Mast cell anaphylatoxin receptor expression can enhance IgE-dependent skin inflammation in mice

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Background: Mast cells express receptors for complement anaphylatoxins C3a and C5a (ie, C3a receptor [C3aR] and C5a receptor [C5aR]), and C3a and C5a are generated during various IgE-dependent immediate hypersensitivity reactions *in vivo*. However, it is not clear to what extent mast cell expression of C3aR or C5aR influences C3a- or C5a-induced cutaneous responses or IgE-dependent mast cell activation and passive cutaneous anaphylaxis (PCA) *in vivo*.
Objective: We sought to assess whether mouse skin mast cell expression of C3aR or C5aR influences (1) the cells' responsiveness to intradermal injections of C3a or C5a or (2) the extent of IgE-dependent mast cell degranulation and PCA *in vivo*.
Methods: We measured the magnitude of cutaneous responses to intradermal injections of C3a or C5a and the extent of IgE-dependent mast cell degranulation and PCA responses in mice containing mast cells that did or did not express C3aR or C5aR.
Results: The majority of the skin swelling induced by means of intradermal injection of C3a or C5a required that mast cells at the site expressed C3aR or C5aR, respectively, and the extent of IgE-dependent degranulation of skin mast cells and IgE-dependent PCA was significantly reduced when mast cells lacked either C3aR or C5aR. IgE-dependent PCA responses associated with local increases in C3a levels occurred in antibody-deficient mice but not in mice deficient in FcεRIγ.
Conclusion: Expression of C3aR and C5aR by skin mast cells contributes importantly to the ability of C3a and C5a to induce skin swelling and can enhance mast cell degranulation and inflammation during IgE-dependent PCA *in vivo*. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: *Anaphylatoxin, anaphylaxis, C3a, C5a, complement, IgE, inflammation, mast cells*

Abbreviations used

BMCMC:	Bone marrow–derived cultured mast cell
C3aR:	C3a receptor
C5aR:	C5a receptor
DNP:	2,4-Dinitrophenol
HSA:	Human serum albumin
PCA:	Passive cutaneous anaphylaxis
PCMC:	Peritoneal cultured mast cell
WT:	Wild-type

Anaphylaxis is a severe acute systemic allergic reaction that can be induced when certain unfortunate subjects previously sensitized to an allergen are later exposed to even very small amounts of that allergen.[1,2] In anaphylaxis the offending antigen interacts with specific IgE antibodies bound to tissue mast cells and peripheral blood basophils, causing them to release histamine and other mediators that induce the local and systemic clinical manifestations of this reaction within minutes.[1-4] Long ago, it was proposed that "humoral factors" derived from the incubation of normal guinea pig serum with washed immune precipitin (these factors were termed "anaphylatoxins" or "serotoxins") might contribute to anaphylaxis.[5] Although this hypothesis was controversial for decades,[6] it is now known that the complement anaphylatoxins C3a and C5a can activate various populations of rodent[7-9] or human[9-16] mast cells *in vitro* and that cutaneous injection of C3a or C5a can induce degranulation of rodent[8] or human[17-19] mast cells *in vivo*. Moreover, the complement system is activated and C3a and C5a are generated during certain immediate hypersensitivity reactions, including anaphylaxis, in human subjects and other mammals,[20-22] and positive correlations have been reported between the extent of complement activation, as assessed by measuring C3a levels[23] or cleavage of C3,[24] and the severity of systemic anaphylaxis in human subjects[23] or in a guinea pig model of immediate hypersensitivity.[24]

However, despite extensive evidence associating anaphylatoxins with anaphylaxis, it is not clear to what extent C3a or C5a responsiveness of mast cells, as opposed to other cells with anaphylatoxin receptors, contributes to C3a- or C5a-induced inflammation or IgE-dependent immediate hypersensitivity responses *in vivo*. Using mice containing mast cells that did or did not express the anaphylatoxin receptors C3a receptor (C3aR) or C5a receptor (C5aR), we measured the extent to which inflammation induced by cutaneous injection of C3a or C5a reflected C3aR and C5aR expression on mast cells and assessed the role of mast cell C3aR and C5aR in regulating the extent of IgE-dependent mast cell activation during IgE-dependent passive cutaneous anaphylaxis (PCA).

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METHODS

Mice

Kit$^{W\text{-}sh/+}$ mice obtained from Peter Besmer (Memorial Sloan-Kettering Cancer Center, New York, NY) were bred to produce mast cell–deficient C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ (*Kit*$^{W\text{-}sh/W\text{-}sh}$) mice and normal congenic wild-type (WT) littermates. C57BL/6J *C3aR*$^{-/-}$ and *C5aR*$^{-/-}$ mice were from the populations reported by Humbles et al[25] and Hopken et al,[26] respectively. C57BL/6 FcRγ-deficient mice (B6.129P2 *Fcer1g*^{tm1Rav} mice backcrossed 12 generations) were from Taconic (New York, NY). Antibody-deficient B6.129S2-Igh-6^{tm1Cgn}/J mice (stock no. 002288) were from Jackson Laboratories (Bar Harbor, Me). For additional details, see the Methods section in this article's Online Repository at www.jacionline.org.

Anaphylatoxin-induced ear swelling

After achievement of isoflurane anesthesia, mice received an intradermal injection of 100 ng of C3a (Calbiochem, San Diego, Calif) or C5a (R&D Systems, Minneapolis, Minn) in 20 μL of HMEM-Pipes buffer (500 mL of Sigma M4642 [MEM] + 0.47 g of Pipes + 0.105 g of NaOH) in the right ear and 20 μL of vehicle in the left ear as a control. Ear thickness was measured with a dial thickness gauge (G-1A; Ozaki, Tokyo, Japan) before and at intervals after intradermal injections.

IgE-dependent PCA

After achievement of isoflurane anesthesia, mice were passively sensitized by means of intradermal injection of 20 ng of 2,4-dinitrophenol (DNP)–specific IgE (α DNP clone ε26[27] from Dr Fu-Tong Liu, University of California–Davis, Davis, Calif) in 20 μL of HMEM-Pipes buffer in the right ear; 20 μL of vehicle was injected into the left ear as a control. Twenty-four hours later, mice were challenged intravenously with 200 μg of DNP-conjugated human serum albumin (HSA; Sigma, St Louis, Mo) in 100 μL of 0.9% NaCl. Ear thickness was measured before and at intervals after intravenous antigen challenge. Mice were killed 6 hours after challenge, and ear pinnae were collected for histologic analysis.

Cell culture and flow cytometry

Bone marrow–derived cultured mast cells (BMCMCs) were obtained by culturing femoral bone marrow cells from female mice in 20% WEHI-3 conditioned medium (as a source of IL-3) for 4 to 6 weeks, at which time greater than 95% of the cells were identified as mast cells by means of May Grünwald-Giemsa (Sigma) staining and flow cytometry (Kit^{+} and FcεRIα^{+}). For details, see the Methods section in this article's Online Repository.

Adoptive transfer of BMCMCs into mast cell–deficient mice

BMCMCs derived from WT (C57BL/6), *C3aR*$^{-/-}$, *C5aR*$^{-/-}$, or *Fcer1g*$^{-/-}$ mice were transferred by means of intradermal injection (2 injections per ear; 1×10^6 cells in 20 μL of Dulbecco modified Eagle medium per injection) into 4-week old female *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice 8 weeks before initiating the experiments.

Quantification of mast cell degranulation

Mast cells were classified by means of histomorphometry in alkaline Giemsa-stained, plastic-embedded 1-μm sections. For details, see the Methods section in this article's Online Repository.

Histologic analysis of leukocytes

Mice were killed by means of CO_2 inhalation, and 4-μm sections of ear pinnae fixed in 10% (vol/vol) buffered formalin and embedded in paraffin were stained with hematoxylin and eosin. Dermal leukocytes were counted (numbers per square millimeter) at a magnification of ×1000 in the entire length of the ear in a blinded fashion.

Measurement of C3a

Ear skin lysates prepared by using sonication of finely chopped ear pinnae (in 300 μL of T-PER EDTA-free lysis buffer [Pierce, Cheshire, United Kingdom] containing protease inhibitors [Roche, Mannheim, Germany]) were frozen at −80°C overnight, thawed, and centrifuged at 16,000*g* for 20 minutes at 4°C, and C3a concentrations in the supernatants were measured by using ELISA. For details, see the Methods section in this article's Online Repository.

Statistical analyses

ANOVA was used to assess the significance of differences in ear thickness. Unless specified otherwise, differences in the numbers of dermal mast cells or leukocytes or levels of C3a in ear lysates were tested for significance by using the 2-tailed Mann-Whitney *U* test. We used the χ^2 test to compare values for the extent of mast cell degranulation. For details, see the Methods section in this article's Online Repository.

RESULTS

Mast cells can enhance ear-swelling responses to C3a and C5a in a complement receptor–dependent manner

Ear-swelling responses after intradermal injection of C3a (Fig 1, *A*) or C5a (Fig 1, *B*) peaked at 30 minutes and were significantly lower in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice compared with those in WT control animals after the injection of either anaphylatoxin (Fig 1). *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice did not detectably respond to C3a (see Fig E1 in this article's Online Repository at www.jacionline.org). There was a small but significant increase in the ear-swelling response observed in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice after injection with C5a versus vehicle ($P < .0001$), suggesting that a mast cell–independent pathway might contribute to this response. We did not observe any ear-swelling responses in WT mice after injection with 1 μg of C3 or C5 (data not shown), suggesting that these precursors need to be cleaved to induce a cutaneous response.

Kit$^{W\text{-}sh/W\text{-}sh}$ mice have several abnormalities beside a profound deficiency in mast cells,[28-31] including increased numbers of blood neutrophils.[29-31] To assess whether the adoptive transfer of mast cells to the skin of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice would enhance their ability to respond to challenge with C3a or C5a, we engrafted these mice with WT BMCMCs. *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice engrafted with WT mast cells (WT BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice) exhibited C3a- or C5a-induced ear-swelling responses similar to those observed in WT C57BL/6J mice (Fig 1). By contrast, *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice engrafted with *C3aR*$^{-/-}$ BMCMCs (*C3aR*$^{-/-}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice) did not exhibit an ear-swelling response to C3a above background values (see Fig E1) or that observed in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (Fig 1, *A*) but exhibited responses to C5a comparable with those seen in WT BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org). Similarly, *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice engrafted with *C5aR*$^{-/-}$ BMCMCs (*C5aR*$^{-/-}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice) did not exhibit ear swelling after injection of C5a (Fig 1, *B*), but their C3a-induced response was similar to that observed in WT BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (see Fig E2, *B*). *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice engrafted with WT, *C3aR*$^{-/-}$, or *C5aR*$^{-/-}$ BMCMCs had similar numbers of ear dermal mast cells, but the number in *C5aR*$^{-/-}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice was significantly lower than that in the WT BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (see Fig E3 in this article's

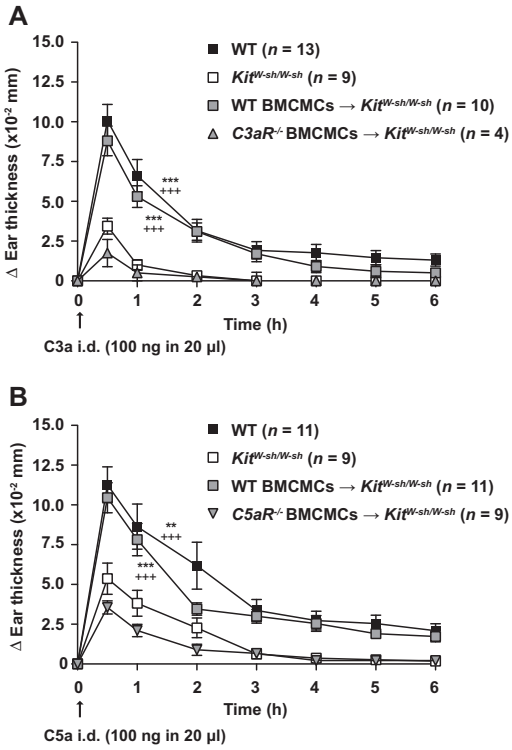

FIG 1. Mast cells enhance C3a- or C5a-induced tissue swelling in a C3aR- or C5aR-dependent manner, respectively. Changes *(Δ)* in ear thickness in mice after intradermal *(i.d.)* injection of 100 ng of C3a **(A)** or C5a **(B)** are shown. $^{**}P < .01$ and $^{***}P < .001$ versus $Kit^{W\text{-}sh/W\text{-}sh}$ mice. $+++P < .001$ versus $C3aR^{-/-}$ BMCMCs→$Kit^{W\text{-}sh/W\text{-}sh}$ mice (Fig 1, *A*) or $C5aR^{-/-}$ BMCMCs→$Kit^{W\text{-}sh/W\text{-}sh}$ mice (Fig 1, *B*).

Online Repository at www.jacionline.org). For all mouse groups studied, the vehicle-induced ear-swelling responses were much weaker than those induced by the anaphylatoxins (see Fig E1).

Although it has been difficult to implicate antibody isotypes other than IgE in the pathogenesis of allergen-induced anaphylaxis in human subjects, both IgE and IgG_1 antibodies can contribute to anaphylaxis in mice.[1,4,30,32] To determine whether C3a- or C5a-induced responses involved effects of endogenous IgE or IgG_1 antibodies mediated through FcεRI or FcγRIII, we assessed such responses in Fc receptor common γ chain–deficient *($Fcer1g^{-/-}$)* mice.[33] Ear-swelling responses to C3a (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org) or C5a (see Fig E4, *B*) in $Fcer1g^{-/-}$ mice were comparable with those in WT control animals and significantly higher than those induced by vehicle (see Fig E4), indicating that such responses can occur independently of the FcεRIγ. Moreover, in $Kit^{W\text{-}sh/W\text{-}sh}$ mice engrafted with $Fcer1g^{-/-}$ BMCMCs, the C3a- or C5a-induced ear-swelling responses were similar to those in WT BMCMCs→$Kit^{W\text{-}sh/W\text{-}sh}$ mice (data not shown).

IgE-dependent mast cell activation is sufficient for local C3a production during PCA reactions

We measured changes in ear thickness, skin mast cell degranulation, and levels of endogenous C3a after intravenous challenge with specific antigen in IgE-sensitized versus vehicle-injected ear pinnae of WT C57BL/6J mice and B6.129S2-Igh-6^{tm1Cgn}/J mice, which lack all endogenous antibodies (Fig 2). In B6.129S2-Igh-6^{tm1Cgn}/J mice, which have levels of ear pinnae (see Fig E5, *A*, in this article's Online Repository at www.jacionline.org) and peritoneal (data not shown) mast cells and peritoneal mast cell surface FcεRI and Kit levels (see Fig E5, *B*) comparable with those of WT mice, IgE-dependent PCA reactions were associated with levels of ear swelling (Fig 2, *A*), skin mast cell degranulation (Fig 2, *B*), and skin C3a (Fig 2, *C*) that were even greater than those in WT mice. These results support the conclusion that C3a is produced locally during IgE-dependent PCA reactions and that functions of native antibodies, including any role in the classical pathway of complement activation, are not required for the tissue swelling, mast cell degranulation, or increase in tissue levels of C3a at sites of IgE-dependent PCA. By contrast, $Fcer1g^{-/-}$ mice did not have tissue swelling, enhanced mast cell degranulation, or increased tissue levels of C3a at sites challenged to assess IgE-dependent PCA (Fig 3). As predicted, we did not observe a swelling response or mast cell degranulation in the absence of IgE (vehicle-treated ears) or FcεRIγ (Fig 3). Taken together, the data in Figs 2 and 3 indicate that IgE-dependent activation of skin mast cells through the FcεRIγ chain is sufficient to activate complement at sites of IgE-dependent PCA.

C3aR and C5aR expression by mast cells can contribute to the features of IgE-dependent PCA reactions

We found that the ear swelling associated with PCA reactions in C3aR- or C5aR-deficient mice were markedly reduced in mice lacking C3aR or the C5aR compared with those in WT mice (Fig 4). However, the ear-swelling responses in the C5aR-deficient mice were slightly ($P < .05$) higher than those in the C3aR-deficient mice (Fig 4). We observed no significant ear swelling in the vehicle-treated ears of these mice (data not shown), and the anatomic distribution of mast cells in the ears of WT, $C3aR^{-/-}$, and $C5aR^{-/-}$ mice were similar (data not shown). Moreover, despite the decreased PCA responses observed in $C3aR^{-/-}$ versus WT mice, we observed slightly higher numbers of mast cells in the ears of these mice compared with those seen in WT or $C5aR^{-/-}$ mice (see Fig E6 in this article's Online Repository at www.jacionline.org). Similar levels of FcεRI and Kit were detected on peritoneal mast cells from $C3aR^{-/-}$, $C5aR^{-/-}$, and WT mice (see Fig E7 in this article's Online Repository at www.jacionline.org).

The PCA-induced ear swelling in WT BMCMCs→$Kit^{W\text{-}sh/W\text{-}sh}$ mice was significantly greater than that in $Kit^{W\text{-}sh/W\text{-}sh}$ mice (Fig 5, *A*) and was similar to the response observed in WT mice (Fig 4). Importantly, the BMCMCs derived from $C3aR^{-/-}$ or $C5aR^{-/-}$ mice that were used in our engraftment studies showed similar levels of FcεRI expression compared with that seen in WT BMCMCs by means of flow cytometry (data not shown). However, engraftment of $Kit^{W\text{-}sh/W\text{-}sh}$ mice with $C3aR^{-/-}$ or $C5aR^{-/-}$ BMCMCs did not restore the PCA-induced ear swelling response to the levels observed in WT BMCMCs→$Kit^{W\text{-}sh/W\text{-}sh}$ mice (Fig 5, *A*). Similar to the results we obtained using the C3aR- and C5aR-deficient mice (Fig 4), the PCA-induced ear-swelling response was greater in $C5aR^{-/-}$ BMCMCs→$Kit^{W\text{-}sh/W\text{-}sh}$ mice than in $C3aR^{-/-}$ BMCMCs→$Kit^{W\text{-}sh/W\text{-}sh}$ mice (Fig 5, *A*). These data suggest that expression of C3aR or C5aR on mast cells contributes significantly to ear-swelling responses during

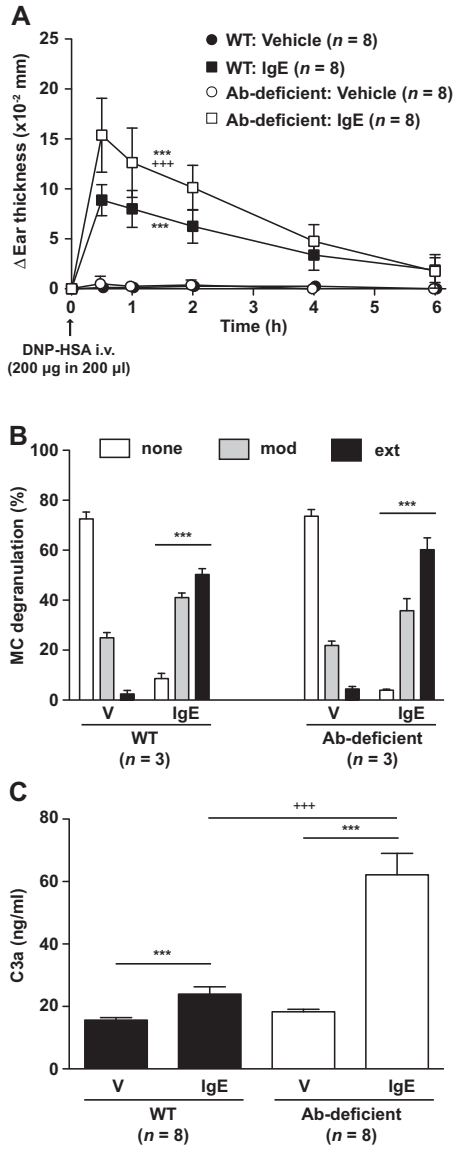

FIG 2. Ear swelling and C3a production during IgE-dependent PCA reactions are not dependent on the presence of native antibodies *(Ab)*. **A,** Changes *(Δ)* in ear thickness after intravenous *(i.v.)* infection of 200 mg of DNP-HSA into WT (C57BL/6) and antibody-deficient (B6.129S2-Igh-6^{tm1Cgn}/J) mice 24 hours after intradermal injection of vehicle (left ears) or 20 ng of anti-DNP IgE (right ears). ***$P < .001$ versus corresponding vehicle control. +++$P < .001$ versus IgE/DNP-HSA–treated WT group. **B,** Percentage of mast cells *(MC)* in vehicle *(V)*– or IgE-injected pinnae exhibiting extensive *(ext)*, moderate *(mod)*, or no *(none)* degranulation 6 hours after intravenous DNP-HSA challenge. ***$P < .001$ versus corresponding vehicle group. $P = .16$ for the IgE/DNP-HSA–treated WT group versus the IgE/DNP-HSA–treated antibody-deficient group. **C,** C3a in ear lysates 2 hours after intravenous injection of 200 μg of DNP-HSA into WT or antibody-deficient mice 24 hours after intradermal injection of vehicle (*V*; left ears) or 20 ng of anti-DNP IgE (right ears). ***$P < .001$ versus vehicle. +++$P < .001$ versus the IgE/DNP-HSA–treated WT group.

IgE-dependent PCA reactions. We also quantified leukocytes in the dermis 6 hours after inducing PCA. The reactions in ears that had been engrafted with C3aR- or C5aR-deficient mast cells contained, respectively, approximately 50% or 60% fewer leukocytes (predominantly neutrophils and mononuclear cells) than did reactions in ears that had been engrafted with WT mast cells (Fig 5, *B*).

We also assessed the extent of mast cell degranulation in IgE-dependent PCA reactions. In WT BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice we observed extensive degranulation in approximately 70% of the ear dermal mast cells 6 hours after challenge with antigen (Fig 5, *C*). The extent of mast cell degranulation in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice engrafted with C3aR- or C5aR-deficient mast cells (approximately 35% or 15% extensively degranulated mast cells, respectively) was significantly reduced compared with that observed in WT BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (Fig 5, *C*). However, in all groups studied the extent of mast cell degranulation observed at sites of IgE-dependent PCA was significantly greater than that in the contralateral vehicle-injected ears (Fig 5, *C*).

DISCUSSION

Our results in mast cell–engrafted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice show that expression of anaphylatoxin receptors by the adoptively transferred mast cells can both markedly increase the skin swelling associated with intradermal injection of C3a or C5a and also markedly increase the mast cell degranulation, skin swelling, and cutaneous leukocyte infiltration associated with IgE-dependent PCA responses. Although our study focused on skin mast cells in mice, various types of rodent or human mast cells can react to C3a, C5a, or both[7-10,12-19]; C3a or C5a can stimulate human skin mast cell chemotaxis,[14] as well as degranulation,[9,10] *in vitro*; and C5a can stimulate such cells to produce the neutrophil chemotaxin IL-8.[9] In addition, injection of C3a or C5a into normal mouse or human skin can result in wheal-and-flare reactions associated with dermal mast cell degranulation and polymorphonuclear leukocyte–rich inflammatory infiltrates.[8,17-19] Our findings in mast cell–engrafted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice indicate that much of the local inflammation induced by the intradermal injection of C3a or C5a requires that the mast cells at the site of the reaction express the appropriate anaphylatoxin receptors.

Our results also show that C3a and C5a, in contrast to bradykinin,[34] induce substantially less edema on intradermal injection in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice than in the corresponding WT mice. We found that C3a-induced skin swelling depended almost entirely on mast cells, whereas both mast cell–dependent and mast cell–independent pathways appeared to contribute to C5a-mediated responses (ie, some C5a-induced tissue swelling was observed in mice whose skin virtually lacked mast cells). These results are in accord with previous findings of neutrophil-rich infiltrates in mast cell-depleted human skin after intradermal challenge with C5a.[19]

We were particularly interested in the extent to which skin mast cell activation by anaphylatoxins reflected mast cell expression of the relevant anaphylatoxin receptors. Many studies have reported the expression of C3aR and C5aR on mast cell populations,[11,13,35,36] and a blocking antibody against C5aR can reduce C5a-mediated degranulation of human skin mast cells *in vitro*, which is evidence that C5a can directly activate human skin mast cells through C5aR.[11] However, other groups have reported that C3a and C5a, which are cationic, can bind nonspecifically to rat peritoneal mast cells through electrostatic interactions with anionic heparin-proteoglycan on the mast cell surface.[37-39] C3a- or C5a-induced ear-swelling responses were significantly reduced in mast cell–engrafted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, the skin mast cells of which lacked C3aR or C5aR, respectively, confirming that the binding of each anaphylatoxin to its specific receptors

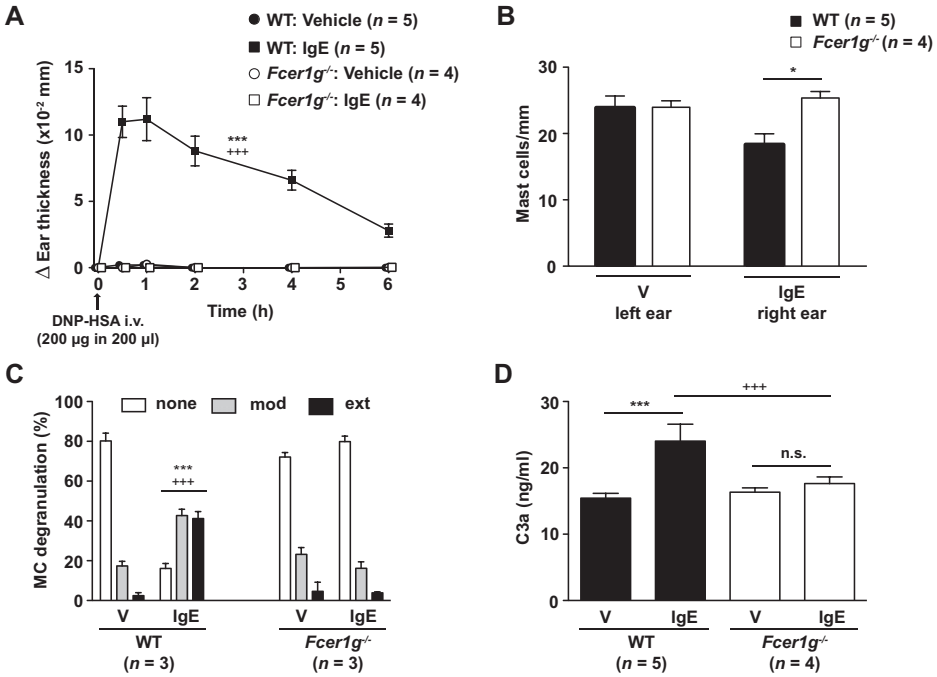

FIG 3. IgE-dependent PCA reactions require the FcR γ chain. **A,** Changes *(Δ)* in ear thickness after intravenous *(i.v.)* injection of 200 μg of DNP-HSA into mice 24 hours after intradermal injection of vehicle (left ears) or 20 ng of anti-DNP IgE (right ears). ***P < .001 versus the corresponding vehicle-injected group. +++P < .001 versus the IgE/DNP-HSA–treated *Fcer1g*$^{-/-}$ group. **B,** Numbers of mast cells per millimeter of ear cartilage were counted in toluidine blue–stained ear pinnae sections prepared from WT or *Fcer1g*$^{-/-}$ mice (data for vehicle *[V]*– or IgE-injected ears). *P < .05. **C,** Percentage of mast cells *(MC)* in vehicle *(V)*– or IgE-injected pinnae exhibiting extensive *(ext)*, moderate *(mod)*, or no *(none)* degranulation 6 hours after intravenous DNP-HSA challenge. ***P < .001 versus the corresponding vehicle group. +++P < .001 versus the IgE/DNP-HSA–treated *Fcer1g*$^{-/-}$ group. **D,** C3a in ear lysates 2 hours after intravenous injection of 200 μg of DNP-HSA into WT or *Fcer1g*$^{-/-}$ mice 24 hours after intradermal injection of vehicle (*V*; left ears) or 20 ng of anti-DNP IgE (right ears). ***P < .001 versus the corresponding vehicle-injected group. +++P < .001 versus the IgE/DNP-HSA–treated *Fcer1g*$^{-/-}$ group. *n.s.,* Not significant (P > .05).

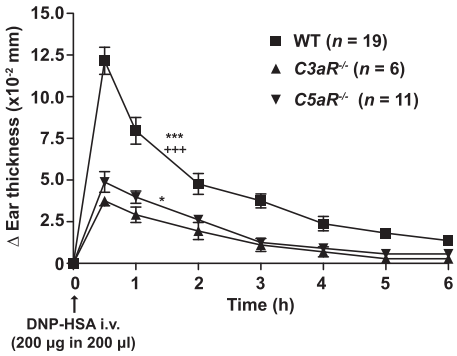

FIG 4. C3aR and C5aR enhance ear swelling during IgE-dependent PCA reactions. Changes *(Δ)* in ear thickness after intravenous *(i.v.)* injection of 200 μg of DNP-HSA into mice 24 hours after intradermal injection of 20 ng of anti-DNP IgE (right ears) are shown *P < .05 and ***P < .001 versus *C3aR*$^{-/-}$ mice. +++P < .001 versus *C5aR*$^{-/-}$ mice.

on skin mast cells can markedly enhance the skin-swelling response induced by that anaphylatoxin *in vivo*.

Activation of the C5aR pathway has been shown to decrease internalization of C3aR *in vitro* when the 2 anaphylatoxins are added simultaneously,[40] and C5L2, a nonsignaling receptor shared by both anaphylatoxins, can positively modulate both C5a and C3a anaphylatoxin–induced responses.[41] We found that the ear-swelling response to C5a was not influenced by a lack of C3aR expression on mast cells and *vice versa* (see Fig E2), indicating that, at least under the *in vivo* conditions examined, a lack of one of the 2 anaphylatoxin receptors did not detectably influence the functionality of the other. Crosstalk between anaphylatoxins and Fc receptors also has been reported.[42,43] However, we found that a general (see Fig E4) or mast cell–specific (data not shown) deficiency in FcεRIγ did not detectably influence C3a- or C5a-mediated ear-swelling responses, indicating that the cutaneous reactions induced by these anaphylatoxins are not dependent on antibody-induced signaling events, such as effects on mast cells of baseline levels of IgE that are mediated through FcεRI.[44]

We especially wanted to assess whether activation of mast cells through anaphylatoxin receptors could influence the extent of mast cell degranulation and the extent of IgE-induced tissue swelling and leukocyte infiltration during the PCA model of IgE- and mast cell–dependent inflammation. We found that C3a was produced locally during IgE-dependent PCA (Fig 2, *C*). We also detected increased levels of C5a at sites of PCA reactions in some experiments but not others (data not shown), perhaps because 2 hours after antigen challenge is not the optimal time to perform such measurements. However, the mechanism by which anaphylatoxins are generated at sites of IgE-dependent PCA

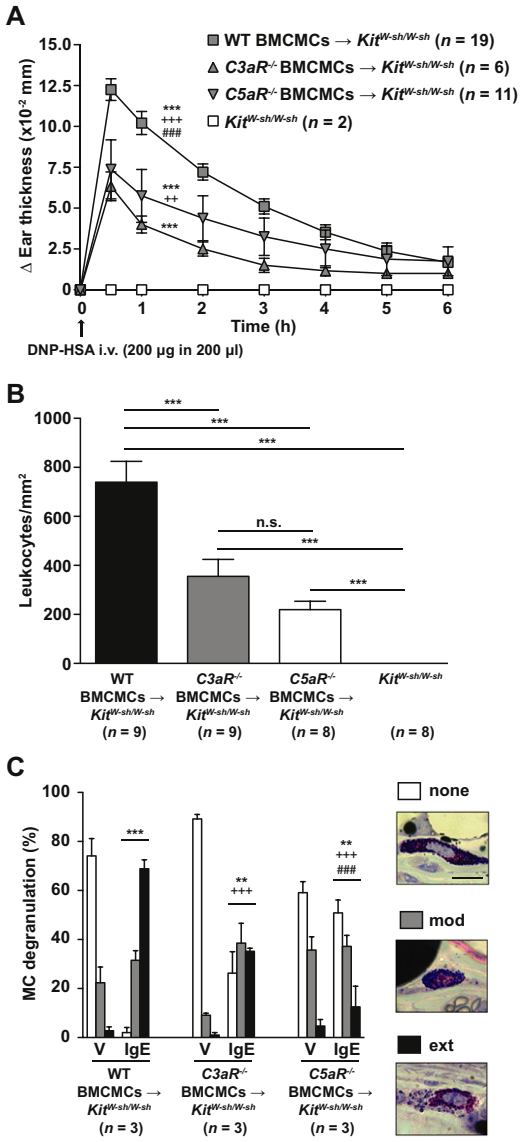

FIG 5. Enhancement of IgE-dependent PCA reactions by mast cell expression of C3aR and C5aR. **A,** Changes *(Δ)* in ear thickness after intravenous *(i.v.)* injection of 200 μg of DNP-HSA into mice 24 hours after intradermal injection of vehicle (left ears) or 20 ng of anti-DNP IgE (right ears). ***$P < .001$ versus $Kit^{W\text{-}sh/W\text{-}sh}$ mice. ++$P < .01$ and +++$P < .001$ versus $C3aR^{-/-}$ BMCMCs→$Kit^{W\text{-}sh/W\text{-}sh}$ mice. ###$P < .001$ versus $C5aR^{-/-}$ BMCMCs→$Kit^{W\text{-}sh/W\text{-}sh}$ mice. No swelling was detected in any of the left (vehicle-injected) ears. **B,** Numbers of leukocytes in the dermis of ear pinnae 6 hours after intravenous DNP-HSA challenge. ***$P < .001$ for the indicated comparisons. *n.s.*, Not significant ($P > .05$). **C,** Percentage of mast cells *(MC)* in vehicle *(V)*– or IgE-injected pinnae exhibiting extensive *(ext)*, moderate *(mod)*, or no *(none)* degranulation 6 hours after intravenous DNP-HSA challenge. *Insets* show mast cells in Giemsa-stained, plastic-embedded 1-μm sections of ear pinnae. *Scale bar* = 10 μm. **$P < .01$ and ***$P < .001$ versus the corresponding vehicle group. +++$P < .001$ versus IgE/DNP-HSA–treated WT BMCMCs→$Kit^{W\text{-}sh/W\text{-}sh}$ mice. ###$P < .001$ versus IgE/DNP-HSA–treated $C3aR^{-/-}$ BMCMCs→$Kit^{W\text{-}sh/W\text{-}sh}$ mice.

remains to be determined. We found no reports indicating that either mouse or human mast cells can produce C3 or C5 and detected neither of the corresponding mRNAs in WT BMCMCs by using real-time PCR (see the Methods section in this article's Online Repository). Cultured WT peritoneal mast cells exhibited only very low levels of transcript for C3 or C5 either at baseline or after stimulation with LPS or IgE and antigen (see the Methods section in this article's Online Repository for details). Thus we think it unlikely that mast cells represent a major source of C3 and C5 at sites of PCA.

Given that IgE does not activate complement and our findings that tissue swelling and C3a levels at sites of IgE-dependent PCA responses actually were enhanced in antibody-deficient mice that lack IgG and IgM (Fig 2), it is unlikely that the classical pathway of complement activation is responsible for the generation of C3a (or C5a) in this model. However, the release of histamine and other vasoactive mediators from mast cells might be sufficient to generate anaphylatoxins because both the injection of histamine into guinea pig skin[45] and IgE-dependent degranulation of cutaneous mast cells in mice[46] result in the rapid local activation of coagulation, which is known in turn to activate complement.[47] Indeed, we have confirmed that intradermal injection of histamine results in the increase in skin levels of C3a in mice (see Fig E8 in this article's Online Repository at www.jacionline.org).

Human β-tryptase can generate anaphylatoxins from C3, C4, and C5 at acidic pH *in vitro,* suggesting that the mast cell protease β-tryptase might generate complement anaphylatoxins *in vivo* at sites of inflammation.[48,49] Although it has not yet been shown that tryptase (of human or mouse origin) can cleave complement components *in vivo*, mast cells contain a number of proteases, and it is tempting to speculate that proteases (or perhaps other mediators) capable of generating anaphylatoxins are generated by activated mast cells at sites of IgE-dependent PCA. In addition to its ability to generate anaphylatoxins, it has been reported that human β-tryptase can degrade C3a[48,49] and C4a[49] *in vitro*. Thus it is possible that anaphylatoxins can be both generated and degraded by tryptase *in vivo*. Similarly, it has been reported that chymase (from rat peritoneal mast cells) is capable of degrading human C3a within minutes *in vitro*[50] and *in vivo*.[51] However, it remains to be determined whether anaphylatoxin degradation by chymase or other mast cell–derived proteases represents a mechanism by which mast cells can regulate tissue levels of anaphylatoxins *in vivo*.

We previously showed that mast cells are required for the local tissue swelling and leukocyte (primarily neutrophil) infiltration at sites of IgE-dependent PCA in mice.[52] In the present study we found that expression of the anaphylatoxin receptors C3aR or C5aR on skin mast cells markedly enhanced IgE- and antigen-induced mast cell degranulation, ear-swelling responses, and leukocyte infiltration at sites of PCA *in vivo* (Fig 5). Although our experiments focused on IgE-dependent PCA, it is possible that anaphylatoxin-dependent augmentation of mast cell function can occur in other settings that involve both complement activation and an effector role for mast cells. For example, a recent article showed that $C5aR^{-/-}$ mice exhibited impaired neutrophil recruitment in a model of immune complex arthritis in which mast cells also have been implicated.[9]

C3aR and C5aR are expressed on antigen-presenting cells and T cells, as well as on mast cells,[53,54] basophils,[54-56] and other hematopoietic cells.[26,57] Accordingly, the mechanism or mechanisms by which the anaphylatoxins C3a and C5a might regulate IgE-dependent allergic inflammation is of interest and has been the subject of debate. Of course, we recognize that there are many known or potential differences between immune responses involving IgE, mast cells, or both in human subjects versus mice.[32,44,58] However, the mast cell C3aR- and C5aR-mediated enhancement of IgE-dependent mast cell

activation observed in our study might explain, at least in part, the positive correlation between complement activation and the severity of anaphylaxis in human and rodent models. It might also shed light on the role of complement activation in patients with asthma and other allergic or pathologic disorders that involve mast cell activation.[53,54,59-62]

We thank Mariola Liebersbach for her support with mouse breeding, Chen Liu for processing slides for histologic analysis, and Jennifer Lilla for help with figure preparation.

Key messages

- **Expression of C3aR and C5aR by skin mast cells significantly increases the ability of intradermal injections of C3a and C5a to induce skin swelling in mice.**
- **C3a is generated locally in the skin at sites of IgE-dependent PCA in mice.**
- **Expression of C3aR and C5aR by skin mast cells significantly increases the ability of IgE and specific antigen to induce mast cell degranulation, skin swelling, and leukocyte infiltration at sites of IgE-dependent PCA in mice.**

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METHODS

Mice

Unless specified otherwise, female mice were used in all experiments. All animal care and experimentation was conducted in accordance with the "Guide for the care and use of laboratory animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press (revised, 1996) and with the approval of the Stanford University Committee on Animal Welfare.

Flow cytometry

BMCMCs or peritoneal cells were incubated for 5 minutes with mAbs to CD16/32 (clone 93) to block nonspecific binding and then stained for 30 minutes with phycoerythrin-conjugated anti-mouse FcεR1α (clone MAR-1; BioLegend, San Diego, Calif) and allophycocyanin-conjugated anti-mouse CD117 (c-Kit, clone 2B8; eBioscience, San Diego, Calif) antibodies. Both antibody stocks were 200 μg/mL, and the antibodies were used at 1 μg/mL for staining. Cells were analyzed on a FACSCalibur flow cytometer (Beckton Dickinson, San Jose, Calif). Dead cells were excluded by means of propidium iodide gating, and data were analyzed (mean fluorescence intensity was calculated with FLOWJO software [TreeStar, Ashland, Ore]). Data for each group were pooled from the 2 or 3 independent experiments performed, each of which provided similar results and are expressed as means + SEMs (Fig E7) or individual data points (Fig E5, *B*).

Quantification of mast cell degranulation

Plastic-embedded 1-μm sections were dried at 60°C overnight. Sides were stained in Giemsa solution at 60°C for 1 hour (Giemsa staining solution: 100 mL of Giemsa stain [SG-28; Fisher Scientific, Hampton] + 450 mL of 2% sodium borate; adjust final pH to 8.2 with 10 N NaOH). Slides were rinsed in tap water for 1 to 2 minutes, destained with 50% ethanol for 3 to 6 dips, and then rinsed with tap water for 3 to 4 dips. (Note: Check the stain intensity at this point and destain more if necessary.) Slides were dried at 60°C (approximately 2 hours), cleared with Histoclear (National Diagnostics, Atlanta, Ga) or Xylene (Sigma-Aldrich, St Louis, Mo), and then cover slipped with Permount (Fisher Scientific). Mast cells were classified by using histomorphometry (at a magnification of ×1000) as extensively degranulated (>50% of cytoplasmatic granules exhibiting staining alterations, fusion, and/or exteriorization), moderately degranulated (10% to 50% of granules affected), or not degranulated (<10% granules affected).[E1] Sections were assessed in a blinded manner.

Measurement of C3a

For C3a ELISA (reagents from BD PharMingen), rat anti-mouse C3a (558250) was used as the capture antibody, biotin rat anti-mouse C3a (558251) was used as the detection antibody, purified murine C3a (558618) was used for the standard, and the detection threshold was approximately 0.8 ng/mL.

Statistical analyses

P values of less than .05 are considered statistically significant. Unless otherwise specified, all data are presented as means ± SEMs or means + SEMs, and, except for the data shown in Figs 2, *B*, and 5, *C*, and the data for $Kit^{W\text{-}sh/W\text{-}sh}$ mice in Fig 5, *A*, each regular figure presents the pooled results of the 2 (Figs 2 and 3) or 3 (all other figures) replicate experiments performed, each of which produced similar results.

Real-time PCR

cDNA isolated from WT BMCMCs and peritoneal cultured mast cells (PCMCs) was analyzed by using real-time PCR with the following primers (written in the 5′-3′ direction): C3 forward, TCTGCCCACCCCT GCCCCTTA; C3 reverse, GGAAGGCCGTACCCCCTCCAT; C5 forward, CTCCGGGTCGGCTCGTCTGA; and C5 reverse, GCGGCTTGTGT GGCTTTGTGC. The comparative cycle threshold method (or $2-\Delta\Delta CT$ method) was used to determine relative levels of C3 and C5 expression (by using glyceraldehyde-3-phosphate dehydrogenase as an internal control); mouse liver RNA was used as a positive control for both C3 and C5 expression. We could not detect mRNA for C3 or C5 in BMCMCs generated from C57BL/6J mice using real-time PCR (data not shown). Similarly, C3 and C5 mRNA levels in PCMCs from C57BL/6J mice were extremely low at rest (8.7535×10^{-5} and 2.7009×10^{-5}, respectively) and 1 hour after activation with LPS (1 μg/mL; 8.7592×10^{-5} and 2.700×10^{-5}, respectively) or IgE plus DNP (2 μg/mL IgE overnight, 10 ng/mL DNP for 1 hour; 1.111×10^{-4} and 1.8566×10^{-5}, respectively). PCMCs were cultured by using a protocol modified from Malbec et al.[E2] Briefly, peritoneal cells were isolated and cultured in RPMI containing 10 ng/mL IL-3 and 30 ng/mL stem cell factor (2.5 mL per mouse). Two days after cultures were started, nonadherent cells were removed, and the media was replaced with an equal volume of fresh media. Fresh media was added on day 5 (2.5 mL per mouse), and nonadherent cells were used for experiments on day 10 (at which point cells were >95% $Fc\varepsilon RI\alpha^{+}$ and Kit^{+}, as analyzed by using flow cytometry). Samples from BMCMCs and PCMCs were run in triplicate, and each experiment was performed twice (by using a 2-fold increase in the amount of cDNA in the second experiment; both experiments for each group of cells produced similar results).

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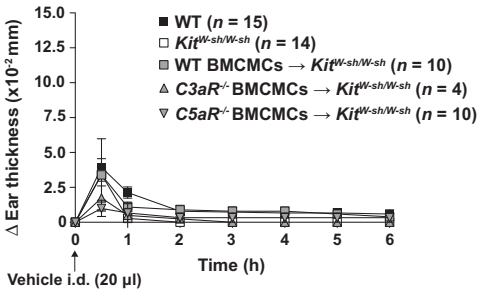

FIG E1. Ear-swelling responses after injection with vehicle. Changes *(Δ)* in ear thickness in WT (C57BL/6) mice, *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, or *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice engrafted with WT (WT BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$), *C3aR*$^{-/-}$ (*C3aR*$^{-/-}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$), or *C5aR*$^{-/-}$ (*C5aR*$^{-/-}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$) BMCMCs before and after intradermal *(i.d.)* injection with vehicle (left ear) are shown. None of the differences between any of the groups achieved statistical significance (defined as $P < .05$) by means of ANOVA. These data are from the same mice whose results for C3a- or C5a-injected right ears are shown in Fig 1.

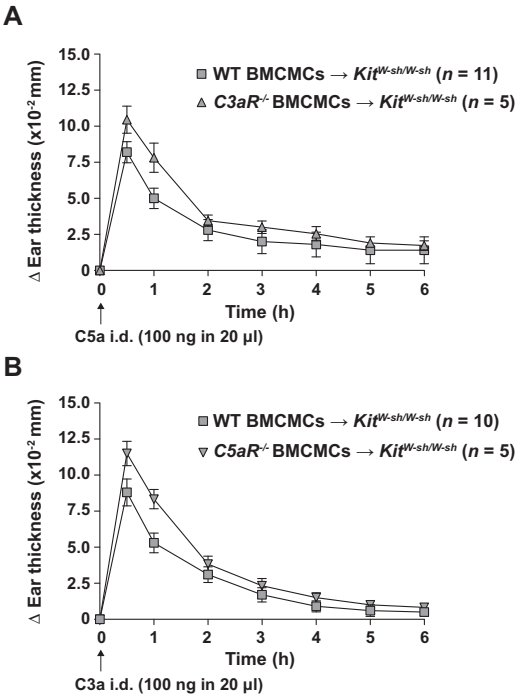

FIG E2. C3a- or C5a-induced ear swelling is not dependent on mast cell expression of C5aR or the C3aR, respectively. **A,** Changes *(Δ)* in ear thickness in $Kit^{W\text{-}sh/W\text{-}sh}$ mice engrafted with WT (C57BL/6; (WT BMCMCs→$Kit^{W\text{-}sh/W\text{-}sh}$) or $C3aR^{-/-}$ ($C3aR^{-/-}$ BMCMCs→$Kit^{W\text{-}sh/W\text{-}sh}$) BMCMCs before and after intradermal *(i.d.)* injection with 100 ng of C5a. **B,** Changes *(Δ)* in ear thickness in $Kit^{W\text{-}sh/W\text{-}sh}$ mice engrafted with WT (WT BMCMCs→$Kit^{W\text{-}sh/W\text{-}sh}$) or $C5aR^{-/-}$ ($C5aR^{-/-}$ BMCMCs→$Kit^{W\text{-}sh/W\text{-}sh}$) BMCMCs before and after intradermal *(i.d.)* injection with 100 ng of C3a. In Fig E2, *A* and *B*, data for each group were pooled from the 3 independent experiments performed, each of which produced similar results, and are expressed as means ± SEMs; neither of the differences between the groups in Fig E2, *A* or *B*, achieved statistical significance (defined as $P < .05$) by means of ANOVA.

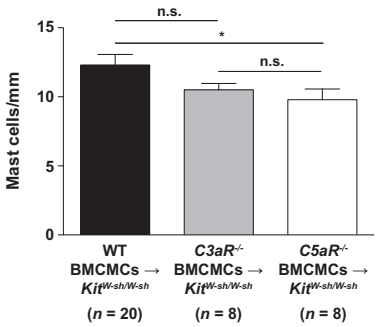

FIG E3. Dermal mast cell numbers in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice engrafted in ear pinnae with WT (C57BL/6; WT BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$), *C3aR*$^{-/-}$ (*C3aR*$^{-/-}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$), or *C5aR*$^{-/-}$ (*C5aR*$^{-/-}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$) BMCMCs. BMCMCs were resuspended at 5×10^7 cells/mL in Dulbecco modified Eagle medium and transferred by means of intradermal injection (each ear received 2 injections; 1×10^6 cells/20 μL into each of 2 sites) into 4-week-old female *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. Numbers of mast cells per millimeter of ear cartilage were counted in toluidine blue–stained ear pinnae sections prepared 8 weeks after mast cell engraftment. Data for each group were pooled from the 3 independent experiments performed, each of which produced similar results, and are expressed as means + SEMs. $*P < .05$ for the indicated comparisons by using the 2-tailed Mann-Whitney *U* test. *n.s.*, Not significant ($P > .05$).

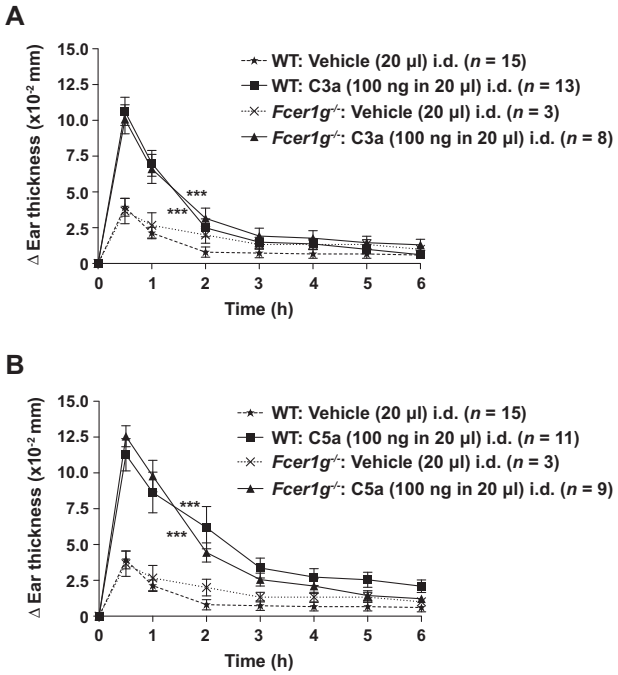

FIG E4. Ear swelling induced by C3a or C5a occurs independently of the Fc receptor γ chain. Changes *(Δ)* in ear thickness in WT (C57BL/6) mice or C57BL/6-Fc receptor γ chain–deficient *(Fcer1g$^{-/-}$)* mice before and after intradermal *(i.d.)* injection with vehicle (left ear) or 100 ng of C3a **(A)** or C5a (**B**; right ear). Data for each group were pooled from the 3 independent experiments performed, each of which produced similar results, and are expressed as means ± SEMs. ***$P < .001$ versus the corresponding vehicle-injected group by means of ANOVA.

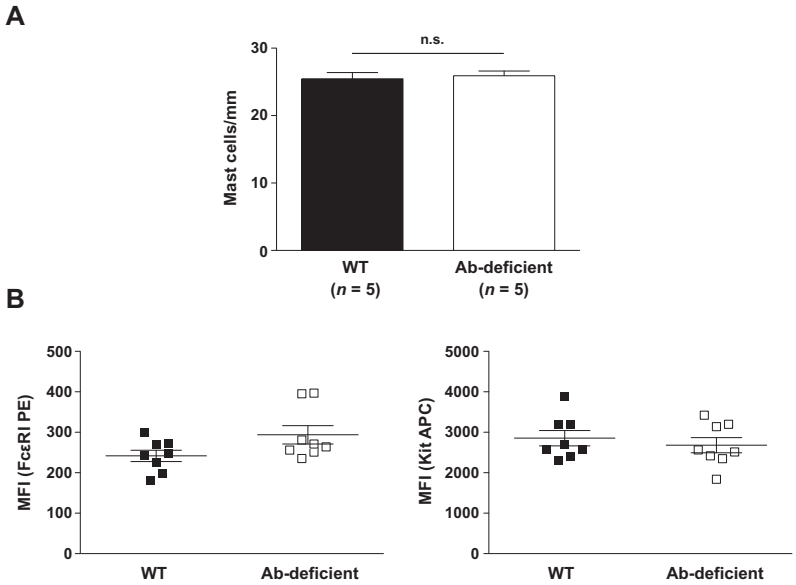

FIG E5. Dermal mast cell numbers in ear pinnae and FcεRI and Kit expression levels on peritoneal mast cells from WT (C57BL/6) or antibody *(Ab)*–deficient (B6.129S2-Igh-6^{tm1Cgn}/J) mice. **A,** Numbers of mast cells per millimeter of ear cartilage were counted in toluidine blue–stained ear pinnae sections prepared from WT or antibody-deficient mice. Data for each group were pooled from 2 independent experiments and are expressed as means + SEMs. *n.s.*, Not significant ($P > .05$). **B,** Surface FcεRI *(left panel)* and Kit *(right panel)* expression levels on peritoneal mast cells from WT or antibody-deficient mice were analyzed by using flow cytometry. *APC*, Allophycocyanin; *MFI*, mean fluorescence intensity; *PE*, phycoerythrin.

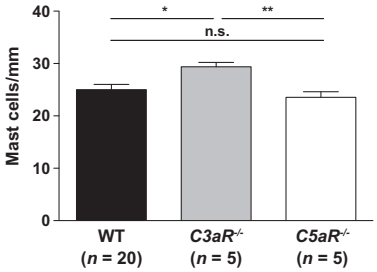

FIG E6. Dermal mast cell numbers in ear pinnae of WT (C57BL/6), *C3aR*$^{-/-}$, or *C5aR*$^{-/-}$ mice. Numbers of mast cells per millimeter of ear cartilage were counted in toluidine blue–stained ear pinnae sections prepared from WT, *C3aR*$^{-/-}$, or *C5aR*$^{-/-}$ mice. Data for each group were pooled from 2 independent experiments and are expressed as means + SEMs. $*P < .05$ and $**P < .001$ for the indicated comparisons by using the 2-tailed Mann-Whitney *U* test. *n.s.*, Not significant ($P > .05$).

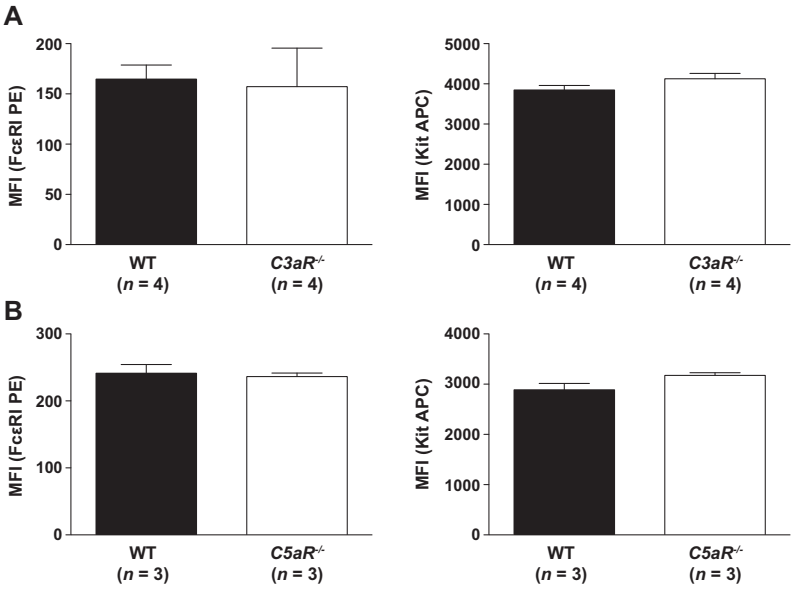

FIG E7. FcεRI and Kit expression levels on peritoneal mast cells from WT (C57BL/6), *C3aR*$^{-/-}$, or *C5aR*$^{-/-}$ mice. Surface FcεRI *(left panels)* and Kit *(right panels)* expression levels on peritoneal mast cells from WT or *C3aR*$^{-/-}$ mice **(A)** and WT or *C5aR*$^{-/-}$ mice **(B)** were analyzed by means of flow cytometry. Data for each group were pooled from the 3 independent experiments performed, each of which produced similar results, and are expressed as means + SEMs. *APC*, Allophycocyanin; *MFI*, mean fluorescence intensity; *PE*, phycoerythrin.

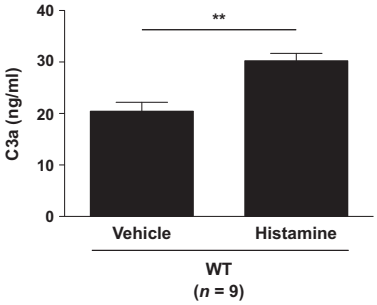

FIG E8. Intradermal injection of histamine results in increased levels of skin C3a. C3a levels in ear lysates 2 hours after intradermal injection of 2 μg of histamine in 20 μL of HMEM-Pipes buffer in the right ear pinna of 9- or 7-week-old male or female WT (C57BL/6J) mice, respectively are shown; 20 μL of vehicle was injected intradermally into the left ear pinna as a control. Mice were under isoflurane anesthesia at the time of injection of histamine and vehicle. The amount of histamine injected was selected based on a pilot experiment showing that the change *(Δ)* in ear thickness observed 30 minutes after intradermal injection of 2 μg of histamine was approximately 10×10^{-2} mm above the change observed in the vehicle-injected ear, which was calculated as follows: [(Ear thickness after intradermal injection of 2 μg of histamine) − (Baseline ear thickness of right ear)] − [(Ear thickness after intradermal injection of vehicle) − (Baseline ear thickness of the left ear)], which is similar to the amount of swelling induced by injecting specific antigen (DNP-HSA) intravenously into WT mice that had been passively sensitized 24 hours earlier by an intradermal injection of IgE (see Figs 2, *A*, and 3, *A*). $^{**}P < .01$ versus vehicle by using the 2-tailed Mann-Whitney *U* test (n = 9 mice, with data pooled from 2 separate experiments that produced similar results).