

Basophils Play a Critical Role in the Development of IgE-Mediated Chronic Allergic Inflammation Independently of T Cells and Mast Cells

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Summary

The recruitment of basophils into the sites of allergic inflammation is often observed. However, no definitive evidence has been provided that basophils are crucially involved in the pathogenesis of chronic allergic disorders. Here, we show that basophils are responsible for the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells. A single subcutaneous injection of multivalent antigens elicited not only immediate- and late-phase ear swelling but also delayed-onset ear swelling with massive eosinophil infiltration in mice sensitized with antigen-specific IgE. Mast cells were essential for the immediate- and late-phase ear swelling but dispensable for the delayed one. T cells were also dispensable for the latter. Transfer of FcεRI-expressing basophils into FcεRI-deficient mice restored the development of the delayed-onset allergic inflammation. These findings indicate a novel mechanism of development of chronic allergic inflammation that is induced by basophils through the interaction of antigen, IgE, and FcεRI.

Introduction

Allergen challenge of sensitized individuals can elicit two types of allergic responses that occur typically in a distinct temporal pattern: the immediate-phase reaction develops within minutes of allergen exposure,

whereas the late-phase reaction develops within hours. The major features of the immediate-phase reaction and at least some features of the late-phase reaction can be explained by the action of chemical mediators, cytokines, and chemokines, released from mast cells, and perhaps also from basophils, which become activated through IgE-mediated antigen stimulation (Galli et al., 2005; Gould et al., 2003; Oettgen and Geha, 1999; Turner and Kinet, 1999). In contrast, there is less agreement about the importance of IgE and mast cells/basophils in chronic allergic inflammation that can persist for days to years as observed in chronic allergic disorders such as asthma and atopic dermatitis (Leung and Bieber, 2003; Oettgen and Geha, 2001; Umetsu et al., 2002; Wedemeyer and Galli, 2000).

Both mast cells and basophils express the αβγ2 form of FcεRI on their surface and can be activated to secrete an array of allergy mediators upon crosslinking of FcεRI bound IgE with antigens (Galli et al., 2005; Gould et al., 2003; Oettgen and Geha, 1999; Turner and Kinet, 1999). Despite these common features, they differ in natural history and localization. Mast cells typically do not circulate in the blood in mature state but complete their differentiation in vascularized tissues and have long life spans. On the other hand, basophils typically mature in the bone marrow, then circulate in the peripheral blood and have a short life span of several days. They represent less than 1% of peripheral blood leukocytes, making them one of the least common cell lineages found in the peripheral blood. Therefore, basophils were often considered as minor and possibly redundant “circulating mast cells.” The recent finding that basophils readily generate large quantities of Th2 cytokines has provided new insights into the possible role of basophils in allergic diseases and immunity to pathogens (Falcone et al., 2000; Min et al., 2004; Schroeder et al., 2001; Seder et al., 1991; Voehringer et al., 2004). However, no clear evidence has been demonstrated that basophils play a crucial role in the pathogenesis of chronic allergic disorders. This is partly due to the limitations of allergy models available to address the issue. Mutant mouse strains deficient only in basophils have not yet been established.

It is generally considered that chronic allergic inflammation is mediated mainly by T cells. Higher levels of serum IgE in atopic patients could simply reflect the increased expression of Th2 cytokines often observed under allergic conditions. Interestingly, however, recent clinical trials have demonstrated that the severity of symptoms was improved by treatment with humanized anti-IgE antibody not only in allergic rhinitis patients but also in some poorly controlled asthma patients (Busse et al., 2001; Holgate et al., 2004, 2005), indicating that IgE may be involved in the pathogenesis of chronic allergic inflammation.

We have previously established an allergy model in mice carrying the transgene encoding IgE specific to the hapten 2,4,6-trinitrophenol (TNP) (Matsuoka et al., 1999). Intravenous injection of the corresponding antigen elicits typical systemic anaphylaxis in these IgE

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transgenic mice (Matsuoka et al., 1999). We found that a single subcutaneous injection of the antigen without any prior immunization elicited not only immediate-type but also delayed-type ear swelling in these mice (Sato et al., 2003). The immediate-phase (within 1 hr after antigen challenge) ear swelling and the late-phase (6–10 hr) swelling were followed by much more intense swelling that began 2 days later and peaked on day 4. Massive infiltration of inflammatory cells, such as eosinophils and neutrophils, as well as hyperplastic epidermis with hyperkeratosis were observed in the third-phase ear swelling. These findings suggested that IgE was involved not only in immediate-type but also in chronic allergic inflammation under these experimental settings. However, it remains to be determined how IgE contributes to chronic allergic inflammation.

In the present study, we defined cells and molecules essential for the development of IgE-mediated chronic allergic inflammation by analyzing a panel of mutant mice and by performing cell transfer experiments. Unexpectedly, both mast cells and T cells were dispensable. We found that high-affinity IgE receptor FcεRI-expressing basophils were essential for the development of delayed-type allergic inflammation even though they constituted a minority of cells infiltrating into the skin lesions. These findings unmasked the critical role of basophils in the development of IgE/FcεRI-mediated chronic allergic inflammation that is independent of T cells and mast cells, raising the potential for new therapeutic strategies in chronic allergic disorders.

Results

T Cells Are Dispensable for IgE-Mediated Chronic Allergic Inflammation

As we reported previously (Sato et al., 2003), a single subcutaneous administration of TNP-conjugated ovalbumin (OVA), but not OVA, elicited triphasic ear swelling in unimmunized BALB/c mice carrying the TNP-specific IgE transgene (Figure 1A). The immediate-phase swelling peaked within 1 hr after antigen challenge. The following late-phase (6–10 hr) swelling was less intense, and the peak of the swelling was often obscure. The third-phase swelling starting 1 or 2 days later was immense and peaked on day 3 or 4, in that ears became congested (Figure 1B) and ear thickness became twice the basal level or that of ears challenged with control OVA (Figure 1A). We found that a comparable triphasic ear swelling was elicited when normal BALB/c or C57BL/6 mice were pretreated with the intravenous administration of TNP-specific IgE one day before antigen challenge (Figures 1C and 2A). This reaction was not due to nonspecific stimulation by TNP-OVA or any immunomodulatory contaminant in the IgE sample, as the TNP-OVA-induced ear swelling was not elicited in mice untreated or pretreated with the same volume of ascites containing comparable amounts of irrelevant IgE (Figure 1C). Histopathological examination of the ear skin in third-phase swelling revealed massive infiltration of inflammatory cells, including eosinophils and neutrophils (Figure 2D, upper panels), as observed in IgE transgenic mice (Sato et al., 2003). The magnitude

of third-phase ear swelling correlated with the dose of IgE injected for sensitization as in the case of immediate- and late-phase ear swelling, and as little as 11 μg of IgE could elicit third-phase ear swelling (Figure 1D). The findings in nontransgenic mice indicated that the third-phase ear swelling was not an artifact derived from the transgenic expression of IgE and made it easier to examine the incidence of the third-phase ear swelling in various mutant mice, avoiding tedious crossbreeding with the transgenic mice. We designated the third-phase ear swelling response as IgE-mediated chronic allergic inflammation (IgE-CAI).

The two observations, the delayed onset of IgE-CAI and the efficient suppression of IgE-CAI by cyclosporin A (Sato et al., 2003), prompted us to examine the possibility that T cells might play an important role in IgE-CAI even though the mice had not been sensitized with the antigen prior to challenge. In contrast to our expectations, RAG-2-deficient mice lacking both T and B cells (Shinkai et al., 1992) developed IgE-CAI comparable to that observed in control normal mice in terms of ear swelling and histopathological features, including eosinophil infiltration (Figures 2B and 2D, lower panels). This was also the case in nude mice (data not shown). Thus, T cells were dispensable for IgE-CAI ear swelling and eosinophil infiltration. IgE-CAI was also elicited in mice deficient for both RAG-2 and γc (Cao et al., 1995) (Figure 2C), indicating that NK and NKT cells were also dispensable.

FcεRI, but Not CD23, Is Essential for IgE-CAI

There are two types of IgE receptors: high-affinity FcεRI and low-affinity CD23 (Gould et al., 2003). To determine which type is involved in IgE-CAI, mice deficient for either receptor were analyzed for their ability to develop IgE-CAI. CD23-deficient mice (Fujiwara et al., 1994) could mount all three types (immediate, late, and third phase) of ear swelling, whereas FcRγ-deficient mice lacking FcεRI expression (Takai et al., 1994) developed none of them (Figures 3A and 3B). Furthermore, mice carrying a transgene encoding either the human FcεRIα chain or humanized TNP-specific IgE did not develop IgE-CAI upon challenge with TNP-OVA, whereas those carrying both of the transgenes did develop IgE-CAI after immediate- and late-phase ear swelling (Figure 3C). These results clearly indicated that IgE and its high-affinity receptor FcεRI played essential roles in the development of IgE-CAI.

Mast Cells Are Dispensable for IgE-CAI

We reported previously that high-valence antigens such as TNP11-OVA, but not low-valence antigens such as TNP2-OVA and TNP5-OVA, elicited IgE-CAI even though both could induce immediate-type hypersensitivity (Sato et al., 2003). We assumed that strong, but not milder, crosslinking of FcεRI might stimulate mast cells in the skin to produce higher levels or distinct types of chemotactic factors that recruit inflammatory cells such as eosinophils to the site of IgE-CAI. However, WBB6F1-W/W^v mice deficient for mast cells developed IgE-CAI comparable to that in control WBB6F1-+/+ mice (Figures 3D and 3E) even though the

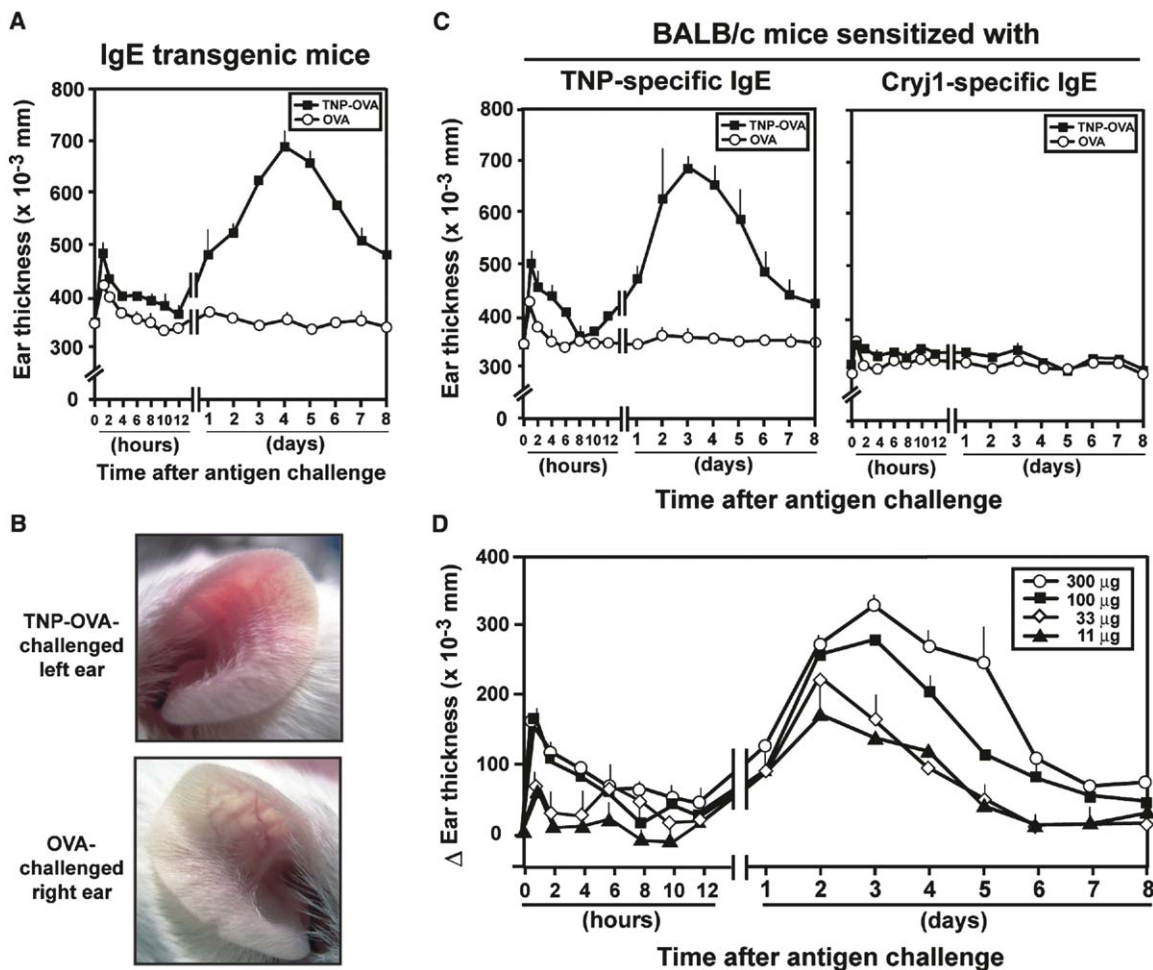


Figure 1. IgE-Mediated, Antigen-Specific Triphasic Ear Swelling

(A) The ear thickness of TNP-specific IgE transgenic BALB/c mice ($n = 5$) was measured at the indicated time points after subcutaneous administration of 10μ g of TNP11-OVA into the left ear (closed squares) and 10μ g of OVA in the right ear (open circles). The kinetics of changes in ear thickness after antigen challenge is shown. Data are expressed as the mean $\pm$ SEM and are representative of five repeated experiments.

(B) Photographs of right and left ears were taken 4 days after antigen challenge.

(C) Normal BALB/c mice ($n = 5$ in each group) were passively sensitized with i.v. injection of 200μ l of diluted ascites containing 300μ g of TNP-specific IgE (left) or similarly diluted ascites containing comparable amounts of Cryj1-specific IgE (right) 1 day before the antigens were administered as in (A). Unsensitized mice responded to the antigen challenge as those sensitized with Cryj1-specific IgE did (Sato et al., 2003).

(D) Normal BALB/c mice ($n = 3$ in each group) were passively sensitized with 300 (open circle), 100 (closed square), 33 (open diamond), or 11 (closed triangle) μ g of TNP-specific IgE 1 day before the antigens were administered as in (A). Thickness of left and right ears was measured at the indicated time points. The values of Δ ear thickness, the differences in ear thickness (left – right) at each time point, are plotted. Data are expressed as the mean $\pm$ SEM and are representative of three repeated experiments.

former did not show immediate- or late-phase ear swelling upon subcutaneous antigen challenge (Figure 3D) or systemic anaphylaxis upon intravenous antigen challenge (data not shown), in contrast to the latter. Thus, mast cells were essential for immediate- and late-phase ear swelling but were dispensable for IgE-CAI. T cell depletion by anti-CD3 mAb treatment did not abolish IgE-CAI in WBB6F1-W/W^v mice (data not shown), indicating that IgE-CAI could be developed in the absence of both T cells and mast cells. Antigens with high valence such as TNP11-OVA and TNP20-OVA, but not those with low valence such as TNP2-OVA and TNP4-OVA, elicited IgE-CAI in WBB6F1-W/W^v mice (Figure 3F, not all data are shown) as observed in the IgE transgenic mice (Sato et al., 2003).

Basophils Are Responsible for the Induction of IgE-CAI

In addition to mast cells, basophils are also known to express Fc ϵ RI. However, in contrast to the case of mast cells, we were not able to directly examine the incidence of IgE-CAI in the absence of basophils, as mutant mouse strains deficient only in basophils have not yet been established. Furthermore, in humans, particularly atopic patients, other cell types such as neutrophils, eosinophils, platelets, dendritic cells, and monocytes/macrophages have also been shown to express Fc ϵ RI (Bieber et al., 1992; Gounni et al., 1994, 2001; Joseph et al., 1997; Maurer et al., 1994). To clarify which cell type is responsible for the induction of IgE-CAI, we performed experiments with bone marrow chimeras.

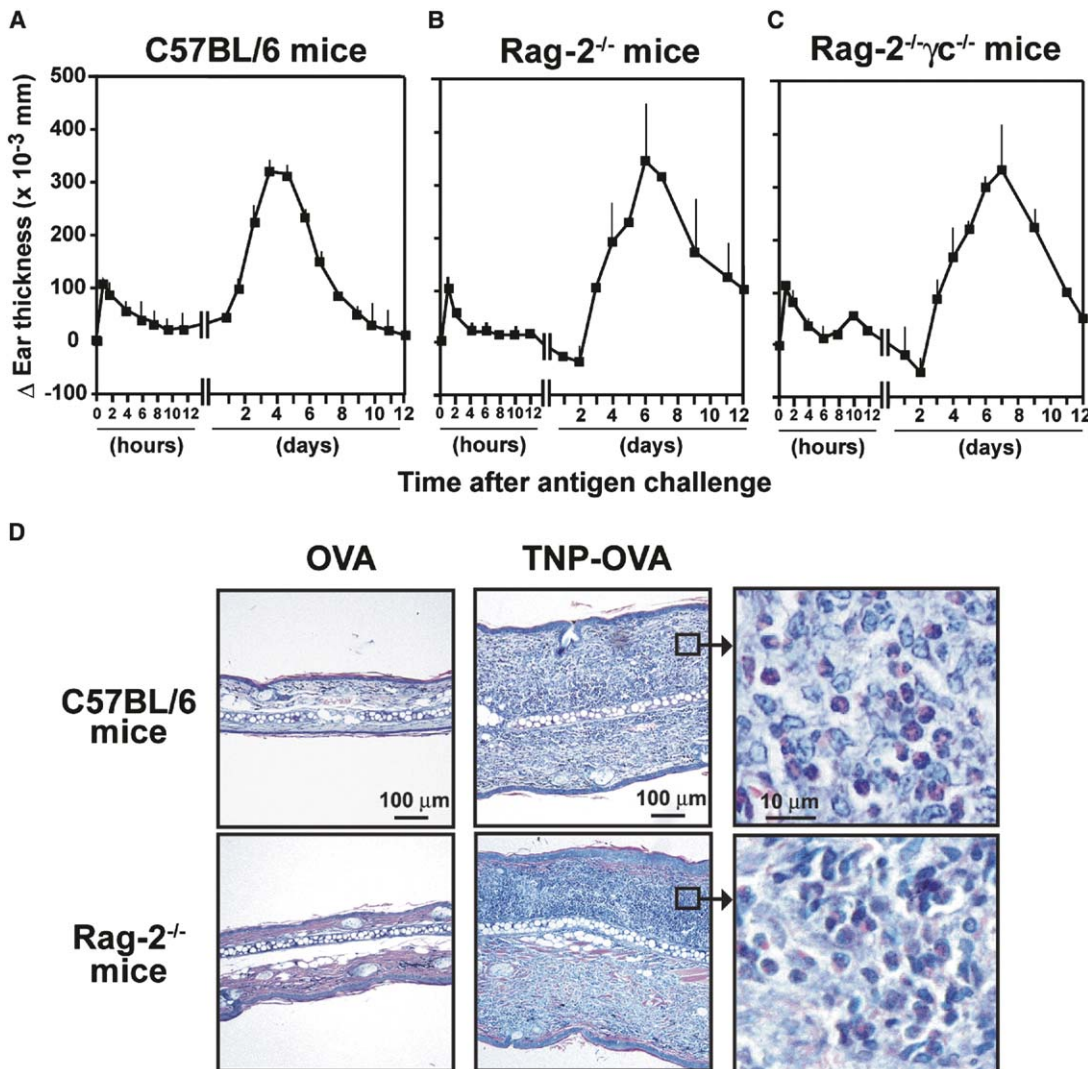


Figure 2. T Cells, B Cells, and NK Cells Are Dispensable for IgE-CAI

(A–C) Normal mice ([A], $n = 5$), RAG-2^{-/-} ([B], $n = 4$), and RAG-2^{-/-}γc^{-/-} ([C], $n = 4$) mice on a C57BL/6 background were passively sensitized with 300 μg of TNP-specific IgE 1 day before subcutaneous administration of the antigens as shown in Figure 1. The kinetics of ΔEar thickness after the antigen challenge is shown. Data are expressed as the mean ± SEM and are representative of three repeated experiments. (D) In parallel experiments performed as described in (A) and (B), ear specimens were prepared from normal and RAG-2^{-/-} mice and stained with Giemsa 4 days after antigen challenge.

Bone marrow cells isolated from normal mice were transferred to irradiated FcγR^{-/-} mice that were incapable of developing IgE-CAI due to the absence of FcεRI. This transfer restored IgE-CAI when recipient mice were analyzed 3 weeks after the transfer, whereas the transfer of bone marrow cells from FcγR^{-/-} mice did not (Figure 4A). These results clearly indicated that FcεRI-expressing hematopoietic cells derived from bone marrow were responsible for the development of IgE-CAI.

We noticed that IgE-CAI could be induced in recipient mice even when the antigen challenge was performed 4 days after transfer of bone marrow cells (Figures 4B and 4C), suggesting that relatively differentiated cells in bone marrow could reconstitute IgE-CAI. Therefore, we fractionated normal bone marrow cells on the basis of expression of lineage markers and transferred each cell fraction to FcγR^{-/-} mice. When bone marrow cells were

depleted of NK cell marker DX5 (CD49b)-positive cells that constituted 3%–4% of nucleated cells, IgE-CAI could not be restored (Figure 4D). In contrast, those depleted of cells positive for CD3, CD19, or NK1.1 could restore IgE-CAI (data not shown). When the fraction enriched for DX5⁺ cells in bone marrow from normal mice was transferred together with total bone marrow cells from FcγR^{-/-} mice that supplied other lineage cells to irradiated recipient mice, IgE-CAI was restored in the recipient mice (Figure 4D, and the titration of transferred DX5⁺ cells is shown in Figure S1 available in the Supplemental Data with this article online). In contrast, normal bone marrow cells depleted of DX5⁺ cells did not restore IgE-CAI even when supplemented with total bone marrow cells from FcγR^{-/-} mice (data not shown). These results implied that the DX5⁺ fraction of bone marrow cells from normal mice included cells

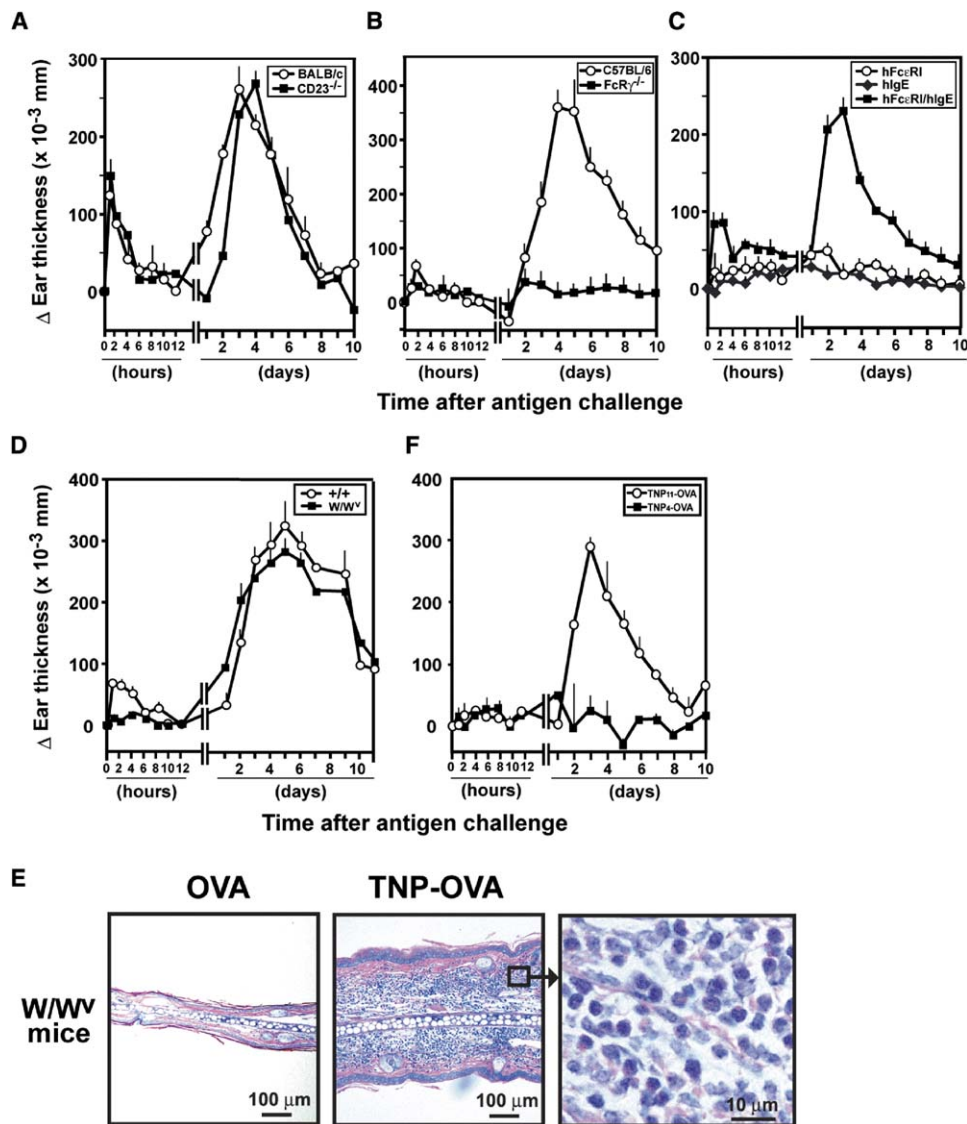


Figure 3. FcεRI Is Essential, but Mast Cells Are Dispensable for IgE-CAI

(A and B) Normal (open circles) and $CD23^{-/-}$ (closed squares) BALB/c mice ($n = 4$ each) (A) and normal (open circles) and $Fc\epsilon R\gamma$ chain- $^{-/-}$ (closed squares) C57BL/6 mice ($n = 5$ each) (B) were treated as in Figure 2.

(C) BALB/c mice carrying the transgene encoding human FcεRIα chain ($n = 5$, open circles), those carrying the transgene encoding humanized TNP-specific IgE ($n = 6$, closed diamonds), and those carrying both of the transgenes ($n = 8$, closed squares) were challenged with the antigens as shown in Figure 1A. The kinetics of Δ Ear thickness after the antigen challenge is shown.

Data are representative of two, five, and three repeated experiments in (A), (B), and (C), respectively.

(D) WBB6F1- $+/+$ (open circles) and WBB6F1- W/W^v (closed squares) mice ($n = 5$ each) were treated as in Figure 2. The kinetics of Δ Ear thickness after antigen challenge is shown. Data are representative of five repeated experiments.

(E) In parallel experiments performed as in (D), ear specimens were prepared from WBB6F1- W/W^v mice and stained with Giemsa 4 days after antigen challenge.

(F) WBB6F1- W/W^v mice were passively sensitized with IgE as in Figure 2 1 day before subcutaneous administration of 10 μ g of TNP11-OVA (open circles) or TNP4-OVA (closed squares) in the left ears and control OVA in the right ears ($n = 3$ each). Data are representative of three repeated experiments.

In (A)–(D) and (F) the error bars represent the mean $\pm$ SEM.

that expressed FcεRI and were responsible for the induction of IgE-CAI.

Flow cytometric analyses revealed that 15%–25% of DX5 $^{+}$ cells in normal bone marrow expressed FcεRI (Figure 5A). These cells were c-kit $^{-}$, NK1.1 $^{-}$, CD3 $^{-}$, B220 $^{-}$, Gr-1 $^{-}$, CD11c $^{-}$, CD11b ^{dull} , 2B4 $^{+}$, and Thy1.2 $^{+}$

(Figure 5B), indicating that they were not mast cells, NK cells, T cells, NKT cells, B cells, neutrophils, eosinophils, or dendritic cells. All these surface phenotypes as well as their relatively low forward and side scatter in flow cytometric analysis were consistent with the characteristics described recently for mouse basophils

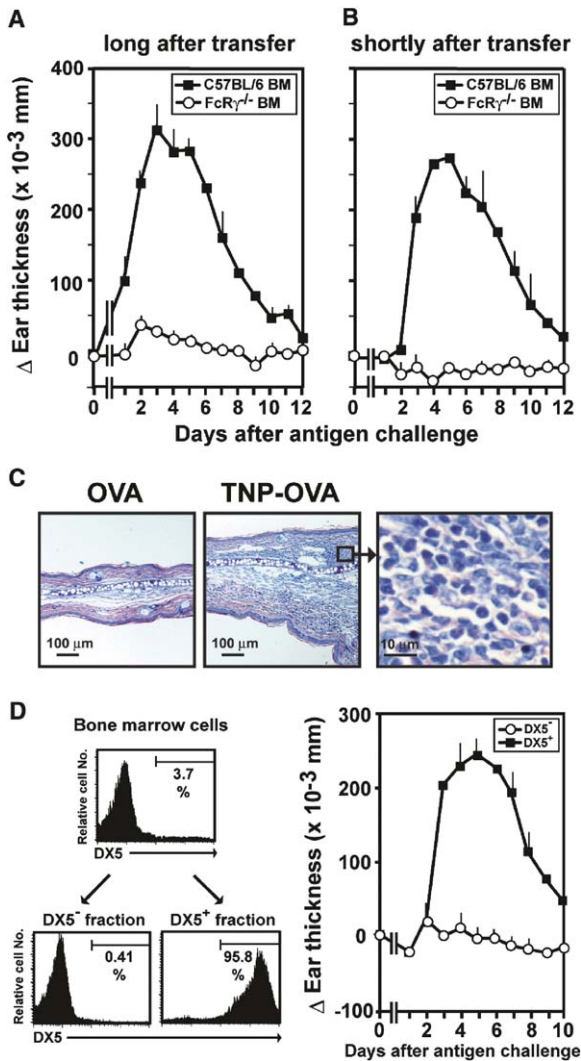


Figure 4. Transfer of DX5⁺ Normal Bone Marrow Cells Restores IgE-CAI in Fcγ Chain^{-/-} Mice

(A and B) Bone marrow cells (5×10^7 cells per recipient) isolated from normal (closed squares) or Fcγ chain^{-/-} (open circles) C57BL/6 mice were transferred intravenously to six Gy-irradiated Fcγ chain^{-/-} C57BL/6 mice. 20 days (A) or 3 days (B) after transfer, the recipient mice were passively sensitized with IgE and challenged with the antigens the next day as shown in Figure 2. The kinetics of ΔEar thickness after antigen challenge is shown. Data are expressed as the mean $\pm$ SEM of five mice in each group and are representative of three repeated experiments. In these experiments as well as those shown in (D), we focused on the incidence of IgE-CAI, and therefore, earlier kinetics of ear thickness is not plotted in the figures.

(C) In parallel experiments performed as in (B), ear specimens were prepared and stained with Giemsa 4 days after antigen challenge. (D) Bone marrow cells from C57BL/6 mice were fractionated with magnetic beads on the basis of the surface expression of DX5. Pre- and postfractionation profiles of DX5 expression are shown on the left. On the right, the DX5⁻ fraction (open circles; 5×10^7 cells per recipient, $n = 5$) or the DX5⁺ fraction (closed squares; 6×10^6 cells per recipient, $n = 3$) was transferred to irradiated Fcγ chain^{-/-} mice. In the case of the DX5⁺ fraction, to supply cells of other lineages to irradiated recipient mice, the total number of transferred cells was adjusted to 5×10^7 by adding 4.4×10^7 Fcγ chain^{-/-} bone marrow cells that were incapable of restoring IgE-

(Min et al., 2004; Voehringer et al., 2004). Light microscopic examination of sorted FcεRI⁺DX5⁺ cells revealed that they possessed lobulated and often ring-like nuclei and few granules in the cytoplasm (Figure 5C). Further electron microscopic examination evidenced their lobulated nuclei and characteristic electron-dense granules (Figure 5D), consistent with their identification as basophils in the mouse (Dvorak, 2000; Min et al., 2004; Seder et al., 1991). Taken together, we concluded that FcεRI⁺DX5⁺ basophils were responsible for the induction of IgE-CAI.

Kinetics of Cellular Infiltrates and IL-4 Expression in the Skin Lesions of IgE-CAI

We examined kinetics of cells infiltrated into the skin lesions of IgE-CAI by using flow cytometry (Figure 6). To distinguish mast cells and basophils among the IgE-bearing FcεRI⁺ population, a surface marker c-kit was used instead of DX5 (Figure 6A), as mast cells in the skin were found to express DX5 in contrast to those in bone marrow (data not shown). Basophils were indeed detected in the skin lesions, and their numbers increased along the development of IgE-CAI: 15–30 cells/ear on day 0 and day 1, 1500–2000 cells/ear on day 2, and 4000–6000 cells/ear on day 4. In terms of frequency, however, basophils accounted for only a minor population among the cells detected in the skin lesions: approximately 0.05%, 0.05%, 2%, 2%, and 2% on days 0, 1, 2, 4, and 7 postchallenge, respectively (Figure 6B). In contrast, eosinophils and neutrophils were the major populations, accounting for approximately 40% and 15%, respectively, on day 4, for example.

We next examined kinetics of IL-4 gene expression in the skin lesions of IgE-CAI, because recent studies have demonstrated that basophils are the major source of IL-4 (Min et al., 2004; Seder et al., 1991; Voehringer et al., 2004). Semiquantitative RT-PCR analysis revealed that IL-4 mRNA became detectable on day 2 postchallenge concomitantly with the increase of basophil numbers in the skin lesions (Figure 6C), suggesting that basophils infiltrated into the skin lesions were activated to produce IL-4 in the IgE-CAI reaction.

Discussion

For many years, basophils were thought to release only preformed histamine and newly synthesized leukotriene C4 after activation by a variety of stimuli (Falcone et al., 2000; Schroeder et al., 2001). There is now firm evidence that basophils are also a major source of Th2 cytokines such as IL-4 and IL-13, key mediators of allergic inflammation (Falcone et al., 2000; Min et al., 2004; Schroeder et al., 2001; Seder et al., 1991; Voehringer et al., 2004). However, no definitive evidence has been provided that basophils are crucially involved in the pathogenesis of chronic allergic disorders. The findings in the present study cast light on the role of

CAI. The recipient mice were treated as shown in (B). The kinetics of ΔEar thickness after antigen challenge is shown. Data are representative of three repeated experiments, and the error bars represent the mean $\pm$ SEM.

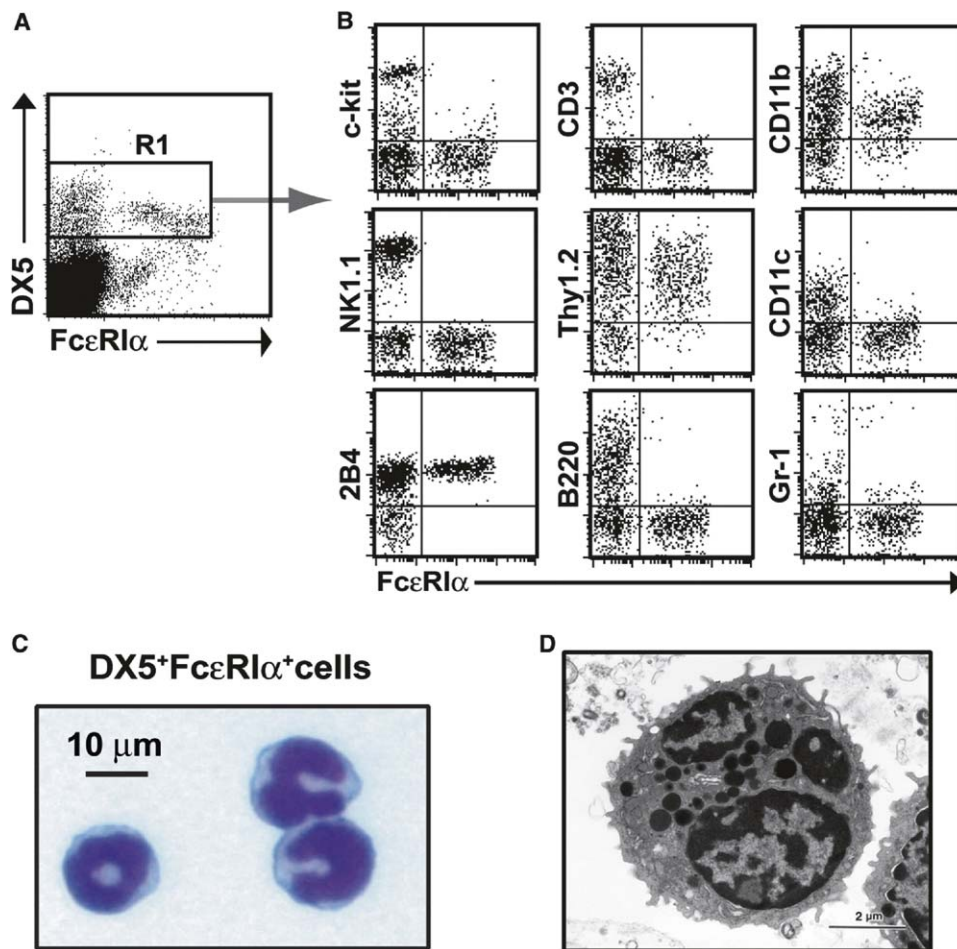


Figure 5. Characterization of FcεRI⁺ Cells among the DX5⁺ Population in the Bone Marrow

(A) Bone marrow cells isolated from C57BL/6 mice were stained for DX5 and FcεRI in combination with the panel of surface markers indicated in (B).

(B) Dot plots were gated on DX5⁺ cells (R1 gate shown in [A]).

(C) DX5⁺FcεRI⁺ cells were sorted from C57BL/6 bone marrow, and cytospin preparations were stained with Giemsa.

(D) DX5⁺ cells were isolated from bone marrow of RAG-2^{-/-}γc^{-/-} mice by using magnetic beads and subjected to electron microscopic examination.

basophils in the development of chronic allergic inflammation.

The IgE transgenic mice and normal mice sensitized with IgE developed delayed-onset and long-lasting allergic skin inflammation (IgE-CAI) characterized by massive infiltration of eosinophils after the immediate- and late-phase ear swelling upon single antigen challenge. Mast cell-deficient mice could develop IgE-CAI without showing the immediate- or late-phase ear swelling. This indicated that mast cells were dispensable for IgE-CAI and that the immediate- and late-phase reactions mediated by mast cells were not prerequisites for IgE-CAI. The time course of ear swelling in the IgE-CAI reaction resembles that observed in the classical delayed-type hypersensitivity (DTH) reaction mediated by T cells. However, there are several differences between the two reactions. The cell types infiltrating the skin lesions are distinct: eosinophils are rarely found in classical DTH (Gaga et al., 1991), whereas they were abundant in IgE-CAI. IgE-CAI could be elicited in the absence of T cells in contrast to classical DTH. On the

other hand, IgE was essential for IgE-CAI, but not for classical DTH. Based on these differences, we concluded that IgE-CAI is developed by a mechanism distinct from that of classical DTH.

IgE-CAI does not seem to belong to the type III hypersensitivity reaction. First, passive Arthus reaction typically occurs several hours after antigen challenge in the skin, much earlier than IgE-CAI. Second, as little as 11 μg of IgE was sufficient for sensitization to elicit IgE-CAI. Third, IgE-CAI could be induced even when the antigen was challenged 4 days after the sensitization with 300 μg of IgE (Figure S2). By that time, serum IgE levels declined to less than 1 μg/ml (Kubo et al., 2003). Thus, high levels of IgE in serum at the time of antigen challenge were not prerequisite for IgE-CAI. Therefore, it is unlikely that the formation and local deposition of immune complexes as observed in the type III hypersensitivity is responsible for the induction of IgE-CAI. Moreover, as IgE usually does not fix complement in contrast to IgG, complement activation is improbable even if the IgE-antigen immune complex is

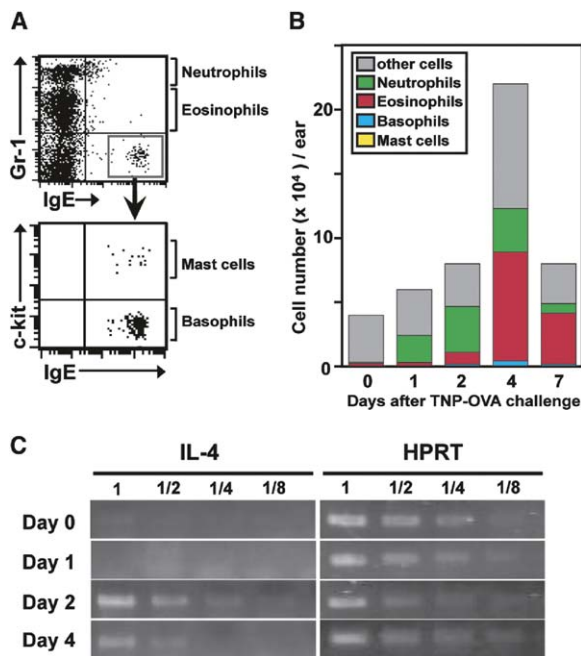


Figure 6. Kinetics of Cellular Infiltrates and IL-4 Expression in the Skin Lesions of IgE-CAI

(A) TNP-specific IgE transgenic mice were challenged with TNP-OVA as in Figure 1A. Cells were isolated from the ears by treatment with collagenase at the time points indicated in (B) and incubated with IgE at 4°C for 30 min to saturate FcεRI. Subsequently, cells were stained for IgE, Gr-1, and c-kit. Dot plots show flow cytometric analysis of ear samples prepared 4 days after the antigen challenge. c-kit staining profile of IgE^{high}Gr-1⁺ cells gated as shown in the upper panel is shown in the lower panel.

(B) Cell numbers of each cell lineage recovered from one ear at the indicated time points after antigen challenge were calculated from the results shown in (A) and are displayed as a bar graph. Mast cells, basophils, eosinophils, and neutrophils were defined as IgE⁺c-kit⁺ cells, IgE⁺c-kit⁻ cells, IgE⁻Gr-1^{low} cells, and IgE⁻Gr-1^{high} cells, respectively. Data are representative of three repeated experiments.

(C) Semiquantitative RT-PCR analysis for IL-4 gene expression was performed by using RNA prepared from cells that were isolated from the ears at the indicated time points as described in (A) and (B). PCR templates were 2-fold serially diluted.

formed, and it may not be crucial for the development of IgE-CAI. Indeed, C5-deficient mouse strains such as A/J and DBA/2J could develop IgE-CAI comparable to that observed in C5-sufficient mouse strains such as BALB/c and C57BL/6 mice (Figure S3).

We found that FcεRI-bearing basophils were essential for the development of IgE-CAI. Mouse basophils have been notoriously difficult to identify due to their few granules in contrast to their human counterparts (Dvorak, 2000). However, the recent characterization of surface markers, including DX5, has made it possible to identify and isolate mouse basophils by flow cytometry (Min et al., 2004; Voehringer et al., 2004). We demonstrated that FcεRI-deficient mice could not develop IgE-CAI but that the transfer of bone marrow cells from normal mice restored the hypersensitivity reaction, indicating that FcεRI-bearing hematopoietic cells were responsible for IgE-CAI. Depletion of DX5⁺ cells from do-

nor cells abolished the restoration, whereas transfer of DX5⁺ bone marrow cells restored IgE-CAI in FcεRI-deficient mice. ~20% of the DX5⁺ bone marrow cells were shown to express FcεRI and other surface markers as well as morphological characteristics that were consistent with their identification as basophils. We could not identify any other lineage cells expressing FcεRI among the DX5⁺ bone marrow cells. Taken together, we concluded that FcεRI⁺DX5⁺ basophils were responsible for the development of IgE-CAI. Cells possessing the phenotype comparable to that of bone marrow basophils were detected in spleen and peripheral blood, albeit much less in number than in bone marrow. Transfer of DX5⁺ spleen cells including basophils from normal mice to FcεRI-deficient mice failed to restore IgE-CAI in the range of cell numbers we could transfer (data not shown). We assume that the average life span of peripheral basophils might be too short to complete IgE-CAI as compared to that of newly generated basophils in bone marrow. Alternatively, peripheral basophils might be more sensitive to the cell damage caused by the cell isolation procedure than bone marrow basophils.

It has been demonstrated that cutaneous DTH containing large basophil infiltrates, termed Jones-Mote hypersensitivity in humans (Jones and Mote, 1934) or cutaneous basophil hypersensitivity (CBH) in guinea pigs (Richerson et al., 1970), can be elicited by immunization with proteins without mycobacterial adjuvants. The reported features of this type of DTH appear to be distinct from those of IgE-CAI. First, basophils constitute as much as 80% of the dermal infiltrates in guinea pig CBH, whereas they account for less than 2% of those in IgE-CAI. Second, CBH peaks at 18–24 hr and fades by 48 hr, much earlier than IgE-CAI. Third, it was originally reported that CBH could be transferred passively with lymphocytes from sensitized animals, but not with their serum as in the case of classical DTH (Dvorak et al., 1971). Later studies indicated that IgG1 or IgE from sensitized guinea pigs could transfer a delayed-onset basophil-containing reaction (Graziano et al., 1983; Haynes et al., 1978), but this appeared to be similar to the late-phase reaction of the IgE-mediated immediate-type allergic response. Taken together, we concluded that IgE-CAI was distinct from CBH. It remains to be determined whether the difference of these two reactions is attributed to species difference or amounts of antibodies used.

The present study unmasked the critical role of basophils in the development of IgE/FcεRI-mediated chronic allergic inflammation that is T cell and mast cell independent. Importantly, basophils accounted for only a minor population of the infiltrating cells in IgE-CAI in contrast to the large numbers of eosinophils and neutrophils. Therefore, we assume that basophils are initiators or propagators rather than effectors of inflammation, even though the detailed mode of action of basophils in IgE-CAI-type chronic allergic inflammation remains to be determined. It has been shown that depletion of basophils diminishes eosinophil infiltration at the site of inflammation in tick infection of guinea pigs (Brown et al., 1982), suggesting that activated basophils secrete cytokines or chemokines to recruit and activate eosinophils and perhaps other inflammatory

cells. Upon stimulation, basophils have been shown to rapidly produce large amounts of Th2 cytokines such as IL-4 and IL-13, which are key mediators of allergic inflammation (Falcone et al., 2000; Min et al., 2004; Schroeder et al., 2001; Seder et al., 1991; Voehringer et al., 2004). Indeed, IL-4 expression became detectable in the skin lesions of IgE-CAI concomitantly with the increase of basophil numbers infiltrated in the skin. The transgenic expression of IL-4 or IL-13 in the mouse lung resulted in eosinophil infiltration (Rankin et al., 1996; Zhu et al., 1999), and both cytokines have been shown to promote the production of eotaxin, an eosinophil chemoattractant, in airway epithelial cells or dermal fibroblasts (Li et al., 1999; Mochizuki et al., 1998). Thus, basophils appear to play an important role in allergic inflammation beyond their recognized role as effector cells (Falcone et al., 2000; Marone et al., 2005; Prussin and Metcalfe, 2003; Schroeder et al., 2001). We are currently analyzing with DNA microarray and a panel of mutant mice what kinds of cytokines, chemokines, and chemical mediators are released from basophils in IgE-CAI and which is crucial for recruitment of other inflammatory cells. Preliminary results using mutant mice suggested that IL-4 was dispensable for IgE-CAI (data not shown).

It remains to be determined how basophils are recruited to the sites of antigen exposure to initiate IgE-CAI. We noticed that the intradermal injection of OVA induced mild infiltration of cells such as neutrophils, which was detected on day 1 but subsided by day 2 (data not shown). Therefore, it is plausible that intradermal injection of antigens nonspecifically stimulates dendritic cells, macrophages, keratinocytes, fibroblasts, or other cells in the skin to transiently produce chemotactic factors that recruit inflammatory cells, including basophils. Then, TNP-OVA, but not OVA, might stimulate such recruited basophils via TNP-specific IgE bound to their surface to initiate IgE-CAI.

High valency of antigens was necessary to elicit IgE-CAI (Figure 3F), whereas both low- and high-valence antigens could elicit immediate-type hypersensitivity (Sato et al., 2003). Therefore, there appears to be a threshold in signal strength to stimulate basophils to develop IgE-CAI. In *in vitro* experiments, binding of TNP11-OVA induced much stronger downmodulation of IgE-Fc ϵ R1 complexes on the surface of bone marrow basophils than that of TNP4-OVA did (data not shown). Thus, we speculate that the stronger crosslinking of Fc ϵ R1 with TNP11-OVA stimulates basophils to secrete higher levels or distinct types of cytokines or chemokines necessary for the development of IgE-CAI. Molecules possessing repetitive epitopes like TNP11-OVA might not be found so frequently among allergens in real life. However, in contrast to mice sensitized with monoclonal IgE, allergic patients could produce polyclonal IgE against a given allergen. A grass pollen allergen has been shown to have at least nine different epitopes recognized by patient serum IgE (Schramm et al., 2001). Thus, it would be intriguing to examine whether such polyvalent antigens can stimulate basophils via polyclonal IgE bound to their surface to induce the IgE-CAI-type reaction.

A number of cohort studies indicated that there was a correlation between the disease severity and serum

IgE levels in patients with asthma or atopic dermatitis, especially in younger people (Burrows et al., 1989; Schafer et al., 1999). Recent clinical trials have indicated that the rapid and pronounced decrease in serum IgE level achieved by treatment with humanized anti-IgE antibody (omalizumab) is correlated with improvement of the severity of symptoms not only in allergic rhinitis patients but also in some poorly controlled asthma patients, leading to a reduction in the requirement for inhaled corticosteroids (Busse et al., 2001; Holgate et al., 2004, 2005). This suggests that IgE may be involved in the pathogenesis of chronic allergic inflammation in humans. Based on the present observations in the mouse model, we speculate that IgE-CAI might contribute to the initiation, prolongation, or deterioration of chronic allergic inflammation in a certain type of asthma and atopic dermatitis.

Corticosteroids and immunosuppressants are widely used for the treatment of chronic allergic disorders. We reported previously that cyclosporin A almost completely suppressed IgE-CAI in IgE transgenic mice (Sato et al., 2003). Although the main action of cyclosporin A has been demonstrated to be suppression of the transcription of cytokine genes in activated T cells, it has also been reported to have effects on basophils (Genovese et al., 2003; Hultsch et al., 1990; Plath et al., 2003). These results suggest that basophils and their products might be good targets to control IgE-CAI-type chronic allergic inflammation. Further detailed studies on IgE-CAI should help to develop novel therapeutic strategies for the treatment of chronic allergic disorders.

In conclusion, to our knowledge, we have defined a previously unknown *in vivo* function of basophils, namely, initiating IgE-mediated chronic allergic inflammation independently of T cells and mast cells. Previous studies suggested that Fc ϵ R1-IgE-antigen complexes on human antigen presenting cells such as Langerhans cells facilitate antigen uptake and antigen presentation to T cells and, hence, contribute to T cell-mediated chronic allergic inflammation (Bieber, 1997; von Bubnoff et al., 2003). The present study proposes a novel mechanism whereby IgE contributes the development or deterioration of chronic allergic inflammation through activation of basophils in a T cell- and mast cell-independent manner.

Experimental Procedures

Mice

BALB/c and C57BL/6 mice were purchased from Charles River Japan (Yokohama, Japan). FcR γ chain^{-/-} C57BL/6 mice (Takai et al., 1994) were from Jackson Laboratory (Bar Harbor, ME). RAG-2^{-/-} γ C^{-/-} C57BL/6 mice (Cao et al., 1995) were from Taconic (Germantown, NY). A/J, WBB6F1- +/+, and WBB6F1-W/W^v mice were from Japan SLC (Hamamatsu, Japan). DBA/2J mice were from CLEA Japan (Tokyo, Japan). RAG-2^{-/-} C57BL/6 mice (Shinkai et al., 1992), CD23^{-/-} BALB/c mice (Fujiwara et al., 1994), and TNP-specific IgE transgenic BALB/c mice (Matsuoka et al., 1999) were described previously. BALB/c mice carrying a humanized IgE transgene were established by replacing the constant region of the ϵ H chain gene encoding TNP-specific mouse IgE with the human counterpart. Human Fc ϵ R1 α chain transgenic mice were established by microinjection of the 17 kb BamHI fragment of the genomic DNA encoding human Fc ϵ R1 α chain into fertilized eggs of BALB/c mice. Mice were maintained under specific pathogen-free conditions in our animal

facility. All the experiments in this study were performed according to the Guidelines for Animal Use and Experimentation as set out by our University.

Antibodies

Biotinylated mAbs specific for mouse IgE (R35-72), Thy1.2 (30-H12), and DX5 (DX5) and FITC-conjugated and PE-conjugated mAbs specific for DX5 (DX5) and c-kit (ACK45) were purchased from BD Pharmingen (San Diego, CA). Biotinylated mAbs specific for CD3 (145-2C11), CD11c (HL3), Gr-1 (RB6-8C5), B220 (RA3-6B2), NK1.1 (PK136), 2B4 (2B4), CD11b (M1/70), and c-kit (2B8), and FITC-conjugated anti-Gr-1 mAb (RB6-8C5) and PE-conjugated anti-mouse Fc ϵ R1 α (MAR-1) were purchased from eBioscience (San Diego, CA).

Elicitation and Measurement of Antigen-Induced Ear Swelling

Antigen TNP-conjugated OVA was purchased from Biosearch Technologies (Novato, CA). TNP11-OVA and TNP4-OVA were used for the experiments. Mice were sensitized with IgE by intravenous injection of diluted ascites (200 μ l) containing indicated amounts of TNP-specific IgE derived from IGELb4 B cell hybridoma (Rudolph et al., 1981) or comparable amounts of irrelevant IgE that is specific to cedar pollen antigen Cryj1 (K.M., H.Y., and H.K., unpublished data) 1 day before antigen challenge. In the case of TNP-specific IgE transgenic mice, this sensitization with exogenous IgE was omitted. The left ears of mice were administered subcutaneously with 10 μ g of TNP-OVA in 10 μ l of PBS, and their right ears were administered an equal amount of OVA by using a microsyringe under light anesthesia with diethyl ether. Ear thickness was measured with a dial thickness gauge (G-1A; Ozaki, Tokyo, Japan).

Fractionation and Transfer of Bone Marrow Cells

Single-cell suspensions prepared from bone marrow were reacted with biotinylated mAbs at 4°C for 30 min, followed by incubation with streptavidin-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min. Positive and negative fractions were isolated separately by autoMACS (Miltenyi Biotec). Total or fractionated bone marrow cells (5×10^7 cells per recipient) from C57BL/6 mice were transferred to six Gy-irradiated FcR $\gamma^{-/-}$ mice. At the indicated time points after bone marrow transfer, the recipient mice were sensitized with IgE followed by antigen challenge.

Histological, Flow Cytometric, and Electron Microscopic Analysis

Ear specimens were fixed with 10% formalin and embedded in paraffin. Sections were stained with Giemsa for histological examination under a microscope. For flow cytometric analysis, ears removed from mice were treated with collagenase type III (125 U/ml, Worthington Biochemical Corporation, Lakewood, NJ) in RPMI complete medium for 2 hr at 37°C to obtain single-cell suspensions. Cells prepared from ears and bone marrow were depleted of RBC by lysis with hypotonic buffer and preincubated with anti-CD16/32 (2.4G2) mAb (BD Pharmingen) and normal rat serum on ice for 15 min to prevent nonspecific binding of other Abs. Remaining cells were stained with the indicated combinations of Abs and analyzed with FACSCalibur (BD Biosciences, Mountain View, CA). DX5⁺Fc ϵ R1⁺ cells were sorted from bone marrow cells with FACS-Vantage (BD Biosciences), and cytospin preparations (Cytospin 2; Thermo Shandon, Runcorn, UK) were stained with Giemsa. DX5⁺ cells were isolated from bone marrow cells with autoMACS (Miltenyi Biotec) and fixed with 2% glutaraldehyde, postfixed with 1% OsO₄, and embedded in Epon. Ultrathin sections were double stained with uranyl acetate and lead citrate and examined with a JEM 1200EX transmission electron microscopy (JEOL, Tokyo, Japan).

Semiquantitative RT-PCR Analysis

Total RNA was prepared from cells that were isolated from the ears of mice as for flow cytometric analysis and subjected to the first strand cDNA synthesis with reverse transcription using oligo-dT primers. PCR was performed with 2-fold serially diluted cDNA templates at 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s for 30 cycles, followed by further extension at 72°C for 5 min. The follow-

ing primers were used for PCR: for IL-4 forward, 5'-GAGCCATATG CACGGATGCGACAA-3' and reverse, 5'-CATGGTGGCTCAGTAC TACGAGTA-3' and for HPRT forward, 5'-GCTGGTGAAAAGGACC TCT-3' and reverse, 5'-CACAGGACTAGAACACCTGC-3'.

Supplemental Data

Supplemental Data include three figures and are available with this article online at <http://www.immunity.com/cgi/content/full/23/2/191/DC1/>.

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