



Eosinophils Mediate Basophil-Dependent Allergic Skin Inflammation in Mice

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The mechanisms leading to allergic skin inflammation, such as atopic dermatitis or urticaria, are poorly defined. Here we used a mouse model for IgE-dependent chronic allergic inflammation to study the role of basophils and eosinophils for induction of pathology. FcεRI expression in basophils was required for the ear swelling response, and basophils promoted the expression of eosinophil-recruiting chemokines in the ear. The ear swelling response could be prevented by prior infection of mice with helminths in an IgE-dependent manner. Impaired skin eosinophilia and reduced ear swelling was further observed in IL-4/IL-13–deficient and STAT6-deficient mice. In addition, eosinophil-deficient Δ dblGATA mice showed only weak ear swelling response, which could be enhanced by eosinophil transfer. This suggests that IgE-activated basophils orchestrate the recruitment of eosinophils by secretion of IL-4/IL-13, which leads to STAT6-dependent expression of CCL24 from endothelial cells and extravasation of eosinophils into the ear pinna. Eosinophils are then the critical effector cells that cause pathology. Therefore, combined therapeutic approaches that block basophil activation and reduce eosinophil numbers could be efficient strategies to improve treatment of chronic allergic disorders of the skin.

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INTRODUCTION

Both allergic responses and helminth infections elicit a strong T helper type 2–dominated immune response, with increased numbers of eosinophils, basophils, mast cells, type 2 innate lymphoid cells, T helper type 2 cells; pronounced expression of T helper type 2–associated cytokines like IL-4, IL-5, and IL-13; and high serum levels of IgE.

Mast cells and basophils are the two main cell populations that express FcεRI and get activated by IgE in vivo. Mast cells are tissue-resident cells with a lifespan of several weeks. In contrast, basophils mature in the bone marrow, circulate in the bloodstream with a lifespan of only 2–3 days, and usually do not extravasate into peripheral tissues under steady-state conditions (Ohnmacht and Voehringer, 2009). During inflammatory conditions, increased numbers of basophils and high levels of IgE can be found, for example, in the lung of asthma patients (Lichtenstein and Bochner, 1991); in allergic skin inflammation like atopic dermatitis; and in other inflammatory skin conditions, such as purigo and urticaria (Irani et al., 1998; Ito et al., 2011; Otsuka and Kabashima, 2015). Basophils in the skin can also have a protective function against secondary encounters with ticks or helminth

larvae (Brown et al., 1982; Obata-Ninomiya et al., 2013; Wada et al., 2010).

A critical pro-inflammatory role of basophils could be discovered in IgE-mediated chronic allergic skin inflammation (IgE-CAI), a model established by Mukai et al. (2005), in which intravenous anti-hapten IgE injection, followed by a single intradermal injection of multivalent antigen into the ear pinna, results in a strong ear swelling response. A relatively mild and transient immediate swelling response occurs within minutes and is mediated by mast cells, followed by a much stronger basophil-dependent and mast cell-independent response. This late-phase swelling and inflammation persists for several days, is characterized by recruitment of basophils, eosinophils, and neutrophils and is reminiscent of human chronic idiopathic urticaria (Mukai et al., 2005; Sato et al., 2003). It has also been shown that basophil-derived proteases can elicit the ear swelling response (Iki et al., 2016; Tsutsui et al., 2017). However, it remains unclear whether basophils themselves or other recruited effector cells, like eosinophils or neutrophils, are responsible for the inflammatory ear swelling response in this model. Furthermore, experimental evidence is missing regarding the requirement of direct basophil activation via FcεRI and whether helminth infections can block the response by either saturating FcεRI on basophils with helminth-elicited IgE or expansion of immunosuppressive cell populations, as described previously in other allergy models (Flohr et al., 2009).

Here, we addressed these points and show that infection of mice with two different gastrointestinal helminths protects against IgE-CAI in an IgE-dependent manner. We further demonstrate that basophils are required for upregulation of CCL24 (eotaxin-2), CCL3 (MIP-1α), and TSLP in the ear pinna. Eosinophil recruitment and the ear swelling response are diminished in IL-4/IL-13–deficient (4-14ko) and

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Abbreviations: BM, bone marrow; IgE-CAI, IgE-mediated chronic allergic skin inflammation; ko, deficient; TNP, trinitrophenyl; WT, wild-type

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STAT6-deficient (STAT6ko) and mice, suggesting that eosinophils are the critical terminal effector cells in this model. Indeed, eosinophil-deficient Δ dblGATA mice showed only weak ear swelling, which could be restored by adoptively transferred eosinophils. These results indicate that IgE-activated basophils elicit the terminal effector phase by orchestrating recruitment of eosinophils, which then cause pathology in the skin.

RESULTS

Expression of activating Fc receptors and IL-4/IL-13 by basophils is required for IgE-CAI

Previous studies have demonstrated a critical role for basophils in IgE-mediated chronic allergic skin inflammation at the late phase (day 2–3 after challenge) (Mukai et al., 2005; Ohnmacht et al., 2010). However, it remained unclear whether basophils have to be directly activated by IgE and whether basophils are required for causing vascular leakage in the skin. To address these points, we injected Mcpt8Cre and wild-type (WT) mice with IgE anti-trinitrophenyl (TNP) intravenously, followed by intradermal injection of TNP-OVA into the ear pinnae on the next day. The ear thickness was measured every day and we observed a prominent ear swelling response in WT mice, peaking on day 2 after challenge and going back to normal at day 5–6, whereas no swelling was detected in basophil-deficient Mcpt8Cre mice (Figure 1a). By intravenous injection of Evans Blue at 20 minutes before harvesting the ears on day 2 after TNP-OVA challenge, we could visualize and quantify the leakage of blood vessels at the site of inflammation of WT mice, while no vascular leakage was found in Mcpt8Cre mice (Figure 1b).

To further investigate whether the ear swelling response requires direct binding and cross-linking of IgE anti-TNP on the cell surface of basophils, we created mixed bone marrow (BM) chimeras lacking Fc γ on basophils by mixing Mcpt8Cre BM with Fc γ ko BM in a 4:1 ratio. As control WT BM was used instead of Fc γ ko BM. Like in the complete absence of basophils, mice lacking Fc γ on basophils show an impaired ear swelling response when compared to Baso-WT chimeras (Figure 1c).

This indicates that basophils have to be directly activated by the high-affinity IgE receptor (Fc ϵ RI) to elicit the ear swelling response. We further investigated the role of IL-4/IL-13 from basophils for the ear swelling response by generating mixed BM chimeras with BM from Mcpt8Cre mice and 4-13ko mice mixed in a 4:1 ratio. Mice lacking IL-4/IL-13 expression by basophils developed a significantly reduced ear swelling response compared to control mice (Figure 1d).

Helminth-elicited IgE ameliorates IgE-CAI

Based on the observation that basophils have to be directly activated by IgE anti-TNP bound to Fc ϵ RI, we next investigated whether high levels of polyclonal IgE can saturate Fc ϵ RI receptors and thereby prevent basophil activation in this model. To address this point, we infected mice with the gastrointestinal helminths *Nippostrongylus brasiliensis* or *Heligmosomoides polygyrus*. Thirteen days later, serum IgE levels were measured by ELISA and IgE anti-TNP was injected into the infected mice or naïve mice as controls, followed by intradermal injection of TNP-OVA into the ear on the next day. Helminth-infected mice showed high serum

IgE levels and were protected from the ear swelling response compared to uninfected control mice (Figure 2a, 2b). The protective role of helminth-elicited IgE in IgE-CAI was further confirmed by analysis of *N. brasiliensis*-infected IgE-deficient mice, which developed a normal ear swelling response (Figure 2c). By staining of basophils with fluorescently labeled IgE anti-TNP antibody, we could demonstrate that binding of this antibody to the surface of basophils isolated from helminth-infected mice was impaired (Figure 2d). Therefore, we can conclude that helminth infection leads to saturation of the Fc ϵ RI on basophils and thereby prevents their sensitization with the administered IgE anti-TNP antibody.

Basophils enhance expression of TSLP, CCL3, and CCL24 in the ear pinna

To further investigate the mechanisms by which activated basophils orchestrate the ear swelling response, we analyzed basophil-dependent expression of chemokines, TSLP, and VCAM-1 in the ear pinna by quantitative reverse transcriptase PCR on day 2 after TNP-OVA challenge. No changes were detected for CCL5 (RANTES), CCL11 (eotaxin-1), and VCAM-1 expression, but expression levels of TSLP, CCL24 (eotaxin-2), and CCL3 (MIP-1 α) were significantly lower in the absence of basophils compared to WT mice (Figure 3a).

We further determined the CCL24 protein level in the ear pinna by ELISA and observed significantly reduced levels in Mcpt8Cre compared to WT mice (Figure 3b). This result suggests that basophils promote the recruitment of other effector cells that could contribute to the ear swelling response.

IL-4/IL-13 and STAT6 signaling promotes ear swelling and effector cell recruitment during IgE-CAI

Basophils are an important source of IL-4 and IL-13, which signal through STAT6 and induce expression of chemokines and adhesion molecules in endothelial cells. To investigate the role of IL-4/IL-13 and STAT6 for the ear swelling response and recruitment of effector cells, we studied the IgE-CAI response in 4-13ko and STAT6ko mice. Ear swelling was significantly decreased in 4-13ko and STAT6ko mice (Figure 4a, 4b). Furthermore, to study the gene expression and recruitment of innate effector cells into the ear pinna, reverse transcriptase PCR and flow cytometric analysis of WT, 4-13ko, and STAT6ko ears was performed on day 2 after TNP-OVA challenge. We observed no significant increase of M2 macrophage marker in WT compared to 4-13ko and STAT6ko mice at this early time point (Supplementary Figure S1 online). We further revealed a trend toward higher CCL24 and TSLP levels in WT compared to 4-13ko and STAT6ko mice (Supplementary Figure S1). No difference in percentages and total numbers of neutrophils and basophils was detected in the ears of WT, 4-13ko, and STAT6ko mice. However, percentage and also total numbers of eosinophils in the ears were significantly reduced in both knockout strains compared to WT mice (Figure 4c–4g).

Eosinophils are required for induction for the ear swelling response in IgE-CAI

Based on the observation that the reduced ear swelling response in 4-13ko and STAT6ko mice was associated with

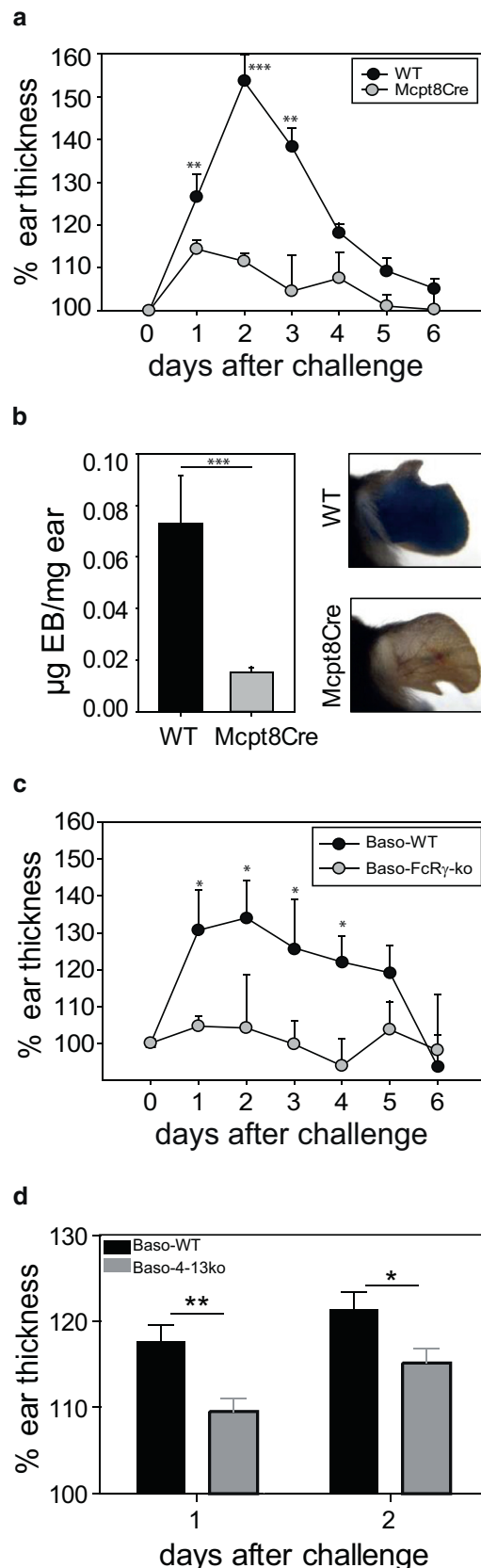


Figure 1. Expression of activating Fc receptors on basophils is required for IgE-mediated chronic allergic skin inflammation. (a) Ear thickness of Mcpt8Cre and WT mice after trinitrophenyl-OVA challenge. Mean $\pm$ standard error of 14 ears of 8 Mcpt8Cre mice and 9 ears of 7 WT mice from 2 experiments. (b) EB extravasation on day 2 after challenge. Mean $\pm$ standard

reduced numbers of eosinophils in the ear pinna, we hypothesized that eosinophils could play an important role in this model of skin inflammation. To prove this hypothesis, we analyzed eosinophil-deficient Δ dblGATA mice and observed that in the absence of eosinophils, no ear swelling was elicited while adoptive transfer of BM-derived eosinophils was sufficient to promote the ear swelling response in Δ dblGATA mice (Figure 5a). This effect could not be explained by reduced basophil numbers or impaired basophil function in Δ dblGATA mice (Supplementary Figure S2 online). The eosinophil cultures contained negligible numbers of basophils so that the transfer did not artificially increase the number of basophils (Supplementary Figure S3 online). On the other hand, Mcpt8Cre mice showed no defect in generation of eosinophils (Supplementary Figure S2).

To visualize the distribution and accumulation of eosinophils in the ear parenchyma, two-photon imaging of unfixed ear of EpxCre_R26^{tdTomato} mice was performed on day 2 after challenge and compared to untreated EpxCre_R26^{tdTomato} mice. Prominent extravascular accumulation of eosinophils was apparent in the ear of challenged mice, while only few eosinophils were found in the ear of untreated control mice (Figure 5b). Next, we addressed the activation status of recruited eosinophils in the ear in comparison to eosinophils in the peripheral blood by flow cytometry. We used IL-4eGFP reporter mice (4get mice) to facilitate the gating of eosinophils as IL-4eGFP⁺SSC^{hi} cells with a parental gate on live CD45^{hi} cells. The samples were stained for CD62L and Siglec-F to differentiate between resting (Siglec-F^{lo} CD62L^{hi}) and activated (Siglec-F^{hi} CD62L^{lo}) eosinophils. As expected, recruited eosinophils in the ear showed an activated phenotype, while blood eosinophils were not activated (Figure 5c). Finally, we performed adoptive transfers of eosinophils into Δ dblGATA mice and analyzed the role of G-protein-coupled chemotactic receptors on eosinophils for recruitment to the ear pinna. Therefore, we transferred 1.5×10^7 eosinophils that had either been treated with pertussis toxin to inhibit G-protein-coupled chemotactic receptors or were left untreated. Right after the eosinophil transfer, mice were subjected to the IgE-CAI model. Efficient recruitment of eosinophils was observed after transfer of untreated eosinophils into TNP-OVA challenged mice, which could be inhibited by prior pertussis toxin treatment (Figure 5d).

Taken together, these experiments demonstrate that eosinophils are recruited to the ear pinna, acquire an activated phenotype, and promote the ear swelling response. Therefore, a two-step model is likely to operate in this setting, where basophils get first activated by cross-linking of IgE on

error of 14 ears of 9 Mcpt8Cre mice and 7 ears of 5 WT mice from two experiments. (c) Ear thickness of mixed bone marrow chimeras lacking FcRγ on basophils (gray) and controls (black). Mean $\pm$ standard deviation of three Baso-FcRγ-deficient chimeras and three Baso-WT chimeras. (d) Ear thickness of mixed bone marrow chimeras lacking IL-4 and IL-13 in basophils (gray) and controls (black). Mean $\pm$ standard error for 18 ears per group analyzed by analysis of variance. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, two-tailed Student *t* test was used if not otherwise indicated. EB, Evans Blue; WT, wild-type.

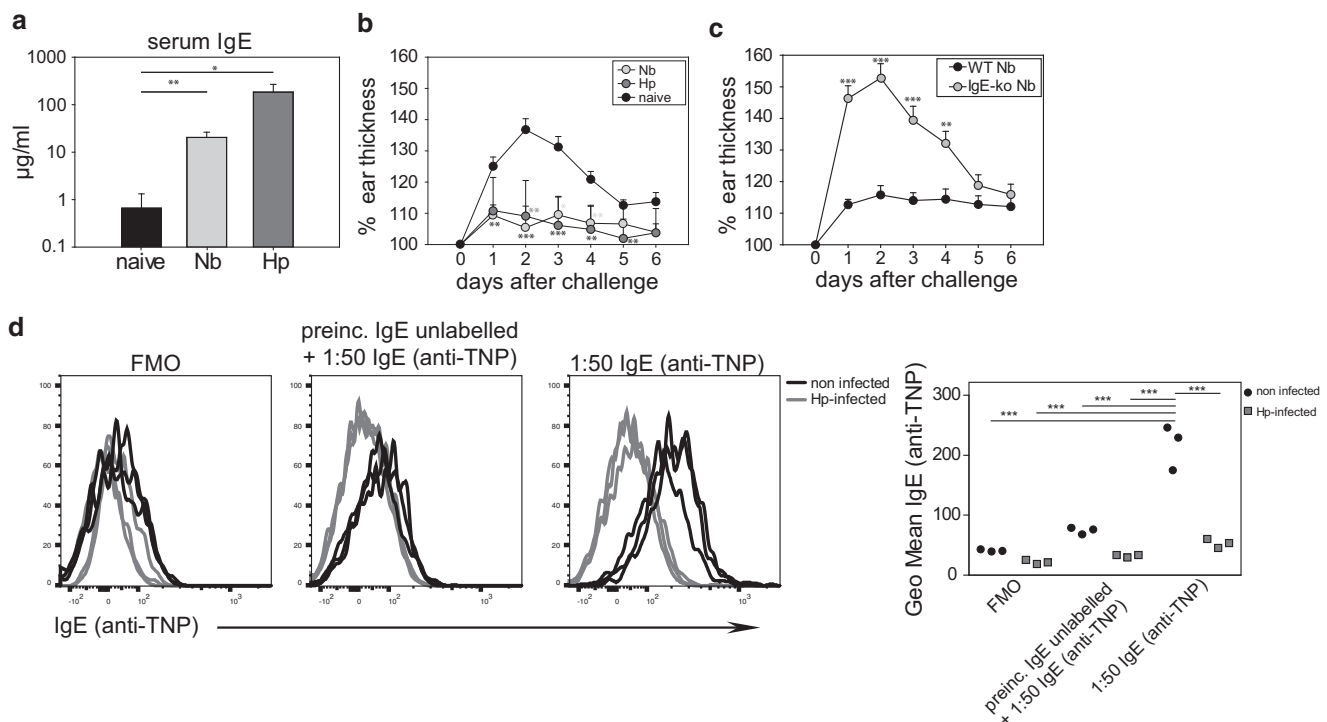


Figure 2. Helminth-elicited IgE ameliorates IgE-mediated chronic allergic skin inflammation. (a) IgE serum levels of naïve Nb-infected and or Hp-infected mice. Mean $\pm$ standard error of 3–7 mice per group. (b) Ear thickness of naïve, Nb- or Hp-infected mice. Mean $\pm$ standard error of 6 ears of Nb- or Hp-infected mice and 35 ears of naïve mice. (c) Ear thickness of Nb-infected IgE-deficient and WT mice. Mean $\pm$ standard error of 14 ears of Nb-infected IgEko mice and 16 ears of Nb-infected WT mice. (d) In vitro binding of A647-labeled IgE anti-TNP to basophils from naïve or Hp-infected mice (right). For FMO, no IgE antibody was added (left). Pre-incubation with unlabeled IgE anti-TNP blocks the binding of labeled IgE anti-TNP (middle). Each curve represents data from one mouse. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ two-tailed Student t test used if not indicated different. FMO, fluorescence minus one; Hp, *Heligmosomoides polygyrus*; Nb, *Nippostrongylus brasiliensis*; TNP, trinitrophenyl; WT, wild-type.

the cell surface and secrete IL-4, which induces expression of CCL24 and probably other chemoattractants in endothelial cells to recruit eosinophils that finally cause pathology in the skin (Figure 6).

DISCUSSION

Basophils are indispensable for IgE-CAI in the ear skin, as we demonstrate by comparing ear swelling and dye extravasation in WT and basophil-deficient *Mcpt8Cre* mice and thereby largely confirm previous studies (Cheng et al., 2015; Mukai et al., 2005; Ohnmacht et al., 2010). It was previously shown that mice deficient for the signal transducing subunit $\text{Fc}\gamma$ of the $\text{Fc}\epsilon\text{RI}$ develop no IgE-CAI and reconstitution of irradiated $\text{Fc}\gamma$ -deficient mice with total or basophil-enriched BM from WT mice was sufficient to restore the response (Mukai et al., 2005). By generating mixed BM chimeras, we could now demonstrate that $\text{Fc}\gamma$ expression on basophils is required for IgE-CAI. We previously observed a similar importance for activation of basophils via this receptor during secondary helminth infections, where only mice with $\text{Fc}\epsilon\text{RI}$ -expressing basophils were able to clear worms efficiently (Schwartz et al., 2014).

Interestingly, not only the lack of $\text{Fc}\epsilon\text{RI}$ on basophils protects against pathology in the IgE-CAI model, but also high levels of helminth-elicited IgE. We observed the ameliorated ear swelling response in two different gastrointestinal helminth infection models. A protective effect was

only observed in WT but not in IgE-deficient mice, which demonstrates that indeed helminth-induced IgE rather than other helminth-elicited suppressive cell types or factors are accountable for the effect. The IgE saturation hypothesis suggests that high levels of helminth-elicited polyclonal IgE saturates the high-affinity receptor for IgE ($\text{Fc}\epsilon\text{RI}$) on mast cells and basophils and prevents binding of allergen-specific IgE (Godfrey and Gradidge, 1976; Stanworth et al., 1967). Here we used a mouse infection model to provide further experimental evidence that helminth-elicited IgE does saturate the available $\text{Fc}\epsilon\text{RI}$ on the surface of basophils so that the sensitizing IgE anti-TNP antibody cannot bind. However other studies could not confirm a blocking effect by helminth-elicited IgE (Flohr et al., 2009). They rather indicate that helminth-induced suppression of allergy is caused by increased numbers of regulatory T cells, IL-10-producing T cells, and alternatively activated macrophages (Flohr et al., 2009; Wilson et al., 2005). Possible explanations for the apparent discrepancies between different studies include the experimental protocols (active vs passive sensitization), investigated organs (skin, lung, vascular system), and differences in serum levels of IgE. With regard to the human population, the low incidence of allergic inflammation in countries with poor sanitary conditions and high prevalence of helminth infections suggests that helminths can protect against allergy, supporting the hygiene hypothesis (Wills-Karp et al., 2001).

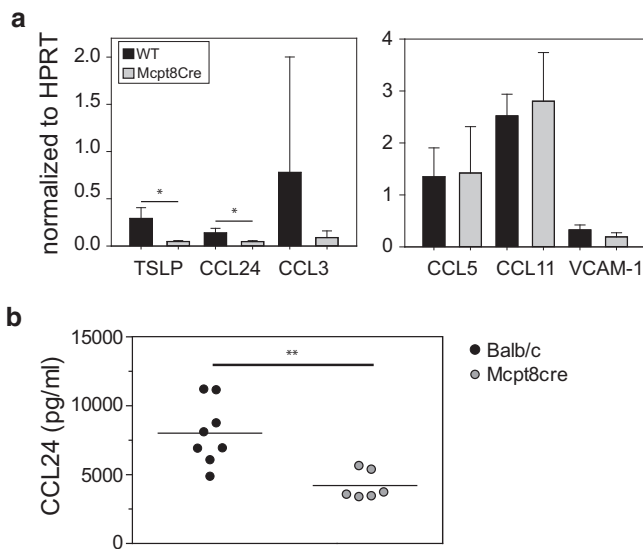


Figure 3. Basophils enhance expression of TSLP, CCL3, and CCL24 in the ear pinna. Analysis of total ear pinnae 2 days after trinitrophenyl-OVA challenge of IgE-anti- trinitrophenyl sensitized Mcpt8Cre and wild-type mice. (a) Quantitative reverse transcriptase PCR analysis normalized to HPRT. Data show the mean $\pm$ standard deviation of 3 Mcpt8Cre and 3 WT mice. (b) CCL24 protein level was determined by ELISA after ear pinna lysis in 1 ml RIPA with protease inhibitors. * $P < 0.05$, ** $P < 0.01$, two-tailed Student t test. WT, wild-type.

A large cohort study demonstrated that de-worming leads to increased incidence of skin prick test positivity against house dust mite allergens (van den Biggelaar et al., 2004). Several other studies have confirmed the effect, but the mechanism of protection remains poorly defined (Flohr et al., 2009).

IgE-mediated basophil activation in the skin causes release of lipid mediators, cytokines, chemokines, and histamine, which can directly or indirectly result in pathology. Moreover, recent studies could show that the release of basophil-specific proteases, like mMCP-8 and mMCP-11, increases microvascular permeability in an activity-dependent manner to induce allergic inflammation (Iki et al., 2016; Tsutsui et al., 2017). Acting on tissue-resident cells, like fibroblasts and endothelial cells, we found that basophils enhance expression of TSLP, CCL3, and CCL24 (eotaxin-2) in the ear pinna. We have previously shown that TSLP is required for the ear swelling response elicited by topical application of the vitamin D analogon MC903 to the ear skin (Schwartz et al., 2016). However, we observed no difference in the IgE-CAI ear swelling response when comparing TSLP-deficient to WT mice, indicating that TSLP is not a major pro-inflammatory factor in this model (data not shown). A recent study demonstrated basophil-dependent upregulation of VCAM-1 in a similar model of ear skin inflammation induced by topical application of DNFB in anti-DNP IgE-sensitized mice (Cheng et al., 2015). Consistent with this report, we observed massive recruitment of eosinophils into the ear pinna, and although VCAM-1 expression was not induced in the IgE-CAI model, eosinophil recruitment could be explained by induction of CCL24 expression in a basophil-dependent manner. The chemokine CCL24 binds to CCR3, which is highly

expressed on resting eosinophils and promotes trans-endothelial migration (Shahabuddin et al., 2000). CCL24 is further known to be regulated by STAT6, which gets activated in response to IL-4 and IL-13 signaling (Nielsen and Bryce, 2010; Walker et al., 2009; Zimmermann et al., 2000). Because basophils are a major source for IL-4 and IL-13, they probably directly induce expression of CCL24 in endothelial cells of the ear. Our results demonstrate an important role of basophil-derived IL-4/IL-13 and expression of STAT6-regulated genes for eosinophil recruitment and the ear swelling response in the IgE-CAI model. Interestingly, basophil and neutrophil recruitment was not affected by the lack of IL-4/IL-13 or STAT6. Despite the profound reduction in eosinophil recruitment, the ear swelling was only partially reduced in STAT6ko and 4-13ko mice, indicating that even low numbers of eosinophils can be sufficient to elicit a weak skin inflammation. Interestingly, others have shown that STAT6 can also dampen the inflammatory skin response to repeated TNP-OVA challenge at later phases of the response, probably by induction of M2-polarized anti-inflammatory macrophages (Hashimoto et al., 2015).

Further analysis of eosinophil-deficient Δ dblGATA mice revealed a critical role for eosinophils in addition to basophils for induction of the ear swelling response in IgE-CAI. In contrast to blood eosinophils, the recruited eosinophils in the skin had an activated phenotype with high expression levels of Siglec-F and downregulation of CD62L, as we previously defined in a helminth infection model (Voehringer et al., 2007). Recently, this subset has been further characterized and termed *inflammatory eosinophils* in a murine model of allergic asthma (Mesnil et al., 2016). In the IgE-CAI model, it is likely that circulating eosinophils get recruited via CCL24 and other chemoattractants that signal via G-protein-coupled chemotactic receptors, because the ear swelling response could be reconstituted in Δ dblGATA mice by transfer of untreated BM-derived eosinophils and pretreatment with pertussis toxin, which blocks G-protein-coupled chemotactic receptor signaling, resulting in an ameliorated response.

In conclusion, we demonstrate that allergic skin inflammation in the IgE-CAI model requires both basophils and eosinophils. Basophils first get activated by crosslinking of IgE bound to Fc ϵ RI, and this response can be blocked by saturating levels of unspecific serum IgE elicited by helminth infections. Basophil-derived IL-4/IL-13 then promotes CCL24 expression to induce recruitment of eosinophils, which are required for downstream execution of the ear swelling response by release of pro-inflammatory mediators. Based on these observations, the role of basophils and eosinophils in human allergic skin diseases should be further investigated, and combined therapeutic interventions that inhibit basophil activation and lower eosinophil levels in the skin might be a promising approach in the future.

MATERIALS AND METHODS

Mice

Basophil-deficient Mcpt8Cre mice (Ohnmacht et al., 2010), eosinophil-deficient Δ dblGATA mice (Yu et al., 2002), IL-4eGFP reporter (4get) mice (Mohrs et al., 2001), IgE-deficient mice

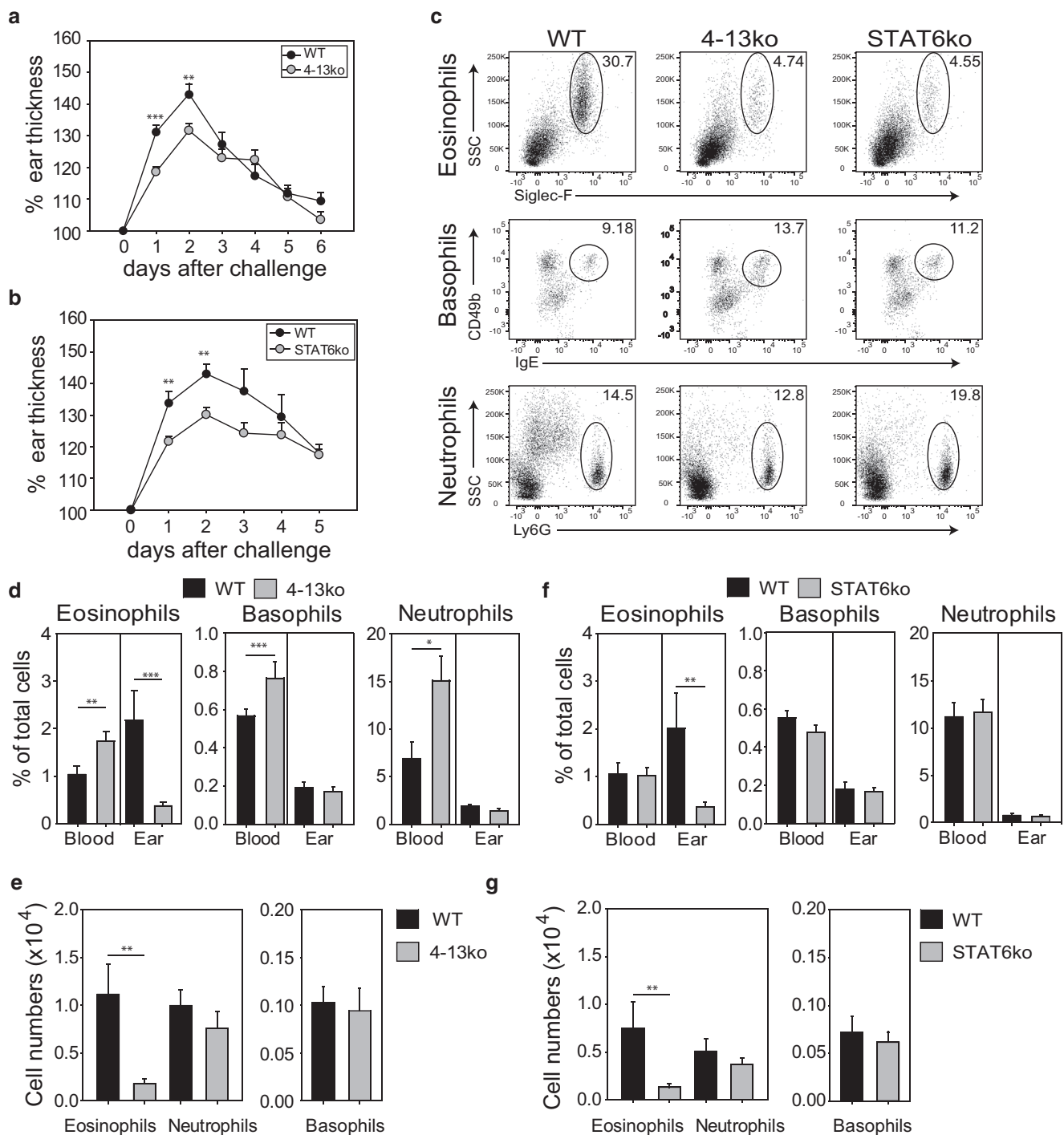


Figure 4. IL-4/IL-13 and STAT6 signaling promotes ear swelling and effector cell recruitment during IgE-mediated chronic allergic skin inflammation. (a, b) Ear thickness of indicated mice. Mean $\pm$ standard error of 30 ears of 4-13ko or STAT6ko mice and 21–28 ears of WT mice. (c) Analysis of the ear 2 days after trinitrophenyl-OVA challenge. Eosinophils (CD49b⁺IgE⁺) are gated on live CD45^{lo} cells, eosinophils (Siglec-F⁺SSC^{hi}), and neutrophils (Ly6G^{hi}) are gated on live CD45^{hi} cells. (d, f) Percentage of indicated populations in blood and ear of indicated mice. (e, g) Total cell counts of ear of indicated mice. Mean $\pm$ standard error of eight 4-13ko mice, 9 STAT6ko mice and 6 WT mice in (d) and (e) and another 6 WT mice in (f) and (g). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, two-tailed Student t test. 4-13ko, IL-4/IL-13-deficient; STAT6ko, STAT6-deficient; WT, wild-type.

(Oettgen et al., 1994), 4-13ko mice (McKenzie et al., 1999), IL-5 transgenic mice (Lee et al., 1997) and STAT6ko mice (Kaplan et al., 1996) have been described. Mice were housed according to institutional guidelines and used at 6–8 weeks of age. All animal experiments were approved by the federal government, Regierung von Unterfranken, Germany.

Generation of mixed BM chimeras

BM cells were obtained from the tibia and femur of Mcpt8Cre mice and mixed at a 4:1 ratio with BM cells from either WT mice or FcR γ -deficient mice or WT and 4-13ko mice; 2×10^6 cells were injected into the tail vein of lethally irradiated (9 Gy) recipient C57BL/6 mice to create Baso-WT and Baso-FcR γ ko or Baso-WT and Baso-4-13ko

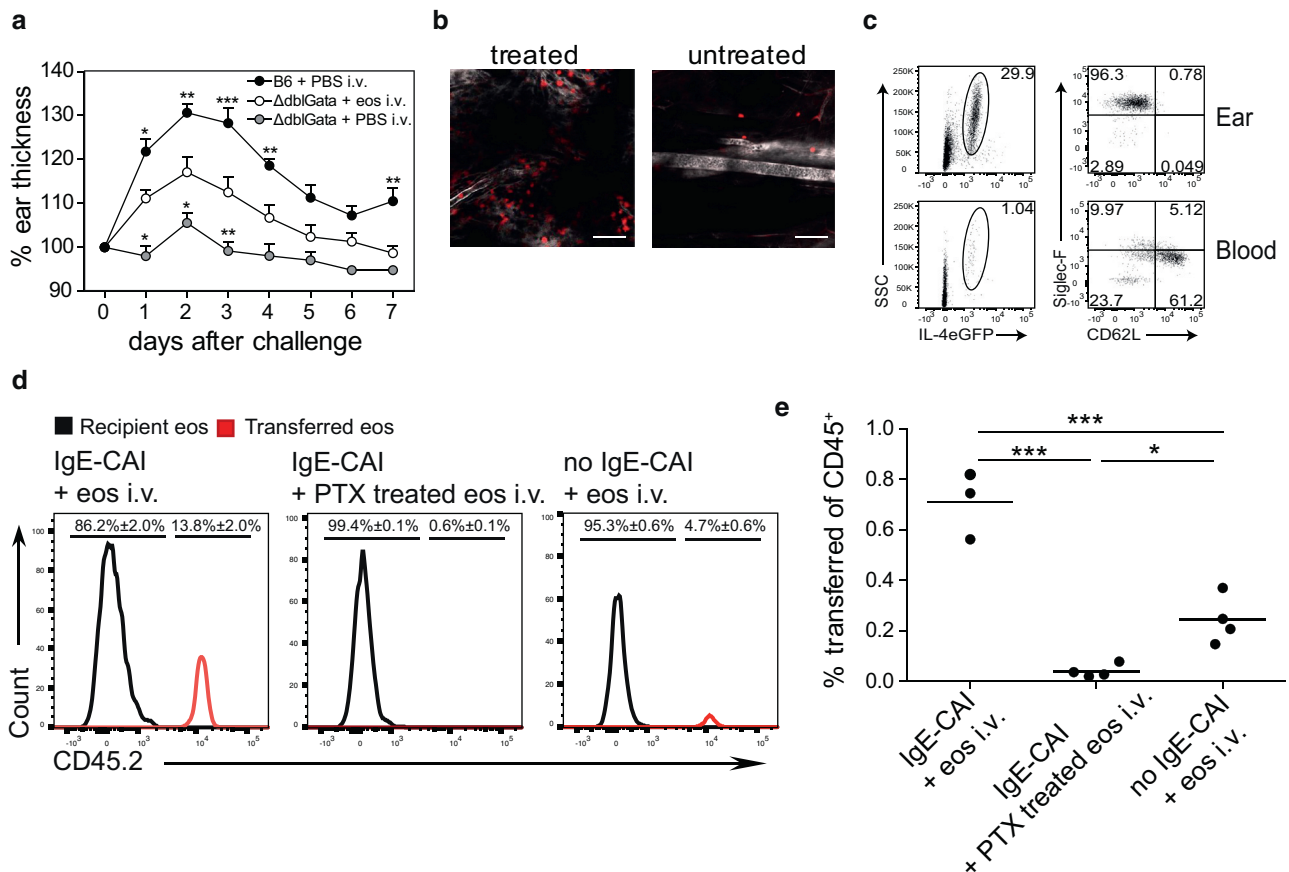


Figure 5. Eosinophils are required for induction for the ear swelling response in IgE-CAI. (a) Ear thickness of WT, Δ dblGATA, or Δ dblGATA mice with transferred eosinophils. Mean $\pm$ standard error of 4–10 ears per group. Analysis of variance was performed to compare Δ dblGATA with eosinophil transfer against the other groups. (b) Two-photon imaging of ear of EpxCre_R26^{tdTomato} mice on day 2 after challenge compared to untreated mice. Scale bar = 100 μ m. (c) Frequency of eosinophils (CD45^{hi}IL-4eGFP⁺SSC^{hi}; left plots) and their activation status based on Siglec-F and CD62L expression (right plots). Representative of 6 mice. (d) Frequency of transferred untreated or PTX-treated eosinophils in the ear of trinitrophenyl-OVA challenged or untreated mice. (e) Percentage of transferred eosinophils among total CD45⁺ cells. Analysis of variance was used to determine significance. * P < 0.05, ** P < 0.01, *** P < 0.001 two-tailed Student t test used if not indicated different. IgE-CAI, IgE-mediated chronic allergic skin inflammation; i.v., intravenous; PBS, phosphate buffered saline; PTX, pertussis toxin.

mixed BM chimeras, respectively. Mice were kept with antibiotics-containing drinking water (2 g/L neomycin sulfate, 100 mg/L polymyxin B sulfate; Sigma-Aldrich, St Louis, MO) for 8 weeks after reconstitution.

Adoptive transfer of eosinophils

Eosinophils were generated from BM as described previously (Dyer et al., 2008) or isolated from IL-5 transgenic mice (Lee et al., 1997). To block signaling of G-protein-coupled receptors mature eosinophils were incubated with 100 ng/ml pertussis toxin (Calbiochem at Merck KGaA, Darmstadt, Germany) for 2 hours at 37°C, washed with phosphate buffered saline and 1.5×10^7 of pertussis toxin treated or untreated cells were transferred intravenously into Δ dblGATA mice simultaneously with starting the IgE-CAI model.

IgE-CAI experiments

Mice were injected via the tail vein with 33 μ g monoclonal IgE-anti-TNP antibody (clone IgElb4) and challenged the next day via intradermal injection of 10 μ g TNP₁₂-coupled OVA (TNP₁₂-OVA; LGC Biosearch Technologies, Petaluma, CA) in 10 μ l phosphate buffered saline into the ear pinna. Ear thickness was measured daily with a digital thickness gauge and the change in percentage compared to the thickness on day 0 was calculated.

In some experiments, 1 mg Evans blue (Sigma-Aldrich) was injected intravenously in 200 μ l phosphate buffered saline on day 2 after challenge and mice were analyzed 20 minutes later. Vascular leakage was measured after dye extraction from ear pinnae with formamide (Sigma-Aldrich) for 24–48 hours at 55°C. Light absorption was measured at 620 nm using a Multiskan FC Microplate Photometer (ThermoFisher Scientific, Waltham, MA).

Helminth infections

Third-stage larvae of *N. brasiliensis* were washed extensively in sterile 0.9% saline at 37°C and 500 third-stage larvae were injected subcutaneously into mice. Mice were provided antibiotics containing water (2 g/l neomycin sulfate, 100 mg/l polymyxin B sulfate) for the first 5 days after infection. For the *Heligmosomoides polygyrus bakeri* infection model, 200 third-stage larvae were administered by oral gavage. Thirteen days later, mice were subjected to IgE-CAI, as described.

ELISA

IgE concentrations were determined by conventional ELISA procedure using anti-IgE clone R35-72 for coating and biotinylated anti-IgE clone R35-118 (both from BD Pharmingen, San Jose, CA) for detection, followed by streptavidin-coupled alkaline phosphatase

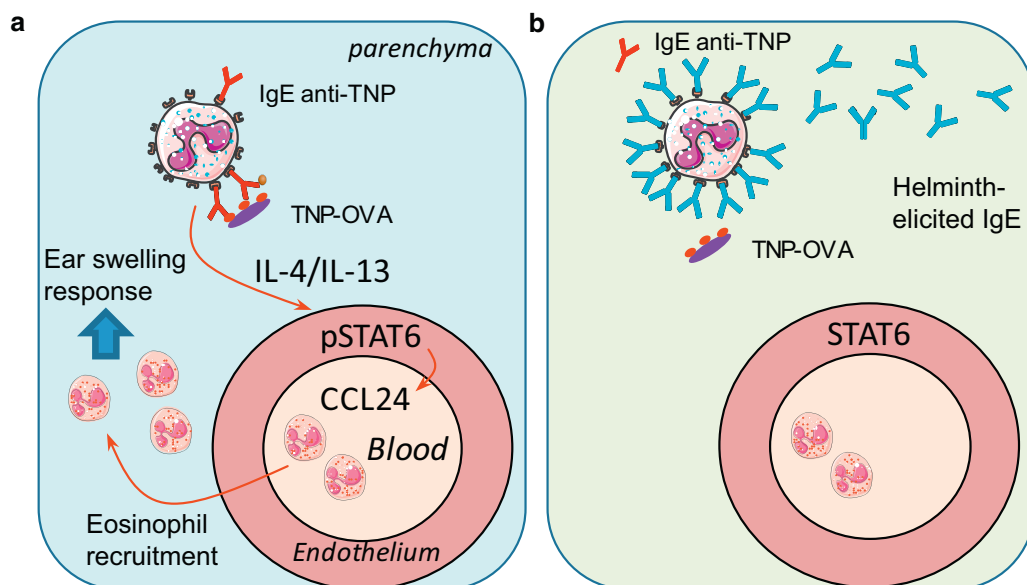


Figure 6. Model for basophil-elicited and eosinophil-dependent IgE-mediated chronic allergic skin inflammation. (a) In the absence of helminth infection, FcεRI on basophils is not saturated and can bind injected hapten-specific IgE. Antigen-induced IgE cross-linking then activates basophils to release IL-4/IL-13, which leads to induction of CCL24 expression in endothelial cells. This results in recruitment of eosinophils, which are required for induction of the ear swelling response. (b) Prior infection with helminths leads to high serum IgE levels, resulting in saturation of FcεRI on basophils so that subsequent injection of hapten-specific IgE will not lead to sensitization of basophils and therefore inhibits the ear swelling response. TNP, trinitrophenyl.

(SouthernBiotech, Birmingham, AL) and para-nitrophenyl substrate (Sigma-Aldrich). Absorbance at 405 nm was determined using a Multiskan FC Microplate Photometer (ThermoFisher Scientific). For CCL24 ELISA, ears were homogenized in RIPA buffer with protease inhibitors (Complete Protease Inhibitor Cocktail; Roche, Basel, Switzerland) with the gentleMACS Octo Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) and suspensions analyzed according to manufacturer's instructions (Mouse Eotaxin-2 ELISA Kit [ab206985]; Abcam, Cambridge, MA).

Quantitative reverse transcriptase PCR

RNA of total ear pinnae 2 days after TNP-OVA challenge of IgE-anti-TNP-sensitized mice was extracted using TRIzol (Biolabs, Luckenwalde, Germany) and gentleMACS Octo Dissociator (Miltenyi Biotec) according to manufacturer's protocol. Quantitative reverse transcriptase PCR was performed with primers listed in [Supplementary Table S1](#) online.

Antibodies, cell preparation, and flow cytometry

Single-cell suspension of total ear pinnae was prepared using the Multi Tissue Dissociation Kit I (Miltenyi Biotec) and gentleMACS Octo Dissociator with heaters (Miltenyi Biotec) according to manufacturer's protocol. Single-cell suspensions from spleen were prepared with 100-μm cell strainers before an erythrocyte lysis was performed. Cell suspensions were stained with diluted antibody mixtures on ice. Antibodies against the following antigens were used: APC anti-mouse CD49b (HMα2; Biolegend, San Diego, CA), PE-Cy7 anti-mouse FcεRIα (MAR-1; eBioscience at Thermo Fisher Scientific, Waltham, MA), BV421 anti-mouse Siglec-F (E50-2440; BD Pharmingen at Thermo Fisher Scientific), PerCP-Cy5.5 anti-mouse CD45 (30-F11; BD Pharmingen), APC/Cy7 anti-mouse Ly-6G (1A8; Biolegend), PE anti-mouse IgE (23G3; eBioscience), PE-Cy7 anti-mouse CD62L (MEL-14; eBioscience), A647 IgE anti-TNP (IgE1b4), PE anti-mouse CD200R3 (Ba13; Biolegend), biotin anti-mouse CD69 (H1.2F3; eBioscience) combined with streptavidin-

A647, and purified rat-anti-mouse CD16/32 (2.4G2; Bioxcell, West Lebanon, NH). To exclude dead cells, fixable ViaDye eFluor 506 (eBioscience) was used. Cell populations in ear tissue and blood on day 2 after TNP-OVA were defined as follows: basophils (CD49b⁺IgE⁺) gated on live CD45^{lo} cells, eosinophils (Siglec-F⁺SSC^{hi}) and neutrophils (Ly6G^{hi}) gated on live CD45^{hi} cells. Flow cytometry data acquisition was performed on a MACSQuant (Miltenyi Biotec) or BD LSRFortessa (BD Biosciences) instrument. Files were analyzed with FlowJo software (TreeStar Ashland, OR).

Two-photon microscopy

Two-photon microscopy of unfixed ear pinnae of EpxCre_R26^{tdTomato} mice was performed on day 2 after challenge and compared to untreated EpxCre_R26^{tdTomato} mice using a Zeiss LSM880 NLO Intravital Microscope (Carl Zeiss, Oberkochen, Germany). Acquired data were analyzed with ZEN (blue edition) 2.5 software (Carl Zeiss).

Statistical analysis

Statistical analysis was performed with SigmaPlot 12.3 (Systat Software, San Jose, CA) or PRISM 5.02 (GraphPad, La Jolla CA). For ear swelling curves, data points at each time point were directly compared between groups of indicated experimental and control mice if not stated differently in figure legends.

DATA AVAILABILITY

All primary data are available from the authors upon reasonable request.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

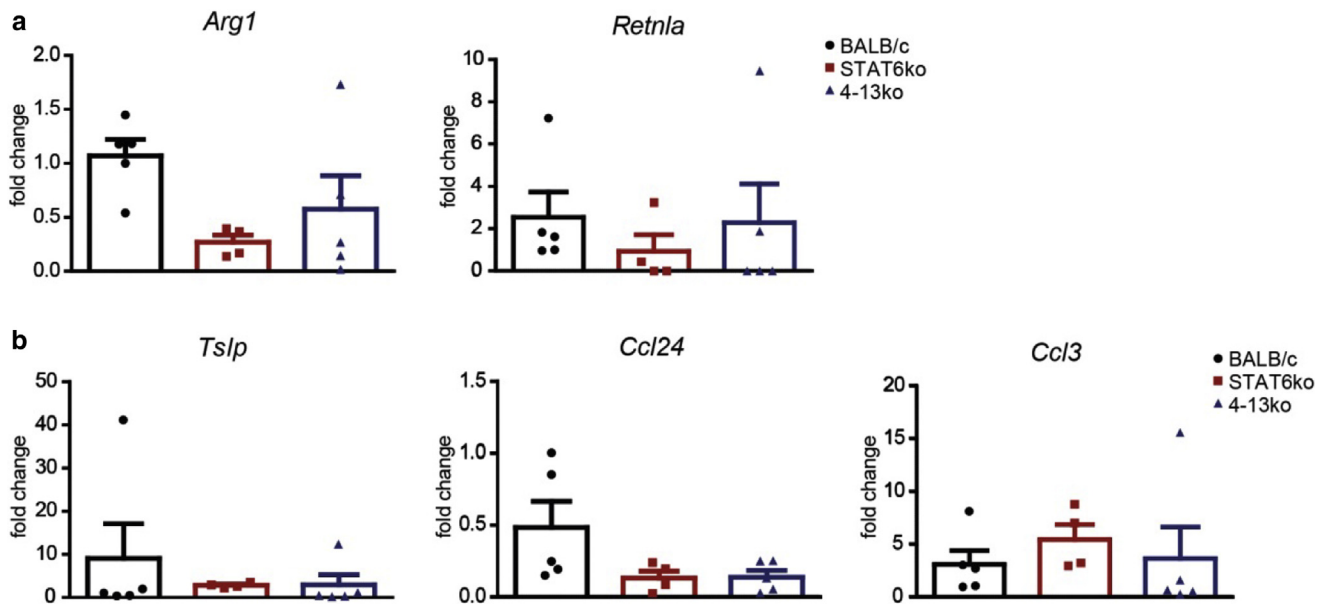
Conceptualization: JUE, DR, DV; Funding Acquisition: DV; Investigation: JUE, DR; Resources: FN; Supervision: DV; Writing – Original Draft Preparation: JUE, DR, DV; Writing – Review and Editing: JUE, DR, DV

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <https://doi.org/10.1016/j.jid.2019.03.1129>.

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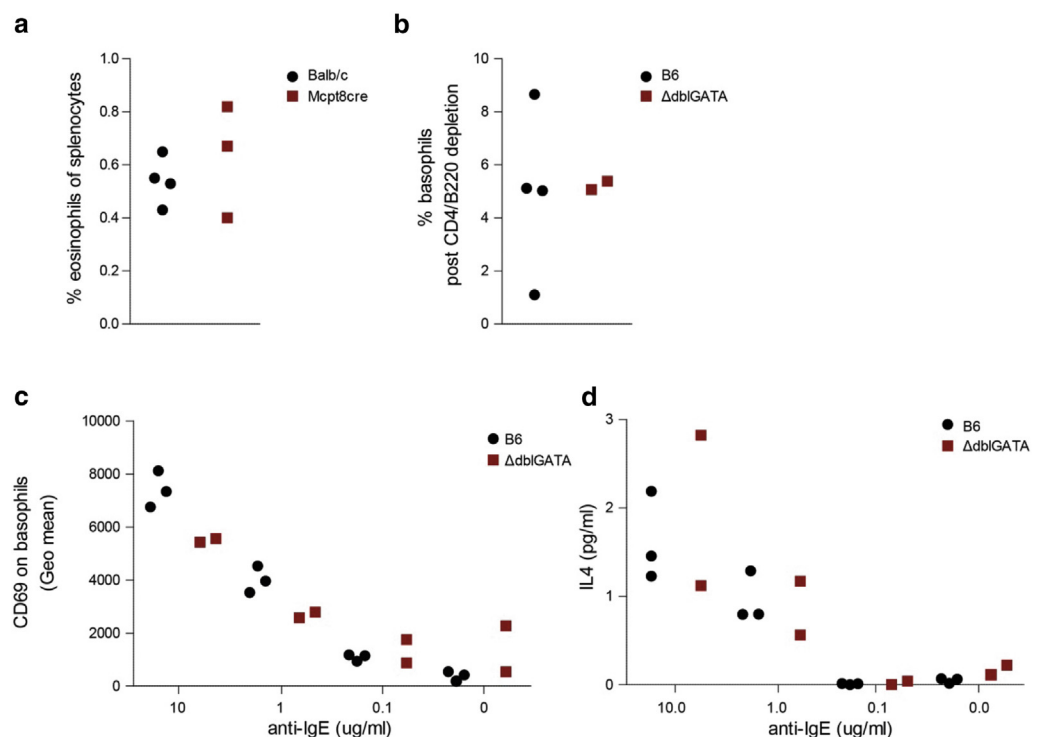
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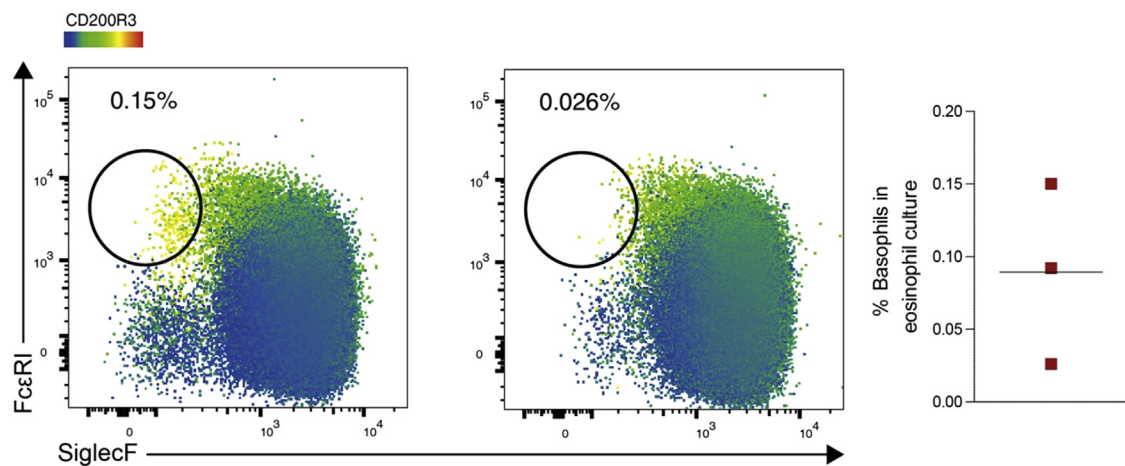


Supplementary Figure S1. Expression of M2 macrophage markers and recruitment related genes in the ear of wild-type, STAT6ko, and 4-13ko mice. (a) Quantitative reverse transcriptase PCR analysis of total ear pinnae 2 days after trinitrophenyl-OVA challenge of IgE-anti-trinitrophenyl sensitized wild-type, 4-13ko, and STAT6ko mice. Results are normalized to HPRT and fold-change was calculated with one wild-type sample as reference. (a) Expression of *Arginase 1* (fwd: 5' GTA TGA CGT GAG AGA CCA CG 3', rev: 5' CTC GCA AGC CAA TGT ACA CG 3') and *Retnla* (fwd: 5' CCA TAG AGA GAT TAT CGT GGA 3', rev: 5' TGG TCG AGT CAA CGA GTA AG 3'). (b) Expression of *Tslp* (fwd: 5' ACG GAT GGG GCT AAC TTA CAA 3', rev: 5' AGT CCT CGA TTT GCT CGA ACT 3'), *Ccl24* (fwd: 5' AGG CAG TGA GAA CCA AGT 3', rev: 5' GCG TCA ATA CCT ATG TCC AA 3') and *Ccl3* (fwd: 5' GTCTTCTCAGCGCCATATGGAGC 3', rev: 5' GTC AGG AAA ATG ACA CCT GGC TGG 3'). Analysis of variance was performed with no significance $P < 0.05$ determined. 4-13ko, IL-4/IL-13-deficient; STAT6ko, STAT6-deficient.

Supplementary Figure S2. Normal eosinophil numbers in Mcpt8Cre mice and normal basophil number and function in Δ dblGATA mice.

(a) Splenocytes of IgE anti-trinitrophenyl intravenously injected Mcpt8Cre mice challenged by trinitrophenyl₁₂OVA ear injection were analyzed 2 days later for eosinophil content by flow cytometry (Siglec-F⁺SSC^{hi}). (b) For Δ dblGATA splenocytes the percentage of basophils (CD49b⁺CD200R3⁺) after erythrocyte lysis and magnetic depletion of CD4⁺ and B220⁺ cells for later functional analysis was determined. (c) Basophils (CD49b⁺CD200R3⁺) were sorted from pre-purified cells described in (b); 6×10^4 cells per well in 100 μ l were seeded into a plate precoated overnight with indicated concentrations of anti-IgE antibody, followed by 12 hours of incubation. The expression of CD69 was analyzed by flow cytometry. (d) IL-4 in the supernatant of cultured basophils was measured by ELISA.





Supplementary Figure S3. Eosinophil cultures contain a minor basophil fractions. Flow cytometric analysis of bone marrow derived eosinophil cultures at day 14 to determine the percentage of contaminating basophils. Two exemplary plots are shown and quantification from three cultures is given.

Supplementary Table S1. Primer sequences used for RT-PCR analysis	
TSLP (fwd.: 5'- ACG GAT GGG GCT AAC TTA CAA-3', rev.: 5'-AGT CCT CGA TTT GCT CGA ACT-3'),	
CCL24 (fwd.: 5'-AGG CAG TGA GAA CCA AGT-3', rev.: 5'-GCG TCA ATA CCT ATG TCC AA-3'),	
CCL3 (fwd.: 5'-GTCTTCTCAGCGCCATATGGAGC-3', rev.: 5'-GTC AGG AAA ATG ACA CCT GGC TGG-3'),	
CCL5 (fwd.: 5'-CAC CAT CAT CCT CAC TGC AGC C-3', rev.: 5'-GCG GTT CCT TCG AGT GAC AAA C-3'),	
CCL11 (fwd.: 5'-GAA TCA CCA ACA ACA GAT GCA C-3', rev.: 5'-ATC CTG GAC CCA CTT CTT CTT-3'),	
VCAM1 (fwd.: 5'-TAC CAG CTC CCA AAA TCC TG-3', rev.: 5'-TCT GCT AAT TCC AGC CTC GT-3'),	
HPRT (fwd.: 5'-GTT GGA TAC AGG CCA GAC TTT GTT-3' , rev: 5'-GAG GGT AGG CTG GCC TAT AGG CT-3'),	

Supplementary Figure S3. [Eosinophil](#) cultures contain a minor [basophil](#) fractions. Flow cytometric analysis of bone marrow derived eosinophil cultures at day 14 to determine the percentage of contaminating basophils. Two exemplary plots are shown and quantification from three cultures is given.

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CCL3 (fwd.: 5'-GTCTTCTCAGCGCCATATGGAGC-3', rev.: 5'-GTC AGG AAA ATG ACA CCT GGC TGG-3'),
CCL5 (fwd: 5'-CAC CAT CAT CCT CAC TGC AGC C-3', rev.: 5'-GCG GTT CCT TCG AGT GAC AAA C-3'),
CCL11 (fwd.: 5'-GAA TCA CCA ACA ACA GAT GCA C-3', rev.: 5'-ATC CTG GAC CCA CTT CTT CTT-3'),
VCAM1 (fwd.: 5'-TAC CAG CTC CCA AAA TCC TG-3', rev.: 5'-TCT GCT AAT TCC AGC CTC GT-3'),
HPRT (fwd.: 5'-GTT GGA TAC AGG CCA GAC TTT GTT-3' , rev: 5'-GAG GGT AGG CTG GCC TAT AGG CT-3'),