al., 2007) or fully (Dombrowicz et al., 1997; Jonsson et al., 2011; Oettgen et al., 1994) develop features of anaphylaxis in these ASA models. Other studies have subsequently shown that mouse IgG antibodies can trigger anaphylaxis in ASA models, through activation of IgG receptors (FcγRs) on the surface of various myeloid cells, including basophils, macrophages and neutrophils (Balbino et al., 2017; Finkelman et al., 2016; Jonsson et al., 2011; Khodoun et al., 2013; Miyajima et al., 1997).

#### 4.1.3. Allergic asthma

Asthma is a chronic inflammatory disease of the airways with continual increasing prevalence (Busse & Lemanske Jr., 2001; Subbarao, Mandhane, & Sears, 2009). In many patients, the asthmatic condition is associated with allergic reactivity to environmental allergens and elevated levels of IgE antibodies (Busse & Lemanske Jr., 2001). In these allergic patients, IgE is thought to contribute to the asthmatic manifestations (Galli & Tsai, 2012). Following antigen exposure in the airways, rapid local IgE/FcεRI-dependent mast cell activation and the immediate hypersensitivity reaction can lead to increased vascular permeability, bronchoconstriction and increased mucus production. A large array of cytokines, growth factors and chemokines secreted by activated mast cells can influence airway remodeling (Galli, Tsai, & Piliponsky, 2008; Moiseeva & Bradding, 2011). Finally, IgE can also act on other cell types that express FcεRI or CD23, such as DCs, B cells, basophils or (in humans) eosinophils, which may potentially affect several biological responses associated with the asthmatic response (Galli et al., 2008; Galli & Tsai, 2012). Supporting the important role of IgE in asthma, the anti-IgE antibody omalizumab has been shown to reduce asthma symptoms in several clinical trials involving patients with moderate-to-severe and severe allergic asthma (reviewed in (Humbert et al., 2014)) (for more detail see Section 5.1, below).

#### 4.1.4. Atopic dermatitis

Eczema, or atopic dermatitis (AD), is a pruritic inflammatory skin disease with dramatically increased incidence over the last decades (Bieber, 2008; Dharmage et al., 2014). AD manifestations are characterized by pruritus (itching), skin inflammatory lesions associated with cellular infiltration and histopathological changes, and atopy. Indeed, the majority of AD patients exhibit increased serum levels of total and antigen-specific IgE (Laske & Niggemann, 2004; Leung & Bieber, 2003; Oyoshi, He, Kumar, Yoon, & Geha, 2009). The function of IgE in development of AD is supported by the beneficial effect of anti-IgE therapy in a number of clinical studies (Belloni, Andres, Ollert, Ring, & Mempel, 2008; Liu, Goodarzi, & Chen, 2011).

Abboud, Staumont-Sallé et al. used a mouse model of AD induced by repeated epicutaneous sensitizations with ovalbumin. They reported that several features of this model (including $T_H1$ and $T_H2$ skin responses, mast cell recruitment into draining lymph nodes and IgE production) were reduced in *FcεRI*$^{-/-}$ mice. In this model, $T_H2$ skin response as well as T cell proliferation and IgG1 production were also reduced in mice lacking the IgG receptor FcγRIII (Abboud et al., 2009). In addition, symptoms of AD were completely absent in mice deficient for FcRγ, a subunit shared by FcεRI and FcγRIII (and several other FcR). The authors therefore concluded that in this model, FcεRI and FcγRIII both contribute to AD but differentially regulate immune responses associated with the disease (Abboud et al., 2009). Ando and colleagues developed a mouse model of AD in which eczematous skin lesions are induced by repeated epicutaneous applications of house dust mite extract and staphylococcal enterotoxin B (Ando et al., 2013; Kawakami, Yumoto, & Kawakami, 2007). The global skin gene expression pattern in this model was very similar to that observed in human AD skin. Mast cell-deficient mice had markedly reduced skin inflammation; and FcεRI expression was required to attain maximal clinical scores in this AD model (Ando et al., 2013). However, some features of the model were reduced in mast cell-deficient mice but not in *FcεRI*$^{-/-}$ mice, which suggests that mast cells can amplify inflammation in the context of AD model though both IgE-dependent and IgE-independent pathways (Ando et al., 2013).

#### 4.1.5. Chronic spontaneous urticaria

Chronic spontaneous urticaria (CSU; also known as chronic idiopathic urticaria) is defined as itchy wheals, angioedema, or both that reoccur for more than 6 weeks without a specific trigger (Zuberbier, Aberer et al., 2014). Antihistamines show clinical benefit for many (but not all) CSU patients, and it is therefore believed that skin mast cells, which are a major source of histamine, play an important role in CSU (Vonakis & Saini, 2008). CSU patients often have high levels of total IgE (Kessel et al., 2010). However, CSU may not be triggered by specific external antigens. By contrast, most CSU patients exhibit autoimmune responses in the form of serum IgE to autoantigens or IgG autoantibodies to IgE or FcεRI (reviewed in (Kolkhir et al., 2017)). 35-45% of adults with CSU develop a wheal when injected intradermally with their own serum, a test called autologous serum skin test (ASST) (Metz et al., 2009). Such positive ASSTs responses have been linked to IgG autoantibodies directed against the high-affinity IgE receptor FcεRI, or less commonly against IgE (Auyeung, Mittag, Hodgkin, & Harrison, 2016; Chang et al., 2015; Hide et al., 1993). Both types of autoantibodies can trigger activation of mast cells (and other FcεRI-bearing cells) through cross-linking of FcεRI. In a recent study, autoreactive T cells specific for FcεRI were also detected in the blood of a large proportion of patients with CSU (Auyeung et al., 2016). The authors therefore proposed that, as for other autoimmune diseases, activation of autoreactive T cells is likely one of the initial events in CSU (Auyeung et al., 2016). Moreover, some CSU patients have high titers of autoreactive IgE directed against dsDNA or thyroid antigens, such as thyroperoxidase (TPO) (Altrichter et al., 2011; Hatada et al., 2013). It was also recently reported that IL-24 is a common autoantigen in patients with CSU (Schmetzer et al., 2017). Such IgE autoantibodies could mediate skin reactions in CSU by inducing mast cell degranulation in response to autoantigens (Altrichter et al., 2011; Chang et al., 2015; Hatada et al., 2013). It should be noted, however, that the presence of

IgE against autoantigens is also documented in diseases other than CSU, such as atopic dermatitis (reviewed in (Hradetzky, Werfel, & Rosner, 2015)), and a direct link between autoantibodies and the clinical manifestations of CSU has not yet been demonstrated. Some reports also indicate the presence of IgE against exogenous antigens, such as *Staphylococcus aureus* enterotoxins, in some CSU patients, which could contribute to the pathogenesis of CSU in a subpopulation of patients (Altrichter et al., 2018; Ye et al., 2008).

In support of a key role of IgE and FcεRI in CSU, the anti-IgE therapeutic antibody omalizumab is now approved for the treatment of CSU (Chang et al., 2015; Maurer et al., 2013; Zhao et al., 2016). Moreover, most patients with CSU who stop omalizumab treatment relapse within a few months, and a recent study indicates that total IgE serum levels before omalizumab treatment correlate negatively with the time to relapse in these patients (Ertas et al., 2017). As reviewed in detail by Chang et al. (2015), the clinical benefits of omalizumab are likely due to a direct blockade of IgE antibodies before they can bind FcεRI and activate mast cells (especially in patients with autoreactive IgE), and/or a downregulation of FcεRI on the surface of mast cells and other effector cells (Chang et al., 2015).

### 4.2. Protective roles of IgE

IgE and the main FcεRI-expressing effector cells, mast cells and basophils, do not only play roles in pathology, but also critically contribute to host defense. This has been convincingly demonstrated using mouse models of host defense against certain parasites and venoms.

#### 4.2.1. Host defense against parasites

Helminth infections are generally associated with a “type 2” immune response, characterized by helper type 2 T ($T_H2$) cells that typically produce IL-4, IL-5 and IL-13, increased numbers of tissue mast cells and eosinophils, and elevated levels of antigen-specific and unspecific IgE (Anthony, Rutitzky, Urban Jr., Stadecker, & Gause, 2007; Finkelman et al., 1997; Grencis, Humphreys, & Bancroft, 2014). Data from epidemiological studies in humans point towards a protective role for IgE in helminth infections, as increased levels of helminth-specific IgE correlate with host resistance (Faulkner et al., 2002; Hagan, Blumenthal, Dunn, Simpson, & Wilkins, 1991; Rihet, Demeure, Bourgois, Prata, & Dessein, 1991). Remarkably, anti-IgE antibody treatment of human patients at high risk of helminth infections did modestly increase parasite infection risk, albeit an effect that did not reach statistical significance (Cruz et al., 2007). Increased IgE levels might, however, simply reflect a strong $T_H2$ cell response in infected individuals, the latter being of unquestionable importance in host defense against parasites. Indeed, the actual contributions of non-specific *vs.* specific IgE antibodies in host defense and parasite clearance are still unclear and numerous experimental studies aiming at addressing this question have led to different, sometimes opposing, conclusions (recently reviewed in (Mukai, Tsai, Starkl, Marichal, & Galli, 2016)). Also, protective *vs.* detrimental roles of IgE antibodies in anti-parasite immunity appear to be parasite-dependent. For instance, data from experiments with IgE-deficient mice indicate beneficial functions for IgE in models of *Trichinella sprialis* (Gurish et al., 2004), *Schistosoma mansoni* (King et al., 1997), *Brugia Malayi* (Spencer, Porte, Zetoff, & Rajan 2003), *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus* (Schwartz et al., 2014). On the other hand, experiments with IgE- or FcεRIα-deficient mice in other studies showed no effect or decreased parasite burden in infections with *H. polygyrus* (McCoy et al., 2008), *Strongyloides venezuelensis* (Matsumoto et al., 2013) or *S. mansoni* (Jankovic et al., 1997). Among the factors potentially contributing to these discrepancies, one could cite differences in experimental approaches (transgenic [IgE- or FcεRIα-deficient mice] or pharmacological [anti-IgE treatments]), the experimental model and/or the genetic background of the mice (Mukai et al., 2016).

#### 4.2.2. Host defense against venoms

Toxic substances, such as venoms, represent an obvious threat for mammals, against which defense mechanisms are needed. In 1991, Margie Profet proposed a theory known as the "toxin hypothesis", suggesting that allergic immune responses (*i.e.*, IgE-associated type 2 immune responses and effector cell-mediated allergic reactions) represent an immunological defense against toxins (Profet, 1991). According to this theory, the purpose of an acute allergic reaction (manifested by, *e.g.*, scratching, vomiting, diarrhea, and, in extreme cases, anaphylaxis) is to respond rapidly and avoid, eliminate and/or neutralize toxic substances indicative of life-threatening situations (Palm, Rosenstein, & Medzhitov, 2012; Profet, 1991).

Recently, Profet's hypothesis was supported by experimental evidence demonstrating that IgE antibodies could contribute to acquired resistance against honeybee and snake venoms (Marichal et al., 2013; Palm et al., 2013; Starkl et al., 2016). Marichal, Starkl et al. characterized the immune response of mice following subcutaneous injection of whole bee venom to mimic bee stings (Marichal et al., 2013). The venom induced a robust adaptive type 2 immune response associated with development of venom-specific $T_H2$ cells and IgE, and this acquired immune response was associated with increased resistance of mice (quantified by survival and body temperature) against a subsequent challenge with bee venom. Experiments involving passive immunization and transgenic animals deficient in IgE or FcεRI demonstrated that IgE antibodies and IgE effector mechanisms played a crucial role in mediating acquired host resistance against bee venom (Marichal et al., 2013). In a complementary study, Palm, Rosenstein et al. provided experimental evidence that a type 2 immune response directed against the bee venom component phospholipase A2 (PLA2) was able to confer protection against a subsequent near lethal dose of PLA2, and that such protection was dependent on FcεRI (Palm et al., 2013). Subsequently, Starkl, Marichal et al. found that IgE effector mechanisms also played a critical role in acquired host defense against the venom of the Russell's viper (Starkl et al., 2016).

The strong evidence for the important protective function of IgE and IgE effector cells in immune defense against venoms in mice challenges the current view of the function of IgE in (venom-) allergic humans (Artis, Maizels, & Finkelman, 2012). Therefore, future investigations are needed to determine whether IgE-associated responses can enhance resistance to other toxins, and to understand why, in some species or individuals, exposure to the same venom or venom component may induce either a protective IgE-dependent adaptive immune response, as in the mouse studies described above (Marichal et al., 2013, Palm et al., 2013, Starkl et al., 2016), or a deleterious and potentially fatal allergic reaction (*i.e.*, anaphylaxis) (Charavejasarn, Reisman, & Arbesman, 1975; Saelinger & Higginbotham, 1974). This question is of great interest and relevance for basic and clinical allergy research.

## 5. Targeted anti-IgE therapies

### 5.1. Anti-IgE antibodies

#### 5.1.1. Omalizumab

Omalizumab is a recombinant humanized IgG1 monoclonal antibody directed against human IgE sold by Novartis and Genentech under the trade name Xolair® (Presta et al., 1993). It binds to the Cε3 domain of free IgE, and thereby impairs binding of IgE to both FcεRI and CD23 (Chang et al., 1990; Davies et al., 2017; Selb et al., 2017) (Fig. 5). Importantly, omalizumab does not recognize IgE already bound to FcεRI or CD23, and therefore cannot induce cell activation by crosslinking of IgE receptors (Chang et al., 1990; Davies et al., 2017).

The IgE binding site of omalizumab has been characterized recently by molecular modeling and crystallography (Davies et al., 2017; Pennington et al., 2016; Wright et al., 2015; Zheng et al., 2008). Omalizumab binds to symmetric sites on the two IgE Cε3 domains: it does not directly mask the FcεRI binding site on IgE, but rather induces

major conformational changes in the Cε3 domains that inhibit interaction with FcεRI (Davies et al., 2017, Pennington et al., 2016, Wright et al., 2015, Zheng et al., 2008). Davies and colleagues reported that, furthermore, IgE binding to CD23 is sterically hindered by Omalizumab due to overlapping binding sites on each Cε3 domain (Davies et al., 2017). While omalizumab is alleged to be unable to bind IgE already bound to FcεRI, *in vitro* data suggest that omalizumab could also facilitate the dissociation of FcεRI-bound IgE (Eggel et al., 2014).

The first randomized, double blind, placebo controlled trials were conducted in 1996 to assess the tolerability and efficiency of omalizumab in patients with allergic asthma (Boulet et al., 1997; Fahy et al., 1997). These trials showed a reduction of free serum IgE levels (but an increase in total serum IgE, *i.e.* free IgE and IgE complexed with omalizumab), and improved responses to inhaled allergens following omalizumab therapy (Boulet et al., 1997, Fahy et al., 1997). In addition to the reduction of free serum IgE levels, treatment with omalizumab also induced a decrease in the expression of FcεRI on the surface of basophils, DCs and mast cells (Lin et al., 2004; Prussin et al., 2003; Saini et al., 1999). In 2003, Xolair® was approved for the treatment of moderate to severe persistent allergic asthma, and is now also approved for the treatment of chronic spontaneous urticaria (CSU) (Chang et al., 2015; Maurer et al., 2013; Zhao et al., 2016). In addition, more than 150 clinical trials of omalizumab are now listed on the website clinicaltrials.gov, in various diseases including food and venom allergies (in combination with allergen-specific immunotherapy), allergic rhinitis or mastocytosis. It is, however, important to note that, although Xolair® is generally well tolerated, it can induce side effects ranging from skin inflammation (at the site of subcutaneous injection) to systemic anaphylaxis (in 0.1–0.2% of patients) (Harrison, MacRae, Karsh, Santucci, & Yang, 2015; Lieberman, Umetsu, Carrigan, & Rahmaoui, 2016).

#### 5.1.2. Ligelizumab

Ligelizumab (QGE031) is a more recent humanized anti-IgE antibody developed by Novartis. It is also directed against Cε3, but is designed to achieve improved IgE suppression, with an equilibrium dissociation constant ($K_D$) of 139 pM (as compared to the $K_D$ of omalizumab, ~6–8 nm) (Arm et al., 2014) (Fig. 5). The first clinical results of ligelizumab treatment indicated that this antibody can reduce free-IgE and basophil FcεRI with an efficiency superior to that of omalizumab (NCT01716754). Although the authors did not observe serious adverse events in this study, one patient treated with ligelizumab developed systemic symptoms (Arm et al., 2014). In 2016, ligelizumab was tested in patients with mild allergic asthma, and was shown to have greater efficacy than omalizumab on inhaled and skin allergen responses in these patients (NCT01703312) (Gauvreau et al., 2016). However, in a more recent phase II field study of asthma patients, ligelizumab was not seen to be superior to omalizumab (NCT01716754), and further development for asthma has been discontinued.

#### 5.1.3. Quilizumab

Quilizumab (MEMP1972A) is a humanized monoclonal antibody developed by Genentech targeting the M1′ epitope which is present on membrane IgE (mIgE) but not on serum IgE (Fig. 5). Brightbill and colleagues demonstrated, using genetically modified mice that contained the human M1′ domain inserted into the mouse IgE locus, that quilizumab could reduce serum IgE and deplete IgE-producing plasma cells *in vivo*, without affecting other immunoglobulin isotypes (Brightbill et al., 2010). Quilizumab has been tested in clinical trials in patients with allergic rhinitis (NCT01160861) and mild allergic asthma (NCT01196039) (Gauvreau et al., 2014). In both studies, reductions in total and allergen-specific serum IgE were observed, as well as improved clinical responses to allergen, suggesting that targeting mIgE can reduce IgE production in humans (Gauvreau et al., 2014). In a subsequent trial (NCT01582503), treatment with quilizumab also reduced total and allergen-specific IgE in patients with allergic asthma uncontrolled by standard therapy. However, treatment with quilizumab had no impact on asthma exacerbations, lung functions, or patient-reported symptoms in this trial (Harris et al., 2016). Similarly, quilizumab reduced IgE levels by about 30% in CSU patients, but it did not lead to clinical improvements in patient's self-reported itch-severity scores (NCT01987947) (Harris et al., 2016).

#### 5.1.4. XmAb7195

XmAb7195 is a monoclonal anti-IgE antibody developed by Xencor through humanization, affinity maturation, and Fc engineering of the murine parental antibody of omalizumab (MaE11) (Chu et al., 2012). XmAb7195 has an IgE-binding affinity 5.3-fold higher than that of omalizumab. In addition, two point mutations in the IgG1 Fc portion of the mAb (G236R and L328R) increase the binding affinity to inhibitory IgG receptor FcγRIIB by 400 times compared to omalizumab (Chu et al., 2012). The authors demonstrated that XmAb7195 could block free IgE and inhibit IgE production in B cells through co-engagement of mIgE and FcγRIIB (Chu et al., 2012) (Fig. 5). In a first-in-human phase 1a trial in healthy volunteers (NCT02148744), XmAb7195 decreased IgE levels below the limit of detection in 90% of subjects that had detectable IgE levels at baseline. Transient thrombocytopenia was observed at a dose of 3.0 mg/kg, but no other major adverse events were reported (Gershman, Goldwater, & Forster, 2016). A phase 1b study on the safety, tolerability and bioavailability of a subcutaneous formulation of XmAb7195 has been recently completed (NCT02881853), but the results of this study have not yet been reported.

#### 5.1.5. MEDI4212

MEDI4212 is a human IgG1 anti-IgE antibody developed by MedImmune. MEDI4212 was generated using phage display technology, combined with targeted mutagenesis of $V_H$ and $V_L$ sequences to increase its affinity for IgE (Cohen et al., 2014). Like omalizumab, MEDI4212 does not recognize IgE already bound to FcεRI, but the authors report that MEDI4212 binds free IgE with an affinity of 1.95 pM, more than 100-fold higher than omalizumab (Cohen et al., 2014) (Fig. 5). Analysis of the crystal structure of IgE Cε3-4 domains in complex with MEDI4212 Fab portion revealed that MEDI4212 recognizes residues in the Cε3 and Cε4 domains, and targets critical residues in Cε3 also involved in binding to FcεRI. This suggests that MEDI4212 directly competes with FcεRI for IgE binding (Cohen et al., 2014).

Since MEDI4212 recognizes residues in the IgE Cε3-4 domains, it can also bind mIgE on the surface of B cells. MEDI4212 was further engineered in order to increase its potential to eliminate IgE-expressing B cells through antibody-dependent cell-mediated cytotoxicity (ADCC) (Nyborg et al., 2016). The authors chose to insert mutations in the Fc portion of MEDI4212 in order to improve its affinity for the IgG receptor FcγRIIIA, as ADCC can be performed by natural killer (NK) cells that express FcγRIIIA. Indeed, *in vitro* experiments revealed that, thus Fc-engineered, MEDI4212 could eliminate class-switched human IgE B cells more efficiently (Nyborg et al., 2016). A phase I study on the pharmacokinetics, pharmacodynamics, and safety of MEDI4212 in subjects with atopy was initiated in 2012 (NCT01544348); and demonstrated that MEDI4212 rapidly reduced free IgE to a greater extent than omalizumab. However, recovery of free IgE to baseline was much faster in patients receiving MEDI4212 as compared as omalizumab, which was attributed to a rapid decrease of serum MEDI4212. Since then, no other study has been initiated with this antibody.

### 5.2. Anti-IgE, anti-FcεRI and anti-CD23 DARPins

Designed ankyrin repeat proteins (DARPins) are engineered small proteins that can recognize targets with high specificity and with affinity in the low nanomolar range (Binz et al., 2004; Pluckthun, 2015). In 2009, Eggel and collaborators reported identification of two monovalent DARPins, termed B-A4-85 and C-A3-30, displaying high affinity for two

different epitopes on human FcεRIα (Eggel, Baumann, Amstutz, Stadler, & Vogel, 2009). They further produced a bispecific anti-FcεRIα DARPin (designated 30/85) by linking sequences of the two monovalent DARPins with a $[Gly_4–Ser]_4$ linker. Remarkably, this bispecific DARPin showed greater affinity than IgE for FcεRIα, and was able to inhibit IgE-FcεRIα interaction and IgE-mediated degranulation of rat basophilic leukemia cells expressing human FcεRIα (RBL-2H3-huα cells), with an effect similar to that of omalizumab (Eggel et al., 2009) (Fig. 5).

Using a similar strategy, the same group reported identification of several DARPins binding human IgE (Fig. 5). Among these, the DARPins E2_79 and E3_54 were able to inhibit binding of IgE to either FcεRIα or omalizumab, and inhibit IgE-mediated activation of RBL-2H3-huα cells with higher efficacy than omalizumab (Baumann, Eggel, Amstutz, Stadler, & Vogel, 2010). It was further demonstrated that E2_79 not only prevented binding of free IgE to FcεRI, but also actively disrupted pre-formed IgE:FcεRI complexes (Kim et al., 2012). Such facilitated IgE dissociation was observed both *in vitro*, *ex vivo* in primary human basophils, and *in vivo* in human FcεRI transgenic mice (Eggel et al., 2014; Kim et al., 2012), suggesting that anti-IgE DARPins might be suitable drug candidates to desensitize allergic patients.

Another DARPin (E3_53) can recognize both free IgE and IgE bound to FcεRI. This DARPin was linked to the Fc portion of human IgG1 (using a $[Gly_4–Ser]_3$ linker) to produce a fusion protein capable of cross-linking FcεRI-bound IgE with the inhibitory receptor FcγRIIB. This molecule, termed DE53-Fc, was able to reduce allergen-induced basophil activation *ex vivo* using whole blood samples from allergic patients (Eggel et al., 2011). Furthermore, by using blocking antibodies against FcγRIIB, the authors demonstrated that binding of DE53-Fc to FcγRIIB was required for full inhibitory properties of the fusion molecule (Eggel et al., 2011). Confirming this mode of action, it was later reported that mutant forms of DE53-Fc displaying enhanced affinity for FcγRIIB also have greater capacity to inhibit basophil activation (Buschor, Eggel, Zellweger, Stadler, & Vogel, 2014). However, while mouse basophils and mast cells and human basophils express high levels of FcγRIIB, it is still ambiguous whether human mast cells also express this inhibitory receptor (Zhao et al., 2006). Therefore, whether cross-linking of FcεRI-bound IgE to FcγRIIB could inhibit IgE- and mast cell-mediated responses in humans remains an open question.

More recently, two DARPins (D86 and D89), which specifically recognize CD23, were also identified. These anti-CD23 DARPins inhibited binding of IgE to CD23 (which suggests that they share a similar binding epitope to IgE), and could inhibit IgE synthesis in human peripheral B cells (Fellmann, Buschor, Rothlisberger, Zellweger, & Vogel, 2015).

### 5.3. Fcε-Fcγ fusion proteins

The human Fcγ-Fcε bifunctional fusion protein consists of the Fc region of human IgG1 (hinge-Cγ2-3) linked to the Fc portion of human IgE (Cε2-4) by a 15 amino acid linker $(Gly_4Ser)_3$ (Zhu, Kepley, Zhang, Zhang, & Saxon, 2002). As first described by Zhu et al., this fusion protein (called GE2) was able to compete with IgE for the binding to FcεRI, and could thereby be used to 'desensitize' mast cells and basophils (Fig. 5). It could also bind to IgG FcγRs through its Cγ2-3 domains, and it was therefore proposed that GE2 could block IgE-mediated mast cell and basophil activation through co-engagement of FcεRI with the inhibitory receptor FcγRIIB (Zhu et al., 2002). Indeed, the authors demonstrated that GE2 was able to inhibit histamine release in primary human blood basophils sensitized with IgE, and could also block IgE-mediated passive cutaneous anaphylaxis (PCA) in transgenic mice expressing human FcεRI (Zhu et al., 2002). In addition to its effect on mast cells and basophils, it was proposed that the fusion protein could also inhibit allergic inflammation through effects on FcεRI-expressing Langerhans cells (Kepley, Zhang, Zhu, & Saxon, 2003), and inhibit IgE class switch recombination in B cells by co-aggregating CD23 and FcγRII (Yamada, Zhu, Zhang, & Saxon, 2003). Several attempts were subsequently made to improve the efficiency of the fusion protein, such as removal of the $(Gly_4Ser)_3$ linker, or mutations in the Cγ portion to improve binding to FcγRIIB and/or decrease binding to FcγRIII (Allen, Kepley, Saxon, & Zhang, 2007). However, most of these modifications altered the effectiveness of the fusion protein to inhibit FcεRI-mediated functions (Allen et al., 2007). Nevertheless, and as described above (Section 5.2), while basophils undoubtedly express FcγRIIB, it is still unclear whether human mast cells express FcγRIIB *in vivo* (Zhao et al., 2006).

The effects of GE2 were also tested in non-human primates. Rhesus monkeys have been reported to exhibit skin test reactivity and serum IgE directed against dust mites (Schelegle et al., 2001; Zhang et al., 2004). Taking advantage of this, Zhang and collaborators showed that GE2 was able to inhibit dust mite allergen-induced skin reactivity in rhesus monkeys in a dose-dependent manner (Zhang et al., 2004). In a subsequent study, GE2 demonstrated efficacy in a model of house dust mite-induced allergic asthma in cynomolgus monkeys (Van Scott et al., 2008). The effects of GE2 lasted for 4 weeks and were associated with reduced numbers of circulating basophils and reduced FcεRI expression on basophils. However, repeated injections of GE2 induced the production of serum antibodies against the fusion protein, and increased occurrence of serious adverse events, including anaphylaxis (Van Scott et al., 2008).

## 6. Concluding remarks

Discovered some 50 years ago, IgE continues to be the focus of extensive academic and industrial research. The clinical benefits of the anti-IgE antibody omalizumab best exemplify the key role of IgE in allergic diseases and chronic spontaneous urticaria. Besides omalizumab, several new anti-IgE therapies are now at various stages of clinical development, with some promising early results. Recent insights from crystallographic studies have also shed light on the mechanisms by which IgE antibodies recognize their main receptors FcεRI and CD23; findings that should help in the design of additional therapeutic approaches aimed at blocking these interactions.

While IgE can undeniably trigger allergic reactions, it is also now clear that not all allergies are IgE-mediated, and evidence from mouse models suggests that IgE may have protective functions in host defense against parasites and venoms. An ongoing effort is therefore necessary to clearly identify the full spectrum of IgE-mediated diseases, but also to address the potential limitations of targeted anti-IgE therapies.

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## Conflict of interest statement

E.C. is an employee of Neovacs SA. L.L.R. reports serving as consultant for Neovacs SA. All other authors have declared no conflicts of interest.

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## 10. Table of Figures