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Atopic Dermatitis, Urticaria and Skin Disease



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Skin-infiltrating basophils promote atopic dermatitis-like inflammation via IL-4 production in mice

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Abstract

Background: Patients with atopic dermatitis (AD) often show the infiltration of basophils in the affected skin. Because basophils represent only a minor fraction among cellular infiltrates in the skin lesion, the functional significance of skin-infiltrating basophils in AD pathogenesis remains ill-defined. In this study, we aimed to clarify the role of basophils and their effector molecules triggering skin inflammation in oxazolone (OX)-induced murine model of AD.

Methods: A panel of mouse strains were sensitized and repeatedly challenged with topical applications of OX to induce AD-like skin inflammation. Both local and systemic Th2 immune responses were analyzed.

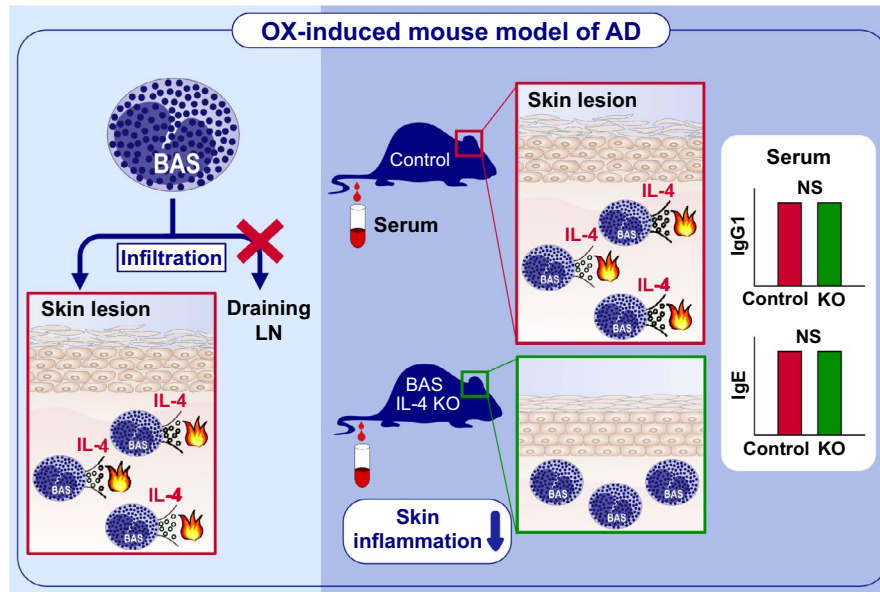
Results: Basophils progressively accumulated in the skin lesion but barely in draining lymph nodes (LNs). When basophils were depleted during the elicitation phase, skin inflammation was ameliorated while Th2 cell differentiation in draining LNs remained intact. The expression of IL-4 was highly upregulated in the affected skin, and basophils turned out to be the major producers of IL-4 among cellular infiltrates, suggesting the involvement of basophil-derived IL-4 in the Th2 skin inflammation. Indeed, basophil-specific IL-4-deficient mice displayed attenuated skin inflammation with a marked reduction of IL-4 in the skin lesion, even though cutaneous basophil infiltration and serum levels of IgE remained intact.

Conclusions: Skin-infiltrating basophils promoted OX-induced AD-like skin inflammation through their local production of IL-4, rather than the induction of Th2 cell differentiation in draining LNs. This study suggests that the selective targeting of basophils could be a beneficial strategy in the treatment of a certain type of AD.

KEYWORDS

animal models, atopic dermatitis, basophil

Abbreviations: AD, atopic dermatitis; DT, diphtheria toxin; IL, interleukin; ILC2, group 2 innate lymphocyte; LN, lymph node; mDT, mutant diphtheria toxin; OX, oxazolone; Th, T helper; TSLP, thymic stromal lymphopoietin; WT, wild-type.



GRAPHICAL ABSTRACT

Basophils infiltrated the OX-induced AD-like skin lesion but barely draining LNs, and they were major IL-4 producers in the affected skin. Basophil-specific IL-4-deficient mice showed ameliorated skin inflammation even though serum levels of IgG1/IgE remained intact. Skin-infiltrating basophils play a crucial role in the development of OX-induced AD-like skin inflammation through local IL-4 production, independently of LN Th2 cell differentiation.

1 | INTRODUCTION

Atopic dermatitis (AD) is a common skin disease characterized by chronic and relapsing eczematous lesions with itching, which substantially impairs the patient's quality of life. The prevalence of AD has increased particularly in industrialized countries over the past four decades, currently approaching 15%–30% of children and 2%–10% of adults.^{1,2} Although both endogenous and exogenous factors are associated with AD pathogenesis, accumulating evidence suggests that T helper (Th) 2 cytokines are pivotal effector molecules for the development of AD.³ Indeed, Th2 cytokines IL-4 and IL-13 are genetically associated with human AD^{4,5} and highly expressed in the lesional skin of AD,^{6,7} implying their significant roles in AD pathogenesis. Moreover, recent clinical trials with an IL-4R α antagonist dupilumab revealed its therapeutic effect on moderate-to-severe AD, clearly demonstrating that IL-4/13 signaling plays a crucial role in the development of AD.^{8,9} However, the cellular source of these Th2 cytokines remains to be clarified.

AD patients often show the infiltration of basophils in the affected skin.^{10–12} Because basophils represent only a minor fraction among cellular infiltrates in the skin lesion, the functional significance of skin-infiltrating basophils in AD pathogenesis remains ill-defined. Otsuka et al examined the possible role for basophils in the oxazolone (OX)-induced murine model of AD.¹³ In this model, a single challenge of OX on the skin induces a Th1-mediated delayed-type hypersensitivity in OX-sensitized mice,^{14,15} while repeated OX challenges over an extended period progressively cause a more chronic Th2 inflammation similar to human AD.¹⁶ Otsuka et al reported that ablation of basophils resulted in reduction of Th2 skin inflammation

and IgG1 induction (as a marker of Th2 cell differentiation). Basophils accumulated in draining lymph nodes (LNs) and expressed MHC class II and co-stimulatory molecules. Moreover, some of them produced IL-4, suggesting that basophils are involved in Th2 cell differentiation in draining LNs as antigen-presenting and IL-4-providing cells.¹³ However, it remained to be determined how basophils contribute to the development of skin inflammation and whether basophil-elicited Th2 cell differentiation in draining LNs are related to the pathogenesis of Th2 skin inflammation.

In the present study, we revisited the role for basophils in the OX-induced AD model. We found that basophils accumulated in the affected skin but barely in draining LNs in the elicitation phase of skin inflammation. Basophil depletion resulted in attenuated skin inflammation whereas Th2 cell differentiation remained intact. Analysis of various genetically engineered mice revealed that skin-infiltrating basophils promoted skin inflammation through local production of IL-4, independently of Th2 cell differentiation in draining LNs.

2 | METHODS

2.1 | Mice

C57BL/6J mice were purchased from Japan SLC. IL-4-deficient mice,¹⁷ *Mcpt8*^{DTR/+} mice,¹⁸ IL-4 reporter G4 (*Il4*^{GFP/+}) mice,¹⁹ and *Mcpt8*^{iCre/+}*Il4*^{fl/fl} mice²⁰ were described previously. The information about the genetically engineered mice used in this study is summarized in Table S1. Mice were maintained under specific pathogen-free

conditions in our animal facilities. All animal studies were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (No. A2019-110C4).

2.2 | Antibodies

The following antibodies were purchased from BioLegend: biotinylated anti-F4/80 (BM8), FITC-conjugated anti-CD11b (M1/70), anti-CD45 (30-F11) and anti-c-kit (2B8), PE-conjugated anti-CD3 ϵ (145-2C11) and anti-CD49b (HM α 2), APC-conjugated anti-CD8a (53-6.7) and anti-CD200R3 (Ba13), PE-Cy7-conjugated anti-CD4 (GK1.5), anti-CD49b (HM α 2) and anti-F4/80 (BM8), APC-Cy7-conjugated anti-CD45 (30-F11) and anti-Ly6G (1A8), Pacific Blue-conjugated anti-c-kit (2B8) and anti-CD11b (M1/70), Brilliant Violet 421-conjugated anti-CD3 ϵ (145-2C11), and streptavidin. Alexa Fluor 647-conjugated anti-Siglec-F (E50-2440) was from BD Biosciences. Both anti-CD200R3 (Ba103)^{21,22} and anti-CD16/32 (2.4G2) were prepared in our laboratory. To measure serum immunoglobulin levels, ELISA MAX Standard Set Mouse IgE, anti-mouse IgG1 (RMG1-1), and HRP-conjugated goat anti-mouse IgG (Poly4053) were purchased from BioLegend.

2.3 | OX-induced mouse model of AD

OX (4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one, Sigma-Aldrich) was dissolved in the mixture of acetone and olive oil (4:1 ratio). Mice at the age of 7-10 weeks old were epicutaneously sensitized with 100 μ l of 3% OX on their shaved abdomen 7 days before the first challenge, and repeatedly challenged every other day with topical applications of either 10 μ l of 0.5% OX or vehicle alone on both the dorsal and ventral surfaces of their right or left ear, respectively. Ear thickness was measured at indicated time points just before the next challenge (Δ ear thickness = right - left).

2.4 | Histopathological analysis of the skin

Ear specimens were fixed with 4% paraformaldehyde (Wako, Osaka, Japan) and embedded in paraffin, and sections were stained with hematoxylin and eosin (Wako, Osaka, Japan) or with a basophil-specific anti-mMCP-8 monoclonal antibody (TUG8, BioLegend) in combination with the appropriate secondary antibody and 3, 3'-diaminobenzidine, followed by hematoxylin counterstaining.

2.5 | Quantitative PCR (Q-PCR)

Total RNA was extracted from tissues or isolated cells by RNeasy Mini Kit (QIAGEN), followed by cDNA synthesis with SuperScript III First-Strand Synthesis System (Invitrogen). Q-PCR of the cDNA was performed with a Fast SYBR Green Master Mix (Applied Biosystems) by using

following primer sets: 5'-ACAGCAAGGCGAAAAAGGATG-3' and 5'-TGGTGGACCACTCGGATGA-3' for *Ifng*; 5'-GGCATTGTAACGAGGTCAC-3' and 5'-AAATATGCGAAGCACCTTGG-3' for *Il4*; 5'-CTCTGTTGACAAGCAATGAGACG-3' and 5'-TCTTCAGTATGTCTAGCCCCTG-3' for *Il5*; 5'-GCCTCTGTTTGTCTTCAGTT-3' and 5'-GCA TTTTGACGGTGGATCA-3' for *Il9*; 5'-GCAACATCACACAAGACCAGA-3' and 5'-GTCAGGGAATCCAGGGCTAC-3' for *Il13*; 5'-TTTAACTCCCTTGGCGCAAAA-3' and 5'-CTTTCCTCCCGCATTGACAC-3' for *Il17a*; 5'-GGCCCTCGACTCTCGCTTTC-3' and 5'-TGCCAGGACGCGCTTGT-3' for *36B4*. Relative gene expression levels were calculated by the $\Delta\Delta$ Ct method with normalization using *36B4* expression.

2.6 | Flow cytometric analysis and cell sorting

The ear skin was treated with 150 U/mL collagenase (Wako) at 37°C for 2 hours. Single cell suspensions were obtained from the treated skin, LN, or spleen. After pretreatment with anti-CD16/32 (2.4G2) and normal rat serum to prevent the nonspecific binding of irrelevant Abs, cells were stained with indicated combination of antibodies for 30 minutes and analyzed by FACSCanto II (BD Biosciences) and FlowJo (Tree star). Dead cells were excluded by staining with propidium iodide (Sigma-Aldrich). Each cell lineages were defined as follows: hematopoietic cells (CD45⁺), basophils (c-kit⁺CD49b⁺CD200R3⁺), eosinophils (Siglec-F⁺ CD11b⁺SSC^{high}), neutrophils (Ly6G⁺CD11b⁺), monocytes/macrophages (Ly6G⁺Siglec-F⁺F4/80⁺CD11b⁺), mast cells (c-kit⁺CD200R3⁺), CD4 T cells (CD3⁺CD4⁺FSC^{low}SSC^{low}), and CD8 T cells (CD3⁺CD8⁺FSC^{low}SSC^{low}). In some experiments, each cell lineages were isolated from tissues by sorting with FACSaria II (BD Biosciences). For measurement of tissue cytokines at the protein levels, the ear skin was lysed with 0.5% Triton X-100 (Sigma-Aldrich) in PBS containing a protease inhibitor cocktail (Sigma-Aldrich). The cytokine levels in the tissue lysates were measured by using cytometric bead array (BD Biosciences).

2.7 | Depletion of basophils in mice

For the DT-mediated basophil depletion, *Mcpt8*^{DTR/+} mice were intravenously injected with 500 ng of DT (Sigma-Aldrich) or its inactive mutant [Glu⁵²]-DT (mDT, Sigma-Aldrich) one day before and four days after the first OX challenge.¹⁸ For the antibody-mediated basophil depletion, mice were intraperitoneally injected with 150 μ g of anti-CD200R3 monoclonal antibody (Ba103)^{21,22} or control rat IgG2b (RTK4530, BioLegend) on days -1, 3, and 7.

2.8 | Statistical analysis

Comparisons between two groups were performed using unpaired Student's *t* test. Comparisons between multiple treatment groups and

a control group were performed using one-way ANOVA with post hoc Tukey HSD test. A P value < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Basophils promote OX-induced AD-like skin inflammation

To elucidate cellular and molecular mechanisms underlying atopic dermatitis (AD), we analyzed a murine AD-like model induced by repeated OX applications.¹⁶ C57BL/6J mice were epicutaneously sensitized with OX and challenged on days 0, 2, 4, 8, and 10 with topical applications of OX on the ear skin. Multiple applications

of OX but not vehicle alone progressively elicited ear thickening (Figure 1A) accompanied by the formation of eczematous skin lesions with excoriations and lichenifications (Figure 1B). Histopathological analysis revealed progressive epidermal and dermal hyperplasia with cellular infiltration in the dermis of the OX-treated skin (Figure 1C). Recently, basophils were reported to be crucial for the development of the OX-induced AD-like skin inflammation.¹³ To examine whether this is also the case under our experimental conditions, we depleted basophils *in vivo* by two different methods that are both distinct from one used in the previous study¹³: diphtheria toxin (DT)-treatment of *Mcpt8*^{DTR/+} C57BL/6J mice¹⁸ and anti-CD200R3 antibody (Ba103)-treatment of wild-type (WT) mice.^{21,22} DT administrations, but not its inactive mutant (mDT), to *Mcpt8*^{DTR/+} mice selectively ablated basophils in the

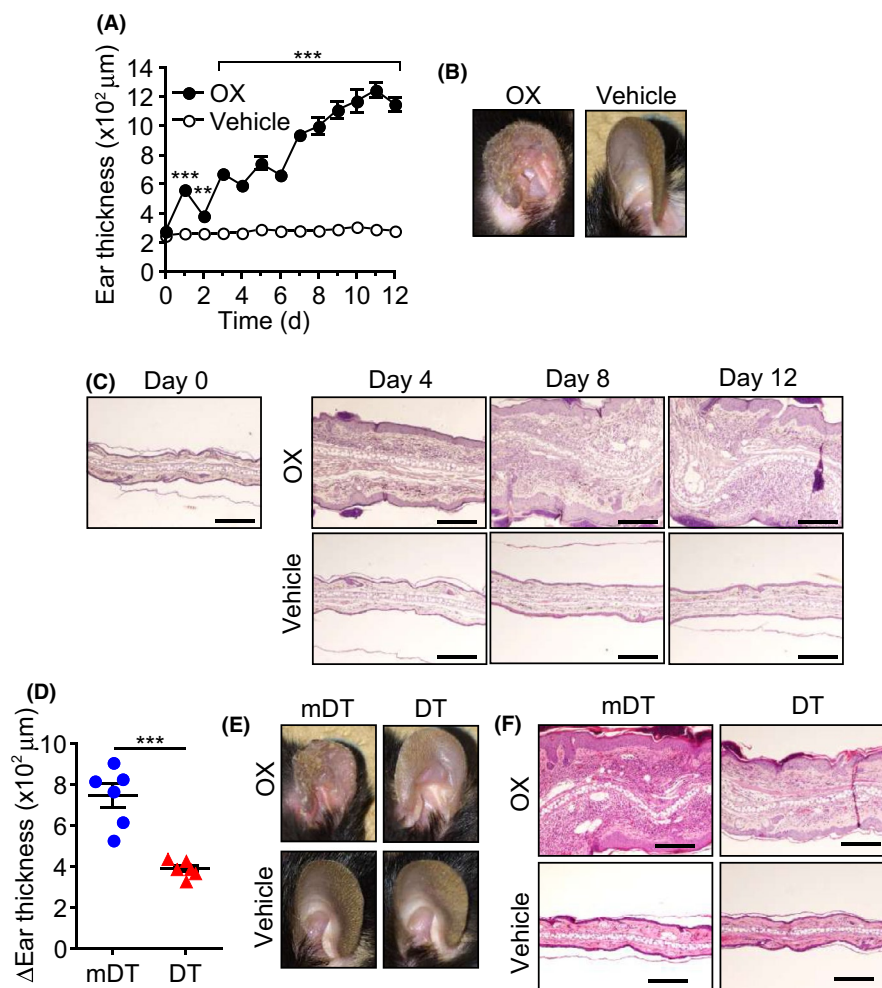


FIGURE 1 Basophils contribute to the development of OX-induced AD-like skin inflammation. (A–C) C57BL/6J mice were epicutaneously sensitized with OX on their shaved abdomen and challenged every other day with topical application of OX and vehicle alone on their right and left ear, respectively. Time course of ear thickening is shown in A (mean $\pm$ SEM, $n = 5$ mice each). The gross appearance of OX- or vehicle-treated ear skin on day 8 is shown in B. Hematoxylin and eosin (HE)-stained specimens of the OX- or vehicle-treated ear skin on days 0 (untreated), 4, 8, and 12 are shown in C. Scale bars, 200 μ m. (D–F) *Mcpt8*^{DTR/+} mice were treated with OX to induce skin inflammation in the presence or absence of basophils by treatment with intravenous administrations of either mDT or DT one day before and four days after the first OX challenge, respectively. The extent of ear thickening on day 8 is shown in D (Δ ear thickness = OX–vehicle, mean $\pm$ SEM, $n = 6$ mice each). The gross appearance and HE-stained specimens of OX- or vehicle-treated ear skin on day 8 are shown in E and F, respectively. Data shown are representative of three independent experiments. ** $P < .01$ and *** $P < .001$ measured by unpaired Student's t test

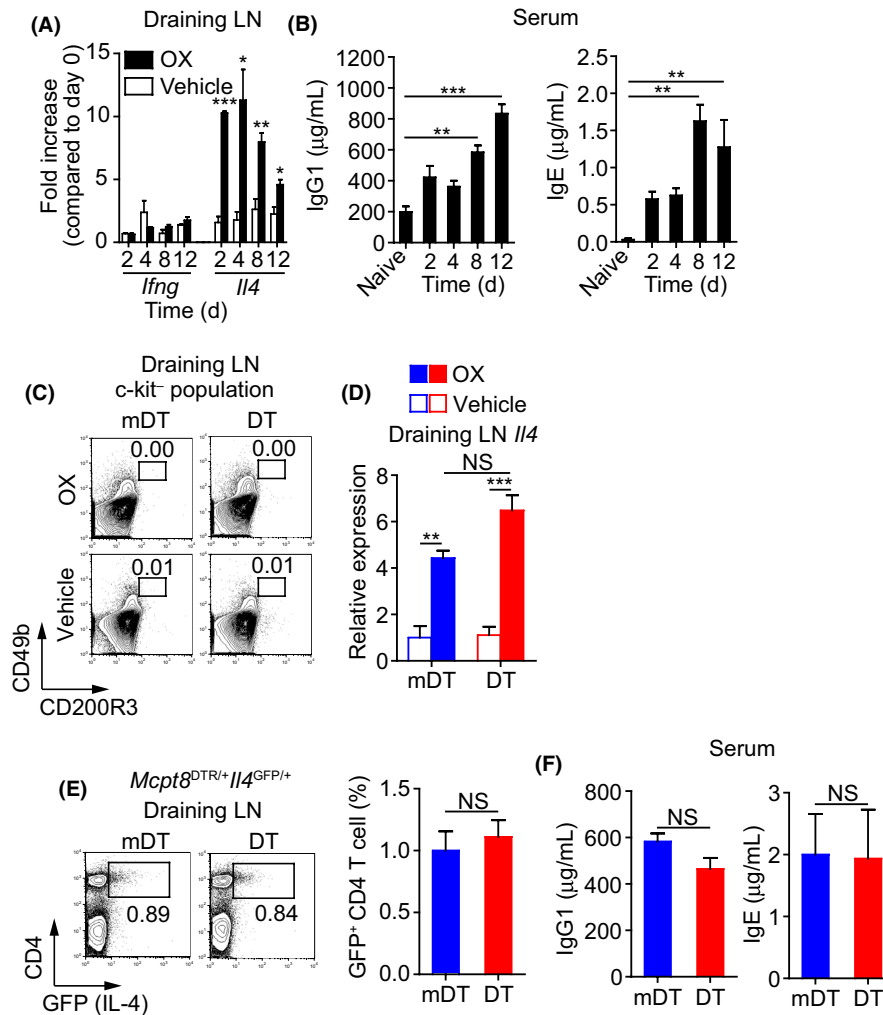


FIGURE 2 Basophils are dispensable for the induction of Th2 cell differentiation and IgG1/IgE production in the OX-induced AD model. (A and B) C57BL/6J mice were treated with OX as in Figure 1 to induce skin inflammation. Relative expression levels of indicated mRNAs isolated from the draining LNs at indicated time points are shown in A (mean $\pm$ SEM, $n = 3$ –4 mice each). The value of mRNA expression on day 0 is set as 1. The serum IgG1 and IgE levels at indicated time points are shown in B (mean $\pm$ SEM, $n = 3$ mice each). (C–F) *Mcpt8^{DTR/+}* mice (C, D, and F) and *Mcpt8^{DTR/+} Il4^{GFP/+}* mice (E) were treated with OX to induce skin inflammation in conjunction with mDT or DT treatment as in Figure 1. In C, contour plots show the flow cytometric analysis of the cervical draining LNs collected from either OX- or vehicle-treated side of DT- or mDT-treated mice on day 8. The c-kit-negative populations are displayed. Numbers above the gated areas indicate percentages of basophils among total live cells. In D, relative expression levels of *Il4* mRNA in the draining LNs were analyzed on day 8 (mean $\pm$ SEM, $n = 4$ mice each). The value in the mDT vehicle is set as 1. In E, contour plots show flow cytometric analysis of the draining LNs collected from OX-treated side of DT- or mDT-treated mice on day 8. Numbers below the gated areas indicate percent of GFP (IL-4)-positive CD4 T cells among total live cells, and their average is shown in the right panel (mean $\pm$ SEM, $n = 3$ mice each). In F, serum levels of total IgG1 and IgE on day 8 were measured by ELISA (mean $\pm$ SEM, $n = 4$ mice each). Data shown are representative of at least three independent experiments. NS: not significant, * $P < .05$, ** $P < .01$, and *** $P < .001$ measured by unpaired Student's *t* test or one-way ANOVA with post hoc Tukey HSD test

spleen (Figure S1A and B), resulting in attenuation of OX-induced ear thickening and eczema formation on day 8 (Figure 1D and E). Histopathological examination revealed the reduction of epidermal and dermal hyperplasia and cellular infiltration in the affected skin collected from DT-treated mice (Figure 1F). Amelioration of both ear thickening and skin pathology was also observed in OX-treated WT mice that had been injected with a basophil-depleting Ba103 antibody (Figure S2A and B). Thus, we confirmed the previous finding that basophils play a crucial role in the development of OX-induced skin inflammation, under our experimental conditions.

3.2 | Basophils are dispensable for the OX-induced Th2 cell differentiation in draining lymph nodes

Otsuka et al reported a potential role for draining LN-infiltrating basophils in promoting Th2 cell differentiation and systemic IgG1 induction in OX-induced AD-like skin inflammation.¹³ Consistent with this, cervical LNs isolated from the OX-treated side of WT mice on day 2, 4, 8, and 12 showed increased mRNA expression of *Il4*, but not *Ifng*, compared to those from vehicle-treated side (Figure 2A). Moreover, OX-treated mice exhibited increased levels of serum IgG1 and IgE

(Figure 2B), suggesting that basophil-mediated Th2 cell differentiation might have occurred in the skin-draining LNs. To examine this possibility, we compared *Mcpt8*^{DTR/+} mice treated with either DT or control mDT in terms of *Il4* mRNA expression and the frequency of IL-4⁺ CD4 T cells in the draining LNs on day 8. Unexpectedly, we found little or no accumulation of basophils in the skin-draining LNs even in control mDT-treated *Mcpt8*^{DTR/+} mice on days 4 and 8 (Figure 2C, not all data shown). In accordance with this, DT treatment of *Mcpt8*^{DTR/+} mice had no significant impact on the level of *Il4* mRNA expression in the draining LN (Figure 2D) and IL-4 expression by LN CD4 T cells (Figure 2E), even though DT treatment sufficiently depleted basophils from the spleen in *Mcpt8*^{DTR/+} mice (Figure S1A and B). This clearly indicated that basophils were dispensable for the OX-induced Th2 cell differentiation in the draining LNs when examined on day 8. Consistent with this, DT-mediated basophil depletion had no apparent effect on the level of serum IgG1 and IgE on day 8 (Figure 2F). Therefore, basophils appeared to contribute to the development of skin inflammation, independently of Th2 cell differentiation in the draining LNs and serum levels of Th2-type Ig isotypes.

3.3 | IL-4 plays a crucial role in the development of OX-induced AD-like skin inflammation

It has been demonstrated that repeated OX challenge elicits Th2 inflammation in the skin.¹⁶ Accordingly, we next analyzed the expression

of Th cytokines in the skin lesions (Figure 3A and B). Expression of Th1 cytokine *Ifng* mRNA was transiently upregulated from day 2 to day 8 to some extent in the OX-treated skin, while Th2 cytokine *Il4* mRNA expression was dramatically increased in the OX-treated skin compared to the nontreated ear skin collected from other mice on day 0 (Figure 3A). In contrast, mRNA expressions of other Th cytokines, including *Il5*, *Il9*, *Il13*, and *Il17a*, were slightly or barely elevated in the OX-treated skin (Figure 3A). Indeed, among Th cytokines examined, IL-4 was the only one detectable at the protein level in lysates from OX-treated ear skins (Figure 3B), suggesting a possible role for cutaneous IL-4 in the development of OX-induced skin inflammation.

Indeed, IL-4-deficient mice showed significant reduction in OX-induced ear thickening, excoriation, and lichenification (Figure 3C and D). Histopathological examination revealed the reduction of epidermal and dermal hyperplasia and cellular infiltration in the OX-treated ear skin collected from IL-4-deficient mice (Figure 3E). Thus, IL-4 plays a crucial role in promoting OX-induced AD-like skin inflammation.

3.4 | Basophils accumulate in the OX-induced skin lesion and are the major producers of IL-4 among skin infiltrates

We then addressed the cellular source of IL-4 in the affected skin. Several cell lineages known to have the potential to produce IL-4,

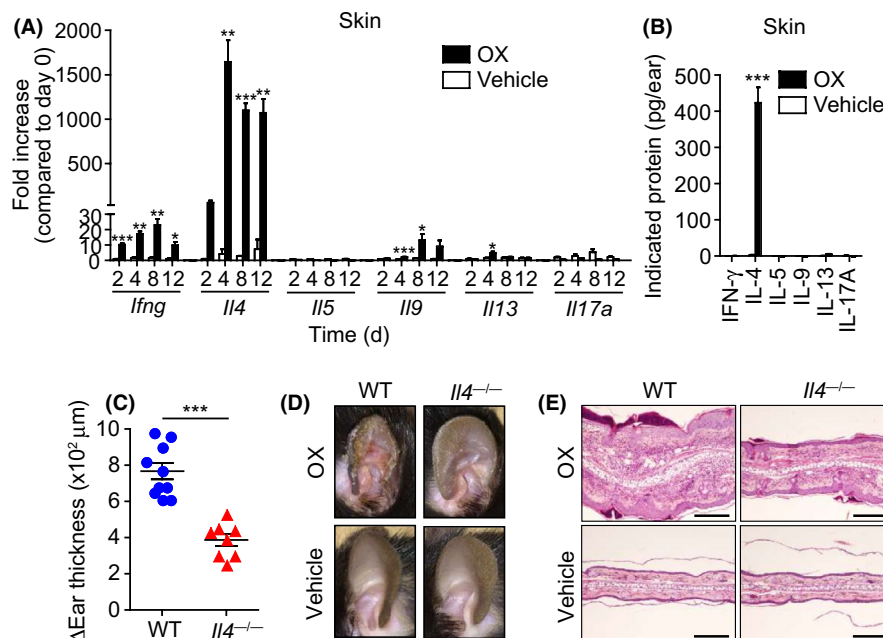


FIGURE 3 IL-4 is highly elevated in the skin lesion and required for skin inflammation. (A–B) C57BL/6J mice were treated with OX as in Figure 1 to induce skin inflammation. In A, relative expression levels of indicated mRNAs isolated from the OX- or vehicle-treated ear skin at indicated time points are shown (mean ± SEM, n = 3 mice each). The value of mRNA expression on day 0 is set as 1. In B, the protein amount of the indicated cytokines in the lysates of OX- or vehicle-treated ear skin on day 8 was measured by cytometric bead array (mean ± SEM, n = 4 mice each). (C–E) WT or *Il4*^{−/−} mice were treated with OX as in Figure 1 to induce skin inflammation. The extent of ear thickening on day 8 is shown in C (Δear thickness = OX–vehicle, mean ± SEM, n = 8–10 mice each). The gross appearance and HE-stained specimens of OX- or vehicle-treated ear skin on day 8 are shown in D and E, respectively. Scale bars, 200 μm. Data shown are representative of at least three independent experiments. **P* < .05, ***P* < .01, and ****P* < .001 measured by unpaired Student's *t* test

including CD4 T cells, basophils, eosinophils, and mast cells,²³⁻²⁶ were significantly increased in the skin lesion of OX-treated mice (Figure 4A). We found that *Il4* mRNA was predominantly expressed by basophils among various cell types isolated from the affected

skin on day 8 (Figure 4B). Moreover, skin-infiltrating basophils expressed much higher levels of *Il4* mRNA than did those residing in the spleen (Figure 4C), suggesting that basophils were activated to produce a large amount of IL-4 in the skin. Flow cytometric analysis

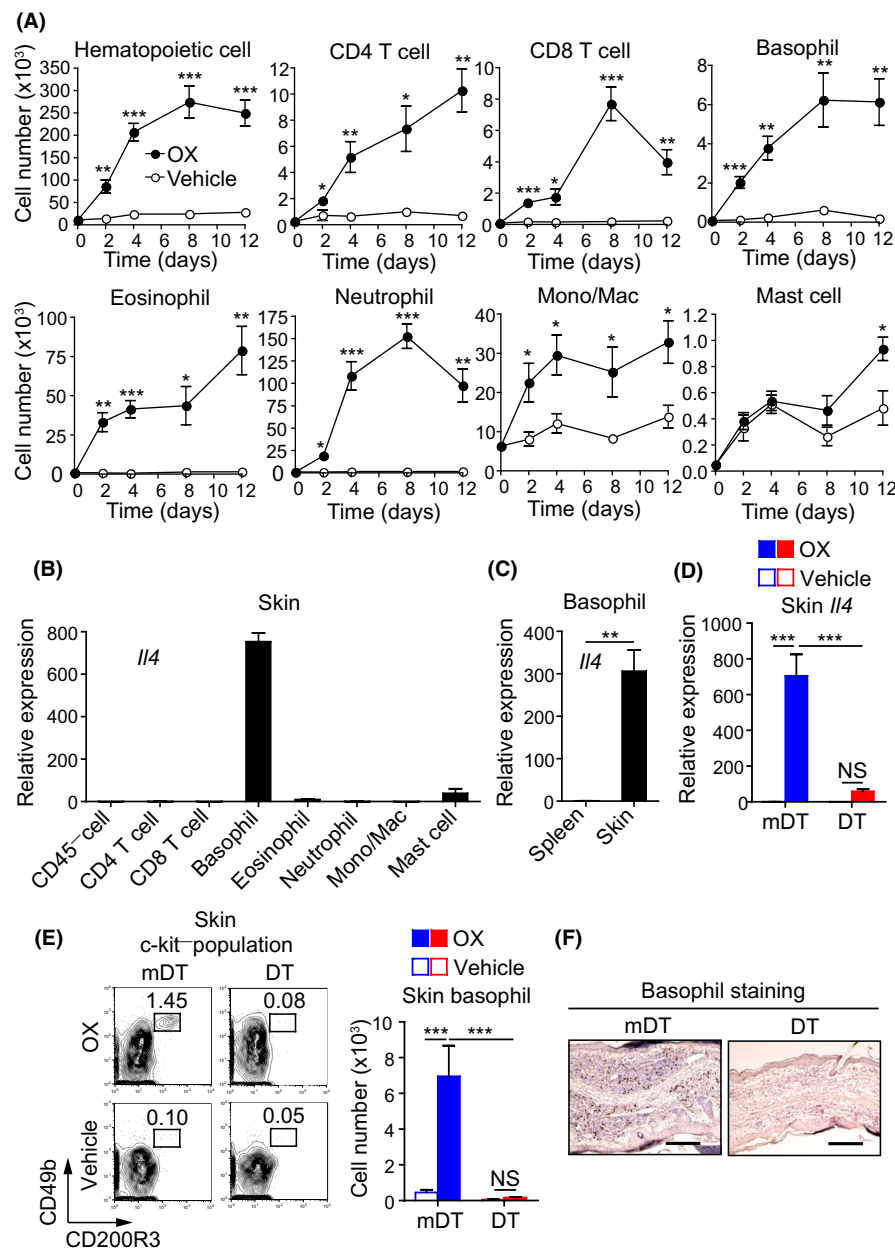


FIGURE 4 Basophils progressively accumulate in the skin lesion and are the major producers of IL-4 among skin-infiltrating cells. (A-C) C57BL/6J mice were treated with OX as in Figure 1 to induce skin inflammation. The numbers of indicated cell types isolated from the OX- or vehicle-treated ear skin at indicated time points are shown in A (mean $\pm$ SEM, $n = 4$ mice each). Relative expression levels of *Il4* mRNA in indicated cell lineages isolated from the OX-treated ear skin on day 8 are shown in B (mean $\pm$ SEM, $n = 3$ mice each). The value in neutrophils is set as 1. Relative expression levels of *Il4* mRNA in splenic or skin-infiltrating basophils on day 8 are shown in C (mean $\pm$ SEM, $n = 3$ mice each). The value in splenic basophils is set as 1. (D-F) *Mcpt8*^{DTR/+} mice were treated with OX to induce skin inflammation in conjunction with mDT or DT treatment as in Figure 1. In D, relative expression levels of *Il4* mRNA in the skin were analyzed on day 8 (mean $\pm$ SEM, $n = 4$ mice each). The value in the mDT vehicle is set as 1. In E, contour plots show cytometric analysis of OX- or vehicle-treated ear skin collected from DT- or mDT-treated mice on day 8. The c-kit-negative populations are displayed. Numbers above the gated areas indicate percentages of basophils among total live cells. The numbers of basophils isolated from the ear skin on day 8 are shown in the right panel (mean $\pm$ SEM, $n = 4$ mice each). In F, OX- or vehicle-treated ear skins on day 8 were subjected to immunostaining with a basophil-specific anti-mMCP-8 antibody. Scale bars, 200 μ m. Data shown are representative of three independent experiments. * $P < .05$, ** $P < .01$, and *** $P < .001$ measured by unpaired Student's *t* test or one-way ANOVA with post hoc Tukey HSD test

of $Il4^{GFP/+}$ mice confirmed the high levels of $Il4$ gene transcription in basophils, but not other cell types, present in the affected skin (Figure S3). Notably, DT treatment of $Mcpt8^{DTR/+}$ mice resulted in significant reduction of $Il4$ mRNA expression in the OX-treated ear skin (Figure 4D) in parallel with the absence of basophil accumulation in the skin lesion (Figure 4E and F). Taken together with the observation that DT-treated $Mcpt8^{DTR/+}$ mice exhibited a marked attenuation of OX-induced skin inflammation (Figure 1D-F), skin-infiltrating basophils appeared to play an important role in the development of OX-induced skin inflammation through the production of IL-4.

3.5 | IL-4 produced by basophils plays a crucial role in the development of OX-induced AD-like skin inflammation

To directly assess the importance of basophil-derived IL-4 in the development of the skin inflammation, we analyzed $Mcpt8^{iCre/+}Il4^{fl/fl}$ mice that are deficient for IL-4 only in basophils.²⁰ $Mcpt8^{iCre/+}Il4^{fl/fl}$ mice exhibited a marked amelioration of OX-induced eczema formation

and skin pathology (Figure 5A and B) in parallel with reduced expression of $Il4$ in the skin (Figure 5C) compared to control $Mcpt8^{iCre/+}$ mice, even though the number of basophils accumulated in the skin lesion was comparable in both mouse strains (Figure 5D). OX-treated $Mcpt8^{iCre/+}Il4^{fl/fl}$ mice showed elevated levels of serum IgG1 and IgE as high as observed in OX-treated control $Mcpt8^{iCre/+}$ mice (Figure 5E), suggesting that IL-4 deficiency in basophils had no apparent impact on Th2 cell differentiation in the draining LNs. Taken together with observations that basophils were the major IL-4 producers in the skin lesion (Figure 4B-D), these results strongly supported the notion that skin-infiltrating basophils play a crucial role in promoting OX-induced AD-like skin inflammation through IL-4 production, independently of Th2 cell differentiation in the draining LNs.

4 | DISCUSSION

The involvement of basophils has been demonstrated in the development of OX-induced allergic inflammation in a murine model of AD.¹³ However, it remained to be clarified how basophils contribute to the

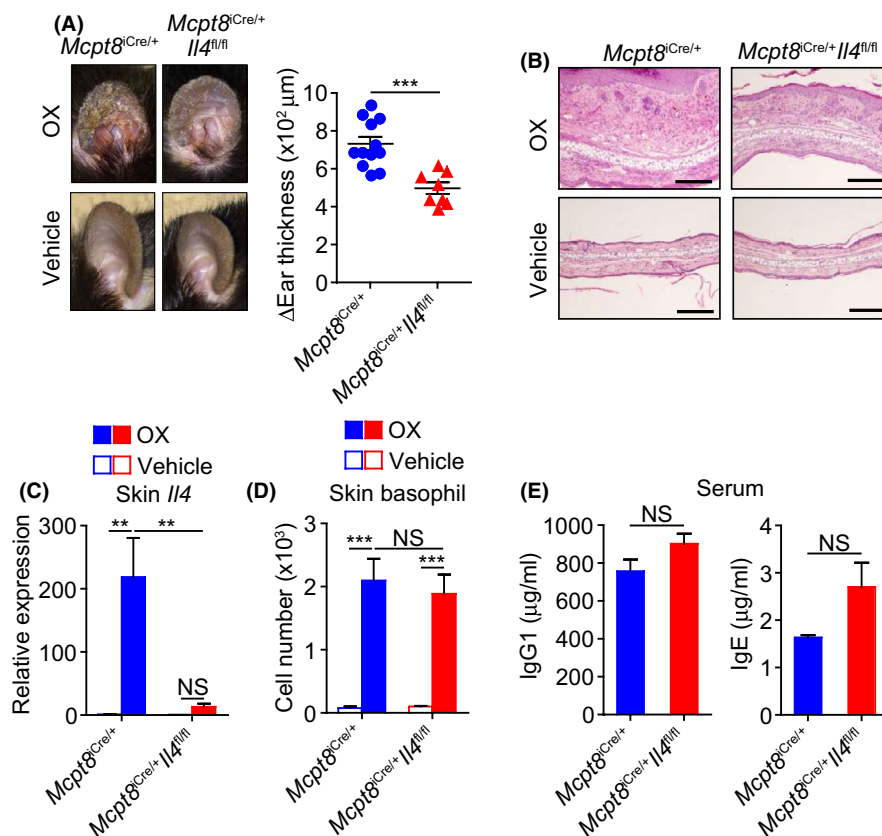


FIGURE 5 Basophil-specific IL-4-deficient mice show ameliorated skin inflammation with reduced expression of IL-4 in the affected skin. $Mcpt8^{iCre/+}Il4^{fl/fl}$ and control $Mcpt8^{iCre/+}$ mice were treated with OX as in Figure 1 to induce skin inflammation. (A) The OX- or vehicle-treated ear skin on day 8 was subjected to photography, followed by measuring the extent of ear thickening (Δ ear thickness = OX-vehicle, mean $\pm$ SEM, $n = 8$ -12 mice each). (B) Photomicrographs show HE-stained ear specimens on day 8. (C) Relative expression levels of $Il4$ in the ear skin on day 8 are shown (mean $\pm$ SEM, $n = 4$ -6 mice each). The value in the $Mcpt8^{iCre/+}$ vehicle is set as 1. (D) The numbers of basophils isolated from the ear skin on day 8 are shown (mean $\pm$ SEM, $n = 4$ mice each). (E) Serum levels of total IgG1 and IgE on day 8 were measured by ELISA (mean $\pm$ SEM, $n = 4$ -5 mice each). Data shown are representative of at least three independent experiments. NS: not significant, ** $P < .01$, and *** $P < .001$ measured by unpaired Student's t test or one-way ANOVA with post hoc Tukey HSD test

allergic inflammation in the skin. In the present study, by analyzing a series of genetically engineered mice, we clearly demonstrated that skin-infiltrating basophils promote allergic inflammation through the local production of IL-4, independently of Th2 cell differentiation in the draining LNs and serum levels of IgE.

The functional significance of basophils in Th2 cell differentiation in the draining LNs, as antigen-presenting and/or IL-4 providing cells, is a matter of debate.^{24,27} Leyva-Castillo et al addressed this controversial issue by analyzing MC903 (a vitamin D3 analog)-induced AD model and demonstrated that basophils functioned as the provider of initial IL-4 but not as antigen-presenting cells in the draining LNs to promote Th2 cell differentiation.²⁸ Otsuka et al studied the role of basophils in OX-induced AD model. The authors detected the accumulation of basophils in the draining LNs on day 28.¹³ Those basophils expressed MHC class II and co-stimulatory molecules, and a fraction of them produced IL-4. DT-mediated basophil depletion in Bas TRECK mice resulted in reduced levels of serum IgG1 on day 28, suggesting the contribution of basophils as antigen-presenting and IL-4-providing cells to Th2 cell differentiation in the draining LNs.¹³ In the present study, we focused on the role of basophils in the elicitation phase of skin inflammation (from day 2 to day 12) in contrast to the study by Otsuka et al (on day 28). On day 8 when levels of serum IgG1 and IgE were significantly elevated, basophils were hardly detected in the draining LNs, and basophil depletion showed no apparent impact on Th2 cell differentiation in the draining LNs and serum IgG1 and IgE levels. Therefore, we concluded that basophils are dispensable for Th2 cell differentiation in the elicitation phase of skin inflammation and that basophils contribute to the development of skin inflammation, independently of Th2 cell differentiation.

Although few or no basophils were detected in the draining LNs in the elicitation phase, we found that basophils progressively accumulated in the OX-treated skin until day 8, and basophils but not CD4⁺ T cells were the major producers of IL-4 in the skin lesion. Indeed, in spite of the fact that basophils accounted for 2%-3% of skin-infiltrating cells, basophil depletion almost completely abolished the IL-4 expression in the skin lesion, in parallel with markedly attenuated skin inflammation. IL-4-deficient mice showed a similar phenotype in the skin lesion, suggesting that IL-4 produced by skin-infiltrating basophils play an important role in the development of allergic inflammation. In accordance of this assumption, both inflammation and IL-4 expression in the skin lesion were markedly attenuated in basophil-specific IL-4-deficient mice, even though basophils normally accumulated in the skin lesion. Thus, skin-infiltrating basophils promoted OX-induced allergic inflammation through local production of IL-4 in the skin lesion, rather than the induction of Th2 cell differentiation.

It remains to be determined what kinds of stimuli activate basophils in the affected skin to produce IL-4. In another model of AD, topical treatment of murine skin with MC903 induces thymic stromal lymphopoietin (TSLP) expression in keratinocytes, leading to AD-like skin inflammation.^{29,30} Similarly, upregulated *Tslp* expression was reported in OX-induced AD model.¹³ We also observed that OX

treatment induced transient upregulation of *Tslp* expression in the skin lesion (Figure S4). Because TSLP has been shown to promote basophilia,³¹ one may assume that TSLP produced by OX-stimulated keratinocytes acts on basophils to promote their recruitment, survival/proliferation, and/or IL-4 production, directly or indirectly. In the MC903-induced AD model, basophil-derived IL-4 reportedly promotes group 2 innate lymphocyte (ILC2) proliferation, leading to AD-like skin inflammation.¹¹ In our present study, the accumulation of ILC2s (data not shown) and upregulation of Th2 cytokines IL-5 and IL-13 characteristic for ILC2s were hardly detected in the skin lesion (Figure 3A and B), suggesting little or no contribution of ILC2s to OX-induced skin inflammation. Further studies are needed to identify target cells of basophil-derived IL-4 and their effector molecules that can promote skin inflammation in the OX-induced AD model.

Considering the clinical observations that basophil accumulate in the skin lesion of AD patients¹⁰⁻¹² and that treatment with dupilumab, a blocking antibody against IL-4R α chain, shows some therapeutic effect on moderate-to-severe AD,^{8,9} our findings in OX-induced allergic inflammation suggest that skin-infiltrating basophils may contribute to promote allergic inflammation through IL-4 production in a certain type of AD patients. If this is the case, the selective targeting of basophils, representing a minor population among cellular infiltrates, could be a beneficial strategy in the treatment of AD.

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CONFLICT OF INTEREST

All the authors declare that they have no relevant conflict of interest.

AUTHOR CONTRIBUTIONS

YY, KM, and KT performed experiments. YY and HK designed and supervised the study. KM and SY provided helpful suggestions. YY and HK wrote the manuscript.

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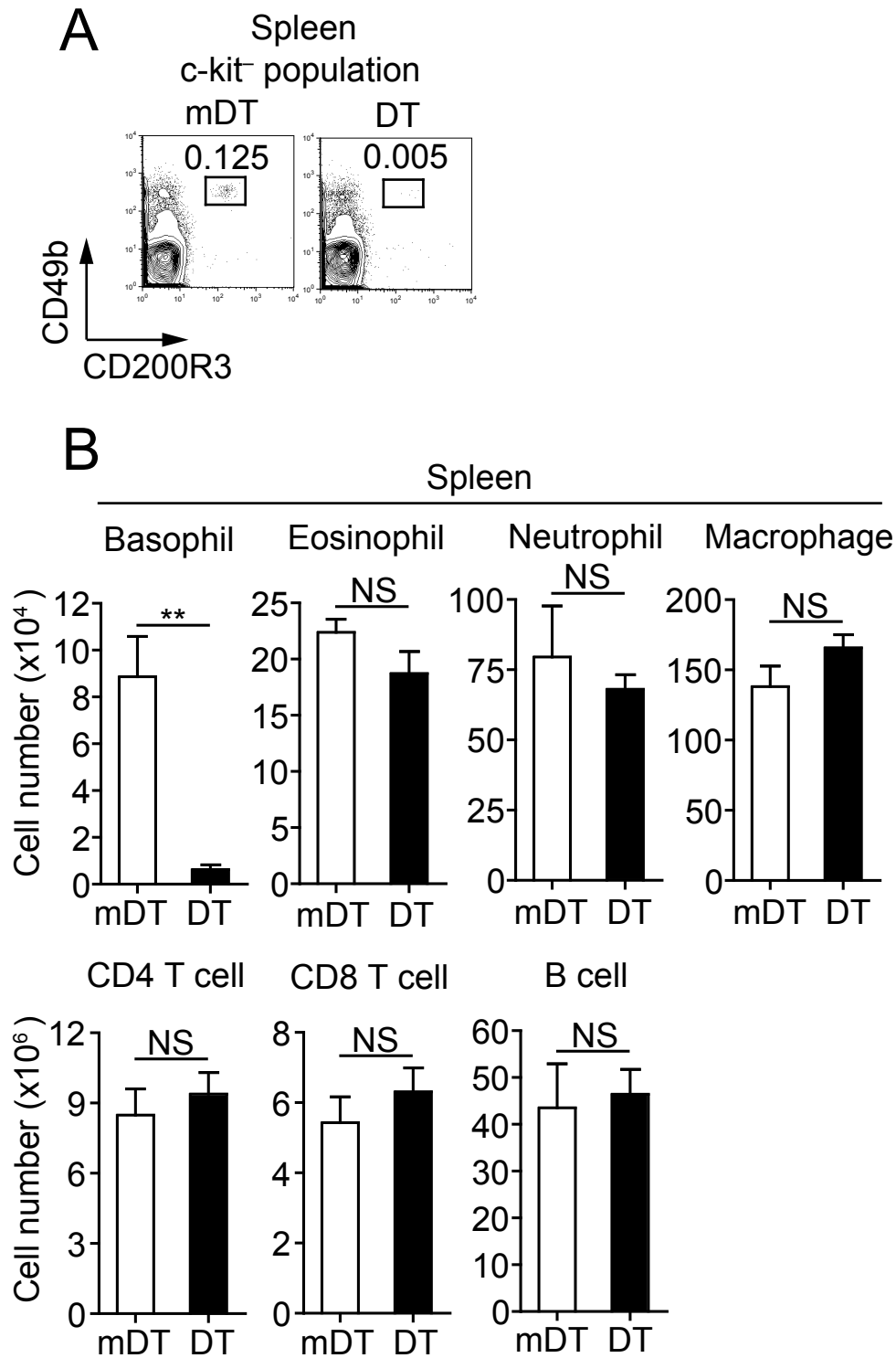
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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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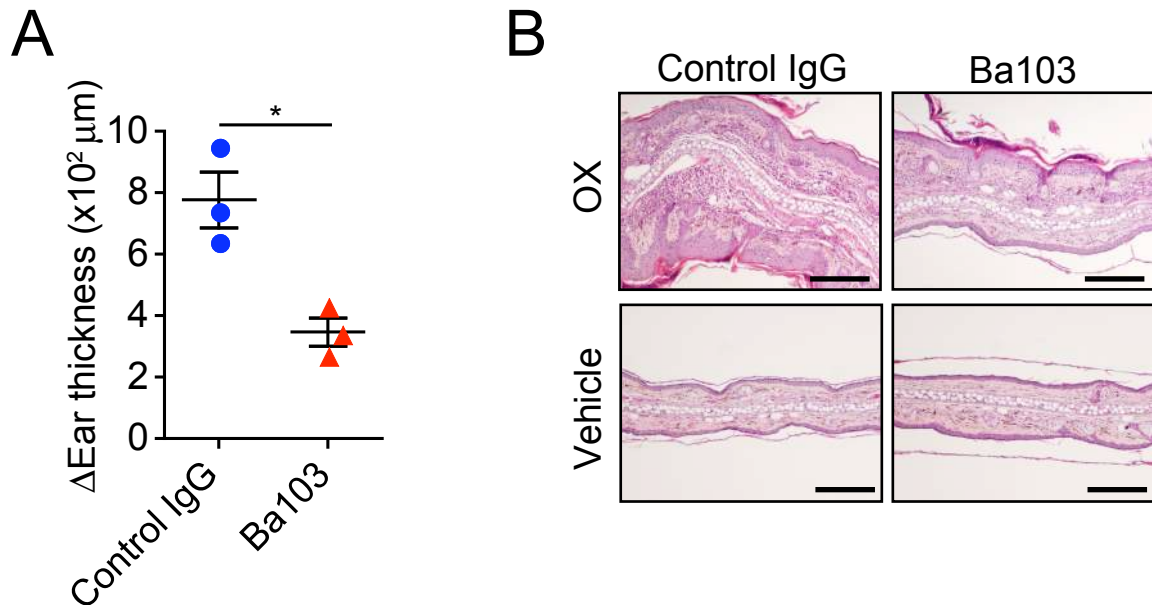
Supplemental Figure 1



Supplemental Figure 1. Intravenous injections of DT, but not mDT, selectively ablates basophils from *Mcpt8*^{DTR/+} mice

Mcpt8^{DTR/+} mice were treated with OX as in Figure 1 to induce skin inflammation in conjunction with intravenous injections with 500 ng of DT or mDT on days -1 and 4. (A) Contour plots show flow cytometric analysis of c-kit-negative populations in spleen collected from mDT- or DT-treated mice on day 8. Numbers above the gated areas indicate percentages of basophils among total live cells. (B) The numbers of indicated cell types isolated from the spleen on day 8 are shown (mean ± SEM, n=3 mice each). Data shown are representative of at least three independent experiments. NS: not significant, **p<0.01 measured by unpaired student's t test.

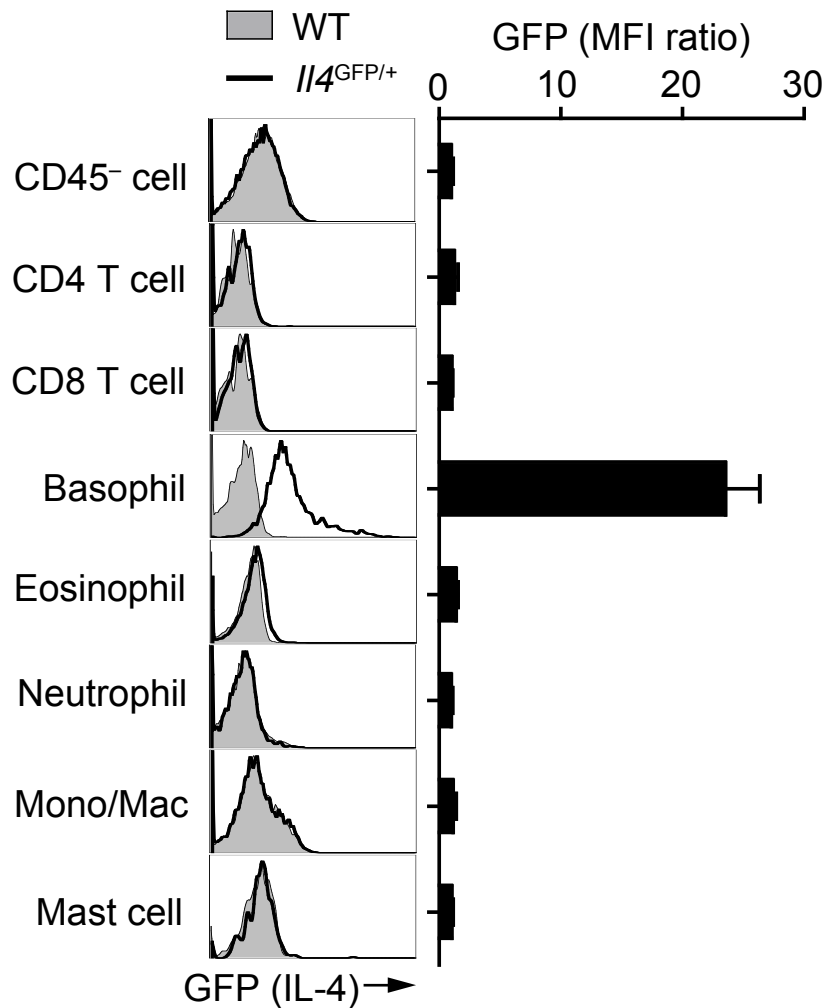
Supplemental Figure 2



Supplemental Figure 2. Anti-CD200R3 antibody (Ba103)-mediated depletion of basophils attenuates OX-induced skin inflammation

C57BL/6J mice were treated with OX as in Figure 1 to induce skin inflammation in conjunction with intraperitoneal injections with 150 μg of a basophil-depleting anti-CD200R3 antibody (Ba103) or control IgG on days -1, 3, and 7. (A) The extent of ear thickening on day 8 is shown (Δ ear thickness = OX – vehicle, mean $\pm$ SEM, $n=3$ mice each). (B) The HE-stained specimens of the OX- or vehicle-treated ear skin on day 8 are shown. Scale bars, 200 μm . Data shown are representative of at least three independent experiments. NS: not significant, * $p < 0.05$ by unpaired student's t test.

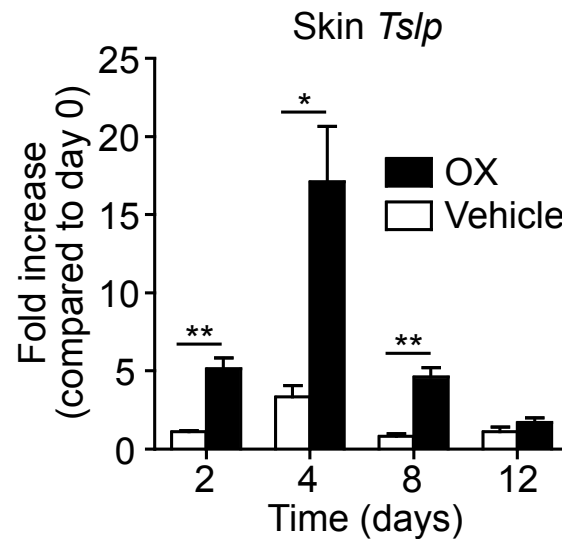
Supplemental Figure 3



Supplemental Figure 3. IL-4 is predominantly expressed by basophils among cells isolated from the OX-induced AD-like skin lesion

WT (shaded histograms) or *Il4*^{GFP/+} (open histograms) mice were treated with OX to induce skin inflammation. Histograms show GFP (IL-4) expression in indicated cell lineages isolated from the OX-treated ear skin on day 8. The ratio of mean fluorescent intensity (MFI) in *Il4*^{GFP/+} cells to that in WT cells was determined (mean $\pm$ SEM, n=3 mice each). Data shown are representative of at least three independent experiments.

Supplemental Figure 4



Supplemental Figure 4. Gene expression of *Ts/p* in the skin is upregulated by repeated OX applications

C57BL/6J mice were treated with OX as in Figure 1 to induce skin inflammation. Relative expression levels of *Ts/p* mRNAs isolated from the OX- or vehicle-treated ear skin at each time point are shown (mean $\pm$ SEM, n=3 mice each). The value of mRNA expression on day 0 is set as 1. Data shown are representative of at least three independent experiments. *p<0.05, **p<0.01 measured by unpaired student's t test.

Supplemental Table 1

Strain name	Background	Details of gene engineering	Used as	References
<i>Il4</i> ^{-/-}	C57BL/6	The neomycin resistance gene was inserted into exon 3 of the <i>Il4</i> gene.	IL-4-deficient mice	17
<i>Mcpt8</i> ^{DTR/+}	C57BL/6	A cDNA-encoding the human diphtheria toxin receptor (DTR) fused to green fluorescent protein (GFP) and internal ribosome entry site (IRES) were inserted into the 3' untranslated region of the basophil-specific <i>Mcpt8</i> gene.	Conditional basophil-deficient mice (DT-mediated selective depletion of basophils)	18
<i>Il4</i> ^{GFP/+}	C57BL/6	The first exon and 178 nucleotides of the first intron of the <i>Il4</i> gene were replaced by the gene specifying enhanced GFP.	IL-4 reporter mice	19
<i>Mcpt8</i> ^{DTR/+} <i>Il4</i> ^{GFP/+}	C57BL/6	<i>Mcpt8</i> ^{DTR/+} × <i>Il4</i> ^{GFP/+}	IL-4 reporter mice under basophil-depleted condition	18, 19
<i>Mcpt8</i> ^{iCre/+}	C57BL/6	The first exon of the basophil-specific <i>Mcpt8</i> gene was replaced with the gene encoding improved Cre (iCre).	Control mice for basophil-specific IL-4-deficient mice	20
<i>Il4</i> ^{fl/fl}	C57BL/6	loxP sites were inserted into the introns upstream of exon 3 and downstream of exon 4 of the <i>Il4</i> gene.	IL-4-floxed mice	20
<i>Mcpt8</i> ^{iCre/+} <i>Il4</i> ^{fl/fl}	C57BL/6	<i>Mcpt8</i> ^{iCre/+} × <i>Il4</i> ^{fl/fl}	Basophil-specific IL-4-deficient mice	20

Supplemental Table 1. The genetically-engineered mice used in this study