NEW INSIGHTS IN ANAPHYLAXIS

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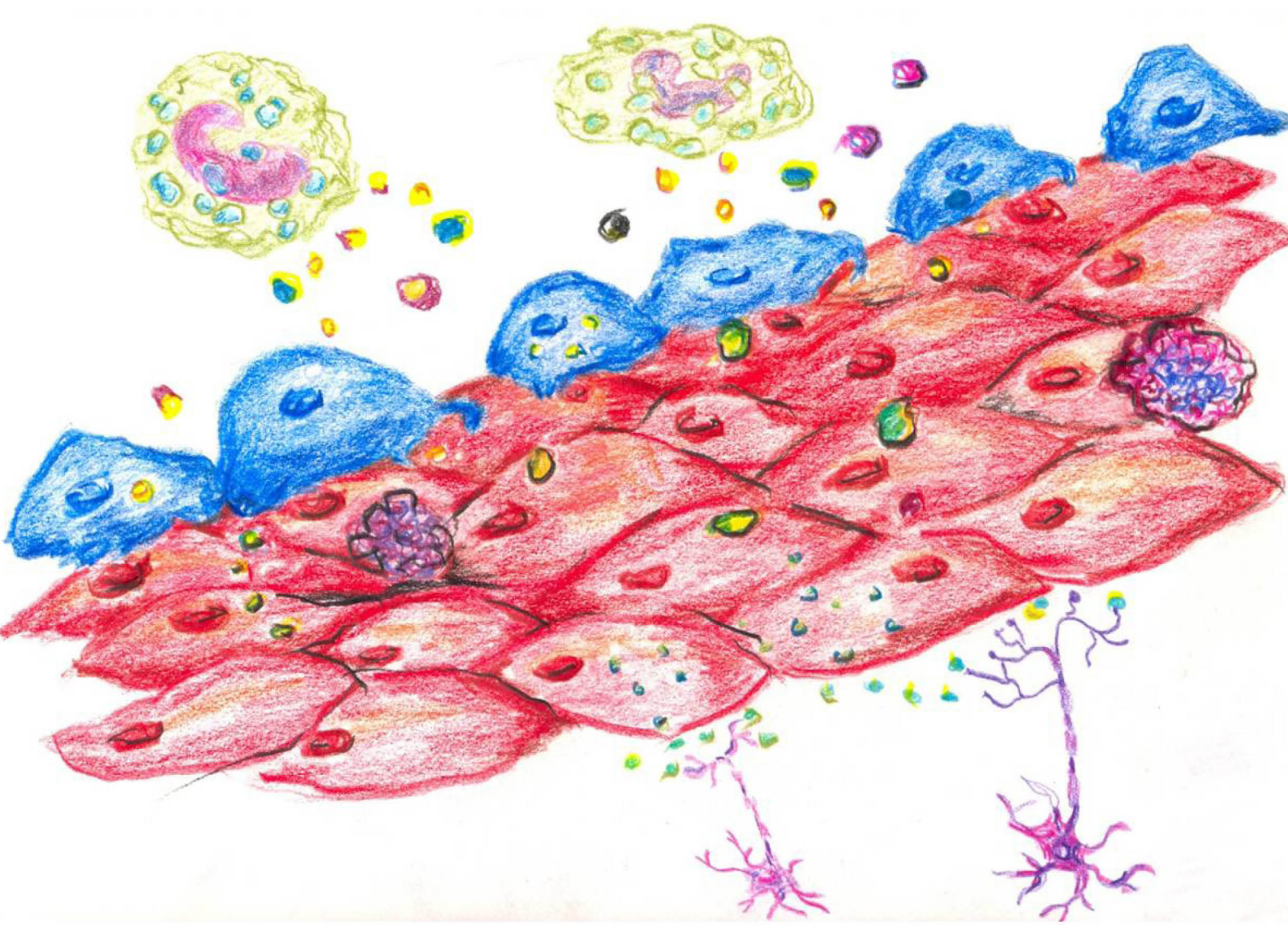

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NEW INSIGHTS IN ANAPHYLAXIS

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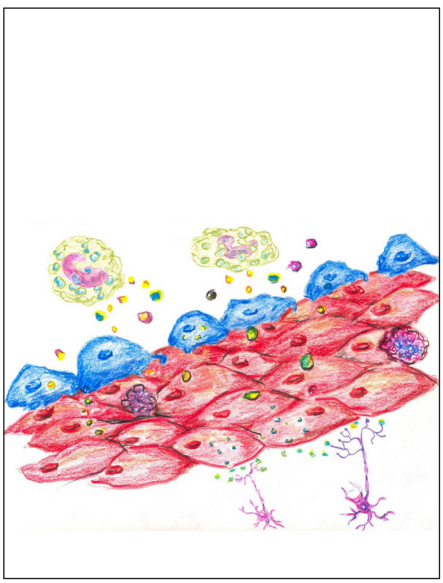

Cellular and molecular mechanisms underlying anaphylaxis.

Image: Vanesa Esteban.

Anaphylaxis has usually been studied and understood from an immunological view and the main lethal effects involve the cardio-vascular system. Despite exhaustive efforts exerted in this area of research, much more investigation is needed to mitigate, recognize or prevent this disease. The scope of this Research Topic is to present and discuss the current knowledge of anaphylaxis, from the clinic to the underlying molecular mechanisms.

We are thankful to the medical doctors, researchers, and colleagues who have contributed to this issue, as it would not have been possible without their highly valuable expertise in allergy and anaphylaxis research.

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EDITORIAL
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Editorial: New Insights In Anaphylaxis

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Keywords: anaphylaxis, drug and food allergy, molecular signaling, augmentine factors, endothelial cells

Editorial on the Research Topic

New Insights In Anaphylaxis

In choosing a theme for this issue of *Frontiers in Immunology*, we settled on anaphylaxis, which is understood as the most aggressive manifestation of allergic disorders, and also one that has often been studied solely from an immunological point of view. Recent insights, however, suggest that different perspectives are necessary to advance the knowledge of this multifactorial and complex clinical condition. The articles published here offer a wide-angle view of anaphylaxis, from diagnosis and treatment, through the augmenting factors and cofactors involved, and including the study of the molecular and cellular mechanisms underlying anaphylactic reactions.

Drugs are the most common cause of anaphylaxis in adults. Here, Montanez et al. present an extensive knowledge update on this issue, providing in-depth analysis of the manifold aspects of the two most common anaphylaxis-causing drugs, i.e., non-steroidal anti-inflammatory drugs (NSAIDs) and antibiotics. The study, which includes the latest epidemiological data, molecular mechanisms, and risk factors, offers a brief guide on drug-allergy diagnosis using both *in vivo* and *in vitro* testing methods. On a related topic, a chapter by Castells details new insights into drug allergy and anaphylaxis in the context of cancer and chronic inflammatory diseases. In this work, current knowledge in personalized desensitization protocols is detailed and aimed at the management of patients who have suffered reactions to their first-line therapy. In this context, personalized protocols are a promising first step to enhance the quality of life and life expectancy of patients.

Food allergy is a serious health concern and its prevalence is on the rise, especially in the pediatric population. Allergy to food products is a frequent cause of anaphylaxis and one that is significantly detrimental to patient quality of life. This paper presents the most relevant aspects of food allergy and anaphylaxis. In this contribution, we see the complex network and multiple factors that can affect food-induced anaphylaxis such as genetic predisposition, environmental factors, and the influence of dietary or intestinal micro biota (Benede et al.). Moreover, key features of the immunological mechanisms of the intestinal mucosa involved in food allergy and food-induced anaphylaxis are illustrated. As the authors remark, despite the efforts exerted in this area of research, much more investigation is needed to mitigate this disease.

In human anaphylaxis, the available information on the cellular and molecular level mainly points to the immunological processes that represent the early stages of the anaphylactic reaction. Overall, these events are triggered by hypersensitivity reactions mediated by FcεR1-bound IgE antibodies in response to allergens and result in activation of mast cells (MCs) and basophils. These immune cells are considered the main cell effectors and amplifiers of the allergic reaction. MCs activation induces the release of mediators, which are the ultimate elicitors of reactions in the resident tissues of the surrounding area. As anaphylaxis in patients with systemic mastocytosis is up to 100 times more frequent in patients with systemic mastocytosis, an overview of the epidemiology, triggers, and risk factors of anaphylaxis in patients with MC activation syndromes is included (Gonzalez-de-Olano

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and Alvarez-Twose). Moreover, the diagnosis and treatment aspects related to this syndrome are given in-depth treatment.

The identification of other cell types participating in anaphylaxis, such as neutrophils or macrophages, is especially relevant to experimental anaphylaxis. Mice deficient in either MCs or IgE still develop anaphylaxis, thereby suggesting the existence of alternative pathways (to date, mainly IgG). Focusing on this molecular and cellular aspect, Escribese et al. report on the potential role of macrophages in anaphylaxis and how these can play an important role in IgG-dependent anaphylaxis induced by allergen-IgG immunocomplex bound to IgG receptors on macrophages, neutrophils, and basophils. Knowledge of other molecular mechanisms that impact the anaphylactic process enables us to speculate as to whether the known classical effector cells are the only meaningful contributors also to the human anaphylactic episodes.

Recent findings on cofactors and augmenting factors are of great importance when establishing degrees of risk in anaphylaxis patients, as these factors may predict or even prevent the occurrence of reactions. Some such factors include estrogens, exercise, statins, alcohol, drugs such as NSAIDs, angiotensin-converting enzyme inhibitors, and β-blockers (Munoz-Cano et al.). This collection contains a number of descriptions detailing the most common immunological mechanisms occurring in anaphylaxis, though special emphasis is given to non-immune mechanisms. One example is the interesting review by Poulsen et al., whose chapter focuses on the importance of internal dose, described as the quantity of the anaphylactic trigger (most often an allergen), allergokinetics as a mechanistic factor, and also the importance of intrinsic and extrinsic cofactors in the pathophysiology and occurrence of anaphylactic reactions.

New findings on the potential of molecular mechanisms are essential to better understand diverse inflammatory-related pathologies linked to allergy and anaphylaxis. This collection of articles includes two studies providing support for the relevance of the contact, complement, and coagulation systems (Bender et al.; Guilarte et al.). Activation of the FXII substrate triggers the kallikrein system, releases the mediator bradykinin, and activates the complement pathway. The relevance of FXII in hereditary angioedema and anaphylaxis highlights this molecule and its related pathways as targets in current diagnosis and treatment. In addition, anaphylaxis mediators are also involved in the activation of the fibrinolytic and coagulation system. Therefore, although only histamine and tryptase can be routinely measured as biomarkers in clinical practice, use of reliable biomarkers to assess the activation of these systems together with standardized assays would ameliorate the diagnosis and management of this disease.

Finally, these relevant pathways connect MCs activation with the mediator's ability to increase inflammation and permeability/contractility processes occurring in vessels. Regarding vessels and anaphylaxis, original data supported from our own studies reveal Rcan1 an endothelial protein synthesized in response to histamine, which contributes to the strengthening of the endothelium in response to anaphylaxis (Ballesteros-Martinez et al.). This new insight underscores the potential of endothelial molecules as regulators of sensitivity to anaphylaxis.

We are thankful to the medical doctors, researchers, and colleagues who have contributed to this issue, as it would not have been possible without their highly valuable expertise in allergy and anaphylaxis research.

AUTHOR CONTRIBUTIONS

CP-V and VE wrote and edited the manuscript.

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MINI REVIEW
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Epidemiology, Mechanisms, and Diagnosis of Drug-Induced Anaphylaxis

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Anaphylaxis is an acute, life-threatening, multisystem syndrome resulting from the sudden release of mediators by mast cells and basophils. Although anaphylaxis is often under-communicated and thus underestimated, its incidence appears to have risen over recent decades. Drugs are among the most common triggers in adults, being analgesics and antibiotics the most common causal agents. Anaphylaxis can be caused by immunologic or non-immunologic mechanisms. Immunologic anaphylaxis can be mediated by IgE-dependent or -independent pathways. The former involves activation of Th2 cells and the cross-linking of two or more specific IgE (sIgE) antibodies on the surface of mast cells or basophils. The IgE-independent mechanism can be mediated by IgG, involving the release of platelet-activating factor, and/or complement activation. Non-immunological anaphylaxis can occur through the direct stimulation of mast cell degranulation by some drugs, inducing histamine release and leading to anaphylactic symptoms. Work-up of a suspected drug-induced anaphylaxis should include clinical history; however, this can be unreliable, and skin tests should also be used if available and validated. Drug provocation testing is not recommended due to the risk of inducing a harmful reaction. *In vitro* testing can help to confirm anaphylaxis by analyzing the release of mediators such as tryptase or histamine by mast cells. When immunologic mechanisms are suspected, serum-sIgE quantification or the use of the basophil activation test can help confirm the culprit drug. In this review, we will discuss multiple aspects of drug-induced anaphylaxis, including epidemiology, mechanisms, and diagnosis.

Keywords: anaphylaxis, drugs, IgE, MAS-related G protein-coupled receptor, IgG, *in vivo* diagnosis, *in vitro* tests

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INTRODUCTION

Anaphylaxis is a severe, potentially life-threatening, generalized, or systemic hypersensitivity reaction that results from the sudden release of mediators derived from mast cells and basophils *via* degranulation (1–3). Drugs are the most common anaphylaxis triggers in adults (4–6), representing up to 10% of overall causes in outpatient studies (7), whereas for emergency department and hospitalized patients the proportion ranges from 27–60% (4, 8, 9).

While the symptoms of anaphylaxis can involve any organ, the most commonly affected are the cutaneous (affecting around 88% of cases), respiratory (76.1%), cardiovascular (41.9%), and

gastrointestinal systems (12.8%) (10). Severe reactions (associated with hypotension) are more likely to be drug induced (4), representing up to 58% of fatal anaphylaxis (11).

Although anaphylaxis usually presents as an acute episode, mast cells can release mediators hours after the initial reaction causing a biphasic or late phase reaction. These biphasic and protracted cases can occur in up to 10% of drug-induced anaphylaxis instances (12).

In this paper, we will review the epidemiology, mechanisms, *in vivo* and *in vitro* diagnosis, and management of drug-induced anaphylaxis.

EPIDEMIOLOGY OF DRUG-INDUCED ANAPHYLAXIS

Estimates of the prevalence of anaphylaxis can vary, mainly due to a lack of consensus on the definition of anaphylaxis, the source of data, and populations evaluated. One study calculated an overall incidence of 3–50 per 100,000 person years and a lifetime prevalence of 0.05–2% (8). The incidence of drug-induced anaphylaxis has been estimated to range from 0.04 to 3.1% (13–15) and to be responsible for one case in every 4,000 emergency department visits (16), with a fatality rate of 0.65% (17). In terms of changes over time, drug-induced anaphylaxis has increased by 150% and mortality rates by 300% in parallel with an increasing incidence of overall anaphylaxis from 1997 to 2005 (4).

DRUGS CAUSING ANAPHYLAXIS

Anaphylaxis can be induced by a range of drugs, being analgesics and antibiotics the most commonly involved, which may be partly explained by their frequent use in current medical practice (9, 10, 18).

Non-Steroidal Anti-inflammatory Drugs (NSAIDs)

Non-Steroidal Anti-inflammatory Drugs are the most frequent triggers of drug-induced anaphylaxis, being responsible for 48.7–57.8% of incidents (10, 18). These are typically immunological reactions (19) that can be driven by an IgE-dependent mechanism with sufferers showing tolerance to other strong COX-1 inhibitors (19, 20). However, anaphylaxis induced by cross hypersensitivity to NSAIDs, driven by an IgE-independent mechanism, has also been described (21–23). The most common culprits are pyrazolones, propionic acid derivatives, diclofenac, and paracetamol (10, 19, 22, 24). The incidence of NSAID-induced anaphylaxis with concomitant asthma, rhinosinusitis, and nasal polyps ranges from 2%, in children, to 97%, in adults (25). The prevalence ranges from 0.06 to 0.9% (26), with acetyl salicylic acid accounting for approximately 3% of all instances of anaphylaxis (27).

Beta-Lactam Antibiotics

Beta-lactams represent the second most frequent cause of drug-induced anaphylaxis, accounting for 14.3% of cases (18), with amoxicillin being the most common trigger (5). Recently, clavulanic acid, usually prescribed in combination with amoxicillin, has also been implicated (28, 29). Cases with cephalosporins, carbapenems, and monobactams are rare (30–32). The rate of anaphylactic reactions to beta-lactams has been estimated to be between 1 and 5 per 10,000 patient courses of treatment (33) and these drugs account for 75% of all fatal anaphylactic episodes in the US each year (34).

Non-Beta-Lactam Antibiotics

Up to 75% of patients with immediate hypersensitivity to fluoroquinolones develop anaphylaxis, with moxifloxacin being the most common culprit, followed by ciprofloxacin (35). As a whole, fluoroquinolones are responsible for 9% of severe antibiotic anaphylaxis (31).

Anaphylaxis to sulfonamides, trimethoprim, and macrolides are rare (36, 37). Cases of vancomycin IgE-mediated anaphylaxis have been occasionally reported (38); however, this drug more commonly induces direct mast cell stimulation, associated with rapid intravenous administration, and characterized by flushing and pruritus, known as "red man syndrome" (24). In addition, this drug may lead to more severe reactions including hypotension and muscle spasms (24).

Radiocontrast Media (RCM)

Reactions to RCM with systemic symptoms have decreased with the introduction of non-ionic, low osmolar agents, down from 12.1 to 0.04% of patients receiving RCM (39, 40). Although these reactions have historically been deemed non-IgE mediated, it should be noted that both ionic and non-ionic RCM may trigger IgE-mediated anaphylaxis (35, 41–43). Anaphylaxis to gadolinium agents is much less frequent with an incidence of 0.004–0.01% (44, 45). Older age and multiple previous exposures to RCM increase the risk of having anaphylaxis associated with hypotension. Fatalities have been reported even after the introduction of non-ionic RCM, with most cases lacking predictable risk factors (46). RCM accounted for 27% of fatal drug-induced anaphylaxis (11).

Proton Pump Inhibitors (PPIs)

Anaphylaxis to PPIs is also becoming more common, representing 36–80% of all hypersensitivity reactions to these drugs (47–50). Lansoprazole is the most commonly involved agent (68.3–26.41%), followed by esomeprazole (30.18–10.0%), pantoprazole (20.0%), omeprazole (18.86–1.7%), and rabeprazole (6.7–3.77%) (51).

Neuromuscular Blocking Agents (NMBAs)

Neuromuscular blocking agents are often considered one of the group of drugs that most frequently cause allergic reactions during the perioperative period (52–54). Reactions may be IgE mediated or due to the non-specific release of histamine (52). There are geographical differences and changes over time in the epidemiology of perioperative anaphylaxis. The incidence of intraoperative anaphylactic reactions has been estimated to be 1 in 1,250–10,000 anesthetics in France (54, 55), being lower in

Australia and New Zealand (1 in 10,000–20,000) (56). Although mortality from perioperative anaphylaxis has been previously reported between 3 and 9% (54), a more recent study put it in the range of 0–1.4% (56). A study from France reported that for 59% of intraoperative anaphylactic reactions, the etiological agent was an NMBA, more specifically suxamethonium, vecuronium, pancuronium, alcuronium, atracurium, or gallamine (57). More recent studies report rocuronium and succinylcholine at higher risk of anaphylaxis, whereas pancuronium and cis-atracurium are reported to be the NMBAs associated with the lowest incidence of anaphylaxis (53, 58–62).

Sugammadex

Sugammadex is a synthetic g-dextrin derivative designed to selectively bind to steroidal NMBAs. Cases of anaphylaxis to sugammadex have been recently reported (63–65) being an IgE-mediated mechanism suggested in several cases as patients gave positive skin tests and flow cytometry results (66, 67). It has been suggested that treatment of rocuronium-induced anaphylaxis should include the administration of sugammadex (68, 69). However, other studies have concluded that sugammadex is unlikely to modify the clinical course of an established allergic reaction (70).

Hypnotics

Barbiturates induce frequent reactions due to the ability to elicit direct histamine release, although IgE-mediated anaphylaxis has also been described (71, 72). Reactions were also frequent with hypnotics using Cremophor EL as solubilizer; however, since propofol was formulated in soybean oil emulsion, the rate of reactions decreased (54, 73, 74). It has been suggested that allergic patients to eggs or soy should avoid propofol because of the presence of lecithins in the propofol vehicle; however, this has not been confirmed (75, 76) and currently is not recommended (77).

Opioids

Hypersensitivity reactions to opioids are rare, and most cases are due to the non-immunologic induction of histamine release, being pruritus the most frequent symptom. Although rare, isolated episodes of IgE-mediated anaphylaxis to opioids have been described (78–80). The most common offenders inducing non-imnunologic reactions are the low-potency opiates (meperidine, codeine, and morphine); interestingly, high-potency opioids such as fentanyl and hydromorphone are less likely to cause histamine release (81).

Chlorhexidine

Chlorhexidine is a skin antiseptic widely used in surgical settings. Perioperative anaphylaxis induced by chlorhexidine is quite frequent in UK or Denmark (82, 83) but rare in France maybe due to its limited use (84). Sensitization to chlorhexidine can occur from home products such as mouthwash, toothpaste, dressings, ointments, and over the counter disinfectant solutions (85).

Dyes

Triarylmethane dyes, methylene blue, patent blue V, and isosulfan blue induce a relatively frequent rate of perioperative anaphylaxis due to their wide use in sentinel lymph node mapping in cancer surgery. Reactions may be induced by direct mast cell and/or basophil activation and specific IgE (sIgE) sensitization (86–88).

Colloids

The incidence of anaphylaxis to colloids has been estimated to range from 0.033 to 0.22% (89). Gelatins and dextrans are more commonly associated with reactions than albumin and hetastarch (90).

FACTORS INCREASING THE RISK OF DRUG-INDUCED ANAPHYLAXIS

Clinical Factors

Older age and intravenous administration have been shown to be associated with higher rates of drug-induced anaphylaxis (11) and an increased risk of severe reaction (91, 92). Other factors associated with the prevalence of fatal drug-induced anaphylaxis include race, with African-Americans being shown to have higher prevalence (11), the interruption of prior therapy creating gaps in administration (93) and decreased platelet-activating factor (PAF) acetylhydrolase activity (92). The role of atopy in predisposing an individual to drug-induced anaphylaxis is controversial (94) and underlying mast cell disease has not been described as a predisposing factor (95). Further research is needed to better identify patients at risk and to design preventive strategies to reduce the frequency of drug-induced anaphylaxis.

Cofactors

The presence of several cofactors can increase the risk of suffering anaphylaxis and are reported to be relevant in up to 30% of anaphylaxis episodes (96). They include treatment with drugs such as NSAIDs, PPIs, or angiotensin-converting enzyme inhibitors; the presence of concomitant diseases (asthma, mastocytosis, and cardiovascular diseases); and other factors such as alcohol, emotional stress, or menstruation (96, 97).

ANAPHYLAXIS MECHANISMS

Anaphylaxis can be classified as immunologic and non-immunologic depending on the underlying mechanism; either type of reaction can be induced by drugs (98, 99). In some cases, the trigger cannot be identified; such reactions are classified as idiopathic anaphylaxis (100). Different mechanisms and pathways may be involved as illustrated in **Figure 1**. Immunologic anaphylaxis can be mediated by an IgE-dependent or -independent mechanism (101), whereas non-immunologic anaphylaxis involves direct mast cell activation (102–104). Independent of the underlying mechanism, allergic symptoms are similar and caused by the release of mediators such as histamine, tryptase, PAF, cysteinyl leukotrienes, and others (1). Histamine is responsible for flushing, pruritus, rhinorrhea, tachycardia, and bronchospasm *via* the induction of smooth muscle constriction and the increase of vascular permeability. Tryptase activates several pathways, including the complement cascade, coagulation pathway, and the kallikrein–kinin system, contributing to the development of

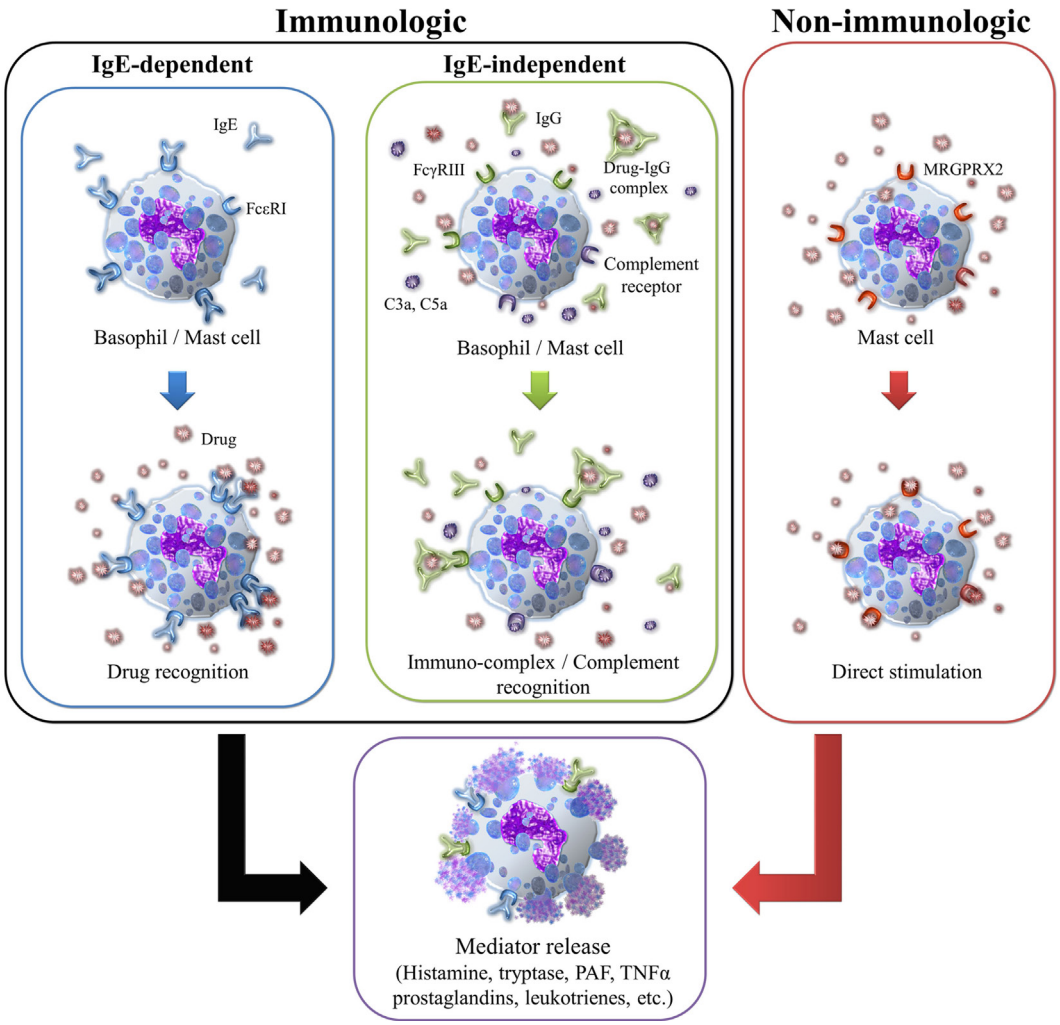

FIGURE 1 | **Different mechanisms of mast cell or basophil activation induced by drugs**.

hypotension and angioedema. PAF and cysteinyl leukotrienes also enhance vascular permeability and the development of hypotension (101).

Immunologic Anaphylaxis

This can be induced by IgE-dependent or -independent mechanisms and mediated by the production of antibodies or the activation of the complement pathway (97).

The IgE-dependent mechanism or classical pathway involves a sensitization process including the activation of Th2 cells by the drug, inducing sIgE. This IgE binds to the FcεRI receptor on mast cells, basophils, or both. The cross-linking of two or more of these receptors by the hapten upon subsequent contact, initiates a complex intracellular signaling cascade that leads to degranulation and the release of preformed mediators such as histamine and tryptase. These cause the allergic symptoms and activate other inflammatory cells that can in turn release additional mediators and stimulate the production of others such as prostaglandin D2 and cysteinyl leukotrienes, which serve to amplify the allergic reaction. Two main mechanisms of degranulation have been recently proposed that may be related to reaction severity: piecemeal and anaphylactic degranulation (105). The former is associated with the upregulation of CD203c on basophils (106) by the formation of small vesicles from the histamine-containing granules, which are rapidly shuttled to the plasma membrane (107, 108). This process may be linked to stimulation by certain drugs and the development of more severe reactions like anaphylactic shock (105, 109). In the second mechanism, the main histamine-containing granules are fused to the plasma membrane, releasing the entire contents to the extracellular space and exposing CD63 on the surface of basophils (106). This second process is slower than piecemeal degranulation and could be related to the development of anaphylaxis (110). Penicillins and NMBA are considered the main triggers of IgE-mediated anaphylaxis induced by drugs (54, 111, 112).

The IgE-independent mechanisms can be mediated by IgG antibodies or by complement (97, 113). IgG-mediated anaphylaxis has been demonstrated in mouse models and involves the release of PAF by basophils, macrophages, or neutrophils after the interaction of the drug with specific IgG (sIgG) bound to FcγRIII. Although this mechanism has not been fully established in humans, some studies have shown that PAF is an essential mediator in anaphylaxis (92, 114). Biological agents have been shown to induce anaphylaxis without the presence of detectable sIgE but with high levels of sIgG, as occurs with patients transfused with IgA (115, 116), treated with infliximab or adalimimab (117, 118), and other biological factors (119–121). Complement activation can be induced through the presence of IgG immunocomplex, but also with drugs solubilized in therapeutic liposomes and lipid-based excipients under physiological conditions. This mechanism leads to the release of C3a, C5a, and C5b-9, which trigger activation of mast cells, basophils, and other cells *via* their specific receptors, causing degranulation and mediator release (97).

IgE-independent mechanism is clinically indistinguishable from IgE-mediated anaphylaxis. Among the most common causes of IgE-independent anaphylaxis are RCM, dextran, and some NSAIDs (20, 122, 123).

Non-Immunologic Anaphylaxis

This type of anaphylaxis does not involve the activation of the immune system, rather the direct stimulation of mast cell degranulation, as has been shown for some drugs (104). This process can be mediated through the MAS-related G protein-coupled receptor-X2 (MRGPRX2) (102–104). The interaction of certain drugs with this mast cell receptor can induce the release of histamine, β-hexosaminidase, TNFα, and PGD2 among others, potentially leading to non-allergic anaphylactic reactions. Medications such as quinolones, opioids, vancomycin, RCM, dextrans, and NMBA have been found to directly stimulate mast cells (104, 124). Whether certain factors may predispose individuals to this type of anaphylaxis needs further research.

DIAGNOSIS OF ANAPHYLAXIS AND IDENTIFICATION OF THE CULPRIT DRUG

The diagnosis of anaphylaxis is based on a thorough examination of patient history and physical evaluation (125). It is important to evaluate various aspects: clinical signs and symptoms of the reaction, grade of severity, drugs administered for treating the reaction, the time needed for the reaction to resolve, age, underlying diseases, and ongoing treatments, such as beta-blockers and angiotensin-converting enzyme inhibitors, and all possible drugs involved in the episode. An accurate identification of the responsible agent is crucial for avoiding anaphylaxis in future treatments (126). The temporal relation of anaphylaxis after the intake of the drug should be ascertained, as most reactions occur within minutes to hours after exposure. However, different drugs are often taken simultaneously, so clinical history is often inconclusive, in which case the work-up of a suspected drug-induced anaphylaxis should also include skin tests, when available and well validated, and in some cases, although not recommended, drug provocation tests (DPTs) (126). *In vitro* tests can complement the diagnosis confirming clinical suspicions of a severe systemic reaction and avoiding the need to conduct DPTs, potentially saving the patient from suffering another reaction. Moreover, they may help to identify the culprit drug and the underlying mechanism (127). We provide a flowchart for diagnosing drug-induced anaphylaxis in **Figure 2**.

In Vivo Diagnosis

To assess IgE-mediated anaphylaxis, skin testing including skin prick tests (SPT) and intradermal testing (IDT) should be performed. For drug-induced anaphylaxis, SPT are typically performed with the undiluted drug. If negative, IDT is performed sequentially with increasing concentrations of the drug, due to the potential risk of inducing systemic symptoms (128). A positive skin test response is defined by the size of the wheal, which should be 3 mm or greater than that of the negative control (129). Testing should be performed as soon as possible to avoid loss of test sensitivity over time reported for IgE-mediated reactions to drugs (130, 131); although it should not be performed less than 6 weeks after the episode, to avoid any possible refractory period in which testing may give a false negative (24) The rate of negativization depends on the drug, ranging from 60% after 6 months for dipyrone (131) to 47% within 4 years for NBMAs (132).

For most drugs, a negative skin test does not rule out allergy. Therefore, DPT is generally accepted as the gold standard; however, it is not recommended in anaphylaxis due to the high risk of inducing another reaction. It is primarily indicated for patients where clinical suspicion is low, and for patients where it is essential that alternatives to an implicated drug are found (24). It can also be recommended for assessing tolerance to potentially cross-reactive drugs (24). It must be performed under expert supervision, where resuscitation facilities are available and early signs of disorders arising from DPT can be detected (133). Although the traditional drug challenge consists of stepwise graduations, one-step and two-step test dose strategies have been suggested recently (134). Nevertheless, since crucial cofactors might be absent during the procedure, its sensitivity may be not optimal.

In Vitro Diagnosis

Mast cell mediator release can be analyzed immediately after symptom onset and can be considered useful for diagnosis. Tryptase is among the early mediators released by mast cells during an acute allergic reaction, often showing elevated serum levels (>11.5 ng/mL) in anaphylaxis. The measure of total serum tryptase is the most widely used laboratory test to confirm anaphylaxis. As its levels peak 1–2 h after symptom onset and normalize after 5–6 h (101), the optimal timing for drawing a tryptase concentration is 1–2 h after the event (24). However, a normal tryptase level does not rule out anaphylaxis, and values obtained at the time of the event should always be compared with a recent baseline serum tryptase (135, 136). Indeed, a relative increase greater than 135% of the baseline value (even below 11.4 ng/mL) has been suggested to improve diagnosis (137).

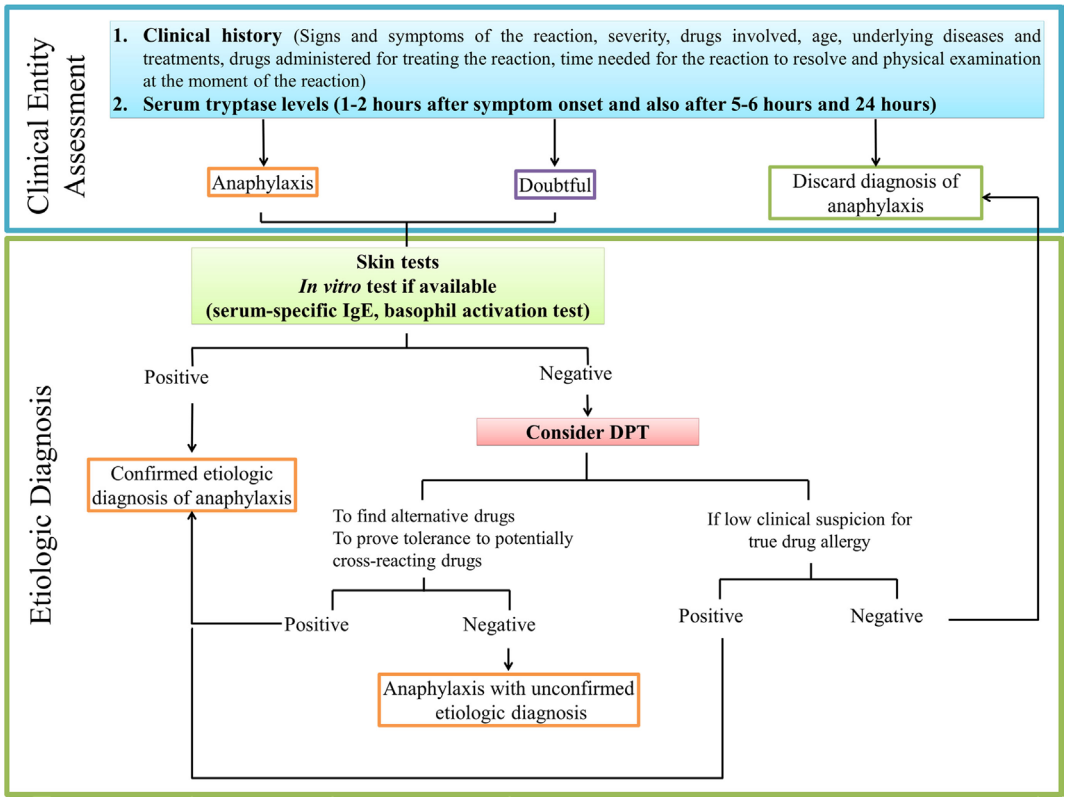

FIGURE 2 | **Recommended practice flowchart for allergy diagnostic work-up in drug-induced anaphylaxis**.

Histamine is the first mediator released by mast cells; any elevation in plasma or urine is consistent with anaphylaxis. However, normal levels do not exclude diagnosis and, like tryptase, the acute level must be compared with baseline (127). However, plasma histamine has short half-life (20 min), which limits the utility of this measurement in the clinical setting (101, 138). An indirect method for the determination of histamine consists of measurement of its metabolites, *N*-methylhistamine or *N*-methylimidazoleacetic acid, in urine. These appear within 30–60 min of the event and stay detectable for a 24-h period (98, 139, 140).

In addition, levels of chymase, mast cell carboxypeptidase A3, PFA, and other mast cell products may prove to be useful as biomarkers for anaphylaxis (141).

When immunologic mechanisms are involved in the reaction, additional laboratory assays, such as serum-sIgE quantification or the basophil activation test, can be useful to confirm the culprit drug. Immunoassays for drug-sIgE determination using ImmunoCAP are available for a handful of drugs, including five beta-lactams, NMBAs, chlorhexidine, and a few other biological agents (127). Although immunoCAP is the most widely used method, custom-made radioimmunoassays can also be used for a wider variety of drugs including quinolones and other beta-lactams (127). The basophil activation test, which can be performed with any suspected drug, measures the activation of basophils after stimulation and is suitable for both IgE-mediated and non-IgE-mediated hypersensitivity (24).

MANAGEMENT

Adrenaline is the first-line treatment for anaphylaxis and should be administered as soon as possible by intramuscular injection into the middle of the outer thigh (142). The patient may require the repeated administration of adrenaline at 5-min intervals if improvement is not observed or symptoms reoccur. Following adrenaline treatment, the trigger should be removed if possible, for example, stopping i.v. medication. The administration of other drugs such as corticosteroids and beta-2 agonists may reduce other features of anaphylaxis and the risk of biphasic and protracted reactions (143, 144). Parenteral administration of glucagon may be useful for treating patients who are unresponsive to adrenaline, particularly in those taking beta-blockers (145).

CONCLUSION

Drug-induced anaphylaxis is a potentially life-threatening reaction that appears to be increasing in both prevalence and incidence, likely due in part to the introduction of new medications. An accurate and prompt diagnosis is necessary to a correct management of this acute reaction, and the identification of the culprit drug is crucial to avoid new future reactions. Further research about mechanisms and risk factors is needed to try to prevent the development of this reaction and to orient therapeutic approaches to patient, based on the culprit drug and the

clinical reactions, which should target the underlying specific mechanisms.

AUTHOR CONTRIBUTIONS

All authors have made substantial intellectual contributions to the preparation of the manuscript and approved it for publication.

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REVIEW
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Drug Hypersensitivity and Anaphylaxis in Cancer and Chronic Inflammatory Diseases: The Role of Desensitizations

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Drug allergy is a rising problem in the twenty-first century which affects all populations and races, children, and adults, and for which the recognition, diagnosis, management, and treatment is still not well standardized. Classical and new chemotherapy drugs, monoclonal antibodies (MoAbs), and small molecules to treat cancer and chronic inflammatory diseases are aimed at improving quality of life and life expectancy of patients, but an increasing number of reactions including anaphylaxis precludes their use in targeted populations. Women are more affected by drug allergy and up to 27% of women with ovarian and breast cancer develop carboplatin allergy after multiple cycles of treatment. Carriers of BRCA genes develop drug allergy after fewer exposures and can present with severe reactions, including anaphylaxis. Atopic patients are at increased risk for chemotherapy and MoAbs drug allergy and the current patterns of treatment with recurrent and intermittent drug exposures may favor the development of drug allergies. To overcome drug allergy, desensitization has been developed, a novel approach which provides a unique opportunity to protect against anaphylaxis and to improve clinical outcomes. There is evidence that inhibitory mechanisms blocking IgE/antigen mast cell activation are active during desensitization, enhancing safety. Whether desensitization modulates drug allergic and anaphylactic responses facilitating tolerance is currently being investigated. This review provides insight into the current knowledge of drug allergy and anaphylaxis to cancer and chronic inflammatory diseases drugs, the mechanisms of drug desensitization and its applications to personalized medicine.

Keywords: desensitization, monoclonal antibodies in cancer, platins, drug allergy, taxanes

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INTRODUCTION

With the unprecedented use of chemotherapies drugs and targeted monoclonal antibodies (MoAbs) and small molecules in the twenty-first century, increased hypersensitivity reactions (HSRs) have emerged worldwide (1, 2). Drug allergic reactions are unexpected, can be severe including anaphylaxis and prevent the use of first-line therapies, with consequent impact in patient's survival and quality of life (3, 4). These reactions range from mild cutaneous manifestations such as pruritus and hives to life-threatening anaphylaxis with hypotension, oxygen desaturation and cardiovascular collapse, and deaths have been reported after re-exposure to allergic drugs (5, 6). The presentation of symptoms can be atypical such as pain, which has been associated with taxenes reactions, and chills and fever which have been seen with oxaliplatin and MoAbs reactions (7, 8). Delayed reactions occurring

more than 24 h after chemotherapy infusions can be due to the prolonged half-life of MoAbs and the presence of premedications, which may mask the acute phase of the reactions (9, 10).

The traditional classification of drug hypersensitivity and allergy into the classical types I–IV does not encompass the current spectrum of reactions and symptoms occurring in cancer patients and patients with chronic inflammatory diseases (11, 12). Some of the reactions have no known underlying mechanism, others have a known mechanism which is not part of the four described types and some drugs can induce mixed reactions with two or more proposed mechanisms (13, 14). Hypersensitivity to rituximab, a chimeric anti-CD20 MoAb, can induce cytokine-like reactions with chills, fevers, hypotension, and oxygen desaturation, which have been attributed to the release of cytokines such as IL-6, IL-1β, and TNF-α and are named cytokine release syndrome or cytokine storm, which is not contemplated in the Gell and Coombs classification (15). In contrast, some patients have classical IgE-mediated reactions to rituximab and have presented positive skin testing demonstrating that IgE and mast cells are part of the underlying mechanism (16). Some patients reactive to oxaliplatin present mixed reactions with Type I features such as hives and hypotension, along with fever and chills as seen in cytokine storm-like reactions, presenting a complex mixed pattern of reactivity which provides challenges to management and treatment (17). During mixed reactions tryptase, the major mast cell protease, and IL-6 can be elevated in serum indicating mast cell activation and cytokine release from unknown cellular sources. Reactions to taxenes can trigger direct mast cell/basophil activation with elevation of serum tryptase with or without evidence of IgE, indicating that more than one mechanism can explain taxane hypersensitivity. A different receptor than FceRI, such as the recently described MrgprX2 for drugs with THIQ motifs such as quinolones and paralyzing agents such as atracuronium could be activated during non-IgE taxane reactions (13, 18–20).

Patients presenting with delayed cutaneous reactions are at a great concern for Stevens–Johnson syndrome and toxic epidermal necrolysis, two life-threatening conditions which can lead to permanent disability, blindness, and dramatic decrease in the quality of life for survivors (16, 21). The underlying mechanisms of the reactions are poorly understood and up to now no predictive markers have been available. Genetic susceptibility and defined HLA haplotypes are thought to be risk factors for some of the reactions, such as HLA-B 5701 in HIV patients reactive to abacavir. In patients with targeted haplotypes, a new role for viral reactivation of HHV6 and other virus have been demonstrated, and the pathogenic role of the virus is under study (22–24).

To provide an operational classification which can adapt to the increasing knowledge of the mechanisms of reactions and to the symptoms and clinical presentations, a recent initiative has provided a new terminology, applicable to precision medicine. In the new categorization drug allergy phenotypes are defined by the underlying endotypes and associated biomarkers and can be used in personalized medicine, with each patient being categorized according to her/his symptoms complex presentation. Current phenotypes include acute and delayed reactions with IgE and non-IgE involvement, cytokine storm, and mixed patterns. The endotypes responsible for the expression of symptoms include mast cell and basophil activation through known receptors (FceRI, FcgR, MRGPRX2) and directly through known receptors: complement, kinin and bradykinin activation and COX-1 inhibition. Associated biomarkers include serum tryptase, skin testing, basophil activation test, specific IgE and patch testing among others (13, 25–28).

Patients presenting with reactions compatible with phenotypes consistent with acute and delayed IgE and non-IgE, mast cell/basophil activation, and T cell activation endoptypes may be prevented from the use of first-line therapies for fear of inducing anaphylaxis or more severe delayed reactions upon re-exposure to the allergenic drug. A groundbreaking procedure, desensitization, has emerged in the last 15 years as a proven effective and safe procedure to maintain patients on their first-line medications.

CLINICAL VIGNETTE

Mrs. MFF is a 49-year-old healthy female who was discovered to have ovarian cancer after a routine gynecology ultrasound and was initially treated with surgery and chemotherapy with six courses of carboplatin and paclitaxel and entered remission. Two years later, the CA125 is increased and new masses are found in her abdomen, a diagnosis of recurrent stage 4 ovarian cancer is made and carboplatin and paclitaxel restarted. After the second course of carboplatin, the patient feels her hands itchy but finished the infusion and did not have any further symptoms. On the day of her third infusion, the patient presented flushing, generalized pruritus, shortness of breath, and sudden dizziness. The blood pressure drops below normal range as well as the oxygen saturation and the patient has a syncopal episode and needs to be resuscitated with epinephrine, fluids, anti-histamines, and steroids. She recovers and her diagnosis is of anaphylaxis, a serum tryptase level during the episode is elevated at 52 ng/ml (normal range 11.4 ng/ml). The patient is evaluated for carboplatin allergy and skin testing is positive. Her options are to change to a second-line agent which is likely to reduce her life expectancy or to remain on first-line therapy with carboplatin but because of her anaphylactic reaction this option is not considered safe unless carboplatin can be introduced through desensitization, a powerful and novel intervention which has shown to protect patients against anaphylaxis and permit re-introduction of allergy drugs.

DRUG DESENSITIZATION

The term drug desensitization is currently used to define a process by which a patient's immune response to a drug is modified to generate temporary tolerance, taking advantage of well characterized inhibitory pathways (6). In the case of IgE-mediated drug allergy, positive skin testing and specific serum IgE can be used as biomarkers along with elevated serum tryptase level during the acute reaction (8, 11, 17). Patients without evidence of IgE mechanism are good candidates for desensitization provided the phenotype of the drug reaction is a type I or a type IV like reaction without features of SJS/TEN (16, 21, 29) (**Table 1**).

TABLE 1 | Indications, contraindications and risk factors for drug desensitization.

Indications	High-risk patients	Contraindications
Reactions type I (mast cells/IgE/basofills) Reaction type IV (except SCARs)	Severe anaphylaxis (intubation)	Severe cutaneous adverse reactions (SCARs) (SJS/TEN, DIHS/DRESS, AGEP)
No alternative drug	Severe respiratory disease	Immunocytotoxic reactions (type II reactions)
Drug is more effective and/or associated with less side effects	Severe cardiac disease	Vasculitis
Drug has a unique mechanism of action	Severe systemic diseases	Serum sickness-like (type III reactions)
	Use of beta-blockers, ACE inhibitors Pregnancy	

SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis; DIHS, drug-induced hypersensitivity syndrome; DRESS, drug reaction (rash) with eosinophilia and systemic symptoms; AGEP, acute generalized exanthematous.

The mechanisms underlying drug desensitization are based on *in vitro* and *in vivo* models which have proposed that mast cells and basophils can be induced to predominantly inhibitory pathways by small incremental antigen doses, deactivating signal transduction and mediators release (13) (**Figure 1**).

Negative skin test is seen following desensitization in patients with IgE-mediated reactions, providing evidence of the powerful mechanisms which turn off skin mast cells (30–32). Partitioning of an optimal dose into 11–16 incremental doses starting at 1/1,000 the target dose and delivering them with sufficient time interval to mast cells; inhibits the acute release of beta-hexosaminidase, a mast cell granule mediator, prevents the generation of arachidonic acid and products such as leukotrienes and prostaglandins and the late generation of inflammatory cytokines (**Figure 1**) (13).

During desensitization, calcium influx is abolished and actin polymerization impaired, providing stability to intracellular granules in an antigen-specific fashion (**Figure 1**) (13). Membrane events that prevent internalization or modify its

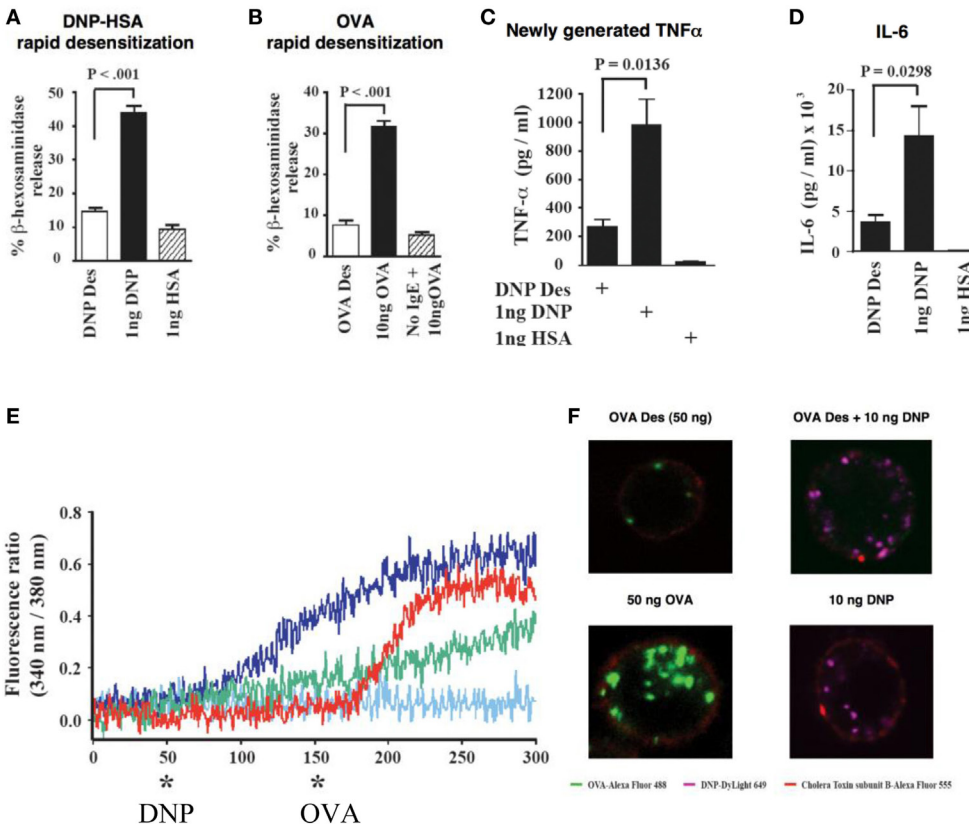


FIGURE 1 | *In vitro* IgE/antigen mouse mast cells activation and desensitization (13). **(A)** Desensitization of *in vitro* DNP-IgE sensitized mouse mast cells with DNP inhibits the release of granule mediators such as beta-hexosaminidase. Instead of one single optimal dose, 11 suboptimal sequential doses are given until reaching the optimal dose. **(B)** Desensitization of *in vitro* OVA-IgE sensitized mouse mast cells with OVA inhibits the release of granule mediators such as beta-hexosaminidase. Instead of one single optimal dose, 11 suboptimal sequential doses are given until reaching the optimal dose. **(C)** Desensitization of *in vitro* DNP-IgE sensitized mouse mast cell mediators with DNP inhibits the *de novo* generation of cytokines TNF alpha and IL-6 **(D)**. **(E)** Calcium entry (blue line) occurs after activation of DNP-IgE sensitized mouse mast cells with DNP but not when cells have been desensitized to DNP (red line, * DNP). Desensitization is specific since cells that were DNP desensitized and OVA-IgE sensitized presented calcium entry after OVA activation (red line, * OVA). **(F)** Membrane events are modified during desensitization with lack of internalization of desensitized antigens: left panels indicate that OVA desensitization does not present internalization of antigen (upper panel) as opposed to activation (lower panel with green labeled OVA internalized) and right panels indicate that after OVA desensitization another non desensitizing antigen such as DNP can be internalized (upper panel) as seen with activation (lower panel).

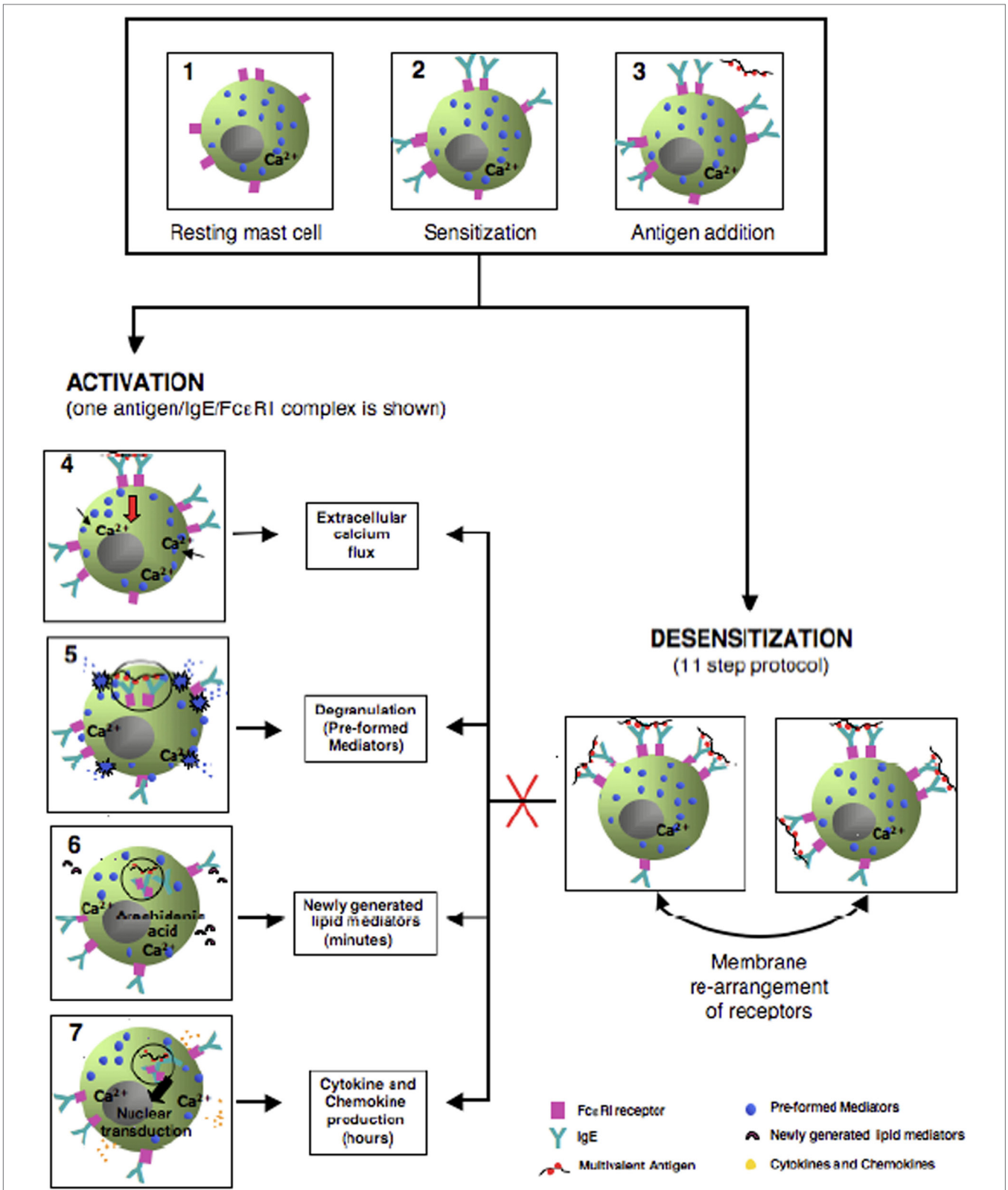
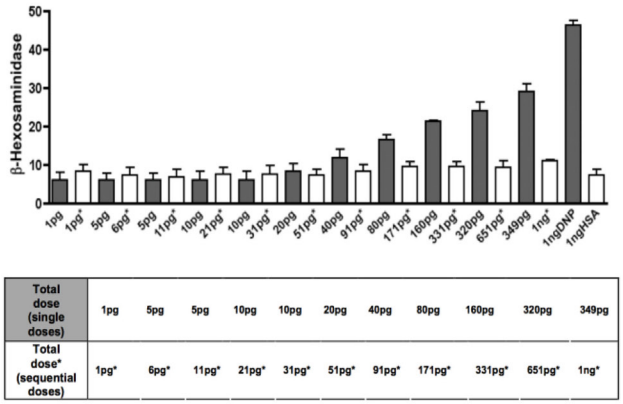


FIGURE 2 | Model of *in vitro* mouse mast cells activation and desensitization. The left side cartoons provide the steps of antigen/IgE/FceRI activation starting from antigen cross-linking, internalization, calcium entry and release of granule mediators, generation of lipd mediators such as prostaglandins and leukotrienes, and production of late phase cytokines. The right sided panel provides the hypothetical membrane capping and rearrangement occurring during the delivery of sequential suboptimal doses of allergen in desensitization preventing internalization of antigen, calcium entry, and mediators release.



Total dose (single doses)	1pg	5pg	5pg	10pg	10pg	20pg	40pg	80pg	160pg	320pg	349pg
Total dose* (sequential doses)	1pg*	6pg*	11pg*	21pg*	31pg*	51pg*	91pg*	171pg*	331pg*	651pg*	1ng*

Step	Solution	Rate (ml/h)	Time (min)	Volume infused per step (ml)	Dose administered with this step (mg)	Cumulative dose (mg)
1	1	2.5	15	0.63	0.0075	0.0075
2	1	5	15	1.25	0.015	0.0225
3	1	10	15	2.5	0.03	0.0525
4	1	20	15	5	0.06	0.1125
5	2	5	15	1.25	0.15	0.2625
6	2	10	15	2.5	0.3	0.5625
7	2	20	15	5	0.6	1.1625
8	2	40	15	10	1.2	2.3625
9	3	10	15	2.5	2.9764	5.3389
10	3	20	15	5	5.9528	11.2916
11	3	40	15	10	11.9055	23.1971
12	3	80	174.375	232.5	276.8029	300
Total time: 5.66 hrs						

FIGURE 3 | *In vitro* and *in vivo* protocols for induction of IgE/antigen desensitization. In the left panel in black, increasing single doses of antigen induce a dose response release of beta-hexosaminidase *in vitro* mouse mast cells. In white, the same doses given sequentially induce a profound inhibition of the beta -hexosaminidase release. In the right panel, a model of desensitization protocol used for human desensitization in which 3 bags and 12 steps (4 steps per bag) are used to administer sequential doubling doses every 15 min which provides the target dose of 300 mg after 5.66 h when the last step is completed.

response to subthreshold doses of antigen occur during desensitization and are associated with incremental unresponsiveness to specific antigen (**Figure 2**) (13). Association of the FceRI to ITIM containing receptors, capable of dephosphorylating ITAMs receptors has been postulated as one of the mechanisms of desensitization. Mast cells desensitized to one antigen are responsive to a second non desensitizing antigen, providing evidence of compartimentalization and highly specialized and regulated intracellular processes (**Figure 1**) (13).

The protocols used for *in vitro* desensitization have been adapted *in vivo* and further adaptations have produced safe protocols for human use (**Figure 3**) with similar dose increments and interval times (25–28). These human protocols have now been used in thousands of cases with remarkable safety since the inhibitory mechanisms of desensitization protect against anaphylaxis (**Figure 4**) (33, 34).

Whether after multiple desensitizations neutralizing antibodies can be generated which may block allergenic drug epitopes has been hypothesized (6). Maintaining drug desensitization state depends upon continued drug exposure and desensitized drugs require administration at regular intervals to maintain a stable pharmacokinetics state. Desensitization needs to be repeated if several half-lives of the medication have elapsed (6, 33).

Protocols for drug desensitization have been successfully used for antibiotics, chemotherapy drugs, and MoAbs among other drugs in patients with IgE and non-IgE-mediated HSRs (**Figure 5**) (6, 14, 33, 35). The phenotypes of reactions amendable to desensitization include immediate and delayed reactions. Typical type I reactions usually begin within minutes of initiation of the infusion to few hours after the infusion due to anti-histamine and steroid premedication. The signs and symptoms include puritus, flushing, urticaria, angioedema, throat tightening, wheezing, nausea, diarrhea, hypotension, syncope, seizures, and cardiovascular collapse which can lead to death. Atypical symptoms include back,

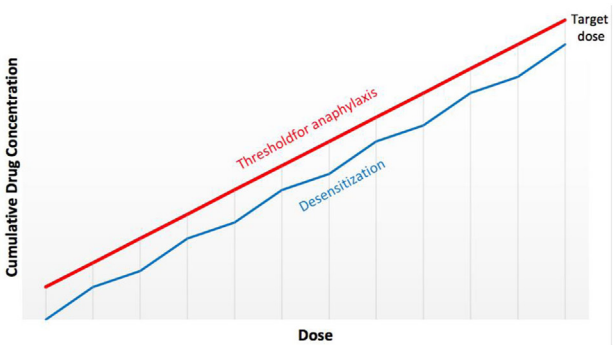


FIGURE 4 | Putative mechanism of protection against anaphylaxis during human desensitizations. By delivering the target dose of the drug by small incremental doubling doses (**Figure 3**), the threshold for anaphylaxis is re-established at each step and never reaches that of the initial triggering dose.

chest, or abdominal pain (such as seen with taxanes, oxaliplatin, MoAbs such as rituximab) (33, 35).

Delayed reactions are attributed to type IV reactions and can occur several days after the infusion and are typically limited to the skin with maculopapular rashes (36). Reactions that involve mucosal membranes and/or are associated with systemic symptoms are not amenable to desensitization due to the risk of inducing a severe systemic reaction with small amounts of drug antigen (16, 21, 29).

Desensitization should be considered in patients with reaction phenotypes consistent with type I and type IV reactions who have no alternative therapy or for whom alternative therapies are of less value or can induce more side effects. The algorithm for the evaluation of these patients is seen in **Figure 6** (6, 34, 37). The

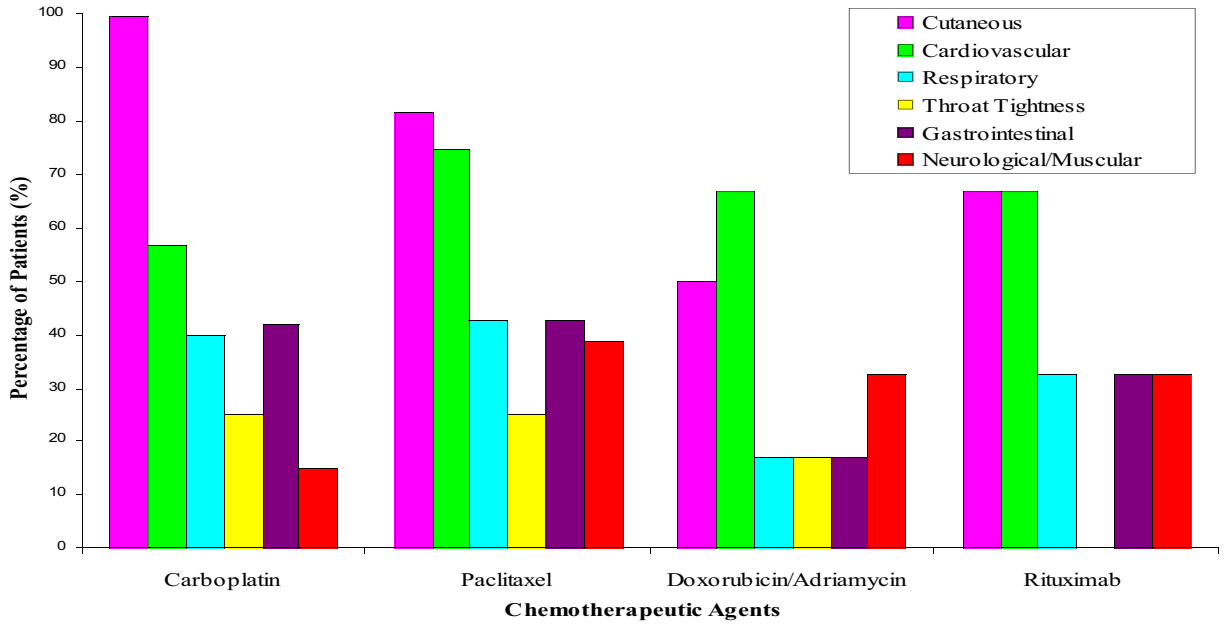


FIGURE 5 | Symptoms and signs of hypersensitivity reactions amendable to desensitization. Carboplatin and other paltins such as cisplatin and oxaliplatin reactions include classical symptoms of anaphylaxis with cutaneous, respiratory, cardiovascular, and gastrointestinal symptoms. Reactions to taxenes including paclitaxel and docetaxel present with pain as a neuromuscular symptoms in up to 4% of the patients. Doxorubicin/adriamycin and other chemotherapies present with sudden onset hypo or hypertension in up to 60% of patients and rituximab and other monoclonal antibodies present with cutaneous and cardiovascular symptoms in 70% of the patients.

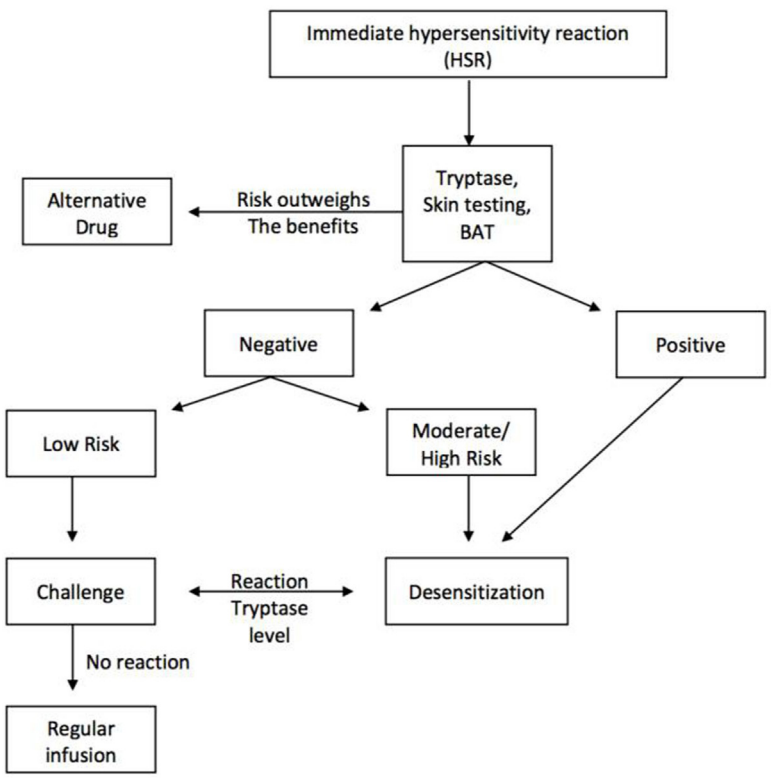


FIGURE 6 | Algorithm for the evaluation of drug hypersensitivity reactions and the role of desensitization for the re-introduction of the first-line medications, when no alternative is available or the alternative does not provide the same benefits or life expectancy as the first line.

nature and symptoms of the initial reactions needs to be established and tryptase and skin test provide evidence of IgE and/or mast cell involvement. BAT is a research tool and cannot be applied to current clinical practice.

Drug desensitization can be performed in patients of any age and in pregnant women when alternative therapies are not possible or when delaying therapy may incur a shortened life span. Anaphylaxis is the major risk during desensitization since patients are exposed to their allergic drug. Large series have demonstrated that most breakthrough reactions during desensitization are mild and less severe than the patient's initial HSR and fatalities have not been reported, but all desensitizations can only be performed by an expert allergist familiar with the personalized protocol and potential reactions (**Table 1**) (17, 33, 35). The safety of desensitizations is paramount and patients with grade 3 severe initial reactions and anaphylaxis can be desensitized with minimal reactions as seen in **Table 2**.

Desensitization is not recommended for type II and type III serum sickness-like reactions or in patients with reactions with skin desquamation, EM, Stevens–Johnson syndrome or toxic epidermal necrolysis, because small amounts of drug can induce irreversible and potentially fatal reactions (**Table 1**) (16, 21, 29).

The most commonly used intravenous desensitization protocols are standardized 12- to 16-step protocols modeled after *in vitro* protocols and can be personalized to all drugs with adjustment of the target dose, time intervals between doses and starting dose (**Figure 3**) (33, 38). Protocols are available for intravenous desensitizations but also for oral, subcutaneous, intraperitoneal, or intravenous routes in the outpatient and inpatient settings (39–41). Desensitization for delayed reactions is also available and may take several days but recent data suggest that some of these reactions may be amenable to shorter time intervals (8, 36, 42). The overall safety of desensitizations is similar for all medications provided the mechanism of the initial reaction is of type I, IgE and non-IgE or type IV. As seen in **Figure 7**, the overall safety indicates that 93% of patients present with no reaction or grade 1 reactions and all completed the desensitization.

Platins, taxanes, and MoAbs are the most common chemotherapy currently used in desensitization and are described below.

Platin Hypersensitivity

Platinum compounds are used in ovarian, colorectal, endometrial, glioblastoma, lung, and pancreatic cancer as initial chemotherapy and in second-line or salvage settings. Carboplatin is the most popular since it is less nephrotoxic and neurotoxic than cisplatin. Allergic reactions to platins are IgE-mediated and require sensitization through multiple exposures, with 27% of women becoming allergic after seven life time exposures (12, 43, 44). Allergic symptoms typically start at the second round of treatment, when the cancer recurs and after 1–2 exposures sensitized patients present with flushing and pruritus which can progress to shortness of breath with further exposure and can lead to anaphylaxis, with hypotension and cardiovascular collapse (2, 4, 7, 33, 45). Patients bearing BRCA 1 and 2 gene mutations have an increased risk for carboplatin reactions, which can occur with fewer exposures (46, 47). Most reactions to platins occur during or shortly after the drug infusion and the phenotype is that of type I reaction. In a study of 60 carboplatin sensitized patients, 100% had cutaneous, 60% pulmonary, 40% respiratory, and 42% gastrointestinal symptoms (6).

The phenotype of reactions to oxaliplatin can be more complex with features including typical IgE-mediated symptoms and atypical symptoms such as back and pelvic pain and cytokine-mediated fever and chills (7, 11, 17, 48, 49). Antibody-mediated thrombocytopenia and immune complex-mediated syndromes with urticaria and proteinuria have also been observed (17, 50).

Skin testing to platins has been safely done (**Table 3**) and is diagnostic tool to demonstrate an IgE/mast cell mechanism in patients with carboplatin and cisplatin reactions (7, 11, 17). For patients exposed to six or more courses of carboplatin in the last 6 months the positive predictive value is up to 86% (11). Oxaliplatin skin testing is negative in up to 50% of patients presenting type I reactions, indicating other than IgE mechanisms or lack of skin test allergenic determinants (17). Circulating serum specific IgE has been demonstrated and patients reactive to oxaliplatin with detectable serum-specific IgE have also demonstrated IgE to carboplatin and cisplatin without exposure, indicating broad cross-reactivity (51). IgE to platins can be short lived since a study has demonstrated that ST is negative in a high proportion of patients with a remote history of HSR to carboplatin, but re-exposure leads to resensitization and severe reactions (11). When platins are considered as first-line therapy, desensitization is a safe option since increased premedications alone do not prevent anaphylaxis and cross-reactivity may prevent the use of other platins (51). Patients with severe cutanoues reactions, SJS and TEN are currently not candidates since the mechanism of the reactions is unknown and small amounts of medications may induce severe symptoms (1, 16, 21, 29). Desensitization provides a similar life expectancy as non-allergic non desensitized patients (**Figure 8**) without increased health costs (33).

TABLE 2 | Safety of first desensitization in patients with grades 1, 2, and 3 initial reactions.

	First desensitization reaction grade				
Initial hypersensitivity reaction grade	**0**	**1**	**2**	**3**	**Total**
1	76 (61%)	38 (30%)	7 (6%)	4 (3%)	125
2	38 (58%)	22 (34%)	4 (6%)	1 (2%)	65
3	122 (60%)	54 (26%)	10 (5%)	19 (9%)	205
Total	236	114	21	24	396

From Sloane et al. (33).

Taxanes

Taxanes are used in gynecologic, lung, breast, and prostate cancers and reactions to taxenes are among the most frequent chemotherapy reactions and fatalities have been reported (52). Paclitaxel and docetaxel have been the more frequently used and more recently other taxenes such as cabacitaxel and abraxene have become popular (53). Paclitaxel is an insoluble compound originally isolated from the bark of the pacific yew tree, Taxus

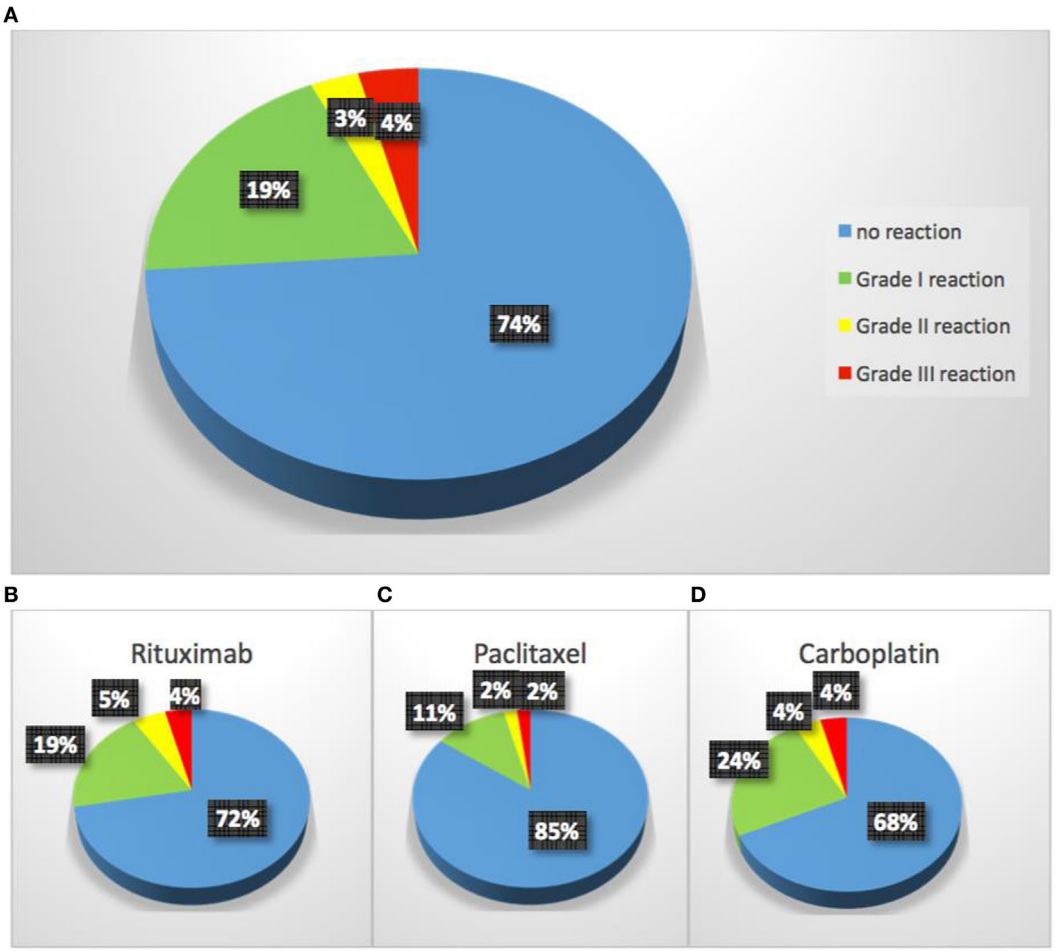


FIGURE 7 | The overall safety of desensitization for common chemotherapy drugs and monoclonal antibodies.

TABLE 3 | Skin testing for the diagnosis of chemotherapy drug allergy including platins, monoclonal antibodies, and paclitaxel.

Medication	Prick (mg/ml)	Intradermal (mg/ml)
Carboplatin	10	0.1, 1, 5, and 10
Cisplatin	1	0.1 and 1
Oxaliplatin	5	0.5 and 5
Rituximab	10	0.1, 1, and 10
Infliximab	10	0.1, 1, and 10
Tocilizumab	20	0.2, 2, and 20
Centuximab	20	0.2, 2, and 20
Traztuzmab	21	0.21, 2.1, and 21
Bevacizumab	25	0.25, 2.5, and 25
Cyclophosphamide	10	0.1, 1, and 10
Methotrexate	25	0.2, 2.5, and 25
Paclitaxel	1–6	0.001 and 0.01

Baccata tree and solubilized in cremophor and docetaxel is a semi-synthetic molecule derived from a precursor found in European yew tree needles and solubilized in polysorbate 80 (36, 54). The solvents can cause complement activation, generating anaphylotoxins C3a and C5a and leading to mast cell activation (55–57). Taxanes are used with premedications including anti-histamines and steroids due to a high rate of reactions in early clinical studies (54). The rate of reaction has decreased to less than 10% and typically occurs during the first or second lifetime exposure in up to 80% of the patients (54). The phenotype of the reactions include type I symptoms such as throat tightness, flushing, hypotension, and dyspnea but atypical symptoms such as chest, back, or pelvic pain (8, 36, 54).

Skin testing has uncovered IgE-mediated reactions to taxanes and a recent study reported that 103 of 145 taxane reactive patients (71%) had positive results. Negative skin test patients who were challenged were likely to tolerated taxane infusions without desensitization. Atopy was present in over 40% of the patients and because patients react at first or second exposure suggested prior sensitization or cross-reactivity with environmental allergens (8). Risk stratification based on biomarkers such as skin testing can safely guide the management to taxane reactions and allows a significant number of patients to resume regular infusions. For patients with positive skin test and significant initial reaction for whom taxanes are first-line therapy, desensitization should be considered (8).

Monoclonal Antibodies

There are over 45 MoAbs currently in use for the treatment of cancer and inflammatory and autoimmune diseases. Reactions to MoAbs depend on their structure and vary from chimeric mouse-human, humanized, to fully human. Some of the most frequently used MoAbs are presented in **Table 4**, including their targets, incidence of overall injection/infusion site reactions, and HSRs (19, 34, 35).

Monoclonal antibodies immunogenicity depends on the human content but fully human MoAbs, such as adalimumab and ofatumumab can induce severe HSRs likely due to the glycosylation patterns *in vitro* and the generation of neo antigens (58). This is best exemplified in reactions to cituximab which can occur at first exposure in patients sensitized through tick bites to the mammalian oligosaccharide epitope, galactose-alpha-1,3-galactose (alpha-gal) (59).

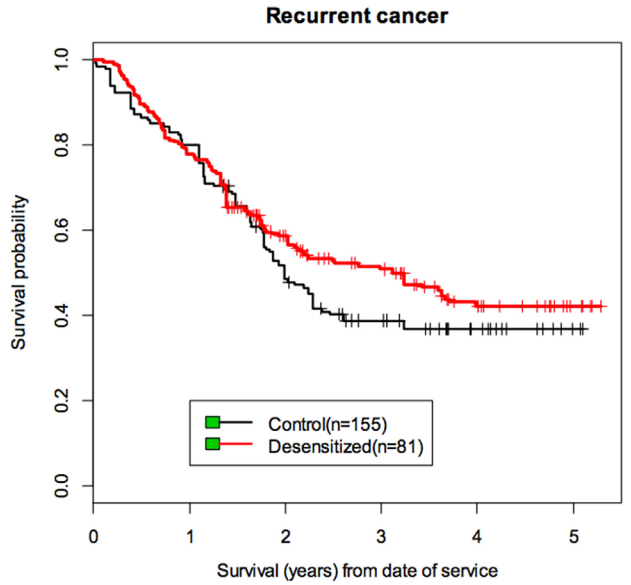


FIGURE 8 | Life expectancy for cancer patients allergic and desensitized to carboplatin and non-allergic to carboplatin [from Sloane et al. (33)]. Allergic and non-allergic ovarian cancer patients treated with carboplatin or carboplatin desensitization presented a similar life expectancy with a non significant advantage for the allergy desensitized patients.

The phenotypes of MoAbs reactions include limited infusion reactions, IgE-mediated reactions, serum sickness-like reactions, cytokine storm-like reactions, and mixed reactions. Infusion reactions are characterized by nausea, chills, fever, and malaise and for trastuzumab these reactions can occur in up to 40% of patients (34, 35). Like cytokine storm-like reactions, which are more severe, can associate with hypotension, oxygen desaturation, and require treatment with steroids and COX-1 inhibitors, proinflammatory cytokines (such as IL-6 and TNF-α) are thought to be involved (60, 61).

Immediate and delayed HSRs can occur with MoAbs and serum sickness-like reactions, such as seen with infliximab and omalizumab, which can present with rash, myalgia, fever, polyarthralgias, pruritus, edema, and fatigue (35).

Monoclonal antibodies used subcutaneously can elicit injection-site reactions few hours after the injection and persisting for several days. The phenotype of these reactions include local redness, warmth, burning, itching, urticaria, pain, and induration, varying in frequency from 0.8 to 4.5% with certolizumab to up to 45% with omalizumab (39).

Reactions to MoAbs can occur during the infusion and should prompt interruption of the treatment and the evaluation of tryptase and inflammatory cytokines to further understand the mechanism of the reactions. Skin testing with the offending agent can be done for type I and mixed reactions 2–4 weeks after the reaction to avoid false negative results, in particular in anaphylactic reactions in which natural desensitization can occur (35). An important consideration is cost of MoAbs; there are no available reagents at the present time for the evaluation of MoAb reactions and using a treatment vial may exceed several thousand dollars, precluding a diagnostic skin test evaluation. The negative

TABLE 4 | Common monoclonal antibodies in use and rate of overall reactions and hypersensitivity reactions (HSR).

Drug	Target	Overall reactions	HSR
Rituximab (Rituxan®) IV	CD20	77% (first infusion) (52)	5–10% (53)
Ofatumumab (Arzerra®) IV	CD20	44% (first infusion) (54) 67% (combination therapy) (55)	2% (55)
Obinutuzumab (Gazyva®) IV	CD20	66% (56,57)	(58)*
Trastuzumab (Herceptin®) IV	HER-2	40% (mild; first infusion) (59)	0.6–5% (60)
Cetuximab (Erbitux®) IV	EGFR	15–21% (61)	1.1–5% (62–65) 14–27% (Southern USA) (43,66,67)
Tocilizumab (Actemra®) IV	IL-6 receptor	7–8% (68)	0.1–0.7% (68)
Infliximab (Remicade®) IV	TNF-α	5–18% (69)	1%* (69)
Etanercept (Enbrel®) SC	TNF-α	15–37% (70)	<2% (70)
Adalimumab (Humira®) SC	TNF-α	20% (71)	1% (71)
Golimumab (Simponi®) SC	TNF-α	4–20% (72,73)	Not reported
Certolizumab (Cimzia®) SC	TNF-α	0.8–4.5% (74,75)	Not reported
Brentuximab (Adcetris®) IV	CD30	12% (76)	(77–79)*
Bevacizumab (Avastin®) IV	VEGF-A	<3% (80)	Not reported
Omalizumab(Xolair®) SC	IgE	45% (81)	00.9–0.2% (81,82)

*$< p = 0.05$

predictive value of skin testing is not known and in a study of 23 patients reactive to trastuzumab, infliximab, or rituximab, 13 patients had positive skin test. Positive and negative skin test patient with significant initial reactions are candidates for desensitization and subcutaneous protocols are available. Successful desensitizations to rituximab, ofatumumab, obinutuzumab, trastuzumab, cetuximab, tocilizumab, infliximab, etanercept, adalimumab, golimumab, certolizumab, brentuximab, bevacizumab, and omalizumab have been reported (33–35).

CONCLUSION

Drug hypersensitivity is an increasing health hazard, which can compromise the quality of life and the life expectancy of cancer patients and patients with chronic inflammatory diseases with reactions to their first-line therapy. Recognition of the mechanisms of the reactions into phenotypes, understanding the underlying endotypes and evaluation of biomarkers is key to personalized medicine and enhanced patient safety allowing for informed decisions regarding drug re-exposure and desensitization. Serum tryptase, skin testing, BAT, and specific IgE are helpful diagnostic tools which will be complemented in the future with genotyping to identify patients at risk before reactions occur. Appropriate treatment of the reactions including epinephrine use and management with personalized desensitization protocols can enhance the quality of life, life expectancy, and safety of an increasing at risk population of patients with cancer and inflammatory diseases allergic to their best medications. Most patients with reactions with phenotypes consistent with type I and type IV reactions are candidates for desensitization, which can provide advancement of personalized treatments. Drug desensitization protects against anaphylaxis and activates inhibitory mechanisms which need further research to uncover cellular and molecular players amendable to pharmacological applications, which can make desensitization safer and more effective.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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MINI REVIEW
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Multifactorial Modulation of Food-Induced Anaphylaxis

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Prevalence of food-induced anaphylaxis increases progressively and occurs in an unpredictable manner, seriously affecting the quality of life of patients. Intrinsic factors including age, physiological, and genetic features of the patient as well as extrinsic factors such as the intake of drugs and exposure to environmental agents modulate this disorder. It has been proven that diseases, such as mastocytosis, defects in HLA, or filaggrin genes, increase the risk of severe allergic episodes. Certain allergen families such as storage proteins, lipid transfer proteins, or parvalbumins have also been linked to anaphylaxis. Environmental factors such as inhaled allergens or sensitization through the skin can exacerbate or trigger acute anaphylaxis. Moreover, the effect of dietary habits such as the early introduction of certain foods in the diet, and the advantage of the breastfeeding remain as yet unresolved. Interaction of allergens with the intestinal cell barrier together with a set of effector cells represents the primary pathways of food-induced anaphylaxis. After an antigen cross-links the IgEs on the membrane of effector cells, a complex intracellular signaling cascade is initiated, which leads cells to release preformed mediators stored in their granules that are responsible for the acute symptoms of anaphylaxis. Afterward, they can also rapidly synthesize lipid compounds such as prostaglandins or leukotrienes. Cytokines or chemokines are also released, leading to the recruitment and activation of immune cells in the inflammatory microenvironment. Multiple factors that affect food-induced anaphylaxis are discussed in this review, paying special attention to dietary habits and environmental and genetic conditions.

Keywords: food-induced anaphylaxis, IgE, allergens, diet, mast cells, basophils

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INTRODUCTION

The widely used definition of anaphylaxis as "an adverse allergic reaction that is rapid in onset and may cause death" is also accompanied by clinical criteria for diagnosis (1). Other definitions of anaphylaxis have been formulated to aid its diagnosis and management (2). It constitutes an alarming medical emergency (1, 3, 4), not only for the patient and members of the family, but sometimes also for the healthcare professionals involved. Death usually occurs because of respiratory or cardiac arrest as an aftershock of an anaphylactic attack (5). Although life-threatening episodes are uncommon,

Abbreviations: FA, food allergy; HIS, histamine; IECs, intestinal epithelial cells; LTP, lipid transfer protein; MCs, mast cells; PAF, platelet-activating factor.

these events constitute an unpredictable risk and their prevalence is steadily increasing affecting up to 2% of the population (6). Hospital and critical care unit admissions are not common but continue to increase, doubling in frequency between 1998 and 2012 (7, 8). An accurate population-based estimate is difficult to obtain due to underdiagnosis and underreporting, as well as by the use of different clinical definitions for anaphylaxis and methods of case diagnosis in populations under study (9).

Molecules such as histamine (HIS), tryptase, leukotrienes, and prostaglandins, among others, mediate the clinical manifestations of anaphylaxis. Secretion of these mediators occurs after an allergen cross-links the IgEs bound to mast cells (MCs) and basophils. However, IgE-independent immune mechanisms may also be involved (3). Physiological state (3), as well as certain diseases and medications (10), are risk factors for anaphylaxis. Cofactors such as drugs or exercise that can exacerbate or trigger acute anaphylactic episodes have been described (11–13). Specialist physicians and patients need to be aware of the relevant risk factors and cofactors in the context of long-term management and treatment of this condition. In this mini review, we summarize the physiological, genetic, and environmental aspects in the field of food allergy (FA) focusing special attention on anaphylactic reactions.

INCIDENCE OF FOOD-DERIVED ANAPHYLAXIS

Food allergy is a serious and often life-threatening health concern that is increasing in frequency especially in the vulnerable pediatric population affecting 4% of children and 2–3% of the adult population worldwide (14). The treatment requires changes in dietary habits and social behavior (15). FA is originated by a reaction of the immune system that results in non-tolerance of specific foods. In most patients, IgE mediates this immune disorder, although there are also IgE-independent cell-mediated allergies that are accompanied by gastrointestinal symptoms (16), but they are not going to be analyzed in this review.

More than 170 foods have been associated with type I allergies, the most common of which are milk, egg, wheat, fish, shellfish, peanuts, soy, and tree nuts, although the prevalence varies geographically (17). The two most frequent food allergens that induce severe and potentially lethal anaphylaxis are milk and egg, while the third differs between countries (18) being peanuts in the USA and Switzerland, wheat in Germany and Japan, tree nuts in Spain, and sesame in Israel (16). Foods and meals containing hidden allergens at restaurants are a serious source of risk for patients with food allergies (19). The prevalence of food-induced anaphylaxis seems to vary with the population selected and the region where they are recruited. The point prevalence of food challenge-confirmed allergy is under 1% (20–23).

Food allergies can be caused by primary sensitization to an eliciting food allergen (class I allergen), or they can be triggering by a primary sensitization to an inhalant allergen and later IgE cross-reactivity to a homologous protein in food (class II allergen) (24). Almost all plant food allergens are either storage or defense-related proteins and three dominating plant allergenic protein superfamilies have been identified as being involved in triggering severe reactions. These are prolamins that include the 2S albumins, lipid transfer proteins (LTPs), and α-amylase/trypsin inhibitors, cupins that comprise the major globulin storage proteins, mainly found in legumes and nuts and the Bet v 1-related protein family (25, 26). Among animal food allergen families, parvalbumins, tropomyosins, and caseins are the three dominant groups (25, 26). The most significant molecular characteristic of these allergens is their resistance to proteolysis, which increases the probability of reaching intact the intestinal mucosa and triggering an immune response (27).

INFLUENCE OF DIET IN FA

The prevalence of FA is growing at an alarming rate (28), and it is not easy to know with precision the reasons behind this growth. Factors such as a sterile pathogen-free environment, changing dietary habits, vitamin D deficiency, intestinal microbiota composition, stability properties of certain allergens, or alcohol consumption may play a role in this increase (29) (**Figure 1**).

Breast milk provides an abundant source of soluble IgA and prebiotic glycans that promote the expansion of species of *Lachnospira*, *Veillonella*, and *Rothia*, which are adapted to this food source (30, 31). In contrast, formula feeding may not be sufficient for neonates with poor immunity, exacerbating the allergic reactions and only amino acid-based formulas should be considered as non-allergenic. Pre- or probiotic supplemented infant formulas are also used but they might not fully replicate the beneficial effect of breast milk.

The correct time to incorporate certain allergenic foods in the diet of infants has also been controversial topic. It has been demonstrated that the early introduction of peanut to high-risk 7-month-old babies reduces the incidence of peanut allergy (32). Moreover, the LEAP study showed that in high-risk infants, sustained consumption of peanut beginning in the first 11 months of life was highly effective in preventing the development of peanut allergy (33).

Nowadays, food processing is an important aspect in our daily diet. The stability of the allergenic proteins during these procedures (34) and the resistance to gastric and intestinal digestion (35) are properties that preserve the integrity of IgE-specific epitopes, as occurs in LTPs allergic patients (36). These processes can alter structural characteristics of allergens and therefore modify their allergenic capacity (37–40). Patients, who possess specific IgE to linear epitopes, have a stronger response to cooked and partially digested antigens, while those patients who recognize conformational or three-dimensional epitopes have a milder clinical response (41, 42).

It has also been proved that some nutrients, such as vitamin D, influence the regulation of the immune system (43–45). In fact, the frequency of food anaphylaxis increases in areas where the exposure to UVB radiation is low and vitamin D is not synthesized in adequate concentrations (46). Moreover, vitamin D deficiency is associated with challenge-proven FA in infants (47). The deficit of this vitamin promotes Th2 responses by reducing the number of Th1 cells and inducing Th2 cell proliferation. However, in correct doses, vitamin D is responsible for reducing the allergic response

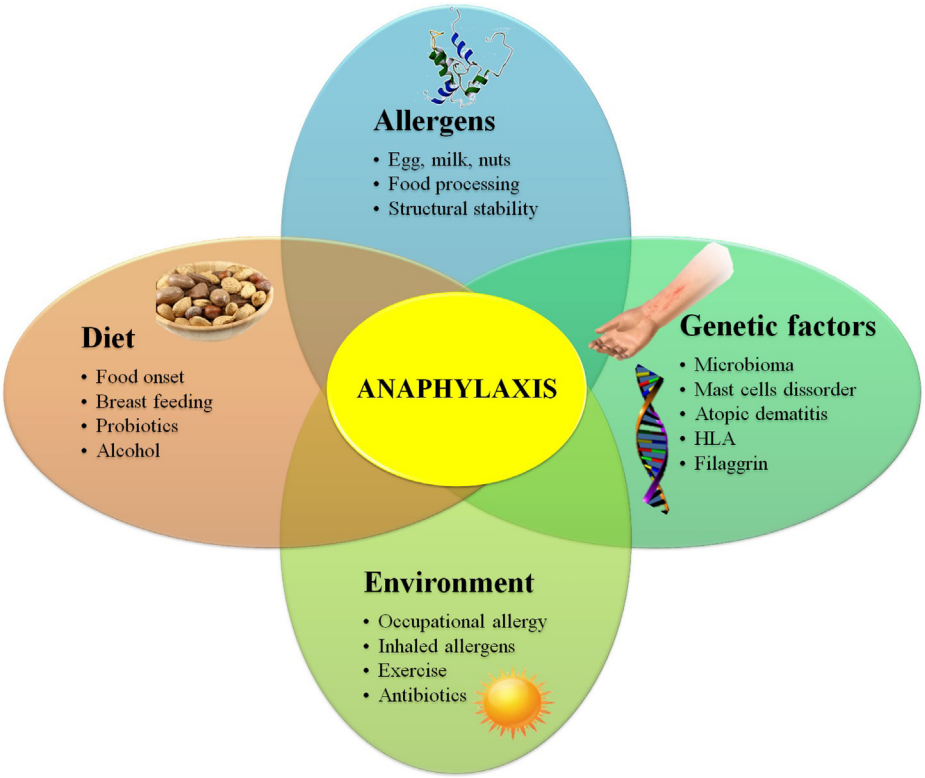

FIGURE 1 | **Diagram showing genetic, environmental, dietary habits, and allergen-related risk factors for food allergy**.

by promoting Treg and suppressing Th17 cells, demonstrating the importance of a correct balance (48).

GENETIC AND ENVIRONMENTAL FACTORS INVOLVED IN FA

A few specific gene mutations have been related to food anaphylaxis. Several studies have identified an association of HLA genes with peanut allergy (49–51). Moreover, filaggrin is a key protein in the function of the epidermic epithelial barrier, and Cabanillas and Novak (52) have shown that the development of peanut allergy in children that carry one or more mutations in the filaggrin genes is provoked even when they are exposed to very small quantities of allergen, a risk that increased with higher dose exposure.

However, exclusively genetic susceptibility cannot explain the rapidly increasing prevalence of food allergies, suggesting that something in our environment is promoting this disease. There is increasing evidence that an early sensitization is occurring in individuals through the skin or even through breast milk and the amniotic fluid. Atopic dermatitis, multiplies by a factor of 10 the risk of suffering peanut allergy (53). The exposure to peanut particles suspended in the environment (54), the absorption of active allergenic components present in topical lotions (55), the damage induced by scratching, or the presence of Staphylococcal enterotoxin B in the areas surrounding a skin lesion have all been associated to peanut allergy (56). Moreover, workers exposed to occupational food allergens can be sensitized through the skin or by inhalation (57, 58).

Allergic reactions are increasingly associated with demographic variables, which are becoming every day more widespread in the twenty-first century such as cesarean births. Delivery by cesarean section may predispose the newborn baby to FA, presumably due to modifications in the establishment of gut microbiota caused by a different initial exposure to microbes (59, 60). Continuous intake of antibiotics has a profound influence on the bacterial composition that colonize the intestinal track, which may affect the allergic response to food and which seems to be essential for the maintenance of homeostasis (61, 62). In a healthy gastrointestinal tract, the epithelium develops immune tolerance to antigens present in the diet, fights invading microorganisms, and limits their presence near the mucosa (63, 64). Alterations in gut microbiota (dysbiosis) and consequent disruption of homeostasis have been linked to the occurrence of allergic reactions (65). However, whether an incremented permeability in the intestine is a cause or a consequence of an allergic reaction is still an open question (66). The finding that Clostridia strains can suppress allergy in mice (67, 68) suggests the potential use of microbial therapies to enhance the development of tolerance when given with allergen immunotherapy (69, 70).

A broad variety of other factors may also contribute to the increased risk of food-induced anaphylaxis. A prospective study reported an association between anaphylaxis and exercise, drug use, acute infection, premenstrual status, or psychological stress

in 20% of patients (13). Food-dependent exercise-induced anaphylaxis has also been observed, although it is found more often in adults than in children (71–73). Studies have also shown that epigenetic variants, primarily in the pattern of DNA methylation, are associated with FA (74). Increased severity of anaphylaxis has also been reported for the elderly, patients with pre-existing cardiovascular disease, MC disorders, or undergoing concomitant treatment with a beta-adrenergic blocker and/or angiotensin converting enzyme inhibitor (75). Non-steroidal anti-inflammatory drugs also seem to enhance some food-allergic reactions (76).

MUCOSAL IMMUNOLOGY OF FA

The gastrointestinal tract has a large surface area that is responsible for the digestion and absorption of food. It is formed by the intestinal epithelium, a cell monolayer that creates a selective barrier separating both outside and inside environments (77) (**Figure 2**). This epithelium is the first line of defense forming a physical and biochemical barrier where the mutualistic relationship between commensal microbial communities and immune cells are in a state of homeostasis (78), thereby preventing the colonization by other pathogenic microorganisms (79). This delicate balance depends on the functions of intestinal epithelial cells (IECs), maintaining a state of non-responsiveness (tolerance) or releasing an antipathogenic immunity.

Enterocytes form the dominant cell population in the epithelial barrier, constituting more than 80% of all IECs with functions that are metabolic and digestive, as well as secretory. Globet and paneth cells, also present in the barrier, release numerous hormone regulators such as mucins and antimicrobial proteins and protect the host from infection (80, 81). The most abundant mucins, MUC2, control macrophage and adaptive T cell responses during inflammation and inhibit pathogen microorganism chemotaxis (82, 83). Microfold cells (M cells) that form concentrations in the epithelium above Peyer's patches mediate the presentation

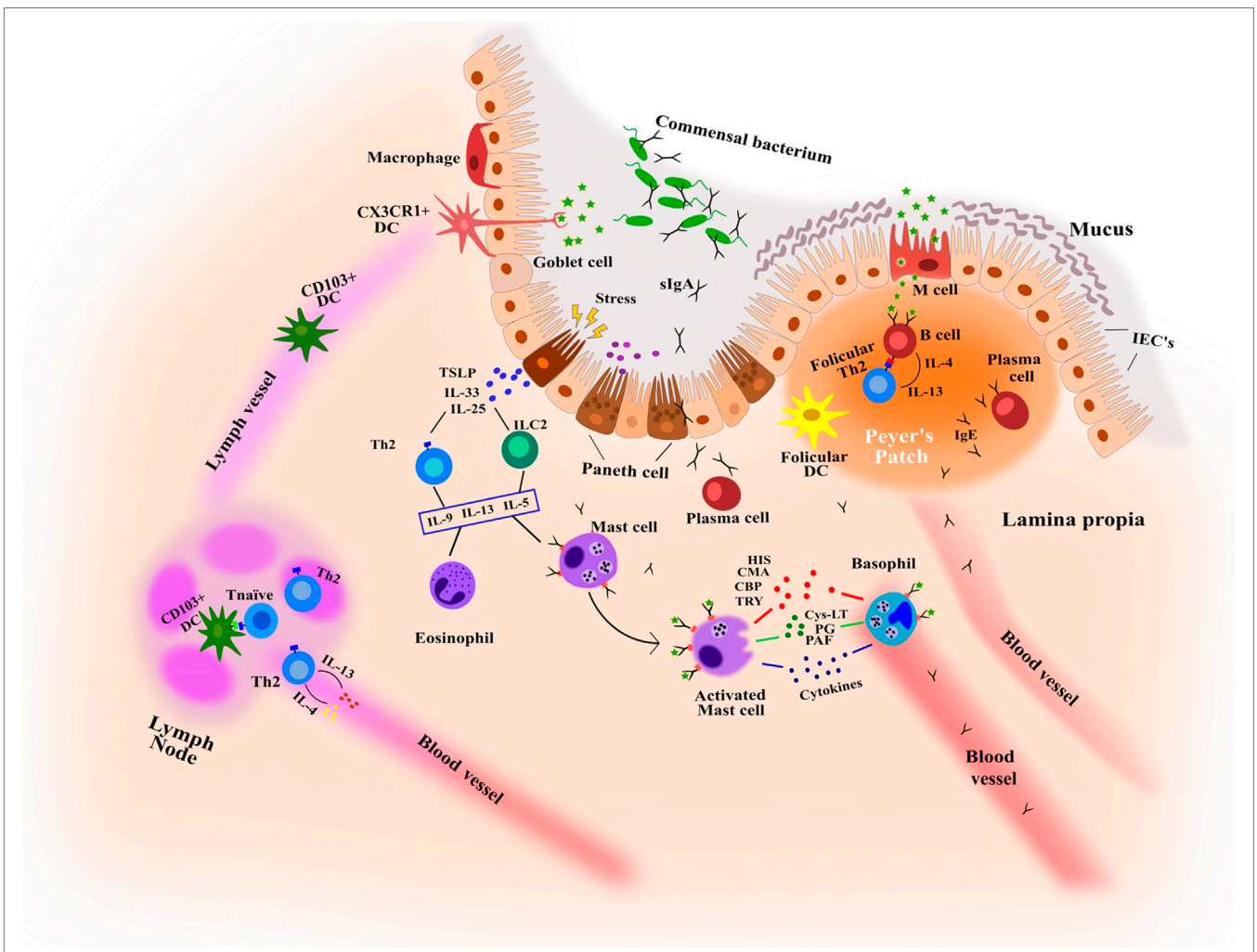

FIGURE 2 | Key features in the immunological mechanisms of intestinal mucosa involved in food allergy and food-induced anaphylaxis. CBP, carboxipeptidase; CMA, chymase; Cys-LT, cysteinyl leukotriene; HIS, histamine; IECs, intestinal epithelial cells; PAF, platelet-activating factor; PG, prostaglandins; TRY, tryptase.

of antigens and microorganisms to the mucosal immune system, appearing as efficient mechanisms of receptor-mediated transport (84). In addition, among the cells of the immune system, dendritic cells and macrophages play an important role as antigen presenting cells. They are able to capture, process, and present the antigen to T lymphocytes, and induce a specific immune response. On the other hand, MCs located in the lamina propria and cell barrier detect antigens through the specific IgEs attached to their membrane and induce inflammatory reactions by secreting cytokines and immune mediators.

CELLS AND MOLECULES INVOLVED IN FOOD-INDUCED ANAPHYLAXIS

Effector Cells of Anaphylaxis

Mast cells and basophils represent the primary effector cells in the pathophysiological process of food-induced anaphylaxis (85, 86), although it has been speculated that they may also participate in late-phase and chronic allergic reactions (87). They express complementary and overlapping roles in both regulatory and effector activities (88) and due to the similarities in histochemical characteristics, IgE receptor expression and the mediators produced, MCs and basophils were supposed to be related. However, transcriptional analysis has shown minimal similarity between MCs and basophils (89), and in recent years, several studies have indicated that although derived from unique hematopoietic progenitors, they are not closely related (90, 91). Both cell types also differ in their development (92). Basophils complete their differentiation within the bone marrow, while MCs circulate in the blood as progenitor cells and enter the tissues to proliferate and maturate (93). Tissue microenvironment regulates the mast cell expression of proteases (94, 95). Accordingly, humans MCs are classified based on their protease composition as MCs containing both tryptase and chymase, typically present in the bowel submucosa within the connective tissue, and those containing only tryptase, which are predominant on mucosal and epithelial surfaces such as bowel mucosa (96). Chymase-only-positive MCs have also been described but they appear to be very infrequent (97).

Mechanism of Effector Cell Signaling

To understand the mechanisms of effector cell activation, it is essential to comprehend the regulation of intracellular signaling pathways that lead cells to release their mediators during the acute phase of anaphylaxis. In the last years, these events have been extensively studied and reviewed in the literature (98–104). However, given the complexity of these processes, here we present only a brief overview of the current state of affairs, focusing primarily on FcεRI. Signaling initiated by FcεRI aggregation triggers phosphorylation of immunoglobulin receptor activation motifs, which is mediated by the Src family tyrosine kinase Lyn and Syk. Subsequently, two major signaling enzymes, phosphoinositide phospholipase C-gamma and phosphoinositide 3-kinase, are activated which causes the release of calcium from intracellular stores, leading to cell degranulation, arachidonic acid metabolism, and activation of transcription factors with the consequent production and release of lipid mediators and the production of cytokine and chemokine. Effector cells also possess receptors characterized by a conserved immunoreceptor tyrosine-based inhibitory motif that when phosphorylated, recruits protein (SHP-1) and lipid phosphatases (SHIP1, SHIP2) that inhibit protein interactions and therefore inhibit the signaling cascade. In the end, despite all these complex cellular signaling pathways, the status of effector cell activation is really a balance between the pathways that upregulate these processes and those that downregulate them.

Mediators of Anaphylaxis

Both MCs and basophils participate in clinical manifestations of anaphylaxis that typically involve the skin and the respiratory and gastrointestinal tracts (105). They release potent inflammatory mediators after an antigen cross-links the specific IgE attached to their surface receptors. Tissue-derived MCs and circulating basophils can immediately release preformed mediators stored in their granules such as HIS, heparin, or proteases that are responsible for many of the acute symptoms such as vascular leak or bronchoconstriction (106, 107). HIS, the main biogenic amine released upon activation of effector cells, has long been proven to be a short half-life factor triggering a variety of symptoms of anaphylaxis, including inflammation, itchiness, and mucus production (108). Recent studies have shown a correlation between plasma HIS levels and anaphylaxis severity (109). The efficiency of blocking HIS H1 and H4 receptors to suppress intestinal anaphylaxis in peanut allergy has also been proved, being this effect mediated through the limitation of mesenteric lymph node and intestinal dendritic cell accumulation and function (110). Tryptase is currently one of the biomarkers to assess MCs activation and levels are enhanced after onset of anaphylaxis (111). Basal tryptase levels in serum may predict moderate to severe anaphylaxis in children with FA (112) and they have also been described as good markers for the diagnosis of food-induced anaphylaxis (113). Chymase and carboxypeptidase levels in serum of patients with anaphylaxis have been found to be significantly greater than those found in healthy subjects, although there is no correlation with tryptase levels (114).

After activation, MCs and basophils can also rapidly synthesize lipid compounds in their membrane. These include prostaglandins, leukotrienes, and platelet-activating factor (PAF) that are mediators of hypotension, bronchospasm, mucus secretion, as well as leukocyte and dendritic cell recruitment during anaphylaxis (115). Increased urinary concentrations of leukotrienes and prostaglandins have been found in patients with food-induced anaphylaxis (caused by seafood, nuts, and soybean milk) compared to healthy individuals, showing good correlation between both mediators (116). PAF correlates better than either HIS or tryptase with severity of symptoms (117), and a deficiency of PAF-AH, the enzyme that inactivates PAF, predisposes patients to severe anaphylaxis (118). Moreover, it has been proven that detection of malfunction of PAF-AH may help identify individuals at risk of anaphylaxis (119).

Upon activation of MCs and basophils, cytokines or chemokines are newly synthesized and released, which are involved in

the recruitment and activation of several cells in the inflammatory microenvironment (106). Many cytokines, chemokines, and growth factors are released from MCs (120) and basophils (121). IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, and IFN-γ are elevated in anaphylactic patients (106), but only IL-2, IL-6, and IL-10 have been correlated with initial reaction severity (106, 109), although it is difficult to determine their origin because they are not exclusively produced by effector cells.

CONCLUSION

Despite efforts to prevent FA and anaphylaxis and to elucidate the fundamental underlying mechanisms that link allergen exposure to symptoms, the incidence is increasing in developed countries. Individual genetic predisposition is an important determinant, although the features of the allergen itself and a variety of environmental factors, including diet and intestinal microbiota may have an important impact on sensitization, severity, and persistence of FA. Preventive strategies, such as dietary interventions, the use of probiotics, or prolonged exclusive breast-feeding, among others, have recently come into question and therefore it is important to identify and study how to control the risk factors to reduce the development of FA. The complex network of factors involved in these disorders makes research in this field difficult and much more investigation needs to be done to identify and manipulate the modifiable risk factors to offer significant benefits to mitigate this disease in the future.

AUTHOR CONTRIBUTIONS

SB, MG-A, LM-P, AD-P, and MV contributed to writing and critically revised the paper. CB contributed to designing and drawing the figures.

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Insights in Anaphylaxis and Clonal Mast Cell Disorders

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The prevalence of anaphylaxis among patients with clonal mast cell disorders (MCD) is clearly higher comparing to the general population. Due to a lower frequency of symptoms outside of acute episodes, clonal MCD in the absence of skin lesions might sometimes be difficult to identify which may lead to underdiagnosis, and anaphylaxis is commonly the presenting symptom in these patients. Although the release of mast cell (MC) mediators upon MC activation might present with a wide variety of symptoms, particular clinical features typically characterize MC mediator release episodes in patients with clonal MCD without skin involvement. Final diagnosis requires a bone marrow study, and it is recommended that this should be done in reference centers. In this article, we address the main triggers for anaphylaxis, risk factors, clinical presentation, diagnosis, and management of patients with MC activation syndromes (MCASs), with special emphasis on clonal MCAS [systemic mastocytosis and mono(clonal) MC activations syndromes].

Keywords: anaphylaxis, clonal, mast cell, mast cell activation syndrome, mastocytosis

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INTRODUCTION

Anaphylaxis occurs as a result of the sudden release of a wide broad of mediators from mast cells (MCs) and basophils. Clinically, it may show heterogeneous symptoms involving different organs and tissues as far as it fulfils the proposed diagnostic criteria (1), but it usually presents as a serious reaction which can be life threatening or fatal (2).

Mast cells are ubiquous immune cells that preferentially reside as mature cells in the connective tissue from body sites acting as natural barriers for exogenous antigens such as the skin and the gastrointestinal or respiratory tracts, among other tissues; despite this, mature MCs derive from a hematopoietic precursor in the bone marrow (BM). The activation of MCs can be mediated by immunological or non-immunological mechanisms that induce the release of preformed proinflammatory substances and also promote the synthesis of many other mediators (3, 4).

The term MC activation syndrome (MCAS) encompasses a heterogeneous group of disorders characterized by the existence of clinical symptoms secondary to the systemic effects of mediators released by MCs upon their activation, including anaphylaxis. Based on a recent consensus proposal (5, 6), MCAS can be classified into three main categories: (1) primary MCAS, which includes systemic mastocytosis (SM) and (mono)clonal MCAS (MMAS), (2) secondary MCAS, and (3) idiopathic MCAS. The key feature that defines primary MCAS is the demonstration of clonal BM MCs, which results into a constitutive hyperactivity of MCs. In most SM and MMAS patients, MC clonality can be established by the detection of activating mutations of the KIT receptor, a protein membrane involved in the regulation of crucial MC functions such as differentiation, activation and survival. On the contrary, MCs in patients with secondary and idiopathic MCAS are normal;

in these latter cases, MC activation symptoms are related with clinical conditions that can secondarily activate MCs such as allergic, neoplastic, inflammatory, or autoimmnune diseases, or with unknown factors.

Herein, we review the main triggers, risk factors, clinical presentation, diagnosis, and management of patients with MCAS, with special emphasis on primary (clonal) MCAS (SM and MMAS).

PATHOPHYSIOLOGY OF ANAPHYLAXIS AND CLONAL MCAS

In allergic reactions, MC activation is due to the interaction of circulating IgE antibodies–antigen complexes with high-affinity Fc receptors for IgE (FcεRI) on the surface of MCs (and basophils). In addition to this mechanism, MCs can also be activated by other non-IgE-mediated immunological mechanisms and by non-immunological mechanisms, such as C3 and C5 (7), nerve growth factor (8), IgG (9–12), and toll-like receptors (13–15), among others. Upon MC activation, the proinflammatory response is further regulated by the balance of both positive and negative multiple molecular events (16), including gp49B1-αvβ3 (17), ITIM and ITAM motifs, kinases, phosphatases, adaptors, and lipids–lipases pathways (16). In parallel, normal and reactive MCs, as well as clonal MCs from patients with primary MCAS, systematically express the stem cell factor receptor (c-kit or CD117) (18), which plays a key role in the regulation of several processes that are crucial for MC function. Similarly to SM patients, the presence of activating *KIT* mutations in clonal MCAS results into a constitutive, ligand-independent hyperactivation of the KIT receptor; this eventually induces the activation of several intracellular downstream signaling pathways involved in differentiation, maturation, migration, activation, and survival of MCs, such as the Ras, Jak, and phosphatidylinositol 3-kinase (PI3K) pathways (17).

SM AND MONOCLONAL MCASs

Mastocytosis is a heterogeneous group of disorders characterized by the presence of abnormal expansion of clonal MCs in organs and tissues (19, 20). The most recent version (2016) of the World Health Organization classification recognizes several categories of mastocytosis that can be grouped into three main categories of the disease: cutaneous mastocytosis, SM, and MC sarcoma (21). Aditionally, SM can be divided into different subtypes depending on the extent of BM involvement, the existence of signs or symptoms due to end-organ dysfunctions and the presence vs. absence of associated hematologic neoplasms. The most frequent subtype of SM (~80% of all SM cases) is indolent systemic mastocytosis (ISM) (22), which can present with or without skin lesions (ISMs+ and ISMs−, respectively). It is widely accepted that the demonstration of typical skin lesions of mastocytosis in adults leads to the suspicion of SM, and such finding usually initiates the diagnostic work-up of the disease, including a BM evaluation. By contrast, ISMs− (~20% of all ISM cases) is frequently underdiagnosed, mainly due to the heterogeneity and the lack of specificity of presenting clinical symptoms that can overlap with those found in more common allergic diseases (23). In this regard, the demonstration of increased levels of serum baseline tryptase (sBT), a protease which is almost exclusively released by MCs, has contributed for a better identification of ISMs− cases; despite this, a subset of patients with ISMs−, particularly those who have a low BM MC burden, may show low (even normal) sBT levels. Altogether, these findings support the need for additional (prediagnostic) criteria that could help to determine the risk of having an underlying clonal MCAS in patients suffering from MC mediator release symptoms, in order to properly select potential candidates for a BM study (24).

In recent years, the term MCAS has emerged to encompass all those clinical entities characterized by MC activation, including SM. In general terms, MCAS is defined by (i) the presence of recurrent signs or symptoms attributable to the release of MC mediators, together with (ii) increased levels of biochemical markers of MC degranulation in blood and/or urine, and (iii) response to MC stabilizers and/or MC mediator-targeted drugs (6). The European Competence Network on Mastocytosis (ECNM) has recently proposed a comprehensive classification of MCAS (25), in which three main categories of MCAS are recognized depending on whether the cause of MC activation is the presence of a clonal expansion of MCs (primary MCAS), the existence of disorders that can potentially induce MC degranulation such as allergy, inflammatory, and autoimmune diseases or tumors (secondary MCAS), or unknown (idiopathic MCAS) (5, 6). As some patients with primary MCAS (e.g., SM) can also present with secondary causes of MC activation (e.g., allergy) or fulfill diagnostic criteria for idiopathic entities of MCAS (i.e., idiopathic anaphylaxis), the Spanish Network on Mastocytosis (REMA) has proposed to classify MCAS in only two main groups (i.e., clonal and non-clonal MCAS) based on the presence vs. absence of clonal BM MCs, respectively. In any case, a complete BM evaluation should be necessary in all patients with suspected MCAS in order to discriminate between entities presenting with clonal (primary) MCAS, including SM and (mono)clonal MCAS (MMAS), and non-clonal (secondary and idiopathic) MCAS. Despite this, non-clonal MCAS are frequently assumed in clinical practice in the absence of BM evaluation; in turn, primary MCAS may represent a diagnostic challenge due to the lack of specificity of their clinical symptoms and the need of highly sensitive diagnostic techniques to establish the clonal nature of MCs, as discussed in detail below.

From a pathogenic point of view, the most relevant biological finding in SM (and also in MMAS) is the presence of activating *KIT* mutations (mostly the Asp816Val -D816V- *KIT* mutation) in the vast majority of cases (26–29), which results into a constitutive, ligand-independent, activation of the KIT receptor. In virtually all patients with SM, the existence of activating *KIT* mutations is accompanied by the aberrant expression of CD25 (and/or CD2) on BM MCs, which is therefore widely considered as a surrogate marker of MC clonality (30). Both genetic and immunophenotypic features suggest a profound alteration in the mechanisms of adhesion, activation and migration of MCs (31). Despite MC mediator release symptoms, MC clonality and increased sBT levels are findings commonly shared by SM and

MMAS, the distinction between both entities can be established by the absence of enough criteria for the diagnosis of SM in MMAS patients, as further discussed herein.

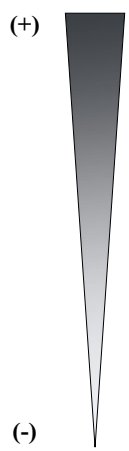
EPIDEMIOLOGY, TRIGGERS, AND RISK FACTORS OF ANAPHYLAXIS IN CLONAL MCAS

Different allergic diseases such as rhinitis, conjunctivitis, asthma, urticaria, and atopic dermatitis have been reported to be present in patients with mastocytosis, with a similar frequency as that found in the general population (32). Nevertheless, the prevalence of anaphylaxis has been reported to be up to 100 times more frequent among patients with SM vs. general population (33, 34) with an overall frequency that ranges from 22 and 49% in adults (32, 35, 36) and between 6 and 9% in children (32, 35). Furthermore, it seems to occur more often in patients with clonal MC disease without cutaneous involvement (24, 37). In fact, anaphylaxis is commonly the presenting symptom in patients with ISMs− and MMAS (18, 34, 38). Although the existence of a clonal MCAS is a predisposing factor for severe MC mediator release episodes by itself, other factors have been associated with an increased risk for the development or the severity of anaphylaxis among patients with SM and MMAS (35). In adults with SM, it has been suggested that the overall BM MC burden is inversely related with the severity of MC mediator release symptoms. Thus, ISM patients (particularly those with ISMs−) suffer from anaphylaxis more frequently than patients with advanced forms of SM (i.e., aggressive SM) (24, 39).

A wide variety of elicitors such as insects, drugs, food, as well as physical, environmental, and emotional factors have been recurrently reported as potential triggers of MC activation episodes in patients with MCAS (18, 24, 40). Among these, the most common trigger of anaphylaxis in adults with ISMs− and MMAS is, by far, hymenoptera sting, followed by unknown cause (idiopathic) and drugs (**Figure 1**). A recent study by the REMA (38) has suggested that ISMs− associated with anaphylaxis exclusively triggered by insects (mostly hymenoptera) represents a subtype of ISMs− that rarely refer anaphylaxis with additional elicitors (38, 40) and displays unique clinical, biological, and molecular features vs. ISMs− triggered by other factors and ISMs+. These features include (1) a clear male predominance, (2) a typical clinical profile of acute MC mediator release episodes characterized by (cardio)vascular symptoms in the absence of urticaria and angioedema, (3) a low BM MC burden, and (4) the detection of the *KIT* mutation restricted to the MC compartment. Importantly, the extent of involvement of hematopoiesis by the *KIT* mutation—restricted to MC or affecting additional hematopoietic cell lineages—has been reported to be the most relevant risk factor for disease progression in ISMs−; for this reason, ISMs− associated with insect-induced anaphylaxis appears to be the subtype of ISM with more favorable long-term prognosis (41).

CLINICAL PRESENTATION OF ANAPHYLAXIS IN CLONAL MCAS

Upon MC activation, a wide variety of symptoms can occur as a result of the systemic effect of proinflammatory and vasoactive mediators released from MCs, which ranges from pruritus, hives, flushing, tachycardia, abdominal pain, or diarrhea, to syncopal or near-syncopal episodes. Several studies by the REMA have shown that MC mediator release episodes in patients with ISMs− (and MMAS) are typically characterized by cardiovascular symptoms (i.e., dizziness and/or syncope) without cutaneo-mucosal symptoms (i.e., urticaria and angioedema) (24). These observations, together with a male predominance of ISMs− and increased levels of sBT, led to the development of a predictive model (REMA



	General population	ISMs+	ISMs-	c-MCAS
	Food (34%)	**Idiophatic** (49%)	**Hymenoptera** (54%)	**Hymenoptera** (50%)
	Drugs (31%)	**Drugs** (30%)	**Idiophatic** (27%)	**Drugs** (30%)
	Idiophatic (21%)	**Hymenoptera** (19%)	**Drugs** (13%)	**Idiophatic** (20%)
	Anisakis (11%)	**Food** (8%)	**Anisakis** (8%)	**Food** (10%)
	Exercise (2%)	**Anisakis** (4%)	**Food** (6%)	**Anisakis** (10%)
	Latex (0.6%)	**Latex** (0%)	**Latex** (0%)	**Latex** (0%)
	Hymenoptera (0.3%)	**Exercise** (0%)	**Exercise** (0%)	**Exercise** (0%)

FIGURE 1 | Main triggers of anaphylaxis in general population vs. clonal MCAS patients. Modified from Ref. (18). ISMs+, indolent systemic mastocytosis with skin lesions; ISMs−, indolent systemic mastocytosis without skin lesions; c-MCAS, (mono)clonal mast cell activation syndromes.

score) (**Table 1**), which showed a high efficiency to discriminate between patients with clonal MCAS and other types of MCAS (24, 42). Interestingly, the REMA score also showed higher specificity and sensitivity compared to the evaluation of sBT levels alone for predicting SM and MMAS. Based on these results, the ECNM has incorporated the REMA score in the most recent consensus algorithm for the diagnosis of SM (25). Moreover, the systematic application of the REMA score in patients presenting with symptoms related with MC activation in the absence of skin lesions evaluated by the REMA has allowed not only to decrease the number of BM studies carried out in these group of patients, but also to identify an increasing number of patients with ISMs− (and MMAS) showing normal sBT levels (~10% of all ISMs− cases in the updated series of the REMA, data not published). These observations, together with the simplicity and the low cost of the method to select for potential candidates for BM evaluation, highlights the clinical and socioeconomic impact of the REMA score and supports its application in a routine basis in clinical practice.

DIAGNOSIS OF CLONAL MCAS

The distinction between primary MCAS (ISMs− and MMAS) and secondary or idiopathic MCAS is based on the demonstration on the clonal nature of MCAS. Given the fact that MCs are produced in the BM and that *KIT* mutation is usually restricted to the MC compartment (24), the establishment of MC clonality requires the study of the BM.

The diagnosis of SM is based on the coexistence of one major criterion and one minor criterion, or ≥3 minor criteria in the absence of the major criterion (21). The major criterion consists on the demonstration of multifocal aggregates of ≥15 MCs in BM sections (or in other extra-cutaneous tissues). In turn, the minor criteria include (i) identification of >25% of morphologically abnormal MCs in BM smears, (ii) demonstration of an aberrant expression of CD25 and/or CD2 on MCs, (iii) detection of the activating mutations in codon 816 of the *KIT* gene, and (iv) presence of sBT levels ≥20 μg/l. In contrast to SM, the diagnosis of MMAS is established when only one or two minor criteria (not including increased sBT levels) are present in the absence of the major criterion (43–45).

Whereas *KIT* mutations and the aberrant expression of CD25 on BM MCs can be already detected in early stages of SM as long as highly sensitive techniques are applied (46), the presence of BM MC aggregates and increased levels of sBT are closely related with the proliferation rate of the clonal MC population in SM and can be absent in a significant proportion of patients with ISMs−. Given the low (frequently very low) BM MC burden that characterizes both ISMs− and MMAS, it must be emphasized that BM studies in patients with symptoms related with MC activation without skin lesions should include highly sensitive diagnostic techniques in order to detect clonal MCs even when they represent only a minority of the nucleated cells in the BM (24). Among these techniques, multiparametric flow cytometry and molecular methods on fluorescence-activated cell sorting-purified BM MCs such as peptide nucleic acid-mediated polymerase chain reaction (PCR) clamping or allele-specific oligonucleotide quantitative PCR, are preferred over other methods (i.e., CD25 immunohistochemistry and conventional PCR) to establish the clonal nature of BM MCs in this clinical setting (47). Accordingly, it is strongly recommended to perform the BM study of patients with suspected SM (particularly those without skin involvement) in highly specialized reference centers for mastocytosis.

TABLE 1 | REMA score proposed to predict clonal MCAS in patients in the absence of skin lesions.

Variable	Score
Gender	
Male	+1
Female	−1
Clinical symptoms	
Absence of urticaria, pruritus, and angioedema	+1
Urticaria, pruritus, and/or angioedema	−2
Presyncope and/or syncope	+3
Baseline serum tryptase	
<15 ng/ml	−1
>25 ng/ml	+2

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MCAS, mast cell activation syndrome.
Score <2: low probability of clonality.
Score ≥2: high probability of clonality.

TREATMENT OF ANAPHYLAXIS IN PATIENTS WITH CLONAL MCAS

Acute Treatment

It is of paramount importance that clinicians early recognize and treat MC mediator release symptoms (48). As among general population, treatment with epinephrine (adrenaline) injected intramuscularly in the mid-outer thigh, as soon as anaphylaxis is diagnosed or strongly suspected, constitutes the first line treatment of anaphylaxis, and repeated doses might be administered after 5–15 min in the absence of optimal response (2). At the same time, life-sustaining treatment including supplemental oxygen or intravenous fluids should be administered as needed (49). H1 antihistamines and H2 antihistamines usually in combination with glucocorticoids are considered as second-line medications in anaphylaxis. In addition, other coadjuvant drugs might be administered depending on the presenting clinical features of the patients.

Baseline Treatment

Treatment strategies for clonal MCAS do not significantly differ from those used in other well-known entities cursing with secondary or idiopathic MC activation, and they are focused on preventing and/or decreasing the effects of mediators released from MCs. These strategies include an adequate information and training of the patient, their relatives, and care providers in order to avoid triggers (18), and the administration of different anti-mediator therapy selected on the basis of the intensity and/or severity of the signs and symptoms linked with the activation of MCs (46) such as histamine receptors blockers, sodium cromolyn, leukotriene antagonists, corticosteroids, and epinephrine, among others.

H1 blockers have been shown to decrease pruritus, flushing, urticaria, tachycardia, hypotension, and abdominal pain related with MC degranulation (50, 51). H2 antihistamines seem to potentiate the effect of H1 antihistamines and can also be of potential utility in patients with suboptimal response to H1 blockers alone (52). Oral sodium cromolyn is a MC stabilizer, which has proven to decrease symptoms such as diarrhea, abdominal cramping, nausea, pruritus, flushing, bone pain, headache, and some cognitive symptoms in patients with mastocytosis (53). In selected cases, aspirin and COX-2 selective inhibitors such as celecoxib might be also useful whenever previous tolerance to such drugs has been demonstrated (52).

In refractory cases despite conventional therapy, treatment with the anti-IgE recombinant humanized monoclonal antibody omalizumab has shown to suppress MC activation episodes in all clinical subtypes of MCAS presenting as idiopathic anaphylaxis (54–56), Meniere's disease (57), and also to prevent reactions related to venom immunotherapy administration (58, 59). The mechanism by which omalizumab decreases the release of MC mediators in asthma (60) and in spontaneous chronic urticaria (61) appears to be related with its ability to block the binding of circulating IgE antibodies to FcεRI receptors on the surface of MCs and basophils resulting into a decrease on receptor expression (62), or by interfering with the release of MC mediator (63). Although it has not been confirmed so far, the response to omalizumab reported in a few primary MCAS patients (54–56) may not differ from the mechanisms referred above.

More rarely, some patients with SM showing high BM MC burden could benefit from cytorreductive or immunomodulatory drugs such as hydroxyurea (64), interferon alpha2b (65), and cladribine (2-CDA) (66) among others. More recently, several tyrosine kinase inhibitors (TKIs) such as midostaurin, masitinib, or dasatinib have shown to improve MC mediator release symptoms in a subset of patients with SM, even in the absence of significant decrease in BM MC numbers (52); despite this, the usage of TKI drugs should be restricted to highly symptomatic SM patients unresponsive to conventional intensive anti-mediator therapy, ideally in the setting of clinical trials.

CONCLUDING REMARKS

Emerging entities with clonal BM MCs as ISMs− and clonal MCAS might sometimes be difficult to recognize. Anaphylaxis is commonly the presenting symptom, and hymenoptera sting is the most common trigger. Both entities involve a great challenge either from the diagnostic or therapeutic point of view. Final diagnosis requires a BM study, but given the low BM MC burden typical of these entities, highly sensitive techniques are mandatory.

AUTHOR CONTRIBUTIONS

Both authors have contributed to the conception, design, and drafting of the paper.

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Alternative Anaphylactic Routes: The Potential Role of Macrophages

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Anaphylaxis is an acute, life-threatening, multisystem syndrome resulting from the sudden release of mediators from effector cells. There are two potential pathways for anaphylaxis. The first one, IgE-dependent anaphylaxis, is induced by antigen (Ag) cross-linking of Ag-specific IgE bound to the high-affinity IgE receptor (FcεRI) on mast cells and basophils. The second one, IgG-dependent anaphylaxis is induced by Ag cross-linking of Ag-specific IgG bound to IgG receptors (FcγRI, FcγRIIA, FcγRIIB, FcγRIIC, and FcγRIIIA) on macrophages, neutrophils, and basophils. Macrophages exhibit a huge functional plasticity and are capable of exerting their scavenging, bactericidal, and regulatory functions under a wide variety of tissue conditions. Herein, we will review their potential role in the triggering and development of anaphylaxis. Thereby, macrophages, among other immune cells, play a role in both anaphylactic pathways (1) by responding to anaphylactic mediators secreted by mast cells after specific IgE cross-linking or (2) by acting as effector cells in the anaphylactic response mediated by IgG. In this review, we will go over the cellular and molecular mechanisms that take place in the above-mentioned anaphylactic pathways and will discuss the clinical implications in human allergic reactions.

Keywords: anaphylaxis, IgG, IgE, macrophages, serotonin

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PATHWAY FOR ANAPHYLAXIS: IgE AND IgG DEPENDENT

IgE-mediated anaphylaxis is well established and is thought to be the main anaphylactic pathway. However, increasing evidence obtained from animal models supports the existence of a second pathway. In this IgG-dependent pathway, macrophages instead of mast cells, and IgGs rather than IgE, are the immunoglobulins involved, and the main mediator released is platelet-activating factor (PAF) instead of histamine. Differences were detailed in **Table 1**. Data from IgG-mediated anaphylaxis were recopilated mainly from previous murine models, while data from IgE-mediated anaphylaxis were obtained from both animal and human previous reports (**Table 1**).

In this review, we will analyze the evidence obtained from murine experimental models supporting the existence of an IgG-dependent anaphylaxis pathway and speculated about the possibility of a similar mechanism in humans, either as a stand-alone pathway or as a synergistic mechanism to IgE-mediated anaphylaxis.

The main body of evidence for IgG-mediated anaphylaxis comes from animal models.

Passive immunization, result of the administration of specific Igs, followed by enteral or parenteral challenge with the appropriate antigen (Ag) supported the relevance of IgE and mast cells in the development of anaphylaxis (1–3). Indeed, animals with depleted mast cells, IgE or FcεRI, subjected

TABLE 1 | Main features in the mechanisms and triggering factors involved in IgE- and IgG-dependent anaphylactic pathways.

	IgE-dependent pathway	IgG-dependent pathway
Ig involved	IgE	IgGs
Antigen concentration	Low	High
Fc receptor	FcεRI	FcγRI, FcγRIIA, FcγRIIB, FcγRIIC, FcγRIIIA, and FcγRIIIB
Effector cells	Mast cells	Macrophages, monocytes, and neutrophils
Mediators	Histamine (leukotrienes, prostaglandin, serotonin, etc.)	Platelet-activating factor (leukotrienes, prostaglandin, serotonin, etc.)
Triggering factors	Food, drugs (e.g., beta-lactam antibiotics), insect sting and bites, exercise (food dependent)	Food, drugs [monoclonal antibodies (omalizumab or infliximab)], or dextrans, others

to active or passive immunization followed by oral challenge, completely suppress the anaphylactic reaction.

However, animal immunization followed parental challenge with the same Ag, revealed that anaphylaxis could even occur in the absence of the IgE/FcεRI/mast cell pathway. This demonstrates the existence of an alternative anaphylaxis pathway that closely resembles IgE-mediated anaphylaxis but involves other key players (3–5).

Both pathways display significant differences in their main features (**Table 1**), such as the requirement of different concentrations of Ag and Ab to induce the reaction.

In fact, studies comparing Ag doses required to elicit IgE- or IgG-mediated anaphylaxis suggested that the IgG-dependent pathway requires approximately 100-fold more Ag than the IgE pathway to induce a similar response (3).

Additionally, anaphylaxis mediated through IgG also appeared to require much more Ab than anaphylaxis mediated through IgE. In fact, IgE-mediated anaphylaxis can even be seen with serologically undetected sIgE levels, in which sIgE bound to mast cells is sufficient (5). In contrast, relatively high levels of serum IgG are required for Ag induction of anaphylaxis through the IgG pathway (3). This could be due to two factors: first, the much higher affinity of FcεRI for IgE than FcγRIII for IgG, and second, the fact that IgE binds directly to mast cell-associated IgE, whereas Ag/IgG complexes are presumably formed in blood and lymph before binding by FcγRIII on other immune cells such as macrophages (6, 7).

In the case of IgG-mediated anaphylaxis, the immunoglobulin subclasses and receptors involved in the reaction also play an important role. Regarding IgG subclasses, IgG1, IgG2a, and IgG2b have been reported to enable the induction of systemic anaphylaxis, inducing mild to severe hypothermia (8, 9). Furthermore, IgGs can bind to six different FcγR, namely, FcγRI, FcγRIIA, FcγRIIB, FcγRIIC, FcγRIIIA, and FcγRIIIB, which have different affinities, downstream signaling routes, and patterns of expression (10, 11). FcγRI is considered as the high-affinity receptor, although FcγRIIIB can bind IgG with high and low affinity depending on the IgG subclass (7).

Another crucial issue for development of either the IgE or IgG pathways of anaphylaxis is the balance between Ag concentration and the levels of IgG or IgE. Usually, both Ag-specific IgE and IgG are present in blood, with IgG levels being higher. Under these conditions, Ag will encounter IgG in blood before it can bind to mast cell-associated IgE, which results in blockage of IgE-mediated anaphylaxis. However, when Ag levels are insufficient to induce IgG-mediated anaphylaxis, high levels of IgG can prevent the development of any anaphylactic response. For a similar reason, larger amounts of Ag trigger anaphylaxis predominantly through the alternative pathway when Ag-specific IgG antibody levels are high, even though Ag-specific IgE is present. In this situation, the anaphylactic pathways will only be triggered simultaneously when the amount of challenge Ag exceeds the capacity of IgG antibody to block Ag binding to mast cell-associated IgE (5).

Taken together, these data clearly support significant differences between both anaphylactic pathways regarding the type of Ig as well as the conditions needed for the development of one pathway or the other (**Figure 1**). However, in humans the relevance of the alternative pathway is still a matter of debate.

EFFECTOR CELLS AND MEDIATORS INVOLVED IN IgE- AND IgG-MEDIATED ANAPHYLAXIS

There is complete segregation of the effector cells and mediators underlying both anaphylactic pathways.

While it is well known that the IgE-dependent pathway of anaphylaxis is triggered by an allergen interacting with allergen-specific IgE bound to the FcεRI on mast cells, which leads to cross-linking and subsequent degranulation of the cells, the exact mechanism underlying the IgG anaphylactic pathway has not been completely elucidated. In fact, there is significant controversy about the effector cells involved in IgG-mediated anaphylaxis, and it seems that the main effector cells, at least in murine experimental models are macrophages/monocytes and basophils. However, some authors also suggest a role for neutrophils (8) and basophils (12). In fact, the latest publication of Khodoun et al., covering all three known effector cell types, concluded that all cells, monocytes/macrophages, basophils, and neutrophils, participate in IgG-induced anaphylaxis (13).

Another point of controversy is the level of FcγR expression and the type of myeloid cell expressing the receptor. In this regard, Beutier et al. showed that the differential expression of inhibitory FcγRIIB on myeloid cells and its differential binding of IgG subclasses control the contribution of basophils, neutrophils, and monocytes to IgG-dependent anaphylaxis, thus revealing novel complexities in cell population regulation mechanisms and, therefore, their relative contribution to IgG-induced reactions in murine models (14).

The outcome of this process of Ab–receptor recognition and subsequent cellular signaling activation is the release of several

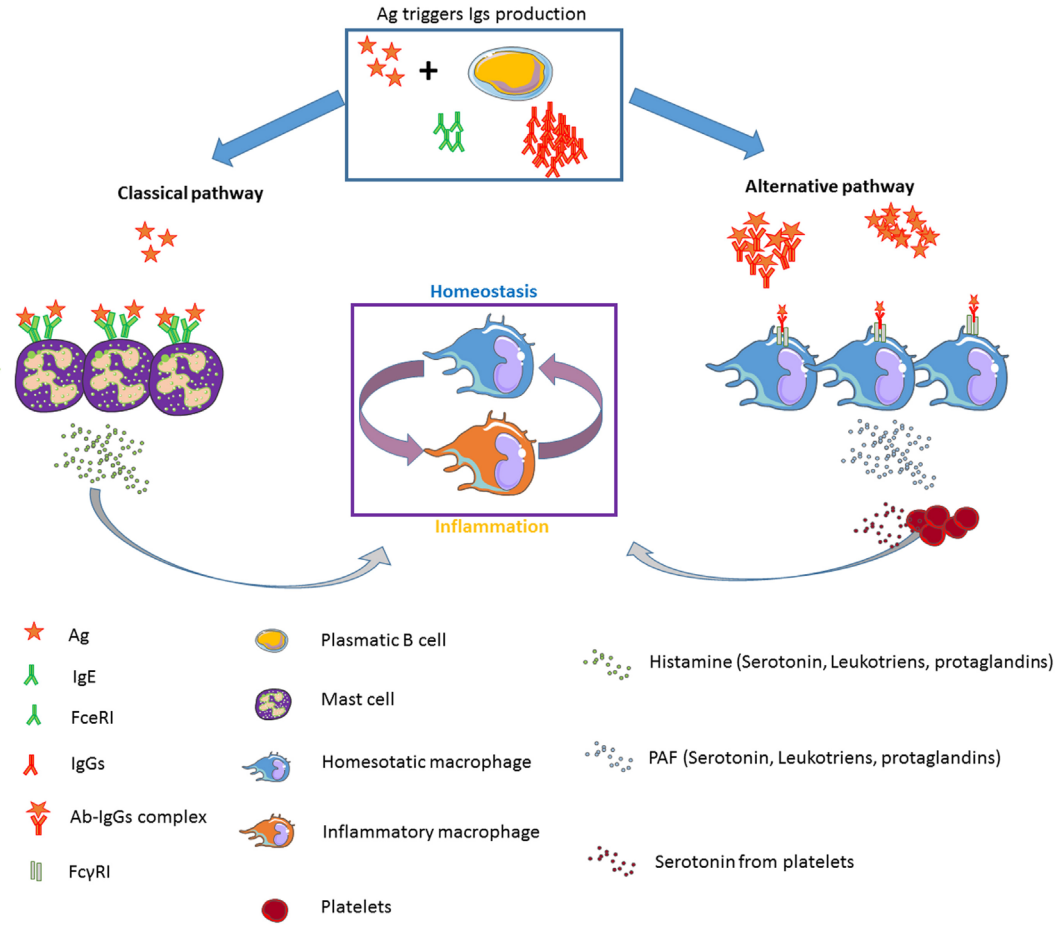

FIGURE 1 | Diagram showing the classical (IgE-dependent) and alternative (IgG-dependent) anaphylactic pathways: effector cells, mediators, Igs, and FcR implicated.

mediators responsible for the hypothermia and hypotension that characterize anaphylaxis.

The main mediator involved in IgE-mediated anaphylaxis is histamine. Histamine is known to play an essential role in the evolution of the anaphylactic process (15–17). Moreover, it is also involved in regulation of the immune response (18, 19). Other mediators released during IgE-mediated anaphylaxis are prostaglandins and leukotrienes (17, 20). Furthermore, a receptor for prostaglandins has also been described in several immune cells, such as macrophages or innate lymphoid cells (ILC2) (21).

Another significant metabolite reported to be released by mast cells upon IgE cross-linking is serotonin (22–24). The role of serotonin in the anaphylactic process is still unknown, although recent reports have suggested that this metabolite is key in immune response regulation (23, 25) and, more specifically, in the regulation of macrophage polarization and inflammatory resolution (26, 27), allergy (28), and hypotension (29). Serotonin participation in the regulation of inflammation and immune response upon anaphylaxis will be further discussed.

In the case of IgG-mediated anaphylaxis, the main mediator is PAF (5, 30, 31). It has been reported that serum PAF levels correlate with the severity of anaphylaxis (32). This metabolite is produced and secreted by several types of cells and is active at concentrations as low as 10^{-12} mol/L despite its short half-life (32).

Platelet-activating factor (PAF) is implicated in platelet aggregation and activation through the release of vasoactive amine during inflammatory responses, thus resulting in an increase in vascular permeability, circulatory collapse, a decreased cardiac output, and other biological effects (33).

Strikingly, platelets have been reported to be the major reservoirs of serotonin outside the nervous system (34), once again suggesting a novel role for serotonin in progression of the anaphylactic pathway as well as in allergic disease progression.

MACROPHAGES AND SEROTONIN: POTENTIAL NOVEL PLAYERS IN ANAPHYLAXIS?

In anaphylaxis, macrophages have been described as effector cells in the IgG-dependent pathway, since they express FcγR and release PAF. This has been demonstrated in mouse models of

anaphylaxis. Apart from this, no specific role has been described for these immune cells in IgE-dependent anaphylaxis in neither human nor mice. However, one could speculate that all the mediators released by mast cells (histamine, leukotrienes, and prostaglandin) might significantly affect macrophage polarization status and, thus, immune response outcome. These mechanisms will probably occur in both mouse and humans.

Macrophages and dendritic cells occupy a prominent position during immune responses, being essential for their initiation (a function primarily displayed by dendritic cells) and for the final effector phases (mostly macrophages) (35). In fact, and regardless of the triggering stimulus, macrophages are usually the final effectors of any given immune response, because they can acquire a continuum of functional states, thus adapting their effector functions to the surrounding environment and to the prevailing T cell-derived cytokines in the extracellular milieu receptor signals (36). By virtue of this plasticity, macrophages are not only critical for maintaining tissue homeostasis but can either display pro- or anti-inflammatory functions, promote or resolve an inflammatory response, and cause tissue damage or help in tissue repair. Results generated in recent years have clearly established the widespread homeostatic functions of macrophages, as they fine-tune physiological parameters as relevant as body temperature and even transit time in the gut (37–39).

Regarding factors with a prominent role in macrophage polarization and anaphylaxis, serotonin has also been shown to modify macrophage polarization in the phenotypic, cytokine, and transcriptional profile (27). Besides its production by mast cells (40), peripheral serotonin is mostly produced by enterochromaffin cells and later stored by platelets in dense granules (34). Serotonin not only promotes proliferation of numerous cell types but also functions as a regulator of immune and inflammatory responses. In fact, the immunomodulatory activity of serotonin is partly mediated through direct actions on macrophages: serotonin favors angiogenesis in colon cancer allografts by acting on tumor-infiltrating macrophages (41), contributes to pulmonary arterial hypertension by altering myeloid cell differentiation potential (42), and limits postoperative bowel inflammation *via* recognition by muscularis and peritoneal macrophages (43). At the molecular level, these actions appear to be mediated by serotonin receptors expressed on the macrophage cell surface. We have previously demonstrated that human anti-inflammatory macrophages specifically express HTR2B and HTR7 serotonin receptors, whose ligation results in altered macrophage transcriptome and inhibition of pro-inflammatory cytokine production (27). In fact, serotonin appears to switch the macrophage transcriptome toward a growth-promoting, anti-inflammatory, and pro-fibrotic gene profile, whose acquisition depends on both HTR2B and HTR7 (27). Therefore, we can speculate that agonists/antagonists of serotonin receptors might be therapeutically useful for limiting the uncontrolled production of pro-inflammatory cytokines that takes place in chronic inflammatory diseases (44). Surprisingly, HTR7 is the receptor responsible for serotonin-induced hypothermia (45), but whether macrophage HTR7 contributes to this response is currently unknown.

A reasonable hypothesis for the role of serotonin in the IgG-mediated anaphylaxis might be the generation of a feedback loop that favors the acquisition of an anti-inflammatory phenotype by macrophages right after the induction of an anaphylactic shock, aiming to restore homeostatic conditions (**Figure 1**).

Another strategy in line with the alternative anaphylactic pathway in humans that also supports the connection between changes in IgG concentration and a regulation of macrophages polarization is treatment with intravenous immunoglobulins (IVIg). IVIg is a preparation of polyclonal poly-specific IgG from the plasma of thousands of donors that is currently used as immunoregulatory and anti-inflammatory treatment in autoimmune and inflammatory disorders (46). The mechanism of action of IVIg has not been completely elucidated, but we have reported that IVIg skews human and mouse macrophage polarization through FcγR-dependent mechanisms (47). IVIg immunomodulatory activity is dependent on the macrophage polarization state, as it limits the pro-inflammatory nature of GM-CSF-dependent macrophages and favors the acquisition of pro-inflammatory properties in anti-inflammatory macrophages (47). In fact, IVIg enhances inflammatory tissue-damaging responses in murine models of stroke and sepsis and reduces tumor growth and metastasis by shifting the polarization state of tumor-associated myeloid cells toward the pro-inflammatory side (47). Since the latter effect was dependent on the expression of Fc receptors, we can conclude that ligation of molecules, such as CD16 and FcRγ, might be useful targets for the modulation of macrophage polarization.

EVIDENCE FOR IgG-MEDIATED ANAPHYLAXIS IN HUMAN

The existence of IgG-mediated anaphylaxis in humans is not clear. In spite of a lack of direct evidence, the findings of some studies imply a possible alternative mechanism to IgE-mediated anaphylaxis (5, 30). PAF, which seems to be associated with the IgG mechanism in mice, is an essential mediator in human anaphylaxis, and its levels are elevated in patients undergoing anaphylaxis compared with a control group (48). The catabolism of this mediator is controlled by the enzyme PAF acetylhydrolase (PAF-AH), which is in charge of PAF inactivation (49). Some studies have correlated the levels of these two markers with the severity of anaphylaxis, with increases in PAF levels and decreases in PAF-AH activity. Moreover, patients with the lowest levels of PAF-AH activity were found to exhibit a 27-times higher risk of developing severe or fatal anaphylaxis than patients with normal levels (48, 50).

Several cases of drug anaphylaxis are classified as non-allergic due to the absence of specific IgE titers (measured in sera or by skin test) and the lack of increased serum tryptase or basophil activation (51), although no study has addressed the IgG-mediated mechanism in these patients.

However, in patients treated with biological drugs, these can induce anaphylaxis without the presence of detectable specific IgE, although they do present high levels of specific IgG (52).

This observation derives from patients with IgA deficiency who developed anaphylaxis after receiving a blood transfusion or treatment with intravenous injections of IgA. In these subjects, increased levels of IgG anti-IgA antibodies were also found (53, 54).

Moreover, in a later study patients with higher levels of IgG were found to present an increased frequency of a gain-of-function allele of the stimulatory FcγRIIA (55), although this study was conducted in a limited number of subjects.

The presence of increased titers of specific IgG has also been observed in patients treated with human, humanized, or chimeric mAbs, such as infliximab or adalimumab (56), and other biological factors (57–59). In the case of infliximab, the presence of high levels of specific IgG has been related to an increased risk of suffering anaphylaxis (60). A common factor to all these reported cases was the administration of high quantities of the suspected Ag, leading to the presence of high levels of specific IgG.

As with drug allergies, evidence for the existence of IgG-mediated anaphylaxis has also been found in cases of food allergy, especially in anaphylaxis induced by lipid transfer proteins (LTP). Increased levels of anti-LTP IgG1 and IgG3 and increased expression of the three genes coding for the activating receptor FcγRI (CD64) have been observed in a group of patients with food anaphylaxis induced by LTP (61). Mast cells can be activated by IgG *via* this receptor (62, 63) and are able to recognize both IgG1 and IgG3 with high affinity (64, 65). Interestingly, both anti-LTP specific IgG and IgE have been found in LTP allergic patients, which could suggest an involvement of both pathways in the anaphylactic mechanism in these subjects (61). The most severe food allergens are milk, egg, and peanut, and all of them share a high allergenic concentration, thus fulfilling the criteria necessary to elicit an alternative anaphylactic pathway.

CONCLUSION AND FURTHER EXPECTATIONS

Anaphylaxis is the most serious allergic reaction that can occur and may even endanger the patient's life. Moreover, epidemiological data indicate that cases of anaphylaxis are increasing worldwide. The mechanisms involved in the pathogenesis of anaphylaxis can be immunological or non-immunological. Classical immunological reactions mediated by IgE are observed in food anaphylaxis, beta-lactam antibiotics, or hymenopteran stings. Immunological reactions mediated by IgG are being observed following administration of certain monoclonal antibodies (omalizumab or infliximab) or dextrans. The role of macrophages is relevant in this type of IgG-mediated immunological anaphylaxis. PAF released by activated macrophages can activate mast cells, explaining the pathogenesis of this anaphylaxis. Given the increased use of different monoclonal antibodies in clinical practice for the treatment of immune-based diseases, an increase in this type of IgG-mediated anaphylaxis might be observed.

AUTHOR CONTRIBUTIONS

All the authors have participated in the writing of the manuscript. DB has written, organized, and revised the manuscript.

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Mechanisms, Cofactors, and Augmenting Factors Involved in Anaphylaxis

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Anaphylaxis is an acute and life-threatening systemic reaction. Many triggers have been described, including food, drug, and hymenoptera allergens, which are the most frequently involved. The mechanisms described in anaphylactic reactions are complex and implicate a diversity of pathways. Some of these mechanisms may be key to the development of the anaphylactic reaction, while others may only modify its severity. Although specific IgE, mast cells, and basophils are considered the principal players in anaphylaxis, alternative mechanisms have been proposed in non-IgE anaphylactic reactions. Neutrophils, macrophages, as well as basophils, have been involved, as have IgG-dependent, complement and contact system activation. A range of cationic substances can induce antibody-independent mast cells activation through MRGPRX2 receptor. Cofactors and augmenting factors may explain why, in some patients, food allergen exposure can cause anaphylaxis, while in other clinical scenario it can be tolerated or elicits a mild reaction. With the influence of these factors, food allergic reactions may be induced at lower doses of allergen and/or become more severe. Exercise, alcohol, estrogens, and some drugs such as Non-steroidal anti-inflammatory drugs, angiotensin-converting enzyme inhibitors, β-blockers, and lipid-lowering drugs are the main factors described, though their mechanisms and signaling pathways are poorly understood.

Keywords: adenosine, anaphylaxis, cofactor, exercise, IgE, IgG, mast cell, non-steroidal anti-inflammatory drug

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INTRODUCTION

Anaphylaxis is an acute, life-threatening, systemic reaction caused by the mediators released from different cells (1). Although the underlying mechanism is frequently IgE-dependent, some other mechanisms there are also involved (2–4). Complement activation, neuropeptide release, T-cell activation, immune complex formation, cytotoxicity, IgG-dependent activation, induction of purinergic metabolism, and activation of the receptor MRGPRX2, are some of those alternative pathways (2, 5). Mast cells are considered the pivotal cells in IgE-mediated anaphylaxis (6), and the role of macrophages and neutrophils has been described in IgE-independent reactions (6, 7). Interestingly, basophil implication has been suggested in both IgE-dependent and -independent reactions, although its role in allergic reactions has been considered as redundant with mast cells for

some time (8). In humans, CD203c and CD63, basophil activation surface markers, are used to confirm allergen sensitization (9). Recently, a decrease on circulating basophil and an increase in serum concentration of the major basophil chemotactic factor (CCL2) during food and hymenoptera-induced anaphylaxis has been observed, suggesting the role of basophil in human IgE-dependent anaphylaxis (10).

IMMUNE-MEDIATED ANAPHYLAXIS

IgE-Mediated Reactions

The more common mechanism involved in an anaphylactic reaction is promoted by an allergen recognized by an allergen-specific IgE bound to the FcεRI receptor on the surface of mast cells and basophils. When the signal is sufficiently powerful, mast cell and basophil activation make progress, releasing mediators (11). Those mediators also lead to the amplification of the allergic reaction through the recruitment and activation of other cells involved in the IgE immunological response (12–14). However, this explanation is too simple to understand what take place in an *in vivo* reaction, and sundry factors could influence allergen-dependent mast cell and basophil activation under specific conditions (15).

IgG-Mediated Reactions

There are six different Fcgamma receptors (FcγRI, FcγRIIA, FcγRIIB, FcγRIIC, FcγRIIIA, and FcγRIIIB), and all of them bind IgG. Among them, FcγRI is considered a high-affinity receptor (16). Most of these receptors induce cell activation, except for FcγRIIB, which induces an inhibitory signal and has been proposed as a key player in IgG subclass-dependent anaphylaxis in a recent study (17).

Mouse models have been used to demonstrate the relevance of IgG in anaphylaxis. A passive systemic anaphylaxis model has suggested that FcγRIII on cells as macrophages and basophils mediates these reactions (3, 4, 18), and platelet-activating factor (PAF) but not histamine (3, 4) is the main mediator involved.

An IgG-dependent mechanism has been also suggested in human anaphylaxis. PAF, mostly associated with an IgG mechanism, has a key role in human anaphylaxis as several authors have suggested. Vadas et al. (19) found increased circulating PAF levels and decreased PAF acetylhydrolase (PAF-AH) activity in proportion to the severity of the anaphylactic reaction (20). Indeed, the lowest levels of PAF-AH activity were related with a 27 times more risk of severe or fatal anaphylaxis compared to normal activity (19, 21).

Several authors have suggested that both IgG and neutrophils may be involved in human anaphylaxis. Muñoz-Cano et al. (5) studied patients with anaphylaxis induced by lipid transfer proteins (LTP) and mediated by IgE. They found an increase of specific anti-LTP IgG1 and IgG3 levels and increased expression of the three genes coding for FcγRI (CD64), an activating receptor (5). It has been shown that FcγRI mediates mast cell and neutrophil activation *via* IgG (22, 23), by both IgG1 and IgG3 (16) also in humans. Interestingly, Muñoz-Cano et al. (5) found specific IgG and anti-LTP IgE in those patients, suggesting that both IgG and IgE pathways may contribute substantially to anaphylaxis. Rispens et al. (24) also found both specific IgE and IgG1 anti-α-gal in patients with galactose-alpha-1,3-alpha-galactose (α-gal) allergy.

Neutrophils, activated through FcγRIV-IgG2, are proposed to play a major role in a mouse model of anaphylaxis (7). They are important PAF producers, and a differential PAF release has been observed in neutrophil-dependent reactions in mice (7, 25). However, PAF is also observed in IgE-mediated reactions in animal models (11). Muñoz-Cano et al. (5) showed that several markers of neutrophil activation and trafficking were highly expressed in patients with IgE-dependent anaphylaxis allergic to LTP. Moreover, the authors found increased levels of reactive oxygen species/reactive nitrogen species, known as a measure of oxidative outburst, suggesting an enhancement of neutrophilic activity. Francis et al. (26) also found increased neutrophil activation markers (myeloperoxidase and CD62L) during an acute anaphylactic reaction.

In the light of these findings, the paradigm of anaphylaxis mediated only by IgE and mast cell/basophil seems not totally accurate. In the LTP particular case, anaphylaxis may be elicited *via* IgE, IgG, or both, with the involvement of neutrophils and not only of mast cells and basophils, although other allergens may act similarly.

Complement Activation in Anaphylaxis

Monomeric IgG and IgG immune complexes can bind FcγRI receptors (27, 28) and are key in the novel paradigm in human anaphylaxis (IgG anaphylaxis). Furthermore, the complement system can also be activated by immune complexes, resulting in the generation of anaphylatoxins such as C3a (23, 29). Interestingly, C3a has demonstrated a direct effect on mast cell and also a synergistic effect (twofold increase) with FcγRI receptor activation (23). Therefore, the combination of IgG and C3a activation results in greater mast cell activation or activation under circumstances in which neither of the stimuli would elicit maximal release on its own.

Large amounts of the anaphylatoxin C3a have been found in peanut severe allergic reactions by Khodoun et al. (30), in both mouse and human plasma. However, allergens such as milk and egg white did not have the ability to activate complement in humans (30). Therefore, several factor as patient susceptibility (5), cofactors (31) and characteristics of a particular allergen may determine the severity of an allergic reaction.

Reactions with drugs solubilized in therapeutic liposomes and lipid-based excipients have been related with the activation of complement in the absence of immune complex. It is the case of Cremophor EL, a diluent used in the older preparations of propofol and paclitaxel, which has been found to induce complement activation (32).

Finally, it has been also demonstrated that lypopolysaccharides (LPS) can induce a strong activation of the complement and trigger an anaphylactic reaction in a mouse model (33). Recently, Rodriguez et al. (34) demonstrated the role of LPS as a co-stimulus triggering anaphylaxis in a mouse model; specific Pru p 3-induced anaphylaxis was generated after nasal sensitization to Pru p 3 in combination with LPS.

NON-IMMUNE-MEDIATED ANAPHYLAXIS

Contact System Activation in Anaphylaxis

It has been identified as direct or indirect activation of the blood coagulation pathway in allergic reactions mediated by IgE (35). During acute anaphylaxis, an increase of the heparin levels and an activation of the factor XII-driven contact system has been observed, which results in the production of bradykinin (36). In fact, after the analysis of more than 150 deaths associated to anaphylaxis induced by oversulfated chondroitin sulfate-contaminated heparin, the possible role of heparin as a trigger of bradykinin formation through contact activation was suggested (37, 38). Therefore, targeting its generation may be a promising strategy for treatment of severe allergic reactions, importantly those with hypotension (39).

New Mast Cell Receptors in Anaphylaxis: MRGPRX2

Mast cells are classically activated by IgE antibodies, although a range of cationic substances, called basic secretagogues, can induce antibody-independent activation. Among those secretagogues, there are peptides with pro-inflammatory effects and several drugs. Recently, Mrgprb2, the ortholog of the human G-protein-coupled receptor MRGPRX2, has been described to mediate this activation in a mouse model. This receptor seems to be the target of many small-molecule drugs involved in non-IgE anaphylactic reactions, such as non-steroidal neuromuscular blocking drugs (tubocurarine, atracurium, or ciprofloxacin). This work identified a chemical motif that is common to several of these molecules and may be linked to some of the observed side effects. In conclusion, MRGPRX2 may be considered a potential therapeutic target to reduce some adverse effects induced by some drugs (40).

COFACTORS AND AUGMENTING FACTORS IN ANAPHYLAXIS

The so-called accompanying factors may explain why an allergen can either be tolerated or trigger a mild reaction or, in the same patients, induce a severe anaphylaxis. In the presence of cofactors, reactions become more severe and/or the amount of allergen eliciting the allergic reaction can be lower. According to published data, the presence of those accompanying factors occurs in up to 30% of episodes of anaphylaxis (31, 41). Niggemann and Beyer (42) postulated three categories of risk factors for anaphylactic reactions: first, the *augmenting factors*, which influence the immunological mechanism, such as physical exercise, acute infections, drugs [non-steroidal anti-inflammatory drugs (NSAIDs), proton pump inhibitors], alcohol, and menstruation; second, *concomitant diseases*, such as asthma, mastocytosis, and cardiovascular disease, which are associated with more severe reactions and/or increased mortality; and third, *cofactors*, which do not have any influence on the immunological mechanism, as psychological factors (e.g., emotional stress) or specific allergens. Nevertheless, the lack of knowledge about the mechanisms underlying these risk factors limits a strict categorization. Therefore, for the purposes of this review, the terms cofactor and augmenting factor are used indistinctively.

Estrogens

Gender differences have been reported in the incidence of anaphylactic reactions, demonstrating that anaphylaxis is more frequent in women than men (43, 44), but only during the reproductive years, suggesting that sexual hormones might play a role. Additionally, like the episodes of asthma or urticaria associated with the menstrual cycle (45, 46), recurrent episodes of anaphylactic reactions around menstruation have been described, pointing at the estrogens or progesterone as the augmenting factors involved (47).

The susceptibility of women to develop anaphylactic reactions observed in clinical studies was also demonstrated in a murine model (48). Female mice were ovariectomized to eliminate the major source of estrogens and the result was the decrease in the severity of anaphylaxis. Moreover, the implant of subcutaneous estradiol-releasing pellets in the ovariectomized mice resulted in an increase in the severity of anaphylaxis. The mechanism involved was not related with the increase in mast cell degranulation, but with an augmentation of the vascular permeability. A higher production of nitric oxide as a result of major expression of endothelial nitric oxide synthase was in fact the cause (48).

Exercise

Exercise is involved up to 10% of anaphylactic reactions, being one of the more frequent augmenting factors (49). However, the knowledge of its pathogenic mechanism still continues to be poorly understood, and some theories have being proposed. Although, according to the literature, the foods involved in food-dependent exercise-induced anaphylaxis are very diverse, wheat is the most frequent one, being ω-5 gliadin the culprit protein in most cases (50). As a result, most of the mechanistic studies have been performed in patients with wheat-dependent exercise-induced anaphylaxis (WDEIA).

One of the theories hypothesizes that exercise induces an activation of tissue transglutaminase (tTG), resulting in a formation of large ω-5 gliadin/tTG complexes that would facilitate the ω-5gliadin–IgE binding. Nonetheless, no direct evidence of this phenomenon has been found in patients with WDEIA (51).

A second hypothesis establishes that exercise would induce an increase in the intestinal allergen absorption, and as a consequence, an increase of blood allergen concentration and the likelihood to develop an anaphylactic reaction (52, 53). Some murine models of food allergy have demonstrated how physical exercise increases the absorption of allergen from the gastrointestinal tract due to mucosa injury (54, 55). An increase of the core temperature in the gastrointestinal tract due to intense exercise would result in epithelial cell damage owing to the phosphorylation state of tight junction proteins. In addition, another mechanism involved in the mucosal damage may be related with the deviation of blood flow away from the splanchnic arteries to the working muscle, resulting in an ischemia/reperfusion cycle that causes the epithelial damage (56, 57).

Another hypothesis establishes that exercise, due to a direct effect on mast cells, would modify the threshold dose of allergen

in patients with WDEIA. It has been described that physical exercise induces an increment of the plasma osmolarity (58), and this increase results in mast cells activation and release of inflammatory mediators (59). Additionally, a previous *in vitro* study showed that IgE activation and hyperosmolar stimuli at the same time have a synergistic effect on IgE-induced mast cell release (60). However, the increase of plasma osmolarity due to physical exercise is only significant when the exercise is strenuous and, in patients with WDEIA reactions, the intensity of the physical exercise is frequently moderate.

Lipid-Lowering Drugs (Statins)

Lipid-lowering drugs can be considered as a risk factor in anaphylaxis since some studies posited that low plasma levels of low-density lipoprotein (LDL) may augment the risk of severe or fatal anaphylaxis. These drugs increase plasma concentration of PAF by lowering PAF-AH activity (19, 61). In this way, Perelman et al. (62) demonstrated a significant direct correlation between PAF-AH activity and LDL levels in patients with peanut allergy. Moreover, a significant correlation between PAF plasma levels and the severity of anaphylaxis has also been demonstrated (33).

Non-Steroidal Anti-inflammatory Drugs

Non-steroidal anti-inflammatory drugs are other well-known augmentation factors in anaphylaxis. They have been reported to be present in up to 22% of cases of food-induced severe anaphylaxis, constituting a risk factor with an odds ratio >11 (63). In the Mediterranean area, NSAIDs are involved up to 58% of cofactor-induced food-related anaphylaxis episodes (64) and in up to 33% of cases of anaphylactic reaction induced by LTP (65). Two hypotheses have been proposed to explain the underlying mechanisms involved in food-dependent NSAID-induced anaphylaxis (FDNIA).

The first hypothesis suggests that the increase of gastrointestinal permeability and allergen absorption may account for the augmentation effect of NSAIDs (53). It is well known that prostaglandins play an important role in gastrointestinal mucosa defense and repair. NSAIDs, through prostaglandin inhibition, leave gastrointestinal tissues more susceptible to the injury caused by gastric acid and bile and with less capacity to retrieve the mucosa function (38). Additionally, NSAIDs induce mitochondrial damage that leads to the malfunction of the intestinal epithelial cells and increase of the intestinal permeability (66, 67).

A second hypothesis suggests that NSAIDs have a direct impact on mast cells and basophils IgE activation, amplifying their activation and degranulation (68, 69). However, the underlying mechanisms involved remain still unknown.

Bartra et al. (70) suggested that the enhancing effect of NSAIDs in food allergic reactions might be related with the cyclooxygenase (COX) pathway. Several authors (71–73) have also shown that this effect is a class effect, therefore COX-dependent. Moreover, it has been demonstrated that selective COX-2 inhibitors (nimesulide and etodolac) (68, 69) did not increase the severity of food allergic reactions. Additionally, prostaglandin E_1, an important prostanoid derived from the COX pathway (74), has been demonstrated to be protective in patients with FDNIA (75).

Adenosine metabolism and A3 receptor (A3R) have been linked with the underlying mechanisms of some diseases exacerbated by NSAIDs, such as NSAID-dependent urticaria (76) and aspirin-induced asthma (77). Interestingly, it has also been demonstrated that the activation of A3R enhances FcεRI-induced granule release in human mast cells (78–80). Moreover, a study that evaluated the transcriptome of patients with FDNIA showed an overexpression of genes related to adenosine metabolism, particularly A3R gene (5). NSAIDs are able to inhibit oxidative phosphorylation of ATP and promote its hydrolysis which entails the release of adenosine (81, 82); therefore, a link between NSAIDs, adenosine, adenosine receptors, and allergic reaction has been suggested.

Angiotensin-Converting Enzyme Inhibitors and β-Blockers

Angiotensin-converting enzyme inhibitors (ACE inhibitors) and β-blockers have been described as augmenting factors in anaphylactic reactions according to several studies (63, 83, 84). The odds ratio established for β-blockers was 6.8 and 13 for ACE inhibitors. However, other studies concluded that the risk of develop anaphylaxis related to ACE inhibitors and β-blockers

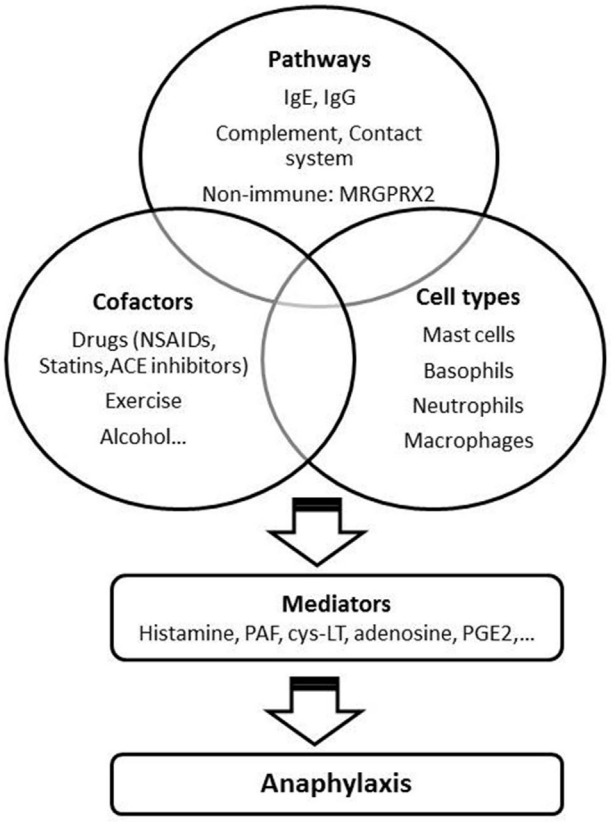

FIGURE 1 | Factors involved in anaphylaxis. NSAIDs, non-steroidal anti-inflammatory drugs; ACE, angiotensin-converting enzyme; PAF, platelet-activating factor; Cys-LT, cysteinyl leukotrienes; PGE2, prostaglandin E2.

does not exist unless both treatments are combined (21, 85, 86). Recently, mast cells were recognized as targets of ACE inhibitors and β-blockers in a murine model, augmenting their activation through FcεRI (86). In spite of the fact of this important data, further epidemiological and *in vivo* and *in vitro* studies in humans are necessary to determinate the real impact of these drugs as a risk factors in anaphylaxis.

Alcohol

Alcohol is involved in up to 15% of cases of anaphylactic reaction according to some series (49, 87), independently of their severity (86). Although, the underlying mechanisms are not well established, alcohol may increase allergen absorption. It has been described that alcohol induces a modification in the expression of the tight junction-associated proteins ZO-1 and claudin-1 of the intestinal epithelium, thereby augmenting the permeability of the intestinal epithelial barrier (88).

An adenosine-related mechanism has also been suggested in IgE-mediated anaphylaxis when alcohol is involved. Alcohol inhibits the adenosine uptake, inducing an increase of the extracellular adenosine, thus enhancing FcεRI-induced mast cells and basophil activation (89).

Another mechanism postulated is based on the capacity of alcohol to boost the serum IgE concentration (90). In a murine model, alcohol intake was linked with a raise in IgE serum levels and a decrease in IgG (90). Nevertheless, this acute alcohol intake has also been linked with lower release of mast cell mediators, such tryptase (91).

CONCLUSION

IgE, mast cells and basophils have been considered the main key players in human anaphylaxis for a long time, although alternative mechanisms have been suggested. Neutrophils and macrophages, IgG-mediated, complement, and contact system activation are some of them. A range of cationic substances can induce antibody-independent activation through the recently described receptor MRGPRX2. The presence of the so-called cofactors (accompanying or augmenting factors) may explain why the intake of some food sometimes lead to anaphylaxis, while in other cases the same allergen induces a milder reaction or is even tolerated. An understanding of the mechanisms underlying the anaphylactic reactions as well as of the predisposing and augmenting factors could help in the development of new prophylactic and therapeutic approaches. These strategies should target the specific pathways involved in anaphylaxis which, in the light of this review, may be more than one (**Figure 1**).

AUTHOR CONTRIBUTIONS

All authors have contributed equally to this review.

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Beyond IgE—When Do IgE-Crosslinking and Effector Cell Activation Lead to Clinical Anaphylaxis?

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Anaphylaxis in humans is inherently difficult to study due to the acuteness of symptoms and the lack of biomarkers serving as risk predictors. Most cases are related to IgE sensitizations to foods, insect venoms, and drugs with mastocytosis patients forming a smaller risk group. However, identifying the relatively small fraction of persons at risk has been exceedingly difficult. In this review, we propose to describe anaphylaxis in a broader context than defined by IgE sensitization alone. Exposure to a trigger, such as an allergen, may lead to anaphylaxis, but in particular, the internal dose sensed by the immune system needs to be established. Moreover, intrinsic patient factors as well as the specific circumstances of the exposure, i.e., the extrinsic factors, need to be thoroughly accounted for. More controversially, other triggers of anaphylaxis, such as increased sensitivity to or reduced catabolism of histamine ("histamine intolerance") or mast cell activation syndrome also named mast cell activation disorder have been suggested, but still with very limited epidemiological evidence that a significant proportion of the observed reactions are caused by these alleged conditions. Thus, when all conditions are considered, it seems as if IgE-mediated reactions are responsible for the vast majority of anaphylactic conditions.

Keywords: anaphylaxis, allergens, mast cells, mast cell activation, cofactors

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INTRODUCTION

Anaphylaxis is a serious allergic reaction that is rapid in onset and may cause death (1). It typically involves one or more of the symptoms: an itchy rash, throat or tongue swelling, shortness of breath, vomiting, lightheadedness, and low blood pressure (BP) appearing in minutes to hours after a stimulus that is mostly believed to be of exogenous origin.

Epidemiological studies are scarce (1), but recent studies suggest that foods (two-thirds), insect venoms (20%), and drugs are among the most frequent triggers, with differences between age groups. A special population at risk seems to be patients with mastocytosis, where various triggers including mechanical stimuli and insect stings—even without the presence of allergic sensitizations to venom allergens—may precipitate severe reactions.

While becoming increasingly popular in quasi-scientific fora on the Internet, other triggers of anaphylaxis, such as increased sensitivity to or reduced catabolism of histamine ("histamine

tolerance") or mast cell activation syndrome (MCAS) also named mast cell activation disorder (MCAD) still have unclear definitions and limited epidemiological evidence exists that a significant proportion of anaphylaxis should be caused by these alleged conditions. Thus, when all conditions are considered, it seems as if IgE-mediated reactions are responsible for the vast majority of anaphylactic conditions.

When looking at the triggers of anaphylaxis, it is clear, however, that only a tiny fraction of patients with food, insect venom, or drug allergy will experience an anaphylactic reaction. Due to the severity and life-threatening nature of anaphylactic reactions, it is of utmost importance to identify the risk factors in these patients that may predict—or ultimately prevent—the occurrence of anaphylactic reactions. While some foods seem to be more allergenic than others, there is little prognostic value in both food sources and sensitization to specific allergens. A similar uncharacteristic pattern has emerged for insect venoms, and even more so for drugs where the diversity and complexity of drugs make it impossible to gather much experience except for large drug groups such as beta lactam antibiotics.

Next in line for the risk analysis comes quantitative factors, such as the IgE-titer—or more indirectly: skin test response—to certain allergens. Also, these parameters have, however, failed to be strongly predictive of anaphylaxis risk, and examples can be found where the IgE-titer in serum over time has fallen below the detection limit, while the patient has retained clinicial reactivity upon reexposure (2). While IgE sensitization is still the best biomarker for risk of anaphylaxis, we aim at identifying additional features of the anaphylaxis pathophysiology that may eventually provide tools for identifying the patients at high risk.

All the above evidence suggests that individual factors to a great extent determine the severity of the allergic response and thereby also the risk of developing anaphylaxis, but knowledge about predictive factors is lacking. In this paper, we propose a theoretical framework for the pathogenesis of anaphylaxis, which by investigating a putative pathway of the mast cell activation, the primary target cells of the mast cell mediators, and of the neurological and other secondary mechanisms in the vasculature display a research paradigm that may help shedding some light on a disease, which by its acute form and unpredictable occurrence has so far eluded a more systematic study approach (**Figure 1A**).

TYPE 1 HYPERSENSITIVITY

The hypersensitivity reaction classified by the British immunologists, Philip Gell and Robin Coombs, as the type 1 reaction (now also named *the IgE-mediated allergic reaction*, but notably IgE had still not been discovered and named in 1963, when Gell and Coombs first suggested their classification of this humoral form of hypersensitivity), still provides the basic theoretical framework for our discussion of anaphylaxis. Centrally in this theory is the involvement of effector cell-bound antibodies, which upon binding to an allergen will induce activation and release of anaphylactogenic mediators. Immunoglobulin E is in humans the only antibody class with a solid evidence of binding to and activating the effector cells, mast cells, and basophil granulocytes, *via* the tetrameric form of the high affinity IgE receptor, the FcεRI comprising one alpha, one beta, and two gamma units (as opposed to the trimeric form of the receptor, lacking the beta-chain, which may be found on other cell types not believed to take part in the acute allergic reaction). So-called homocytotrophic antibodies, i.e., antibodies binding to effector cells, of other isoforms such as IgG4 (3), IgD (4), and even isolated light chains (5) have been suggested, but not supported by a solid body of evidence. There is likely to be marked differences between man and rodents, and while the human isotype IgG4 has similarities to mouse, rat, and guinea pig IgG1, there is little evidence of functional effector cell receptors playing an important clinical role in the human system. Thus, it seems fair to conclude that most anaphylactogens are in fact allergens, as defined as an antigen to which an IgE-immune response is mounted.

As mentioned above, there is a large body of literature discussing the quality and quantity of IgE, in relation to anaphylaxis caused by foods, insect venoms, and drugs. Many such studies suggest that not all allergens may necessarily be anaphylactogens, but since our aim is to go beyond this discussion, we will not dwell further on this aspect, but refer the reader to the vast amount of literature most recently reviewed in Ref. (6).

ALLERGOKINETICS AND INTERNAL DOSE

Much less studied are the pathways before and after the sensitized effector cell meets the allergen (**Figure 1A**). We have used the term *allergokinetics* (derived from the terms and concepts of pharmacokinetics and toxicokinetics) to describe the mechanisms by which an allergen source such as a food, an insect venom, or a drug is taken up by the body, how the allergenic (=IgE-binding) molecules are solubilized, absorbed over biological membranes, and eventually distributed in the organism where it is able to meet and activate effector cells, causing mediator release. Even less studied are the subsequent events where the allergen is potentially metabolized and excreted by the organism.

We propose to use the concept of *internal dose*, to describe the quantity of the anaphylactic trigger (most often an allergen) that becomes available to the systemic circulation, from where it is believed to activate the vascular system.

Old studies using the method of passively sensitizing healthy persons with a hyperimmune serum from a sensitized person, followed by ingestion of the culprit allergenic food, has demonstrated a much faster uptake than would be expected by normal gastrointestinal physiology (7). This was confirmed by studies using sensitive biological tests (8, 9) for detection of allergen in plasma of non-allergic persons ingesting foods to which hyperimmune sera could be used for detection. Finally, an elegant revival of the old Prausnitz–Küstner method has been used after a rigorous ascertainment of the safety of the sensitizing serum. Here, an inverse relationship between oral dose and onset of skin reaction was demonstrated (10). The overall conclusions from these studies suggest that less than one part per million of food protein (and thus almost out of reach with the present status of analytical allergen detection) is absorbed systemically. However, so far, it has not been

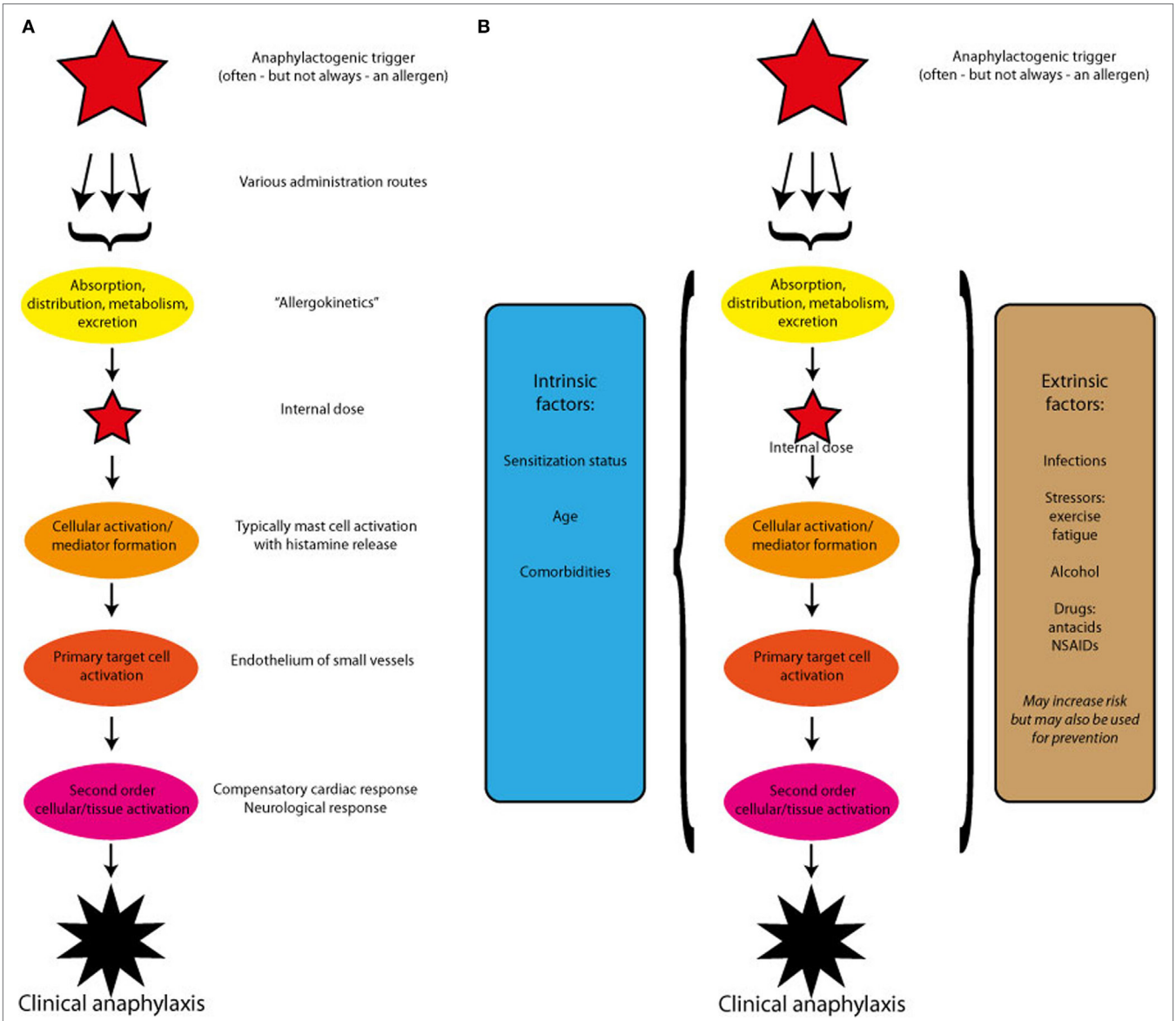

FIGURE 1 | **(A)** A theoretical outline of the pathogenesis of anaphylaxis induced by a single trigger. After absorption and redistribution, the trigger reaches and activates the effectors cells. The ensuing mediator release induces the primary target cells to react, which may in turn activate compensatory or enhancing mechanisms. **(B)** A theoretical outline of the pathogenesis of anaphylaxis induced by a single trigger, but modified by host-specific intrinsic and extrinsic factors. The modifying factors may alter each of the steps: cellular activation, primary target cell activation, or secondary cellular or tissues activation.

possible to study whether similar mechanisms of limited intact food allergen absorption is taking place in allergic patients, or whether they might have qualitatively different mechanisms operating.

Some foods are known to potentially induce severe anaphylactic reactions, such as egg and milk in early childhood, and peanut, tree nuts, fish, and shellfish persistently throughout life. It is well-accepted that a "dangerous" food contains allergens that are sufficiently resistant to gastrointestinal proteolysis allowing them to cross the gut epithelial barrier and cause systemic reactions. Legume and tree nut storage proteins such as peanut Ara h 2 and lipid transfer proteins such as peach Pru p 3 are probably the best characterized molecules in this category. They are both highly resistant to proteolysis and have been identified as risk factors for severe symptoms. Nevertheless, a very significant number of patients sensitized to such stable allergens only experience mild symptoms, usually limited to the oral cavity. This means that, besides protease-resistance, other factors determine the clinical outcome of exposure to potentially severe (stable) allergens.

The relationship between the *internal dose* reaching the immune system, and the actual dose administered *via* ingestion, inhalation, skin absorption, or *via* parenteral routes is not only determined by absorption. In particular, many food allergens are

digested in the stomach but may also react or become absorbed intact in the buccal mucosa (8). The clinical studies of threshold values to allergenic challenges suggest that there are large interindividual (of 3–5 decades) differences between patients' threshold dosages when challenged with food allergens. Although insufficient data are available, it seems most likely that each individual has his/her own threshold value, and the more the given dose exceeds this threshold, the higher likelihood of an anaphylactic reaction.

MEDIATOR RELEASE AND PRIMARY TARGET CELL ACTIVATION

Following mediator cell activation, different mediators, the most prominent being histamine, but likely also involving tryptase, prostaglandins, the sulphido-leukotrienes, LTC4, LTD4, and LTE4; platelet aggregating factor and other lipid-derived mediators are released to the surrounding tissue to act locally and systemically. Both primary triggers such as allergens and mediators released by these may be systemically distributed, but it is rarely clear which of the two distribution mechanisms that are most important for the systemic nature of anaphylaxis.

SECONDARY EFFECTS: LOCALIZED OR SYSTEMIC RESPONSE?

Anaphylaxis can affect multiple organ systems and results in a broad range of symptoms from the skin and mucous membranes, upper and lower respiratory tract, gastrointestinal tract, and cardiovascular and nervous systems. Cutaneous symptoms are present in more than 80% of episodes and are often transient including erythema, pruritus, rash, and urticaria/angioedema (11, 12). During skin and mucosal symptoms, the dermal microvasculature is highly unbalanced and important extravasations of fluid occurs. However, the vascular system extends well beyond of the microvasculature. The majority of severe alterations described in human anaphylaxis involve the vascular system and hypotension is one of three important criteria for diagnosing anaphylaxis, with resulting hypoxia being a key feature contributing to the severity of the reaction (13, 14). Overall hemodynamic defects are fundamental for the sudden fall in the BP and may directly cause some of the neurologic symptoms as dizziness, fainting, and seizures associated to severe anaphylaxis. Moreover, involvement of the gastrointestinal system (abdominal pain, cramping, nausea, vomiting, incontinence, and diarrhea) has been strongly correlated with hypotension and hypoxia too. Low BP during anaphylaxis might result in decreased myocardial perfusion, which in turn causes arrhythmias and cardiovascular collapse. The respiratory system is commonly affected giving rise to symptoms such as dyspnea, wheezing, stridor, deep cough, upper airway obstruction, asphyxia, and respiratory arrest (15, 16).

The extravasation of fluid leads to decreased venous return, which in turn causes insufficient filling of the heart, reduced cardiac output, and ultimately cardiac arrest. Respiratory obstruction/arrest, cardiovascular collapse, or a combination of these might be fatal. Moreover, there is increasing evidence of the human heart as a target of cardiac anaphylaxis involving human heart mast cells (17), but this area deserves further studies.

PATIENT-ASSOCIATED INTRINSIC FACTORS

Clearly, allergen dose and the patients IgE titer are not enough to predict the severity of an anaphylactic reaction, and other contributing factors termed cofactors may be described as intrinsic or extrinsic (**Figure 1B**).

Patients with, e.g., severe food allergy often report an almost immediate tingling sensation in the mouth, already upon exposure to tiny quantities of the offending food. An anaphylactic reaction can occur within a few minutes, so symptoms are initiated long before the food allergen has had the chance to pass the stomach into the intestinal tract and interact with the relevant epithelial mast cells. This suggests that the process of anaphylaxis already starts when the allergen is exposed to the oral (or esophageal) mucosal/epithelial barrier. The kinetics of uptake of food proteins over the oral mucosal barrier is a poorly understood process that may differ between patients with mild and severe food allergy. Anaphylactic patients may have a more permeable mucosal/epithelial barrier in the oral cavity but possibly also in the intestinal tract. In addition to differences in the physical barrier function, the innate immune function of epithelial cells may also differ, and the number of subepithelial tissue mast cells and/or their sensitivity to allergen may distinguish between patients with mild and severe reactions. Also, differences in the phenotype and thus responsiveness of mast cells (18) and basophils (19) may play a role.

In addition, much more crude physiological parameters, such as age, hormonal status, and comorbidities may determine the reactivity of the organism being exposed to an anaphylactic trigger, and it is important to emphasize that the intrinsic, as well as the extrinsic factors described below, may interact differently with each of the steps in the anaphylactic pathway.

EXTRINSIC FACTORS: FOOD, LIFESTYLE, ENVIRONMENT, AND INFECTIONS

Thresholds for allergic reactions to food also have considerable intraindividual variability. It is nevertheless assumed that all patients can develop severe reactions, given the right combination of factors and events and if the dose is high enough. Apart from the dose of a food, there is also the way it is consumed, i.e., pure unprocessed, processed, and/or as part of a composite food. The matrix in which a food allergen is presented to mucosal surfaces, both with respect to its composition and its way of processing, is an extrinsic factor that has significant impact on release and uptake of food allergens (**Figure 1B**).

Other extrinsic factors that may influence allergen uptake are exercise shortly after food consumption (exercise-induced anaphylaxis), alcohol-use, and the use of non-steroidal

antiinflammatory drugs and antacids (20, 21). Exercise-induced anaphylaxis is most commonly but not exclusively associated with wheat allergy. All these extrinsic factors are thought to increase gut permeability or increase allergen solubility, thereby lowering the threshold for severe reactions. Quantitative data are, however, not available. Some of these extrinsic factors may very well synergize into the perfect storm of a "party challenge" (dinner including potential allergens, alcohol, dance, preventive aspirin, antacid), of which the quantitative impact is of course even more complex. Stress and sleep deprivation are also on the list of extrinsic factors that increase the risk for severe reactions. Last but not least, infections such as common cold (rhinovirus) and flu (influenza virus) have been implicated to increases hyperresponsiveness and may thus lower thresholds or increase the severity of a reaction. As of yet, the effects attributed to most of these extrinsic factors have not yet been confirmed by clinical, prospective studies.

TRIGGERS OF THE ANAPHYLACTIC REACTION

After having discussed the potential host-modifying (intrinsic and extrinsic) factors (**Figure 1B**), we can move back to the actual triggers (**Figure 2**) of the reaction. Clearly, not all clinical cases of anaphylaxis may be fully explained and diagnosed, but as previously cited from the literature, food seems to be most important trigger, followed by insect venom stings and drugs, the latter also including allergic side effects of allergen-specific immunotherapy, and systemic reactions caused by diagnostic (iatrogenic) allergen challenges.

Food

By definition, the oral route is the only relevant exposure to foods, even though food proteins are known to elicit (and perhaps sensitize) *via* inhalation or *via* dermal exposure. This does not preclude, however, that the primary sensitization to a food allergen has taken place *via* another administration route.

Insect Venoms

Insect stings often occur in body areas with thin skin or on mucous membranes that allow a rapid and relatively unhindered systemic absorption of the individual venom components. By nature, these are toxic in their own right, and even if the patients is not allergic, large local reactions may be seen.

Drugs

Anaphylactic reactions to drugs are divided into allergic reactions (including IgE-mediated reactions) and non-allergic reactions, which may have several other mechanisms, such as

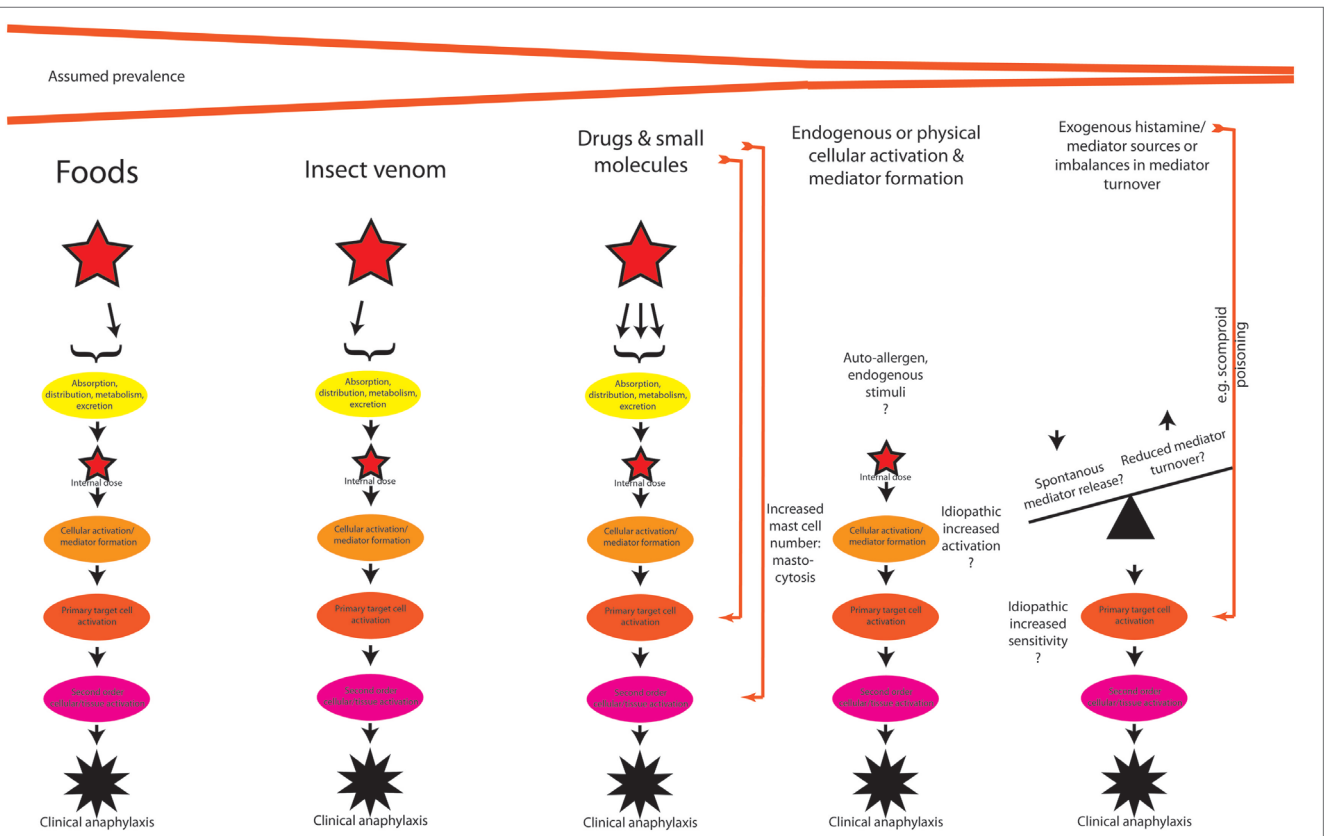

FIGURE 2 | A theoretical outline of the pathogenesis of anaphylaxis induced by different triggers, with potentially different pathways. See text for discussion.

activation *via* the complement system or direct actions of a drug on receptors of the target cell, e.g., opioid drugs activating mast cells directly, or more recently, the suggestion that some drugs may directly activate the mast cell *via* the MRGPRX2 surface receptor (22).

In practice, it is important not to limit the scope to the active component in prescription drug. Excipients and additives such as, e.g., methylcelluloses or polyethylene glycols (23) may also have allergenic potential. In addition, chemicals used for disinfection or sterilization such as chlorhexidine and ethylene oxide may be important to consider. Also, many drugs have potent effects in their own right, and the differentiation between pharmacological effects, side effects, and allergic symptoms is important for reaching the correct diagnosis (24).

Within the realm of perioperative anaphylaxis and postsurgical recovery, the administration of blood products may also give rise to anaphylactic reactions, and here, two immune systems, the host's and donor's, may be on collision course with other mechanisms than IgE being involved. Such mechanisms includes an immune-complex-mediated (with possible complement-system involvement also including the contact system) reaction between anti-IgA auto-antibodies of IgA-deficient donors and IgA of the recipient (25).

Endogenous Cellular Activation: Mastocytosis and Other Mast Cell-Related Disorders

Mastocytosis is a well-known cause of anaphylaxis (26), which can often be diagnosed *via* the signature KIT D816V mutation (27). Additionally, condition(s) described as MCAS or MCAD have been suggested, but it has been difficult to establish firm criteria for this or these conditions. One important step forward was the suggestion to first eliminate primary MCADs such as mastocytosis and secondary activation disorders such as allergic reactions and other conditions caused by receptor-mediated mast cell activation before considering MCAS. Further, strict criteria have been proposed including (a) symptoms from two or more organs; (b) response to anti-mediator therapy; and (c) evidence of increased mediator turnover (28). It seems fair to state that the clinical significance of such reactions are still controversial and very limited epidemiological evidence exists where the above-described criteria have rigorously been applied. Thus, when all conditions are considered, it seems as if IgE-mediated reactions followed by mastocytosis-related anaphylaxis are responsible for the vast majority of anaphylactic conditions.

Exogenous Mediator Intake and/or Reduced Catabolism, i.e., Histamine Intolerance

Scombroid poisoning—an allergy-like intoxication after ingestion of fish or other foods with high histamine content due to microbial degradation—can be considered as an overdose of histamine. Intake of more than 50 mg histamine/100 g food is considered toxic, but intake of histamine 20 mg/100 g food has in some cases elicited symptoms. None of the symptoms are pathognomonic to histamine poisoning, and the only reliable way to diagnose this condition is to measure histamine content in the food (29, 30).

It is likely that sensitive individuals exists, who may react to lower dosages of histamine, either because of an increased sensitivity at the receptor level or because of a reduced catabolism of histamine, which is mainly degraded by the enzyme, diamine oxidase [see p. 89 in Ref. (31)]. In principle, similar syndromes may exists for other mediators. A considerable literature is available, but the diagnosis, *histamine intolerance,* is controversial (32) and not always easily differentiated from extrinsic or intrinsic factors described above.

CONCLUSION

Experimental evidence, both clinically and at laboratory level, is needed to facilitate better understanding of the mechanisms by which severe reactions occur, but also to be able to quantify their impact on threshold doses. Severe, potentially life-threatening anaphylactic reactions have great impact on the quality of life of patients. They are the basis of the anxiety and fear common to these patients and their relatives. An important knowledge gap relevant to evidence-based risk management of anaphylaxis is our poor ability to predict whether, and if so when, patients will develop such severe systemic, potentially life-threatening reactions. Here biomarkers are essential, and biomarkers that are bedded in mechanisms of disease are more powerful than markers identified simply by association (33). On the other hand, the difficulties in performing clinical research on patients with acute but fast remitting symptoms may also have inspired the proposal of a number of controversial disease mechanisms.

AUTHOR CONTRIBUTIONS

LP made the original outline after common discussions. BJ, VE, and LG read and commented the manuscript, which was finalized by LP.

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REVIEW
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Factor XII-Driven Inflammatory Reactions with Implications for Anaphylaxis

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Anaphylaxis is a life-threatening allergic reaction. It is triggered by the release of pro-inflammatory cytokines and mediators from mast cells and basophils in response to immunologic or non-immunologic mechanisms. Mediators that are released upon mast cell activation include the highly sulfated polysaccharide and inorganic polymer heparin and polyphosphate (polyP), respectively. Heparin and polyP supply a negative surface for factor XII (FXII) activation, a serine protease that drives contact system-mediated coagulation and inflammation. Activation of the FXII substrate plasma kallikrein leads to further activation of zymogen FXII and triggers the pro-inflammatory kallikrein–kinin system that results in the release of the mediator bradykinin (BK). The severity of anaphylaxis is correlated with the intensity of contact system activation, the magnitude of mast cell activation, and BK formation. The main inhibitor of the complement system, C1 esterase inhibitor, potently interferes with FXII activity, indicating a meaningful cross-link between complement and kallikrein–kinin systems. Deficiency in a functional C1 esterase inhibitor leads to a severe swelling disorder called hereditary angioedema (HAE). The significance of FXII in these disorders highlights the importance of studying how these processes are integrated and can be therapeutically targeted. In this review, we focus on how FXII integrates with inflammation and the complement system to cause anaphylaxis and HAE as well as highlight current diagnosis and treatments of BK-related diseases.

Keywords: contact system, factor XII, kallikrein–kinin system, bradykinin, mast cells, heparin, polyP, anaphylaxis

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BACKGROUND OF THE PLASMA CONTACT SYSTEM

The factor XII (FXII)-driven contact system is a network of proteases and inhibitors that integrates four major pathways: (1) the complement system, (2) the coagulation cascade, (3) the fibrinolytic system, and (4) the kallikrein–kinin system (1). The name "plasma contact system" comes from FXII being activated when it comes into "contact" with anionic surfaces, which leads to a conformational rearrangement resulting in the active protease factor XIIa (FXIIa). FXIIa initiates a series of downstream events that mediate the interface between of inflammation and coagulation (2, 3). FXIIa activates two serine proteinases, factor XI (FXI) and plasma prekallikrein (PK) that drive the coagulation and kallikrein–kinin systems, respectively. The

non-enzymatic cofactor, high-molecular-weight kininogen (HK) is cleaved by activated plasma kallikrein (PKa) to release the pro-inflammatory oligopeptide bradykinin (BK) (4). Recent data have linked FXIIa-driven formation of BK and the downstream activation of the G-protein-coupled receptor B2 (B2R) potentially signaling to anaphylaxis and other immunologic disorders (**Figure 1**) (5, 6).

Proteins and Molecules of the Contact System

Factor XII

Factor XII circulates in plasma as a zymogen with a concentration of 40 µg/ml (375 nM) (1). Coming into contact with anionic surfaces causes the zymogen form of FXII to undergo a conformational change in the presence of zinc ions. Conformational rearrangements induce auto-activation, which leads to small amounts of FXIIa (7). Due to activation of FXII zymogen, the single-chain polypeptide is converted into a two-chain molecule, composed of a heavy chain [353 amino acid (aa)] and a light chain (243 aa). The two chains stay connected with each other by a disulfide bond between Cys340 and Cys367 residues. The heavy chain is responsible for binding to anionic surfaces and, similar to HK heavy chain (8), links the zymogen to proteoglycans of cell surfaces (9). The catalytic domain is located within the C-terminal light chain of the protease. In humans, single-chain (sc)FXII has measurable, although much lower, proteolytic activity than complete FXIIa and its potential importance *in vivo* remains to be shown (10). FXIIa initiates the intrinsic coagulation cascade, which leads to the generation of thrombin and fibrin to produce clots in the blood (11). Furthermore, FXIIa converts PK to the active protease PKa, which reciprocally activates more FXII (7). In addition, PKa can initiate a further proteolysis of FXIIa into a ~30 kDa light chain fragment, termed β-FXIIa. The cleavage takes place at the peptide bond Arg353–Val354 and consequently, the active site released from the heavy chain and thus from surfaces. This small, soluble β-FXIIa variant retains its proteolytic activity toward PK, but not to FXI (**Figure 1**), offering an explanation

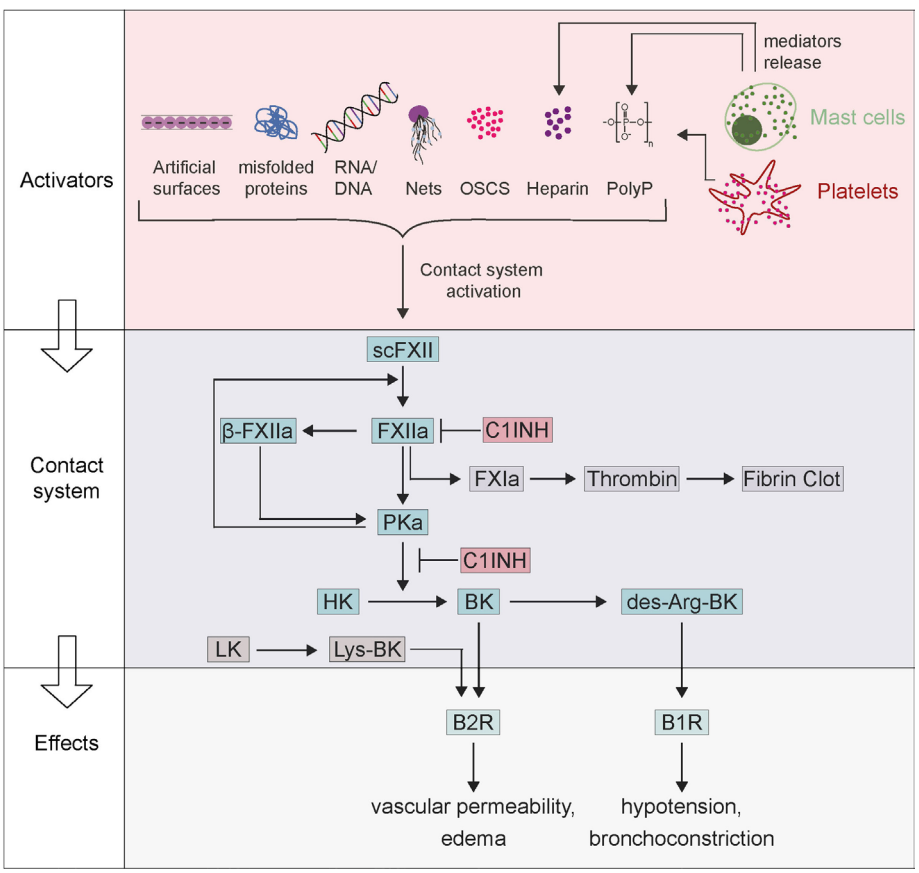

FIGURE 1 | Factor XII (FXII)-driven contact system in activation of anaphylaxis. Zymogen scFXII becomes activated to FXIIa either by endogenous activators [misfolded proteins, RNA/DNA, neutrophil extracellular traps (NETs), polyP, oversulfated chondroitin sulfate-contaminated heparin (OSCS-heparin) and heparin] or by artificial surfaces. Anaphylaxis can activate mast cells with the release of their mediators (polyP and heparin), which also leads to FXIIa. FXIIa proceeds to activate prekallikrein, which reciprocally cleaves both FXIIa into β-FXIIa and high-molecular-weight kininogen (HK) to bradykinin (BK). BK binds receptor B2 (B2R) and triggers inflammation, edema, and symptoms of anaphylaxis. BK can be further proceeding to des-Arg-BK and mediates B1 receptor (B1R) activation resulting in hypotension and bronchoconstriction. The contact system can be inhibited by the C1INH that inhibits both FXIIa and plasma kallikrein.

for selective activation of the kallikrein–kinin pathway in the absence of coagulation (12).

Plasma Kallikrein

Prekallikrein has a plasma concentration of 35–50 µg/ml (580 nM) and exists as two different glycosylated forms with molecular weights of 85 and 88 kDa, respectively. Similar to FXII, a limited proteolysis activates zymogen PK and the active form is composed of a heavy chain (residues 1–371, 55 kDa) linked by a disulfide bond and a light chain (residues 378–619, 30 kDa). The heavy chain contains four apple domains and PK/PKa binding to HK is mediated by apple domains 1, 2, and 4 (13, 14). The PK light chain contains the peptidase domain with the substrates being HK, FXII, plasminogen, and urokinase-type plasminogen activator. Interestingly, the kallikrein–kinin system is linked to thrombosis, fibrinolysis, and the rennin–angiotensin system through the conversion of plasminogen to plasmin by PKa (7).

High-Molecular-Weight Kininogen

In humans, the non-enzymatic cofactor HK is generated from a single gene but undergoes alternative splicing to form high- (HK) and low-molecular (LK) weight kininogen. Murine HK contains two kininogen genes and both transcripts undergo alternative splicing which results in four kininogens. HK, but not LK, binds to cell surface glycosaminoglycans and the interaction is improved by zinc ions (15, 16). There is no detectable spontaneous HK activation due to HK protection from proteolytic cleavage by glycosaminoglycans binding. Therefore, cell surface presents a reservoir for BK production (7, 17).

Activation of BK *via* the FXII-Driven Contact System

Bradykinin is a nonapeptide composed of the sequence Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg and functions as an inflammatory mediator. BK is the product of the kallikrein–kinin system following activation of FXII. FXIIa leads to proteolysis of PK, and the resulting PKa cleaves HK to generate BK (**Figure 1**). In contrast to PK, tissue kallikrein liberates kallidin (Lys-BK) from LK (18). Released BK binds with high-affinity (8–12 nM) to B2R. Upon binding of BK or kallidin, the activated B2R induces an increase of intracellular calcium ($[Ca^{2+}]_i$) that stimulates the endothelial nitric oxide synthase resulting in increased protein kinase G activity (19, 20). B2R signaling triggers vasodilatation, increase of vascular permeability, mobilization of arachidonic acid, and chemotaxis in granulocytes (21). BK increases vascular permeability *via* opening tight junctions of endothelial cells (22). B2R is constitutively expressed in multiple tissues such as endothelial cells, sensory fibers, smooth muscle cells, and epithelial cells, among others. Furthermore, expression of the B2R is enhanced by cytokines, cyclic adenosine monophosphate, estrogen, and glucocorticoids. Pathologic B2R activation contributes to various allergic, inflammatory, and infectious diseases such as sepsis, anaphylaxis, traumatic brain edema, rhinitis, capillary leak syndrome, or ischemia/reperfusion injuries (6, 23, 24). BK has a short half-life (<30 s) in plasma because it is quickly degraded by both plasma and endothelial peptidases. To overcome limitations in analyzing BK in patient samples elegant assays that measure BK-free HK (cleaved HK) have recently been developed (25). The angiotensin-converting enzyme (kinase 2), carboxypeptidases M and N (kininase 1), and the neutral endopeptidase (Neprilysin) process BK at two distinct sites (Pro7–Phe8 and Phe5–Ser6) leading to the inactive peptides BK1–7 and BK1–5 (26). Carboxypeptidase N removes the C-terminal BK arginine residue resulting in the metabolite des-Arg9-BK. This peptide stimulates the G-protein-coupled kinin B1 receptor (B1R) (27). Under normal physiological conditions, B1R is minimally expressed, but expression is rapidly upregulated in response to stimuli such as tissue injury or an increase in inflammation (20). Pharmacological inhibition of some mitogen-activated protein kinases and NF-κB interfere with B1R expression. Interestingly, all kallikrein–kinin system components are found within the central nervous system (CNS), and BK is formed and contributes to brain trauma and ischemia (28). Recently, a role for B1R in brain immune inflammation in a mouse model of Alzheimer's disease was identified, possibly with microglial/macrophage involvement (29). Blocking B1R reduces brain infarction and edema formation in mice, while B2R deficiency had no effect on stroke outcome in mice (30). Furthermore, murine models indicate a role of FXIIa and BK in CNS autoimmunity, including multiple sclerosis (31) and pharmacologic interference with BK formation and/or signaling might ameliorate secondary brain injury (32).

THE CONTACT SYSTEM INTEGRATES WITH ACTIVATED MAST CELLS, THE COMPLEMENT SYSTEM AND MEDIATES ANAPHYLAXIS

Anaphylaxis is a multisystem syndrome of a rapid onset of symptoms and an immunologic response to allergens (33) that is predominantly driven by activated mast cells. Mast cells are found near blood vessels and areas susceptible to foreign antigens, such as tissue mucosa, and serve as multifunctional effector cells in the immune system (34). In most cases, the initiation of anaphylaxis is due to an antigen (allergen) that interacts with high-affinity receptors for immunoglobulin E (FcεRI), which are located on mast cells and basophils. Allergen-binding leads to intracellular signaling that results in the release of granules (35). These components, which are synthesized by mast cells and other immune cells such as macrophages or neutrophils, interact with circulating plasma proteins or tissue factors. Among the liberated compounds is histamine, which increases vascular permeability and vasodilation, leads to plasma leakage and reduced intravascular volume (36). This induces a drop in blood pressure that can lead to a lethal outcome.

Mediators of the Mast Cells and Activators of FXII-Driven Contact System

Mast cells critically contribute to anaphylaxis. The link between mast cells and anaphylaxis was established once it was discovered that mast cells were abundant in protein and mediators such as tryptase, chymase, and other cytokines, as well as newly synthesized lipid-derived molecules such as prostaglandins,

platelet-activating factor (PAF), cytokine tumor necrosis factor α, and leukotrienes (37). These mediators play an important role in the development of anaphylaxis; however, the mechanisms of inducing anaphylaxis vary widely (6). For instance, PAF activates inflammatory and thrombotic pathways by causing platelet activation and liberates vasoactive substances, resulting in increased endothelial permeability. Uncontrolled PAF activities can result in sepsis, shock and are important in disseminated intravascular coagulation (38, 39). Prostaglandins lead to smooth muscle relaxation and act as vasodilators. Interestingly, they can also inhibit platelet adherence. Levels of urinary prostaglandin D2 correlate with severity of anaphylaxis (40) and leukotriene production accompanies histamine and prostaglandin production. Their release triggers smooth muscle contractions and vasodilation, leading to bronchoconstriction and hypotension. Cysteinyl leukotrienes are termed slow-reacting substance of anaphylaxis and are up to 1,000-fold more potent than histamine but have a slower onset and long-lasting activities (41–43). The overlap of these pathways leads to synergistic pathologic effects that also result in activation of complement and contact system pathways, highlighting the importance of developing effective therapeutics for this potentially lethal condition. In this section, some of the main mediators that induce inflammation and/or coagulation through contact system-mediated pathways will be discussed in greater detail.

Histamine

Released histamine causes increased angioedema, anaphylaxis, or chronic spontaneous urticaria and is also involved in allergic responses. Histidine decarboxylase is the only enzyme capable of producing histamine (44). Upon mast cell release, histamine promotes recruitment of T_H2 helper cells and dendritic cells along with antigen presentation (35). Mast cell secretory granules also contain heparin and proteoglycans, which are heavily negatively charged, in contrast to histamine, which is positively charged. Both components can interact within granules and upon mast cell activation, heparin proteoglycans and histamine are released with similar kinetics (45). Furthermore, histamine and heparin have been shown to interact in purified systems (34, 46), but there is no evidence for a physiologically relevant interaction *in vivo*. In urticaria patients, the occurrence of angioedema was reduced with antihistamine therapy (47). In addition, there were no increased plasma BK levels in four patients with an acute histamine-sensitive angioedema (48) arguing that BK and histamine have the capacity for inducing edema by independent pathways. For angioedema with unknown derivation (idiopathic angioedema) and for hereditary angioedema (HAE), histamine receptor antagonists are clinically applied, but approximately one in six patients exhibiting idiopathic angioedema do not respond to antihistamine treatments (49). This suggests that other mediators are involved in the trigger and the outcome of hereditary forms of angioedema.

Serotonin

Serotonin, a biogenic amine, is a mast cell granule constituent. However, confocal microscopy revealed that distinct mast cell granules contain both histamine and serotonin (50, 51). In absence of endogenous histamine, serotonin is increased in immune cells including mast cells (44). One explanation could be that mast cells can selectively release serotonin without releasing histamine (52) *via* high-affinity serotonin-binding proteins used to sequester serotonin from secretory vesicles (53). Serotonin functions as a regulator of immune and inflammatory responses and is partially mediated through direct interactions with macrophages (54).

Heparin

Another major component of mast cell granules is heparin, which is released following IgE/antigen activation (5). Heparin-driven FXII contact activation triggers the kallikrein–kinin system, releases BK to stimulate B2R in human plasma and leads to edema *in vivo* (55). Heparin levels are elevated in patients with anaphylaxis while PK and HK plasma levels are low in anaphylaxis, indicating that the contact system is indeed activated. In contrast to other contact system activators, mast cell heparin does not activate the coagulation pathway, possibly because heparin binds to antithrombin III, thereby increasing its inhibitory activity toward thrombin (6).

In 2007, heparin contaminated with synthetic oversulfated chondroitin sulfate-contaminated heparin (OSCS-heparin) was accidentally given to patients in the United States and Germany. This commercially available contaminated heparin resulted in adverse clinical events in the heparin therapy for hundreds of individuals (56). Within several minutes of intravenous infusion of contaminated heparin, there was a drastic reaction in patients causing edema, hypotension, swelling of the larynx and other related symptoms including death (56). The OSCS-contaminated heparin potently activates FXII *via* the kallikrein–kinin system through BK formation in human plasma (57), demonstrating the importance of understanding the mechanisms that induce BK in patients.

Polyphosphate

Polyphosphate (polyP) is a polymer of linear linked phosphate units *via* energy-rich phosphoanhydrous bonds. PolyP is pro-inflammatory and procoagulant and is found in secretory granules of platelets, basophils, and mast cells that resemble acidocalcisomes in prokaryotes (51, 58). Mast cell activation leads to a release of polyP that activates the FXII-driven contact system (51) while FXII- or B2R-deficient mice do not exhibit activated mast cell-induced edema and hypotension (5).

Polyphosphate was first found in prokaryotes and is involved in metabolism, structural behavior and stress responses. The polymer can be from a few up to thousands of residues long (11). In artificial systems, dissolved long-chain polyP (>500 residues) activates FXII more potently than short-chain polyP (<100 residues); however, these long-chain polymers have low solubility under physiological conditions (59). The hypothesis that size determines the activity of polyP for activating FXII has been challenged by the fact that polyP form calcium-rich nanoparticles *in vivo*. Independent of the size of the individual, polyP monomer polyP, packed into particles potently activates FXII (60). PolyP is unstable in plasma (61) and technology to specifically analyze the polymer has been developed (62). Recently, intravital microscopy visualized release of polyP nanoparticles

from platelet dense granules. PolyP nanoparticles accumulate on the procoagulant platelet surface *in vivo*. The polyP particles are retained on the platelet surface where they potently initiate FXII contact activation (63, 64). FXII activation by exposed procoagulant polyP offers a rationale for the critical role of FXIIa in mediating platelet driven coagulation/clot formation that is well established since decades the field (65–70). In addition to polyP particles, small amounts of short-chain soluble polymers are released into the supernatant from activated platelets (71) and activate an array of procoagulant mechanisms (72). The role of these FXII-independent mechanisms, however, remains enigmatic *in vivo*. PolyP colocalizes with serotonin and calcium in the acidic secretory granules of mast cells (51). Taken together, polyP in mast cells is released in a mechanism similar to that of platelets. These data suggest that the release of heparin coupled with polyP inhibits the procoagulant properties of polyP while retaining the pro-inflammatory capability.

Contact System Cross Talk with the Complement System

An important component of the immune response is the complement system, which is composed of soluble proteins circulating as precursors in the plasma. There are three distinct pathways that can activate the complement system: (1) the classical pathway, (2) the lectin pathway, and (3) the alternative pathway. The classical pathway is activated *via* binding of C1q to antibodies complexed with antigens. In some cases, the interaction of C1q with certain pathogens can lead to a direct surface binding without the presence of antibodies. The C1 complex contains C1q, which is further bound to two molecules each of the zymogens C1r and C1s (73). The mannose-binding lectin (MBL) pathway is initiated when mannan-binding lectin-associated serine proteases (MASP-1 and MASP-2) bind and are activated *via* MBL, ficolins or collectins to carbohydrates on the bacterial cell wall (12). The alternative pathway is initiated *via* spontaneous activation of C3b that leads to binding on the pathogenic surface. The activation of all three pathways is driven by a series of limited-proteolysis reactions that convert the proenzymes to an active enzymes (74) culminating in generation of C3 convertase (73). The convertase cleaves C3 to C3a and C3b and can generate more C3 molecules to amplify production of C3b. C3b is involved in the production of C5 convertase, which functions as an opsonization marker for bacteria to be phagocytosed by macrophages and neutrophils (75). The cleavage of C5 by C5 convertase yields C5a and C5b in a similar fashion as C3. C3a and C5a, known as anaphylatoxins, are pleiotropic inflammatory mediators and proteolytically released from C3 and C5 (74). In host defense responses, the membrane attack complex (MAC) is produced by C5b-mediated formation of C5b-9 complex. This MAC induces lysis of pathogens or cells *via* incorporation into the cell membranes (12).

The complement system has the capacity to trigger anaphylactic shock, mainly *via* C3a and C5a activity. These anaphylatoxins induce degranulation of mast cells, which leads to the release of histamine. In addition, they also increase vascular permeability and induce contraction of smooth muscle cells (76, 77). Complement activation was found to trigger anaphylactic shock in mice exposed to peanut extract through C3 activation. In accordance with this finding, the authors showed that mice deficient in C3 or its receptor C3aR had almost no response to the peanut extract (78).

There is extensive cross talk between the complement and contact systems at several levels (**Figure 2**). They share the major endogenous inhibitor, C1INH that inhibits the initial step of both cascades. While C1INH inhibits FXIIa activation of the contact system, all three-activation pathways of the complement are also inhibited by distinct mechanisms. The classical pathway is inhibited by C1INH-mediated inactivation of C1r and C1s (79), two subunits of the C1 complex that is also known to be activated by FXIIa (**Figure 2**) (80, 81). By covalent binding to MASP-1 and MASP-2, C1INH also inhibits the lectin pathway (82). Finally, the alternative pathway is inhibited by reversible binding of C1INH to C3b (83). Interestingly, *in vitro* activation of FXII by OSCS activates C3 and C5 in human plasma in addition to the kallikrein–kinin system. In FXII-deficient plasma, activation was abrogated with no effect on normal complement activation, an effect that was rescued by addition of purified FXII to FXII-deficient plasma (57). There are multiple inhibitors of the complement system that are expressed on cell surfaces. The inhibitory effect of antithrombin, however, is much enhanced by glycosaminoglycans, such as heparin and heparan sulfate (84, 85). Further interactions between the complement and the kallikrein–kinin system have been discovered. For example, PKa has been found to trigger the generation of C3a fragments in humans (86) and C5a due to limited proteolysis of C5 in rabbits (87). While this last reaction was confirmed using anti-PK IgG

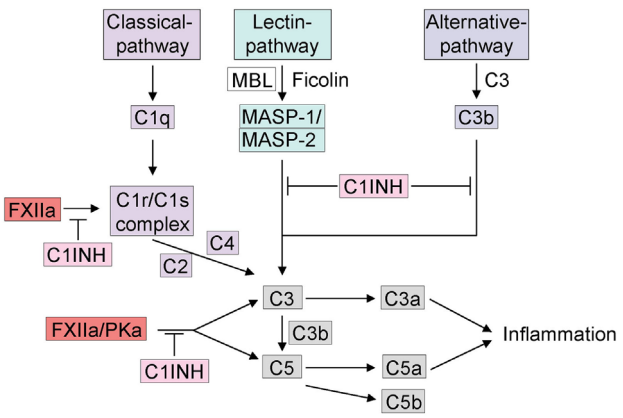

FIGURE 2 | Cross talk between the kallikrein–kinin system and the complement system. The complement system can be activated by three different pathways: (1) C1q initiates the activation of the classical pathway, (2) the mannose-binding lectin (MBL) or ficolins trigger the lectin pathway for glycosylation on the surface of pathogens. Activation of either the classical pathway or MBL generates C3 convertase. (3) If C3 is spontaneously hydrolyzed, the alternative pathway is activated and generates activated C3b. C3 and C5 release C3a and C5a, which can trigger inflammation. The activation of the complement system *via* the kallikrein–kinin system is indicated, mainly FXIIa and plasma kallikrein are involved in activation of C3 and C5. FXIIa can trigger the C1r/C1s complex. C1INH inhibits the complement system in all three different pathways and furthermore *via* the inhibition of the kallikrein–kinin system.

or soybean trypsin inhibitor, this result has yet to be confirmed in humans. The cross talk between complement and the contact system has become increasingly more relevant because many types of molecules are produced that play an important role in pathologies such as angioedema (12) and anaphylaxis.

BK IN ANAPHYLAXIS AND INFLAMMATORY DISEASES

Anaphylaxis

Anaphylaxis can result from serious allergic reactions and immunologic response to allergens and may lead to life-threatening swelling episodes (33). Its onset is in the range of a few minutes if the allergen entered *via* the circulatory system to a couple of hours if the allergen was ingested. Depending on the type of the response, symptoms of anaphylaxis include hypotension, vascular leakage, or even cardiac arrhythmia and bronchial constriction in severe cases (6). There are common triggers for anaphylactic reactions such as food, medications or insect venom with 1–15% of the population being susceptible to anaphylaxis (88).

Recent work from our group has shown that this increased vascular permeability was mediated by heparin-initiated BK formation in mice (5). In this study, it was shown that targeting FXII or B2R abrogated heparin-mediated leukocyte adhesion to the endothelium and inhibited mast cell-triggered hypotension. Ablation of FXII or B2R protected against mast cell-mediated leakage in response to allergens and heparin-induced edema. Furthermore, our group has also demonstrated that deficiency or targeted inhibition of FXII, PK, HK, B2R, but not B1R, resulted in a protective effect against anaphylaxis in an allergen/IgE mice model. In *F12*$^{-/-}$ mice, this protective effect could be abolished by restoration of plasma FXII levels, confirming the involvement of the contact system in this model of anaphylaxis. Analysis of human plasma from anaphylactic patients revealed activation of the contact system. The degree of anaphylaxis associated with levels of mast cell degranulation, heparin levels in the plasma, the amount of contact activation, and subsequent BK formation (6).

Abnormal blood coagulation as a result of IgE-triggered hypersensitivity has been known for years. Activated partial thromboplastin time, a measure of FXIIa-driven coagulation is delayed in patients with anaphylaxis and anaphylactic shock (89, 90). In contrast, the prothrombin time, which utilizes the FXII-independent extrinsic pathway of coagulation, remains unchanged in patients with allergen-mediated anaphylaxis, suggesting that they mediate their effect only *via* the intrinsic coagulation pathway (11). The plasma of IgE/Ag-challenged mice does not clot due to a heparin concentration of >4 μg/ml, which is sufficient for initiation of BK formation (5). Therefore, minute amounts of heparin may produce BK on the mast cells surface.

Hereditary Angioedema

Dysregulation of the contact system leads to HAE, an autosomal dominant disorder that results in recurrent episodes of angioedema of the skin or tissue mucosa. Before the use of prophylactic drugs, laryngeal edema and upper airway obstruction were lethal in up to one-third of patients (91). It is unknown how prevalent HAE is across the world but current estimates propose as many as 1/10,000–1/150,000 individuals in Europe (92). HAE is caused by either reduced C1INH levels (HAE type I) (93), a defective C1INH protein (HAE type II) (94), or hyperactive FXII (HAE type III). In HAE type III patients, C1INH functions normally and circulates at a normal concentration in plasma. However, a single point mutation in FXII (position 309) leads to enhanced FXIIa activity by a mechanism that recently has been unraveled (95). A defective FXII glycosylation at that single site (Thr309 that is mutated to Arg or Lys) is the underlying cause of excessive FXII activation in HAE type III, suggesting that HAE type III is a disease model for gain of function FXII contact activation (96). Edema in HAE type III is not associated with thrombosis (96), supporting a role of mast cell heparin in activating mutant FXII similar to anaphylactic reactions (6). C1INH deficiency increases the ability of FXIIa to convert PPK to PKa (97), since C1INH inhibits over 90% of plasma FXIIa (94). In murine models, cross-breeding C1INH-null with B2R-null mice completely rescues the leakage phenotype, confirming that BK triggers edema formation (98). During acute swelling attacks, C1INH infusions, B2R antagonists, and PK inhibitors have all been shown to effectively block generation of BK (20).

Inflammatory Diseases

Vasodilation and vascular permeability are two processes are involved in many inflammatory diseases (48), leading to local swelling attacks of the dermis and submucosa (99). As an important regulator of those processes, the contact system has been studied in several inflammatory diseases. In rheumatoid arthritis and irritable bowel diseases, for example, high levels of PKa and BK have been observed. Furthermore, it was shown in rodent rheumatoid arthritis models that inhibition of the contact system interferes with arthritis. Moreover, HK deficiency in rats resulted in less acute and chronic arthritis (100). The precise role of the kinin receptors in rheumatoid arthritis has remained a matter of discussions. While B2R receptor deficiency did not affect arthritis in a mouse model of anti-collagen antibody-induced arthritis, combined deficiency of B2R and B1R attenuated arthritis (101). In support of these observations, there are similar findings in irritable bowel disease. Patients with ulcerative colitis (UC) showed decreased plasma levels of PKa and HK, which indicated proteolysis of these precursors and therefore contact system activation (102). Both kinin receptors are expressed in UC patients in intestinal epithelial cells. During active UC, however, B1R is significantly upregulated and seems to be the main receptor by which BK exerts its deleterious effect in UC (18). Interestingly, in a murine dextran sulfate induced colitis model C3, deficiency conferred protection from disease development indicating a role for the complement system in the disease (103). In the same study, the authors showed that treatment with C1INH would also reduce the severity of the disease in WT mice. A rat enterocolitis model confirmed the clinical observation of decreased plasma PKa and HK and intestinal inflammation could be reduced by treatment with BK antagonists or HK deficiency in a PG-PS model. There are many different animal models that display intestinal inflammation and contact system inflammation but use different triggering agents (56, 104, 105), suggesting that the

contact system is an integral part of the process. Taken together, these data indicate that contact activation can be detected in most inflammatory diseases and is mostly mediated through BK production and its receptors.

Diagnostics Related to the Kallikrein–Kinin System

Diagnostics for anaphylaxis are well described in Montanez et al. (106). In the case of BK-related anaphylaxis, there are some more *in vitro* assays available. But measuring the concentration of BK is very challenging, due to rapid degradation of BK and des-Arg9-BK (27 ± 10 and 643 ± 436 s, respectively) (107). Therefore, a number of enzymatic assays have been designed to circumnavigate this issue by measuring more stable BK-related products such as cleaved HK levels (108, 109). Other assays focus on C1INH inhibitory capacities by measuring free C1s activity (110) and C1INH-protease complexes levels (111).

Amidase Activity Assay

This assay measures the activity of free, active C1s amidase (e.g., not bound to C1INH) by the kinetic or endpoint colorimetric assay, using the substrate H-D–Pro–Phe–Arg–pNA (110). It was demonstrated that spontaneous amidase activity was increased in plasma from patients with BK-dependent disorders compared to plasma from normal patients. They confirmed increased BK production by detecting HK cleavage *via* Western blot, which also correlated with increased kininogenase activity (112).

Cleaved Kininogen Assay

A direct indicator of BK release is cleaved HK (25, 113). The reconstitution of liver-synthesized, novel protein is slow. Due to the slow recovery of plasma HK levels, the observed distribution of HK and HK degradation products gives a robust readout of the *in vivo* BK production and allows for the detection of active angioedema (114). The cleaved kininogen assay could be used in injury cases where the role of contact system is developing, such as in transfusion-related acute lung injury and other detrimental blood reactions (115).

Treatments with Drugs against BK Formation

The standard treatment for anaphylaxis is adrenaline, but since mast cell and contact system activation correlate with the severity of the response (116), other drugs inhibiting BK formation could be also considered. For example, specific inhibitors of the kallikrein–kinin system have been shown to be effective at preventing BK-mediated HAE attacks. Some severe side effects can exist, such as a hypersensitivity to the drug that can induce anaphylaxis. The variations in physiological responses demonstrate the need for detailed mechanistic studies of therapeutics that target the contact system. Some of the current therapeutics in clinical trials will be discussed in more detail.

Icatibant

Icatibant (Firazyr®; Shire) is a synthetic decapeptide containing five non-proteinogenic amino acids (H–D-Arg–Arg–Pro–Hyp–Gly–Thi–Ser-D–Tic–Oic–Arg–OH) which resembles the BK-peptide and selectively blocks B2R. In contrast to BK, icatibant has a relatively long half-life (1–2 h) (117). Several *in vitro* and *in vivo* pharmacological assays showed that icatibant binds with a high-affinity to B2R in guinea pig models (118). Furthermore, the anaphylaxis associated BK-induced bronchoconstriction in guinea pig models was inhibited with icatibant (119). Consistent with animal model data, clinical trials showed the efficacy of icatibant and a strong decrease of HAE attacks in treated patients (114, 120). Some side effects were observed in 90% of the patients treated with icatibant, such as temporally local pain, swelling, and erythema at the injection site.

Ecallantide

Ecallantide (Kalbitor®; Dyax, USA) is a potent recombinant protein modeled after the human tissue factor pathway inhibitor Kunitz 1 domain that inhibits PK (121). To test the efficacy and safety in acute attacks, there were two double-blind, placebo-controlled studies performed in 160 patients with HAE. The results of these studies were comparable but the measurement of the patients' reported outcomes was different (122, 123). A known risk of ecallantide treatment for acute HAE attacks is hypersensitivity and subsequent anaphylaxis. The clinical relevance and post-marketing surveillance are required to determine the therapeutic and clinical value (121).

C1INH

Some drugs are available to cover the impropriate function of C1INH or C1INH deficiency. These are plasma-derived (pd)C1INHs [Berinert® (CSL Behring), Cetor® (Sanquin), and Cinryze® (ViroPharma)] or recombinant human (rh)C1INH (Ruconest® in Europe, Rhucin® in the USA, Pharming Group NV). The pdC1INHs prepared and pasteurized from fractionated plasma. rhC1INH is expressed in the mammary gland of transgenic rabbits. Interestingly, both synthetic proteins produced C1INHs differ in their glycosylation pattern. rhC1INH contains less glycosylation than pdC1INH due to its production in a heterologous system. Because of the differences in glycosylation patterns, the rhC1INH can be cleared within 3 h from the circulation, in comparison to pdC1INH, which takes more than 24 h. It is important to know if patients have a rabbit allergy since this could induce anaphylaxis upon treatment with rhC1INH (121). To confirm the safety of these products, more long-term data are necessary.

Avoralstat

Avoralstat (BCX4161) is developed by BioCryst Pharmaceuticals Ltd. (Durham, NC, USA) and is a small molecule kallikrein inhibitor of oral administration. It is an effective and specific inhibitor of PK, as indicated in preclinical studies. One promising study in phase IIa was performed with statistically significant mean attack reduction for HAE type I and type II (124).

DX-2930

DX-2930 is a recombinant human monoclonal antibody against PK produced by Dyax Corp (Burlington, MA, USA) that was

developed using phage display. DX-2930 acts as a long-acting inhibitor and could be used to prevent HAE attacks (124).

Anti-FXIIa Antibody (3F7)

3F7 is a recombinant, fully humanized antibody (3F7) which neutralizes FXIIa by blocking the protease activity of the catalytic domain (125). 3F7 blocks the intrinsic clotting cascade in human plasma and thrombosis formation in mouse models. Consistent with the selective role of FXII in thrombosis but not in hemostatic mechanisms, 3F7 thromboprotection is similar to that of heparin but there is no change in bleeding. 3F7 interferes with FXII activation in response to an array of contact activators including polyP and heparin (126). In humanized mouse models of HAE type III, 3F7 inhibits FXIIa and as a consequence prevents edema in animal models. Supporting a potential use of 3F7 to treat anaphylaxis and HAE, the addition of the antibody abolished BK formation in patients' plasma of HAE type III (96).

CONCLUSION

The FXII-driven contact system plays a role in anaphylaxis and angioedema *via* its ability to increase inflammation and vessel permeability. During the onset of these pathologies, the mast cell activation releases pro-inflammatory mediators including polyP and heparin that can activate the contact system. This contact system activation triggers the kallikrein–kinin system and the complement pathways that intertwine at many levels, for example frequently used control mechanisms, cross-activation, and commonly used binding proteins. The abnormal production of BK leads to HAE and also plays a role in anaphylaxis that both can lead to acute, life-threatening attacks of edema. Therefore, it is of interest to study the common pathways between these pathologies. There are several novel drugs emerging to interfere with contact system activation and possibly other pathologies involving HK, BK, and C1INH. Further clinical studies of the contact system are required to better understand the connection between the contact system and inflammatory-related pathologies like HAE and anaphylaxis.

AUTHOR CONTRIBUTIONS

All authors have made significant intellectual contributions of the review. LB, HW and ATL drafted the original manuscript. SRJ and TR critically analyzed and gave suggestions for the concept and revision that improved content of the text and figures. All authors approved the final version of the manuscript.

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MINI REVIEW
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The Mast Cell, Contact, and Coagulation System Connection in Anaphylaxis

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Anaphylaxis is the most severe form of allergic reaction, resulting from the effect of mediators and chemotactic substances released by activated cells. Mast cells and basophils are considered key players in IgE-mediated human anaphylaxis. Beyond IgE-mediated activation of mast cells/basophils, further mechanisms are involved in the occurrence of anaphylaxis. New insights into the potential relevance of pathways other than mast cell and basophil degranulation have been unraveled, such as the activation of the contact and the coagulation systems. Mast cell heparin released upon activation provides negatively charged surfaces for factor XII (FXII) binding and auto-activation. Activated FXII, the initiating serine protease in both the contact and the intrinsic coagulation system, activates factor XI and prekallikrein, respectively. FXII-mediated bradykinin (BK) formation has been proven in the human plasma of anaphylactic patients as well as in experimental models of anaphylaxis. Moreover, the severity of anaphylaxis is correlated with the increase in plasma heparin, BK formation and the intensity of contact system activation. FXII also activates plasminogen in the fibrinolysis system. Mast cell tryptase has been shown to participate in fibrinolysis through plasmin activation and by facilitating the degradation of fibrinogen. Some usual clinical manifestations in anaphylaxis, such as angioedema or hypotension, or other less common, such as metrorrhagia, may be explained by the direct effect of the activation of the coagulation and contact system driven by mast cell mediators.

Keywords: mast cell, heparin, tryptase, bradykinin, coagulation system, factor XII, fibrinolysis, anaphylaxis

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INTRODUCTION

Anaphylaxis is a severe, life-threatening reaction that results from the systemic effect of mediators and chemotactic substances (1). Pathophysiological mechanisms of human anaphylaxis are not fully understood, but classically mast cells and basophils are considered to play a pivotal role. Allergen crosslinking of specific IgE bound to the high affinity receptor, FcεRI, leads to the activation of mast cells and basophils, inducing cellular degranulation and release of mediators, both preformed or *de novo* synthesized. Mediators of human mast cells comprise pro-inflammatory molecules, such as histamine, leukotriene (LT) B4 and LTC4, or prostaglandin D2 (PGD2), cytokines, vascular endothelial growth factor (VEGF), proteases such as tryptase and chymase, and the highly sulfated polysaccharides, heparin and chondroitin sulfate, being the last two especially abundant in mast

cell secretory granules (2). Besides IgE-mediated activation of mast cells and basophils, further mechanisms are involved in the occurrence of anaphylaxis. In mouse models, the role of IgG has been demonstrated. The IgG/antigen complex crosslinking of FcγRIII on macrophages and basophils results in the release of platelet-activating factor (PAF) and the induction of symptoms resembling anaphylaxis (3, 4). Neutrophils have also been suggested as relevant cells in anaphylaxis through IgG1 and 2 in mice (5). In humans, the existence of IgG-mediated anaphylaxis remains unclear. However, studies, showing that PAF— a mediator linked to IgG-mediated anaphylaxis—is essential in human anaphylaxis, reinforce the role of this potential mechanism (6). Recently, the implication of IgG1 and neutrophils in human anaphylaxis has been suggested (7, 8). Other mechanisms linked to severe allergic reactions are the Ig-dependent and independent activation of the complement system, with anaphylatoxin (C3a, C5a) production and binding to their receptors on mast cells, basophils, and other myeloid cells (9, 10) and the direct activation of mast cells by drugs interacting with receptors on these cells.

A myriad of clinical symptoms involving the skin (erythema, itching, urticaria, angioedema), the respiratory tract (bronchospasm, dyspnea, or laryngeal edema), the digestive (diarrhea, vomiting, nausea, pain), or the cardiovascular systems (dizziness and hypotension) can be present during an anaphylactic episode (1). Unusual symptoms include diffuse alveolar hemorrhage, thrombocytopenic purpura, vasculitis, or metrorrhagia, especially in honey bee venom-induced anaphylaxis (11–13). Clinical symptoms of anaphylaxis have classically been attributed to the effects of mast cell/basophil mediators. For instance, histamine binding to its receptors is responsible of pruritus, rhinorrhea, tachycardia, bronchospasm, or hypotension (14) while vasodilation and edema formation, as well as abdominal pain, may be related to tryptase or chymase effects on its target cells (15, 16). On the other hand, mast cell mediators can secondarily promote the activation of different pathways, leading to the release of molecules affecting the clinical expression of anaphylaxis. In this line, it is currently known that the kallikrein–kinin system, the clotting cascade, and the fibrinolytic system may be activated during anaphylaxis (17, 18). The purpose of this review is to give new insights on the implication of the contact and the coagulation systems in anaphylaxis, focusing on the central role of mast cell/basophil mediators on their activation.

THE CONTACT SYSTEM IN ANAPHYLAXIS

The contact system integrates the plasma bradykinin (BK) formation pathway (the kallikrein–kinin system) and the intrinsic coagulation cascade. In addition, it is involved in thrombus formation, fibrinolysis, and complement activation (19). The contact system is configured by three serine proteases, coagulation factor XII (FXII) or Hageman factor, coagulation factor XI (FXI), and plasma prekallikrein (PK), and by a non-enzymatic cofactor, the high molecular-weight kininogen (HK). FXII, the initiating protein in the kallikrein–kinin system auto-activates to form activated FXII after binding to certain negatively charged surfaces or to macromolecular complexes formed during an inflammatory response or to proteins along cell surfaces (20). There are two plasma substrates for activated FXII, PK, and FXI, and each of these circulates as a complex with HK. Kallikrein is liberated from PK, its plasma precursor, by activated FXII (21). BK, the final product of the system, is released after cleavage of HK by kallikrein (**Figure 1**). Although the mechanisms of *in vitro* contact system activation have been elucidated, the *in vivo* effects of this complex system in some instances are still unclear.

The effects of BK include peripheral vasodilatation, enhancement of vascular permeability and subsequent vascular leakage, and the contraction of gastrointestinal and uterine smooth muscle, resulting in angioedema, hypotension, and abdominal pain (21), all clinical manifestations of anaphylaxis. The role of kinins in allergy was first established more than 30 years ago from the observation than BK was produced in parallel with histamine release in patients with allergic rhinitis during allergen nasal challenge (22) and in the lungs of asthmatic patients (23), suggesting a link between mast-cell-related conditions and the contact system.

A strong consumption of contact system factors has been observed in patients with anaphylaxis (17, 24, 25) and in IgE-mediated mouse models of anaphylaxis (26). Deficiency or pharmacologic inhibition of FXII, plasma kallikrein, HK, or the bradykinin B2 receptor (BK2R), largely attenuated allergen/IgE-mediated mast cell hyperresponsiveness in mice (17). Interestingly, in this study, both FXII or BK2R-deficient mice, that are resistant to BK signaling, were protected from systemic hypotension during anaphylactic reactions (17), indicating that the contact system is active and contributes to systemic anaphylaxis. In patients with anaphylactic shock to bee venom immunotherapy, intravascular coagulation and diminution of plasma HK has been reported (25) and the sting-challenge test in patients with sting-induced anaphylaxis has been described to promote the generation of FXIIa-C1INH and kallikrein-C1INH complexes, as well as cleavage of HK (24). Recently, activation of FXII and plasma kallikrein has been shown in patients with food or drug-induced anaphylaxis, as well as a 60% cleavage of HK. Furthermore, this activation of the contact system was associated with mast cell degranulation and increased plasma heparin levels (17). Mast cells are an important source of heparin, which contributes to the morphology and storage capacity of their secretory granules (27). *In vivo*, this proteoglycan is exclusively synthesized in mast cells (28). Therefore, heparin derived from human mast cells seems to represent the physiological macromolecule capable of activating the contact system. In experimental models, when heparin either isolated from peritoneal or from human lungs mast cells is added to plasma, contact system activation and BK generation occurs (26, 29). Heparin provides the negatively charged surface for binding and activation of plasma FXII and initiates the kallikrein–kinin cascade. In 2007, several cases of heparin-induced anaphylactic shocks occurred after the use of heparin contaminated with oversulfated condroitin sulfate (30). This contaminated heparin was shown to activate FXII and trigger BK generation (31). Mast cell heparin is a potent FXII activator as it has been observed in mouse models and in humans (22, 32, 33). In acute anaphylaxis, the activation of the contact system correlates with the severity of the episode and the degree of mast cell activation (17).

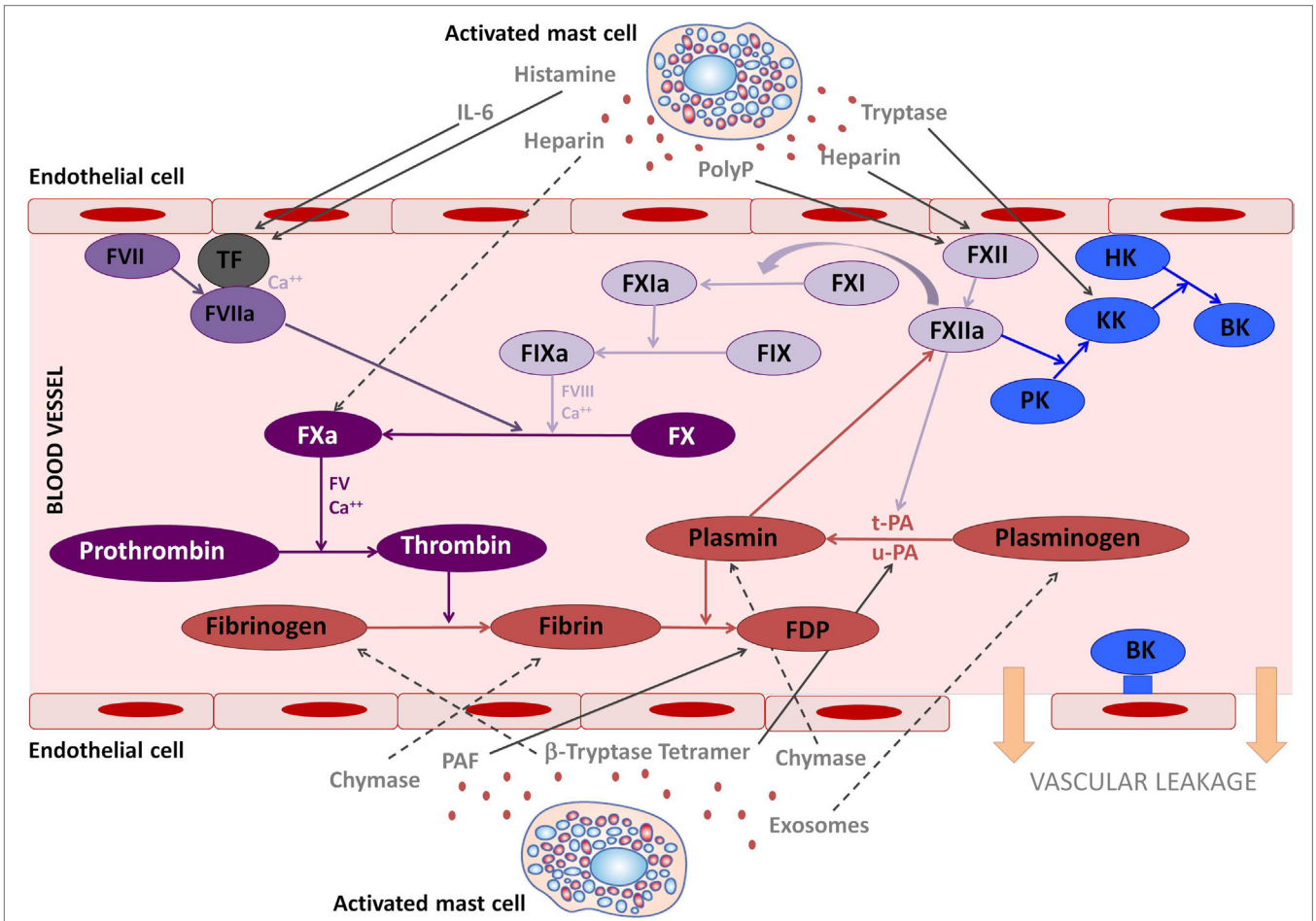

FIGURE 1 | The involvement of mast cell mediators in the coagulation and kallikrein–kinin system. The figure illustrates the effects that mast cells mediators released upon activation during anaphylaxis exert in the kallikrein–kinin, coagulation, and fibrinolytic systems. Solid lines represent activated pathways. Dashed lines are inhibitory pathways. Kinin-forming system factors are represented in blue; the fibrinolytic system is represented in red; the common coagulation pathway in dark purple; the extrinsic coagulation pathway in medium purple; the intrinsic coagulation pathway in light purple. PolyP, polyphosphates; TF, tissue factor; PK, prekallikrein; KK, Kallikrein; BK, bradykinin; HK, high molecular-weight kininogen; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; FDP, fibrin degradation products; PAF, platelet-activating factor.

Tryptase, a trypsin-type serine protease released from mast cells upon activation, may also contribute to kinin production in allergic diseases and in anaphylaxis. Tryptase derived from human lungs was able to release BK mainly through plasma kallikrein activation and to enhance vascular permeability (34). Furthermore, tryptase levels are correlated to plasma HK cleavage during human anaphylaxis (17). Other mast cell mediators potentially capable of activating the contact system are polyphosphates (polyP) and elastase. PolyP are pro-inflammatory agents and potent modulators of the human blood-clotting system, released from activated platelets (35). In mast cells activated through IgE-binding, polyP levels greatly decrease (36). Elastase, a serine protease mainly released by neutrophils but also by basophils and mast cells granules (37), has the capacity to cleave the light chain of HK leaving the kinin sequence untouched and seems to be a positive regulator of the contact system activation (38).

Taken together, these findings indicate that mast cell degranulation during anaphylaxis may trigger FXII activation and the generation of BK through the release of heparin, tryptase and possibly polyP, elastase, or other mediators. However, the role of other mast cell mediators in contact system activation needs to be further explored.

THE COAGULATION SYSTEM IN ANAPHYLAXIS

In addition to contact system activation, mast cell mediators may also be involved in the activation of the coagulation system. Hemostasis is maintained by complicated interactions between the coagulation and fibrinolytic systems as well as platelets and vessel walls (39). During anaphylaxis, the release of mast cell

mediators may break this hemostasis and the subsequent effects may explain the findings described in these patients.

The finality of the coagulation cascade is to form the clot. It involves the extrinsic, intrinsic, and common pathways. The key initiating factor of the extrinsic pathway is tissue factor (TF), present in the circulation and expressed by cells around the vessels when the endothelial layer is compromised. The intrinsic pathway is activated by FXII, which activates both the contact system *via* PK, and the coagulation system *via* FXI, converting prothrombin to thrombin. Then thrombin further cleaves fibrinogen to insoluble fibrin and activates factor XIII, which will crosslink fibrin polymers (40). Fibrinolysis is activated simultaneously as the coagulation cascade and limits the size of the clot. Plasmin dissolves the fibrin clot into fibrin degradation products. Thus, D-dimers are specific indicators of fibrinolysis. This step is mediated by tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA) release from vascular endothelium (40) (see **Figure 1**). The release of tPA is stimulated by tissue occlusion, epinephrine, thrombin, vasopressin, and strenuous exercise (41). Plasminogen is also activated by FXII, although in a weaker manner than tPA and uPA (42). This may suggest a protective role of activated FXII for cardiovascular disease. In addition, plasmin is able to cleave and activate FXII (43), making the interaction between the fibrinolytic and the contact system bidirectional. In fact, angioedema, probably BK-mediated, as a side effect of plasminogen activators administered to patients with thrombotic conditions, has been widely reported (44, 45), indicating contact system activation. Recently the possible role of human tissue mast cells as another important source of tPA has been described (46). Moreover, mast cell tryptase has been shown to activate tPA and pro-urokinase (47, 48).

Few studies have evaluated the interaction of mast cell mediators released during anaphylaxis with the coagulation system. In a case of wasp-sting anaphylaxis, unclottable activated partial thromboplastin time (aPTT) that measures intrinsic coagulation pathway, and a significant anti-Xa activity, with extremely low fibrinogen level with only a slight increase of D-dimer was observed (49). The authors hypothesized that this effects were caused by tryptase tetramers stabilized by heparin released from mast cells during anaphylaxis. On the one hand, heparin acts as an anticoagulant by binding to antithrombin and is responsible of anti-Xa activity and unclottable aPTT. On the other hand, tryptase tetramers act directly on the fibrinolytic pathway by activating pro-urokinase and subsequently degrading fibrinogen. This may explain the low fibrinogen level and the increase in D-dimer reported in the mentioned case (49). In this line, a hyperfibrinolytic state in anaphylactic shock to suxamethonium has been reported (50). Our group also evaluated the coagulation system in anaphylaxis by measuring anti-Xa and aPTT (17). Only 20% of patients had a prolonged aPTT, greater than 100 s, and almost all of them showed a high anti-Xa. These changes occurred concomitantly to HK cleavage. These findings were probably due to mast cell heparin release, which activates FXII leading to the activation of the intrinsic coagulation and the kallikrein–kinin systems, at the same time that it inhibits FXa. Intriguingly, none of these patients developed bleeding (17). In fact, FXII deficiency is an asymptomatic condition and is not associated with bleeding (51). Therefore, it is not surprising that in anaphylaxis, despite activation of FXII usually no hemorrhagic events occur.

The coagulation system has also been assessed in relation to conditions which share some features with anaphylaxis, and in which, mast cells plays a crucial role, such as urticaria. In chronic spontaneous urticaria it was found that coagulation factors (D-dimer, factor VIIa, and fibrinogen) were increased compared to controls and were significantly correlated with disease severity (52).

In a murine model, the tetramer-forming β-tryptases cleaved the α and β-fibrinogen chains and, therefore, the thrombin-initiated clot formation was inhibited (18). This is an additional potential mechanism that could explain why some rare cases of anaphylaxis may develop hemorrhagic disorders. PAF is another mast cell mediator known to interact with the coagulation system. Despite PAF is released by different cell types (neutrophils, endothelial cells, eosinophils, platelets, macrophages, monocytes, and mast cells), in anaphylaxis mast cells are the main source (6). A mice study demonstrated that the release of mast cell PAF could explain disseminated intravascular coagulation symptoms (thrombocytopenia, prolongation of prothrombin time and hypofibrinogenemia, and increase levels of D-dimer), since PAF antagonists could prevent these effects while intravenous PAF was able to reproduce some of the symptoms (53). In addition, another experimental murine study demonstrated that blocking PAF prevents life-threatening peanut-induced anaphylactic reactions (54).

Moreover chymase, a protease exclusively of mast cell origin, has also been shown to affect the coagulation pathway. Mast cell chymase, whose activity depends on heparin (55), is responsible for the degradation and, therefore, inactivation of both thrombin and plasmin, suggesting that mast cell chymase-heparin complexes have a potential function in regulating extravascular coagulation processes, as well as the plasminogen activator/plasmin system (56, 57). Both human chymase and its mouse homolog, mouse mast cell protease-4, had the ability to reduce factor XIIIA, the fibrin-stabilizing factor, levels, and function *via* proteolytic degradation (58). In addition, chymase deficiency led to an increase in the levels and activity of factor XIIIA reducing bleeding times in homeostatic conditions and during sepsis (58).

Histamine has been shown to induce the expression of TF in vascular cells activating the H1, but not the H2, receptor (59, 60), revealing the potential effect of histamine on the extrinsic coagulation pathway. Some newly generated cytokines from mast cells, such as IL-6, also affect the coagulation system. It has been described that IL-6 may amplify activation of coagulation through upregulation of TF (61). Finally, it has also been shown that mast cell-derived exosomes activate endothelial cells to secrete plasminogen activator inhibitor type 1 and induce procoagulant states (62).

Thus, as it occurs with the contact system, several mast cell mediators have a potential role in the regulation of coagulation and fibrinolysis. When a massive release of mast cells mediators occurs, as in anaphylaxis, the activation of these two systems may contribute to the pathophysiology of the multisystemic reaction. In addition, depending on the relative release of each mediator, the clinical effects may differ.

BIOMARKERS TO ASSESS THE CONTACT AND COAGULATION SYSTEM INVOLVEMENT IN ANAPHYLAXIS

Currently, the only biomarkers that can be measured routinely in anaphylaxis are histamine and tryptase. Reliable biomarkers to assess contact system activation and the generation of BK have not been established.

Measurement of BK *in vivo* is practically impossible due to its rapid degradation by kininases once released. As BK is present in the circulation for only few seconds, the measurement of activation products of the contact system can be valuable biomarkers as they may reflect recent BK production. Thus, we speculate that the most suitable biomarkers to assess contact activation are circulating cleaved HK and kallikrein activity but these measurements are very labor intensive since standardized assays are lacking. To assess the coagulation system, the observation of prolonged aPTT reveals activation of the intrinsic coagulation pathway. Increased levels of anti-Xa reflect the possible effect of heparin and the inhibition of the common coagulation pathway. Regarding the fibrinolytic system, increased levels of plasmin complexes and also increased levels of D-dimer, in patients without a thromboembolic event, should raise the suspicion that the contact and fibrinolytic systems are activated.

CONCLUSION

Anaphylaxis is a complex allergic reaction where multiple biological systems are involved. Further mechanistic studies to discern the involvement of molecules from the contact and coagulation systems are warranted to completely understand the pathophysiology and subsequent clinical effects during anaphylaxis. In order to assess all these complex connections between mast cell mediators and the activation of the contact and coagulation systems, a wide array of potential biomarkers are needed and should be monitored at multiple time-points together with their functional effects. Only then, will we be able to have a complete overview of the interactions and subsequent effects of each mediator and pathway, and maybe also offer a closer insight to new potential diagnostic markers or therapeutic targets for anaphylaxis.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design of the review. MG and AS-C drafted the manuscript. ML-H, OL, and VC participated in revising it critically for important intellectual content. All authors approved the final version of the manuscript.

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Endothelial Regulator of Calcineurin 1 Promotes Barrier Integrity and Modulates Histamine-Induced Barrier Dysfunction in Anaphylaxis

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Anaphylaxis, the most serious and life-threatening allergic reaction, produces the release of inflammatory mediators by mast cells and basophils. Regulator of calcineurin 1 (Rcan1) is a negative regulator of mast-cell degranulation. The action of mediators leads to vasodilation and an increase in vascular permeability, causing great loss of intravascular volume in a short time. Nevertheless, the molecular basis remains unexplored on the vascular level. We investigated Rcan1 expression induced by histamine, platelet-activating factor (PAF), and epinephrine in primary human vein (HV)-/artery (HA)-derived endothelial cells (ECs) and human dermal microvascular ECs (HMVEC-D). Vascular permeability was analyzed *in vitro* in human ECs with forced Rcan1 expression using Transwell migration assays and *in vivo* using Rcan1 knockout mice. Histamine, but neither PAF nor epinephrine, induced Rcan1-4 mRNA and protein expression in primary HV-ECs, HA-ECs, and HMVEC-D through histamine receptor 1 (H1R). These effects were prevented by pharmacological inhibition of calcineurin with cyclosporine A. Moreover, intravenous histamine administration increased Rcan1 expression in lung tissues of mice undergoing experimental anaphylaxis. Functional *in vitro* assays showed that overexpression of Rcan1 promotes barrier integrity, suggesting a role played by this molecule in vascular permeability. Consistent with these findings, *in vivo* models of subcutaneous and intravenous histamine-mediated fluid extravasation showed increased response in skin, aorta, and lungs of *Rcan1*-deficient mice compared with wild-type animals. These findings reveal that endothelial Rcan1 is synthesized in response to histamine through a calcineurin-sensitive pathway and may reduce barrier breakdown, thus contributing to the strengthening of the endothelium and resistance to anaphylaxis. These new insights underscore its potential role as a regulator of sensitivity to anaphylaxis in humans.

Keywords: anaphylaxis, endothelial cells, vascular permeability, regulator of calcineurin 1, histamine

INTRODUCTION

Anaphylaxis is a potentially lethal, rapid-onset allergic reaction, and is known to be the most aggressive manifestation of allergic disorders (1). Anaphylactic reactions trigger a broad range of symptoms affecting different organs and bodily systems. The severe alterations described in human anaphylaxis involve the cardiovascular system and include loss of peripheral vascular resistance (vasodilation) and exacerbated vascular extravasation. These physiological processes are associated with low blood pressure, reduced venous return, and decreased cardiac output. Furthermore, bronchoconstriction and pulmonary/coronary-artery vasoconstriction commonly occur in the thoracic cavity, which contributes to widespread circulatory collapse (2–4). The clinical features of anaphylaxis have been well-described and classified, though investigations into its molecular signaling dynamics remain scarce in humans. To date, this hypersensitivity event has been considered a disorder of the immune system. However, immunological mechanisms do not fully explain the versatility of the events that take place during a reaction (5). In order to obtain a better understanding of anaphylaxis, we searched for new strategies, including the study of vascular mechanisms.

Molecules released mainly by active mast cells and basophiles interact with the vascular endothelium and the smooth muscle layers, destabilizing the endothelial barrier and modifying essential vascular contractile functions (tone) in vessels and airways (6). Tryptase, histamine, and platelet-activating factor (PAF) are only two such relevant biochemical mediators found in the serum of anaphylaxis patients (7). In cells, histamine binds to four types of receptors coupled to G proteins (H1-4R), and these receptors are widely distributed across tissues and leads to signaling through numerous molecular pathways. Type-1 and type-2 histamine receptors (H1R and H2R) have been widely investigated due to the fact that they mediate the intracellular signaling associated with second messengers such as Ca^{2+} and cAMP, which regulate vascular permeability, vasodilation, and bronchoconstriction (8, 9). In order to control these homeostatic disorders, the first-line treatment for anaphylaxis is intramuscular injection of epinephrine, which acts *via* α- and β-adrenergic receptors, triggering intracellular mechanisms in cardiac and smooth vascular cells (10). Their potent vasoconstrictor actions combined with fluid therapy are effective when administered promptly (11). Moreover, the second messenger cAMP is mediated by activation of β-adrenergic receptor signaling and contributes to the maintenance of endothelial barrier properties under baseline conditions (12).

There is growing interest in understanding the vascular permeability and vasodilation that occur during anaphylaxis. Different endothelial molecular pathways have been described as key targets for anaphylaxis due to their implication in the disruption of endothelial integrity or vascular tone modulation (13, 14). In humans, changes in vascular permeability during anaphylaxis may lead to a transfer of 50% of the intravascular fluid into the interstitial space within 10 min (3). At the molecular and cellular level, it is well known that histamine induces rapid and transient processes, which disrupt the endothelial barrier, thereby allowing the leakage of fluids, mainly in venules (15). Furthermore, endothelial cells (ECs) participate in physiological processes that regulate not only the capillary component but also peripheral vascular resistance and homeostasis. This fact is a focus of research in vascular permeability modulation (16), and vascular wall components are also essential in regulating leakage and peripheral vascular resistance in anaphylaxis. Mechanistically, a cellular counterbalance between contractile and adhesive forces must exist to maintain the stability between cells and prevent the rupture of the endothelial barrier (17). It has been reported that ECs contribute to the widespread effects observed in anaphylaxis through synthesis and the release of substances, including nitric oxide (NO) and mediators generated from the arachidonic acid cascades (18). However, mast cells are the main cellular source recognized to date, and are major releasers of prostaglandins and leukotrienes eliciting anaphylaxis reactions (19, 20).

Histamine binding to H1 receptors activates PLCβ and elevates intracellular Ca^{2+}, both of which determine the signaling pathways which regulate inflammatory processes. *Via* Ca^{2+}-dependent mechanisms, changes take place in cytoskeleton proteins or junction structures that determine cellular permeability and contractility (21). One of the most sensitive downstream effectors of Ca^{2+} is the ubiquitously expressed serine/threonine protein phosphatase calcineurin (22). Activation of calcineurin contributes to immune response signaling by members of the nuclear factor of activated T-cells (NFAT) family (23). Calcineurin activity can be inhibited by the immunosuppressant cyclosporine A (CsA) which forms a complex with cyclophilin A to bind and competitively inhibit calcineurin phosphatase activity (24). Endogenous regulation of calcineurin is mediated by members of the regulator of calcineurin (Rcan) family, and Rcan1 is the only such molecule regulated by Ca^{2+}/calcineurin (25). The RCAN1 gene contains seven exons that can generate several transcripts resulting from differential promoter use and first exon choice. The two major transcriptional products for Rcan1 are isoforms, including exons 1 + 5–7 (Rcan1-1) and isoform 4 (Rcan1-4) with exons 4 + 5–7, which produce proteins with 252 and 197 amino acids, respectively (26, 27).

Divergent functions have been reported for both Rcan1-1 and Rcan1-4. While different inducers of Ca^{2+} selectively upregulate Rcan1-4, few stimuli have been described as modulators of Rcan1-1 expression. A role for apoptosis is attributed to Rcan1-1 in response to glucocorticoids, and relevant studies have linked Rcan1-1 to Huntington disease (28, 29). Rcan1-4 is upregulated by increases in Ca^{2} or in response to a variety of signals, including cytokines, hormones, hydrogen peroxide, and stress (30). Functionally, it has been widely described as an anti-inflammatory, anti-angiogenic agent and modulator of cardiovascular pathologies (31, 32). Due to its influence in regulating calcineurin activity, Rcan1 is involved in a broad range of cellular systems and biological processes. Extensive investigations have provided insights into EC signaling, describing Rcan1 as a potential therapeutic target in vascular inflammation (33). VEGF and thrombin have been reported to be the major inducers of Rcan1 in ECs, while angiotensin II induces Rcan1 expression in vascular smooth muscle cells (34–36).

Given the crucial role exerted by mediators on the vascular wall in anaphylaxis, we assessed the impact of anaphylaxis on Rcan1 expression in human ECs, as well as its functional involvement in vascular permeability and cell dilation. This study evaluates

Rcan1 expression in human ECs in response to mediators of anaphylaxis and, more specifically, the involvement of histamine receptors involved in Rcan1 expression. Moreover, we studied the permeability effects of Rcan1 causing either endothelial barrier rupture or strengthening in response to mediators of anaphylaxis and also analyzed the plausible endothelial mechanisms exerting these functions.

MATERIALS AND METHODS

In Vitro Human Cell Cultures

Human dermal microvascular ECs (HMVEC-D) were acquired from Lonza. Human vascular endothelial vein and artery cells were isolated from the macroscopically healthy part of intact saphenous veins harvested from patients undergoing bypass surgery/high ligation of varicose veins. The study was approved by the research ethics committees of the Gentofte and FJD hospitals, and written informed consent was obtained from all patients. Briefly, after removal of the connective tissue, the vein was opened longitudinally and the endothelium was isolated by digestion with 0.1% type I collagenase (Gibco) in PBS for 30 min at 37°C. Similarly, artery specimens were incubated with the digestion buffer O.N at 37°C. Reactions were stopped and cells were collected by centrifugation and grown in DMEMF12 media supplemented with 10 U/ml heparin, 30 g/ml ECGF, 100 U/ml penicillin, 100 g/ml streptomycin, and 15% (v/v) heat-inactivated fetal bovine serum. Cells were seeded in a plate previously coated with 0.5% sterile gelatin and maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. After 5–7 days of incubation, human vein or artery ECs (abbreviated herein as HV-EC/HA-ECs) were selected with human CD31 antibody. Next, the secondary antibody associated with magnetic beads (Dynabeads® anti-mouse IgG from CELLection ™ Pan Mouse IgG Kit) was incubated for 30 min at 4°C with constant shaking. ECs were seeded on plates previously treated with 0.5% gelatin. Once in confluence, primary cell cultures were FBS 0.5% starved for 18 h before the experiments were performed. All experiments were performed during passages 3–7. A similar protocol was applied to the artery specimens.

Reagents

Histamine, PAF, epinephrine, and the histamine receptor antagonists diphenhydramine hydrochloride (H1RB) and famotidine (H2RB) were obtained from Sigma.

Protein Extraction and Immunoblot Analysis

Endothelial cells were lysed with buffer containing Tris, NaCl, EDTA, EGTA, Triton X-100, NP40, protease inhibitors, PMSF, and DTT. Cellular lysates were shaken for 15 min and centrifuged at 12,000 rpm at 4°C. After stimulation with reagents or sera, ECs were washed with ice-cold PBS and protein extracts obtained as previously described (36). Rabbit polyclonal anti-Rcan1 primary antibody was used at a ratio of 1/1,000 (Sigma). Analysis of images was carried out using the ImageJ program. Protein extracts from animal tissues were previously disaggregated and processed in similar fashion.

mRNA Extraction, RT, and Real-time PCR Analysis

RNA extraction was performed using Tri Reagent (Molecular Research Center). Two micrograms of total RNA were reverse transcribed following the instructions for the high-capacity cDNA RT (Thermo Fisher) protocol, and samples were stored at −70°C (36). The following TaqMan FAM/MGB probes were purchased from Applied Biosystems: human Rcan1 (Hs01120954_m1), human Rcan1.1 (Hs01120956_m1), human PTGS2 (Hs00153133_m1), human MYLK (Hs00364926_m1), human NOS3 (Hs01574659_m1), human ROCK1 (Hs01127699_m1), and human CAMK2B (Hs00365799_m1). Human Rcan1.4 was purchased customized according to primer forward: GCAAACAGTGATATCTTCAG CGAAA, primer reverse: GTGATGTCCTTGTCATACGTCCTAA, and the labeled CAGGGCCAAATTT (5 NFQ). Reactions were incubated in the presence of AmpliTaq Gold DNA polymerase (Applied Biosystems) for 2 min at 50°C followed by 10 min at 95°C. Reactions were then run over 40 cycles of 95°C for 15 s and 60°C for 1 min. Human beta actin VIC/MGB or 18s VIC/MGB rRNA transcripts were used as an internal control, which were amplified in the same tube to normalize for variation in input RNA. The amount of target mRNA in the samples was estimated by the 2CT relative quantification method. Ratios were calculated between the amounts of mRNA from stimulated and/or transfected vs nonstimulated control ECs.

In Vitro Vascular Permeability Assays

Endothelial barrier integrity was evaluated by using Transwell 24-well cell culture inserts (TW) including a membrane pore size of 0.4 μm (Corning). ECs were seeded at a density of 10^5 cells/well in TWs previously coated with 0.5% gelatin diluted in sterile water and grown in DMEMF12 media supplemented as described above. After several days, EC monolayers were observed and starved for 18 h before the experiments were performed. Stimulus together with a final concentration of 1 mg/ml of FITC-Dextran (Sigma) was added to the upper chamber. Vascular endothelial-permeability measurements were determined by measuring fluorescence of the recipient at 5–30 min and 2 h. All samples were evaluated at least in duplicate.

Lentiviral Production and Infection

Lentiviruses expressing Rcan1-1-IRES-GFP, Rcan1-4-IRES-GFP, and IRES-GFP were obtained by transient calcium phosphate transfection of HEK-293. The supernatant containing the lentiviral particles was collected 48 h after removal of the calcium phosphate precipitate, filtered through a 45-μM PVDF membrane (Steriflip; Millipore), and ultracentrifuged for 2 h at 26,000 rpm at 4°C (Ultraclear Tubes, SW28 rotor, and Optima l–100 XP Ultracentrifuge; Beckman Coulter). Viruses were resuspended, titrated, and infection efficiency (GFP-expressing cells) was monitored by flow cytometry (36). Lentiviral infection was performed in subconfluent primary cultures of ECs with a 1:1 mix including Rcan1-IRES-GFP (Rcan1-1-IRES-GFP + Rcan1-4-IRES-GFP) or IRES-GFP. Cells were exposed to the lentiviruses in the presence of 10% FBS during 5 h (37). After 4 days, cells were starved of FBS (0.5%) during for 18 h and the experiments of interest were performed.

Animal Experimental Designs

Animal procedures were carried out in accordance with the European Union guidelines for the care and experimental use of animals. Protocols with reference PROEX: 391/15 received prior approval from the IIS-FJD Ethics Committee and the competent authorities in the region of Madrid.

Two-month-old C57BL/6J mice were intravenously (i.v.) injected with Evans blue (0.04 µg/g in NaCl) followed by subcutaneous (s.c.) injection with histamine and PAF at 5–50 ng/ml for 10 min. To evaluate systemic vascular permeability, histamine at 10 mg/kg or PAF 2 µg/g were i.v. injected together with Evans blue dye. Once i.v. administrated, mice were sacrificed after 15 min, and most PAF animals died spontaneously. The skin pieces, aortas, lungs, and hearts of these animals were incubated in 500 µl of formamide at 55°C for 48 h, and the Evans blue content was determined by absorption at 595 nm. To test passive systemic anaphylaxis (PSA) using an experimental *in vivo* model, mice were i.v. injected with 20 µg of anti-DNP IgE. After 24 h, the mice were challenged with an i.v. injection of 1 mg DNP-HSA (human serum albumin) for the development of anaphylaxis. To test for active systemic anaphylaxis (ASA), we used the classical model, sensitizing mice with i.p injection of 1 mg BSA and 300 ng pertussis toxins as adjuvant in normal saline. After 14 days, the mice were challenged with i.v. injection of 2 mg BSA (38). Following administration of s.c. and i.v. histamine, PSA and ASA mice were sacrificed 30 min after the challenge by cervical dislocation, and blood sampling and biopsies (organ collection) were collected for molecular analysis. All studies were repeated at least once to assure reproducibility. *Rcan1*-deficient (−/−) mice were generated as previously described (39). All mice were genotyped by PCR of tail samples using the following primers: Rcan1, 5′-GGTGGTCCACGTGTGTGAGA-3′, 5′-ACGTGAACAAAG GCTGGTCCT-3′, and 5′-ATTCGCAGCGCATCGCCTTCTAT CGCC-3′. Control littermates were used in all experiments.

Statistical Analysis

All values are expressed as the mean ± SEM. Differences were evaluated with GraphPad Prism 7.3 program using one-way ANOVA analysis followed by Bonferroni's *post hoc* test (experiments ≥3 groups) or Student's *t*-test (experiments with two groups). Statistical significance was set at $p < 0.05$.

RESULTS

Histamine Increases Rcan1-4 Expression in HV-EC

Previous studies have demonstrated the stimulation of Rcan1-4 protein induced by agents that cause the mobilization of Ca^{2+} (33, 34, 36). We observed that histamine stimulation induces a specific increase in Rcan1-4 but not Rcan1-1 protein after a short period (30 min of stimulation); the increase was considerably greater after 60 min of exposure in HV-ECs (**Figures 1A,B**). Parallel to these results, *Rcan1-4* mRNA expression also increased during the same time frame in response to histamine (**Figure 1C**). Moreover, as histamine is not the sole mediator recognized for anaphylaxis, we addressed the effect of PAF on HV-ECs. Over the last decade, evidence based primarily on animal models has indicated the existence of an anaphylaxis reaction mediated by IgG- FcγRIII (5, 40), and PAF has been recognized as essential in those non-IgE-mediated reactions (41). In our studies, PAF did not modify Rcan1-4 protein expression in HV-ECs (**Figures 1A,B**). Additionally, since epinephrine/adrenaline is the first-line treatment in anaphylaxis (1, 42), we set out to determine whether epinephrine could modulate Rcan1-4 expression in ECs. Incubation of epinephrine from 15 min to 1 h did not modify Rcan1-4 protein expression; however, histamine and epinephrine coincubation increased the expression of the Rcan1-4 protein in HV-ECs (**Figures 1D,E**). As with the protein findings, *Rcan1* mRNA expression increased in response to histamine and epinephrine (**Figure 1F**). In all cases, the Rcan1-1 protein and mRNA isoform was unchanged in its expression.

Histamine Increases Rcan1-4 Expression in HA-EC, HMVEC-D, and Lungs of Experimental Anaphylaxis

The cellular heterogeneity of the whole endothelial compartment has been recognized for some time. Both intracellular mechanisms and functional abilities may be different between the micro-, artery, or vein ECs (43, 44). For this reason, we next checked Rcan1-4 modulation by histamine and epinephrine in other endothelial vascular microenvironments. By using primary artery-derived ECs (HA-EC), our results showed that the contact with histamine induced a marked increase in Rcan1-4 protein expression after 30 min and 1 h (**Figures 2A,B**), which was correlated with elevated levels of *Rcan1-4* mRNA expression (data not shown). As we previously observed in HV-ECs, epinephrine had no effect on Rcan1-4 expression when incubated alone; however, coincubation with histamine induced a high Rcan1-4 increase in HA-ECs. Additionally, experiments addressing Rcan1 expression in human dermal microvascular ECs (HMVEC-D) showed similarly increased levels of Rcan1-4 protein and mRNA expression in response to histamine. Contrary to this, no effect was observed upon epinephrine stimulation within the studied time frame in HMVEC-D (**Figures 2C,D**). These results suggest that histamine modulates Rcan1-4 expression in ECs of large vessels regardless of whether they are veins, arteries, or the microvasculature.

The molecular and physiological differences found between studies in animals and studies in humans have sparked debate in anaphylaxis as well as other fields of research (5). Using three experimental designs simulating different degrees of allergic sensitivity, we looked for Rcan1 expression in target organs in mice undergoing anaphylaxis. Experimental passive anaphylaxis (PSA) is based on systemic IgE anti-DNP sensitization over 24 h, while ASA was induced by BSA followed by challenge 14 days later. Analysis of Rcan1 expression in lung extracts of mice treated with histamine for 30 min or undergoing PSA or ASA showed increased Rcan1-4 levels compared to lungs of control mice (**Figures 2E,F**).

Rcan1-4 Protein Expression Is Induced by Histamine *via* Its H1 Receptor and a Calcineurin-Dependent Mechanism

Studies were performed to evaluate the relevant receptors (H1R/H2R) involved in histamine-induced Rcan1-4 expression.

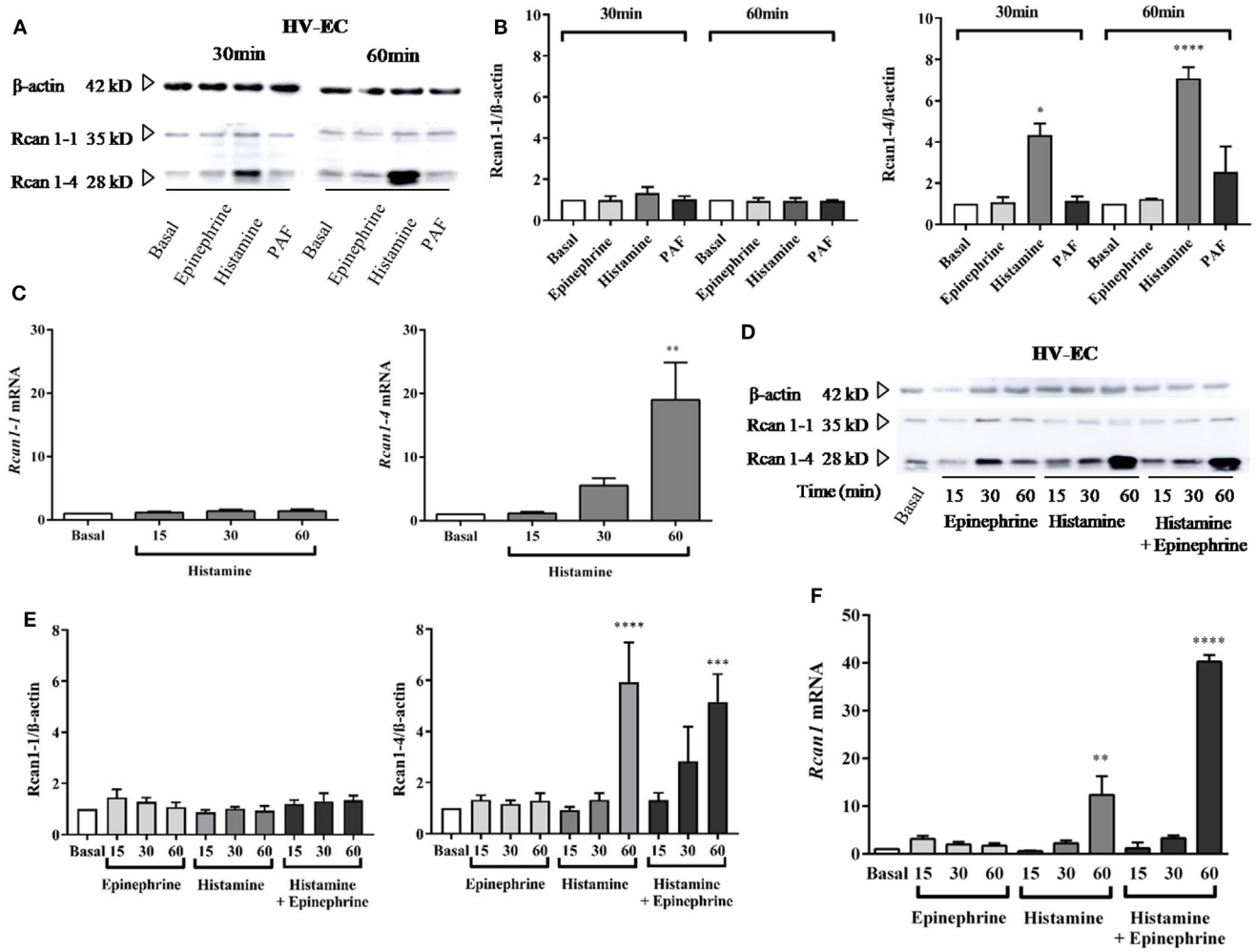

FIGURE 1 | Histamine increases Rcan1-4 protein and mRNA expression in human vein (HV)-endothelial cells (ECs). HV-ECs were treated with epinephrine (1 μM), histamine (1 μM), or platelet-activating factor (PAF) (0.1 μM) at indicated times. **(A)** Representative immunoblots show Rcan1-1 (35 kDa) and Rcan1-4 (28 kDa) expression in stimulated extracts. **(B)** Quantifications were normalized to β-actin and expressed as times of relative change to nonstimulated (basal) cells. Data represent means ± SEM of three and four experiments performed at 30 and 60 min, respectively. One-way ANOVA followed Bonferroni's multiple comparisons test was performed for each time studied [*$P = 0.0375$ vs basal (30 min), ****$P < 0.0001$ (60 min)]. **(C)** qPCR analysis of *Rcan1-1* and *Rcan1-4* mRNA with indicated stimulus and times normalized to the endogenous 18s gene. Data represent means ± SEM of four experiments performed at 15, 30, and 60 min. One-way ANOVA followed Bonferroni's multiple comparisons test was performed (**$P = 0.0052$ vs basal). **(D–E)** HV-ECs were treated with epinephrine, histamine, and histamine in the presence of epinephrine at indicated times. **(D)** Representative immunoblots. **(E)** Quantifications were normalized to β-actin and expressed as times of relative change to nonstimulated (basal) cells. Data represent means ± SEM of five experiments performed at 15, 30, and 60 min. One-way ANOVA followed Bonferroni's multiple comparisons test was performed (****$P < 0.0001$, ***$P = 0.0009$ vs basal). **(F)** qPCR analysis of total *Rcan1* mRNA with indicated stimulus and times normalized to endogenous gene expression. Data represent means ± SEM of six experiments performed at 15, 30, and 60 min. One-way ANOVA followed Bonferroni's multiple comparisons test was performed (****$P < 0.0001$, **$P = 0.0019$).

Prior to stimulation with histamine, HV-ECs were incubated with increasing concentrations of diphenhydramine hydrochloride (type 1 receptor antagonist), determining the optimal concentration of use at 10^{-5} M. Cellular pre-incubation with diphenhydramine hydrochloride completely blocked Rcan1-4 expression induced by histamine in HV-ECs (**Figures 3A–C**). In contrast, no inhibitory effect mediated by famotidine (type 2 receptor antagonist) was observed; indeed, an additional increase in Rcan1-4 expression induced by histamine stimulation was noted when the receptor type 2 was blocked (**Figures 3A–D**). Additionally, coincubation with antagonists for both receptors (diphenhydramine hydrochloride plus famotidine) abolished Rcan1-4 expression induced by histamine in HV-EC and HA-ECs (**Figures 3C,D**).

Next, we investigated the potential role of calcineurin in mediating Rcan1-4 expression induced by histamine. Cellular pre-incubation with CsA completely blocked Rcan1-4 expression induced by histamine in HV-ECs and HMVEC-D (**Figures 3E,F**). Similar to the protein findings, *Rcan1-4* mRNA expression was significantly diminished in the presence of CsA (**Figure 3G**).

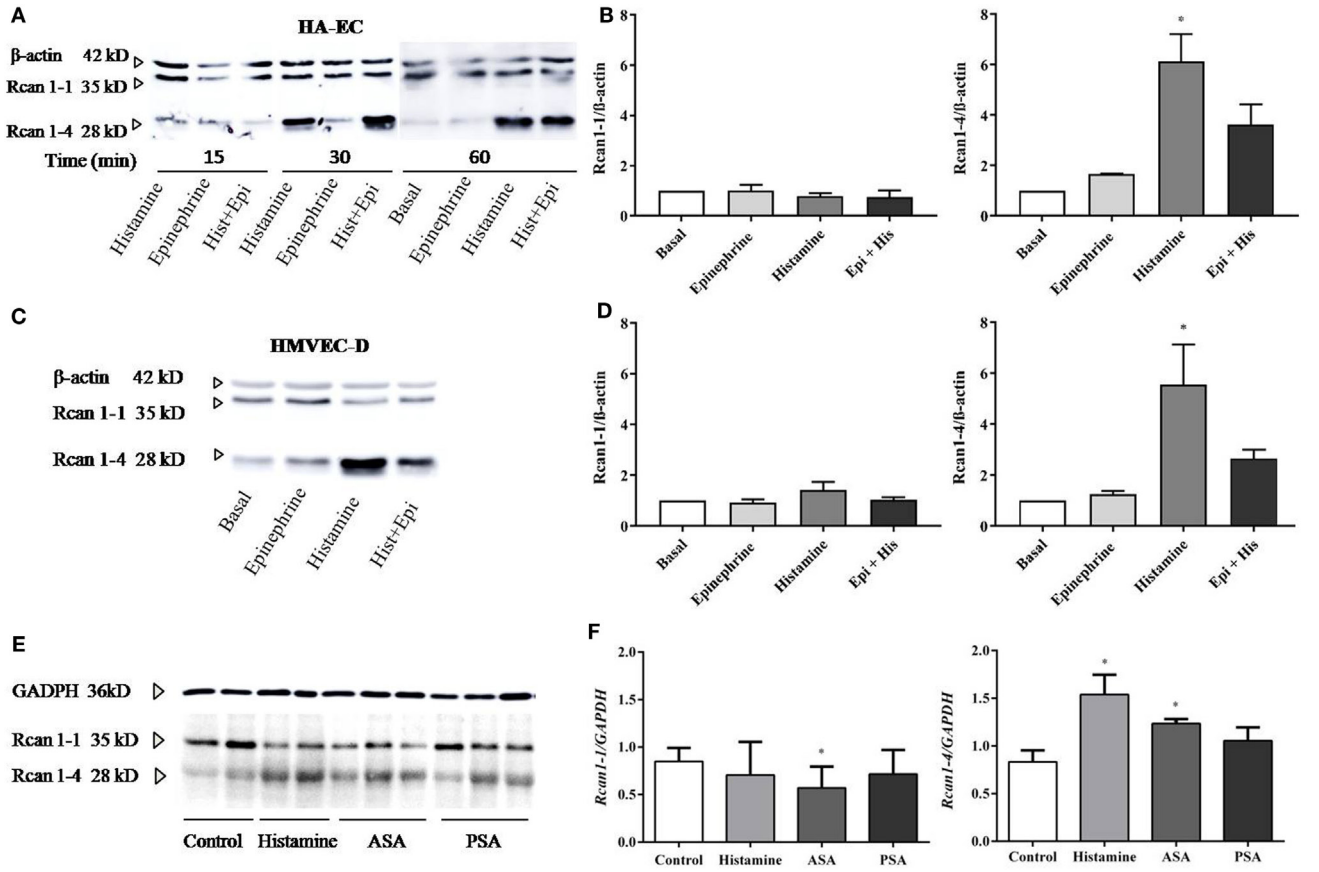

FIGURE 2 | Rcan1-4 expression is modulated in response to histamine in other cellular microenvironments—HA-ECs, HMVEC-D, and lungs from experimental anaphylaxis. HA-ECs and HMVEC-D were treated with epinephrine (1 μM) and/or histamine (1 μM). **(A)** Representative immunoblots show Rcan1-1 (35 kDa) and Rcan1-4 (28 kDa) expression in stimulated HA-ECs for 15, 30, and 60 min. **(B)** Quantifications were normalized to β-actin and expressed as times of relative change to nonstimulated (basal) cells. **(C)** Representative immunoblots show Rcan1-1 (35 kDa) and Rcan1-4 (28 kDa) expression in HMVEC-D for 60 min. **(D)** Quantifications were normalized to β-actin and expressed as times of relative change to nonstimulated (basal) cells. Data represent means ± SEM of five experiments performed per cellular type at 60 min. One-way ANOVA followed Bonferroni's multiple comparisons test was performed for each cellular type studied (HA-ECs; $^*P = 0.0115$, vs basal, HMVEC-D; $^*P = 0.0135$, vs basal). **(E)** Immunoblots include representative lung extracts from wild-type control mice C57BL6, injected i.v. with Hist, active systemic anaphylaxis (ASA), or passive systemic anaphylaxis (PSA). **(F)** Quantification was normalized to GAPDH and expressed as times of relative change to control mice. Data represent means ± SEM of five animals per group. Unpaired *t*-tests were performed vs control (Rcan1-1 $^*P = 0.0462$; Rcan1-4 $^*P < 0.02$).

In addition, an increase in calcineurin phosphatase activity is observed in response to histamine in HMVEC-D (data not shown).

Human Rcan1 Induces Marked Prevention in Vascular Permeability, Strengthening the Endothelial Contact

In vitro ECs adhere to coated surfaces, making up the endothelial monolayer. To evaluate vascular permeability *in vitro*, we employed available assay systems (Transwell, TW) based on measurements of extravasations of fluids through an endothelial monolayer. These cells supported on permeable membranes are inserted in individual containers that allow the transport of molecules through them (**Figure 4A**). First, it was verified that the system allows a measurable increase of FITC-Dextran over time. EC monolayer blocks the passage of the dye in resting conditions (and only endothelial permeability is increased throughout the course of the experiment). As expected, short incubations (minutes) with the vasoactive mediators histamine and PAF induced a rapid increase in vascular permeability on HMVEC-D, which was not observed by incubation with epinephrine (12). The response duration and sensitivity induced by histamine may vary depending on ECs (45). After 15–30 min, we observed a clear tendency to block the passage of molecules across the endothelial barrier both in the presence of histamine and epinephrine, indicating a transient effect of histamine on vascular permeability followed by cell dilation. At this time, only PAF continued destabilizing the endothelial barrier (**Figure 4B**). Following this, we checked the histamine-induced barrier effects in ECs of big vessels. Our studies did not show modification in barrier properties after short periods of stimulation with histamine in HV-ECs or HA-ECs. However, a clear cell-dilating effect induced by histamine is

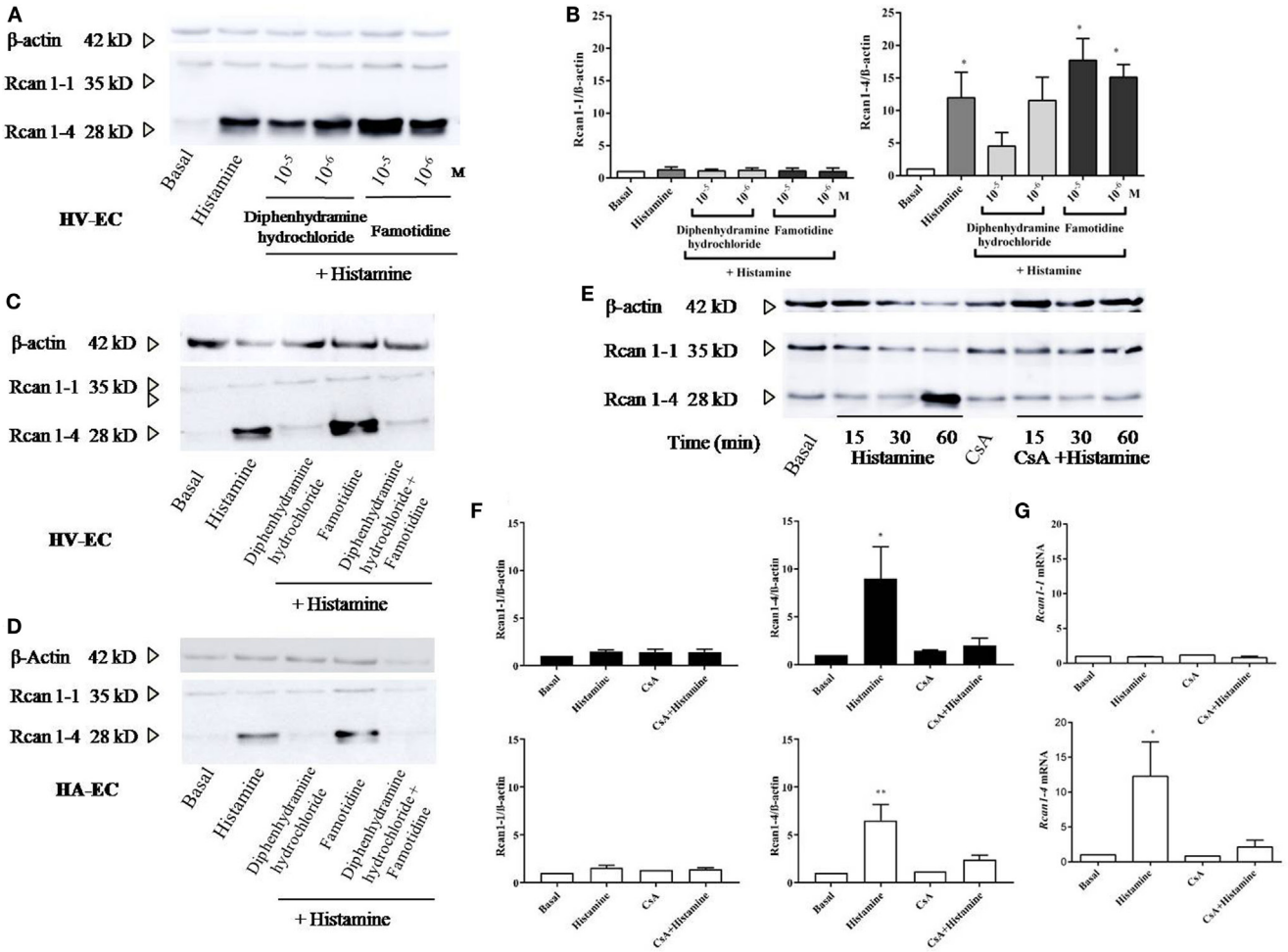

FIGURE 3 | Histamine induces Rcan1-4 protein expression *via* its H1 receptor and CsA sensitive manner. Cells were preincubated with diphenhydramine hydrochloride (H1 receptor antagonist) and famotidine (H2 receptor antagonist) for 30 min at indicated concentrations previously to histamine 1 μM stimulation. **(A)** Representative immunoblots show Rcan1-1 (35 kDa) and Rcan1-4 (28 kDa) expression in stimulated human vein (HV)-endothelial cells (ECs). **(B)** Quantifications were normalized to β-actin and expressed as times of relative change to nonstimulated (basal) cells. Data represent means ± SEM of five experiments performed. One-way ANOVA followed Bonferroni's multiple comparisons test was performed (*$P < 0.05$). **(C,D)** Representative immunoblots show Rcan1-1 (35 kDa) and Rcan1-4 (28 kDa) expression in stimulated HV-ECs and HA-ECs preincubated with diphenhydramine hydrochloride, famotidine and a combination of both at a concentration of 10^{-5} M. **(E,F)** Rcan1 immunoblot in extracts from HV-ECs stimulated with histamine 1 μM at indicated times after pretreatment as indicated (30 min) with 200 ng/ml CsA (cyclosporine A). Representative immunoblots show Rcan1-1 (35 kDa) and Rcan1-4 (28 kDa) expression in stimulated HV-ECs. **(F)** Quantifications were normalized to β-actin and expressed as times of relative change to non-stimulated (basal) cells. Data represent means ± SEM of five and six experiments, respectively, performed on HV-ECs (black bars) and HMVEC-D (white bars) at 60 min. One-way ANOVA followed Bonferroni's multiple comparisons tests were performed (HV-ECs *$P = 0.0232$; HMVEC-D **$P = 0.0022$). **(G)** qPCR analysis of *Rcan1-1* and *Rcan1-4* mRNA with indicated stimulus normalized to the endogenous 18s gene. Data represent means ± SEM of four experiments performed at 60 min. One-way ANOVA followed Bonferroni's multiple comparisons test was performed (*$P = 0.0197$ vs basal).

observed in HV-ECs, and a tendency toward this same dynamic was seen in HA-ECs (**Figure 4C**). In order to determine whether Rcan1 has a functional contribution in these processes, we used lentiviral constructs aimed at exogenously modifying Rcan1 expression on ECs. HV-ECs were transduced with Rcan1-IRES-GFP or the lentiviral control IRES-GFP. The efficiency of infection in human cell cultures was analyzed by GFP expression using flow cytometry (data not shown), fluorescence microscopy, and Western blotting (**Figure 4D**). Lentiviral infection does not modify the effects of histamine on cells, as the Rcan1 pattern expression showed the increase of Rcan1-4 protein resembling that seen in non-transduced cells for both IRES-GFP and Rcan1-IRES-GFP HV-ECs (**Figures 1**–**3**).

To evaluate the role of Rcan1 in vascular permeability, IRES-GFP and Rcan1-IRES-GFP HV-ECs were seeded in TW and stimulated with or without histamine for 30 min. Data show that Rcan1 overexpression prevents basal FITC extravasation in HV-ECs, indicating a marked role of Rcan1 in blocking cell permeability. In addition, HV-ECs infected with control vector and exposed to histamine produced an effect resembling the one observed previously in non-transduced cells. However, no additional effects of histamine were observed when the cells

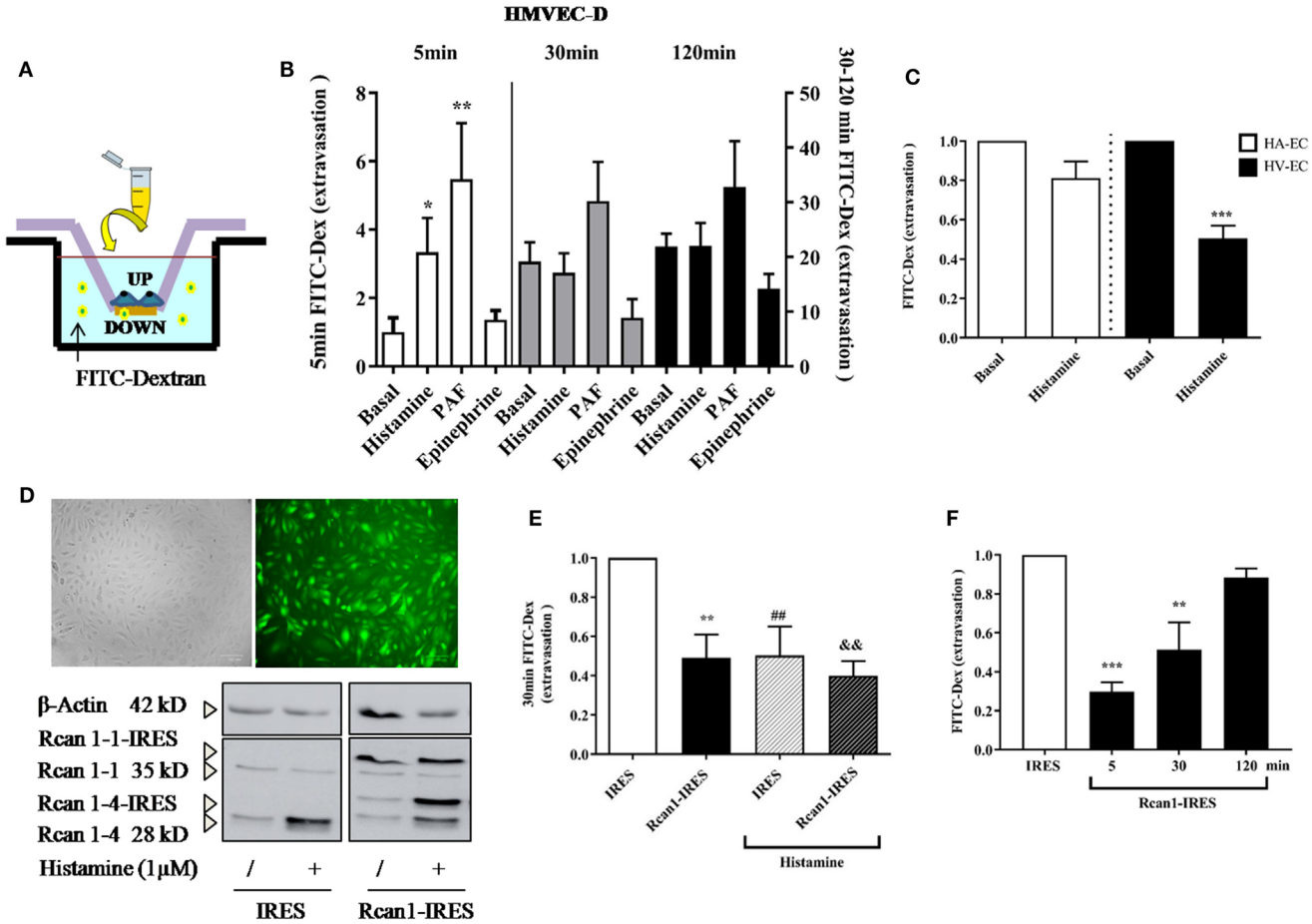

FIGURE 4 | Regulator of calcineurin 1 (Rcan1) maintains the endothelial barrier integrity in human vein (HV)-endothelial cells (ECs). **(A)** Integrity of endothelial monolayers was evaluated using TW assays. **(B)** Quantification of FITC-Dextran molecules extravasated to the TW container and expressed as times of relative change to untreated (basal) cells for 5 (left bars), 30, and 120 min (right bars). Data represent means ± SEM of duplicates determined by TW in five independent experiments performed on HMVEC-D. One-way ANOVA followed Bonferroni's multiple comparisons tests were performed for each time studied (*$P < 0.05$, **$P < 0.001$). **(C)** Quantifications expressed as times of relative change to untreated HV-ECs or HA-ECs, respectively, for 30 min. Data represent means ± SEM of duplicates determined by TW in five independent experiments performed in each genotype. Unpaired *t*-tests were performed vs basal cells (***$P = 0.0003$ vs basal HV-ECs). **(D)** The correct infection with lentiviral constructions expressing IRES-GFP or Rcan1-GFP were detected by fluorescence microscopy and analyzed by Western blot. The panel shows Rcan1-IRES-GFP extracts modified by infection expressing both exogenously overexpressed isoforms (Rcan1-1 and Rcan1-4). **(E)** Quantification of FITC-dextran molecules extravasated to the TW container and expressed as times of relative change to IRES-GFP cells not treated with histamine 1 μM for 30 min. Data represent means ± SEM of duplicates determined by TW in six (IRES-GFP) and seven (Rcan1-IRES-GFP) independent experiments performed on HV-ECs. One-way ANOVA followed Bonferroni's multiple comparisons tests was performed (**$P < 0.0057$, ##$P < 0.0094$, &&$P < 0.0012$ vs IRES-GFP control). **(F)** Quantification of FITC-dextran molecules extravasated to the TW container and expressed as times of relative change to IRES-GFP cells for 5, 30, and 120 min. Data represent means ± SEM of duplicates determined by TW in three independent experiments performed on HV-ECs. One-way ANOVA followed Bonferroni's multiple comparisons tests was performed (***$P = 0.0007$, **$P = 0.0069$ vs IRES-GFP).

overexpressed Rcan1 endogenously (**Figure 4E**). To determine whether Rcan1 can also affect the earliest phases of extravasation and its stability over time, time frames of 5 and 120 min were used for the measurements (**Figure 4F**). Our results indicate that Rcan1 stabilizes the endothelial barrier during short incubation times, though this effect is weakened when incubation is longer.

We, therefore, speculated that increased expression of Rcan1 in ECs could in turn modulate other primordial factors involved in anaphylaxis. To test this hypothesis, we analyzed the expression of some genes involved in vascular homeostasis and barrier stability: cyclooxygenase-2 (*COX-2*), calmodulin-dependent protein kinase II-dependent (*CaMKII*), nitric oxide synthase 3 (*NOS3*), MLC-kinase (*MLCK*), and Rho-kinase 1 (*ROCK1*) in resting and histamine-stimulated IRES-GFP and Rcan1-IRES-GFP HV-ECs. Resting Rcan1-IRES-GFP HV-ECs showed a marked inhibition of *COX-2* mRNA, *CaMKII* mRNA, and *NOS3* expression. However, similar levels of *MLCK* and *ROCK1* were observed compared to IRES-GFP HV-ECs (**Figure 5**). This brief screening suggests that consequent generation of newly formed proinflammatory phospholipid-endothelial derived COX-2 could contribute to vascular contractile processes concomitant to

anaphylactic reactions. Additionally, a direct effect on the production of the NO is exerted when Rcan1 is overexpressed.

Mouse *Rcan1*$^{-/-}$ Tissues Present Higher Leakage in Response to s.c. and i.v. Histamine Administration

The *in vitro* results described above led us to investigate the role of Rcan1 in experimental vascular permeability *in vivo*. We examined the effect exerted by histamine and PAF on vascular permeability in *Rcan1*-deficient vs *WT* mice. NaCl s.c. injection does not modify basal extravasation in *WT* and *Rcan1*$^{-/-}$ mice (data not shown). However, an increase of leakage was seen in response to s.c. injection of histamine in both types of mice, and this increase was dose-dependent and significantly higher in *Rcan1*$^{-/-}$ mice (**Figures 6A,B**). Moreover, histamine administrated intravenously increased extravasation in aortas and lungs, though not in the hearts of *Rcan1*$^{-/-}$ mice, while no significant difference was observed in response to PAF between genotypes (**Figures 6B,C**). As in the *in vitro* human cells, endothelial Rcan1 may prevent or recover the loss of endothelial barrier function induced by histamine.

DISCUSSION

The functional heterogeneity between different EC niches and their importance in the release of certain physiological factors contributing to pathological situations is a topic of high relevance in important fields of research (16, 46). Therefore, in anaphylaxis affecting a large number of organs, it is plausible that the vascular system is a critical participant in the evolution of symptoms and one that may condition the progress of reactions. This study provides the first evidence that histamine, a relevant mediator involved in anaphylactic reactions, modulates Rcan1-4 expression in different human vascular niches (HV-ECs, HA-ECs, and HMVEC-D). Different authors have previously reported that factors such as VEGF and thrombin induce Rcan1-4 expression in cultured ECs (34, 47). Given the major role of histamine when released by immune cells as a mediator of anaphylactic reactions, our results extend previous findings, showing that histamine upregulates Rcan1-4 expression in several types of ECs, whereas Rcan1-1 is not modulated. In agreement with our findings, a number of studies performed on this cellular type did not observe significant Rcan1-1 variation in response to stimuli (34) in spite of its involvement in Huntington disease and mitochondrial autophagy (29, 48). Additionally, epinephrine has no effect on Rcan1 expression, and although its potent actions as a dilator or constrictor through its adrenergic receptors have been widely observed, its role in the endothelium has been poorly investigated. However, the contributions of epinephrine to the maintenance of the endothelial barrier, through its β-adrenergic receptors, have been evaluated in ECs (12). Similarly, a substantial number of studies have demonstrated the role of PAF in anaphylaxis. The data shown here do not support a role for PAF in modulating Rcan1-4 expression in ECs, at least in our experimental conditions. However, given the relevance of PAF, more thorough studies focused on human vascular cells would be of interest to clarify unexplored aspects that thus far have been mostly related to reactions mediated by IgE or IgG antibodies in anaphylaxis (5, 41).

The human H1R acts mainly by coupling to Gq/11 proteins; in fact, experimental anaphylaxis is prevented in endothelial Gq/11-deficient mice (38). Through its specific signal, histamine is one of the most potent vasoactive substances, inducing relaxation or contraction (tissue- and species-dependent) and participating in anaphylactic responses, mainly through its H1R and H2R receptors (8, 9). The data shown here demonstrate that histamine increases Rcan1 expression through the H1R receptor in ECs due to a calcineurin-dependent mechanism, as the calcineurin inhibitor CsA inhibits its expression.

Anaphylaxis is widely recognized by the presence of increased vascular permeability, mediating a shift of intravascular fluid into the extravascular space within minutes, which results in hypotension and hemoconcentration (3). Some studies in human subjects have revealed transient impairment of the microvasculature during severe acute anaphylaxis (49). Thus, the leakage occurring in anaphylaxis requires cellular retraction of ECs as a result of increased cell contractile pathways in response to external stimuli or agents (15). Subsequently, a biological counterbalance between contractile and adhesive forces must exist to maintain the stability between cells or allow cells to recover from the rupture of the endothelial sheet. Thus, most of the mediators described in anaphylaxis elicit vascular EC permeability signaling through specific receptors and molecular pathways. However, some stabilizing molecules preserve the rupture of the endothelial barrier (as cAMP or sphingosine 1 phosphate). The functional evaluation of Rcan1 by using *in vitro* permeability assays and *in vivo* extravasation mice models show Rcan1 as a stabilizer of the endothelial barrier or as a cellular dilator agent. Our studies demonstrate that histamine-induced Rcan1 contributes to the stability of the endothelial barrier and also indicate that this mechanism is a late-secondary response to prevent the loss of fluids and/or the regulation of vascular tone.

Our studies demonstrate that exogenous overexpression of Rcan1 downregulates the expression of calcineurin-related genes such as *COX2, NOS3*, and *CaMKII*. H1R stimulation increases NOS3 synthesis through a mechanism that involves CaMKII in human vascular ECs (24, 50). NOS3 upregulation and consequent NO production is protective under normal conditions, though may be deleterious in a model of experimental anaphylaxis (51, 52). Our studies correlate the stability of the endothelial barrier with a decreased *NOS3* expression in Rcan1 overexpressed cells, as well as a tendency to prevent the increase of *NOS3* induced by histamine, suggesting a protective vasodilator role for Rcan1 in ECs. Interestingly, CaMKII has been reported as a suppressor of intracellular cAMP accumulation (53). The inhibited CaMKII may induce PKA-mediated responses, increasing the levels of cAMP, which in turn could contribute to barrier stability. In fact, direct evidence has demonstrated the ability of Rcan1 to increase the phosphorylation of cAMP response element-binding protein through the negative regulation of the calcineurin signaling pathway (54). Accordingly, Rcan1 overexpression decreases CaMKII levels in our EC system, supporting its role in the strengthening of the endothelial barrier.

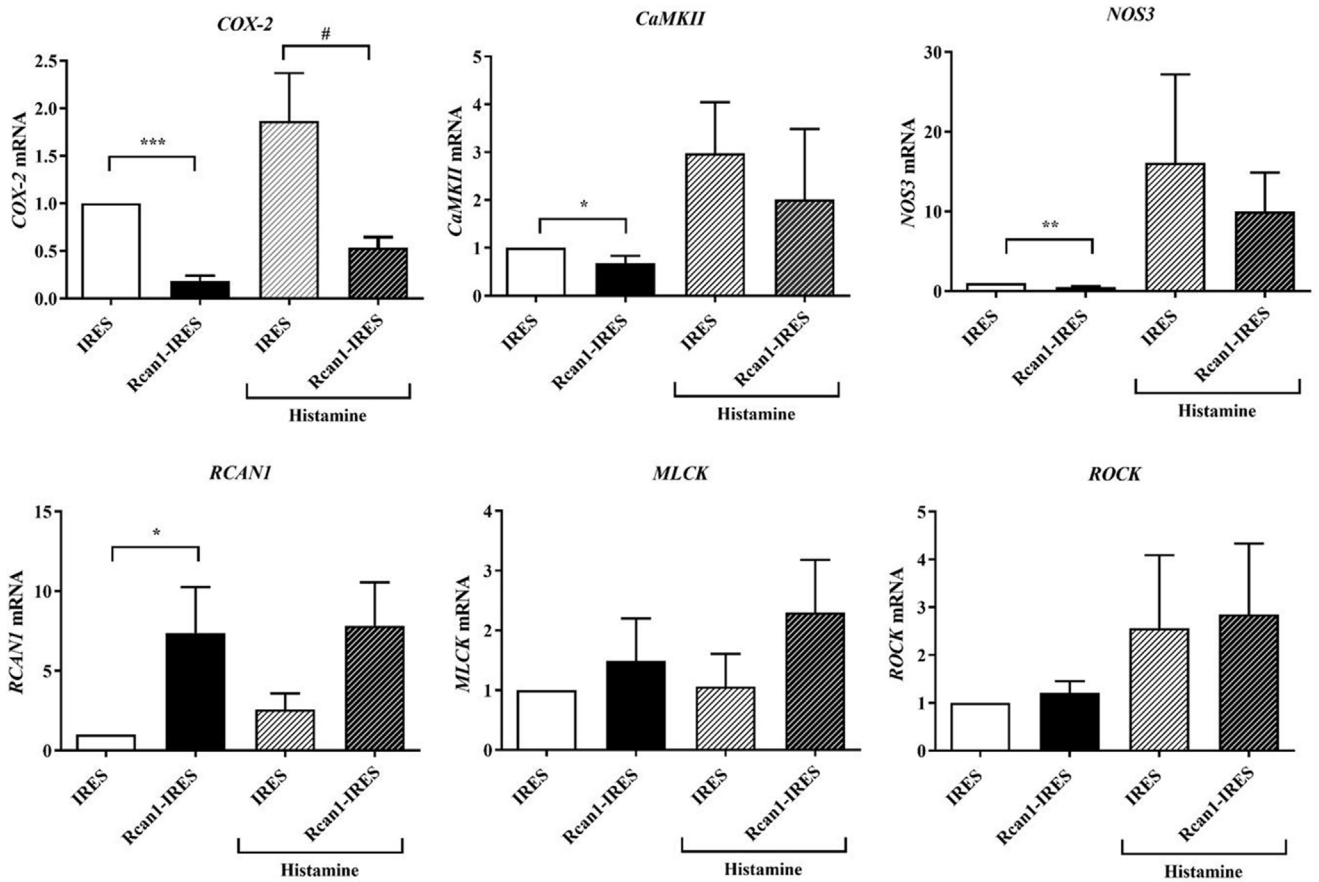

FIGURE 5 | Regulator of calcineurin 1 (Rcan1)-IRES-GFP inhibits *COX2, CaMKII*, and nitric oxide synthase 3 (*NOS3*) expression in human vein (HV)-endothelial cells (ECs). Figure shows the qPCR analysis of *COX2, CaMKII, NOS3,* MLC-kinase (*MLCK*), *ROCK1*, and *RCAN1* mRNA of IRES-GFP and Rcan1-IRES-GFP HV-ECs treated or not with Hist 1 μM for 60 min. Values represent amounts of mRNA normalized to the endogenous gene. Unpaired *t*-test analysis was applied to untreated or histamine-treated cells. Data represent means ± SEM of duplicates determined by qPCR from indicated independent experiments: *COX-2*; $^{****}P < 0.0001$, Rcan1-IRES-GFP control ($n = 13$) vs IRES-GFP control ($n = 12$); $^{\#}P = 0.0130$, Rcan1-IRES-GFP histamine ($n = 14$) vs IRES-GFP histamine ($n = 15$); *CaMKII*; $^{*}P = 0.0454$, Rcan1-IRES-GFP control ($n = 9$) vs IRES-GFP control ($n = 8$); Rcan1-IRES-GFP histamine ($n = 6$) vs IRES-GFP histamine ($n = 6$); *NOS3*; $^{**}P = 0.0044$, Rcan1-IRES-GFP control ($n = 6$) vs IRES-GFP control ($n = 6$); Rcan1-IRES-GFP histamine ($n = 5$) vs IRES-GFP histamine ($n = 5$); RCAN1; $^{*}P = 0.0392$, Rcan1-IRES-GFP control ($n = 10$) vs IRES-GFP control ($n = 10$); Rcan1-IRES-GFP histamine ($n = 11$) vs IRES-GFP histamine ($n = 9$); *MLCK*; Rcan1-IRES-GFP control ($n = 9$) vs IRES-GFP control ($n = 10$); Rcan1-IRES-GFP histamine ($n = 8$) vs IRES-GFP histamine ($n = 6$); *ROCK1*; Rcan1-IRES-GFP control ($n = 10$) vs IRES-GFP control ($n = 11$); Rcan1-IRES-GFP histamine ($n = 11$) vs IRES-GFP histamine ($n = 9$).

Molecular pathways based on disruption of cell–cell contacts and which are regulated by phosphorylation and de-phosphorylation of the myosin light chain are also relevant to this field of research (17, 55, 56). Although RhoA and ROCK have been recently recognized as key targets mediating histamine-induced vascular leakage and anaphylactic shock (14), our results do not reveal significant differences in *MLCK/ROCK1* when Rcan1 is overexpressed in HV-ECs. Nevertheless, the identification of *COX2* as a downstream target of the CN/Rcan1 molecular pathway is a meaningful observation, and one which supports previous studies (57). COX-2 expression is induced by inflammatory stimuli and other mediators in the vascular wall, such as histamine, generating prostaglandin I2, and E2 production in HA-ECs (58). Proinflammatory phospholipid-derived mediators are critical modulators of vascular tone in physiological and pathological situations. COX-2 production induced by histamine has been reported in mast cells (19, 20). In addition to its products, prostaglandin D2, leukotrienes, thromboxane A2, and PAF are released rapidly in anaphylactic events (59, 60). In our studies, *COX2* mRNA was substantially decreased upon histamine stimulation in Rcan1 overexpressed HV-ECs, suggesting a major relevance of the axis histamine/Rcan1-4/*COX2* in anaphylaxis. In general, it can be speculated that both cell types (endothelial and mast cells) may contribute to prostanoid/eicosanoid generation, which greatly render the vasoconstrictor/vasodilator effects that occur in anaphylaxis. Our findings suggest that, through endothelial Rcan1 expression, histamine could also contribute to the regulation of important molecular pathways related to anaphylaxis, although further investigations need to be performed to confirm this.

Knowledge of the consequences of human Rcan1 overexpression is limited to evidence of genetic dysfunctions secondary to

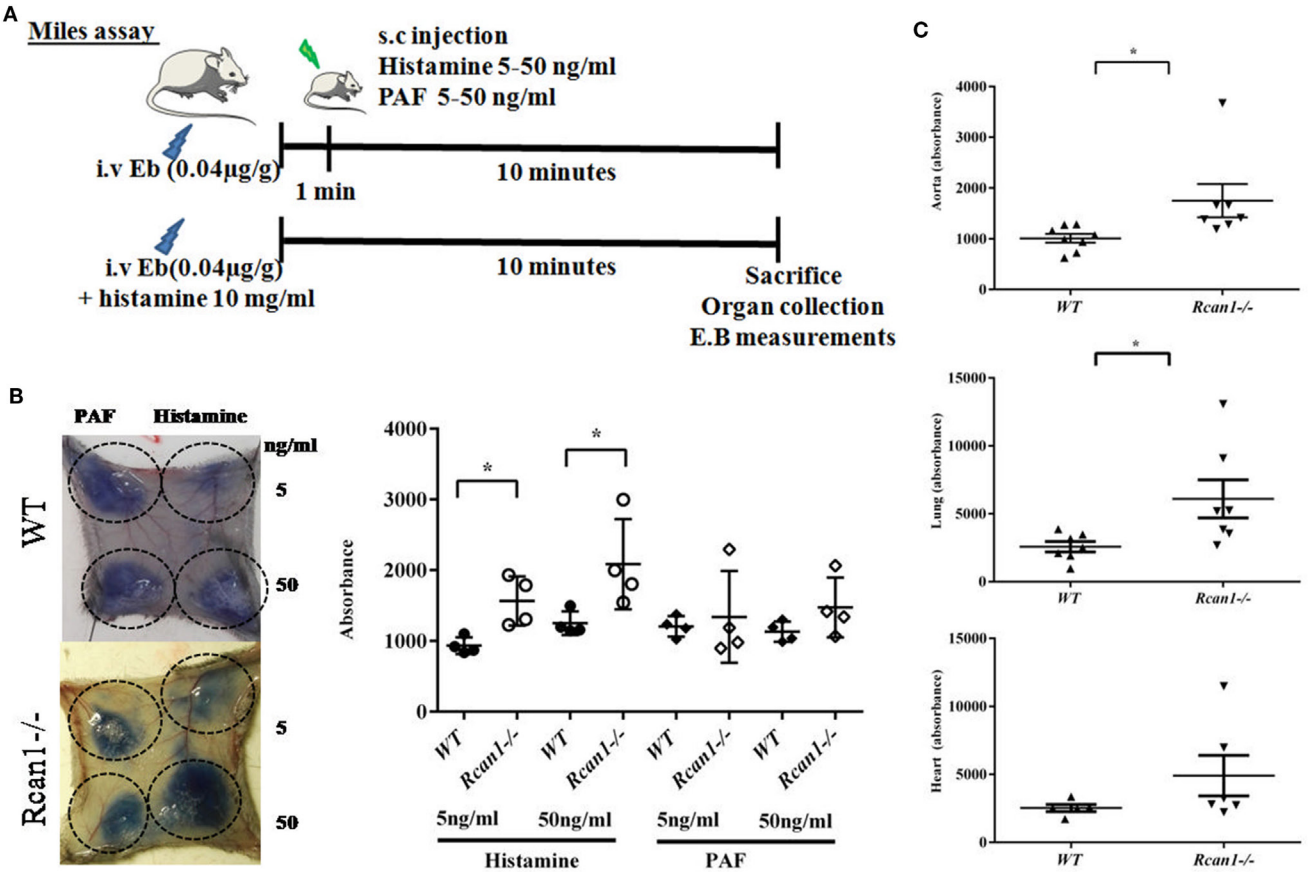

FIGURE 6 | Regulator of calcineurin 1 (Rcan1)-deficient mice prevent histamine-induced vascular subcutaneous and systemic permeability but not systemic anaphylaxis. **(A)** Experimental mice models addressed in *WT* and *Rcan1*$^{-/-}$ mice. **(B)** Representative skin pictures of *WT* and *Rcan1*$^{-/-}$ mice s.c. injected with mediators at indicated concentrations. Evans blue extravasation was determined in four mice per genotype after subcutaneous injection of 20 µl of the indicated doses of histamine or PAF. Figure shows the amounts of Evans blue determined in skin dorsal punches as described in the Section "Materials and Methods." Data represent means ± SEM. Unpaired *t*-tests were performed vs *WT* mice for each treatment and doses, **P* = 0.0138 vs *WT* (5 ng/ml), **P* = 0.0441 vs *WT* (50 ng/ml). **(C)** Graphic shows individual values of aorta and lung extravasation determined in seven (*WT*) and eight (*Rcan1*$^{-/-}$) mice after 15 min of i.v. histamine injection together with Evans blue. Figure shows the amounts of Evans blue determined in tissues as described in the Section "Materials and Methods." Data represent means ± SEM. Unpaired *t*-tests were performed vs *WT* mice. Aorta; **P* = 0.0357, Lung; **P* = 0.0319, Heart; ns = 0.1879 compared with Histamine *WT* mice.

trisomy of chromosome 21 in patients with Down syndrome, who present defects of the immune system, though the prevalence of allergy is not high in these individuals (61). Previous studies using experimental anaphylaxis in Rcan1-deficient mice have found a role for Rcan1 in regulating Fc-εRI-mediated signaling and mast-cell function (62). Rcan1$^{-/-}$ bone-marrow-derived mast cells show increased transcriptional activation of NF-κB and NFAT and calcineurin activity following stem-cell factor stimulation (63). On the other hand, Rcan1 seems to be required for the development of pulmonary eosinophilia in allergic inflammation in mice (64). Additionally, Rcan1 plays a protective role for respiratory infections and sepsis in experimental mice models (33, 65). Consistent with this, studies performed on Rcan1 transgenic mouse have found that Rcan1 gives rise to cancer protection by inhibiting the calcineurin pathway in the vascular endothelium (66). Moreover, as an endogenous inhibitor of calcineurin, Rcan1 has been involved as a negative regulator in inflammatory molecular pathways belonging to ECs (47). Our results in human ECs and an experimental mice model support the immunosuppressive function of Rcan1 for vascular permeability and anaphylaxis.

We, here, show that endothelial Rcan1 maintains the integrity of the endothelial barrier in HV-ECs. This effect is closely correlated with the cellular dilatory process that must occur in the resident cells once the primordial effect of endothelial barrier breakdown has been induced by histamine. Extravasation or vascular permeability in micro-ECs may be best understood in terms of cellular contraction and dilatation, though cellular processes do not always correlate with physiology in vessels, and it is plausible that a similar process would occur in the context of anaphylaxis. Our results suggest that Rcan1 overexpression produces cell dilation, at least in HV-ECs, which correlates with the stabilization of the HMVEC-D barrier.

In summary, this work contributes to the knowledge of anaphylaxis, showing endothelial Rcan1 as an inducible molecule which is modulated in the endothelial compartment

upon contact with histamine through a calcineurin-sensitive pathway. Additionally, human functional assays and *in vivo* experimental mice models suggest a role for endothelial Rcan1 controlling vascular permeability, most likely to recover the loss of fluids, and pointing to Rcan1 as a plausible regulator of sensitivity to anaphylaxis in humans from the endothelial compartment.

ETHICS STATEMENT

The study was approved by the research ethics committees of the respective Gentofte and FJD hospitals, and written informed consent was obtained from all patients. Animal procedures were carried out in accordance with the European Union guidelines for the care and experimental use of animals. Protocols with reference PROEX: 391/15 received prior approval from the IIS-FJD Ethics Committee and the competent authorities in the region of Madrid.

AUTHOR CONTRIBUTIONS

CB-M, NM-B, and VE contributed to the performance of the experimental work; AM-Y and CP-V contributed to the performance of the experimental work included in the reviewed version. BMJ, LHG, LKP and VE as leaders of the overall project "molecular mechanisms in anaphylaxis"; LK, JC-H, and AG-C provided clinical support; NM-B and VE assisted in results interpretation; VE coordinated the work, designed the experiments, and wrote and edited the manuscript; NM-B, BMJ, MG-A, GA-L, CP-V, FV, JC-H, LH-G, and LKP reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01323/full#supplementary-material.

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