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Involvement of Complement in IgG2a-mediated Anaphylaxis

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ABSTRACT

Anaphylaxis is an acute-onset and potentially fatal systemic allergic reaction affecting approximately 0.05 to 2% of the population worldwide and can be induced by a wide range of allergens from foods, medications and insect venoms. Although IgE is commonly involved in human anaphylaxis, other mediators, such as non-IgE antibodies and complement are also reported to be involved. Thus we sought to evaluate the role of complement in IgE-independent anaphylaxis. To approach this, we have studied an IgG2a-mediated passive systemic anaphylaxis model in mice with different backgrounds and gene deficiencies. We demonstrate that complement component 3 (C3) plays a major role in anaphylaxis in C57BL/6 and Balb/c mice sensitized with a low dose of IgG2a\(^a\) mAb Hy1.2 but not in mice sensitized a higher dose of Hy1.2, and that C5 is not involved in IgG2a-mediated anaphylaxis.
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<tr>
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<td>Active systemic anaphylaxis</td>
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<tr>
<td>C3</td>
<td>Complement component 3</td>
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<tr>
<td>C5</td>
<td>Complement component 5</td>
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<tr>
<td>FcεR</td>
<td>Fc-epsilon receptor</td>
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<td>FcγR</td>
<td>Fc-gamma receptor</td>
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<td>KO</td>
<td>Knock out</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>mAb</td>
<td>Monoclonal antibody</td>
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<td>OVA</td>
<td>Ovalbumin</td>
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<td>PSA</td>
<td>Passive systemic anaphylaxis</td>
</tr>
<tr>
<td>TNP</td>
<td>2,4,6-trinitrophenol</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
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CHAPTER 1

Introduction
A.1 Anaphylaxis

A.1.1 Epidemiology of anaphylaxis

Anaphylaxis is currently defined as a severe, acute-onset and potentially fatal generalized or systemic allergic reaction [1]. According to the definition from the National Institute of Allergy and Infectious Disease (NIAID) and the Food Allergy and Anaphylaxis Network (FAAN), anaphylaxis is considered highly likely if any one of three criteria is satisfied: (1) acute onset illness with involvement of the skin, mucosal tissue (e.g., generalized hives, pruritus or flushing, and swollen lips-tongue-uvula), or both and respiratory compromise or hypotension; (2) occurrence of two or more of the following symptoms after exposure to a likely allergen within hours: involvement of the skin-mucosal tissue, respiratory compromise, hypotension, persistent GI symptoms; (3) significant (>30%) systolic blood pressure drop after exposure to a likely allergen within several hours [2].

The lifetime rate of anaphylaxis is estimated to be 0.05 to 2% [2], with most cases in children and adolescents [3]. Acquiring an accurate community-based population estimate is difficult, because data studying frequency of anaphylaxis are sparse and often imprecise. This is because of the lack of a universal accepted definition of anaphylaxis and differences in methods of case ascertainment [3]. In addition, the current estimate may be too low because a patient’s first episode is often mild and/or transient [4]. However, a common conclusion from most studies is that the rate of anaphylaxis has increased in the last decade; probably as much as 350% for food-induced anaphylaxis and 230% for other types of anaphylaxis [5-7].
A.1.2 Triggers for anaphylaxis

The three most common triggers for anaphylaxis are foods, insect venoms and drugs. Food allergens, such as peanuts, tree nuts, shell fish, fish, eggs, milk and sesame are the most common causes of anaphylaxis [8]. These foods contribute to 33.2-56% of total anaphylaxis cases [9], and this number is reported to be increasing, especially during the first twenty years of life [10]. Other food allergens including additives, such as spices and dyes; contaminants, such as dust mites, which are often found in contaminated wheat flour; and parasites, such as nematodes in live fish, can also induce anaphylaxis and thus are considered as food triggers [11, 12]. Insect stings are the second leading trigger for anaphylaxis and account for about 18.5% of total anaphylaxis cases [13]. Venom from stinging insects, such as honeybees, yellow jackets, hornets and paper wasps, and saliva from biting insects, such as mosquitoes and ticks, trigger most of these anaphylaxis cases[14-16]. Medications, the third greatest cause of anaphylaxis, are responsible for 13.7% of total anaphylaxis cases [13]. Medication-triggered anaphylaxis is common in middle-aged and older adults [17]. Most of these cases were induced by penicillin [3], which induced anaphylaxis with a frequency of 0.01~0.05% during treatment, and may account for 75% of fatal anaphylaxis cases in US each year; non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin and ibuprofen; and chemotherapeutic agents[18]. Other medication triggers for anaphylaxis includes loperamide [19] (an opioid drug that is used to treat diarrhea), vitamins and supplements containing folic acid [20], herbal therapeutics [21], and biological agents such as monoclonal antibodies that are used in immunotherapy [22-24].
Other triggers, such as latex, seminal fluid, animal dander or grass pollen, exercise, exposure to cold, sunlight or UV radiation and ethanol, can also induce anaphylaxis. About 20% of anaphylaxis cases are idiopathic (i.e. no triggers have been found) [1, 17].

A.1.3 Mechanism of anaphylaxis in human

Human anaphylaxis can be divided into IgE-dependent anaphylaxis and IgE-independent anaphylaxis. [17, 25]. Most anaphylaxis triggered by food, medications, insect venom, latex and inhaled allergens is IgE-mediated [17]. Allergen-specific IgEs are produced during responses to allergen exposure, then become bound to surface high-affinity IgE receptor (FcεRI) on mast cells and basophils. Upon second exposure to the same allergen, these receptor-bound IgEs are cross-linked by antigen, leading to aggregation of FcεRI, which activates Syk and other tyrosine kinases, including Lyn, Fyn and Bruton’s tyrosine kinase (Btk) [26]. These kinases phosphorylate tyrosine-containing motifs that recognize Src homology 2 (SH2) domains [27] and immunoreceptor tyrosine-based activation motifs (ITAMs), which recruit and activate Syk kinase, that in turn, phosphorylates the scaffold proteins LAT and LAT2 [26]. These adaptors and/or scaffold proteins then recruit and activate phospholipase C 1 (PLCγ1), PLCγ2 and phosphoinositide 3-kinase (PI3K), resulting in the activation of protein kinase C (PKC) and intracellular calcium release. These events lead to mast cell degranulation and contribute to later cytokine and chemokine production [28, 29]. The mediators released by mast cells and basophils during anaphylaxis include preformed histamine, tryptase, carboxypeptidase A, proteoglycans and some cytokines, as well as newly synthesized leukotrienes, prostaglandins, platelet-activating factor (PAF), and other cytokines [17]. Among these mediators, histamine and PAF are the key
contributors to anaphylaxis [30]. Histamine contributes to anaphylactic shock by leading to coronary vasoconstriction and increased vascular permeability, cardiac depression and systemic vasodilatation [31, 32] while PAF causes decreased coronary blood flow and myocardial contractility, increased vascular permeability and peripheral vasodilatation.

Non-IgE antibodies such as IgG, and rarely, IgA and IgM may also trigger anaphylaxis in humans. Anaphylaxis possibly mediated by IgG has been reported in children receiving a brand of MMR vaccine called Morupar (Chiron, Siena, Italy). High titers of dextran-specific IgG but not IgE were found in the sera from children who developed anaphylaxis after receiving this vaccine [33]. In addition, IgG may also be involved in anaphylaxis in patients receiving therapeutic mAbs, such as infliximab [24]. IgE-independent anaphylaxis may also be mediated by complement activation. For example, over-sulfated chondroitin sulfate (OSCS), which can be a contaminant in heparin, is reported to be responsible for at least one syndrome of heparin-induced anaphylaxis [17]. OSCS may contribute to anaphylaxis by activating complement, leading to production of the anaphylatoxins C3a and C5a, which increase vascular permeability and cause smooth muscle contraction [35]. IgE-independent anaphylaxis can also occur when mast cells or basophils are directly activated by physical factors such as exercise, exposure to cold; ethanol; and opioids [17]. IgG immune complexes or complement activation may also be involved in anaphylactic reactions in patients who are considered to have idiopathic anaphylaxis because they have negative immediate hypersensitivity skin test results and undetectable serum antigen-specific IgE.
A.1.4 Mouse model of anaphylaxis

In the study of anaphylaxis, animal models are useful for screening for allergens and for study of possible mechanisms. A variety of animals have been used in models for anaphylaxis, such as guinea pigs, dogs, rats and mice, among which mouse models have distinctive advantages. Firstly, mice are available in a wide range of background with deficiency or enhancement of genes involved in anaphylaxis, such as IgE; FcRs including FcεRI, FcγRI, FcγRIIb, FcγRIII and FcγRIV; complement and its receptors; and c-kit, which is crucial for mast cell development. Another advantage of mouse models is the similarity in anaphylaxis between humans and mice. Both species produce IgE and IgG that can activate mast cells and macrophages, respectively, to release vasoactive mediators, such as PAF and histamine, both of which can cause anaphylaxis by increasing vascular permeability in both species [25].

Commonly used mouse anaphylaxis models include intestinal anaphylaxis models, passive cutaneous anaphylaxis models and systemic anaphylaxis models. In intestinal anaphylaxis models, mice are sensitized with a certain antigen by ingestion, intraperitoneal injection or transdermal immunization, and then the mice are challenged orally with the same antigen; anaphylaxis in this model is experienced as diarrhea and/or shock [34-36]. In passive cutaneous anaphylaxis models, mice are sensitized by intradermal injections of antibody or serum from an immunized animal and, after 24 to 72 hours, injected with the appropriate antigen and Evans blue dye. Reaction of the antigen with skin-fixed antibody causes histamine production that leads to vascular leak at the site of the intradermal injection; this causes the formation of a blue spot, which is the sign of anaphylaxis in this model [37, 38]. Systemic anaphylaxis can be divided into
active systemic anaphylaxis (ASA) and passive systemic anaphylaxis (PSA). In ASA, mice are immunized with one or more injections of antigen ± adjuvant and challenged with the same antigen after natural development of antibodies (usually two or more weeks). In PSA, mice are sensitized by intravenous injection of antibody or serum from an immunized animal and challenged later with antigen. ASA is more natural than PSA in how the animal acquires antibodies; however the antibodies generated during immunization typically include multiple isotypes. In contrast, PSA facilitates control of antibody concentration and isotype, but it is somewhat artificial.

A.1.5 Mechanism of anaphylaxis in mice

Mouse anaphylaxis can be mediated by at least two distinct mechanisms [39], the classic pathway and the alternative pathway [30]. The classic pathway is dependent on IgE, mast cell associated FcεRI, mast cells and histamine, and is similar to the best known mechanism of human anaphylaxis. In this pathway, mouse mast cells are activated by crosslinking of their surface FcεRI; then mediators and enzymes including histamine and PAF [36, 39, 40] are released, which cause anaphylaxis by increasing vascular permeability. Among all the mediators released during this process, histamine from mast cells is the main contributor to anaphylactic shock while mast cell PAF contributes to a lesser extent.

The alternative pathway of mouse systemic anaphylaxis, in which mast cells, IgE and FcεRI are not needed [41-43], is mediated by IgG antibodies. The IgG-mediated alternative pathway depends on FcγRIII and occasionally, FcγRIV. FcγRIII is important in the goat anti-mouse IgD (GAMD) ASA model [44] and in an IgG1 PSA model while FcγRIV is important in IgG2b-mediated PSA [45]. PAF, rather than histamine, plays a
major role in this pathway, and can be released by a wide range of cells including macrophages, neutrophils, eosinophils, basophils, platelets and endothelial cells [46]. The major cell type responsible for IgG-mediated anaphylaxis remains controversial: In the GAMD model in Balb/c mice, anaphylaxis is blocked by the macrophage inhibitor gadolinium, indicating that macrophages are important in anaphylaxis pathogenesis in this model. Notably the IgG isotypes in this model are mainly IgG1 [47]; In contrast, in a IgG1 PSA model in C57BL/6 mice, basophils, but not macrophages, neutrophils or NK cells, are the necessary cell type for anaphylaxis [48]. Another more recent paper showed that neutrophils mediated FcγRIV-dependent anaphylaxis in an ASA model (mice were primed with BSA) in which considerable IgG2a and IgG2b were secreted; and in an IgG2b PSA model [45]. Therefore, the major contributing cell type in IgG-mediated anaphylaxis may depend on the mouse model used. Another important feature of IgG mediated anaphylaxis is that the induction of IgG-mediated anaphylaxis requires much more antibody and antigen than IgE-mediated anaphylaxis. Because the affinity of FcγRIII and FcγRIV for IgG is much lower than the affinity of FcεRI for IgE, cells that capture antigen through IgG bound to FcγRIII or FcγRIV in the alternative pathway (e.g., macrophages, basophils and neutrophils) require more antibody to capture antigen than mast cells, which capture antigen with IgE bound to FcεRI in the classic pathway.

A.2 Complement system and anaphylaxis

A.2.1 Activation pathways of the complement system

The complement system consists of over 25 small proteins and protein fragments, which circulate as inactive precursors (pro-proteins) in blood. Complement is activated
through a cascade of enzymatic cleavages, and the net result of this activation cascade is massive amplification of pro-inflammatory molecules and activation of the membrane attack complex (MAC). There are three major complement activation pathways, the classic pathway, the alternative pathway and the lectin pathway. These three pathways differ mainly in formation of the C3 convertase, which is able to cleave C3 into C3a plus C3b. However, the pathways share later steps of complement activation, such as production of pro-inflammatory peptides, activation of C5 convertase and formation of the MAC.

The classic pathway is activated by antigen-antibody complexes. In this pathway, C1q interacts with the Fc part of IgM and certain IgG immune complexes and recruits C1r and C1s to form C1, which enzymatically cleaves C4 and C2 in turn into C4a plus C4b and C2a and C2b, respectively. Then the newly formed C4b and C2a assemble into C4b2a, the C3 convertase.

The alternative pathway starts with C3b, which is normally produced by spontaneous hydrolysis of C3, but is usually quickly inactivated by inhibitory proteins. However, when bacteria or other foreign materials are present, C3b can bind to their surface and escape inactivation. This active C3b recruits factor B, which is later enzymatically cleaved into its active form Bb by factor D, and is later assembled into the alternative pathway C3 convertase C3bBb. This pathway also functions as an amplification loop for other complement activation pathways.

The lectin pathway is activated by the binding of mannose-binding lectin (MBL) to mannose residues on the surface of pathogens. This activates MBL-associated serine
proteases, such as MASP-1 and MASP-2 (which function like C1q and C1s, respectively). These proteases then enzymatically cleave C4 and C2 into C4b and C2a, respectively, and assemble them into a C3 convertase.

A.2.2 Complement activation in human anaphylaxis.

Complement has been shown to be directly or indirectly involved in humans anaphylaxis caused by a variety of triggers including insect stings, foods and medications. In vitro studies have showed that venoms from Hymenoptera, such as honeybees, yellow jackets, yellow hornets, white-faced hornets, and wasps, can activate complement through the alternative pathway, leading to production of C3a [49]. In this way, these venoms may contribute to anaphylaxis. For example, in one report regarding 8 patients with wasp-venom-induced anaphylaxis, 7 had increased C3a (3 mildly increased and 4 substantially increased) in their blood [50], indicating that complement activation may be involved in wasp-venom induced anaphylaxis.

Complement activation is possibly involved in anaphylaxis triggered by medications, such as OSCS-contaminated heparin, protamine neutralization of heparin, and polyethylene glycols[17]. For example, over-sulfated chondroitin sulfate (OSCS), which is a compound contaminating heparin supplies worldwide, may contribute to heparin-induced anaphylaxis by promoting production of C3a and C5a from the complement system[51]. Another example is complement activation-related pseudoallergy (CARPA), which is closely associated with complement activation and is IgE-independent. CARPA can be caused by radio contrast media, some liposomal drugs (such as Doxil, Ambisome and DaunoXome) or micellar solvents containing amphiphilic lipids (e.g., Taxol) [52]. These agents activate complement through both the classic pathway and
the alternative pathway, and therefore give rise to C3a and C5a which trigger mast cells and basophils to release vasoactive mediators, leading to hemodynamic, respiratory and cutaneous manifestations and sometimes, anaphylaxis [52].

Last but not least, complement may also be involved in exercise-induced anaphylaxis, which often involves food co-triggers [53], since evidence for complement activation through the alternative pathway has been found in patients who had experienced such anaphylaxis[54].

A.2.3 Function of anaphylatoxin during anaphylaxis

The term anaphylatoxin was first noted by Friedberger in 1910 to describe substances that could be generated by immune complexes in normal guinea pig serum. Intravenous injection of high doses of these substances caused lethal shock and bronchospasm in guinea pigs [55]. Not until the 60s did the researchers identify the nature of anaphylatoxins as C3a, C4a and C5a. Among them, C5a is 10 to 100 times more potent than C3a, and C5a is 1,000 times more potent than C4a [56, 57]. Anaphylatoxin can contribute to anaphylaxis by increasing smooth muscle contraction and vascular permeability, and to a lesser extent, mediating chemotaxis, inflammation and generation of cytotoxic oxygen radicals [58]. C3a and C5a can also contribute to anaphylaxis indirectly by activating macrophages and basophils to produce PAF, or by activating mast cells to produce histamine [57]. Both in vivo and in vitro experiments showed that C3a and C5a can induce cardiac anaphylaxis with symptoms including tachycardia, impairment of atrioventricular conduction, left ventricular contractile failure and coronary vasoconstriction [59, 60]; and that such anaphylaxis was dependent on histamine release [60]. The same group also showed that C3a played a minor role in hypotension
during systemic anaphylaxis in an OVA ASA model in C3aR-deficient guinea pigs [61]. In addition, Marat Khodoun in our lab showed that peanut extract (PE) contributed to antibody-independent, anaphylaxis-like shock by causing production of C3a, which stimulates macrophages, basophils and mast cells to produce PAF and histamine. The shock induced by PE was mostly blocked in C3aR knock-out (KO) mice but was not affected in C5aR deficient mice, suggesting that C3a, but not C5a, is important for anaphylaxis in this model [62].

A.2.4 Role of complement in IgG induced systemic anaphylaxis

Given the ability of IgG immune complexes in activating complement and the potential ability of C3a and C5a in inducing anaphylaxis, it is reasonable to suggest a role for complement in IgG-mediated anaphylaxis. However, a previous study of involvement of complement in a GAMD model suggested otherwise, because anaphylaxis was not affected in complement deficient mice (mice treated with cobra venom factor or C3 KO mice) [63]. However, because these studies also identified IgG1 as the predominant IgG isotype in the GAMD model, this result may be explained by IgG1’s inability to activate complement through the classical pathway[64]. Although complement is not involved in the GAMD model, it is still possible that complement contributes to anaphylaxis that is mediated by complement-fixing IgGs, such as IgG2a and IgG2b. Therefore, we hypothesized that complement is involved in IgG2a-mediated anaphylaxis.
CHAPTER 2

Materials and Methods
B.1 Mice

Wild type Balb/c and C57BL/6 mice were bred at the Cincinnati Children’s Research Foundation (CCRF) from mice purchased from Taconic. C57BL/6 background C3 KO mice were a gift of M. Wills-Karp, CCRF. B10.2 background C5 deficient mice and Balb/c background C3 KO mice were purchased from Taconic. All mice were age- and sex-matched within experiments and were used at 8 to 12 weeks of age. All experimental procedures were performed with approval from the Institutional Animal Care and Use Committees of the Cincinnati Children’s Hospital Research Foundation and the Department of Veterans Affairs Medical Center (Cincinnati, Ohio, USA).

B.2 Reagents

C4010 or Hy1.2 hybridoma cells were injected into the peritoneal cavity of nude mice that had been injected at least 1 week earlier with 0.2 ml of Pristane. Ascites from these mice was harvested after a minimum of 7 days. The IgG fraction from ascites was purified by ammonium sulfate fractionation (25–50% saturated cut) followed by DEAE-cellulose (DE-52; Whatman International Ltd.) ion exchange chromatography, in which the IgG fraction of the ammonium sulfate cut, which bound to the column at pH 8.3, 0.05 M Tris, was stepwise eluted with 0.08M Tris, pH 8.3; 0.10M Tris, pH 8.3, 0.10M Tris, pH 8.0, 0.15 M Tris, pH 8.0 and 0.20M Tris, pH 8.0. The IgG fraction from ascites was also purified by Protein A column affinity chromatography, in which the fraction bound to the column at pH8.0, 0.1M Tris, and was eluted with 3.5M MgCl₂, pH8.0. Eluted fractions that contained the desired Ig isotype were identified by gel double diffusion analysis with commercial isotype-specific antisera.
TNP-OVA was prepared by mixing 50 mg of OVA in 5 ml of bicarbonate buffer, pH 8.0, with serial 4-fold dilutions of TNP-succinyl-Osu (starting concentration, 25 mg/ml) in DMSO, and incubation of the mixture overnight in the dark at room temperature. The incubated solution was dialyzed against 5 changes of 0.15M NaCl/0.01 M NaHCO3, pH 8.0. The TNP: OVA molar ratio was determined as shown in 1:

\[ \text{OD}_{280} = [\text{TNP}] \times \varepsilon_{280}^{\text{TNP}} + [\text{OVA}] \times \varepsilon_{280}^{\text{OVA}} \]

\[ \text{OD}_{340} = [\text{TNP}] \times \varepsilon_{340}^{\text{TNP}} + [\text{OVA}] \times \varepsilon_{340}^{\text{OVA}} \]

\[ [\text{TNP}]/[\text{OVA}] = (\varepsilon_{280}^{\text{OVA}} \times \text{OD}_{340} - \varepsilon_{340}^{\text{OVA}} \times \text{OD}_{280}) / (\varepsilon_{340}^{\text{TNP}} \times \text{OD}_{280} - \varepsilon_{280}^{\text{TNP}} \times \text{OD}_{340}) \]  

The molar extinction coefficient \( \varepsilon \) was determined as shown in 2:

\[ (\text{at given length}) = \varepsilon / ([\text{TNP or OVA}]*L) \]

, in which A is absorbance (OD) at given length, and L is length of the light path.

The TNP-OVA we used in our experiment had a TNP:OVA molar ratio of 10.4.

**B.3 Passive anaphylaxis model.**

Mice were primed i.v. with different amounts of C4010 (mouse IgG2a anti-TNP antibody of the \( b \) allotype) or Hy1.2 (mouse IgG2a anti-TNP antibody of the \( a \) allotype), and were then challenged i.v. 2 hours later with 100 ug of TNP-OVA. The severity of anaphylactic shock was assessed by decrease in temperature and activity level. Activity levels: N=normal, S=slow, I=immobile, O=on side, D=dead.
B.4 Statistics.

Differences in temperature between groups of mice were compared using the one way ANOVA turkey test and the Mann-Whitney t test (GraphPad Prism 5.0). A P value less than 0.05 was considered significant.
CHAPTER 3

Results
C.1 IgG2a induces potent systemic anaphylaxis in Balb/c, B10.D2 and C57BL/6 WT mice

To study IgG2a-mediated systemic anaphylaxis, mice were treated with an appropriate dose of IgG2a anti-TNP mAb (Hy1.2 or C4010) i.v. and 100 ug of TNP-OVA 2 hours later. We detected the development of hypothermia, a quantitative characteristic of shock, by using an electronic probe to determine rectal temperature every 5 minutes for the initial 30 minutes after antigen challenge, then every 15 minutes afterwards up to 2 hours or until rectal temperature had returned to normal.

Because the amount of IgG immune complexes in the circulation is important in mediating anaphylaxis and the dose of TNP-OVA used was relatively large compared to that of IgG2a, we adjusted the amount of immune complexes by sensitizing the mice with different amounts of IgG2a mAb. As shown in Figure 1, wild type Balb/c and C57BL/6 mice were sensitized with 100, 300 or 500 ug of the IgG2a anti-TNP mAb C4010 and challenged with 100 ug of TNP-OVA two hours later. Only 500 ug of C4010 could mediate anaphylaxis with a >2°C temperature decrease in C57BL/6 mice (Figure 1A and 1B), while in Balb/c mice, anaphylaxis could be mediated by 300 or 500 ug of C4010, with no significant difference in the severity of anaphylaxis between the two doses (Figure 1C and 1D). These results suggest Balb/c mice have a lower threshold for IgG2a mediated anaphylaxis than C57BL/6 mice.

A similar experiment using IgG2a anti-TNP mAb Hy1.2 sensitization was also performed in age and sex matched Balb/c WT, C57BL/6 and B10.D2 mice. We set the sensitization dose at 100 ug because our previous study showed that Hy1.2 was able to mediated anaphylaxis in Balb/c and C57BL/6 mice at that dose (data not shown). All three mouse stains developed anaphylaxis with a comparable temperature drop
(C57BL/6, 4.88±1.083; Balb/c, 3.280±0.9039; B10.D2, 5.9±2.135) and kinetics (Figure 2). These results show that Hy1.2 can also mediate anaphylaxis, and appears to be more potent than C4010.

**C.2 C3 plays a major role in anaphylaxis in C57BL/6 mice sensitized with 100 µg, but not with 500 µg of Hy1.2 or C4010 mAb**

Complement can be activated in anaphylaxis induced by insect venom or drugs [17], and the anaphylatoxins C3a and C5a produced as a result of complement activation can activate macrophages to secrete PAF and histamine. A recent report from our lab also showed that complement component 3 (C3) can contribute to peanut extract induced anaphylaxis [62]. To determine whether C3 is involved in IgG2a induced anaphylaxis, we treated C57BL/6 WT and C3 KO mice with 100 µg or 500 µg of Hy1.2 and challenged those mice with 100 µg of TNP-OVA 2 hours later. Severe hypothermia developed in the WT, but not the C3 KO mice primed with 100 µg of Hy1.2 (Figure 3A and 3B), while 4 out of 5 mice WT and C3 KO mice died rapidly when primed with 500 µg of the same mAb (Figure 3C and 3D). Thus, anaphylaxis can still be triggered in C3 KO C57BL/6 mice by a large amount of IgG2a immune complex. This is not surprising, because the most important pathway of antibody-dependent anaphylaxis is Fc receptor-mediated. This suggests that the observed C3 dependence in anaphylaxis in the 100 µg group was overridden by massive FcγR activation in the mice sensitized with 500 µg of Hy1.2. Taken together, these results show that C3 plays a major role in anaphylaxis induced by 100 µg of HY1.2 in C57BL/6 mice, but is not required for anaphylaxis mediated by 500 µg of Hy1.2 in these mice (i.e.; C3-independent IgG-mediated
anaphylaxis requires a higher concentration of immune complexes than does C3-dependent anaphylaxis).

Because the heavy chain constant regions of IgG2a^a (Hy1.2) and IgG2a^b (C4010) are 10% and 15% different at the gene and protein level, respectively, and most of this difference is within the hinge region, the CH3 domain and the 3' untranslated region, these two antibodies may act differently in mediating anaphylaxis. With this in mind, we tested C4010 in a PSA model in C57BL/6 WT and C3 KO mice. Because 100 and 300 ug of C4010 failed to induce anaphylaxis in C57BL/6 WT mice, we sensitized the mice with 500 ug of C4010. As shown in Figure 4A, there was no difference in severity of anaphylaxis between the WT and C3 KO C57BL/6 mice, indicating that C3 is dispensable in anaphylaxis mediated by 500 ug of C4010 in C57BL/6 mice. Because C4010 may mediate anaphylaxis in C57BL/6 mice in a similar way to Hy1.2, in which C3 becomes unnecessary for anaphylaxis mediated by a high dose Hy1.2, we lowered the dose of C4010 to 400ug. However, we still saw no difference between C3 KO and WT C57BL/6 mice (Figure 4B). In addition, there was no difference between groups sensitized with these two doses (Figure 4C). These results show that C3 is not required for C4010-mediated anaphylaxis in C57BL/6 mice.

**C.3 C3 is also involved in Hy1.2-mediated anaphylaxis in Balb/c mice**

To test if C3 is involved in Hy1.2-mediated anaphylaxis in Balb/c mice, we induced systemic anaphylaxis in WT and C3 KO mice with 100 or 500 ug of Hy1.2. To our surprise, we found no difference in anaphylaxis between wild type and C3 KO mice at either dose, (Figure 5A and 5B). This result shows that anaphylaxis in Balb/c mice sensitized with 100 or 500 ug of Hy1.2 is not dependent on C3. However, because
Balb/c mice have a lower threshold for C4010-mediated anaphylaxis than C57BL/6 mice, it was possible that 100 ug of Hy1.2 sensitization was sufficient to activate the C3-independent mechanism. Therefore, we examined C3’s role in anaphylaxis mediated by a lower dose Hy1.2 in Balb/c mice. As shown in Figure 6, 10 ug of Hy1.2 failed to induce anaphylaxis in WT Balb/c mice (Figure 6A), but 33 ug Hy1.2 was able to induce anaphylaxis in these mice, with reduced severity compared to the group sensitized with 100 ug of Hy1.2 (Figure 6A). Anaphylaxis induced by 33 ug of Hy1.2 in C3 KO mice was significantly and substantially less severe (Figure 6B), suggesting that C3 is a major contributor to anaphylaxis mediated by a low dose of Hy1.2. These results show that C3 is also involved in Hy1.2-mediated anaphylaxis in Balb/c mice.

Notably, in both WT and C3 KO Balb/c mice, there was no significant difference in the severity of anaphylaxis between groups treated with 100 or 500 ug of Hy1.2 (Figure 5C), and the same result was obtained from Balb/c WT mice sensitized with 300 or 500 ug of C4010. However, in C57BL/6 mice, 500 ug of Hy1.2 led to fatal anaphylaxis in both WT and C3 KO mice. This result suggests that C57BL/6 mice are more susceptible than Balb/c to fatal anaphylaxis when challenged with a large amount of Hy1.2-TNP immune complexes.

C.4 IgG2a induced anaphylaxis of similar severity in B10.D2 WT and C5 KO mice

Activation of C3 can give rise to both C3a and C5a. C5a is derived from C5 and might also contribute to IgG2a-mediated anaphylaxis in our model. To test this, we induced IgG2a-mediated PSA in B10.D2 WT and B10.D2 C5 deficient mice using both Hy1.2 and C4010. As shown in Figure 7, when sensitized with 100 ug of Hy1.2, both WT and C5-deficient mice developed anaphylaxis, and the temperature drop was comparable
(3.325±1.431 in wild type mice and 2.940±1.268 in C5 deficient mice). In mice sensitized with 500 ug of C4010, no difference in anaphylaxis between WT and C5 deficient groups was found either. This result shows that C5 may not be involved in IgG2a mediated anaphylaxis in B10.D2 mice and suggests that in C3 KO C57BL/6 and Balb/c mice, the protection from anaphylaxis may result from lack of C3a activity, but not C3’s downstream target C5a.
Figure 1. Systemic anaphylaxis mediated by different doses of C4010 sensitization. C57BL/6 WT mice (A, B) and Balb/c WT (C, D) mice were sensitized by 100, 300 or 500 ug of C4010 (IgG2a \textsuperscript{b} anti-TNP) and challenged 2 hours later with 100 ug TNP-OVA, and rectal temperature were taken as indicator of anaphylaxis. (A and C) Development of anaphylaxis over time. (B and D) Maximum temperature drop in each group. Data represent means ± SE in one experiment. In all groups, n=5, one-way ANOVA with Tukey’s comparison; *P<0.05, **P<0.01, ***P<0.001.
Figure 2. Systemic anaphylaxis mediated by Hy1.2 sensitization. C57BL/6 WT mice (A), Balb/c WT (B) and B10.D2 WT mice (C) were sensitized by 100 ug of Hy1.2 (IgG2a anti-TNP) and challenged 2 hours later with 100 ug TNP-OVA, and rectal temperature were taken as indicator of anaphylaxis. (A, B and C) Development of anaphylaxis over time. (D) Maximum temperature drop in each group. Data represent means ± SE in one experiment. In all groups, n=5. One-way ANOVA with Tukey’s comparison, no significant difference was found between groups.
Figure 3. Involvement of C3 in Hy1.2-mediated anaphylaxis in C57BL/6 mice. Wildtype and C3 KO C57BL/6 mice were sensitized with 100 ug (A and B) or 500 ug (C) Hy1.2 and then challenged with 100 ug TNP-OVA, and their rectal temperature was monitored. (A and C) Development of anaphylaxis over time. (B) Maximum temperature drop in each group. Data represents mean ± SE, n=5. One-way ANOVA with Tukey’s comparison *P<0.05. **P<0.01. Data are representative of two independent experiments.
Figure 4. C3 is not necessary in C4010-mediated anaphylaxis in C57BL/6 mice. Wildtype and C3 KO C57BL/6 mice were sensitized with 400 ug or 500 ug of C4010 and then challenged with 100 ug TNP-OVA, and their rectal temperature was monitored. Data represent mean ± SE, n=5. One-way ANOVA with Tukey’s comparison *P<0.05. No significant difference was found in any pair of groups.
Figure 5. C3 is not involved in 100 or 500 ug of Hy1.2 mediated anaphylaxis in Balb/c mice. Wildtype and C3 KO Balb/c mice were sensitized with 100 ug (A) or 500 ug (B) Hy1.2 and then challenged with 100 ug TNP-OVA, and their rectal temperature was monitored. (A and B) Development of anaphylaxis over time. (C) Maximum temperature drop in each group. Data represents mean ± SE, n=5. One-way ANOVA with Tukey’s comparison *P<0.05. No significant difference was found in any pair of groups. Data are representative of two independent experiments.
**Figure 6.** C3 plays a major role in low dose Hy1.2 mediated anaphylaxis in Balb/c mice. Wildtype or C3 KO Balb/c mice were sensitized with 10, 33 or 100 ug of Hy1.2, challenged with 100 ug of TNP-OVA 2h later, and their rectal temperature was monitored. (A) Development of anaphylaxis in WT Balb/c mice sensitized with 10, 33 or 100 ug of Hy1.2 over time. (B) Development of anaphylaxis in WT and C3 KO Balb/c mice sensitized with 33 Hy1.2 over time. (C) Maximum temperature drop in each group. Data represent mean ± SE, n=5. One-way ANOVA with Tukey’s comparison *P<0.05.
Figure 7. Involvement of C5 in IgG2a-mediated anaphylaxis in Balb/c mice. Wildtype and C5 deficient B10.D2 mice were sensitized with 500 ug of C4010 (A) or 100 ug of Hy1.2 (B) and then challenged with 100 ug TNP-OVA, and their rectal temperature was monitored. (A and B) Development of anaphylaxis over time. (C) Maximum temperature drop in each group. Data represent mean ± SE, n=5. T-Test *P<0.05.
CHAPTER 4

Discussion and Future Directions
Anaphylaxis is an acute-onset and potentially fatal systemic allergic reaction affecting 0.05% to 2% of the population worldwide. Many factors may contribute to the development of anaphylaxis, including complement. Our data showed that: (1) IgG2a can mediate systemic anaphylaxis in at least 3 different inbred mouse strains; (2) C3 plays a major role in low dose Hy1.2 mediated anaphylaxis in both C57BL/6 and Balb/c mice; (3) There is no evidence supporting C5’s involvement in IgG2a mediated anaphylaxis.

D.1 Systemic anaphylaxis mediated by Hy1.2 and C4010 in multiple backgrounds

Mouse models for IgG-mediated anaphylaxis have shown that: (1) 2.4G2, a rat anti-mouse FcγRIIb/FcγRIII IgG2b antibody, can trigger IgG mediated anaphylaxis by cross-linking and activating FcγRIII [63]; (2) IgG-rich anti-TNP antiserum primed mice develop severe FcγRIII-dependent anaphylaxis when challenged with a high dose of TNP-BSA [40]. In addition, unpublished data from Marat Khodoun in our lab regarding IgG1-mediated anaphylaxis showed that IgG1 can trigger anaphylaxis in both Balb/c and C57BL/6 mice by activating FcγRIII on macrophages and basophils, while anaphylaxis in C57BL/6 mice is more dependent on basophils. Compared to IgG1, IgG2a is a better FcγR agonist with approximately two-fold greater binding affinity to FcγRIII, and can bind FcγRI and FcγRIV with relatively high affinity (association constant: IgG2a to FcγRI, $4.5 \times 10^7$; IgG2a to FcγRIV, $2.9 \times 10^7$) [65, 66], suggesting that IgG2a could mediate anaphylaxis in a similar FcγR-dependent pattern. In addition, IgG2a can activate complement through the classical pathway, while IgG1 cannot. Thus, it was possible that IgG2a could also induce anaphylaxis by activating complement to produce C3a and C5a, which might contribute to the onset of anaphylaxis by promoting the release of
PAF and histamine, which increase vascular permeability, from macrophages and basophils [57].

Our dose response data using a C4010 PSA model showed that Balb/c mice have a lower threshold than C57BL/6 mice for IgG2a-mediated anaphylaxis, because C57BL/6 mice developed anaphylaxis (reflected as hypothermia [63]) only when they were sensitized with 500 ug of C4010, while Balb/c mice sensitized with 300 ug of C4010 had anaphylaxis of similar severity. Because Balb/c mice have about two times more FcγRIII expression on monocytes, basophils and neutrophils than C57BL/6 mice (unpublished data from Marat Khodoun), the mechanism of the lower threshold for IgG2a-mediated anaphylaxis in Balb/c mice may be as follows: in mice sensitized with 300 ug of C4010, the amount of immune complexes formed is too small to sufficiently activate macrophages and/or basophils in C57BL/6 mice to induce anaphylaxis; however, the quantity of immune complexes formed at this dose of antibody activates macrophages and/or basophils in Balb/c mice sufficiently to lead to anaphylaxis. More experiments examining anaphylaxis in mice with partial FcγRIII deficiency, such as FcγRIII+/− mice and mice treated with Fc blocking antibodies, are needed to test this hypothesis.

We also induced anaphylaxis in mice using Hy1.2 and found that priming with Hy1.2 at a dose as low as 100 ug could induce a potent anaphylactic response to TNP-OVA. This result suggests that IgG2a\(^a\) Hy1.2 is a more potent inducer of anaphylaxis than IgG2a\(^b\) C4010. When we increased the Hy1.2 dose to 500 ug, C57BL/6 WT mice developed more severe anaphylaxis than those in the 100 ug sensitization group, and four out of five mice died within 20 min of TNP-OVA challenge. However such a difference was not observed in Balb/c WT mice. These results suggest that C57BL/6
mice are more susceptible to a large amount of immune complexes than Balb/c mice. This may reflect the greater sensitivity to PAF of C57BL/6 mice, which may, in turn result from higher PAFH activity in Balb/c mice (unpublished data from Marat Khodoun). Future investigations investigating PAF level and PAFH activity in both strains during IgG2a-mediated anaphylaxis could test these hypotheses.

D.2 C3 only makes an important contribution to anaphylaxis mediated by a low dose of IgG2a mAb Hy1.2 in both C57BL/6 and Balb/c mice.

C3 can be enzymatically cleaved by C4b2a in the classical and the lectin pathway or by C3bBb in the alternative pathway, giving rise to C3b and the anaphylatoxin C3a; C3b further contributes to the enzymatic cleavage of C5, leading to production of another anaphylatoxin C5a [67, 68]. IgG immune complexes can activate complement by binding C1q in the classical pathway and also facilitate the alternative pathway by binding to nascent C3b, promoting generation of C3-convertase and inhibiting inactivation of C3b [69].

Our results demonstrated that, compared to C57BL/6 WT mice, C57BL/6 C3 KO mice were mostly protected from anaphylaxis mediated by 100 µg of Hy1.2, indicating that C3 plays a major role in anaphylaxis mediated by 100 µg of Hy1.2 in C57BL/6 mice. When we repeated this experiment in Balb/c WT and C3 KO mice, we found that Balb/c C3 KO mice were mostly protected from anaphylaxis mediated by 33 µg, but not 100µg, Hy1.2, indicating that C3 is also a major contributor to anaphylaxis mediated by low dose Hy1.2 in Balb/c mice. The difference in anaphylaxis mediated by 100 µg of Hy1.2 between Balb/c and C57BL/6 mice is possibly a result of the higher expression of
FcγRIII in Balb/c mice (unpublished data from Marat Khodoun as mentioned previously). We suggest that when C57BL/6 mice are sensitized with 100 µg of Hy1.2, the activation of macrophages and/or granulocytes by FcγRIII is insufficient, by itself to induce anaphylaxis, so that development of shock also requires C3; However in Balb/c mice, which have two times more FcγRIII expression on monocytes and granulocytes (unpublished data from Marat Khodoun), the relatively FcγRIII-rich macrophages and/or granulocytes can be sufficiently activated when the mice are sensitized with 100 µg of Hy1.2, leading to anaphylaxis regardless of C3. When Balb/c mice are sensitized with 33 µg of Hy1.2, the amount of immune complexes is insufficient, by itself, to activate macrophages and/or granulocytes enough to induce anaphylaxis, making anaphylaxis again C3-dependent.

When C57BL/6 WT and C3 KO mice were sensitized with 500 ug of Hy1.2, 80% of the mice in both the WT and C3 KO groups died from anaphylactic shock within 20 minutes of TNP-OVA challenge. In addition, when Balb/c WT and C3 KO mice were sensitized with 500 ug of Hy1.2, no difference in severity of anaphylaxis was observed. These results suggest that at a higher sensitization dose, the FcγR-dependent activation of macrophage and/or granulocytes is sufficient for Hy1.2-mediated anaphylaxis.

D.3 Anaphylaxis is not dependent on C3 in C57BL/6 mice primed with C4010

To determine if C3 is involved in anaphylaxis mediated by another IgG2a mAb C4010, we examined anaphylaxis mediated by 400 or 500 ug of C4010 in C57BL/6 mice (since 300 ug of C4010 sensitization did sensitized for anaphylaxis). We found no difference in severity of anaphylaxis between WT and C3 KO mice at either dose, indicating anaphylaxis mediated by these doses of C4010 is C3 independent. These results show
that, unlike Hy1.2, C4010 is unable to induce C3-dependent anaphylaxis in C57BL/6 mice. One report showed that, to acquire the same absorbance value in a C1q binding assay, the amount of C4010 needed is 10~20 fold more than Hy1.2 [70], suggesting that Hy1.2 binds to (and probably activates) complement better than C4010. The difference between these two mAbs may result from: (1) different binding affinity to complement components caused by the structural difference between IgG2a\(^a\) and IgG2a\(^b\) [71]; (2) a difference in N-glycosylation in the CH2 domain between the two allotypes, which is shown to be essential to sustain functional activities, including complement activation [72]; (3) a difference in affinity to TNP between the two antibodies. Future investigation regarding C3a level during anaphylaxis mediated by both antibodies and affinity analysis are needed to evaluate the difference in the C3-dependence of anaphylaxis caused by Hy1.2 versus C4010.

**D.4 C5 is not required in IgG2a-mediated anaphylaxis**

C5 is enzymatically cleaved by C5 convertase during complement activation into C5b and the anaphylatoxin C5a, which is 10 to 100 times more potent than C3a. Our data showed that C5 is not necessary in anaphylaxis mediated by 100 ug of Hy1.2 in B10D.2 mice (Figure 7B), which is a strain very similar to C57BL/6 (identical to the closely related C57BL/10 mouse, except that it expresses MHC of the \(d\) haplotype). In addition, we found no difference in the severity of anaphylaxis between WT and C5 deficient mice in a repeat of this study using 500 ug of C4010 for sensitization (Figure 7A). Therefore, C5 may not be required in IgG2a-mediated anaphylaxis, suggesting that complement contributes to anaphylaxis in our model mainly through C3. Further studies examining anaphylaxis using C3aR KO mice and/or a C3aR antagonist are needed to
determine if C3a/C3aR signaling is responsible for C3’s contribution in IgG2a-mediated anaphylaxis.

D.5 Potential implication of complement for anaphylaxis in humans

Evidence supporting the existence of IgG-mediated anaphylaxis in humans is relatively scarce and scattered and the importance of this anaphylaxis mechanism in humans remains unproven. It is suggested that the importance of IgG-mediated anaphylaxis in humans may be limited by the high levels of antigen and antigen-specific IgG antibodies that are required to trigger IgG-mediated anaphylaxis. An exception may be patients who are repetitively treated with high doses of partially foreign (chimeric) immunoglobulin, such as the anti-TNF-α mAb infliximab [36]. However, there is correlative evidence supporting IgG-mediated anaphylaxis in humans by showing that complement activation by IgG complexes may mediate anaphylaxis: (1) complement, mostly C3, activated by immune complexes of L-asparaginase and L-asparaginase-specific IgM and IgG correlates with the development of anaphylaxis in 2 to 15 year old children [73]; and (2) C4a and C3a activated by IgG1 and IgG4 immune complexes with von Willebrand factor (vWF) are associated with anaphylaxis in patients with infusion of vWF-containing preparations [74]. This evidence, as well as our result, supports the possibility that IgG can mediate anaphylaxis in humans, in part, by activating complement to produce anaphylatoxins.

D.6 Conclusion

Anaphylaxis is an acute-onset and potentially fatal systemic allergic reaction affecting approximately 0.05% to 2% of the population worldwide. With a passive systemic
anaphylaxis model using IgG2a anti-TNP monoclonal antibody as the sensitizing agent and TNP-OVA as the challenge antigen in three mouse strains, we have shown that the complement protein C3 plays a major role in IgG2a mediated anaphylaxis in mice sensitized with a low, but not high, antibody dose. Further studies are needed to ascertain the role of C3a/C3aR signaling in this process, as well as the key difference between Hy1.2 and C4010 that is responsible for the differences in anaphylaxis mediated by these two mAbs.
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