IMMUNE-EFFECTOR PATHWAYS LEADING TO PEANUT-INDUCED ANAPHYLAXIS

IMMUNE-EFFECTOR PATHWAYS UNDERLYING PEANUT-INDUCED ANAPHYLAXIS IN MICE

By

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— ABSTRACT —

Among food allergies, peanut has attracted the most research attention because the allergy is typically lifelong, often severe and potentially fatal. Furthermore, other than epinephrine, there are no treatments available to date. A decade of research has provided a great deal of insight into the factors that promote and regulate the *development* of allergic responses. However, less in known about the factors involved in the *elicitation* of the most common and severe manifestation of peanut allergy, namely anaphylaxis. The research in this thesis centers on the investigation of cellular and molecular pathways leading to peanut-induced anaphylaxis (PIA) as well as potential therapeutic targets. Specifically presented are: i) the development and characterization of a mouse model of PIA (Chapter 2), ii) the role of molecules including histamine, leukotrienes (LT) and platelet-activating factor (PAF) (Chapter 3) and, iii) the relative contribution of mast cells, basophils and macrophages as well as IgE and IgG_1 (Chapter 4). Our data show that oral sensitization to peanut in C57BL/6 mice generated local and systemic markers of type-2 immunity that was associated with robust and consistent clinical anaphylaxis following antigen challenge. In this context, concurrent blockade of PAF and histamine receptors markedly decreases the severity of these reactions. Moreover, they demonstrate that distinctive immune effector pathways involving activation of mast cells (via IgE and IgG_1) and macrophages (via IgG_1) cooperate to elicit a broad range of systemic reactions to peanut. These findings highlight that concomitant and progressive recruitment of immune-effector pathways leads to a full range of anaphylactic reactions and therefore, therapeutic strategies for PIA may need to target several pathways or, alternatively shared components within these pathways. Combination therapy blocking both PAF and histamine may represent such as a therapeutic approach.

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— List of Abbreviations —

Ab	antibody
Ag	antigen
ASA	active systemic anaphylaxis
BSA	bovine serum albumin
CysLT	cysteinyl leukotrienes
Cε	constant region of the heavy chain epsilon
CT	cholera toxin
FcR	Fc receptor
FcεRI	high affinity IgE receptor
FcγR	immunoglobulin G Fc receptor
5-LO	5-lipoxygenase
GI	gastrointestinal
Ig	immunoglobulin
IL	interleukin
LT	leukotrienes
mAb	monoclonal antibody
OVA	ovalbumin
PAF	platelet-activating factor
PAF-AH	platelet-activating factor acetylhydrolase
PAF-R	platelet-activating factor receptor
PBMC	peripheral blood-derived mast cells
PGD_2	prostaglandin D_2
PIA	peanut-induced anaphylaxis
PSA	passive systemic anaphylaxis
Pen-V	penicillin V
TNF-α	tumor necrosis factor-alpha
Th2	type-2 T cells
WT	wild-type

— PREFACE —

The research documented in Chapters 2-4 of this doctoral thesis represents three independent although conceptually related bodies of work that, as of June 2011, have been published. The work conducted in each manuscript required a collaborative effort with several colleagues resulting in multiple authors.

CHAPTER 2 — Sun J,* Arias K,* Alvarez D, Fattouh R, Walker T, Goncharova S, Kim B, Waserman S, Reed J, Coyle AJ, Jordana M. Impact of CD40 Ligand, B Cells, and Mast Cells in Peanut-Induced Anaphylactic Responses. *The Journal of Immunology.* 2007 Nov 179 (10): 6696–6703. * Joint first authors.

This study was conducted during September 2005 – February 2007. As primary authors, Kevin Sun and I jointly designed and performed all of the experiments, analyzed all data and wrote the manuscript. D. Alvarez (graduate student), R. Fattouh (graduate student), T. Walker (technologist), S. Goncharova (technologist), B. Kim (undergraduate student) provided me with experimental assistance. S. Waserman (McMaster University collaborator), J. Reed (MedImmune collaborator) and A.J. Coyle (MedImmune collaborator) contributed valuable scientific input and critically appraised the manuscript.

CHAPTER 3 — Arias K, Baig M, Colangelo M, Chu DK, Walker T, Goncharova S, Coyle AJ, Vadas P, Waserman S, Jordana M. Concurrent Blockade of Platelet Activating Factor and Histamine Prevents Life-threatening Peanut-induced Anaphylactic Reactions. *J Allergy Clin Immunol.* 2009 Aug 124 (2): 307–14. Epub 2009 May 5.

This study was completed between June 2006 – July 2008. As the lead author I designed and performed all of the experiments, analyzed all data and wrote the manuscript. M. Baig (undergraduate student), D.K. Chu (graduate student), T. Walker (technologist) and S. Goncharova (technologist) provided technical assistance with the experiments. M. Colangelo (graduate student) developed the mathematical model. A.J. Coyle (MedImmune collaborator), P. Vadas (University of Toronto collaborator) and S. Waserman (McMaster University collaborator) provided helpful scientific input and critically appraised the manuscript.

CHAPTER 4

Arias K, Chu DK, Flader K, Botelho F, Walker T, Arias N, Humbles AA, Coyle AJ, Oettgen HC, Chang HD, Van Rooijen N, Waserman S, Jordana M. Distinct Immune-effector Pathways Contribute to the Full Expression of Peanut-induced Anaphylactic Reactions in Mice. *J Allergy Clin Immunol.* 2011 Jun 127(6): 1552-1561.

This study was conducted between the period of April 2008 – June 2010. As the principal author I designed and performed all of the experiments, analyzed the data and wrote the manuscript. D.K. Chu (graduate student), K. Flader (graduate student), N. Arias (undergraduate student) and T. Walker (technologist) provided me with technical assistance. F. Botelho (post-doctoral fellow) provided expert scientific input. A.A. Humbles (MedImmune collaborator) and A.J. Coyle (MedImmune collaborator), H.C. Oettgen (Harvard University collaborator), HD Chang (Deutsche Rheuma-Forschungszentrum Institute collaborator), N. Van Rooijen (Vrije Universiteit collaborator) provided us with different mutant mouse strains and/or reagents, contributed

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— Chapter 1 —

INTRODUCTION

The past few decades have witnessed the emergence of food allergy as a significant health problem in so-called 'westernized' regions such as North America, the United Kingdom and Western Europe [1, 2]. This is a result of the dramatic increase in the prevalence of this disease and the severity of the reactions that it elicits. Recent survey data from the Center for Disease Control and Prevention indicate that the current prevalence of food allergy in US children is approximately 4%, an increase of almost 20% over the last decade [3]. The increased prevalence in food allergy parallels the greater number of emergency department (ED) visits and hospitalizations for food-induced anaphylaxis over the last ten years [4, 5]. Food allergic reactions account for one-third to one-half of anaphylaxis cases treated in EDs worldwide [4, 6, 7]; hospitalizations have increased by 350% during the last decade [6, 8]. Studies on peanut allergy in the US and United Kingdom indicate that the number of children affected has doubled in the past decade, with a prevalence now exceeding 1% in school-aged children [9, 10]. A similar prevalence rate was reported in a population-based study recently conducted in Canada [11]. In contrast to other food allergies, peanut allergy is a long-lasting allergy that resolves in only 20% of children by 8 years of age [12]. Among the foods associated with anaphylaxis, peanuts and tree nuts account for approximately 80% of fatal and near-fatal anaphylactic episodes [13]. At the present time, there are no available treatments for peanut allergy other than epinephrine to rescue patients undergoing anaphylaxis. Intervention remains limited to strict allergen avoidance. In addition, current diagnostic testing for food allergy cannot predict a patient's risk for anaphylaxis or determine a subject's threshold dose to trigger symptoms [14, 15]. Therefore, affected subjects and families must be constantly vigilant to

avoid inadvertent exposure; unfortunately, accidental ingestion is a major concern. It has been reported that about 60% of children with peanut allergy experience an accidental peanut exposure within a 5-year period. [16] Remarkably, at least 87% of patients experiencing food-related fatalities (90% triggered by peanut) had a prior history of a reaction to the responsible food allergen [13]. Thus, the need for diligent research into the underlying mechanisms of and therapeutic approaches for peanut allergy is imperative.

Food Allergy As an Immune/allergic Reaction

The gastrointestinal tract is continuously exposed to a myriad of food antigens towards which the default response is tolerance[1]. It is thought that the failure to induce tolerance or the loss of oral tolerance results in food allergy. From an immune perspective, food allergic responses encompass two phases: i) sensitization and ii) elicitation. *Sensitization* (or inductive phase) refers to the immunological process whereby an allergic immune response (i.e. food-specific immunoglobulins) develops following first antigen (Ag) encounter. The *elicitation* (or *effector*) phase is initiated upon allergen re-exposure and is characterized by clinical symptoms that can range from mild to severe and, in some cases anaphylaxis, a severe and potentially life-threatening allergic reaction.

Sensitization (inductive phase): The prevailing notion is that sensitization occurs once dietary Ags contact the intestinal surface. They are sampled and processed by dendritic

[1] A state of specific and intended immunological hyporesponsiveness

cells (DCs) in the lamina propria and carried through the lymph to the draining lymph nodes (i.e., mesenteric lymph nodes) where DCs present allergen peptides to T and B cells[17, 18]. Recognition of peptides by these cells, in conjunction with additional costimulatory signals from DCs and the cytokine environment, triggers T/B cell activation, differentiation and proliferation. For T cells, this typically involves acquisition of a type-2 phenotype [19]. T helper-2 cells aid B cells to undergo immunoglobulin isotype switching by producing IL-4 and IL-13, thereby facilitating B cell production of allergen-specific IgE. Circulating IgE quickly binds to the high affinity receptor, FcεRI, on the surface of mast cells and engages an immune response when that particular food allergen is reencountered [20]. The mechanisms driving food sensitization and the development of allergic immunity, as opposed to oral tolerance, are at this time poorly understood.

Elicitation (effector phase): Once sensitized, elicitation of the allergic response occurs upon subsequent food allergen exposure. Food allergen binding to, and cross-linking of surface IgE triggers mast cell activation and the release of various granules-associated [including vasoactive amines (e.g., histamine and serotonin), proteases and some cytokines] as well as newly generated molecules [i.e., leukotrienes, prostaglandins and PAF] [21, 22]. These mediators act on target cells such as endothelial cells to increase vascular permeability, and smooth muscle cells to induce bronchial constriction and intestinal peristalsis. The combined effect of these events leads to the clinical hallmarks of food allergy, the most severe being anaphylaxis [23, 24]. Skin and mucosal tissues are affected in about 80-90% of the cases, with manifestations including urticaria and/or

angioedema. The smooth muscle of the lower airway, the stomach and the intestine are often involved, resulting in wheezing/dyspnea and abdominal cramps/vomiting, respectively. Notably, increased vascular permeability may allow for the rapid transfer of a large volume of intravascular fluid (50%) into the extravascular space, hence leading to rapid hemodynamic collapse [23]. These responses can occur within minutes (acute) to hours (protracted) and, in some cases, follow a biphasic course after allergen ingestion [25-27]. In comparison with our understanding of the events underlying allergic sensitization much less is known about the effector components leading to anaphylactic reactions to peanut.

Central Aim

Activation of mast cells via IgE has long been envisioned as the typical effector pathway leading to the clinical hallmarks of anaphylaxis in humans. However, compelling evidence in rodents and humans argues for the existence of alternative pathways of anaphylaxis. The central aim of this thesis was to investigate effector pathways leading to peanut-induced anaphylaxis (PIA). With this in mind, we first developed a mouse model that mimicked immunological and physiological features of peanut allergy in humans (Chapter 2). This model allowed us to investigate the roles of histamine, leukotrienes and PAF in PIA and, importantly, evaluate potential therapeutic approaches for this condition (Chapter 3). Lastly, we delineated immune-effector pathways contributing to PIA (Chapter 4).

The remaining sections of the introduction are structured to provide a thorough overview of the immune components thought to mediate systemic anaphylaxis. As animal models of systemic anaphylaxis are a fundamental component of the work presented in the subsequent chapters, a brief critical appraisal of the models most relevant to this thesis is discussed immediately below.

Modelling Systemic Anaphylaxis

The ultimate goal is to understand and treat human anaphylaxis. However, performing clinical investigations, including randomized controlled trials, in anaphylaxis is a formidable task. In particular reference to food-induced anaphylaxis, studies entail intentional oral challenges that must be conducted at a medical facility with expert medical supervision and appropriate medicines and devices on hand. Unfortunately, there are inherent limitations to the oral challenge. A major concern is the unpredictable nature of the reaction, involving sudden onset, multisystem symptoms and nonspecific signs which vary in chronology, severity and duration from one person to another, and in the same person from one anaphylaxis episode to another [15, 28, 29]. In some cases, patients refuse to continue the oral challenge because of fear and anxiety. This likely explains why most anaphylaxis research in humans has been confined to *ex vivo*/*in vitro*- and observational-based investigations. Undoubtedly, these studies have furthered our knowledge of factors involved in anaphylaxis and aided in its diagnosis/prognosis. Nonetheless, their nature does not permit an in depth dissection of the cellular and molecular processes mediating the anaphylactic response. In this regard, animal models

that mimic physiologic and immunologic features of human anaphylaxis enable researchers to conduct detailed mechanistic studies that are simply not feasible in humans.

Models of Ag-Induced Systemic Anaphylaxis

Systemic anaphylaxis has long been induced in animals, mostly mice, by means of either passive or active sensitization. **P**assive **S**ystemic **A**naphylaxis (**PSA**) involves the i.v. administration of Ag-specific mAbs (i.e. IgG_1 or IgE) followed by an i.v. challenge with the same Ag. Most models of PSA use anti-trinitrophenyl (TNP) as a surrogate allergen [30-34]. In models of **A**ctive **S**ystemic **A**naphylaxis (**ASA**), sensitization is achieved by the intraperitoneal injection of either i) chicken egg ovalbumin (OVA)[31, 32], bovine serum albumin (BSA) [35, 36] or penicillin V (PenV) [33, 37] along with an adjuvant [e.g. aluminum potassium sulphate (alum) and/or *Bordetella Pertussi* toxin], or ii) goat anti-mouse IgD alone [38]. Mice are subsequently challenged with the relevant Ag intravenously. In both cases, Ag challenge leads to clinical manifestations including cardiovascular compromise, respiratory distress, hypothermia and, in some cases, death. While both strategies lead to anaphylaxis, models of ASA are associated with many *natural* processes that are not mimicked in mice passively sensitized with antibodies including elevated levels of Ag-specific immunoglobulins of different isotypes, increased number of various effector cells and Fc receptor expression on these cells as well as in elevated concentrations of immunomodulatory cytokines.

Models of Peanut Allergy/Anaphylaxis

An ideal goal of food allergy researchers has been to develop adjuvant-free animal models of allergic sensitization that are responsive to oral re-challenge with allergen, resulting in immediate hypersensitivity reactions representative of human disease [39]. However, a major challenge to this goal is that the normal immune response to Ags encountered via the GI tract is tolerance [40]. Over the last decade, researchers have taken several approaches to break oral tolerance, most of which can be extrapolated to the human condition, including the use of mucosal adjuvants and manipulation of the epithelial barrier function by different means. For example, cholera toxin (CT) is a potent mucosal adjuvant that abrogates oral tolerance to coadministered Ags [41]. For example, Snider *et al* [42] showed that oral administration of hen egg lysozyme, or OVA, along with CT resulted in Ag-specific immunoglobulin production, and immediate hypersensitivity reactions including respiratory distress, edema and, in some cases, death following *systemic* challenge with Ag. Li *et al*. [43] subsequently modified this protocol to develop a model of peanut allergy in which mice are sensitized and challenged through the oral route. They showed that multiple feeds of ground peanut plus CT elicited antigen-specific Th2-polarized immunity. Anaphylaxis was assessed following oral challenge with peanut using a clinical scoring system. Of note, this oral challenge involved 10 mg of ground peanut per mouse, which would be equivalent to a challenge in a human with 90 peanuts [44]. Importantly, several investigators including our group have not been able to reliably evaluate clinical responses following these experimental conditions. Interestingly, objective measurements (i.e. mild-to-severe drop in core body temperature) required the

use of extraordinarily large amounts of Ag. In fact, the same group reported a few years later oral challenges using 200 mg of ground peanut (i.e. 20 times more than the amount initially utilized) [45, 46].

A few years ago, we developed an experimental mouse model of peanut-induced anaphylaxis that established allergic sensitization through the oral route and anaphylaxis by systemic exposure to peanut [47]. We demonstrated that four weekly oral gavages of peanut protein with CT elicited local and systemic markers of type-2 immunity that was associated with robust and consistent clinical anaphylaxis following intraperitoneal challenge with peanut. In particular, anaphylaxis was characterized by severe clinical signs (i.e. pronounced hypothermia), marked vascular leakage and extensive mast cell degranulation. This model development encompasses the first body of work presented in this thesis (Chapter 2), and was instrumental to the second and third bodies presented in Chapters 3-4.

Pathways of Anaphylaxis

As previously mentioned, anaphylaxis is typically referred to as an allergic reaction mediated by vasoactive mediators released in response to Ag cross-linking of IgE bound to FcεRI on mast cells. However, anaphylaxis has also been reported in the absence of detectable allergen-specific IgE in serum and mast cell degranulation. For instance, allergen-specific IgG instead of IgE has been observed in individuals who manifested systemic anaphylaxis against various triggers such as protamine [48], infliximab

(chimeric mouse/human anti-TNF mAb) [49], insect stings [50] and dextran contained in vaccines or high-molecular weight iron-dextran formulations [51]. Of particular interest, a study evaluating the effect of anti-IgE therapy in patients with peanut allergy reported that the treatment increased the threshold of sensitivity to peanut from a level equal to approximately half a peanut to one equal to almost nine peanuts. This occurred in about 50 to 75% of patients treated with the highest dose of Ab; however, the treatment was completely ineffective in 25% of the patients. The partial and/or negligible responses to this therapy foretell the existence of IgE-independent pathways of anaphylaxis to peanut [52]. Moreover, a number of studies have failed to detect elevated levels of tryptase, a reliable marker of mast cell degranulation, in individuals going through fatal and near fatal food-induced anaphylaxis [53, 54].

In the mouse, two distinct pathways leading to anaphylaxis were initially proposed [38]. One, referred to as the classical pathway, involves activation of mast cells sensitized by IgE bound to FcεRI. This pathway is thought to be mediated largely by histamine and, to a lesser extent, platelet-activating factor (PAF). Similarly, anaphylaxis can be induced via an alternative pathway. This pathway is mediated by macrophages, IgG and the activating low-affinity IgG receptor FcγRIII. In this case, PAF, rather than histamine, is thought to be the main contributor to the anaphylactic reaction. More recent data strongly implicated basophils as a major cell type leading to IgG_1-mediated anaphylactic responses via PAF in a model of PenV-induced anaphylaxis [33].

While both pathways share many clinical features, there are some important differences. The involvement, intensity and/or kinetics of some of the pathophysiological changes induced via these pathways differ. For instance, mast cells are required for the development of the tachycardia response, may contribute to but are not essential for decreases in lung function, and are dispensable for the development of hypotension or death [55, 56]. Conversely, some of the cardiopulmonary changes, as well as the mortality associated with anaphylaxis appear to be mediated by IgG_1 antibodies and FcγRIII [31]. Given the implications of these observations to our understanding of how anaphylaxis occurs and, consequently, how it may be treated, a detailed examination of the evidence pertaining the contribution of these, as well as more recently described pathways to anaphylaxis is warranted.

FcεRI and Fcγ Receptors

FcεRI is a cell-surface receptor that binds the Fc fragment of IgE with high affinity. In its complete tetrameric form, FcεRI consists of a single FcεRIα chain, a single β chain, and two disulfide-linked γ chains. The FcεRI α chain binds the Fc portion of IgE, whereas the β chain promotes the assembly and cell surface expression of the FcεRI and helps to amplify signal transduction through the FcεRI receptor's two γ chains (main and indispensable FcεRI signaling units) [57]. The central role of FcεRI in mediating anaphylaxis was investigated in mice with a targeted disruption of the gene encoding the FcεRI α chain (FcεRI α chain –/– mice). These mice failed to undergo IgE-dependent PSA [58]. However, this finding was later challenged by studies using models of ASA to

OVA. Indeed, mice with a homozygous null mutation of the Cε gene (IgE –/– mice) displayed anaphylactic responses (i.e. tachycardia, pulmonary function changes and increases in vascular permeability) similar to those observed in WT mice [59]. Furthermore, fatal ASA was observed in FcεRI α chain –/– mice, but not in mice that lack the common γ chain of the FcεRI and the FcγRI/FcγRIII (FcR γ –/– mice) [30], suggesting that other Ig/Fc receptor pathway/s may contribute to systemic anaphylaxis.

To date, four different classes of Fc receptors for IgG, known as FcγRI, FcγRIIB, FcγRIII and FcγRIV, have been identified in mice. Proteins corresponding to the mouse FcγRs have been identified in humans. Three of these Fcγ receptors (i.e. FcγRI, FcγRIII and FcγRIV) share the same γ chain as the FcεRI [60]. Functionally, there are two different classes of Fc receptors: activating and inhibitory. The paired expression of activating and inhibitory receptors on the same cell is key for the generation of a *balanced* immune response [61]. Additionally, it has been increasingly appreciated that Fcγ receptors show significant differences in their affinity for individual antibody isotype, rendering certain isotypes more strictly regulated than others. For example, FcγRIII is the only activating Fc receptor that binds IgG_1-immune complexes (IgG-IC); IgG_{2a} and IgG_{2b}, despite able to bind FcγRI (in the case of IgG_{2a}) or FcγRIII (in the case of IgG_{2a} and IgG_{2b}), are functionally dependent on FcγRIV. These same principles also apply for humans, where it has been shown that human FcγRIIIA has a higher affinity for IgG_1 as compared to FcγRIIA [61].

The involvement of Fcγ receptors in anaphylaxis has been investigated mostly through the use of mice with targeted mutations. Initial observations indicated that sensitization to OVA resulted in the production of antigen-specific IgE as well as IgG_1 antibodies [62]. Subsequent studies demonstrated that passively administered IgG_1 antibodies were sufficient to induce anaphylaxis [63]. More recent investigations showed that IgG_1 and FcγRIII, rather than IgE and FcεRI, are critical for most of the physiological changes, as well as the death, associated with active anaphylaxis to OVA. In this regard, Miyajima *et al* [31] reported that, unlike mice that had a selective deficiency of FcεRI, mice that lacked the FcRγ subunit were profoundly hampered in their ability to express active anaphylaxis or IgE– and IgG_1–dependent PSA. However, it is relevant to note that while FcRγ chain –/– mice completely failed to express active anaphylaxis to OVA challenge and exhibited neither mortality nor pulmonary changes in response to challenge with IgG_1 antibodies and specific Ag, IgG_1-sensitized FcRγ chain –/– mice did express significant tachycardia following Ag challenge. In addition, these mice manifested levels of mast cell degranulation that were indistinguishable from those in the IgG_1-sensitized and antigen-challenged FcRγ chain +/+ control group, suggesting that additional effector mechanisms may contribute to these responses. Thus, these findings indicate that sensitization with IgG_1 followed by challenge with the relevant Ag induces mast cell degranulation and tachycardia; yet, all the cardiopulmonary changes, and the mortality, associated with IgE-dependent PSA or ASA require adequate signaling through the FcRγ chain. Further studies using FcγRIIIα chain –/– mice provided evidence that FcγRIII, not FcγRI, is critical for the expression of these responses [30].

In addition to regulating IgG-mediated responses, Fcγ receptors have also been shown to modulate IgE-mediated responses (i.e. IgE-mediated PSA) [34]. Indeed, mice deficient in the inhibitory receptor for IgG, FcγRIIB, display enhanced IgE-mediated anaphylactic responses, whereas mice deficient in the IgG activating receptor FcγRIII display attenuated IgE-dependent responses. These observations led investigators to propose that the IgE immune complex–mediated response represents the sum of three components, i.e. an FcεRI-mediated major positive factor, an FcγRIIB negative response, and an FcγRIII-mediated positive component, respectively. Thus, Fcγ, particularly FcγRII and III, receptors play important roles in the modulation of IgE-mediated responses.

Effector Cells

Mast cells have long been thought to be central effector cells in anaphylaxis. This view was established through experiments in mice with spontaneous genetic mutations that perturb mast cell differentiation. Mast cells at all stages of maturation express the receptor tyrosine kinase Kit (CD117). Most laboratory strains deficient in mast cells arise from mutations affecting the Kit gene [64]. For example, the WBB6F1-$Kit^{W}/Kit^{W\text{-}v}$ (W/W^{v}) mouse bears a compound mutation (one allele null, the other impaired) at the Kit locus W (white spotting), while the $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ (W^{sh}) mouse carries an incompletely characterized inversion upstream of Kit that affects a key regulatory element [65-67].

The major role of mast cells in anaphylaxis was first described in a model of anti-IgE-induced anaphylaxis [56]. Specifically, intravenous infusion of goat anti-mouse IgE to

mice induced extensive degranulation of tracheobronchial mast cells, as well as significant elevation of heart rate, significant reductions in pulmonary mechanics parameters (i.e. dynamic compliance and lung conductance), and, in some cases, death. In contrast, W/W^v mice exhibited little or no pathophysiological responses and no mortality after anti-IgE treatment. Moreover, W/W^v mice reconstituted with bone marrow-derived mast cells from congenic +/+ mice (+/+ BM→ W/W^v mice), manifested similar cardiopulmonary changes and mortality to those observed in WBB6F+/+ mice following anti-IgE infusion. These data strongly supported the concept that mast cells are a key effector component in IgE-dependent anaphylaxis. Shortly after, studies in W/W^v mice showed that mast cell degranulation contributes to rapid changes in pulmonary mechanics parameters and the rapid onset of tachycardia typically occurring during ASA [55].

The central role of mast cells in the classical pathway of anaphylaxis was demonstrated in a model of ASA. In this model, mice were immunized with goat anti-mouse IgD (GaMD) antibody, which activates B cells by cross-linking their mIgD. These activated B cells process and present goat IgG (GIgG) to GIgG peptide–specific $CD4^+$ T cells, which respond by secreting cytokines that induce mastocytosis and helping B cells to mount massive IgG_1 and IgE responses [68]. Approximately 10 to 20% of this antibody response is IgG specific [69]. The classical pathway was demonstrated in GaMD-immunized mice by challenging them 14 days after immunization (when serum IgE levels had returned to baseline but IgE remained bound to mast cell FcεRI) with a mAb that efficiently cross-links IgE. Anti-IgE mAb–induced anaphylaxis was dependent on IgE,

FcεRI, and mast cells [38].

Anaphylaxis induced by challenging GaMD-primed mice with Ag (GIgG) elicited a response that was indistinguishable in its kinetics, severity, target organ involvement, and challenge dose requirement from that induced by anti-IgE mAb. However, it differed remarkably in its physiologic mechanism as it was IgE, FcεRI, and mast cell *independent*. These findings clearly illustrate that mast cells are not the sole effector cells in ASA and suggest the existence of an alternative cellular pathway. There is indeed substantial evidence that cells other than mast cells can bind IgG_1 and elaborate sufficient mediators to provoke fatal anaphylactic reactions [31, 33]. In GaMD-primed mice, Ag-induced anaphylaxis was primarily mediated through macrophages and FcγRIII. Anaphylactic responses were nearly abrogated by pre-treating mice with anti-FcγRII/RIII mAb or inhibiting macrophage function [38]. This is in agreement with early studies showing that ASA to OVA occurs largely via FcγRIII-expressing cells [30, 31]. In these studies, FcγRIII-dependent responses were more severe in normal than in congenic W/W^v mice, clearly indicating that mast cells may enhance the intensity of these responses.

Seemingly at variance, findings in a model of PenV-induced anaphylaxis prompted investigators to propose basophils as the pre-eminent FcγRIII-expressing cell type mediating IgG-dependent anaphylaxis [33]. Specifically, the data showed that IgG_1-mediated PenV-induced PSA, although present in mast cell-deficient mice, was nearly abrogated in basophil-depleted mice. However, it is relevant to point out that PenV-

induced fatal ASA was not prevented in mast cell-deficient mice or basophil-depleted WT mice clearly suggesting that neither mast cells nor basophils are indispensable for these responses. Furthermore, ASA-induced decreases in body temperature (a hallmark of anaphylaxis in mice) still occurred in the absence of both cell types, although mortality was abolished. Although these findings denote the contribution of basophils and mast cells to PenV-induced ASA, they clearly suggest the involvement of other cell type(s) in these responses. Collectively, these data challenge the dichotomous model of immune pathways leading to systemic anaphylaxis. Within this framework, we have been particularly interested in delineating the effector pathways leading to anaphylactic reactions to, specifically, peanut. In chapter 2 and 4, we examined the relative contribution of different effector immune components to peanut-induced anaphylaxis.

Effector Molecules: Biomarkers vs. Mediators

The current thinking is that the molecules released following activation of effector cells via Fc receptors play a causative role in anaphylaxis. However, this view requires a few considerations. This paradigm has an empiric, rather than evidential, basis. Indeed, the actual relationship(s) between *most* of these molecules and anaphylaxis is yet to be formally examined. As previously discussed, ethical issues associated with studying anaphylaxis in humans constrain the investigation of the role of these molecules to the pathogenesis of this condition. Thus, these studies provide largely correlative data that considerably limits their significance. In this regard, chapter 3 provides data on the roles of histamine, leukotrienes and PAF in PIA.

In the early 20th century, histamine was considered the primary molecule involved in anaphylaxis. Since then a number of other molecules have been implicated through observations made in human studies, *in vitr*o stimulation assays and animal models. Thus far, these include those released from mast cell and basophil storage vesicles (serotonin, tryptase, and chymase); newly synthesized cytokines (TNF- α, IL-1, IL-4, IL-5, IL-6, IL-9, IL-13) and lipid-derived mediators (platelet activating factor [PAF], prostaglandin D2 [PGD2], leukotriene [16] B4, cysteinyl leukotrienes [CysLT] LTC4, LTD4, and LTE4); complement breakdown products and products of eosinophil activation [70, 71].

Histamine. The detection of mast cell–derived mediators, particularly histamine (and tryptase) in humans was initially thought to provide confirmatory evidence of an anaphylactic episode [72, 73]. In this regard, Lin *et al*. [53] reported that increased levels of histamine (>10 nmol/L) correlated with clinical signs (such as urticaria, extensive erythema and abdominal symptoms) of acute allergic reactions. Nonetheless, this was observed in less than 50% of the subjects (97 patients). This may be explained by two likely complementary explanations: 1) the 'snapshot' nature of this study may have missed the interval of detection of histamine in plasma, and 2) additional (mast cell- or non mast cell-derived) molecules significantly contribute to these reactions. Experimentally, we have shown that concurrent blockade of H_1 and H_2 receptors did not have a significant impact on the extent and/or kinetics of anaphylactic reactions to peanut, thus suggesting that, indeed, other molecules may play a critical role in the pathogenesis

of these reactions (Chapter 3).

Lipid Mediators. Baseline levels of lipid mediators (or byproducts) have been reported to increase following an anaphylactic reaction. The production of these lipids is initiated by the release of arachidonic acid (AA) after the hydrolysis of membrane phospholipids by phospholipase A2 and other enzymes. The byproducts of the reaction include lysophospholipids (Lyso-PL), some of which might be further metabolized to PAF. Unmetabolized AA and Lyso-PL can become reincorporated into the phospholipid pool following re-acylation reactions. AA can then be processed in a variety of ways. It can be metabolized by the 5-lipoxygenase [5-LO] pathway enzyme to the intermediate LTA4, which is unstable and is rapidly converted into LTB4 or into the CysLT: LTC4, LTD4 and LTE4 [74, 75]. Thus, inhibitors of the 5-LO enzyme can prevent the synthesis of all LT.

CysLT are produced by many cells including mast cells, basophils and eosinophils. Historically, they were initially referred to as the principal biological slow-reacting substances of anaphylaxis (SRS-A), because of their ability to induce the gradual progression of the contractile response of smooth muscle, ultimately resulting in bronchoconstriction [76]. Urinary LTE4 measurements have been successfully used to monitor LTC4 production *in vivo*, particularly following anaphylaxis [77, 78]. While the findings insinuate a causative role for CysLT in this response, direct evidence is lacking. In fact, we propose that leukotrienes serve as important clinical biomarkers rather than

mediators of the anaphylactic event. This line of thought is further discussed in Chapter 3.

PAF is a phospholipid involved in many physiological and pathological conditions. It is synthetized by a variety of hematopoietic cells including mast cells, basophils, platelets and monocytes/macrophages. PAF acts through a unique G protein-coupled seven transmembrane receptor (PAFR) that is expressed in the plasma and nuclear membranes of leukocytes, endothelial cells and platelets. By binding to its receptor, PAF mediates cellular responses including Ca^{2+} mobilization, platelet aggregation and vasodilatation [79, 80]. Aside from its role as a physiological mediator, PAF has been associated with several inflammatory events including increase of vascular permeability and lung edema, and leukocyte recruitment [79, 81, 82]. Indeed, blockade of the PAFR has been shown to decrease edema formation and/or leukocyte recruitment in several models of inflammation [83-85].

PAF has also been implicated in the pathogenesis of anaphylactic shock. In fact, PAF is known to contribute to hypotension and cardiac dysfunction during hemorrhagic, traumatic or septic shock [79, 86]. Moreover, administration of PAF to mice can lead to bronchoconstriction, hypotension and increased vascular permeability causing pulmonary edema and impaired cardiac and renal function [82, 85]. On the other hand, PAF-R deficiency and administration of PAF-R antagonists can prevent PAF-induced lethal anaphylaxis in animal models [86, 87]. Importantly, Vadas *et al* [88] have recently highlighted PAF as a marker of severity in human anaphylaxis triggered by different allergens

including foods (i.e. peanut). They noted that PAF levels increased from 4% in the control groups to 20% in the group with grade 1 (acute allergic reaction with cutaneous manifestations but no other organ system involvement) anaphylaxis, 71% in the group with grade 2 (mild-to-moderate manifestations) anaphylaxis, and 100% in the group with grade 3 (severe manifestations, with cutaneous, gastrointestinal, and potentially life-threatening respiratory or cardiovascular signs) anaphylaxis. Moreover, the authors reported an inverse correlation between PAF levels and PAF-AH, the enzyme that degrades PAF. Interestingly, serum PAF-AH activity was significantly lower in patients with fatal peanut anaphylaxis than in control patients. Thus, these findings propose a firm association between PAF levels and the severity of anaphylaxis. Nonetheless, it should be noted that causality was not ascertained in this study. In this regard, as part of our interest to investigate effector molecules in PIA, we have examined the role of PAF in the severity of peanut-induced anaphylactic reactions (Chapter 3).

Objectives

The research presented in Chapters 2, 3 and 4 advances our knowledge of the immune-effector components and pathways that mediate anaphylactic reactions to peanut and explore potential therapeutic approaches for PIA.

In the first manuscript in this thesis (Chapter 2), we report on the development and characterization of a mouse model of peanut-induced anaphylaxis. To this end, we evaluated the impact of the number of administrations of peanut along with CT on the

induction of local and systemic markers of peanut-specific adaptive immunity, including Th2-associated cytokine and immunoglobulin production. We subsequently examined whether the number of oral sensitizations correlated with the severity of the anaphylactic reactions following systemic challenge with peanut. We assessed anaphylaxis based on the following clinical and biological parameters: presence of clinical signs (i.e. drop in core body temperature), release of mast cell-associated molecules, increases in vascular permeability and induction of late-phase inflammatory responses. Lastly, we investigated the impact of immunoglobulins, FcεRI and mast cells on PIA.

In the second manuscript (Chapter 3) we used this model to evaluate the role of histamine, leukotrienes and PAF in PIA. This project was partly based on our previous observations that histamine and leukotriene levels are significantly increased in mice undergoing anaphylaxis. On the other hand, a large body of literature attested to PAF's involvement in several hallmarks of anaphylaxis. The investigation proceeded along four lines. Separate groups of peanut-sensitized mice were treated with either a (A) 5-LO inhibitor, (B) a PAF-R antagonist, (C) histamine receptor antagonists or (D) a PAF-R antagonist along with histamine receptor antagonists prior to peanut challenge. To compare the efficacy of these interventions, we developed a mathematical model that allowed us to comprehensively grade the severity of the anaphylactic responses.

The third manuscript builds on the work presented in Chapter 1. Here, we examined the relative contribution of different immune-effector pathways to PIA. We

investigate the hypothesis that distinct pathways cooperate to the full expression of the clinical manifestations of anaphylaxis. To this end, we used three separate approaches either individually or in combination. These include mice with spontaneous and targeted mutations (i.e. W/W^v, W^{sh}, IgE– and IgG–deficient mice), cellular depletion strategies (i.e. basophil-depleting mAb and liposomes clodronate) and an anti-FcγRIII mAb. When applicable, preliminary experiments were conducted in order to establish optimal protocol regimens. The impact of these approaches on the anaphylactic reactions to peanut was assessed mostly as described in Chapter 1. In an extension of this work, evaluation of hematocrit was incorporated as an indicator of increased vascular permeability.

— Chapter 2 —

Impact of CD40 Ligand, B Cells, and Mast Cells in Peanut-Induced Anaphylactic Responses

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See *Preface* for details regarding authorship

Summary and Central Message: This article focuses on the development of a mouse model that mimics physiologic and immunologic features of peanut allergy in humans. This model allowed us to investigate the impact of immunoglobulins, the IgE high affinity receptor, FcεRI and mast cells on PIA. We found that four weekly oral gavages of peanut protein with cholera toxin elicited local and systemic markers of type-2 immunity that was associated with robust and consistent clinical anaphylaxis following intraperitoneal challenge with peanut. In particular, anaphylaxis was characterized by severe clinical signs (i.e. pronounced hypothermia), marked vascular leakage, extensive mast cell degranulation and significant late-phase allergic responses. Furthermore, we showed that while CD40 ligand-, B cell- or mast cell-deficient mice did not exhibit any measurable anaphylaxis, FcεRI-deficient mice manifested partial anaphylactic responses. Thus, these data demonstrate the critical contribution of immunoglobulins and mast cells to PIA and underscore the involvement of other Ig/Fc pathways in this process.

Impact of CD40 Ligand, B Cells, and Mast Cells in Peanut-Induced Anaphylactic Responses[1]

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The effector immune mechanisms underlying peanut-induced anaphylaxis remain to be fully elucidated. We investigated the relative contribution of Igs, mast cells (MCs), and FcεRI in the elicitation of anaphylaxis in a murine model. Assessment of peanut hypersensitivity reactions was performed clinically and biologically. Our data show that wild-type (WT; C57BL/6 strain) mice consistently developed severe anaphylaxis (median clinical score: 3.5/5), an ~8°C drop in core body temperature, and significantly increased plasma levels of histamine and leukotrienes. CD40 ligand- and B cell-deficient mice presented evidence of allergic sensitization as demonstrated by production of Th2-associated cytokines by splenocytes and a late-phase inflammatory response that were both indistinguishable to those detected in WT mice. However, CD40 ligand- and B cell-deficient mice did not exhibit any evidence of anaphylaxis. Our data also show that MC-deficient ($Kit^{W}/Kit^{W\text{-}v}$) mice did not suffer, unlike their littermate controls, anaphylactic reactions despite the fact that serum levels of peanut-specific Igs were similarly elevated. Finally, FcεRI-deficient mice experienced anaphylactic responses although to a significantly lesser degree than those observed in WT mice. Thus, these data demonstrate that the presence of peanut-specific Abs along with functional MCs comprise a necessary and sufficient condition for the elicitation of peanut-induced anaphylaxis. That the absence of FcεRI prevented the development of anaphylaxis only partially insinuates the contribution of an IgE-independent pathway, and suggests that strategies to impair MC degranulation may be necessary to improve the efficacy of anti-IgE therapy. *The Journal of Immunology,* **2007, 179: 6696–6703.**

Anaphylaxis is an acute, life-threatening, allergic reaction. Food hypersensitivity is the leading cause of anaphylactic episodes treated in hospital emergency departments in western countries (1–3). Food allergy affects 6–8% of children under 4 years of age and ~2% of the U.S. population beyond the first decade of life (4–6). It is estimated that allergy to peanut and tree nut accounts for ~80% of the fatal and near-fatal food hypersensitivity reactions (7, 8). Of concern, the prevalence of childhood peanut allergy (PA)[4] has doubled between 1997 and 2002, and although most food allergies are outgrown, peanut hypersensitivity is usually lifelong (9, 10). At this time, there are no proven effective therapies for PA and, unlike other allergic diseases, immunotherapy strategies are presently not available.

The recent development of murine models of peanut-induced anaphylaxis (PIA) that mimic physiologic and immunologic features of human PA has facilitated both understanding of the underlying mechanisms and evaluation of potential therapeutic approaches (11–13). An important focus of these models has been on the role of Th2 responses in the regulation of sensitization (14–16) and, subsequently, therapeutic strategies to redirect immune responses away from Th2 (17, 18). Comparatively, studies investigating effector events that mediate systemic anaphylaxis have received less attention.

We have developed a murine model of PIA and investigated the involvement of key immunological components in the elicitation of peanut hypersensitivity reactions. Our data show that oral sensitization to peanut in C57BL/6 mice generated peanut-specific Th1- and Th2-associated Igs and cytokines and provoked Th2-effector responses, namely systemic anaphylactic shock and late-phase allergic responses upon in vivo peanut recall. Examination of secondary lymphoid organs provided evidence of pervasive immune activation that was associated with the development of late-phase respiratory and cutaneous responses upon airway and skin re-exposure to peanut, respectively. In addition, our data demonstrate that CD40 ligand (CD40L)-, B cell- or mast cell (MC)-deficient mice failed to mount measurable anaphylactic responses, indicating that the presence of peanut-specific Igs along with functional MCs is necessary and sufficient for the elicitation of a peanut-induced anaphylactic event.

Materials and Methods

Animals

Female C57BL/6 mice (6–8 wk old) were purchased from Charles River Laboratories. B cell-deficient (B6.129S2-*Igh-6*^{tm1Cgn}/J), CD40L-deficient (B6.129S2-*CD40lg*^{tm1Imx}/J), MC-deficient (WBB6F1/J-$kit^{W}/Kit^{W\text{-}v}$), and

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[4] Abbreviations used in this paper: PA, peanut allergy; PIA, peanut-induced anaphylaxis; CD40L, CD40 ligand; PP, peanut protein; PL, peritoneal lavage; CPE, crude peanut extract; CT, cholera toxin; LN, lymph node; BAL, bronchoalveolar lavage; GI, gastrointestinal; i.d., intradermal; i.n., intranasal; DIG, digoxigenin; DC, dendritic cell.

Table I. *Anaphylactic symptom score table*

Score	Symptoms
0	No clinical symptoms
1	Repetitive mouth/ear scratching and ear canal digging with hind legs
2	Decreased activity; self isolation; puffiness around eyes and/or mouth
3	Periods of motionless for more than 1 min; lying prone on stomach
4	No response to whisker stimuli; reduced or no response to prodding
5	Endpoint: tremor; convulsion; death

FcεRI α-chain-deficient mice (C.129S2-*Fcer1a*^{tm1Knt}/J), along with the age-matched congenic normal mice, were purchased from The Jackson Laboratory. The mice were housed under a specific pathogen-free environment and maintained on a 12-h light-dark cycle. All experiments described in this study were approved by the Animal Research Ethics Board of McMaster University.

Model of PIA

Sensitization protocol. Mice were sensitized with oral gavage. To this end, mice were held in the supine position and orally administered 1 mg of peanut protein along with 10 μg of cholera toxin (CT; List Biological Laboratories) in 100 μl of sterile water by gavage once a week for 4 wk. Oral gavages were performed using intragastric feeding needles (01-290–2B; Fisher Scientific). We used peanut butter (Kraft) as a source of peanut protein (PP). In an initial series of experiments, four separate groups of mice were gavaged once a week for 1, 2, 3, or 4 wk.

Intraperitoneal challenge. Sensitized mice were challenged by injecting 5 mg of crude peanut extract (Greer Laboratories) in 500 μl of PBS into the peritoneal cavity 2 wk after the last sensitization. The mice were carefully observed for 40 min immediately following challenge. Anaphylactic responses during this lapse of time were assessed as follows:

1) Symptom score. Clinical symptoms were evaluated using the scoring system shown in Table I.

2) Core body temperature. Rectal temperature readings were performed every 10 min with a rectal probe digital thermometer (VWR).

3) Vascular permeability. Vascular leakage was determined by measuring albumin levels in the peritoneal lavage (PL) fluid 40 min after challenge. Briefly, the peritoneal cavity of anesthetized mice was injected with 3 ml of PBS, 10 mM EDTA. After a 1-min abdominal massage, a small incision was made on the peritoneal membrane and the PL fluid was gently aspirated. The aspirate was centrifuged at 600 rpm for 6 min at 4°C and the supernatant was collected and frozen at −70°C until used. The content of albumin was quantified in the supernatants using a commercial Bicinchoninic Acid kit (Sigma-Aldrich). Albumin levels of naive mice challenged with the same amount of crude peanut extract (CPE) were used as background levels. The concentration of albumin was calculated by comparison with a BSA standard curve.

4) MC mediator release. Histamine and leukotriene levels were determined in the plasma 40 min following challenge using enzyme immunoassay kits (Beckman Coulter and Cedarlane Laboratories, respectively) according to the manufacturer's specifications. Plasma was obtained using K_2-EDTA-containing tubes (BD Biosciences).

Intranasal challenge. Sensitized mice were lightly anesthetized and a 10-μl suspension containing 500 μg of CPE was delivered intranasally once daily for 5 consecutive days. Mice were sacrificed 2 days after the last challenge, and bronchoalveolar lavage (BAL) was performed as previously described (19). Briefly, the lungs were dissected and the trachea was cannulated with a polyethylene tube (BD Biosciences). The lungs were lavaged twice with PBS (0.25 ml, followed by 0.2 ml); ~0.3 ml of the instilled fluid was consistently recovered. Total cell counts were determined in a blinded manner using a hemocytometer. The cell pellet was resuspended in PBS, and smears were prepared by cytocentrifugation (Thermo Shandon) at 300 rpm for 2 min. BAL smears were stained with the Protocol Hema 3 stain set (Fisher Scientific). Differential cell counts of BAL smears were determined in a blinded manner from at least 500 leukocytes using standard hemocytological criteria to classify the cells as neutrophils, eosinophils, or mononuclear cells. When applicable, lung tissue was fixed in 10% formalin and embedded in paraffin. Sections, 3-μm thick, were stained with H&E.

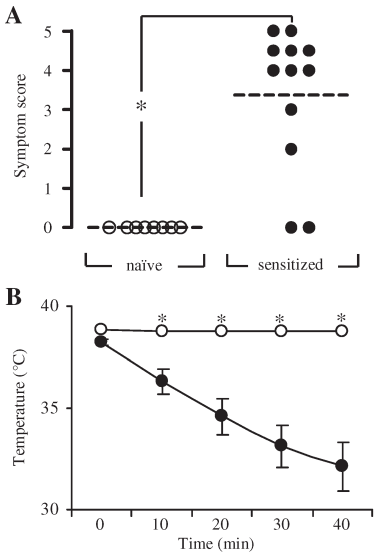

FIGURE 1. Induction of systemic anaphylaxis in orally sensitized mice following i.p. challenge with CPE. Mice were sensitized with peanut protein along with CT by intragastric gavage once a week for 4 wk. Two weeks after the last sensitization, mice were challenged with CPE. *A*, Peak anaphylactic symptom score of each individual mouse from naive (○) and sensitized (●) groups within 40 min after challenge. *B*, Changes in rectal temperature (mean ± SEM of naive and sensitized groups) following i.p. challenge at the indicated time points. Data are representative of three independent experiments. *, $p < 0.05$ compared with naive controls.

Late-phase peritoneal and cutaneous responses to CPE

Peritoneal responses. Late-phase responses were evaluated in the peritoneal cavity from 40 min to up to 72 h after challenge. Briefly, PL fluid was collected and total cell counts were determined using a hemocytometer. Peritoneal cell smears were prepared by cytocentrifugation (Thermo Shandon) at 300 rpm for 2 min and stained with the protocol Hema 3 stain set (Fisher Scientific). Differential cell counts were performed in a blinded manner from at least 500 leukocytes using standard hemocytological criteria to classify the cells as neutrophils, eosinophils, MCs, and mononuclear cells.

Cutaneous responses. Late-phase responses were induced by intradermal (i.d.) injection with 10 μg of CPE in 10 μl of sterile saline into one ear, and 10 μg of control Ag (chicken egg albumin, OVA) into the opposite ear. Ear thickness was measured before and at several time points after injection using a pocket thickness gauge (Mitutoyo) equipped with a preloaded spindle to provide a constant closing force of <0.2 Newtons. Mice were sacrificed 48 h later, and the ears were fixed in 10% formalin, sectioned, and stained with H&E for microscopic examination.

Measurement of Ag-specific Igs

Peripheral blood was collected by retro-orbital bleeding 1 wk after the last sensitization. Serum peanut-specific IgG1 and IgG2a were measured by ELISA. Briefly, 96-well plate Maxi-Sorp plates (Nunc; VWR) were coated with 2 μg/ml CPE or with purified rat anti-mouse IgE Ab (BD Pharmingen) in 50 nM carbonate-bicarbonate buffer (pH 9.6; Sigma-Aldrich) overnight at 4°C.

Coated plates were blocked with 1% BSA/PBS for 2 h at room temperature. Plates were washed and serum samples were incubated overnight at 4°C. Each test sample was diluted as follows: for IgG1 detection 1/20, 1/200, 1/2,000, and 1/20,000 and for IgG2a detection 1/2, 1/20, 1/100, and 1/500. The reactions were developed with biotinylated goat anti-mouse IgG1, IgG2a, and IgE (Southern Biotechnology Associates), respectively, for 2 h at room temperature. Plates were washed and incubated with alkaline-phosphatase streptavidin (Sigma-Aldrich) for 1 h at room temperature. The color reaction was developed with *p*-nitrophenyl phosphate tablets and stopped with 2 M H_2SO_4. Ab titers were calculated using the formula $1/(x \times \text{ODX}) \times 0.05$, where x equals the dilution factor closest to but

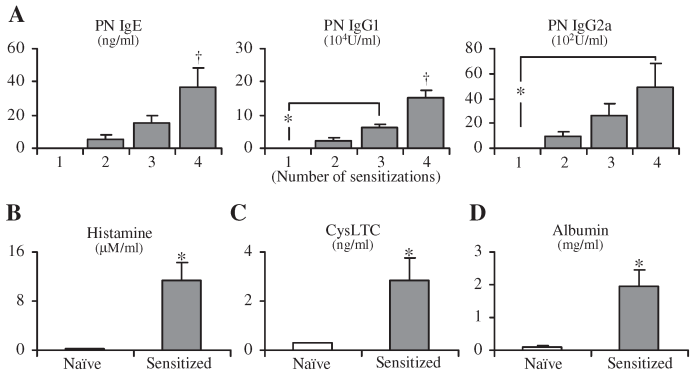

FIGURE 2. CPE-induced anaphylaxis is preceded by a polyisotypic peanut-specific Ig response and accompanied by release of biological markers of anaphylaxis. *A*, Impact of the number of sensitizations on the production of peanut-specific Igs. Four groups of mice were sensitized by oral route once, twice, three and four times, respectively. Figures show levels of peanut-specific IgE, IgG1, and IgG2a in serum samples collected 1 wk after the last sensitization. †, $p < 0.05$ compared with all other groups; ∗, $p < 0.05$ between the two linked groups. Histamine (*B*), Cys-leukotrienes (*C*) in plasma and albumin (*D*) in the peritoneal lavage fluid of naive and sensitized mice 40 min following CPE challenge. ∗, $p < 0.05$ compared with naive controls. Data are expressed as mean ± SEM; $n = 8–12$ mice/group. Results are representative of three independent experiments.

greater than twice the background OD reading, and ODX is the OD reading of *x*. Sample Ig levels were expressed as units per milliliter. Serum peanut-specific IgE was measured by sandwich ELISA. Briefly, 96-well plate Maxi-Sorp plates (Nunc; VWR Canlab) were coated with 2 μg/ml purified rat anti-mouse IgE Ab (BD Pharmingen) in PBB overnight at 4°C. Coated plates were washed and blocked with 10% normal serum 1% BSA/PBS/0.5% Tween 20 for 1 h at 37°C. After washing, serum samples (four serial dilutions) were incubated for 2–3 h at room temperature. Each test sample was diluted as follows: 1/10, 1/50, 1/250, and 1/500. Subsequently, a CPE-digoxigenin (DIG) conjugate solution was added (1 h at 37°C). The coupling of DIG to CPE was performed according to the manufacturer's instructions (Roche). After incubation (1 h at 37°C) with peroxidase-conjugated anti-DIG fragments (Roche), a tetramethylbenzidine substrate (0.1 mg/ml) solution was used and the color reaction was stopped with 2 M H_2SO_4. Absorbance was measured at 450 nm and results expressed as nanograms of DIG-CPE bound times the dilution factor.

Splenocyte and lymph node (LN) cell cultures

Spleens or LNs (inguinal, mediastinal, and mesenteric) were excised from naive and sensitized mice 1 wk after the last sensitization and placed into sterile tubes containing sterile HBSS on ice. Spleens/LNs were triturated between the ends of sterile frosted slides and the resulting cell suspension was filtered through a 70-μm nylon cell strainer (Falcon; BD Biosciences), then washed at 1200 rpm for 10 min at 4°C. RBC were lysed from spleens by adding 1 ml of ACK lysis buffer (0.5 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 nM Na_2EDTA (pH 7.2–7.4)) for 1 min. Splenocytes and dispersed LN cells were washed once with HBSS and then resuspended in RPMI 1640 medium supplemented with 10% FBS (Sigma-Aldrich), 1% L-glutamine, 1% penicillin/streptomycin (Invitrogen Life Technologies), and 0.1% 2-ME (Invitrogen Life Technologies). Cells were seeded at 8×10^5cells/well (spleen) or 5×10^5 cells/well (LNs) in a flat-bottom, 96-well plate (BD Biosciences) and cultured in medium alone or with 50 μg/ml CPE/well. Following 120 h of culture incubation, supernatants were harvested and stored at −20°C for cytokine measurement.

Cytokine measurements

Levels of IL-4, IL-5, IL-10, IL-13, and IFN-γ were determined using Beadlyte mouse multicytokine fluorescent bead-based FLEX assays (Upstate Biotechnology) as previously described, and quantified using a Luminex 100 instrument according to the manufacturer's instructions. In some instances, cytokine levels were determined using ELISA kits for murine IL-4, IL-5, and IL-13 purchased from R&D Systems. Each of these essays has a threshold of detection between 1.5 and 5 pg/ml.

Data analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using SigmaStat software (SPSS). When applicable, results were analyzed using one- or two-way ANOVA with repeated measures followed by Tukey post-hoc test. In cases of raw data failing normality test, the ANOVA test on ranking was performed followed by pairwise comparisons using Dunn's methods. An unpaired Student *t* test (two tailed) was used when only two sets of data were compared. A *p* value of <0.05 was considered statistically significant.

Results

Induction of acute systemic anaphylaxis by peanut challenge in sensitized wild-type (WT) mice

To assess the development of systemic anaphylaxis, clinical and biological responses were carefully monitored in sensitized and challenged mice. We observed clinical symptoms within a few minutes after challenge (Fig. 1*A*). Starting with continuous muzzle scratching and ear canal digging with hind legs (score 1/5, see Table I), symptoms rapidly progressed to puffiness around eyes and/or mouth and self-isolation (score 2/5). Most mice experienced prolonged periods of motionless that gradually worsened to no response to whisker stimuli and only slight reaction to prodding (average score 3.5/5). The most severe reactions provoked loss of consciousness, tremor, and death. The kinetics and severity of the anaphylactic reactions were consistent among mice. Naive mice did not develop any clinical symptoms upon CPE challenge. As shown in Fig. 1, core body temperature readings were inversely correlated with symptom scores. Sensitized mice, but not naive

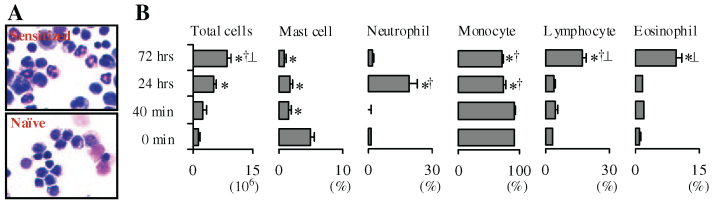

FIGURE 3. Intraperitoneal CPE challenge evokes a late-phase inflammatory response in the peritoneal cavity of orally sensitized mice. *A*, Representative photomicrographs of slide-mounted inflammatory cells collected from the peritoneal lavage fluid of naive and sensitized mice 72 h following i.p. challenge and stained with H&E. *B*, Total inflammatory and differential cell counts at the indicated time points following i.p. challenge. Data represent mean ± SEM; $n = 4–6$ mice/group. ∗, †, and ⊥, $p < 0.05$ compared with 0 min, 40 min, and 24 h, respectively. Results are representative of three independent experiments.

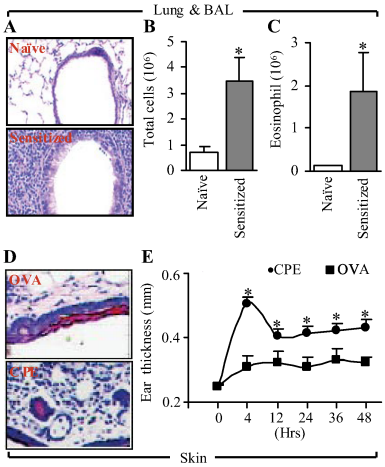

FIGURE 4. Respiratory and cutaneous exposure to CPE leads to allergic inflammatory responses in the lungs and skin of orally sensitized mice, respectively. *A*, Representative light photomicrographs of lung tissues following intranasal challenge with CPE for 5 consecutive days. Total inflammatory cell (*B*) and eosinophil (*C*) counts in BAL 2 days following the last i.n. challenge. *D*, Representative light photomicrographs of ear skin 48 h following i.d. injection with OVA into one ear and CPE into the opposite ear. *E*, Changes in ear thickness measured before and within 48 h following OVA or CPE injection. Histological tissue sections were fixed in 10% formalin and stained with H&E. *, $p < 0.05$ compared with naive. Results are representative of two independent experiments.

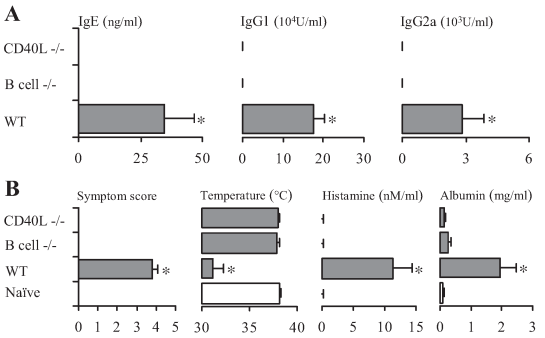

FIGURE 6. B cell- and CD40L-deficient mice fail to develop peanut-induced anaphylactic responses. *A*, The graph depicts serum levels of peanut-specific IgE, IgG1, and IgG2a from CD40L-deficient (CD40L$^{-/-}$), B cell-deficient (B cell $^{-/-}$), and WT mice 2 wk after the fourth oral sensitization *B*, Peak anaphylactic symptom score, rectal temperature, plasma histamine, and albumin content in the peritoneal cavity of naive (□) and sensitized (■) mice 40 min following i.p. CPE challenge. Igs were measured by ELISA. Data represent mean ± SEM; $n = 4–6$ mice/group, and representative of two independent experiments. *, $p < 0.05$ compared with all groups.

controls, experienced a marked drop (up to 9°C) in body temperature during the 40-min observation period following i.p. challenge (Fig. 1*B*). Mice sensitized to peanut but challenged with saline did not manifest any anaphylactic reactions (data not shown).

To investigate the impact of oral sensitization on humoral responses, we evaluated serum peanut-specific Igs generated in response to increased number of oral sensitizations. To this end, we sensitized four groups of mice with one, two, three or four oral administrations of PP and CT. Serum samples were obtained by retro-orbital bleeding one day before i.p. challenge with CPE. We observed that repeated delivery of PP and CT significantly increased the levels of peanut-specific IgE, IgG1, and IgG2a with the highest Ig titers achieved following four oral gavages (Fig. 2*A*). Importantly, the number of oral sensitizations and, thus, the level of peanut-specific Igs closely correlated with the severity of the anaphylactic reactions. Peanut-specific Abs were not detected in naive mice.

The development of clinical signs and symptoms of anaphylaxis was accompanied by a marked increase in biological markers of anaphylaxis. We detected significantly greater histamine (~50-fold increase) and Cys Leukotriene C (LTC) (~10-fold increase) levels in the plasma of peanut-sensitized mice following CPE challenge compared with naive mice (Fig. 2, *B* and *C*). Moreover, increased vascular permeability was demonstrated by a near 20-fold increase in the content of albumin in the PL fluid (Fig. 2*D*) of sensitized challenged mice compared with naive challenged mice.

Late-phase inflammatory responses at the site of immunological challenge

Next, we examined late-phase cellular responses in the peritoneal cavity at various time points following i.p. challenge. We observed a marked and time-dependent cellular inflammatory response in the PL fluid of sensitized, but not naive, mice following CPE challenge (Fig. 3). Peak cellular infiltration occurred 72 h after challenge, with a near 8-fold increase in total cell number compared with naive mice. As shown in Fig. 3*B*, inflammation was initially characterized by a considerable, albeit transient, accumulation of neutrophils (before challenge: 0.2 ± 0.2% vs 24 h after challenge: 25 ± 2%) followed by a marked increase in the proportion of both lymphocytes (before challenge: 3 ± 1% vs 72 h after challenge: 18 ± 2%) and eosinophils (before challenge: 0.4 ± 0.2% vs 72 h after challenge: 12 ± 2%). The increase in the proportion of inflammatory cells consistently correlated with the increase in the overall number of cells in the peritoneal cavity (data not shown).

Impact of intranasal and cutaneous Ag challenge, in sensitized mice

We next investigated whether oral sensitization would also give rise to allergic inflammatory responses in distant compartments (i.e., lungs and skin) upon Ag re-exposure in vivo. Specifically, we examined the impact of respiratory and cutaneous exposure to CPE

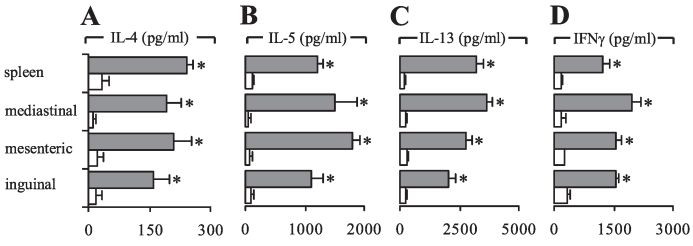

FIGURE 5. Oral peanut sensitization induces peanut-specific Th1- and Th2-associated cytokines. Spleens and GI- and non-GI draining LNs of naive (□) and sensitized (■) mice were harvested 2 wk after the last oral sensitization and stimulated with CPE for 120 h. The supernatants were assessed for IL-4 (*A*), IL-5 (*B*), IL-13 (*C*), and IFN-γ (*D*) by multicytokine Luminex assay. Data represent mean ± SEM; $n = 4–6$ mice/group, and representative of two independent experiments.

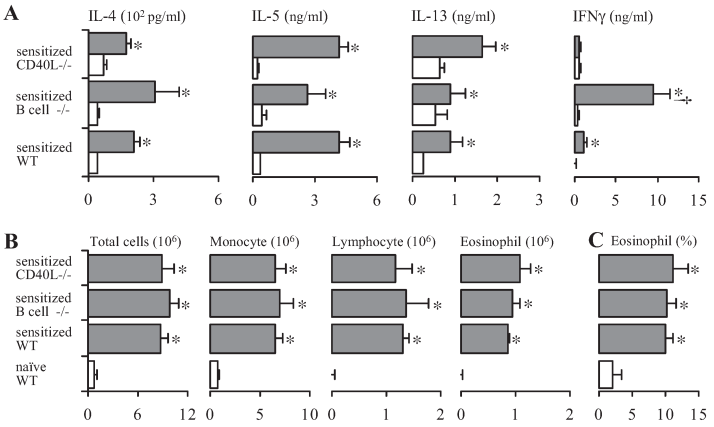

FIGURE 7. CD40L and B cell deficiencies do not impair peanut-specific cellular responses. *A*, Splenocytes from CD40L-deficient (CD40L$^{-/-}$), B cell-deficient (B cell $^{-/-}$), and WT mice were harvested 1 wk following the fourth oral sensitization and stimulated for 120 h with CPE or medium alone in vitro. The graphs depict peanut-specific production of cytokines (IL-4, IL-5, IL-13, and IFN-γ) measured in the supernatants of cultures stimulated with medium alone (□) or CPE (■). *, $p < 0.05$ compared with medium alone; †, $p < 0.01$ compared with all other groups. *B*, Number of total inflammatory cells, monocytes, lymphocytes, and eosinophils in the peritoneal cavities of (■) sensitized CD40L $^{-/-}$, B cell $^{-/-}$, and WT and (□) naive mice 72 h after i.p. CPE challenge. *C*, Percentage of eosinophils among total peritoneal inflammatory cells. Data represent mean ± SEM; $n = 4–6$/group, and are representative of two independent experiments; *, $p <$ 0.05 compared with naive mice.

following oral peanut sensitization. To this end, mice were sensitized to peanut via oral gavage and exposed to CPE intranasally once daily for 5 consecutive days. Histological analysis of lung tissues demonstrated that intranasal (i.n.) challenge of orally sensitized mice resulted in marked peribronchial and perivascular inflammation (Fig. 4*A*) that was accompanied by pronounced eosinophil infiltration, goblet cell hyperplasia, and mucus production (data not shown). Analysis of the airway inflammatory infiltrate revealed an ~4-fold increase in total BAL cells compared with naive mice, with a predominant increase in eosinophils (Fig. 4, *B* and *C*). In a separate series of experiments, mice were sensitized orally and 1 wk later injected i.d. with CPE or control Ag (OVA) into the right and left ears, respectively. CPE, but not OVA, injection led to a significant late-phase cutaneous response as assessed by an increase in ear thickness at 4 h compared with baseline, and OVA control mice (Fig. 4*E*). Histological examination of ear tissue 48 h following i.d. CPE, but not OVA, injection revealed a pronounced mononuclear and eosinophilic infiltrate (Fig. 4*D*). Importantly, mice challenged with CPE, but not OVA, experienced prolonged recovery time from anesthesia and a marked drop in core body temperature (up to 8°C, data not shown).

To evaluate peanut-specific markers of systemic sensitization, we harvested various secondary lymphoid organs before challenge and measured cytokine production in the supernatant of CPE-stimulated cultures. Our data show a considerable increase in the production of IL-4, IL-5, IL-13, and IFN-γ from splenocytes and gastrointestinal (GI)-draining LN cells obtained from sensitized mice compared with naive controls (Fig. 5). Interestingly, a similar cytokine profile was induced following in vitro CPE stimulation of non-GI-draining mediastinal and inguinal LN cells (Fig. 5).

Impact of CD40L and B cell deficiencies on PIA

To evaluate the role of humoral immunity in the elicitation of PIA, we used a system where Ig responses are impaired due to selective gene-targeted mutations, namely CD40L- and B cell-deficient mice. First, we ascertained that both genetically deficient mice were unable to produce peanut-specific Igs (Fig. 6*A*). Importantly, when challenged with CPE, these mice failed to show any overt indication of anaphylaxis (Fig. 6*B*). To address the impact of CD40L and B cell deficiencies on the induction of peanut-specific cellular responses, we harvested splenocytes from sensitized WT, CD40L$^{-/-}$, and B cell$^{-/-}$ mice and subsequently measured the production of peanut-specific cytokines following in vitro stimulation with either CPE or medium alone. We found that oral sensitization resulted in similar production of IL-4, IL-5, and IL-13 by CPE-stimulated splenocytes from both transgenic and WT mice (Fig. 7*A*). Of interest, CPE stimulation led to significantly higher IFN-γ production in splenocytes cultures from B cell$^{-/-}$ mice compared with WT or CD40L$^{-/-}$ mice. Thus, sensitized CD40L$^{-/-}$ and B cell$^{-/-}$ mice, albeit clinically unresponsive to i.p. CPE challenge, still exhibited clear evidence of peanut sensitization. Furthermore, the late-phase inflammatory response in the peritoneal

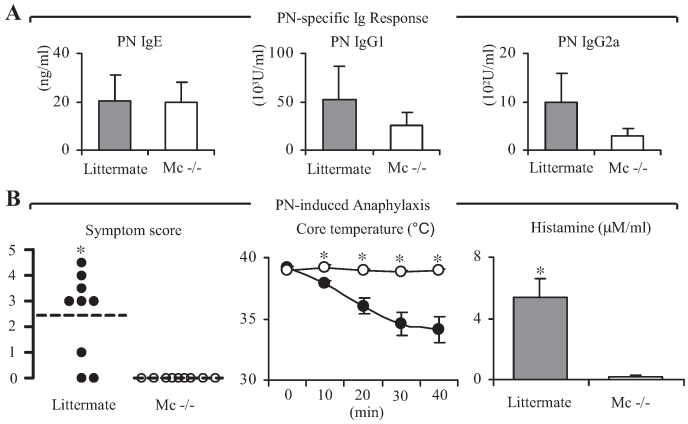

FIGURE 8. MC deficiency abrogates PIA. *A*, The graphs depict serum levels of peanut-specific IgE, IgG1, and IgG2a from MC-deficient (Mc$^{-/-}$, □) and littermate control (■) mice following four oral sensitizations. *B*, Peak anaphylactic symptom score, rectal temperature, and plasma histamine in MC $^{-/-}$ and littermate control mice 40 min following i.p. CPE challenge. Data represent mean ± SEM; $n = 9$ mice/group, and are representative of two independent experiments; *, $p < 0.05$ compared with littermate control mice.

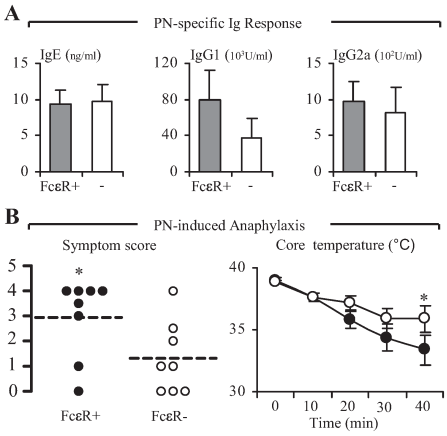

FIGURE 9. FcεRI deficiency partially disrupts PIA. *A*, The graph depicts serum levels of peanut-specific IgE, IgG1, and IgG2a from FcεRI-deficient ($FcεR^{-/-}$, □) and littermate control ($FcεR^{+/+}$, ■) mice 2 wk following four oral sensitizations. *B*, Peak anaphylactic symptom score and rectal temperature in FcεRI $^{+/+}$ and $FcεR^{-/-}$ mice 40 min following i.p. CPE challenge. Data show mean ± SEM and are representative of two independent experiments; $n = 8$ mice/group; *, $p < 0.05$ compared between the two groups.

cavity of $CD40L^{-/-}$ and B $cell^{-/-}$ mice was statistically indistinguishable to that observed in WT mice (Fig. 7*B*), with an ~8-fold increase in total cells compared with naive mice, and significantly higher eosinophils (~10%).

Impact of MC deficiency on PIA

That murine studies have previously reported the induction of active systemic anaphylaxis in the absence of MCs prompted us to investigate the role of MC activity as an effector mechanism in PIA. To this end, genetically MC-deficient (WBB6F1/J-Kit^{W}/$Kit^{W\text{-}v}$) mice, which are virtually devoid of tissue MCs, and the congenic normal (WBB6F1$^{+/+}$) mice were sensitized and challenged as per our standard protocol. As illustrated in Fig. 8, whereas littermate controls exhibited signs and symptoms of systemic anaphylaxis, MC-deficient mice experienced no measurable anaphylactic reactions. In addition, no histamine was detected in the plasma of Kit^{W}/$Kit^{W\text{-}v}$ mice. Importantly, we detected almost identical serum levels of peanut-specific IgE in both strains. The serum levels of peanut-specific IgG1 and IgG2a were statistically not different between MC-deficient and littermate control mice (Fig. 8*A*). Moreover, both strains showed indistinguishable late-phase inflammatory responses in the peritoneal cavity following peanut challenge (data not shown).

Impact of FcεRI deficiency on PIA

To investigate the relative contribution of the high-affinity IgE receptor, FcεRI, in PIA, we used mice with a targeted mutation that results in failure of production of the α-chain of the FcεRI. As shown in Fig. 9*A*, both FcεRI α-chain −/− mice and FcεRI α-chain +/+ mice (WT) controls produced identical levels of peanut-specific IgE following peanut oral sensitization. Despite the presence of peanut-specific Abs, clinical markers of systemic anaphylactic responses were partially abrogated in FcεRI α-chain −/− mice compared with those observed in FcεRI α-chain +/+ mice.

Discussion

The precise mechanisms underlying the pathogenesis of PIA remain to be fully elucidated. The objective of this study was to evaluate the relative contribution of humoral and MC responses in the elicitation of this event. To this end, we developed a murine model in C57BL/6 mice that involved oral sensitization with peanut Ag along with CT followed by an i.p. challenge with CPE. This protocol consistently evoked the rapid onset of severe anaphylactic responses as indicated by severe clinical symptoms, a significant drop in core body temperature, marked MC mediator release (i.e., histamine and leukotrienes) and pronounced vascular leakage. Moreover, we examined immune-inflammatory responses in the peritoneal cavity at various time points following CPE challenge. We observed a marked and time-dependent cellular inflammatory response that was initially characterized by a considerable, albeit transient, accumulation of neutrophils followed by a significant increase in the number of both lymphocytes and eosinophils. Of note, i.p. challenge resulted in a rapid and sustained decrease in the number of MCs detected within the peritoneal cavity. In this regard, Claman et al. (20) showed that immunological processes in chronic graft-vs-host disease cause a slow release of MC granules that leads to an apparent disappearance of dermal MCs, as assessed by toluidine blue staining. As this stain is a marker for mature MC granules, the authors suggested that the loss of granule substances may account for the failure to identify MCs by toluidine blue. Importantly, ultrastructural analysis demonstrated that MCs are present in graft-vs-host disease skin and showed granule depletion and expansion, the first steps in degranulation. These MCs, invisible by toluidine blue stain but visible with the electron microscope, were referred to as "phantom MCs" (20). We used H&E staining to evaluate inflammatory responses including MC responses. However, we confirmed mast numbers with toluidine blue staining. Thus, we suggest that a phenomenon similar to that proposed by Claman et al. (20) may very likely explain the apparent decrease in the number of MCs following peanut challenge.

It may be of significance to note that induction of systemic anaphylaxis in mice sensitized orally to peanut and subsequently challenged through the oral route has been previously reported (12); however, the responses documented are, compared with the responses that we observed, relatively modest and considerably more variable. Indeed, our data show that 80% of mice reached a clinical score of ~3.5 and a very robust ~8°C drop in core body temperature. We did perform a number of detailed experiments using previously reported protocols and explored a series of methodological variations including: an increase in the number of oral sensitizations to up to eight, executing two oral challenges rather than one (17, 21), and increasing gut permeability and absorption of Ag with ethanol (22) at the time of challenge. However, we were not able to elicit consistent anaphylactic reactions not only in C57BL/6 mice but also in C3H/HeJ mice. Thus, we currently do not have an explanation for our inability to generate systemic anaphylaxis in orally sensitized mice upon oral exposure to peanut. Despite this, the robustness and consistency of systemic anaphylactic responses that are elicited with i.p. challenge represent a notable experimental advantage. Moreover, because the peritoneal cavity is a closed anatomical compartment, it provides a most useful site for the evaluation of both immediate and late-phase biomarkers of anaphylactic responses. In this respect, the assessment of albumin and late-phase inflammatory responses after challenge that we furnish here illustrate this point. Of particular interest, that this model was established in C57BL/6 mice, the most commonly used background to breed gene-targeted mice, facilitates the use of knockout mice to decisively investigate the contribution of molecular pathways involved in peanut hypersensitivity.

In addition to investigating systemic anaphylaxis, we examined the consequences of re-exposing orally sensitized mice to peanut in compartments other than the peritoneum, namely lung and skin.

Our data demonstrate that CPE re-exposure via the respiratory mucosa elicited pronounced late-phase peribronchial and perivascular inflammation characterized by marked eosinophilic infiltration. Similarly, exposure to peanut in the skin by way of i.d. ear injection of CPE evoked late-phase cutaneous responses, as demonstrated by ear thickness with a concomitant influx of eosinophils into the ear tissue. The current understanding is that the initial site of Ag priming, i.e., the draining LNs, where dendritic cells (DCs) carrying Ag first interact with T cells, determines the future homing of memory/effector T cells and, hence, the site at which immune-inflammatory responses will manifest upon Ag re-exposure (23–27). This notion along with the observations that we made in the lung and skin prompted us to comprehensively seek for evidence of peanut-specific immunity in a variety of LNs. Interestingly, our data demonstrate production of peanut-specific cytokines not only in GI-draining (mesenteric) but also in distant (non-GI-draining such as inguinal and thoracic) LNs following in vitro peanut stimulation, suggesting that peanut oral sensitization is, under these experimental conditions, pervasive.

The presence of eosinophilia in the peritoneum, lung, and ear after peanut challenge of sensitized mice is a hallmark of Th2 immunity. Hence, we further investigated the peanut-specific effector profile generated following oral peanut sensitization. Our data show that in vitro peanut recall induced a similar cytokine profile in splenocyte, GI-, and non-GI-draining LN cultures, which was characterized by both Th1 (IFN-γ) and Th2 (IL-4, IL-5, IL-13) associated cytokines. Moreover, sensitization led to the production of both peanut-specific Th1 (IgG2a) and Th2 (IgE and IgG1) associated Igs. The detection of both Th1 and Th2 effector biomarkers following oral priming in the model presented here in C57BL/6 mice is in agreement with previous reports in BALB/c (16), C3H/HeJ (15), and C3H/HeOuJ (28) mice. Collectively, these findings intimate that there is not a linear relationship between the mere detection of Th1 and/or Th2 markers and the expression of Th1 and/or Th2 immunity (29–34).

To investigate the role of Igs in PIA, we performed experiments in mice unable to generate Igs, namely CD40L- and B cell-deficient mice, both of which have a C57BL/6 background. That these mice failed to mount any measurable anaphylactic responses demonstrates that Igs are critically required in this process. Importantly, the inability to elicit anaphylaxis cannot be attributed to defective sensitization. Indeed, splenocyte cultures from WT, CD40L$^{-/-}$, and B cell$^{-/-}$ mice produced similar levels of Th2-affiliated cytokines upon in vitro recall. Of note, peanut-specific IFN-γ was significantly elevated in B cell$^{-/-}$ mice compared with WT and/or CD40L$^{-/-}$ mice. This is in accordance with observations made in studies where B cell-deficient mice were infected with *Schistosoma mansoni* eggs (35). In particular, a study by Hernandez et al. (35) showed that LN cells from schistosome-infected B cell-deficient mice produce significantly more Th1-associated cytokines (IFN-γ and IL-12) than cells from control mice following in vitro stimulation with soluble schistosomal egg Ag. Similarly, irradiated splenocytes from B cell-deficient mice elicited stronger Th1 responses in schistosomal egg Ag-specific CD4$^+$ cells than splenocytes from normal mice (35). These findings may be explained by the ability of B cells to regulate the Th1/Th2-polarized effector function of DCs (36). In fact, it has been shown that increased production of IL-12 by DCs from B cell-deficient mice results in the induction of Th1-polarized responses (37). Further evidence of sensitization in CD40L$^{-/-}$ and B cell$^{-/-}$ mice is indicated by the development of a late-phase allergic inflammatory response, including eosinophilia, in the peritoneal cavity following peanut challenge that is comparable to that observed in WT mice. The generation of late-phase responses observed in CD40L$^{-/-}$ mice is in agreement with studies showing a comparable degree of pulmonary inflammation in presensitized CD40$^{-/-}$ and WT mice following i.n. challenge with OVA (38) and *Aspergillus fumigatus* Ag (39). Thus, our findings provide evidence of systemic sensitization in CD40L$^{-/-}$ and B cell$^{-/-}$ mice and suggest that while Th2-associated cytokines are an inherent marker of allergic sensitization, they are not directly required to elicit the anaphylactic event.

Presently, two distinct mechanisms have been identified to induce systemic anaphylaxis in the mouse (40). The classical pathway is mediated by Ag cross-linking of IgE bound to the high-affinity receptor (FcεRI) on MCs. Activation of MCs results in the rapid release of preformed granule-associated molecules such as histamine and serotonin, and newly synthesized lipid-derived mediators such as PGD_2, platelet-activating factor, and leukotrienes. The alternative pathway is thought to involve macrophages, FcγIII, IgG Abs, and platelet-activating factor (41). The finding that treatment of peanut allergic individuals with a humanized IgG1 mAb against the Fc portion of IgE increases the threshold of sensitivity only partially (42) suggests the potential contribution of both pathways in PIA. However, the relative contribution of the individual components involved in the elicitation of food-induced anaphylaxis, and specifically PIA, remains to be explored. To investigate the role of MCs in this process, we evaluated clinical and biological markers of systemic anaphylaxis in orally sensitized MC-deficient (WBB6F1/J-kit^W/Kit$^{W\text{-}v}$) mice following peanut challenge. Our data show that despite elevated levels of peanut-specific Igs, MC-deficient mice failed to develop peanut-induced anaphylactic responses, hence establishing a preeminent effector role for MCs in this experimental model. The absence of measurable anaphylactic responses in MC-deficient mice was very likely due to lack of MCs; however, this was not formally proven via reconstitution.

To investigate the relative contribution of FcεRI in this model, we evaluated clinical and biological markers of systemic anaphylaxis in orally sensitized FcεRI α-chain-deficient mice following peanut challenge. It is noteworthy that, in these mice, the genes encoding the common γ-chain of the FcεRI and the FcγRI/III are intact. Previous studies have demonstrated that the absence of FcεRI α-chain leads to up-regulation of FcγRIII-dependent MC degranulation (43). This is relevant to our findings because we observed that absence of the FcεRI α-chain did not entirely abrogate anaphylactic responses. It has been reported that mouse macrophages do not express FcεRI (40, 44, 45) and, in addition, there is evidence that both IgE and IgG1 can bind to the FcγRIII on MCs (46–49). Hence, the partial protection observed in FcεRI-deficient mice may suggest that the remaining anaphylactic response may be mediated via IgG1-dependent activation of FcγRIII on MCs. It is important to note, however, that the partial responses observed in FcεRI-deficient mice do not demonstrate that the FcγRIII pathway is important in the native animal. This notion may be of therapeutic relevance given that, in humans, the use of Abs that block IgE binding to FcεRI has been shown to be of clear but partial benefit to abrogate anaphylactic responses; strategies to additionally impair MC degranulation might be necessary to increase the efficacy of this therapy.

In short, our data provide direct experimental evidence that peanut-specific Igs along with functional MCs constitute the necessary and sufficient effector requirements for the elicitation of peanut-induced systemic anaphylaxis. In addition, our findings encourage further investigation into the role of FcRs as it may contribute to a better understanding of the mechanisms underlying PIA and, hence, to the design of novel therapeutic approaches.

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Disclosures

The authors have no financial conflict of interest.

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— Chapter 3 —

Concurrent blockade of platelet-activating factor and histamine prevents life-threatening peanut-induced anaphylactic reactions

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Summary and Central Message: This article is the first to investigate the relative contribution of effector molecules to the pathogenesis of food-induced anaphylaxis, particularly PIA. Specifically, we examined whether histamine, leukotrienes and PAF were associated with the presence, severity and/or persistence of anaphylactic responses to peanut. To this end, we evaluated the impact of pharmacological interventions targeting either metabolic pathways or mediator receptors in our mouse model of PIA. We found that treatment targeting either leukotrienes or histamine alone had negligible effects. In contrast, PAF antagonism significantly prevented severe and prolonged anaphylactic reactions. Interestingly, combination therapy targeting PAF and histamine receptors had a synergistic effect in the prevention of PIA. Thus, concomitant blockade of PAF and histamine may represent a *life-saving* therapy for peanut and, likely, other food-induced anaphylaxis as well as a "risk-reducing" approach that facilitates the implementation of other therapies.

Concurrent blockade of platelet-activating factor and histamine prevents life-threatening peanut-induced anaphylactic reactions

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Background: Food anaphylaxis is an acute and life-threatening systemic allergic reaction. Fatality registries place peanut as the most common culprit of fatal and near-fatal reactions in North America. Because prophylaxis and treatment have advanced little in recent years, it is imperative to evaluate novel therapies.
Objective: To investigate the impact of blocking mast cell mediators in a mouse model of peanut-induced anaphylaxis.
Methods: Mice were sensitized with peanut protein and cholera toxin via oral gavage weekly for 4 weeks. One week after the last sensitization, separate groups of mice were treated with either a (1) 5-lypoxygenase inhibitor, (2) a platelet-activating factor (PAF) receptor antagonist, (3) histamine receptor antagonists, or (4) a PAF receptor antagonist along with histamine receptor antagonists before peanut challenge.
Results: Treatment targeting either leukotrienes or histamine alone had no beneficial effects. In contrast, PAF antagonism significantly attenuated the magnitude and duration of the anaphylactic reactions. Particularly, it prevented severe reactions. Moreover, 83% of PAF-treated versus 43% of untreated mice reached recovery within 120 minutes after peanut challenge. Notably, combined blockade of PAF and histamine had a clearly greater beneficial effect. In fact, all but 1 mouse developed mild, if any, anaphylactic reactions. In addition, combination therapy was associated with a significant decrease in vascular leakage and release of vasoactive mediators after peanut challenge.
Conclusion: Combination therapy blocking both PAF and histamine markedly reduces the severity of peanut-induced anaphylaxis, and thus it may be a potential life-saving therapeutic approach in peanut and, likely, other food-induced anaphylaxis. (J Allergy Clin Immunol 2009;124:307-14.)

Key words: *Peanut allergy, anaphylaxis, mast cells, histamine, PAF, leukotrienes, mathematical modeling*

Immediate hypersensitivity reactions to foods account for one third to one half of anaphylaxis cases worldwide.[1,2] Anaphylaxis is an acute and life-threatening systemic allergic reaction, and food anaphylaxis fatality registries in the United States implicate peanuts as the major culprit of fatal reactions.[3] Recent studies in North America and the United Kingdom have reported that prevalence rates of peanut allergy (PA) among schoolchildren are currently higher than 1%.[4,5] Although other food allergies often resolve during the first years of life, peanut hypersensitivity usually persists.[6] Prophylaxis and treatment for PA have advanced little in recent years, and intervention remains limited to strict avoidance. However, accidental ingestion is common, with a reported annual incidence rate of 14.3% among schoolchildren in Montreal, Quebec, Canada.[7]

Mechanistically, anaphylaxis is a hypersensitivity reaction involving the release of mediators from mast cells (MCs) and basophils after allergen interaction with cell-bound IgE. Mediators include vasoactive amines (eg, histamine), proteases, and lipid-derived mediators such as leukotrienes, prostaglandins, and platelet-activating factor (PAF). It is thought that the extent of mediator release closely correlates with the severity and persistence of the anaphylactic reaction. However, the relative contribution of these mediators in the physiopathology of food-induced anaphylaxis is unknown.

Platelet-activating factor is a phospholipid secreted by MCs, monocytes, and fixed tissue macrophages.[8] By binding to a G-protein–coupled transmembrane receptor, PAF mediates cellular responses, including Ca^{2+} mobilization, platelet aggregation, and vasodilatation.[9,10] In addition to its role as a physiological mediator, PAF has been associated with the pathogenesis of anaphylactic shock. Indeed, administration of PAF to mice can lead to bronchoconstriction, hypotension, and increased vascular permeability, causing pulmonary edema and impaired cardiac and renal function. Importantly, PAF-R deficiency and administration of PAF-R antagonists can prevent PAF-induced lethal anaphylaxis in animal models.[10-12] However, the contribution of PAF to food-induced anaphylaxis remains to be elucidated. Of interest, a human study showed that the severity of peanut-induced anaphylaxis (PIA) correlates with the levels of PAF in serum.[13] Here, we examined the impact of pharmacologic interventions targeting either metabolic pathways or mediator receptors in an experimental mouse model of PIA. We observed that blockade

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Abbreviations used
5-LO: 5-Lipoxygenase
AU: Area under response
cysLT: Cysteinyl leukotriene
MC: Mast cell
PA: Peanut allergy
PAF: Platelet-activating factor
PAF-R: Platelet-activating factor receptor
PIA: Peanut-induced anaphylaxis
PL: Peritoneal lavage

of PAF activity significantly prevented prolonged and life-threatening PIA reactions. Interestingly, combination therapy targeting PAF and histamine receptors had a synergistic effect in the prevention of PIA.

METHODS

Animals

Female C57BL/6 mice (6-8 weeks old) were purchased from Charles River Laboratories. All experiments described in this study were approved by the Animal Research Ethics Board of McMaster University.

Grading of the severity of PIA responses

Mice were sensitized and challenged as previously described.[14] Mice were carefully monitored for either 40 or 120 minutes immediately after peanut challenge, and anaphylactic responses were evaluated as previously described.[14] To grade the severity of the anaphylactic response, we used a 3-point grading scheme. Accordingly, anaphylactic reactions were classified as *no reaction* (score 0 and 37.5°C $\leq$ T $\leq$ 40°C), *mild reaction* (score $\leq$2 and 34.5°C $\leq$ T $\leq$ 37.5°C), or *severe or life-threatening reaction* (score $\geq$3 and T $\leq$ 34.5°C).

Determination of vasoactive mediators

Histamine and leukotriene levels were determined in plasma by using enzyme immunoassay kits (Beckman Coulter Canada, Inc, Mississauga, and Cedarlane Laboratories Ltd, Hornby, Canada, respectively) according to the manufacturers' specifications.

Analysis of vascular leakage

Vascular permeability was determined by measuring albumin levels in the peritoneal lavage (PL) fluid as previously described.[14] Albumin content was quantified in the supernatants by using an enzyme immunoassay kit (Immunology Consultants Laboratory, Inc, Newberg, Ore) following the manufacturer's instructions.

Measurement of peanut-specific IgG_1 and IgE

Peripheral blood was collected 1 week after the last sensitization, and levels of serum peanut-specific IgG_1 and IgE were measured by using a previously described sandwich ELISA.[14]

Pharmacologic interventions

Inhibition of leukotrienes. Peanut-sensitized mice were administered a 5-lipoxygenase (5-LO) inhibitor, Zileuton (50 mg/kg; Cayman, Ann Arbor, Mich), twice, 24 hours and 1 hour before challenge orally. The control group received 0.5% hydroxyethyl cellulose (vehicle) in 100 μL saline at equivalent time points.

Blockade of PAF and histamine receptors. Peanut-sensitized mice were treated with a PAF receptor antagonist (50 mg/kg), ABT491 (Sigma, St Louis, Mo), in 100 μL PBS orally 1 hour before challenge. A separate group of sensitized mice were injected histamine receptor antagonists (mepyramine [3 mg/kg], an H1 receptor antagonist; and cimetidine [10 mg/kg], an H2 receptor antagonist; Cedarlane, Mississauga, Ontario, Canada) in 200 μL PBS intravenously 30 minutes before challenge. In some cases, treatments were administered concurrently.

Flow cytometry of peritoneal MCs

Peritoneal cells were harvested as previously described.[14] For cytometric analysis, the cells were preincubated with B3B4 and 2.4G2 mAbs for 10 minutes at 4°C.[15] The cells were then incubated with 10 μg/mL IgE mAb (Sigma, Oakville, Ontario, Canada) for 20 minutes at 4°C and subsequently washed and stained with anti-IgE (fluorescein isothiocyanate conjugated), anti–c-kit (phycoerythrinconjugated), and anti-CD11b (phycoerythrin-Cy7 conjugated) mAbs at 4°C for 30 minutes. All antibodies were purchased from BD PharMingen, Mississauga, Ontario, Canada. Data were collected by using a LSRII (BD Biosciences, San Jose, Calif) flow cytometer and analyzed by using FlowJo 6.4.2 software (Tree Star Inc, Ashland, Ore). Peritoneal MCs were identified as c-kit$^+$IgE$^+$ cells.

Statistical analysis

Statistical analysis was performed by using SIGMAStat (Systat Software Inc, San Jose, Calif). When applicable, results were analyzed using 1-way or 2-way ANOVA with repeated measures followed by the Tukey *post hoc* test. An unpaired Student *t* test (2-tailed) was used when only 2 sets of continuous data were compared. A P value <.05 was considered statistically significant.

RESULTS

PIA is characterized by the acute and release of vasoactive mediators

Oral sensitization with peanut protein and cholera toxin followed by an intraperitoneal challenge with crude peanut extract led to an acute and robust anaphylactic response as indicated by severe clinical symptoms and a significant drop in core body temperature (Fig 1, *A*). We previously demonstrated that the elicitation of PIA is mediated, to a large extent, by MCs, IgE, and FcεRI (IgE high-affinity receptor).[14] Here, we show that sensitization with peanut resulted in a 3-fold increase in the number of peritoneal c-kit$^+$FcεRI$^+$ cells (Fig 1, *B and C*) along with significant upregulation of FcεRI expression (Fig 1, *D*). Moreover, we detected significantly greater levels of histamine (Fig 1, *E*) and cysteinyl leukotrienes (cysLTs; Fig 1, *F*) in the plasma of peanut-sensitized mice after challenge compared with control mice. Interestingly, the kinetics of mediator production are distinct. Although histamine peaked early after challenge (10 minutes), remained substantially elevated for 1 hour, and slowly decreased over time, cysLT progressively increased on challenge, reaching maximum levels at 40 minutes and gradually declining within 2 to 3 hours after challenge (Fig 1, *E and F*). Together, these data suggest that sensitization with peanut results in IgE-mediated upregulation of MC FcεRI expression, ultimately decreasing the threshold for degranulation and, consequently, amplifying the release of vasoactive mediators.

Impact of leukotrienes and histamine blockade in PIA

To assess the role of leukotrienes in PIA, peanut-sensitized mice were administered a 5-LO inhibitor twice before challenge. As shown in Fig 2, *A and B*, blockade of this pathway did not

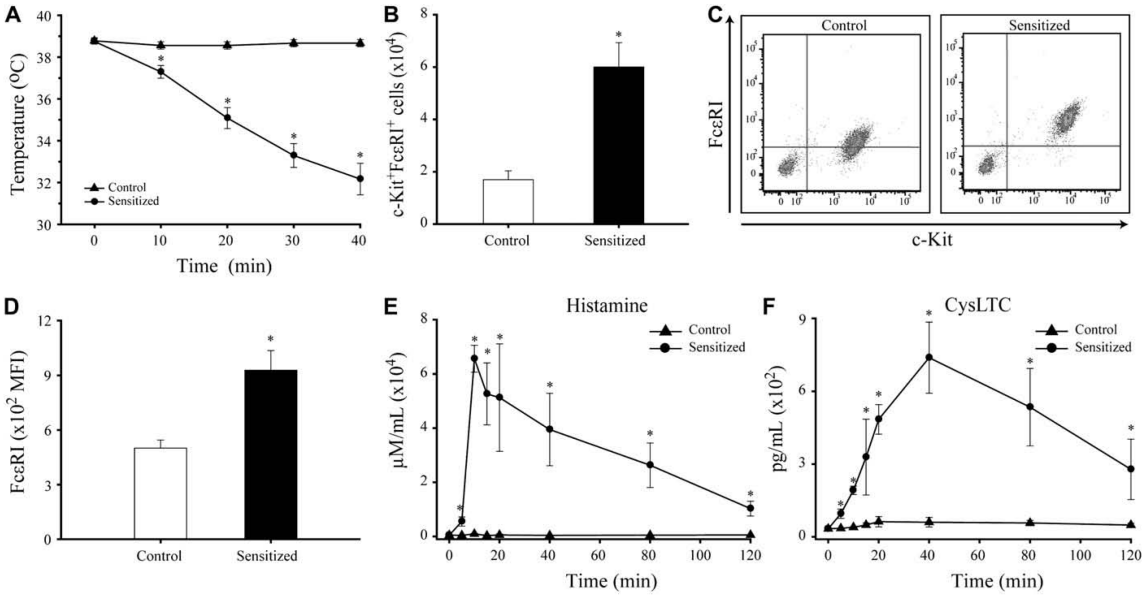


FIG 1. Peanut-induced anaphylaxis is preceded by upregulation of FcεRI on c-kit$^+$ cells and accompanied by release of mediators. **A,** Rectal temperature of control (▲) and sensitized (●) mice after challenge. **B,** FcεRI$^+$ and c-kit$^+$ peritoneal cells. **C,** Representative fluorescence-activated cell sorting plot. **D,** Mean fluorescence intensity *(MFI)* of FcεRI on c-kit$^+$ cells. **E,** Histamine levels. **F,** Leukotriene levels. Means ± SEMs from 3 independent experiments; n = 8 mice/group. *P < .05 relative to control.

affect the extent and/or kinetics of the anaphylactic reactions. Moreover, we observed comparable levels of histamine in plasma from untreated versus treated mice (Fig 2, *C*). Conversely, cysLTs were nearly undetected in plasma from treated, but not untreated, mice, indicating that pharmacologic inhibition of the 5-LO pathway was successful (Fig 2, *D*). In agreement with these findings, mice with a targeted disruption in the 5-LO gene manifested the same degree of anaphylaxis as their wild-type counterparts after challenge (data not shown).

To examine the role of histamine, peanut-sensitized mice were administered H1 and H2 receptor antagonists 30 minutes before challenge. Our data indicate that blocking H1 and H2 receptors did not change the severity or the course of the anaphylactic reaction (Fig 2, *E and F*). Moreover, we observed comparable levels of histamine and cysLT (Fig 2, *G and H*) in plasma between treated and untreated mice after challenge, suggesting that the extent of MC degranulation was equivalent in both groups. Thus, antihistamines, at least when given alone, do not ameliorate the clinical manifestations of PIA.

Impact of PAF-R antagonism on the severity and duration of PIA responses

Peanut-sensitized mice were treated with a PAF-R antagonist once before challenge and monitored for 40 minutes. Targeting PAF activity considerably lessened the severity of the reactions (Fig 3, *A, B, and E*). In fact, ~50% of treated mice developed either no or mild anaphylactic reactions. Notably, there was a considerable difference in the number of mice whose core temperature dropped by more than 4°C (87% in untreated vs 48% in treated), suggesting that PAF antagonism considerably prevented life-threatening reactions. Moreover, although 15% of untreated mice developed convulsions, no seizures were observed in the treated group. Of note, the levels of peanut-specific IgE and IgG_1 were similar among groups, indicating equivalent sensitization (data not shown). Together, our data demonstrate that the blockade of PAF-R substantially prevents severe anaphylactic reactions to peanuts.

To investigate whether PAF-R antagonism had an impact on the time of recovery from an anaphylactic reaction, we monitored clinical responses for 120 minutes after peanut challenge. We observed a clear difference in the number of mice that reached recovery temperature (≥37°C) within 120 minutes after challenge (83% in treated vs 43% in untreated; Fig 3, *C and D*). It should be noted that mice experiencing prolonged severe reactions showed a slower rate of recovery and, in some cases, did not show any clinical improvement during the observation period (Fig 3, *F*). Importantly, ~30% of untreated, but only 9% of treated, mice experienced severe hypothermia 120 minutes after challenge, thus decreasing their likelihood of recovering fully and, correspondingly, increasing the probability of death. In fact, ~20% of untreated, but none of the treated, mice died within 48 hours after challenge. Collectively, these data provide evidence that PAF-R antagonism attenuates the magnitude and duration of PIA reactions.

To assess the anaphylactic response more comprehensively, we developed parameters to grade severity as *global*, *moderate*, and *life-threatening*. To this end, we constructed a mathematical model describing the overall behavior of the anaphylactic response for each experimental group (see this article's

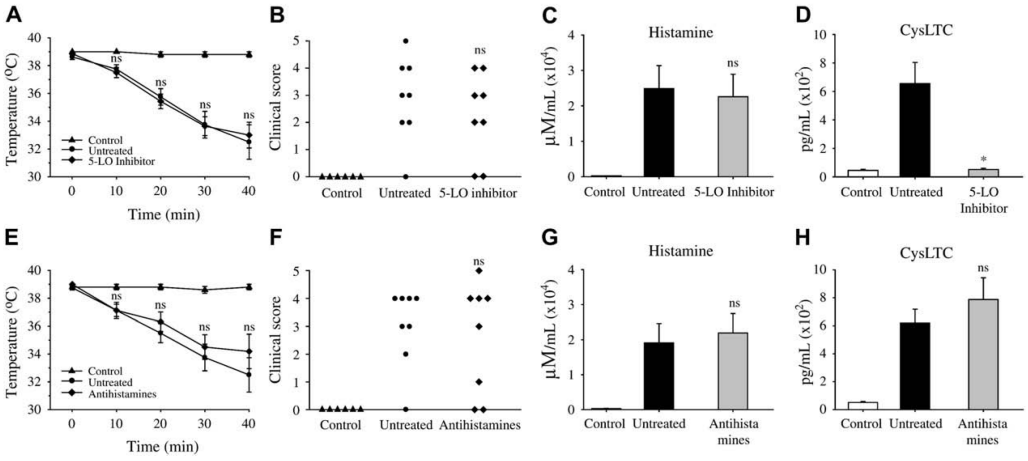


FIG 2. Histamine and leukotrienes are nonessential mediators but are biomarkers of PIA. Mice were treated with **(A-D)** a 5-LO inhibitor or **(E-H)** histamine receptor antagonists. *(A and E)* rectal temperature and *(B and F)* clinical symptoms from control *(▲)*, untreated *(●)*, and treated *(♦)* groups. *(C and G)* Histamine and *(D and H)* leukotriene levels after challenge. Representative data from 3 independent experiments; n = 8-12 mice/group. *$P < .05$ relative to control. *ns,* Not significant.

Supplementary Methods in the Online Repository at www.jacionline.org). By using mean experimental data for untreated mice, we derived an equation that represents the pattern of change in core temperature as long as 120 minutes after challenge. The same equation was then fit to the other experimental group (mice treated with PAF-R antagonist) to derive the numerical coefficients that accurately depict the response. These equations were subsequently used to generate modeled data and validated (Fig 3, *G*). As shown in Fig 3, *H,* we defined *global severity* as the area of the response under 39°C ($AU_{39°C}$), whereas *life-threatening severity* was depicted as the area of the response under 35°C ($AU_{35°C}$). *Moderate severity* was deemed as the difference between global and life-threatening severity ($AU_{39°C} - AU_{35°C}$). Given that these areas were calculated using mean group temperatures at each time point, they describe the overall trend of the response. Hence, the measure of $AU_{35°C}$ does not accurately differentiate life-threatening conditions from the overall response. In other words, the $AU_{35°C}$ is not representative of those mice that experienced life-threatening anaphylaxis exclusively but rather encompasses the response of the entire population. Therefore, we introduced an additional parameter, "events under 35°C", to provide a more exclusive assessment of life-threatening severity. Finally, we evaluated the duration of life-threatening conditions by calculating the time each response was below 35°C.

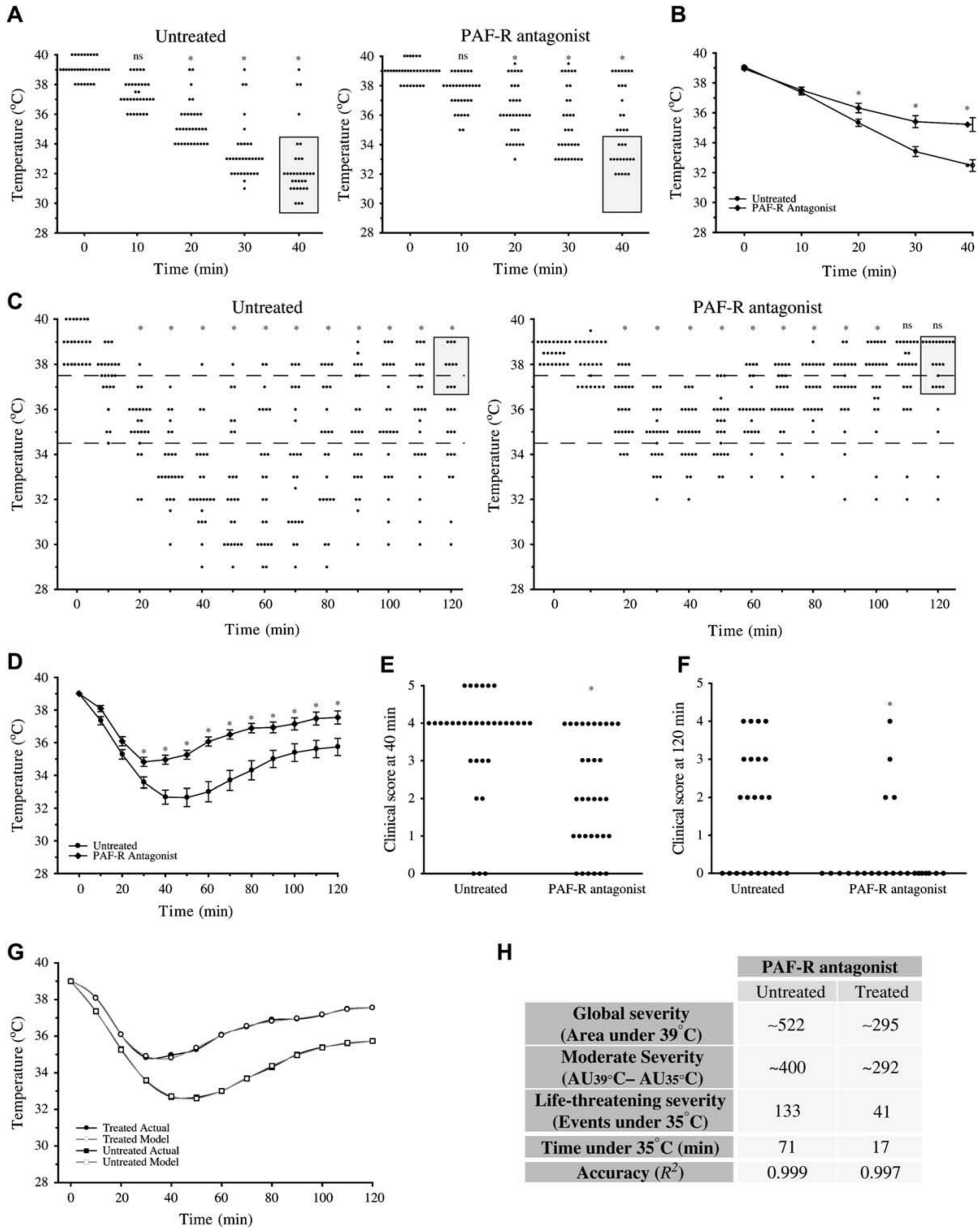
The model shows that treatment with a PAF-R antagonist resulted in ~50% decrease in global severity. In addition, the number of "events under 35°C" was 41 in treated mice versus 133 in untreated mice, indicating that untreated mice approached life-threatening conditions to a much greater extent (Fig 3, *H*). This is relevant because mice experiencing a severe drop in core temperature are less likely to recover and thus succumb to challenge. Moreover, treated mice were under life-threatening conditions for ~75% less time compared with untreated mice (Fig 3, *H*).

Systemic anaphylactic reactions are often associated with excessive vascular permeability and fluid extravasation.[2,16,17] In accordance with this, we observed that the blockade of PAF-R substantially reduced vascular leakage, as indicated by a 57% decrease in the content of albumin in the PL fluid of treated mice compared with untreated mice after challenge. The treatment was also associated with a significant decrease in the levels of histamine and cysLTs in plasma after challenge (see this article's Table E1 in the Online Repository at www.jacionline.org).

Impact of concurrent blockade of histamine and PAF receptors in peanut-induced anaphylaxis

The complexity of the interactions among vasoactive mediators involved in allergic disease has led to the suggestion that control of clinical manifestations may require the blockade of various mediators.[18,19] Hence, we treated peanut-sensitized mice with a PAF-R antagonist along with H1 and H2 receptor antagonists (Fig 4, *A-E*). As shown in Fig 4, *D* all but 1 mouse developed mild, if any, anaphylactic reactions, indicating that concurrent blockade of PAF and histamine has the greatest beneficial effect in the prevention of PIA.

To compare the efficacy of the various interventions, we used the aforementioned mathematical model (see this article's Supplementary Methods in the Online Repository at www.jacionline.org). The model showed that the global response to 120 minutes ($AU_{39°C}$) in the group treated with the combination therapy never fell below 35°C (Fig. 4 *F*). Similarly, the measure of moderate severity reflects the success of the combination therapy in the prevention of severe drops in core temperature. In fact, although PAF antagonism had a partial effect on moderate severity, histamine blockade did not. Importantly, the number of "events under 35°C" was 75, 33, 16, and 0 for the untreated, antihistamines, PAF-R antagonism, and combined treatment groups, respectively, indicating that combination therapy was the most successful intervention to prevent drastic reductions in temperature and, hence, development of life-threatening reactions (Fig. 4 *F*). Similarly, the proportion of mice reaching partial or full recovery at 120 minutes was greatest when treated with

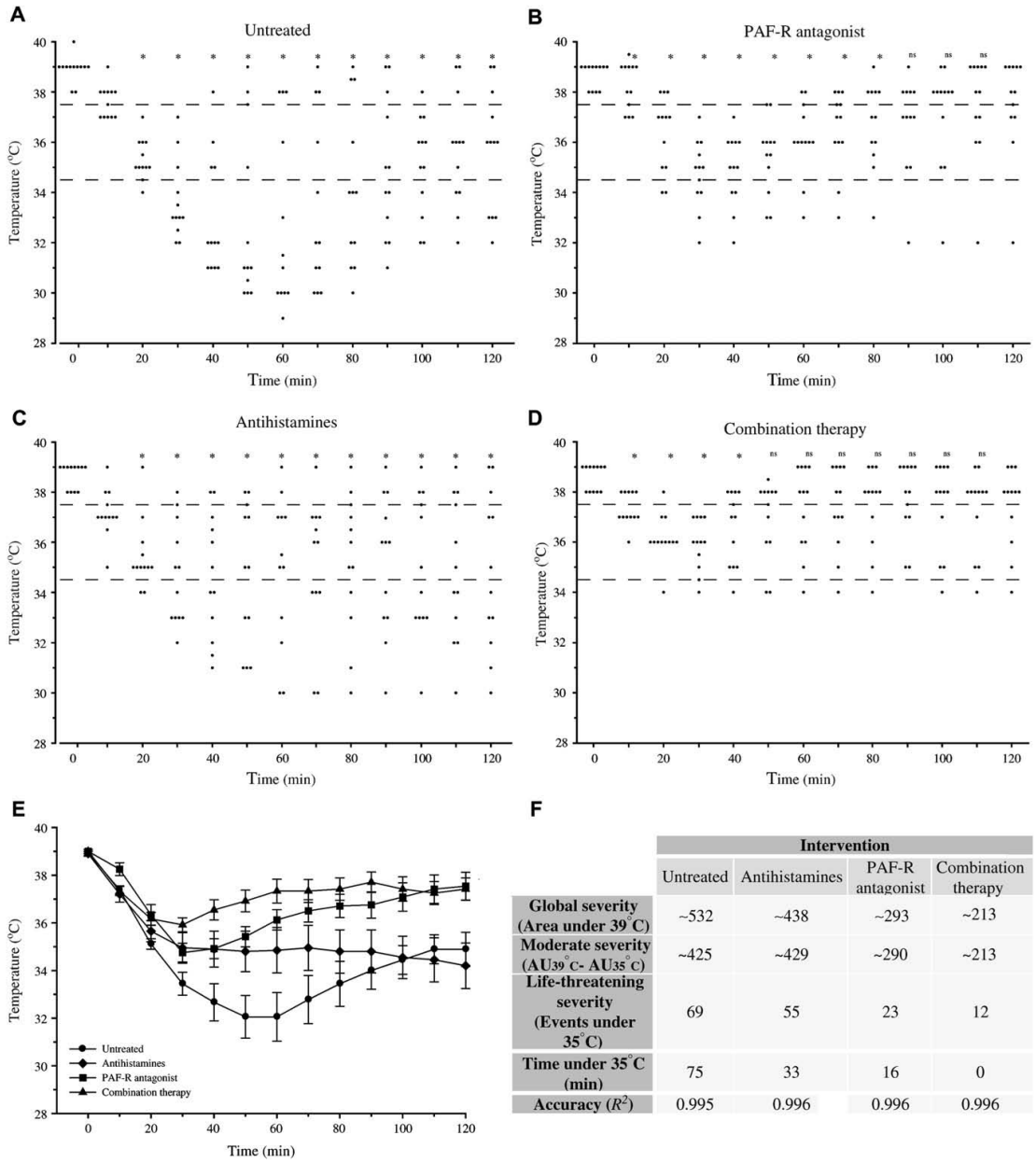


	PAF-R antagonist	
	Untreated	Treated
Global severity (Area under 39°C)	~522	~295
Moderate Severity (AU39°C– AU35°C)	~400	~292
Life-threatening severity (Events under 35°C)	133	41
Time under 35°C (min)	71	17
Accuracy (R^2)	0.999	0.997

FIG 3. Treatment with a PAF-R antagonist diminishes the severity of peanut-induced anaphylaxis and accelerates recovery. **(A and B)** Rectal temperature and **(E)** anaphylactic symptoms as long as 40 minutes after challenge. **(C and D)** Rectal temperature and **(F)** anaphylactic symptoms as long as 120 minutes after challenge. The severity of the reaction was graded using a mathematical model. **G,** Comparison between actual *(■, untreated actual and ●, treated actual)* and modeled *(⊟, untreated model and ⊖ treated model)* data. **H,** Assessment of the anaphylactic response after treatment. Pooled data from 3 independent experiments; **P* < .05 relative to time 0. *ns,* Not significant.

combination therapy. Of note, all of these mice achieved some level of recovery, with 66% of them exhibiting full recovery.

Last, we observed that combined treatment resulted in significantly less vascular permeability, as indicated by an 82% decrease in the content of albumin in the PL fluid of treated mice compared with untreated mice after challenge. Likewise, combination therapy was associated with a considerable decrease in the levels of histamine and CysLTs in plasma (see Table E1).



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	Intervention			
	Untreated	Antihistamines	PAF-R antagonist	Combination therapy
Global severity (Area under 39°C)	~532	~438	~293	~213
Moderate severity ($AU_{39°C}$- $AU_{35°C}$)	~425	~429	~290	~213
Life-threatening severity (Events under 35°C)	69	55	23	12
Time under 35°C (min)	75	33	16	0
Accuracy (R^2)	0.995	0.996	0.996	0.996

FIG 4. Concomitant treatment with PAF-R antagonist and antihistamines prevents life-threatening and prolonged PIA. Mice were treated as follows: **(A)** drug vehicle, **(B)** PAF-R antagonist, **(C)** histamine receptor antagonist. **D,** Treatments *B and C* administered concurrently. **E,** Mean rectal temperatures. **F,** Mathematical parameters to compare the efficacy of the interventions. **P* < .05 relative to time 0. *ns,* Not significant.

DISCUSSION

In North America, food allergies are the most common cause of severe anaphylactic reactions, with peanuts and tree nuts accounting for ~80% of the life-threatening episodes.[3] Unfortunately, pharmacologic interventions for food allergies have advanced little in the past decades. Several lines of evidence indicate that the release of vasoactive mediators is associated with the clinical manifestations of anaphylaxis.[17] Nevertheless, the relative

contribution, if any, of these mediators in the pathogenesis of PIA is not well understood. Hence, the objective of this study was to investigate the impact of the pharmacologic blockade of MC-associated mediators, particularly leukotrienes, histamine, and PAF in a mouse model of PIA.

The protocol used evokes the rapid onset of severe anaphylaxis as indicated by severe clinical symptoms, a significant drop in core temperature, marked release of vasoactive mediators, and pronounced vascular leakage. As MC-deficient (kitW/kitW-v) mice do not experience any measurable anaphylactic reactions in this model, we speculate that the mediators responsible for the elicitation of anaphylactic symptoms are primarily produced by MCs. Our data demonstrate that sensitization with peanut increased the number of peritoneal MCs and FcεRI surface expression. We have previously reported that the number of oral sensitizations progressively enhances the level of circulating peanut-specific IgE.[14] Because IgE is a major regulator of FcεRI expression,[15] it is likely that increased levels of peanut-specific IgE resulted in greater FcεRI density and occupancy. This is relevant to our findings because previous studies have shown that IgE-dependent upregulation of FcεRI expression significantly enhances the ability of MCs to release proinflammatory mediators in response to challenge with IgE and specific antigen.[15]

Recent studies by Khodoun et al[20] in which peanut was delivered intravenously to IL-4 + propranolol-pretreated mice suggest that innate mechanisms such as complement (ie, C3a) act synergistically with IgE/FcεRI-dependent MC degranulation to exacerbate anaphylaxis. In this model, peanuts might contribute to shock by causing production of C3a, which stimulates macrophages, basophils, and MCs to produce mediators including histamine and PAF.

We investigated the relative contribution of some vasoactive mediators in the elicitation of PIA. Our data indicate that pharmacological inhibition of cysLT did not have a significant effect on the extent of PIA. These findings do not negate the potential value of leukotriene levels in the management of anaphylaxis. Indeed, laboratory tests to support the clinical suspicion of anaphylaxis such as serum levels of histamine and tryptase have been rather disappointing to date.[21] Because leukotriene levels gradually increase in plasma after peanut challenge and remain elevated for a considerable period of time, they may represent a useful biomarker of anaphylaxis. In this regard, the presence of leukotrienes and their metabolites has been previously reported in urine during and after anaphylaxis.[22]

Presently, antihistamines are often given as adjuvant therapies. However, the benefit of antihistamines in systemic anaphylaxis has not been conclusively assessed.[23] Antihistamines have a slow onset of action and cannot block events that occur subsequent to histamine binding to its receptors. Because plasma histamine peaks early in anaphylaxis and rapidly returns to baseline, it is thought that the timing of administration of antihistamines is the main factor limiting their effectiveness. We treated peanut-sensitized mice with H1 and H2 receptor antagonists. This treatment did not have an impact on the severity or the course of the anaphylactic reaction. Antihistamines were administered before challenge; thus, the lack of effect cannot be attributed to inadequate timing. There is accumulating evidence linking PAF to the pathology of anaphylactic shock in animals.[10,11,19] Importantly, Vadas et al[13] recently showed that circulating PAF levels correlate with the severity of anaphylactic reactions in human beings. However, causality between PAF levels and severity of anaphylaxis could not, understandably, be ascertained in this study. Our data demonstrate that treatment with a PAF-R antagonist substantially reduces the severity and length of PIA reactions. Moreover, we observed that blockade of PAF-R was associated with decreased vascular leakage and a reduction in histamine and cysLT levels in plasma. *In vitro* studies have shown that PAF can directly induce histamine release and *de novo* synthesis of leukotriene C4 from various cell types.[24,25] It is, therefore, plausible that counteracting PAF may have resulted in a concurrent decrease in other unidentified mediators involved in the anaphylactic response. Last, we evaluated whether administration of H1 and H2 receptor antagonists had a further effect in the prevention of PIA. Our data demonstrate a remarkable benefit when PAF and histamine receptors are blocked concurrently.

The beneficial effect of concomitant blockade of vasoactive mediators has been previously demonstrated. Brandt et al[18] showed that complete inhibition of ovalbumin-induced allergic diarrhea required the targeting of 2 mediators, PAF and serotonin. Of note, neutralization of either mediator alone did not have any significant effect. Interestingly, we observed that the absence of serotonin in the periphery does not ameliorate PIA reactions. In fact, mice with a targeted disruption in the tryptophan hydroxylase 1 gene (tph1$^{-/-}$ mice) manifested the same degree of anaphylaxis as their wild-type counterparts after peanut challenge (data not shown). Nevertheless, we cannot rule out the potential benefit of concurrent inhibition of PAF and serotonin in our model. It is, however, possible that serotonin has a rather distinctive role in the elicitation of local allergic intestinal permeability and, thus, differentially contributes to the etiology of allergic diarrhea.

In summary, our data demonstrate that treatment with a PAF-R antagonist significantly diminishes the severity of PIA and accelerates the recovery from anaphylactic reactions. In addition, the combination of a PAF-R antagonist and antihistamines resulted in dramatic beneficial results. We should note that these findings were made in an experimental model that elicits robust anaphylactic reactions. Expectedly, then, a greater benefit may be anticipated in less severe reactions. We suggest that these findings have implications beyond their inherent, direct, therapeutic value. Indeed, the evaluation of promising strategies such as conventional immunotherapy or anti-IgE treatment is substantially limited by the potential of serious adverse effects. In this context, the pharmacologic strategy that we have investigated may also be viewed as a risk-reducing therapy—in other words, an ancillary therapy that may facilitate the implementation of other therapeutic strategies for PA and, most likely, other food allergies.

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Clinical implications: Concomitant blockade of PAF and histamine may represent a life-saving therapy for food-induced anaphylaxis and a risk-reducing approach that facilitates the implementation of other therapies (ie, conventional immunotherapy or anti-IgE).

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SUPPLEMENTARY METHODS

Mathematical Model

Mathematical equations were generated from the mean experimental data from curve-fitting techniques by using FindGraph 1.821 (UNIPHIZ Lab, Vancouver, British Columbia, Canada). For each experimental group, temperature (T) is expressed as a function of time (t):

$$T = 39 + x_a t + x_b t^2 + x_c t^3 + x_d t^4 + x_e t^5 + x_f t^6 + x_g t^7 + x_h t^8$$

where x_n represents numerical coefficients unique to individual group. The areas above each curve were derived by using Mathematica 6.0.2.1 (Wolfram Research Inc, Champaign, Ill). Accuracy of the model was measured through linear regression, comparing actual and simulated data for each group.

TABLE E1. Mice were treated as follows: histamine receptor antagonists (antihistamines), PAF-R antagonist, or both (combination therapy). Histamine and CysLT levels were analyzed in plasma and albumin was quantified in peritoneal lavage. Means ± SEMs from 3 independent experiments; n = 8-12 mice/group

	Intervention			
	Untreated	**PAF-R antagonist**	**Antihistamines**	**Combination therapy**
Albumin ($\times 10^5$ ng/mL)	3.41 ± 1.16	1.45 ± 0.52	3.78 ± 1.39	0.60 ± 0.45*
Histamine ($\times 10^4$ μM/mL)	1.88 ± 0.55	1.03 ± 0.32	1.65 ± 0.50	0.51 ± 0.17*
CysLT ($\times 10^2$ pg/mL)	5.70 ± 0.97	1.87 ± 0.39*	7.36 ± 1.56	0.70 ± 0.22*†

*$P < .05$ to untreated mice.
†$P < .05$ to PAF-R antagonist.

— Chapter 4 —

Distinct immune effector pathways contribute to the full expression of peanut-induced anaphylactic reactions in mice

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See *Preface* for details regarding authorship

Summary and Central Message: This article examines the relative contribution of immunoglobulin-dependent effector pathways to PIA. To this end, we used three separate approaches either individually or in combination. These include mice with spontaneous and targeted mutations, cellular depletion strategies and blocking mAbs. Our data demonstrate four scenarios in which PIA is fully abrogated: combined mast cell and macrophage (but not basophil) depletion, combined deficiency of IgE and IgG_1, or IgG_1 signalling, (but not of each immunoglobulin pathway alone) and combined absence of mast cells and IgG_1 signalling. However, they also show that the single elimination of mast cells, macrophages or basophils prevents fatal and near fatal reactions. Lastly, our findings indicate that whereas mast cell responses occur via IgE and IgG_1, macrophage responses are fully mediated through IgG_1 exclusively. Collectively, we provide for the first time evidence that distinct pathways cooperate to the full expression of the clinical manifestations of anaphylaxis and thus, full inhibition of PIA requires targeting several cellular and/or humoral effector components.

Distinct immune effector pathways contribute to the full expression of peanut-induced anaphylactic reactions in mice

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Background: Food-induced anaphylaxis is often a severe allergic reaction characterized by multiorgan dysfunction and a potentially fatal outcome.
Objectives: We sought to investigate the relative contribution of immunoglobulin-dependent effector pathways to anaphylactic responses to food (ie, peanut).
Methods: Wild-type and various mutant mice were sensitized with peanut protein and cholera toxin by means of oral gavage weekly for 4 weeks. Mice were subjected to different cellular depletion and Fc receptor blocking strategies before challenge with peanut 1 week after the last sensitization.
Results: Our data indicate that pathways other than the classical mast cell (MC)–IgE pathway contribute to the full spectrum of anaphylactic reactions to peanut. We show that the single deletion of MCs, basophils, or phagocytes (ie, macrophages) prevents the most significant clinical outcome: death. Remarkably, the combined deficiency of MCs and phagocytes, but not MCs and basophils, averted nearly all clinical and physiological signs of anaphylaxis. Furthermore, blockade of both IgE and IgG1 signaling was necessary to abolish anaphylactic responses to peanut. Although MC responses occurred through IgE and IgG1, phagocyte responses were fully mediated through IgG1.
Conclusions: Peanut-induced anaphylaxis is a process that involves the concerted action of multiple immune effector pathways, and thus interventions targeting a single pathway (eg, MC-IgE) might not be sufficient to fully prevent anaphylactic responses. (J Allergy Clin Immunol 2011;127:1552-61.)

Key words: *Peanut allergy, anaphylaxis, mast cells, macrophages, basophils, IgE, IgG1, FcεRI, FcγRIII, Kit$^{W\text{-}sh}$/$^{W\text{-}sh}$*

Anaphylaxis is a life-threatening hypersensitivity reaction commonly triggered by allergens, including foods, insect venoms, and medications.[1,2] Food-induced allergic reactions account for one third to one half of anaphylactic cases in emergency departments worldwide,[3-5] and fatality registries in the United States implicate peanut as a major culprit of food-induced fatal reactions.[6] Peanut allergy develops early in life and persists in 80% of patients.[7,8] The potential lethality of peanut allergy is compounded by the lack of treatments other than epinephrine to rescue patients undergoing anaphylaxis.

Mechanistically, anaphylaxis is thought to involve the massive release of mediators from mast cells (MCs) after allergen interaction with cell-bound IgE. However, numerous studies have demonstrated that anaphylactic responses can occur in mice deficient in MCs,[9] IgE,[10] or the FcεRIα chain,[11] thus arguing for the involvement of alternative pathways. In fact, studies with surrogate allergens (eg, ovalbumin) indicate that the death associated with systemic anaphylaxis, as well as the decrease in body temperature and other physiological changes associated with the reaction, might largely reflect IgG1-dependent activation of FcγRIII.[11,12]

Presently, the contribution of specific effector cells in IgG-mediated anaphylaxis remains a subject of investigation. Studies in BALB/c mice immunized with goat anti-mouse IgD antiserum and then challenged with goat IgG suggested that macrophages are the major cell type contributing to IgG-mediated responses.[13] Seemingly at variance, findings in a model of penicillin V–induced anaphylaxis in C57BL/6 mice demonstrated a central role for basophils, thus illustrating the selective contribution of distinct FcγRIII-expressing cells to IgG-mediated anaphylaxis.[14]

We have investigated immune effector pathways that mediate systemic anaphylaxis to peanut in mice. Our data demonstrate that effector cells, including MCs, phagocytes (ie, macrophages), and, to a lesser extent, basophils contribute to this process. In fact, absence of any one cell lineage prevented mortality. Remarkably, concomitant deficiency of MCs and phagocytes, but not MCs and basophils, abrogated almost completely a broad range of anaphylactic responses. Furthermore, we show that MCs contribute to anaphylaxis through IgE and IgG1, whereas phagocytes

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Abbreviations used
MC: Mast cell
PAF: Platelet-activating factor
PIA: Peanut-induced anaphylaxis
WT: Wild-type

contribute through IgG1 exclusively, suggesting that in patients with peanut-induced anaphylaxis (PIA), IgG-dependent responses occur through various FcγRIII-expressing cells. Collectively, our findings indicate that the full expression of anaphylactic responses to peanut in this model involves cooperation between distinct immune effector pathways.

METHODS

Animals

Female C57BL/6 mice (6-8 weeks old) were purchased from Charles River Laboratories (Ottawa, Ontario, Canada). MC-deficient (WBB6F1/J-Kit^{W}/Kit^{W-v} and C57BL/6J-Kit^{W-sh}/$^{W-sh}$) and age-matched congenic wild-type (WT) mice were purchased from Jackson Laboratory (Bar Harbor, Me). IgG1 (C57BL/6)–[15] and IgE (BALB/c)–deficient[10] mice were bred in house. The mice were housed in a specific pathogen-free environment and maintained on a 12-hour light-dark cycle. All experiments described in this study were approved by the Animal Research Ethics Board of McMaster University.

Reagents

Ba103 and control rat IgG antibodies were purchased from Hycult Biotechnology B.V. (Uden, The Netherlands) and Sigma-Aldrich (Oakville, Ontario, Canada), respectively. Monoclonal rat IgG2b anti-FcγRII/III antibody (2.4G2) specific for the extracellular domains of murine FcγRIIB and FcγRIII was prepared in house from hybridoma culture supernatants and was a gift from Dr Kathy McCoy, McMaster University, Canada. Clodronate was a gift of Roche Diagnostics GmbH (Mannheim, Germany) and was encapsulated in liposomes in the laboratory of Dr Nico van Rooijen (Amsterdam, The Netherlands). Fluorescein isothiocyanate–conjugated mAbs specific for CD3 (145-2C11) and FcεRI (MAR-1) and phycoerythrin–Cy7–conjugated mAbs specific for CD11b (M1/70), phycoerythrin–Cy5–conjugated mAbs specific for CD3e (145-2C11) and F4/80 (BM8), and allophycocyanin-conjugated CD117 (2B8) were purchased from eBioscience (San Diego, Calif). Fluorescein isothiocyanate–conjugated anti-IgE (R35-72) and phycoerythrin-conjugated mAbs specific for CD117 (2B8) and CD49b (DX5) were purchased from BD Biosciences (Mississauga, Ontario, Canada). Appropriate isotype controls were used.

PIA model

Mice were sensitized as previously described,[16] with slight modifications. Specifically, oral gavages were performed with intragastric feeding needles (191183; DELVO, Zurich, Switzerland). Sensitized mice were challenged with 3.75 mg of crude peanut extract (Greer Laboratories, Lenoir, NC) in 500 μL of PBS either intraperitoneally or intravenously 1 to 2 weeks after the last gavage.

Anaphylaxis measurements

Mice were monitored for 40 minutes after challenge, and responses were assessed as follows. First, the clinical score was determined. Clinical signs were evaluated by using the scoring system shown in Table I. In some instances mice died or were killed before the 40-minute time point as they reached end-point conditions (≥2 seizures, loss of consciousness, or both; moribund condition). Second was measurement of core body temperature, as previously reported.[16] Third was measurement of hematocrit values. Peripheral blood was collected by means of retro-orbital bleeding into heparinized microcapillaries. The tubes were centrifuged for 1 minute by using a microhematocrit centrifuge. The hematocrit value was expressed as a percentage of cell volume. Fourth was determination of vascular leakage. Mice were injected with crude peanut extract in Evans blue dye intravenously, and footpads were examined for dye extravasation 10 minutes after challenge. Fifth was measurement of intestinal bleeding. The small intestines were removed and examined for macroscopic hemorrhage 40 minutes after challenge. Finally, we performed measurement of histamine values, as previously described.[17]

In vivo depletion of cell lineages

For basophil depletion, mice were given an intravenous injection of 100 μg of the basophil-depleting mAb Ba103[18] or control rat IgG 1 day before peanut challenge. For phagocyte depletion, mice were given an intraperitoneal injection of 0.5 mL of clodronate or PBS liposomes 2 days before challenge, followed by an intravenous injection of 0.2 mL the next day.[19]

Blockade of IgG-mediated anaphylaxis

Mice were injected intraperitoneally with 500 μg of anti-FcγRII/III mAb in PBS 24 hours before challenge. We used a dose of anti-FcγRII/III mAb previously reported to block FcγRII/III-dependent anaphylaxis.[20-22] However, we validated this dose with dose-response experiments to determine comparable anaphylactic responses to those seen in IgG1-deficient mice (data not shown).

Measurement of peanut-specific IgG1 and IgE levels

Peripheral blood was collected 24 hours before challenge, and levels of serum peanut-specific IgG1 and IgE were measured as previously described.[16]

Flow cytometry

Single-cell suspensions from spleen, blood, and peritoneal lavage fluid were preincubated with anti-CD16/32 mAb at 4°C for 15 minutes before incubation with the indicated combination of antibodies. To detect IgE receptors, we incubated cells with anti-CD23 (FcεRII) at 4°C for 15 minutes and then IgE for 30 minutes to saturate the IgE receptors and then stained them with anti-IgE. Stained cells were analyzed with an LSRII flow cytometer (Becton Dickinson), and the data were analyzed with FlowJo 6.4.2 software (TreeStar, Inc, Ashland, Ore). At least 500,000 events were collected.

Statistical analysis

Statistical analysis was performed with SigmaStat software (Systat Software, Inc, San Jose, Calif). When applicable, results were analyzed with 1- or 2-way ANOVA with repeated measures followed by the Tukey post hoc test. The unpaired Student *t* test (2 tailed) was used when only 2 sets of continuous data were compared. A *P* value of less than .05 was considered statistically significant. Data are expressed as means ± SDs.

RESULTS

Effect of MC deficiency on the elicitation of anaphylactic responses to peanut

We previously reported that MC-deficient WBB6F1/J-Kit^{W}/Kit^{W-v} mice sensitized orally manifested no measurable anaphylactic responses after intraperitoneal challenge with peanut.[16] Given that recent studies have described the involvement of other effector cells in anaphylactic reactions to peanut after intravenous challenge,[23] we evaluated the response of Kit^{W}/Kit^{W-v} mice to this route of challenge (see Fig E1 in this article's Online Repository at www.jacionline.org). We found that Kit^{W}/Kit^{W-v} mice challenged intravenously exhibited mild clinical signs, a small but significant decrease in temperature, and a modest but

TABLE I. Anaphylactic symptom score table

Score	Symptoms
0	No clinical symptoms
1	Repetitive mouth/ear scratching and ear canal digging with hind legs
2	Decreased activity; self-isolation; puffiness around eyes, mouth, or both; pilar erection; labored breathing
3	Prolonged periods of motionlessness; lying prone on stomach
4	No response to whisker stimuli; reduced or no response to prodding; complete paresis
5	End point (>2 seizures or loss of consciousness, moribund condition); death

significant increase in hematocrit values compared with values seen in control mice. It is noteworthy that hematocrit values in naive $Kit^{W}/Kit^{W\text{-}v}$ mice were lower than in littermate control animals likely because of the anemic phenotype of these mice.[24] Histamine was undetectable in $Kit^{W}/Kit^{W\text{-}v}$ mice after challenge. In littermate control animals intravenous challenge elicited more severe anaphylaxis than intraperitoneal challenge. Indeed, the percentage of mice reaching fatal and near-fatal reactions among those challenged intravenously or intraperitoneally was 100% versus 60%, respectively. Core temperatures were monitored in surviving mice; hypothermia was consistently more pronounced in survivors challenged intravenously. Similarly, increases in hematocrit values were more marked after the intravenous challenge. These data indicate that anaphylactic reactions were significantly abrogated but still clearly evident in $Kit^{W}/Kit^{W\text{-}v}$ mice subjected to intravenous challenge.

To further investigate the degree of MC dependence in PIA, we used a genetically different MC-deficient strain, $Kit^{W\text{-}sh}/^{W\text{-}sh}$. Oral sensitization led to increased levels of peanut-specific IgE and IgG1 comparable with those observed in WT control animals (Fig 1, *A*). $Kit^{W\text{-}sh}/^{W\text{-}sh}$ mice exhibited mild clinical signs (Fig 1, *B*) and a significant decrease in temperature after either intraperitoneal or intravenous challenge (Fig 1, *C*). Hemoconcentration was observed after intravenous but not intraperitoneal challenge (Fig 1, *D*). Increased vascular permeability was ascertained by means of Evans blue extravasation in the footpads (Fig 1, *E*). Consistent with our findings in $Kit^{W}/Kit^{W\text{-}v}$ mice, anaphylactic responses in WT control animals were more severe after intravenous challenge. In particular, signs of circulatory collapse, including macroscopic intestinal bleeding, were consistently observed only in mice after intravenous challenge (occurrence, 70%; Fig 1, *F*). As in $Kit^{W}/Kit^{W\text{-}v}$ mice, histamine was undetectable in $Kit^{W\text{-}sh}$ mice after challenge (Fig 1, *G*). Collectively, these data indicate that PIA is substantially mediated by MC. However, MC deficiency does not fully prevent anaphylaxis, thus suggesting the contribution of other cell lineages to this process. Because intravenous challenge resulted in more severe and consistent responses, all subsequent experiments were conducted by using this route.

Role of basophils and macrophages in patients with PIA

Because basophils play a critical role in some types of anaphylaxis,[14] we investigated the contribution of these effector cells to PIA. We treated peanut-sensitized $Kit^{W\text{-}sh}/^{W\text{-}sh}$ mice and WT control animals with a basophil-depleting mAb (Ba103) or an isotype control antibody before challenge. Ba103 treatment depleted 80% to 85% of the basophils from the peripheral blood and spleen (blood: control, 2.3 $\pm$ 0.48 cells/mm^3; Ba103, 0.4 $\pm$ 0.11 cells/mm^3; spleen: control, 8.4 $\pm$ 0.7 cells $\times$ 10^4; Ba103, 1.3 $\pm$ 0.18 cells $\times$ 10^4; Fig 2, *A*). The depletion was selective because no other cell types, including MCs, were affected (Fig 2, *A*, and data not shown). Basophil depletion in WT mice prevented death (approximately 38%), end-point conditions (approximately 63%), or both (Fig 2, *B*). However, absence of basophils did not change the extent of hypothermia (Fig 2, *C*) or hemoconcentration (Fig 2, *D*) in either WT or $Kit^{W\text{-}sh}/^{W\text{-}sh}$ mice, suggesting that basophils contribute only to the most severe expression of anaphylactic responses.

Given that macrophages can mediate systemic anaphylaxis,[25] we examined the role of these cells in patients with PIA. We treated peanut-sensitized $Kit^{W\text{-}sh}/^{W\text{-}sh}$ and congenic control animals with clodronate liposomes before challenge. This treatment almost completely eliminated $F4/80^{+}$ cells from the peritoneal cavity (99.9%) and spleen (95%; Fig 3, *A*). Phagocyte depletion in WT mice consistently prevented severe clinical signs. Indeed, the absence of phagocytes significantly prevented fatal or near-fatal reactions (12.5% in phagocyte-depleted vs 71.4% in nondepleted mice; Fig 3, *B*). Decreases in temperature and increases in hematocrit values were consistently milder in phagocyte-depleted mice compared with those seen in nondepleted mice, although these differences did not reach statistical significance. Remarkably, double deficiency of MCs and phagocytes (ie, macrophages) resulted in a nearly complete abrogation of all responses measured (Fig 3, *C*). Indeed, there was no increase in hematocrit value (Fig 3, *D*), Evans blue extravasation in the footpads (Fig 3, *E*), or macroscopic intestinal bleeding in doubly deficient mice (Fig 3, *F*).

Role of different immunoglobulin isotypes in anaphylactic reactions to peanut

We previously reported that immunoglobulin responses are absolutely required for the elicitation of PIA.[16] However, the relative contribution of different immunoglobulin isotypes to this process has not been formally investigated. To evaluate the role of IgE, we used mice with a homozygous null mutation of the IgE gene, which cannot synthesize IgE but produces other immunoglobulin isotypes normally.[10] Indeed, the levels of peanut-specific IgG1 in IgE-deficient mice were almost identical to those in control mice (data not shown). Despite the IgE deficiency, sensitized mutant mice still displayed mild clinical signs (Fig 4, *A*), a considerable decrease in body temperature (Fig 4, *B*), and significantly increased hematocrit values (Fig 4, *C*). Moreover, histamine levels were lower in IgE-deficient mice than in WT control mice but markedly augmented compared with those in naive mice (Fig 4, *D*). These findings suggest that the remaining responses observed in these mice might be mediated through IgG1-dependent activation of effector cells.

To examine the involvement of IgG1 in this process, we used mice that lacked the splice donor site in the Iγ1 exon necessary for switch recombination to IgG1.[26] Importantly, the frequency of cells switching to IgE is not affected.[27] Indeed, we detected equivalent serum levels of peanut-specific IgE in WT and IgG1-deficient mice after sensitization (data not shown). The absence

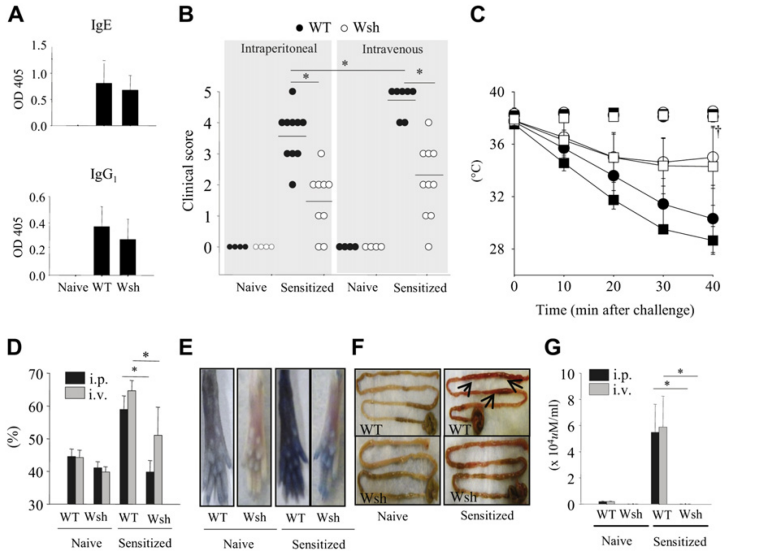

FIG 1. Partial abrogation of PIA in MC-deficient $Kit^{W\text{-}sh}/^{W\text{-}sh}$ *(Wsh)* mice. **A**, Levels of peanut-specific IgE and IgG1 in serum. **B**, Peak anaphylactic symptom score of each mouse. **C**, Rectal temperature of naive (intraperitoneal: WT, ---●---; Wsh, ---o---; intravenous: WT, ---■---; Wsh, ---□---) and sensitized (intraperitoneal: WT, —●—; Wsh, —o—; intravenous: WT, —■—; Wsh, —□—) mice. **P* < .05, Wsh intraperitoneal versus corresponding WT control mice. †*P* < .05, Wsh intravenous versus corresponding WT control mice. **D** and **G**, Percentage of hematocrit (Fig 1, *D*) and histamine levels (Fig 1, *G*). *i.p.*, Intraperitoneal; *i.v.*, intravenous. **E** and **F**, Representative pictures showing Evans blue extravasation in footpads (Fig 1, *E*) and intestinal bleeding (Fig 1, *F*). Data are presented as means ± SDs (4-11 mice per group from at least 3 independent experiments).

of IgG1 did not significantly prevent clinical signs (Fig 4, *A*) or the initial decrease in temperature. However, it avoided prolonged severe hypothermia. Furthermore, hematocrit (Fig 4, *C*) and histamine (Fig 4, *D*) values were significantly lower in IgG1-deficient mice compared with those in WT control mice yet substantially increased compared with those seen in naive mice. These data, along with our observation that FcγRIII-deficient mice are only partly protected from PIA (data not shown), provide evidence of the contribution of IgG1 to this process. To assess the combined contribution of IgE and IgG1 to PIA, we blocked FcγRII/III-dependent responses in IgE-deficient mice. Our data show that mice treated with an anti-FcγRII/III mAb exhibited no measurable anaphylactic responses (Fig 5), hence demonstrating that systemic anaphylactic reactions to peanut are fully mediated by dual IgE and IgG1 actions.

Effect of MCs and other FcγRII/III-expressing cells on anaphylaxis to peanut

To examine the contribution of IgE versus IgG1 to MC-mediated anaphylaxis, we used different approaches. Because engagement of FcγRIV by IgE immune complexes can activate macrophages,[28] we assessed the contribution of MCs to IgE-dependent responses by depleting phagocytes (ie, macrophages) in IgG1-deficient mice. Our data show that these mice manifested anaphylactic responses comparable with those seen in nondepleted IgG1-deficient mice (Fig 6), strongly suggesting that the residual anaphylaxis observed in these mice is dependent on IgE binding to MCs. Given that MC activation can occur after cross-linking of low-affinity IgG receptors (ie, FcγRIII),[29] we investigated whether this pathway is relevant in patients with PIA by depleting phagocytes in IgE-deficient mice. Removal of phagocytes (ie, macrophages) did not further reduce anaphylactic reactions in these mice (Fig 6), suggesting that the remaining responses are elicited through IgG1-dependent activation of MCs. To examine whether the concurrent absence of MCs and FcγRII/III signaling (on other effector cells) abrogated PIA, we blocked FcγRII/III signaling in MC-deficient mice. Remarkably, MC-deficient mice treated with an anti-FcγRII/III mAb did not show any indication of anaphylaxis (Fig 7), demonstrating cooperation between MCs and FcγRII/III-expressing cells in patients with PIA.

DISCUSSION

In this study we have investigated immune effector pathways mediating PIA. We previously showed that orally sensitized $Kit^{W}/Kit^{W\text{-}v}$ mice subjected to a peanut challenge intraperitoneally did not display any indication of anaphylaxis,[16] suggesting that MCs were essential for this process. However, because recent studies with intravenous challenge have shown that food-induced anaphylaxis can occur in the absence of MCs,[23] we investigated anaphylactic responses in both $Kit^{W}/Kit^{W\text{-}v}$ and $Kit^{W\text{-}sh}/^{W\text{-}sh}$ MC-deficient mice challenged either intraperitoneally or intravenously. We confirmed our previous findings in $Kit^{W}/Kit^{W\text{-}v}$ mice challenged intraperitoneally but also showed that intravenous

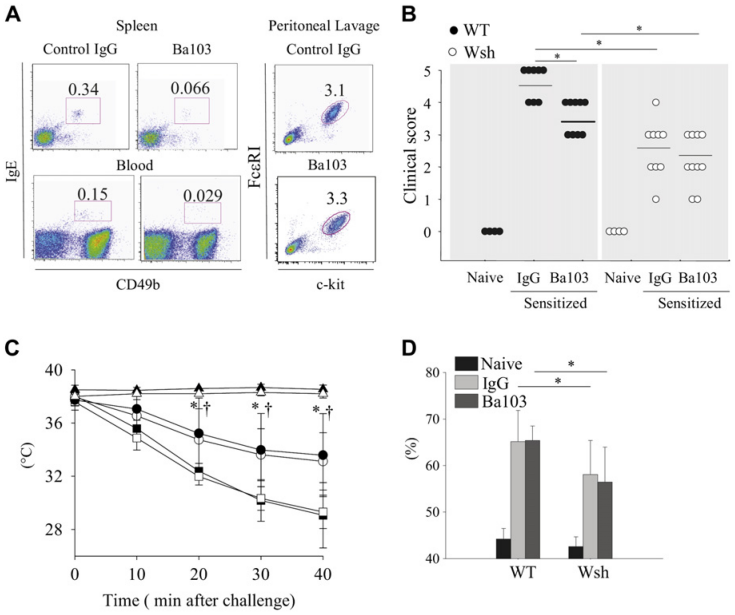

FIG 2. Effect of basophil depletion on peanut-induced anaphylactic reactions. **A**, Percentage of basophils and MCs from sensitized mice after antibody administration. **B**, Peak anaphylactic symptom score of each mouse. **C**, Rectal temperature of naive (WT, —▲—; $Kit^{W\text{-}sh}/^{W\text{-}sh}$ *[Wsh]*, —△—) and sensitized (WT/rat IgG, —■—; WT/Ba103, —□—; Wsh/rat IgG, —●—; Wsh/Ba103, —o—) mice. **P* < .05, Wsh/rat IgG versus corresponding WT control mice. †*P* < .05, Wsh/Ba103 versus corresponding WT control mice. **D**, Hematocrit percentage. Fig 2, *C* and *D*, Data are presented as means ± SDs (4-8 mice per group from at least 2 independent experiments).

challenge elicited significant anaphylactic responses. Anaphylactic responses in $Kit^{W\text{-}sh}/^{W\text{-}sh}$ mice were similar to those in $Kit^{W}/Kit^{W\text{-}v}$ mice, although modest increases in clinical scores and decreases in core temperatures were observed even in mice challenged intraperitoneally. Consistent with our observations in $Kit^{W}/Kit^{W\text{-}v}$ mice, intravenous challenge induced more severe clinical and biological responses. Thus oral sensitization and intravenous challenge elicit a broad range of anaphylactic reactions, including the most severe ones in MC-deficient mice.

Other investigators have studied mechanisms underlying peanut allergy in murine models in which the oral route is used for both sensitization and challenge. At first glance, the oral route of challenge would be preferred; however, there are some considerations that merit reflection. For example, the amount of peanut antigen necessary to induce objective clinical measurements in mice (eg, decrease in core body temperature) is extraordinarily high (up to 200 mg)[30,31] and likely not comparable with the amount of peanut antigen to which patients with peanut allergy might be exposed. Indeed, in many cases trace amounts are known to induce severe anaphylactic reactions in humans.[6] Thus such experimental exposure likely favors distinctive responses that might or might not fully reflect the clinical phenotype observed in these patients (eg, severe reactions, including death).

Furthermore, exposure to peanut by patients with peanut allergy can lead to symptoms that can occur very rapidly after ingestion.[32] However, the site or sites of antigen absorption have not been clearly defined. The small intestine, particularly the jejunum, is thought to be the site of greatest absorption in the gastrointestinal tract.[33] However, it has also been reported that antigen uptake (ie, peanut) occurs in the mouth across the oral or sublingual mucosa, reaching the systemic circulation rapidly after exposure.[32] From this perspective, intraperitoneal or intravenous challenge might be viewed not as an unusual route but as a route that facilitates immediate access to the systemic circulation.

The recognition that there are likely different routes of peanut exposure might allow for the investigation of pathways that contribute to PIA because it is increasingly evident that the IgE-dependent pathway is not be the sole effector pathway relevant to human disease. Indeed, a study involving systemic exposure to peanut showed the contribution of immunoglobulin-independent activation of the anaphylatoxin C3a to anaphylactic shock.[23] In this study the authors reported that a single intravenous administration of peanut into IL-4/propranolol–pretreated BALB/c mice induced severe shock that was largely independent on MCs. In this system because peanut-induced shock occurred in the absence of adaptive immunity, the contribution of immune pathways involving activation of cells through Ig/Fc receptors could not be investigated. In contrast, in the model we used here, allergic sensitization and effector responses (ie, anaphylaxis) require an adaptive immune system (CD4- and CD40 ligand–deficient mice or μMT mice do not show any measurable anaphylaxis[16]; unpublished data), thus allowing for the examination of antibody-mediated pathways leading to PIA.

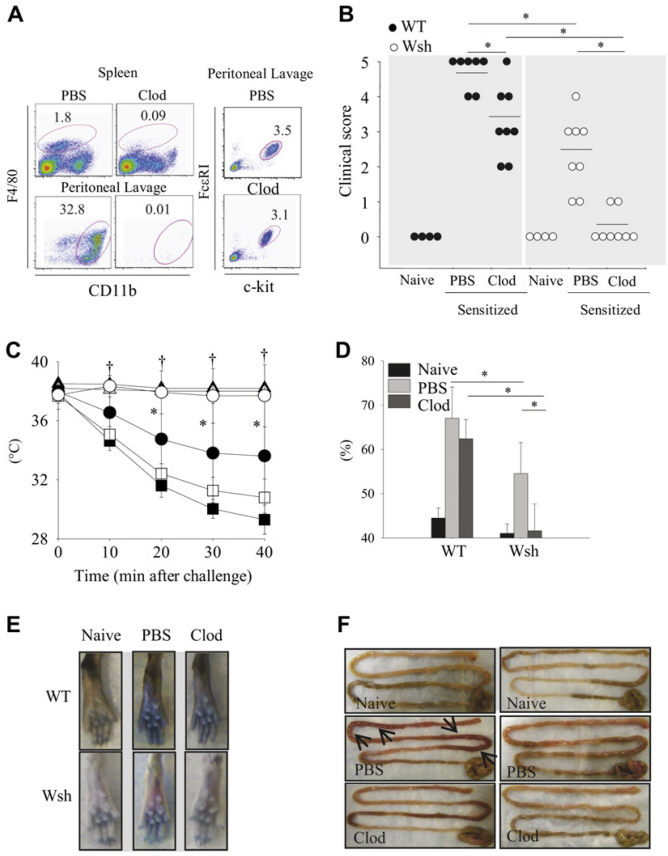

FIG 3. Dual contribution of MCs and phagocytes to PIA. **A**, Percentage of macrophages from sensitized mice after liposome delivery. **B**, Peak anaphylactic symptom score of each mouse. **C**, Rectal temperature of naive (WT, —▲—; $Kit^{W\text{-}sh}/^{W\text{-}sh}$ *[Wsh]*, —△—) and sensitized (WT/PBS liposomes, —■—; WT/clodronate liposomes, —□—; Wsh/PBS liposomes, —●—; Wsh/clodronate liposomes, —o—) mice. **P* < .05, Wsh/PBS liposomes versus corresponding WT control mice. †*P* < .05, Wsh/clodronate liposomes versus corresponding WT control mice. **D**, Hematocrit percentage. **E** and **F**, Representative pictures showing Evans blue extravasation in footpads (Fig 3, *E*) and intestinal bleeding (Fig 3, *F*). Fig 3, *C* and *D*, Data are presented as means ± SDs (4-8 mice per group from at least 4 independent experiments). *Clod*, Clodronate.

Two main pathways leading to systemic anaphylaxis were initially proposed: a classical pathway mediated by IgE, FcεRI, MCs, histamine, and platelet-activating factor (PAF) and an alternative pathway mediated by IgG, FcγRIII, macrophages, and PAF.[25] Our data indicate that anaphylaxis was still present in MC- or IgE-deficient mice. Additionally, we found that absence of phagocytes, IgG1, or both or FcγRIII (unpublished data) reduced the severity of the reactions. Notably, the concomitant deficiency of MCs and phagocytes abrogated all clinical and physiological responses measured, thus demonstrating the critical involvement of these 2 pathways in PIA. Although our data suggest that activation of phagocytes through FcγRIII/IgG1 contributes to anaphylactic responses to peanut, FcγRIII-mediated anaphylaxis can occur through phagocyte–independent mechanisms.[11] Our observation that IgE-deficient mice depleted of phagocytes exhibited some degree of anaphylaxis likely reflects the contribution of MCs to FcγRIII/IgG-mediated responses. Indeed, increased histamine levels after challenge provide evidence of MC degranulation, which likely occurred through IgG.

Recently, Tsujimura et al[14] showed that basophils are a preeminent player in IgG1-induced systemic anaphylaxis to penicillin V in C57BL6 mice. Importantly, depletion of basophils in WT mice, in a model of active systemic anaphylaxis, did not prevent fatalities but rescued MC-deficient mice from death. However, absence of basophils in the latter mice did not fully prevent hypothermia (peak decrease of approximately 4°C), suggesting that other effector cells contribute to this process. In our study we observed that basophils contribute to the most severe manifestations of PIA. Indeed, the absence of basophils prevented fatal (approximately 38%), life-threatening (end-point condition, approximately 63%), or both types of reactions. However, it did not

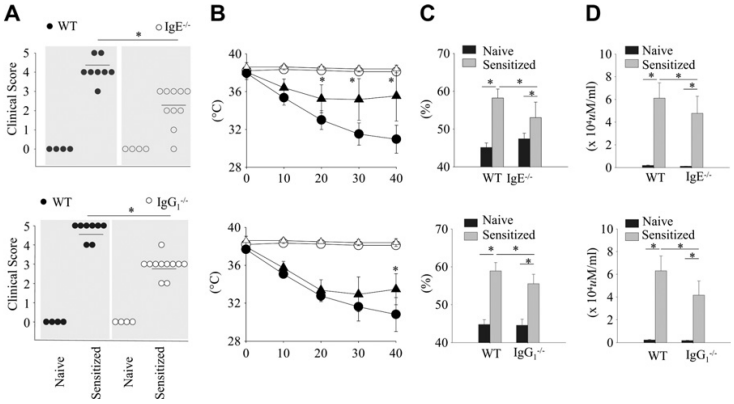

FIG 4. Double, but not single, absence of IgE and IgG1 signaling abolishes PIA. **A**, Peak anaphylactic symptom score of each mouse. **B**, Rectal temperature of naive (WT, —o—; immunoglobulin-deficient, —△—) and sensitized (WT, —●—; immunoglobulin-deficient, —▲—) mice. *$P < .05$ relative to corresponding WT control mice. **C** and **D**, Hematocrit percentage (Fig 4, *C*) and histamine levels (Fig 4, *D*). Fig 4, *B* through *D*, Data are presented as means ± SDs (4-8 mice per group from at least 3 independent experiments).

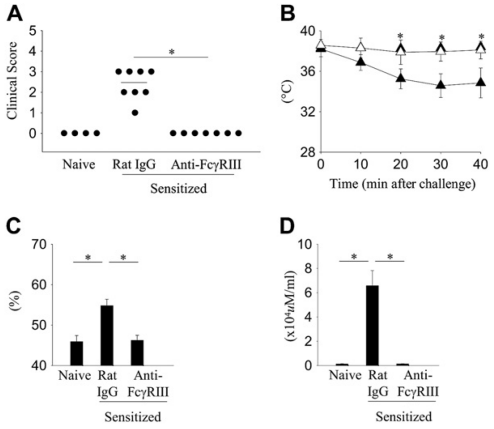

FIG 5. Blockade of IgG1 signaling in IgE-deficient mice abrogates PIA. **A**, Peak anaphylactic symptom score of each mouse. **B**, Rectal temperature of naive (—▲—) and sensitized (rat IgG, —▲—; anti-FcγRII/III, —△—) mice. *$P < .05$ relative to corresponding WT control animals. **C** and **D**, Hematocrit percentage (Fig 5, *C*) and histamine levels (Fig 5, *C*). Fig 5, *B* through *D*, Data are presented as means ± SDs (4-8 mice per group from at least 3 independent experiments).

change the extent of hypothermia or increase in hematocrit values. Clearly, hypothermia, hemoconcentration, and severe end points are all related in this system. However, there is no evidence to indicate that the relationships between these outcomes are linear; in fact, it is quite likely that they are not. This is an important issue in a system in which great pathophysiologic changes occur within a short period of time (40 minutes). Moreover, it is very likely that other pathophysiologic changes (eg, hypotension) play a very significant role in fatal or near-fatal responses in this model. These changes might have additive or independent effects and, possibly, a different kinetics. Importantly, our data demonstrate that treatment of MC-deficient mice with an anti-FcγRII/RIII mAb to temporarily block IgG binding on macrophages/basophils fully abolished anaphylactic responses. Specifically, MCs and phagocytes (ie, macrophages), but not basophils, mediate most of the clinical and physiological signs of anaphylaxis in this model. However, at the far end of the spectrum of severity (mortality), the single elimination of MCs, macrophages, or basophils prevents this outcome, suggesting that anaphylactic death is a catastrophic event that requires the concomitant contribution of these 3 cell types.

We previously showed that peanut-specific antibodies are required for PIA.[16] Here we investigated the relative contributions of IgE and IgG1 to this process. We observed that anaphylaxis is substantially but not completely mediated through IgE and that IgG1-deficient mice manifested some anaphylactic reactions. Importantly, IgE-deficient mice treated with an anti-FcγRII/RIII mAb displayed no measurable reactions at all, hence demonstrating the dual contribution of both IgE and IgG1 to PIA. We should note that involvement of IgG1 in food allergy/anaphylaxis has not been previously reported. Given that IgG1 levels are low in the gastrointestinal tract, it is plausible that within the intestinal mucosa initial responses are primarily mediated through the IgE-MC pathway.[34] However, allergen entry into the systemic circulation is likely to result in activation of effector cells through IgG1. This pathway might actively contribute to the development of both more severe and protracted, biphasic, or both reactions. Of relevance, our data indicate that the contribution of IgG1 is most significant at late time points. Thus we hope that our findings will encourage further studies in other murine strains.

The lack of available evidence for the involvement of the IgG1 pathway in human anaphylaxis might merely be a reflection of not having explored this possibility comprehensively. In particular, in reference to food allergy, it is increasingly clear that the IgE pathway is likely not the only immunoglobulin pathway involved. Indeed, approximately 25% of patients do not respond whatsoever to anti-IgE therapy.[35,36] Moreover, the relationship between levels of food (peanut)–specific IgE and clinical expression, phenotypes, or both is not linear; various studies suggest that there is an adequate relationship for levels of peanut-specific IgE of

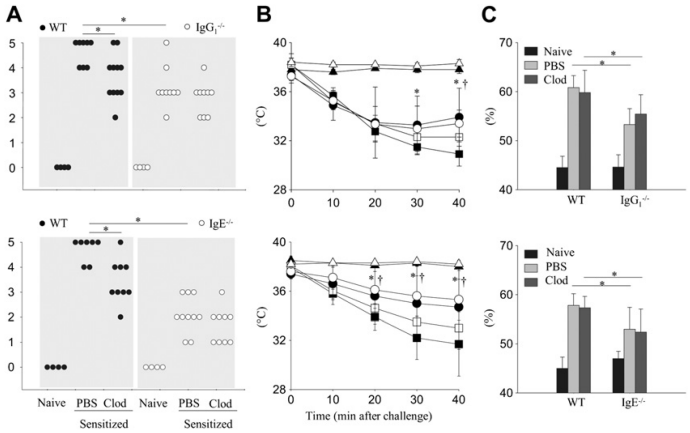

FIG 6. MC-dependent anaphylaxis to peanut occurs through both IgE and IgG1. **A**, Peak anaphylactic symptom score of each mouse. **B**, Rectal temperature of naive (WT, —▲—; immunoglobulin deficient, —△—) and sensitized (WT/PBS liposomes, —■—; WT/clodronate liposomes, —□—; immunoglobulin-deficient/PBS liposomes, —●—; immunoglobulin-deficient/clodronate liposomes, —○—) mice. **P* < .05, immunoglobulin-deficient/PBS liposomes versus corresponding WT control animals. †*P* < .05, immunoglobulin-deficient/clodronate liposomes versus corresponding WT control animals. **C**, Hematocrit percentage. Fig 6, *B* and *C*, Data are presented as means ± SDs (4-8 mice per group from at least 3 independent experiments). *Clod*, Clodronate.

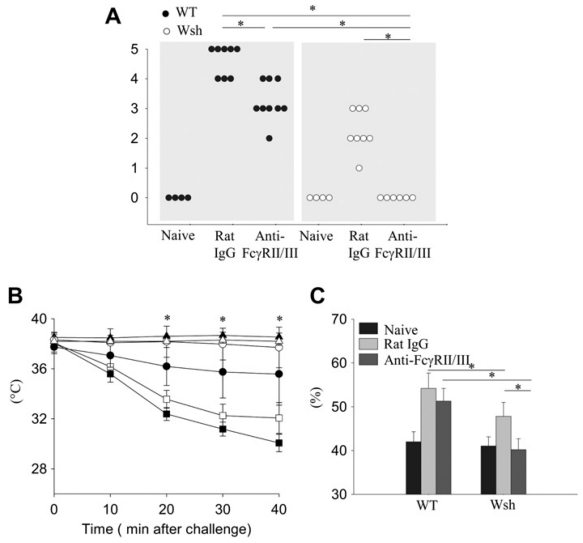

FIG 7. Synergism between MCs and other FcγRIII-expressing cells in the elicitation of PIA. **A**, Peak anaphylactic symptom score of each mouse. **B**, Rectal temperature of naive (WT, —▲—; $Kit^{W\text{-}sh}/^{W\text{-}sh}$ *[Wsh]*, —△—) and sensitized (WT/rat IgG, —■—; WT/ anti-FcγRII/III, —□—; Wsh/rat IgG, —●—; Wsh/anti-FcγRII/III, —○—) mice. **P* < .05, Wsh/anti-FcγRII/III versus Wsh/rat IgG. **C**, Hematocrit percentage. Fig 7, *B* and *C*, Data are presented as means ± SEMs (3-9 mice per group from at least 2 independent experiments).

greater than 15 kU/L but poor for levels of less than this.[37-39] Lastly, Scott-Taylor et al[40] have recently reported increased levels of food-specific IgG1 in 23 children with multiple food allergies compared with those seen in 20 healthy control subjects. Interestingly, levels of food-specific IgG1 in sensitized children correlated with cytokine responses as an indication of T_H2 polarization, a central marker in atopy. As the authors state, compared with IgE, "much less is known about the role of other immunoglobulin classes."

A clinically relevant observation in our study is that anaphylaxis can occur in the absence of histamine, indicating that other vasoactive mediators mediate PIA. In human subjects circulating

PAF levels correlate with the severity of anaphylactic reactions to peanut. In mice blockade of PAF activity significantly reduces the severity and duration of peanut-induced anaphylactic reactions, whereas isolated H1/H2 pharmacologic blockade has no effect.[17] Other studies have demonstrated that blockade of PAF partially inhibits IgE-mediated anaphylaxis but fully prevents IgG-mediated anaphylaxis, suggesting a greater role of PAF in IgG- than in IgE-dependent anaphylaxis. Furthermore, macrophages and basophils contribute to IgG-mediated anaphylaxis by producing PAF.[13,14] These observations suggest that PAF, which is produced largely by macrophages and basophils activated through the IgG pathway, might substantially contribute to the severity of PIA. This notion would explain why the single elimination of MCs, macrophages, or basophils prevented end-point events.

Our data support the conjecture that PIA is a complex process in which distinct immune pathways act in concert to bring about a full range of pathological responses. Our findings suggest that whereas the classical pathway plays a prominent role, progressive recruitment of additional pathways expand clinical expression, culminating ultimately in death. Thus therapeutic strategies to fully prevent peanut-associated anaphylactic reactions might need to target several pathways or alternatively shared components within these pathways. This construct might have clinical and therapeutic implications to human subjects because there is no reason to think that the complexity of food-induced anaphylaxis in human subjects is any less than in mice.

We thank Marie Colbert for secretarial assistance and gratefully acknowledge Julia Cahenzli and Jeanette Boudreau for technical assistance. We thank Cecilia Berin for critical review of the manuscript.

Key Messages

- **Single depletion of MCs, macrophages, or basophils prevents death from PIA. Deletion of both MCs and phagocytes, but not MCs and basophils, fully prevents anaphylactic reactions.**
- **Both IgE and IgG1 signaling contribute to peanut-induced anaphylactic reactions. MC responses occurred through IgE and IgG, whereas phagocyte responses were fully mediated through IgG1.**

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Correction

With regard to the April 2011 article entitled "House dust mite sublingual immunotherapy: Results of a US trial" (J Allergy Clin Immunol 2011;127:974-81.e7), Mary Morris, MD, was inadvertently omitted as an author. Dr Morris should have been included as the second-to-last author in the author list. She is affiliated with Allergy Associates of LaCrosse, Onalaska, Wisconsin, and discloses that she is a shareholder and medical director of Allergychoices.

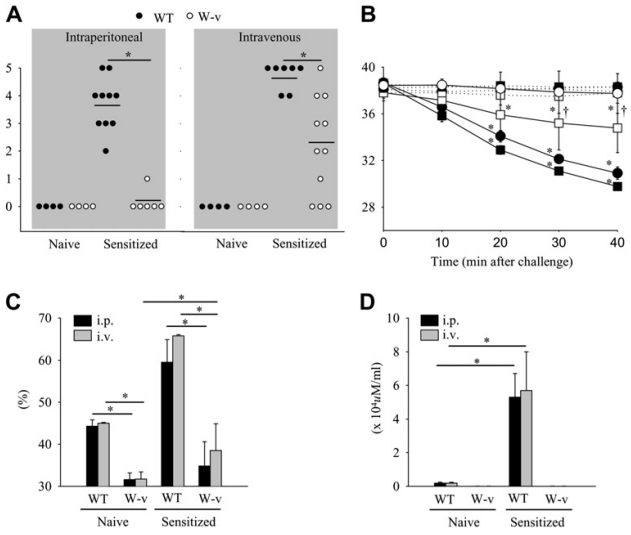

FIG E1. Partial abrogation of PIA in MC-deficient *Kit^{W}/Kit$^{W\text{-}v}$ (W-v)* mice after intravenous but not intraperitoneal challenge. **A**, Peak anaphylactic symptom scores of each WT and W-v mouse. **B**, Rectal temperature of naive (intraperitoneal: WT, —●—; W-v, —○—; intravenous: WT, —■—; W-v, —□—) and sensitized (intraperitoneal: WT, —●—; W-v, —○—; intravenous: WT, —■—; W-v, —□—) mice. **P* < .05 relative to corresponding naive mice. †*P* < .05 relative to corresponding WT control animals. **C** and **D**, Hematocrit percentage (Fig E1, *C*) and histamine levels (Fig E1, *D*). Fig E1, *B* through *D*, Data are presented as means ± SDs (4-11 mice per group from at least 2 independent experiments).

— Chapter 5 —

DISCUSSION

The aim of this chapter is to recapitulate the major findings presented in the preceding chapters and to discuss them in the context of the current literature. The significant results and discoveries have been discussed in each individual manuscript included in this thesis. Accordingly, this chapter will elaborate on the overall significance and implications of the research contained herein, as well as the limitations and potential areas for improvement.

Peanut allergy is typically lifelong, often severe and potentially fatal. The past decade has uncovered considerable knowledge about the factors that promote and regulate sensitization and allergenicity of peanut proteins. Comparatively, the cellular and molecular basis underlying the most severe manifestation of peanut allergy, namely anaphylaxis is less understood. Consequently, no innovative treatments have been developed and, indeed, therapy remains limited to epinephrine. The reason for this may stem, at least in part, from the difficulties associated with conducting clinical studies in peanut allergy, and also to the unavailability of appropriate model systems, which only recently have been emerging. The work presented in this thesis focused on the development and characterization of a mouse model of PIA (Chapter 2) and the use of this experimental platform to investigate potential therapeutic targets (Chapter 3) as well as immune-effector pathways leading to this condition (Chapter 4).

Mouse model of peanut allergy/anaphylaxis

Initial research on peanut allergy/anaphylaxis was conducted in mice of a C3H/HeJ and, to a lesser extent, BALB/c genetic backgrounds. Unfortunately, these strains significantly limit the use of genetically modified mice to explore immune mechanisms underlying disease. Thus, a major implication of the model we developed in a C57BL/6 genetic background was the opportunity to use many mutant strains to investigate immune-effector pathways leading to PIA. This is clearly exemplified in Chapters 1 and 4 of this thesis. For instance, B cell-, CD40L- and IgG_1- deficient mice, all of which are available only on a C57BL/6 background, allowed us to outline the contribution of immunoglobulins to PIA. Other studies showing the critical role of C-type lectin receptor, SIGNR1, in a model of food allergy necessitated the use of mice on a C57BL/6 background [36]. Thus, a mouse model of peanut allergy/anaphylaxis in a C57BL/6 background provides a notable experimental advantage.

The model we established in C57BL/6 mice exhibits central clinical and biological features common to those observed in models using other strains of mice. In agreement with reports in C3H/HeJ [89] and BALB/C [90], oral peanut sensitization in C57BL/6 mice led to systemic antigen-specific Th1- and Th2-associated cytokine responses as well as polyisotypic humoral responses in serum. Furthermore, anaphylaxis was characterized by a rapid onset of clinical symptoms, significant hypothermia and increases in hematocrit, marked mast cell degranulation and pronounced vascular leakage.

In short, the phenotype observed in C57BL/6 is remarkably similar to that observed in other strains of mice.

That a large body of research in peanut allergy has been conducted in C3H/HeJ mice may be explained, at least in part, to the increased susceptibility of this strain to develop anaphylactic responses. In this regard, we made some intriguing and relevant observations not reported in the body of this thesis. For example, the number of peritoneal and skin (i.e. ear) mast cells (c-kit^{+}IgE^{+}) is about three times greater in C3H/HeJ mice compared to C57BL/6 mice. Similarly, the baseline levels of total IgE as well as the expression of FcεRI on peritoneal mast cells are significantly higher in C3H/HeJ than in C57BL/6 mice. These differences in mast cells (number and function) and IgE responses may influence the increased sensitivity of C3H/HeJ to anaphylaxis. Whether this phenotype in C3H/HeJ resembles, or not, steady baseline conditions in humans is unknown, but it may point out to the existence of a sub-phenotype in particularly sensitive individuals with peanut allergy.

An important feature of the model we developed is that mice are sensitized through the oral route and challenged intraperitoneally (Chapter 2). This is in contrast to other models (i.e. C3H/HeJ mice) where the oral route is used for both sensitization and challenge. At a first glance, the oral route of challenge would be preferred; however, the conditions required to elicit an anaphylactic response in C3H/HeJ mice deserve attention.

As discussed in Chapter 1, the amount of orally delivered peanut used to trigger anaphylaxis is exceptionally high (i.e. 200 mg per mouse) [45, 46]. In fact, lower doses elicit anaphylactic responses that are inconsistent, mild and, in most cases, not feasible to assess using objective measurements (i.e. drop in body temperature) [43]. Importantly, we observed that C57BL/6 mice sensitized through the oral route and challenged with a low dose of peanut intraperitoneally developed anaphylactic reactions that were consistent, severe and amenable to objective assessment. Furthermore, we suggest that the intraperitoneal route is not an "ackward" route of exposure. Relevant to this point, Dirks *et al* [91] reported that, in humans, peanut absorption occurs in the mouth across the oral or sublingual mucosa reaching systemic circulation rapidly after antigen uptake. From this perspective, a systemic route of challenge in the mouse may be viewed as a route that facilitates immediate access of peanut to the systemic circulation and, thus, a useful experimental approach to study systemic effector mechanisms underlying PIA. The later is comprehensively illustrated in Chapter 4.

A major finding of this thesis was that the absence of the FcεRI α chain (FcεRIα –/– mice) prevented the anaphylactic response only partially (Chapter 2), thus suggesting that other Ig/Fc pathways could contribute to the anaphylactic reaction. However, there is evidence that the absence of the FcεRI α chain leads to upregulation of FcγRIII-dependent mast cell degranulation and anaphylaxis [30]. In other words, potential mast cell activation via FcγRIII could be not true indication that this pathway contributes to anaphylaxis but, rather, an artifact of the genetic deletion of the FcεRI α chain. This

concern was subsequently dismissed by our findings that direct blockade of the FcγRIII signaling was in fact required to fully prevent PIA in IgE-deficient mice (Chapter 4). This is particular relevant as it is increasingly evident that the IgE-dependent pathway, albeit critical, is not the sole pathway leading to human anaphylaxis. For instance, there is clear evidence of a poor correlation between levels of antigen-specific IgE and incidence, severity or threshold of responsiveness in food allergy [92, 93], including peanut allergy [94, 95]. In addition, anti-IgE therapy increases the threshold of reactivity but does not fully prevent anaphylactic reactions to peanut. Moreover, at least 25% of the patients experienced no change in their threshold of reactivity [52]. Collectively, these findings cast doubt to the notion that the IgE/FcεRI pathway is the single driving effector mechanism in PIA and strongly suggest that both FcεRI– and FcγRIII–mediated responses contribute to this process.

PAF: a therapeutic target in anaphylaxis

One of main objectives of this thesis was to investigate the role of molecules including histamine, leukotrienes and PAF in PIA (Chapter 3). Indeed, the contribution of most molecules produced during anaphylaxis to its pathogenesis remains undefined. This is, to a great extent, because anaphylaxis in humans is generally an unanticipated and life-threatening event requiring emergency management. Here, we determined whether each of the molecules above was implicated with the presence, severity and/or persistence of PIA in the mouse.

A number of studies have documented the presence of leukotrienes and their metabolites in body fluids in the course of anaphylaxis in humans. For instance, two studies have reported that urinary LTE4 concentrations significantly increase within the first few hours following anaphylaxis with an immediate decline during clinical remission [77, 78]. Interestingly, one of these studies reported that LTE4 levels were considerably higher in patients with anaphylactic shock (i.e. hypotension with systolic blood pressure less than 90 mmHg; n=6/32) than in those without [78]. In accordance with this, we observed that the levels of CysLTC in mice undergoing PIA steadily increased following challenge, reached a peak at 40 min, and gradually waned within 2-3 h post-challenge. While these findings suggest a contributing role, we showed that disruption of the 5-LO pathway, via either pharmacological blockade or targeted mutation in the 5-LO gene, did not ameliorate the clinical manifestations of PIA. These data suggest that leukotrienes may serve as clinical biomarkers but do not mediate the anaphylactic event.

In sharp contrast to our observations following the blockade of the 5-LO pathway, we found that impairing PAF activity (i.e. PAFR antagonism) significantly prevented prolonged and life-threatening anaphylactic reactions to peanut (Chapter 3). These findings are particularly relevant as studies in humans showed that the serum levels of PAF closely *correlate* with the severity of food-induced anaphylaxis, including PIA [88]. However, this was a retrospective study. In this context, our study provided for the first time direct evidence of the causative role of this molecule in PIA. An interesting finding in this study was that PAFR antagonism was accompanied by a significant reduction in

blood levels of histamine and CysLT. One possible interpretation of this finding is that PAF activates mast cells, or other effector cells, *in vivo*, thus resulting in the release of these, and likely other, effector molecules. In this regard, Kajiwara *et al* [96] recently reported that PAF induces the release of histamine as well as other inflammatory mediators (e.g. PGD_2, IL-8, etc) from human primary lung and peripheral blood-derived mast cells (PBMCs). Moreover, PAF has been reported to be one of the few mediators with the potential to quickly (within minutes) raise vascular permeability in the pulmonary circulation [82]. Together, these observations support the concept that PAF may mediate an amplification loop involving mast cell activation. For example, PAF-mediated activation of airway mast cells may contribute to bronchospasm and laryngeal edema. Importantly, the ability of PAF to stimulate degranulation is not restricted to mast cells. Indeed, *in vitro* studies have shown that PAF induces histamine release from human basophils of both normal and allergic subjects [97]. Interestingly, *ex vivo* studies have demonstrated that PAF induces contraction of smooth muscle cells isolated from guinea pig ileum [98]. On the basis of these findings, it is plausible that PAF contributes to the severity of anaphylaxis through direct effects on the vasculature and smooth muscle as well as indirect effects involving the stimulation of several amplification loops.

In Chapter 3, we also showed that antihistamine (H1 and H2) treatment did not significantly impact the severity of anaphylactic reactions to peanut. However, we observed that concomitant blockade of PAF and histamine receptors led to greater beneficial effects than PAFR antagonism alone. The idea that concurrent targeting of

mediators is required to successfully control anaphylactic reactions has been previously proposed. For example, studies in a mouse model of PSA showed that mast-cell-derived histamine controls body temperature and respiratory function (i.e. frequency and expiration time) but has negligible effects on the regulation of blood pressure [99]. In other words, inhibition of several mediators, released by mast cells and, likely, other effector cells, may be necessary to express the full spectrum of the pathophysiology underlying an anaphylactic reaction.

The findings reported in Chapter 3 have significant implications. PIA is a life-threatening event in dire need of therapeutic options. Targeting PAF and, possibly, other mediators concurrently presents a therapeutic option that not only has an inherent value but can also be envisioned as a risk-reducing approach that facilitates the implementation of other therapeutic strategies (i.e. conventional immunotherapy).

Pathways of PIA

It is becoming increasingly evident that the cellular and molecular basis of anaphylaxis is heterogeneous, i.e. different pathways might have different contributions depending on the trigger (PenV, OVA, food antigens…). In this context, the research in Chapter 4 provided a comprehensive and integrated investigation of the role of mast cells, basophils and macrophages as well as IgE and IgG_1 to PIA. These studies make a significant contribution to our understanding of the effector mechanisms underlying PIA and convey a message that also carries therapeutic implications

A number of effector cells have been proposed to contribute to ASA. Tsujimura *et al* [33] reported that absence of basophils rescued mast cell-deficient mice from death associated with PenV-induced ASA. However, depletion of basophils did not fully prevent hypothermia in these mice or affect mortality in WT mice, thus suggesting the involvement of additional effector cells in this model. Most recently, Jönsson *et al* [35] showed that depletion of basophils prolonged survival but did not significantly avoid fatalities in a model of BSA-induced ASA. Conversely, absence of neutrophils abolished death and had a significant effect on hypothermia. Of note, concomitant deficiency of neutrophils and basophils had a greater beneficial effect on core body temperature drop. Interestingly, absence of these two populations along with mast cells did not completely prevent the fall in core temperature. These studies support the notion that several effector cell types contribute to ASA. In this regard, studies in the late 19th century suggested that macrophages might contribute to this process. In essence, they showed that FcγRIII-dependent systemic anaphylaxis can occur in the absence of mast cells [30, 31]. Since monocyte/macrophages represent potential sources of many biologically active mediators and were, at the time, the only cell type known to express FcγRIII, the authors proposed a contributing role of macrophages in the pathogenesis of anaphylaxis. This conjecture was later explored in a particular model in which mice were immunized with a goat anti-mouse IgD antibody and then challenged with antigen (goat IgG). Under these experimental conditions, anaphylaxis was largely dependent on macrophages [38]. Thus, the extent to which individual effector cell types contribute to anaphylaxis may vary depending on the experimental system, ranging from little, if any, to considerable.

Collectively, the implication from these studies is that anaphylaxis can occur via several pathways and that blockade of one pathway is not sufficient to prevent ASA.

In Chapter 4 we provided evidence for the first time that distinct pathways cooperate to elicit a broad range of systemic reactions to peanut, thus suggesting that complete inhibition of anaphylaxis requires targeting multiple pathways. In particular, we reported that the single elimination of mast cells, macrophages or basophils prevented mortality associated with PIA; however, concomitant absence of mast cells and macrophages, but not of mast cells and basophils, abrogated the full spectrum of anaphylactic reactions. Our study also showed that while mast cell responses occured via IgE and IgG_1, macrophage responses were fully mediated through IgG_1. On this basis, we demonstrated that blockade of FcγRIII signaling in the context of either IgE or mast deficiency fully abrogated anaphylactic reactions to peanut. This illustrates that full inhibition of PIA requires targeting several cellular and/or humoral effector components.

In particular reference to food allergy, it is increasingly clear that the IgE pathway is likely not the only immunoglobulin pathway involved. In this regard, we reported that IgG_1 (or FcγRIII) plays a significant role in PIA. Of relevance, our data indicate that the contribution of IgG_1 is most significant at late time points Interestingly, This is particularly significant as the presence of peanut-specific IgG_1 has been repeatedly found in the serum of peanut allergic individuals [100, 101]. The significance and implications of our findings can be summarized as Lowell, CA recently pointed out: “Therapeutically,

this could translate into more effort put toward blocking IgG or FcγR binding … in patients with severe allergy" [102].

Summary and Concluding Thoughts

In summary, the research documented in Chapters 2-4 of this thesis has centered on the identification of effector components leading to PIA and the study of their relative contribution to the pathophysiological responses associated with anaphylaxis. The underlying goal of this research was to inform future therapeutic strategies.

The initial objective was to develop a mouse model of PIA that mimicked physiologic and immunologic features of peanut allergy in humans. This model allowed us to investigate key immune-effector pathways leading to anaphylactic reactions to peanut. Similarly, we were able to identify potential therapeutic targets to prevent or ameliorate anaphylaxis. Lastly, this model has also served as an experimental platform for other investigators in our lab to examine immunological mechanisms involved in sensitization to peanuts. Thus, the availability of this mouse model of PIA has and will continue to advance our understanding of the mechanisms underlying peanut allergy and anaphylaxis.

The work presented in Chapter 3 pertaining to the role of histamine, leukotrienes and PAF in PIA illustrate three important have important messages: 1) the presence of a

molecule does not imply a causative role; 2) the potentially remarkable benefit of targeting PAF in humans; and 3) the importance of identifying targets that act as a 'common checkpoints' in distinct effector pathways. The studies showing that *concomitant* or *progressive* recruitment of immune-effector pathways are involved for the full expression of anaphylactic reactions reveals the complex nature of this process. This understanding brings to fore two central messages, both of which have been recently illustrated in the Journal of Clinical Investigation. First, it is evident that " …somebody has to sit down and update Wikipedia…" on the classical teaching of anaphylaxis; second, "…we have to reevaluate our models for anaphylaxis in humans, which will have a direct impact on our therapeutic approaches for the prevention of this potential deadly hypersensitivity reaction" [102]. Indeed, the idea of complexity leads to the recognition of heterogeneity, understood as the existence of distinct immune-effector pathways able to converge onto a final common event, anaphylaxis. This concept, we think, has direct therapeutic implications.

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(FOR CHAPTERS 1 AND 5)

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— Appendix I —

Management of Food-induced Anaphylaxis: Unsolved Challenges

Management of Food-Induced Anaphylaxis: Unsolved Challenges

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Abstract: Anaphylaxis is an acute and often severe systemic allergic reaction. The prevalence of food allergy has been increasing and is currently estimated at ~3.5%. Food allergic reactions account for one-third to one-half of anaphylaxis cases worldwide. It is estimated that approximately 30,000 food-related anaphylactic reactions are treated in United States emergency departments (ED) every year resulting in ~ 2000 hospitalizations and 150 deaths. The increasing rate of food-induced anaphylactic episodes in the last few decades underlines the existence of major challenges. This review will critically appraise current guidelines for the diagnosis as well as the acute and long-term management of food-induced anaphylaxis (FIA). Importantly, it will outline existing challenges and suggest measures to improve outcomes in patients with FIA. We propose that the discovery of novel diagnostic (i.e. biomarkers and predictors) and therapeutic approaches is a major challenge that may be overcome as the mechanisms underlying FIA are better delineated. We further propose that better dissemination, implementation and compliance with the consensus management guidelines are urgently needed. This will require education of ED personnel, patient empowerment as well as effective multilateral communication among patients, emergency and family physicians, allergists and specialized volunteer organizations.

Key Words: Food allergy, anaphylaxis, acute management, long-term management, measures.

INTRODUCTION

Anaphylaxis is an acute, often severe and potentially life-threatening systemic allergic reaction [1, 2]. It is a medical emergency that requires immediate intervention, yet paradoxically, a stepwise management approach with evidence-based guidelines is still to be in place. Indeed, most of the current anaphylaxis guidelines are based on ''clinical experience'' rather than randomized controlled trials. Similarly, the implementation of guideline recommendations represents a significant challenge in anaphylaxis.

This review will evaluate the latest literature on the epidemiology, clinical presentation and management of anaphylaxis, with particular emphasis on food-induced anaphylaxis (FIA). Importantly, it will highlight the suggested guidelines and existing challenges in the management of FIA. Since attention is often focused on managing the acute episode, this review will also appraise long-term management plans that may help improve outcomes in patients with FIA.

ANAPHYLAXIS

Definition and Epidemiology

Anaphylaxis is an old condition. The first documented evidence of anaphylaxis may have been in 2641 BC when the Egyptian pharaoh Menes suddenly died from a reaction to a wasp sting. However, it was not until 1902 that Portier and Richet formally coined the term "anaphylaxis" to describe a systemic shock reaction unexpectedly induced by immunization. The lack of a universally accepted definition of anaphylaxis has hampered research on the epidemiology, pathophysiology and management of this disorder. In an effort to clarify these concepts, the Second National Institute of Allergy and Infectious Diseases (NIAID) and Food Allergy and Anaphylaxis Network (FAAN) Symposium recently recommended the following definition: "Anaphylaxis is a serious allergic reaction that is rapid in onset and may cause death" [1]. There are 3 proposed clinical diagnostic criteria (Box **1**). Anaphylaxis is highly likely when any of the 3 criteria is fulfilled following exposure to an allergen. Note that as the majority of anaphylactic reactions include cutaneous manifestations [3-7], criterion 1 likely allows for the identification of most cases. However, such manifestations might be absent in up to 20% of anaphylactic reactions, particularly in children reacting to foods or insect stings [8-10]. In these cases, an allergic history and knowledge of probable exposure, along with symptoms consistent with criterion 2 would establish the diagnosis. Criterion 3 would identify the rare subset of patients experiencing acute hypotension following exposure to a known allergen [11]. The NIAID/FAAN Symposium acknowledged that no set of criteria will provide 100% sensitivity and specificity; however, there was agreement that the aforementioned criteria should accurately identify anaphylactic reactions in more than 95% of cases. Nonetheless, this proposal needs to be validated by a prospective multicenter clinical trial to determine if further refinements are needed.

Because anaphylaxis has been carefully defined only relatively recently, the "true occurrence rate" of anaphylaxis remains uncertain. Also since the symptoms of anaphylaxis mimic those of other conditions, anaphylaxis is felt to be underdiagnosed and/or underreported [12]. A recent review of available epidemiological data estimated the incidence of anaphylaxis to be approximately 50 to 2000 episodes per 100,000 persons, with a lifetime prevalence of 0.05 to 2% [13]. Of great concern, 600-800 deaths each year in the United

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Anaphylaxis is likely when any one criterion is fulfilled	
Criterion 1	Acute onset of an illness (minutes to several hours) with involvement of the skin and/or mucosal tissue (eg. generalized hives, pruritus, or flushing, swollen lips/tongue/uvula) and at least one of the following: ▪ Respiratory compromise (eg. dyspnea, wheeze/bronchospasm, stridor, reduced PEF, hypoxemia) ▪ Reduced BP or associated symptoms of end-organ dysfunction (eg. hypotonia [collapse], syncope, incontinence)
Criterion 2	Two or more of the following that occur rapidly after exposure to a likely allergen for that patient (minutes to several hours): ▪ Involvement of the skin/mucosal tissue (eg, generalized hives, itch/flush, swollen lips/tongue/uvula ▪ Respiratory compromise (eg, dyspnea, wheeze/bronchospasm, stridor, reduced PEF, hypoxemia) ▪ Reduced BP or associated symptoms (eg, hypotonia [collapse], syncope, incontinence) ▪ Persistent gastrointestinal symptoms (eg, crampy abdominal pain, vomiting)
Criterion 3	Hypotension after exposure to a known allergen for that patient (minutes to several hours): ▪ Infants and children: systolic BP <70 mm from 1 month to 1 year, [<70 mm + (2 x age)] from 1 to 10 years and <90 mm from 11 to 17 years. ▪ Adults: systolic BP <90 mm or greater than 30% decrease from that person's baseline BP.
A less common presentation includes sudden isolated hypotension without a known allergen exposure. Additional signs and symptoms that may occur during anaphylaxis are provided in Table 1. PEF: peak expiratory flow; BP: blood pressure Adapted from Sampson et al [1].	

Box 1. Clinical criteria for the diagnosis of anaphylaxis.

States are attributed to anaphylaxis [14, 15], the majority of which may be potentially preventable.

Pathogenesis

When originally described in 1902 [16], understanding of the events occurring between allergen contact and the manifestation of anaphylaxis was enigmatic. In recent years, studies in animal models have provided important insights into the underlying molecular mechanisms of anaphylaxis [17, 18]. It appears that both immunological and nonimmunological mechanisms are involved. The distinction between these basic mechanisms does not affect the clinical diagnosis and acute treatment of anaphylaxis, but it may be extremely relevant for long-term risk reducing measures.

A critical, and perhaps the most rigorously investigated, immunological mechanism involves the immunoglobulin E (IgE)/FcεRI pathway. IgE is synthesized in response to allergen exposure and binds to FcεRI, a receptor on the surface membrane of mast cells and basophils. Aggregation of receptor-bound IgE molecules on re-exposure to the allergen results in cell activation, mediator release, and the immediate hypersensitivity response. IgE may also contribute to anaphylaxis through mechanisms other than priming and activation; for example, it enhances expression of FcεRI on mast cells and basophils [19, 20]. This may sensitize target cells with larger numbers of IgE molecules increasing cross-linking of FcεRI and, thus, responsiveness.

In addition to FcεRI, receptors such as G protein–coupled receptors [21, 22] and Toll-like receptors [23] have also been implicated in anaphylaxis. Additional immunological factors thought to be involved in anaphylaxis include immune aggregates, IgG, IgM and platelets; a shift in eicosanoid metabolism toward leukotriene formation; neuropeptidase release (substance P) and activation of the complement (C5a and C3a) or coagulation systems [24, 25]. However, the precise mechanisms by which these products may participate in the anaphylactic reaction remain to be largely elucidated. It is highly probable that more than one mechanism is involved in a given event [26]. In addition, in idiopathic anaphylaxis, FcεRI receptors may be aggregated through autoimmune mechanisms (i.e. presence of anti-IgE autoantibodies). Particularly, anti-IgE autoantibodies may act on mast cells to crosslink the FcεRI or IgE bound to this Fc receptor. Furthermore, an imbalance between histamine-releasing factors and histamine-releasing inhibitory factors, and a greater number of activated T and B cells have also been proposed as potential contributory mechanisms [27, 28]. However, there is poor evidence and understanding of the role of these

in the pathogenesis of idiopathic anaphylaxis. Nonimmunologic factors that can activate mast cells by mechanisms not yet fully understood include exercise, cold air or water exposure, radiation, ethanol, insect venom constituents, radiocontrast media, and medications such as COX-1 inhibitors, opioids and vancomycin [24].

Whether the triggering mechanisms are immunological or nonimmunological, mast cells and, likely, basophils play a central role in initiating and amplifying the acute allergic response. Once activated, the mast cell response is regulated by a balance of positive and negative intracellular molecular events involving Fc receptors and cell-associated tyrosine kinases and phosphatases. The role of these receptors as well as their potential as therapeutic targets has been comprehensively reviewed [29-31]. Differential activation of intracellular signals results in the release of several mediators, including vasoactive amines (e.g., histamine and serotonin), proteases such as tryptase, mast cell carboxypeptidase A3 and chymase, lipid-derived mediators such as platelet-activating factor (PAF), prostaglandins (PGD_2), and leukotrienes (LTC_4), as well as chemokines and cytokines. These mediators act on target cells to increase smooth muscle contraction, mucin secretion, vascular permeability and vasodilation, resulting in the cardinal clinical features of anaphylaxis including laryngeal edema, airway obstruction, hypotension, tachycardia, vascular leakage and hypothermia [24, 25]. Importantly, the extent of mediator release is thought to closely correlate with the severity and persistence of the anaphylactic reaction [24]. However, further basic and clinical research is needed to uncover the relative and specific contribution of individual mediators to the severity of the reaction. Clearly, this would be of great therapeutic utility.

While we now appreciate the role of IgE, Fc receptors and regulatory pathways upstream and downstream of IgE, we are not much further ahead than a century ago in uncovering the mediators and specific pathways that distinctively lead to anaphylaxis. Indeed, the basic processes involved in triggering mediator release from mast cells and basophils do not distinguish anaphylaxis from other allergic responses such as sneezing, itchy eyes or wheezing. A better understanding of the distinct mechanisms leading to anaphylaxis is evidently needed.

Clinical Presentation

Clinical Features

Anaphylaxis often affects several organ systems. Symptoms include, but are not limited to, pruritus, flushing, urticaria, angioedema, dyspnea (bronchospasm or upper airway swelling), gastrointestinal symptoms (e.g., nausea, vomiting, diarrhea and abdominal cramps), syncope and hypotension (Table **1**) [3]. Persistent gastrointestinal symptoms and/or hypotension have been associated with severe outcomes [5, 8]. Notably, markedly increased vascular permeability, a hallmark of anaphylaxis, may allow the transfer of 50% of the intravascular fluid into the extravascular space within 10 minutes in the absence of cutaneous or respiratory manifestations, hence leading to rapid hemodynamic collapse [32, 33]. Other determinants of severe outcomes will be discussed later. A number of differences have been documented between children and adults. Anaphylaxis is most frequently caused by injected drugs and insects venom in adults. In contrast, foods are the most common triggers in children, likely due to the greater prevalence of food allergies in this age group [34]. Interestingly, although allergic reactions are more common in children, adults experience anaphylaxis more often [4]. Regarding the clinical expression, children often experience respiratory symptoms, whereas adults frequently have cardiovascular compromise [8]; the reasons for this are uncertain but a higher frequency of co-morbid conditions in adults may be a significant contributory factor.

Table 1. Clinical Features of Anaphylaxis

Organ System	Signs and Symptoms
Skin/mucosal tissue	Urticaria, hives, angioedema, pruritus, flushing, morbilliform rash, swelling of lips, tongue or palate
Respiratory	Upper airway – sneezing, rhinorrhea, stridor Lower airway – cough, wheeze, dyspnea, chest cough, cyanosis
Cardiovascular	Tachycardia, arrhythmia, syncope, hypotension, shock
Gastrointestinal	Metallic taste in the mouth, nausea, vomiting, trouble swallowing, abdominal cramps, diarrhea
Neurologic	Anxiety, headache, seizure, syncope, loss of consciousness
Ocular	Pruritus, conjunctival injection, lacrimation
Hypotension	Systolic blood pressure < 100 mm Hg

Differential Diagnosis

Many signs and symptoms of anaphylaxis overlap with other diseases and conditions [35]; examples are provided in Table **2**. Cutaneous manifestations may be a result of acute or chronic urticaria while respiratory symptoms are seen in asthma exacerbations, foreign body aspiration (especially in children) or vocal cord dysfunction. Other conditions to rule out include acute anxiety (e.g., panic attack or hyperventilation syndrome), myocardial dysfunction, pulmonary embolism, systemic mast cell disorders, food poisoning (in particular from scombroid fish), hypoglycaemia and seizure disorder [36]. A diagnosis of anaphylaxis should be considered in any patient presenting with unexplained syncope or shock.

Symptom Evolution and Biphasic Reactions

The onset of symptoms of anaphylaxis after exposure ranges from within seconds to a few hours, but the majority of reactions manifest within 1h of exposure to the causal allergen [37]. In general, the longer it takes for anaphylactic symptoms to develop, the less severe the overall reaction. Reactions to ingested allergens have a slower onset than in-

Table 2. Differential Diagnosis of Anaphylaxis

Presentation	Differential Diagnosis
Cutaneous symptoms	▪ Acute generalized urticaria and/or angioedema
Hypotension	▪ Shock: septic, cardiogenic, hypovolemic ▪ Vasovagal reaction
Respiratory distress with wheezing or stridor	▪ Airway foreign body ▪ Asthma and COPD exacerbation ▪ Bronchiolitis ▪ Laryngospasm ▪ Vocal chord dysfunction syndrome ▪ Asphyxiation/suffocation
Postprandial collapse	▪ Airway foreign body ▪ Monosodium glutamate, saurine or sulfite ingestion ▪ Scombroid fish poisoning
Flush syndrome	▪ Carcinoid syndrome ▪ Postmenopausal hot flushes ▪ Red man syndrome [infusion of vancomycin] ▪ Medullary carcinoma of the thyroid ▪ Tumor secreting VIP or substance P
Miscellaneous	▪ Panic attacks ▪ Increased intracranial pressure ▪ Systemic mastocytosis ▪ Leukemia with excess histamine production

COPD, chronic obstructive pulmonary disease; VIP, vasoactive intestinal peptide Modified from Tang, AW [35].

jected allergens. In fact, while fatal reactions due to foods can occur within 25-35 min of ingestion, the median time interval for fatal anaphylaxis to injected drugs and insect venom ranges from 5 to 12 min [34]. A particular concern is that once initial symptoms have resolved, some patients may later experience a second phase of symptoms [38-40]. The occurrence of biphasic reactions has been reported to vary between 3 and 20% (Table **3**). The interval between the initial and the second reaction is highly variable, from 1 to 78 h, although the majority occur within 8 h of the initial presentation. The severity of these secondary reactions is also highly variable, being either more or less severe than the initial reaction [41]. Currently, there is no consensus on what features of the primary response might predict the occurrence of a secondary reaction. However, it is thought that the severity of the initial reaction, the delayed onset of symptoms, the presence of hypotension or laryngeal edema, a history of a previous biphasic reaction and, most importantly, delayed administration, inadequate dosing, or need for large doses of epinephrine may be risk factors for a secondary reaction [41-44]. Additionally, there is no agreement on the optimal observation time following the initial reaction. In this regard, the NIAID/FAAN Symposium recommended that observation periods be individualized according to the severity of the initial reaction, reliability of the patient and access to care [1]. For patients whose initial reaction is less severe, four to six hours of observation is reasonable, but extended observation times or hospital admission is advisable for patients with severe or refractory symptoms. Patients with asthma might also need extended care as most fatalities occur in this group of patients.

Risk Factors for Anaphylaxis

A detailed clinical history of any patient experiencing anaphylaxis is a crucial step in the determination of future anaphylactic reactions. Individuals with a history of anaphylaxis are at increased risk of a subsequent episode [11, 45, 46]. Similarly, it is thought that severe past reactions predispose to future severe reactions [47]. In contrast, there is evidence that children with mild reactions may suffer severe reactions later on [48]. A history of asthma is considered a major risk factor for life-threatening anaphylactic reactions to food [11, 37, 46, 49, 50]. Even though life-threatening anaphylaxis has been reported in children without asthma most fatal cases of anaphylaxis occur in patients with asthma [10, 34, 51]. Thus, a history of previous anaphylactic reactions or coexistent asthma can identify food-allergic individuals at higher risk of *severe* anaphylaxis.

A number of factors may increase, not necessarily the risk of anaphylaxis, but the severity of anaphylaxis or the efficacy of epinephrine. These include the use of aspirin, β-blockers, angiotensin converting enzyme inhibitors, monoamine oxidase inhibitors, tricyclic antidepressants and concurrent infection [34, 52]. Therefore, patients at high risk for anaphylaxis should, when possible, avoid these drugs.

FOOD ALLERGY

Food allergy is the most frequent cause of outpatient anaphylaxis [7, 37, 53, 54]. Despite the severity of the reactions that they elicit and the apparently rising prevalence over the past decades, the epidemiology of food allergies remains uncertain [4, 12, 55, 56]. This is likely due to the lack of standardized population-based methodologies that incorporate the gold-standard diagnostic method, a double-blind, placebo-controlled oral challenge. Recently, a meta-analysis of 51 studies examining the prevalence of food allergy, particularly to milk, egg, peanut and seafood showed a wide variation in prevalence estimates [57]. For instance, the prevalence of self-reported allergy to any food ranged from 3 to 35%. This variation likely reflects the shortcomings of self reporting, dissimilar definitions of food allergy and true population heterogeneity. Similarly, approaches such as specific IgE levels and/or positive skin prick test (SPT) or self-reported food allergy along with specific IgE levels or positive SPT also showed broad ranges in prevalence. It is noteworthy that specific IgE levels and/or SPT cutoff values might not accurately predict clinical reactivity. Studies based on oral food challenges were few in this meta-analysis, but

Table 3. Biphasic Reactions

Study	No. of Patients with Biphasic Reactions/ Total No. of Patients	Frequency of Biphasic Reactions (%)	Time from Initial to Biphasic Reaction (h)
Brady *et al.* [38]	2/67	3	26-40
Brazil and MacNamara [39]	6/34	18	4.5-29.5
Ellis and Day [40]	20/103	19	2-38
Lee and Greenes [43]	6/105	6	1.3-28.4
Smit *et al.* [44]	15/282	5	1-23
Douglas *et al.* [99]	6/13	6	1-72
Stark and Suvillan [100]	5/25	20	1-8

Adapted from Lieberman, P [41].

prevalence estimates from 6 such studies that evaluated allergy to ''any food'' ranged from 1% to 10.8%, with a random-effects model showing a prevalence of ~3.5% [57]. The range of prevalence could reflect genuine variation between populations but it may have been influenced as well by differences in study design and/or methodology.

The prevalence of food allergy is greatest in the first 2 years of life and declines with age [58]. The most common food allergens causing reactions in children include milk, egg, wheat, soy peanuts, tree nuts, fish and shellfish [7, 49, 59, 60]. Milk and egg allergies are typically considered transient as they are thought to resolve by age 3 to 5 years. However, recent reports have prompted a reconsideration of this view. A retrospective chart review of 807 patients by Skripak *et al.* revealed that milk allergy resolved in 19% of patients by age 4, 42% by age 8, 64% by age 12, and 79% by age 16 [61]. A similar study that included 881 patients with egg allergy showed that resolution rates were 4% by age 4, 12% by age 6, 37% by age 10, and 68% by age 16 [62]. The latter study also reported that individuals with concomitant atopic diseases and higher levels of antigen-specific IgE were less likely to achieve tolerance to these foods. While the majority of children became tolerant, this relatively recent recognition that the acquisition of tolerance may be slow is disturbing. It is relevant to mention that both studies were carried out by the same center (Johns Hopkins University, Baltimore, MD). While the results may partly reflect a selection bias, they clearly underscore the need for continued attention and monitoring of egg and milk allergies well into the teenage years.

Peanut allergy often has an early onset but is typically life-long. Reports indicate that peanut allergy resolves in only ~20% of children by school age [63, 64]. A study on the natural history of peanut allergy in 35 subjects showed 25 with early onset at a median age of 3, and 10 with late onset, at a median age of 25 years with previous tolerance to peanut [65]. Patients with late onset had lower serum peanut-specific IgE levels than those with early onset. While anecdotal, the case of a 53-year-old subject with late-onset peanut allergy warrants attention because she had routinely tolerated peanut but had been instructed to avoid it after a positive skin test response was detected. Three years after strict avoidance, a food challenge elicited lower airway symptoms, suggesting that *unnecessary* avoidance might have resulted in loss of tolerance.

Epidemiological studies suggest that environmental (i.e. diet, frequency and/or onset of food consumption, etc) and genetic factors might influence the epidemiology of food allergies [66-69]. There is geographic variability in the prevalence of food allergies across the globe. For instance, peanut, tree nut and shellfish allergies are common in North America and the United Kingdom [7, 49, 60, 70-72]. Seafood and royal jelly allergies are very frequent in Hong Kong [44], as is mustard allergy in France [67] and sesame allergy in Israel [73].

FOOD-RELATED ANAPHYLAXIS

Food allergic reactions are the leading cause of anaphylactic reactions treated in emergency departments (ED) [4, 37, 44]. They account for one-third to one-half of anaphylaxis cases in ED in North America, Europe, Asia and Australia. Estimates suggest that approximately 30000 food-related anaphylactic reactions are treated in US ED resulting in ~2000 hospitalizations and ~150 deaths [37, 56]. In the paediatric population, food reactions are responsible for a large proportion of anaphylaxis cases [74, 75]. In Lee and Greenes's report of 108 anaphylaxis episodes in children, almost half (n=51) were caused by food allergens; of these, 27% involved tree nuts, 24% peanuts, and 16% were due to seafood [43]. Other types of foods included fruit, eggs, and seeds.

Fatalities in FIA occur in <1% of cases; however, it has been described with first episodes. A study of 202 cases of fatal anaphylaxis in the United Kingdom reported that food allergens accounted for up to 30%, over half associated with nuts [76]. A US study of 32 patients with fatal anaphylaxis showed that peanuts and tree nuts were responsible for 94% of these fatalities, the majority involving adolescent and young adults [49]. Recent case series in the US [59] and the UK [71] indicated that 78% (25/32) and 38% (18/48) of fatal cases were caused by peanut and tree nuts, respectively. These case series also revealed that milk is an important cul-

prit, responsible for 10% of the deaths attributed to FIA. Consistent findings from most of these reports are that fatalities are associated with untimely use of epinephrine, co-existing asthma, and being a teenager/young adult. Distressingly, numerous studies in the US and Europe concur that most individuals who experienced food-related fatalities were aware of their food allergies and/or had a prior history of a reaction to the triggering food [49, 60, 77]. In this regard, accidental ingestion of the culprit food is not uncommon. For example, a study by Vander Leek *et al.* [48] reported that 58% (31/53) of children with peanut hypersensitivity experienced adverse reactions from accidental peanut exposure during a 5-year study period. In addition, Yu *et al.* [72] reported an annual incidence rate of accidental ingestion of 14.3% among schoolchildren in Montréal, Canada.

Diagnostic Tests for Food-Induced Anaphylaxis

A significant challenge in FIA is the lack of a readily available laboratory test to support the clinical diagnosis. It was initially thought that the measurement of mast cell–derived mediators, such as histamine and tryptase, would provide confirmatory evidence of an anaphylactic episode, including anaphylactic reactions triggered by food. In fact, Lin *et al.* [78] reported that increased levels of histamine (>10 nmol/L) correlated, to a great extent, with clinical signs (such as urticaria, extensive erythema, abdominal symptoms, and wheezing) of acute allergic reactions. Nonetheless, elevated levels of histamine were observed in less than 50% of the overall population (97 patients). Plasma histamine levels typically peak within 5 to 10 minutes from the onset of anaphylactic symptoms and, then, decline to baseline within 60 minutes as a result of rapid metabolism by N-methyltransferase and diamine oxidase [79]. This may limit the value of the test in many clinical circumstances as histamine levels may have returned to baseline by the time the patient arrives to the ED.

Similarly, there are concerns regarding the reliability and usefulness of tryptase measurements as evaluation of serum tryptase levels has both low sensitivity and specificity. For instance, Lin *et al.* [78] reported raised levels (>15 ng/mL) in only 20 of 97 patients with acute allergic reactions. Of particular importance, elevations in total tryptase are not commonly detected in individuals with FIA [10] or in those with positive food challenge tests [80]. A possible explanation for this finding is that in some individuals – for example, those whose primary symptom is laryngeal edema – localized rather than generalized mast cell degranulation may predominate, and, hence, the amount of tryptase entering the circulation may be insufficient to raise levels in serum.

It is evident that sensitive and specific biomarkers of anaphylaxis are needed, especially in cases where medical history is not available or symptoms are atypical. New proteomic and/or metabolomic approaches might prove valuable in identifying relevant biomarkers. Given the often fulminant nature of this disease, these biomarkers are unlikely to be useful to guide immediate interventions; yet, they may assist in the identification of patients at risk of persistent or late phase reactions. Recently, the usefulness of mediators such as carboxypeptidase A3 and PAF to support the diagnosis of anaphylaxis has been explored [79]. Of particular interest, a study by Vadas *et al.* [81] highlighted PAF as a potential biomarker of anaphylaxis triggered by foods, medications and insect stings. They observed that circulating PAF levels were significantly greater among patients with anaphylactic reactions (n=41) than in control individuals (n=23) and correlated with the severity of the reaction. In fact, the proportion of subjects with elevated levels increased from 4% in the control group to 20% in the group with grade 1 (acute allergic reaction with cutaneous manifestations but no other organ system involvement) reactions, 71% in individuals with grade 2 (systolic blood pressure, >90 mm Hg; respiratory rate, <25 breaths per minute; and a normal score on the Glasgow Coma Scale) reactions, and 100% in patients with grade 3 (severe manifestations, with cutaneous, gastrointestinal, and potentially life-threatening respiratory or cardiovascular signs and symptoms) reactions.

The analysis of other biomarkers including leukotrienes may also be of value in the diagnosis of anaphylaxis. Recent data from our laboratory using a mouse model of peanut-induced anaphylaxis show that leukotrienes gradually increased following anaphylactic shock and remained elevated for a considerable period of time (manuscript under revision). Importantly, the presence of urinary leukotrienes and their metabolites, particularly leukotriene E4 and N-acetyl leukotriene E4, has been previously reported in patients for up to 11 days following recovery from anaphylaxis [82].

Management of Food-Induced Anaphylaxis

The available evidence for the recommended management of anaphylaxis is limited. This is due, at least in part, to the ethical and practical difficulties inherent in performing randomized clinical trials in medical emergencies. While adrenaline remains the mainstay of treatment, there is no clear worldwide agreement on the use and role of other commonly used first-line approaches and agents [83, 84]. Algorithms outlining key steps for the acute management of anaphylaxis have been published elsewhere [3, 85], and a summary of the most frequently recommended guidelines are provided below. It should be noted that these recommendations are also pertinent to the acute management of FIA.

Acute Management

Adrenaline

Patients fulfilling any of the 3 criteria for anaphylaxis outlined in Box **1** should receive adrenaline immediately. There are patients who present with symptoms not fulfilling these criteria, but in whom it would be appropriate to initiate therapy with epinephrine. For example, a patient with a history of anaphylaxis to peanut who suspects exposure to peanut and experiences urticaria and generalized flushing within minutes.

The α_1-adrenergic effects of adrenaline increase peripheral vascular resistance, blood pressure and coronary artery perfusion, while reducing angioedema and urticaria [86]. The β_1-adrenergenic effects increase heart rate and contraction, whereas the β_2-adrenergic effects mediate bronchodilation and reduce the release of inflammatory mediators [86].

No trials have been performed in humans to determine the optimal dosage of adrenaline; however, epinephrine [1

mg/ml] at a dose of 0.01 ml/kg body weight (maximum single dose 0.5 mg) is the recommended dosage for controlling symptoms and maintaining blood pressure [87]. This equates to 10 µg of adrenaline per kg body weight. This dosage can be repeated at short intervals (every 5–10 min) until the patient's condition stabilizes. A shorter interval between injections might be used if deemed necessary by the physician. Studies in children [88] and adults [89] not experiencing anaphylaxis have demonstrated that intramuscular injection leads to more rapid absorption and higher plasma concentrations than the subcutaneous route. In particular, peak plasma concentrations were attained more quickly following intramuscular injection into the mid-point of the anterolateral thigh (the bottom right of the right trouser pocket). In addition, these studies showed comparable results using both an ampule of epinephrine and a spring-loaded (e.g. EpiPen [Dey, Napa, Calif]) automatic epinephrine device. Currently, adult autoinjectors contain 300 µg of adrenaline, and junior/paediatric versions 150 µg. An adult autoinjector is recommended for children weighing >30 Kg [86]. The main limitations of currently available adrenaline autoinjectors include restricted range of premeasured doses, needle lengths and high cost. It should be noted that studies regarding the optimal route of administration have not been performed in patients experiencing anaphylaxis.

Intravenous epinephrine should be considered for patients with severe hypotension or cardiac arrest unresponsive to intramuscular doses of epinephrine and fluid resuscitation [90, 91]. There is no precise dosage for intravenous epinephrine in anaphylaxis; however, doses of 5 to 10 µg (0.2 µg/Kg) for hypotension and 0.1 to 0.5 mg for cardiovascular collapse have been suggested [92].

Adrenaline has a relatively narrow therapeutic window (benefit/risk ratio) which should be considered when planning treatment. Administration of adrenaline carries risks, including myocardial ischaemia and cardiac dysrhythmias; however, anaphylaxis itself can cause these problems. Since the potential for lethal arrhythmias following i.v. delivery of epinephrine exist, continuous cardiac monitoring is recommended.

Oxygen and Adrenergic Agonists

High-flow oxygen *via* a nonrebreathing mask or endotratracheal tube should be administered to any patient experiencing respiratory symptoms, hypoxemia or hypotension associated with anaphylaxis [1, 3, 93]. Inhaled β_2-agonists *via* a spacer devicer or nebulizer are a useful adjunct to treat associated airflow obstruction; however, drug delivery may be compromised by acute bronchospasm. At any rate, systemic adrenaline must still be considered the first line of therapy.

Fluid Resuscitation

Anaphylaxis often causes tachycardia and decreased arterial blood pressure. Patients who remain hypotensive despite epinephrine should receive fluid support [94]. A crystalloid solution or a colloid expander can be used, starting with a volume of 20 ml/Kg over 10-20 min. This can be repeated; however, the volume given must be tailored to the clinical situation; persistent hypotension may require an aggressive approach with multiple fluid boluses including colloid, as well as crystalloid, whereas a largely respiratory reaction or one that responds promptly to initial treatment require less aggressive fluid management.

Vasopressors

Potent vassopresors such as noradrenaline, vasopressin or metaraminol might be required to overcome vasodilation if epinephrine and fluid resuscitation have failed to maintain a systolic blood pressure of greater than 90 mm Hg [90].

H_1- and H_2-Antihistamines

Most protocols recommend the use of antihistamines; however, evidence for their efficacy in anaphylaxis is weak [95]. They have a slow onset of action (1-2 h) and little effect on blood pressure and, thus, should be viewed as second-line treatment. Antihistamines are particularly useful for the treatment of associated urticaria-angioedema and pruritus. In these instances, treatment with a combination of H_1- and H_2-antagonists has been shown to be more effective in attenuating the cutaneous manifestations of anaphylaxis than treatment with H_1-antagonists alone [78, 96]. The ideal antihistamine should be in liquid form, rapid-in-onset, nonsedating and long-lasting. Diphenhydramine, administered intravenously or intramuscularly (or orally for mild symptoms), can be given at a dose of 25 to 50 mg for adults and 1 mg/kg (up to 50 mg) for children. Ranitidine and cimetidine have been most studied, but no controlled studies have demonstrated superiority of one H_2-antagonist over another.

Corticosteroids

The effectiveness of corticosteroids in anaphylaxis has never been determined in placebo-controlled trials. The action of corticosteroids requires *de-novo* protein synthesis. Thus, their biological activity is delayed by at least some hours and they are of no immediate benefit. It has been suggested that corticosteroids may be beneficial in preventing or ameliorating biphasic or protracted anaphylactic reactions. However, the evidence for this is weak. In some instances, failure to administer corticosteroids appeared to predispose patients to such a response [97], but this was not confirmed in other reports [98]. If given, the dosing of intravenous corticosteroids should be equivalent to 1 to 2 mg/kg per dose of methylprednisolone every 6 hours. Oral administration of prednisone, 1 mg/kg, up to 50 mg might be sufficient for milder reactions.

It is important to emphasize that a rigorous evaluation of most of the therapeutic measures and medications currently used is needed. To this end, systematic reviews of commonly utilized drugs are being conducted to document the existing evidence. It is noteworthy that the therapeutic strategies suggested by the NIAID/FAAN Symposium are largely based on ''clinical experience and expert opinion''. There is a virtually complete agreement that epinephrine should be the initial treatment of choice for acute anaphylaxis; yet, there are limited data on the appropriate dose, timing or frequency of administration. Similarly, there are practically no studies investigating either the functional role or effectiveness of H_1- and H_2-antihistamines as well as corticosteroids in the treatment of anaphylaxis. Prospective randomized controlled

multicenter trials to establish the appropriate dosing of these medications and the role of other therapeutic interventions, such as the optimal type and rate of fluid replacement, and the use of vasopressors, glucagon and nebulized albuterol or epinephrine are also desirable. Randomized controlled trials of any pharmacological intervention would be difficult to conduct in individuals in the ED as no baseline measurements would be available, symptoms and signs of anaphylaxis might be resolving as a result of first aid treatment, and patients may be too unstable for randomization. Nevertheless, such studies might be feasible in well equipped healthcare settings with appropriately trained personnel in which anaphylaxis can be elicited and readily controlled – for example, in selected patients undergoing a physician supervised controlled food challenge as part of their anaphylaxis risk assessment.

Long-Term Management

Emergency treatment must be followed by outpatient follow-up and a long-term management plan to prevent recurrences and help patients manage potential future reactions. The key elements of a long-term management plans should include education, a prescription for self-injectable epinephrine, and referral to an allergy specialist (Box **2**). Importantly, the effectiveness and cost-effectiveness of management plans need to be formally evaluated [84, 99].

Education
▪ Instructions for food allergen avoidance
▪ Written emergency action plan
▪ Education resources
Prescription of self-injectable epinephrine
▪ Training for proper use
▪ Emphasis on carrying in-date autoinjector
▪ Encouragement for prescription refill
Referral to an allergist specialist
▪ Thorough evaluation
▪ Specialized follow-up care
▪ Intensive education on food allergic reactions

Box 2. Long-term management of food-induced anaphylaxis.

Education

Education should include instructions for relevant food allergen avoidance, a written emergency action plan, and a list of educational resources. Additional information on food labelling, approaches to restaurants and food service, and management of anaphylaxis of children in schools and other settings is critical to prevent reactions in food allergic individuals [100]. The importance of a thorough knowledge of all food ingredients before consumption cannot be overemphasized. Studies have shown that the majority of fatal reactions occurred in individuals who were aware of their allergy, but felt that they were eating something safe. [34]. New food labelling laws in North America [101], requiring less complicated terminology, should facilitate food allergen awareness and avoidance. Patient educational resources (e.g. www.foodallergyalliance.org or www.foodallergens.info) are also recommended for better management of food allergy.

Prescription of Self-Injectable Epinephrine

Despite these measures, accidental exposure to food allergens still occurs [72]. Therefore, individuals with a known food allergy should be prescribed self-injectable epinephrine. The importance of carrying autoinjectors permanently is supported by a US report on fatal cases where ~ 90% of patients did not have epinephrine available at the time of the reaction [59]. Since retrospective studies indicate that 16 to 35% of reactions require a second dose of epinephrine [102, 103], carrying multiple doses is advisable. In addition to providing the prescription, it is imperative to instruct patients and their families on its proper use, reinforce the importance of carrying an up-to-date autoinjector at all times and encourage patients to refill the prescriptions. For instance, a study by Sicherer *et al.* showed that 86% of parents of food allergic children reported carrying self-injectable epinephrine at *all times*; however, only 71% had the device during the study visit, and, significantly only one-third were able to correctly demonstrate its use [104]. Of note, the same study reported that less than half of general paediatricians were knowledgeable on the use of the self-injectable epinephrine [104].

Epinephrine prescriptions should always be accompanied by a written, personalized, and regularly updated anaphylaxis emergency action plan (an example is provided in [2]). Such plan should list the most common symptoms and signs of anaphylaxis and emphasize prompt epinephrine injection, followed by transportation of the individual to the nearest hospital ED.

There are still limitations and concerns regarding self-injectable epinephrine. Autoinjectors are currently available only in 2 fixed doses, 0.15 and 0.3 mg. The recommendation for children with anaphylaxis is 0.01 mg/Kg up to 0.3 mg. Thus, a quandary exists with regard to dosing children who do not weigh approximately 15 Kg (33 lbs [for whom the 0.15 mg-dose is ideal]) or 30 Kg (66 pounds or more [for whom the 0.3 mg-dose is recommended]). This issue, along with some pertinent suggestions, is discussed in a comprehensive report by Sicherer *et al.* [86]. The length of the needle of currently available adrenaline autoinjectors is also a subject of concern, especially in obese or overweight adults, as it may not be long enough to penetrate the fat layer and reach the intramuscular space; this is further compounded in women who have more subcutaneous fat than men [105]. Another concern relates to the fears of patients and/or parents of using needles. This has created another barrier to autoinjector use, and therefore, alternatives to injectable epinephrine are now under investigation. The use of metered dose inhalers has been shown to be suboptimal because of the high number of inhalations required and the unpleasant taste of the drug [106]. More recently, sublingual epinephrine has shown some promising results. In fact, studies in rabbits showed that 40 mg of sublingual epinephrine resulted

in peak plasma concentrations, and time to peak levels, similar to the ones following i.m. administration of 0.34 mg of epinephrine [107].

Furthermore, there is a concern that individuals and caregivers are often reluctant to use self-injectable epinephrine [86]. Potential explanations for this include failure to recognize anaphylaxis, spontaneous recovery from a previous episode, reliance on oral H_1 antihistamines or asthma-relief inhalers and concerns about adverse effects of epinephrine [108].

Referral to an Allergy Specialist

Finally, confirmation of the diagnosis and anaphylaxis management plans must include referral to an allergy specialist. Allergy specialists will demonstrate sensitization to the suspected allergen with skin prick and/or blood tests, and educate patients how to self manage in the community. Comprehensive education should include education on early recognition of signs and symptoms and use of epinephrine autoinjectors.

Compliance with Recommended Management Guidelines

As with many medical conditions, compliance with established management guidelines by both patients and health care personnel is as or more important, than the guidelines themselves. A number of studies have documented that compliance by medical personnel with the emergency management of anaphylaxis remains poor. Low compliance appears to be pervasive as it occurs as much in North America as in Europe.

A 1996 British study in eight patients with severe allergic reactions to known causes reported that none of these patients had been given advice about avoidance of the offending allergen, prescribed self-injectable epinephrine, or referred to an allergy specialist [109]. Similarly, a 1995 US study evaluated 326 patients presenting to the ED with an allergic event; of these, 45 had a food-induced allergic reaction most often to seafood, peanuts, and nuts. None of these patients at discharge was given a prescription for self-injectable epinephrine or referred to an allergy specialist [110]. In 2003, Bellou *et al.* reviewed the charts of 324 patients in France admitted to the ED for an allergic event and found that 31 (9.5%) patients were diagnosed with food allergy. Six (19%) of these patients were observed to have a severe episode of anaphylaxis. Only 42 patients (12.9%) were referred for an allergy consultation after ED discharge [111].

A recent multicenter study conducted by the Emergency Medicine Network reviewed the ED charts of 678 patients who presented with a food-related allergic reaction in 21 North American hospitals [60]. Among this group of patients, 51% were identified as having a severe food-induced acute allergic reaction. Even among these patients, compliance with recommended guidelines for the emergency management of anaphylaxis was extremely low. For example, only 24% of patients with anaphylaxis were treated with epinephrine in the ED. At discharge, only 35% were given instructions to avoid the offending allergen, 22% a prescription for self-injectable epinephrine, and 13% were referred to an allergy specialist. Only 2% of these severe cases received all three recommended preventive interventions [60].

Given recent estimates that FIA is the leading cause of anaphylactic reactions treated in the ED [54] it is evident that better dissemination and implementation of the consensus guidelines for the short- and long-term management of FIA are urgently needed.

Measures to Improve the Management of Food-Induced Anaphylaxis

The effective implementation of action plans will require education and participation of both ED personnel and patients. A number of initiatives have been proposed to improve short- and long-term management of FIA (Box **3**).

Timely and accurate diagnosis
▪ Simulation-based training
▪ Electronic forms with algorithms in ED
▪ Specific and sensitive biomarkers
Patient empowerment
▪ Computerized ED discharge sheets
Medical identification
▪ Alert bracelet or necklace
▪ Folding wallet cards
Protective measures
▪ Sabrina's law
▪ Food Allergy Labeling Consumer Protection Act
Communication between ED personnel and primary care providers
▪ Automatic e-mail notification system
ED, emergency department

Box 3. Measures to improve the management of food-induced anaphylaxis.

1. Timely and Accurate Diagnosis

The evidence discussed above reveals the shortcomings of traditional continuing medical education practices in both the diagnosis and management of patients with anaphylaxis, in the ED and at discharge. Improvements in the management of anaphylaxis might benefit from methods that incorporate simulation-based training. This approach has been proven to significantly improve performance of healthcare professionals in the management of medical crises. In essence, simulation-based training involves highly realistic simulation scenarios requiring complex decision making and interaction with multiple personnel. Scenarios are followed by detailed debriefing sessions. This approach has been applied to a number of situations that involve complexity and dynamism, such as emergency and trauma medicine, intensive care, and cardiac arrest response teams and has been

adopted at major health care institutions around the world [112].

An independent and complementary strategy is the development of an emergency-management algorithm to help healthcare professionals in ED quickly recognize and treat anaphylactic reactions. As Clark *et al.* [113] propose, one approach to facilitate the implementation of this algorithm would be the development of electronic diagnosis and order forms that rapidly assess signs and symptoms if an allergic reaction is suspected, and to initiate treatment. The identification of multi-organ system involvement might be especially useful to recognize severe food-related allergic reactions and anaphylaxis. The aim would be not to create a definition with 100% sensitivity (with an inevitable number of false positives), but rather a definition that identifies most cases. The clinical criteria suggested by the NIAID/FAAN Symposium [1] as well as the severity grading system proposed by Brown *et al.* [5] might be useful in this regard. Since there is evidence that a delay in the initiation of treatment is associated with worse outcomes, the rapid recognition of food-related allergic reactions will not only ensure timely and appropriate treatment but also, particularly in severe episodes, prevent fatalities.

2. Patient Empowerment

Further improvements in patient management would greatly benefit from direct patient involvement. For instance, the distribution of computerized ED discharge sheets has been shown to improve compliance with ED referral recommendations [114] and modify patient behaviour [115]. These computerized discharge sheets could also include contact information from patient advocacy organizations (i.e. the Food Allergy and Anaphylaxis network (US) or Anaphylaxis Canada).

3. Accurate Medical Identification

All individuals at risk for anaphylaxis should be equipped with accurate medical identification listing their confirmed trigger(s) and their relevant comorbidities and medications. One available option includes MedicAlert® identification (i.e. bracelet, necklace, or keychain) [116]. Another approach is to carry folding wallet cards with embedded medical record (an example is provided in [2]). These devices should include personal identification, confirmed triggers, relevant comorbidities, concurrent medications, anaphylaxis symptoms flagging a life-threatening condition and the immediate management plan.

4. Protective Measures

The risk for anaphylactic reactions has been shown to negatively affect quality of life of both patients and their families [117-120]. As most episodes of anaphylaxis occur in the community, measures to ensure prompt and appropriate management of anaphylactic reactions would be exceedingly beneficial. Legislators appear to be receptive toward changing public policy and improving medical services for individuals with anaphylaxis. An inspiring example of such measures is the legislation that recently came into effect in Ontario, Canada, Sabrina's law, requiring school boards to include an anaphylaxis policy that provides training to staff, plans for dealing with students at risk and emergency plans. In the future, legislations such as "Sabrina's Law" may serve as a model for other communities worldwide. An additional landmark legislation implemented in 2006 includes the National Food Allergy Labeling Consumer Protection Act in the United States, which mandates clear food labelling.

5. Communication Between Emergency Personnel and Primary Care Providers

Lastly, better communication between ED staff and primary care providers could also improve patient care following an ED visit for food-related allergic reactions or anaphylaxis. This might occur through automatic e-mail notification systems, which have been implemented in some hospitals. These e-mail alerts notify the primary care physician about the visit itself, the discharge diagnosis, and the management plan. Primary-care involvement would permit close follow-up and additional referral to specialist and augment the likelihood that self-injectable epinephrine prescriptions are refilled.

CONCLUDING REMARKS

Improving the management of anaphylaxis, in particular food-induced anaphylaxis, to reduce morbidity and mortality faces many challenges. We would propose that these can be aggregated into essentially two: *discovery* and *communication*.

The challenge of *discovery* embodies issues pertaining to diagnosis, prognosis (spectrum of severity) and management, acute as well as long term management. For example, there are no specific biomarkers of anaphylaxis, or its evolution, nor specific indicators that might inform about the risk and/or the severity of potential anaphylactic reactions. With respect to treatment, there are no proven therapeutics to date beyond epinephrine. There may not be better treatments than epinephrine for the acute and severe presentation of anaphylaxis. However, there is a need for therapies that could ameliorate the evolution of anaphylaxis or abort recurrent or protracted reactions. From a broader perspective, it may be argued that the greatest challenge might be the development of therapeutics, for food allergic patients, that could either prevent anaphylaxis or, at least, substantially limit the risk of severe reactions. Otherwise, patients will remain unprotected. This goal will only be achieved through a major commitment to research of the basic mechanisms underlying food allergy and, particularly, anaphylaxis.

The challenge of *communication* has been made, we believe, explicit in this review and is supported by extensive primary data. Even an improved pharmacological armamentarium may fail unless decisive efforts are implemented to substantially improve communication among all the participants involved in the process. The closest individual to the anaphylactic reaction is the patient itself. Unique to anaphylaxis is that patients live in an environment with abundant risks that can precipitate fulminant reactions. It thus seems intuitive that empowering patients and their families with knowledge about the disease, its risks, triggers and therapeutic tools is a direction that might yield obvious benefit. This will demand a much finer effort to institute effective multilateral communication among patients, families, emergency

and family physicians, allergists, specialized volunteer organizations, and the community at large. Paraphrasing Winston Churchill, *we should always trust the system, to do the right thing, once all the other possibilities have been exhausted*. The evidence of what has gone wrong is widely available.

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