Basophils regulate the recruitment of eosinophils in a murine model of irritant contact dermatitis

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ABSTRACT

Background: Although eosinophils have been detected in several human skin diseases in the vicinity of basophils, how eosinophils infiltrate the skin and the role of eosinophils in the development of skin inflammation has yet to be examined.

Objective: Using a murine irritant contact dermatitis (ICD) as a model, we sought to clarify the roles of eosinophils in ICD and the underlying mechanism of eosinophil infiltration of the skin.

Methods; We induced croton oil-induced ICD in eosinophil-deficient ΔdblGATA mice with or without a reactive oxygen species (ROS) inhibitor. We performed co-cultivation using fibroblasts and bone marrow-derived basophils and evaluated eosinophil migration using a chemotaxis assay.

Results: ICD responses were significantly attenuated in the absence of eosinophils or by treatment with the ROS inhibitor. ROS was produced abundantly by eosinophils and that both basophils and eosinophils were detected in human and murine ICD skin lesions. In co-culture experiments, basophils attracted eosinophils especially in the presence of fibroblasts. Moreover, basophils produced IL-4 and TNF-α in contact with fibroblasts and promoted the expression of eotaxin/CCL11 from fibroblasts *in vitro*.

Conclusion: Eosinophils mediated the development of murine ICD possibly via ROS production. Recruitment of eosinophils into the skin was induced by basophils in cooperation with fibroblasts. Our findings raise a novel concept that basophils promote the recruitment of eosinophils into the skin via fibroblast in the development of skin inflammation.

Key Messages:

Basophils initiate the recruitment of eosinophils into the skin via fibroblasts.

Interference of this system may control several skin inflammations.

Capsule Summary:

Eosinophil infiltration into the skin by basophils in cooperation with fibroblasts promotes irritant contact dermatitis via ROS production, which raises a new concept that interaction between basophils and fibroblasts induces skin inflammation via eosinophil recruitment.

Key Words:

Eosinophil, basophil, fibroblast, eotaxin/CCL11, RANTES/CCL5, irritant contact dermatitis, reactive oxygen species, tumor necrosis factor

Abbreviations:

BM, bone marrow; BMBa, bone marrow-derived basophil; BMEo, bone marrow-derived eosinophil; CBA, cytometric bead array; CHS, contact hypersensitivity; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; cRPMI, complete RPMI; DCF, 2',7'-dichlorofluorescein; DT, diphtheria toxin; FCS, fetal calf serum; Flt3-L, fms-related tyrosine kinase 3 ligand; IgE-CAI, IgE-mediated chronic allergic inflammation; IL, interleukin; MCP-8, mast cell serine protease-8; MEF, mouse embryonic fibroblast; NAC, N-acetylcysteine; RANTES, regulated on activation, normal T cell expressed and secreted; ROS, reactive oxygen species; SCF, stem cell factor; Tg, transgenic; WT, wild-type

INTRODUCTION

Contact dermatitis is one of the most common inflammatory skin diseases and comprises both irritant contact dermatitis (ICD) and allergic contact dermatitis.[1] ICD is more common than allergic contact dermatitis and is responsible for approximately 80% of all contact dermatitis.[2] It is defined as a locally arising reaction that appears after chemical irritant exposure.[2] The chemical agents are directly responsible for cutaneous inflammation because of their inherent toxic properties, which cause tissue injury.[3, 4] This inflammatory response is known to activate innate immune system cells, but the precise mechanism of ICD remains largely unknown.

Eosinophils are one of the bone marrow (BM)-derived innate immune leukocytes that normally represent less than 5% of leukocytes in the blood, but are frequently detected in the connective tissues and BM.[5] Eosinophils regulate local immune and inflammatory responses, and their accumulation in the blood and tissue is associated with several inflammatory and infectious diseases.[6, 7 8] The recruitment of activated eosinophils from the blood stream into tissues occurs under numerous conditions, and leads to the release of preformed and synthesized products such as cytokines, chemokines, lipid mediators, cytotoxic granule proteins, and reactive oxygen species (ROS).[7, 9] ROS are mainly produced by NADPH oxidase and lead to tissue injury at the inflamed site during allergic inflammation.[10] The differentiation, migration, and activation of eosinophils are mainly enhanced by interleukin (IL)-5.[11] It has been reported that the IL-5-targeted therapy can reduce airway and blood eosinophils and prevent asthma exacerbations[12]; however, the roles of eosinophils in the development of cutaneous immune responses

remain largely unknown. It has been recently reported that basophils have been detected in skin diseases including contact dermatitis where eosinophils were present. [13, 14]

Basophils are one of the least abundant granulocytes, representing less than 1% of peripheral blood leukocytes.[15] Their specific physiological functions during immune responses have been ignored until recently. Basophils play key roles in the development of acute and chronic allergic responses, protective immunity against parasites, and regulation of acquired immunity, including the augmentation of humoral memory responses.[16, 17]

In this study, we observed the infiltration of eosinophils in human and murine ICD. Murine ICD responses were attenuated in eosinophil-deficient mice or in mice treated with an ROS inhibitor. ROS was produced by eosinophils, which were attracted by chemokines produced via interaction between basophils and fibroblasts. Our findings may raise an important concept that the interaction between basophils and mesenchymal fibroblasts induces the development of ICD via recruitment of eosinophils.

Methods

Mice

ΔdblGATA mice on BALB/c background were purchased from the Jackson Laboratory (West Grove, PA, USA). IL-5 transgenic (Tg) mice on BALB/c background[18] were kindly provided by Dr. K. Takatsu (University of Toyama, Toyama, Japan). Basophil-specific enhancer-mediated toxin receptor-mediated conditional cell knock-out (Bas TRECK) mice on BALB/c background were generated as reported previously. Briefly,

basophils use a specific 4 kb enhancer fragment containing the 3'-untranslated region and HS4 elements to regulate *Il4* gene expression.[19] Utilizing this system, we generated mice that express human diphtheria toxin (DT) receptor under the control of HS4.[17, 20] Using these mice, basophils have been reported to play an essential role for the induction and promotion of Th2 immunity.[17, 21] C57BL/6N and BALB/c wild-type (WT) mice were purchased from Japan SLC (Shizuoka, Japan). Eight-to-ten-week-old female mice were used for all the experiments and bred in specific pathogen-free facilities at Kyoto University. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine (Kyoto, Japan).

Reagents, antibodies, and flow cytometry

We purchased croton oil and N-acetylcysteine (NAC) from Sigma-Aldrich (St. Louis MO). 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) was purchased from Invitrogen (Carlsbad, CA, USA). Recombinant murine stem cell factor (SCF), fms-related tyrosine kinase 3 ligand (Flt3-L), and IL-3 were purchased from PeproTech (Rocky Hill, NJ, USA). Recombinant mouse IL-5 was purchased from R&D Systems (Minneapolis, MN, USA). FITC-, PE-, PE-Cy7, APC, APC-Cy7, Pacific Blue-conjugated , anti-Gr-1 (RB6-8C5), anti-CD117 (c-Kit) (2B8), anti-FcεRIα (MAR-1), anti-CD49b (Dx5), anti-CD69 (H1.2F3), anti-CD86 (GL1), anti-CD11b (M1/70), and anti-CD45.1 (A20) mAbs were purchased from eBioscience (San Diego, CA, USA). APC-, and PE-conjugated anti-Siglec F (E50-2440) mAbs were

purchased from BD Biosciences (San Jose, CA). FITC-conjugated anti-intercellular adhesion molecule-1 (ICAM-1(CD54)) (3E2) mAb was purchased from BD Biosciences (Franklin Lakes, NJ, USA). BV-conjugated anti-CD45 (30-F11), purified anti-CD200R3 (Ba13) mAbs, and rat anti-mast cell serine protease-8 (MCP)-8 (TUG8), were purchased from BioLegend (San Diego, CA, USA). For fluorescence labeling, purified anti-CD200R3 mAb was labeled with the HiLyte Fluor 647 Labeling Kit (Dojindo, Kumamoto, Japan). Functional grade purified anti-FcεRIα (MAR-1), anti-tumor necrosis factor (TNF)-α (MP6-XT22), and anti-IL-4 (11B11) mAbs were purchased from eBioscience. Single-cell suspensions from skin were prepared for flow cytometric analysis as follows. Skin/ear samples were collected using 8 mm skin biopsy (= ~100 mm^2), cut into pieces and then digested for 1 h at 37 °C in 1.6 mg/ml collagenase type II (Worthington Biochemical Corp., Freehold, NJ) and 0.1 mg/ml DNase I (Sigma-Aldrich) in complete RPMI medium (cRPMI; RPMI 1640 medium (Sigma-Aldrich) containing 10% heat-inactivated fetal calf serum (FCS) (Invitrogen), 0.05 mmol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 25 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin). Samples were passed through a 40-μm pore size nylon mesh, and cells were stained for the indicated markers. Samples were acquired on a FACSFortessa system (BD) and analyzed with FlowJo software (Tree Star, San Carlos, CA). The numbers of each cell subset were calculated by flow cytometry and presented the numbers per mm^2 of the skin surface.

ICD and basophil-depletion models

Mice were anesthetized with diethyl ether and 20 μl of 1% (v/v) croton oil in acetone was applied to ear skin. To deplete basophils *in vivo*, mice were injected twice daily for 3 days with anti-FcεRIα (MAR-1).[22] The efficiency of basophil depletion was analyzed in peripheral blood on day 4. To block ROS production, mice were intraperitoneally injected with NAC (500 mg per kg body weight) and given 20 μl of 50 mM NAC in 100% ethanol on ear skin 1 h before application of croton oil.

Bas TRECK Tg mice were treated with DT for basophil-depletion. BALB/c mice with DT were used as control.[20] For DT treatment, mice were injected intraperitoneally with 100 ng of DT per mouse.

Histology and immunohistochemistry

Skin samples for hematoxylin-eosin (HE) staining were collected from ICD patients (n=10) and healthy control subjects (n=6). The number of eosinophils was counted in five fields (× 20 objective). HE staining and histological scoring were evaluated as reported.[23] In brief, samples were scored for the severity and character of the inflammatory response on a subjective grading scale. Responses were graded as follows: 0, no response; 1, minimal response; 2, mild response; 3, moderate response; 4, marked response. The slides were blinded, randomized, and reread to determine the histology score. All studies were read by the same pathologist using the same subjective grading scale. The total histology score was calculated as the sum of scores, including

inflammation, neutrophils, mononuclear cells, edema, and epithelial hyperplasia. The evaluation of eosinophils was performed with Papanicolaou staining.

For the identification of basophils by immunohistochemistry, tissue sections were immunostained as previously reported.[24]

Staining of ROS in ear skin

Mice were applied with 1% croton oil and cells from the ear skin were isolated 6 h later, and incubated for 30 min at 37°C with a solution of 1 μM CM-H_2DCFDA in phosphate buffered saline (PBS). After being washed twice with PBS, cells were labeled with anti-SiglecF and anti-CD11b. We detected production of ROS as indicated by an increase in 2',7'-dichlorofluorescein (DCF) fluorescence.

Preparation of BM-derived basophils (BMBas), BM-derived eosinophils (BMEos) and mouse embryonic fibroblasts (MEFs)

Complete RPMI medium (cRPMI) was used as culture medium. For BMBas induction, 5×10^6 BM cells were cultured in cRPMI supplemented with 20% FCS, in the presence of 10 ng/ml recombinant mouse IL-3 (PeproTech) for approximately 9 to 14 days. For BMEos induction, 1×10^6 BM cells of Ly5.1 mice were cultured in cRPMI supplemented with 20% FCS, in the presence of 100 ng/ml recombinant mouse SCF and 100 ng/ml rmFlt3-L (PeproTech) from days 0 to 4. On day 4, the medium containing SCF and Flt3-L was replaced with medium containing 10 ng/ml recombinant mouse IL-5 (R&D) thereafter. [25]

MEFs were obtained from embryos on embryonic day 15 by using standard methods in complete DMEM medium (Sigma-Aldrich). [26]

Chemotaxis assay and cell culture

Cells were tested for transmigration to the lower chamber across uncoated 5-μm transwell filters (Corning Costar Corp., Corning, NY, USA) for 3 h and were enumerated by flow cytometry.

BM cells of IL-5 Tg mice and starved BMBas were co-cultured at a density of 2×10^5 cells in 200 μl per well in a 96-well microplate at a BM: BMBa ratio 1:4 in cRPMI supplemented with 10 ng/ml recombinant mouse IL-3 for 24 h. Separation of BM cells and BMBas was performed by using transwell culture plates with a 3-μm pore size.

MEFs were cultured in 24-well plates to 80% confluences. For co-culture, the medium of MEFs was replaced with cRPMI and the co-culture was performed supplemented with 10 ng/ml recombinant mouse IL-3 for 24 h.

For inhibition assays, BMBas and MEFs were co-cultured with or without 5 μg/ml isotype control Ab (Rat IgG2b, eBioscience), 10 μg/ml anti-IL-4 mAb (11B11, eBioscience) or 5 μg/ml anti-TNF-α mAb (MP6-XT22, eBioscience) for 24 h.

For chemotaxis toward the supernatant of co-culture of BMBas and MEFs, 1×10^6 BM cells were transferred into the upper chamber of transwell containing 5-μm pore filters. The supernatant of cultivation of MEFs with or without BMBas was added to the lower chamber and incubated for 3 h at 37°C. Gr-1^{int+} SiglecF^+ $CD11b^+$ eosinophils which migrated to the lower chambers were counted by flow cytometry.

ELISA and cytometric beads array

The amount of eotaxin/CCL11 in the culture medium was measured by ELISA (eBioscience). The amount of regulated on activation, normal T cell expressed and secreted (RANTES)/CCL5 was measured using a cytometric bead array (CBA) system according to the manufacturer's instructions (BD Biosciences). For the measurement of eotaxin and RANTES, a total of 3×10^5 BMBas and 1×10^5 MEFs were cultured with recombinant mouse IL-3 (10 ng/ml) for 24 h, and the supernatants were collected for ELISA assay and CBA.

Statistical analysis

Unless otherwise indicated, data are presented as the means ± standard error of the mean and a representative of at least three independent experiments. P-values were calculated with Wilcoxon signed-rank test. *P*-values < 0.05 are considered to be significantly different and are marked by an asterisk in the figures

Results

Eosinophils play some roles in the development of ICD

We first evaluated whether eosinophils were detected in the lesional skin of patients with ICD. In comparison with healthy donors, the number of eosinophils was significantly higher in patients with ICD (Fig. 1A). To further investigate the role of

eosinophils in ICD, we used eosinophil-deficient ΔdblGATA mice.[27] In a croton oil-induced ICD model, the ear swelling response in ΔdblGATA mice was significantly attenuated compared with that in WT mice 6, 24, and 48 h after application (Fig. 1B). To confirm the role of eosinophils in ICD, we used IL-5 Tg mice, which demonstrate eosinophilia in peripheral blood as well as infiltration of eosinophils into various tissues. The ICD response in IL-5 Tg mice was significantly enhanced compared with that in WT mice at 1, 3, 6, 24, and 48 h after application (Fig. 1C). Consistent with the ear swelling responses, lymphocyte infiltration including eosinophils and edema in the dermis 6 h after application were lower in ΔdblGATA mice and higher in IL-5 Tg mice than in WT mice (Fig. 1D, Table E1 and 2). In addition, major eosinophil chemoattractants, such as RANTES and eotaxin, were detected in the skin after croton oil application (Fig. E1).

Eosinophils produce ROS in ICD

ROS is known to induce the development of some inflammatory conditions. [28] To assess the role of ROS in the ICD model, we used an ROS-inhibitor NAC. Ear swelling significantly decreased after the NAC treatment in both WT and ΔdblGATA mice (Fig. 2A). Of note, ear swelling in NAC-treated WT mice was comparable to that in NAC-treated ΔdblGATA mice, suggesting that ROS produced from eosinophils play a major role in the induction of ICD. In addition, using the ROS-sensitive fluorescent dye CM-H_2DCFDA, we detected a significant amount of ROS production by eosinophils in the skin under steady states. In addition, eosinophils in the ICD lesional skin expressed

higher amounts of ROS, which was also higher than infiltrated $CD4^+$ T cells [29] (Fig. 2B, C).

Basophils enhance eosinophil recruitment into the skin

Basophils tended to be detected in skin diseases where eosinophils were present.[30] We next analyzed the distribution of eosinophils and basophils using Papanicolaou staining and immunohistochemistry, respectively, in the ICD model. $Mcp8^+$ basophils were localized in the vicinity of eosinophils in the inflamed skin (Fig. 3A). We further evaluated whether basophils were detected in the lesional skin of patients with ICD and demonstrated the coincidental presence of basophils and eosinophils in inflamed skin of human ICD (Fig. E2). In addition, the numbers of neutrophils and basophils in ΔdblGATA mice were comparable to those in WT mice 6 h after croton oil application (Fig. 3B, C), which suggests that eosinophils do not affect the recruitment of neutrophils and basophils into the skin.

We further analyzed the kinetics of recruitment of eosinophils and basophils in the lesional skin in ICD. The number of basophils increased 3 h after croton oil application. On the other hand, eosinophils increased 24 h after croton oil application, the timing of which was later than that of basophils (Fig. 3D).

Therefore, we hypothesized that basophils affect eosinophil infiltration during ICD. To address this hypothesis, we depleted basophils using anti-FcεRIα (MAR-1) antibody.[22] The administration of anti-FcεRIα antibodies significantly suppressed ear

swelling and infiltration of eosinophils but not neutrophils or mast cells into the skin (Fig. 3E, F, and Fig. E3). To confirm these results, we next used Bas TRECK Tg mice to deplete basophils conditionally.[17, 20] Consistently, the ear swelling response and the number of infiltration of eosinophils but not neutrophils in DT-treated Bas TRECK Tg mice were significantly attenuated compared with those in DT-treated WT mice (Fig. 3G, H, and Fig.E4). Similar findings were observed in mast cell-deficient $WBB6F_1$-*Kit*$^{W/Wv}$ (W/Wv) mice (Fig. E5). These findings suggest the potential overlap of mast cells and basophils.

Basophils augment eosinophil activation

Impaired eosinophil recruitment as a result of depletion of basophils suggests that basophils promote eosinophil infiltration into the skin. To address this issue, we prepared BM cells from IL-5 Tg mice that included numerous eosinophils and incubated them with or without BMBas for 24 h. Co-cultivation of BM cells with BMBas significantly enhanced the expression levels of activation markers, CD69, CD86, and ICAM-1, on eosinophils among BM cells (Fig. 4A, B).[31-33] On the other hand, the incubation of BMBas and BM cells of IL-5 Tg mice separately using transwells did not induce up-regulation of the above activation markers on eosinophils (Fig. 4A, B). These findings suggest that basophils require direct cell-to-cell interaction to activate eosinophils.

In cooperation with fibroblasts, basophils promote recruitment of eosinophils

Next we evaluated whether basophils were capable of attracting eosinophils. We prepared BMBas and BMEos for chemotaxis assay. The chemotaxis of BMEos applied to the upper chamber was significantly enhanced when BMBas were added to the lower chamber (Fig. 5A). On the other hand, BMBas applied to the upper chamber did not migrate to the lower chamber where BMEos were added (Fig. 5A). These results suggest that basophils attract eosinophils, but not vice versa.

We then sought to identify how basophils recruit eosinophils. CCR3 is known to mediate eosinophil chemotaxis in response to eotaxin and RANTES.[34] The number of eosinophils in anti-CCR3 antibody-treated mice was attenuated compared to that in control antibody-treated mice 24 h after croton oil application (Fig. E6A). In addition, the amounts of RANTES and eotaxin in the skin after croton oil application were reduced in basophil-depleted mice using a Bas TRECK transgenic system (Fig. E6B). RANTES was detected in the supernatant medium of IL-3-stimulated BMBa cultures (Fig. 5B), but eotaxin was not detected therein (data not shown).

Fibroblasts are known to produce chemoattractants such as RANTES and eotaxin.[35] We observed that BMBas expressed only RANTES mRNA, which was consistent with the findings in Fig. 5B, and that MEFs expressed RANTES and eotaxin mRNA by quantitative PCR (Fig. E7). Since basophils infiltrated into the dermis where

mesenchymal fibroblasts localize abundantly, we then evaluated the effect of the interaction between basophils and fibroblasts on chemokine production. Although MEFs expressed marginal RANTES in the culture supernatant, BMBas expressed pronounced RANTES. Co-cultivation of MEFs significantly increased RANTES levels in the culture supernatant of BMBas (Fig. 5C).

It has been reported that TNF-α promotes migration of immune cells, such as dendritic cells and mast cells,[36] and that IL-4 is an inducer for several chemokines.[37] We next hypothesized that TNF-α or IL-4 might mediate the production of RANTES by basophils. To address this issue, we examined whether enhanced RANTES production in co-cultivation of BMBas and MEFs was inhibited by anti-TNF-α or IL-4 antibody. Although anti-IL-4 antibody did not inhibit RANTES production, anti-TNF-α antibody inhibited RANTES production in the culture supernatant of both basophils and co-cultivation of basophils and MEFs (Fig. 5C).

Next, we sought to reveal the mechanism by which eotaxin is induced in the skin. In contrast to RANTES, eotaxin mRNA was strongly detected in MEFs (Fig. E7), whereas eotaxin protein levels in the culture supernatant of MEFs were marginal (Fig. 5D). Interestingly, eotaxin protein was induced by co-cultivation of BMBas and MEFs (Fig. 5D). Differently from RANTES induction, both anti-IL-4 antibody and anti-TNF-α antibody inhibited the induction of eotaxin. We found that the main producer of TNF-α and IL-4 was IL-3-stimulated BMBas (Fig. 5E). Consistently, the amounts of IL-4 and TNF-α in the skin were reduced by depletion of basophils after croton oil application

(Fig. E8). In addition, co-cultivation of BMBas and MEFs in the presence of IL-3 to the lower chamber attracted eosinophils applied to the upper chamber, when compared to only BMBas or only MEF incubation to the lower chamber (Fig. 5F).

DISCUSSION

In this study, we have demonstrated that eosinophils mediate the development of the ICD reactions possibly via ROS production. Eosinophils accumulate into the human and murine ICD skin lesions in the vicinity of basophils. Basophils are detected in the skin lesion prior to the infiltration of eosinophils, suggesting that basophils promote eosinophil accumulation into the skin. Consistently, BMBas promote the migration and activation of eosinophils *in vitro*. RANTES is produced by IL-3-stimulated basophils and even more by co-cultivation of MEFs in a TNF-α dependent manner. On the other hand, eotaxin is produced by co-cultivation of BMBas and MEFs, which is inhibited by anti-TNF-α and anti-IL-4 antibody. Basophils attracted eosinophils via CCR3. And direct cell-to-cell interaction was required for activation of eosinophils by basophils. Taken together, our findings suggest that basophils infiltrating into the skin attract eosinophils directly or indirectly via interaction of mesenchymal fibroblasts, and that basophils activate eosinophils in situ, which contributes to the development of skin inflammation (Fig. E9).

Basophils are thought to be major early producers of IL-4 and IL-13, which are critical for triggering and maintaining allergic responses.[38, 39] In this report, we have

demonstrated that basophils rapidly infiltrate into the inflamed skin and subsequently attract eosinophil therein for the development of ICD.

IgE-mediated chronic allergic inflammation (IgE-CAI) is a long-lasting inflammation that follows immediate-type reactions and late-phase responses. It is histopathologically characterized by massive eosinophil infiltration into the skin.[40] Basophils are considered as cells responsible for initiating inflammation of IgE-CAI. Consistently with our results using ICD, the number of eosinophils increased in the lesional skin after basophil infiltration in IgE-CAI. [41] In addition, basophils co-localize with eosinophils in human skin diseases such as atopic dermatitis and eosinophilic pustular folliculitis.[42] These findings suggest that our novel findings might be applicable to more general skin inflammatory diseases both in mice and in human.

In this study, we have clarified that basophils recruit eosinophils into the skin. The next question is how basophils infiltrate into the lesional skin. It has been reported that α(1,3) fucosyltransferases IV and VII are essential for the initial recruitment of basophils in chronic allergic inflammation.[43] We are currently working to understand the underlying mechanism of how basophils infiltrate into the lesion as an inducer of skin inflammation in our model.

Both basophils and eosinophils express the common chemokine receptor CCR3. Ligands for CCR3, such as eotaxin, are produced by dermal fibroblasts in response to Th2-type cytokines in humans.[44] We demonstrated herein a new network for eosinophil infiltration into the skin as summarized in Fig. E9. Activated basophils produced RANTES, which was dependent on TNF-α that was possibly produced by basophils

themselves. In addition, basophils that have infiltrated into the lesional skin were activated via contact with dermal fibroblasts and produced IL-4 and TNF-α, which promoted eotaxin expression from fibroblasts. These cytokine-chemokine networks may support recruitment of eosinophils from the blood stream into the skin.

It has been reported that ICD is IgE-independent [45] In addition, recent studies showed that thymic stromal lymphopoietin (TSLP), which is produced by keratinocytes and fibroblasts in the skin, activated basophils.[17, 46] We have demonstrated that croton oil application promoted the induction of TSLP in ICD (data not shown). Therefore, we assume that TSLP is one of the candidates for the activator in this assay.

In this study, we also examined the role of mast cells in ICD using mast cell-deficient *Kit*$^{W/Wv}$ mice. Interestingly, similar phenotypes, such as the attenuation of ICD and eosinophil infiltration, were found in a mast cell-deficient model (Fig. E5). These findings suggest the potential overlap of mast cells and basophils, which seems to be intriguing. We demonstrated that TNF-α was decreased partially but IL-4 was almost completely diminished by basophil depletion (Fig. E8). These findings suggest that TNF-α and IL-4 might be released by mast cells and basophils, respectively, for the development of ICD.

TNF-α is a potent pro-inflammatory cytokine and immunomodulatory cytokine implicated in inflammatory conditions. Treatment with anti-TNF-α antibody is effective for several diseases including psoriasis, Crohn's disease, and rheumatoid arthritis (RA). On the other hand, peripheral blood eosinophilia can be observed in patients with active inflammatory RA.[47] We demonstrated herein that anti-TNF-α antibody inhibited the

production of eosinophil chemoattractant such as RANTES and eotaxin from basophils and fibroblasts (Fig. 5C, D). Since synovial fibroblasts and basophils have been reported to play important roles in the pathogenesis of RA,[48] anti-TNF-α might also block the interaction of basophils, eosinophils and fibroblasts to regulate RA activity. Further understanding of the relationship between basophils, eosinophils, and fibroblasts in the immune organs may lead to the development of new therapeutic strategies to control eosinophil-associated diseases, such as ICD, atopic dermatitis, and allergic asthma.

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Figure Legends

Figure 1. Eosinophils play some role in the development of ICD.

(A) Histology of the skin of ICD (I; n=10) and healthy donors (H; n=6). Scale bars, 50 μm. The number of eosinophils per field (left panel). (B, C) Ear swelling of WT and ΔdblGATA mice (n=9 per group; B) and of WT (n=10) and IL-5 Tg (n=7) mice (C) after application of croton oil. (D) HE staining of ears 6 h after application. Hisotology scores of the skin before and 6 h after the application. Scale bars, 100 μm.

Figure 2. ICD is mediated by eosinophil-derived ROS

(A) Ear swelling of WT (n=11) and ΔdblGATA mice (n=7) pretreated with or without antioxidant NAC measured 24 h after application. (B, C) Histogram (B) and MFI of

DCF (C) on SiglecF$^+$ CD11b$^+$ eosinophils and CD4$^+$ T cells before (i.e., steady states; 0 h) or 6 h after application.

Figure 3. Basophils accumulate into the skin prior to eosinophil infiltration in ICD.

(A) Mcp8$^+$ basophils (black arrowhead) and Papanicolaou staining$^+$ eosinophils (red arrowhead) 24 h after croton-oil application. Scale bars, 50 μm. (B, C) FACS plots (B) and numbers (C) of infiltrating eosinophils (Eo), basophils (Baso), and neutrophils (Neu) of WT and ΔdblGATA mice per mm^2 of surface of the skin 6 h after croton-oil application. (D) Kinetics of the numbers of eosinophils and basophils in skin. (E) Ear swelling and (F) cell infiltration in ICD by depletion of basophils by MAR-1 antibody (n=5 per group). (G, H) Ear swelling in ICD (G) and cell infiltration (H) of WT (n=5) and Bas TRECK Tg (n=4) mice 24 h after croton-oil application.

Figure 4. Basophils promote the activation of eosinophils via direct cell interaction.

Histogram (A) and MFI (B) of CD69, CD86, and ICAM-1 expression on the eosinophil subset in BM cells of IL-5 Tg mice cultured with or without BMBas directly or indirectly.

Figure 5. Basophils promote eosinophil recruitment directly or indirectly via fibroblasts.

(A) Migration of eosinophils and basophils. BMBas or BMEos were applied to the upper or lower chambers with or without IL-3, and cell number was evaluated. (B)

Amount of RANTES in the supernatants of BMBa cultures with or without IL-3. (C) Amount of RANTES and (D) eotaxin in supernatants of MEF, BMBas (Baso), or MEF plus BMBas for 24 h with or without neutralizing anti-IL-4 or TNF-α antibodies. (E) Amount of TNF-α and IL-4 in supernatants of BMBa or MEF cultures. (F) The number of migrating eosinophils. Chemotaxis of eosinophils in lower chamber that was incubated with MEFs, BMBas, or MEF plus BMBas in the presence or abasence of IL-3 was evaluated.

Figure 1

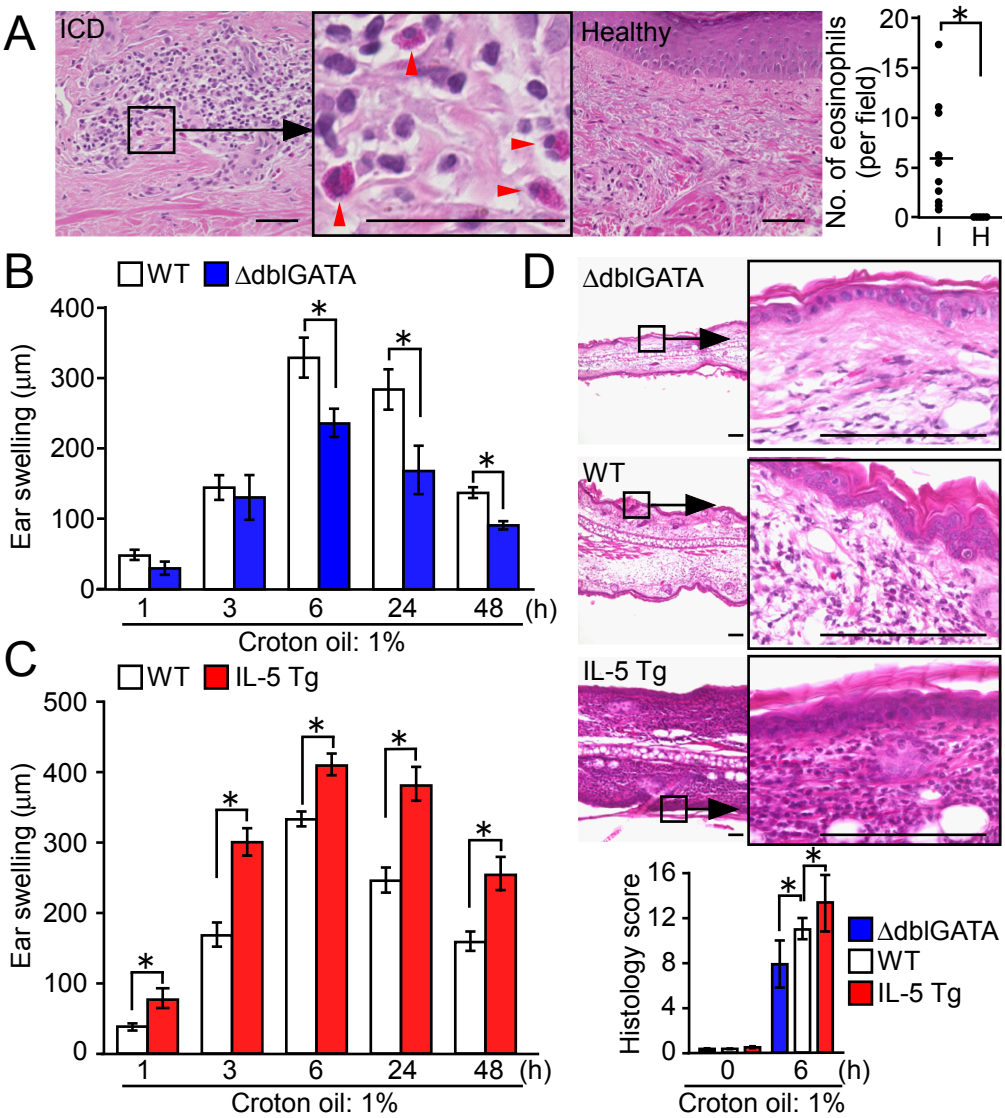

A
ICD
Healthy
No. of eosinophils (per field)
20
15
10
5
0
I
H
*
B
WT
ΔdblGATA
Ear swelling (μm)
400
300
200
100
0
1
3
6
24
48
(h)
Croton oil: 1%
C
WT
IL-5 Tg
Ear swelling (μm)
500
400
300
200
100
0
1
3
6
24
48
(h)
Croton oil: 1%
D
ΔdblGATA
WT
IL-5 Tg
Histology score
16
12
8
4
0
0
6
(h)
Croton oil: 1%
ΔdblGATA
WT
IL-5 Tg

Figure 2

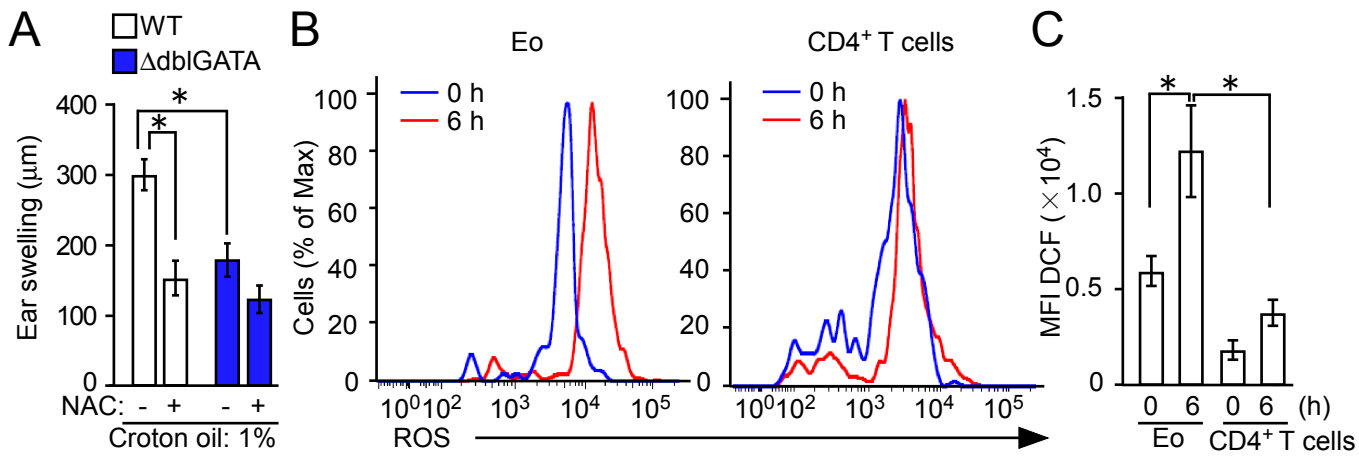

A
WT
ΔdblGATA
Ear swelling (μm)
400
300
200
100
0
*
*
NAC: - + - +
Croton oil: 1%
B
Eo
CD4+ T cells
0 h
6 h
Cells (% of Max)
100
80
60
40
20
0
10^0 10^2 10^3 10^4 10^5
ROS
C
MFI DCF (×10^4)
1.5
1.0
0.5
0
0 6 0 6 (h)
Eo
CD4+ T cells

Figure 3

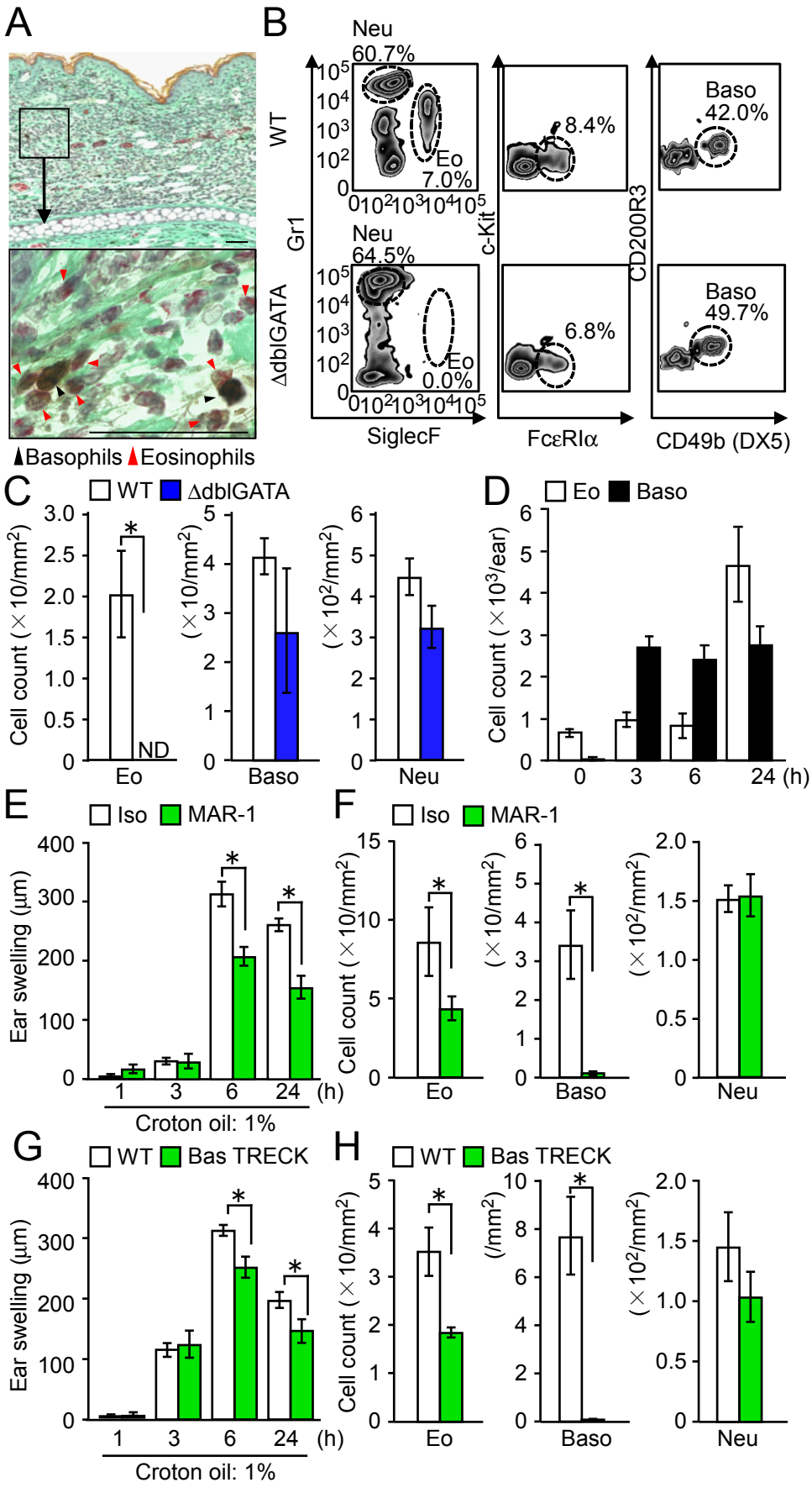

A
Basophils Eosinophils
B
WT
ΔdblGATA
Neu
60.7%
Eo
7.0%
Neu
64.5%
Eo
0.0%
Gr1
SiglecF
c-Kit
FcεRIα
8.4%
6.8%
CD200R3
CD49b (DX5)
Baso
42.0%
Baso
49.7%
C
WT ΔdblGATA
Cell count (×10/mm²)
(×10/mm²)
(×10²/mm²)
ND
Eo
Baso
Neu
D
Eo Baso
Cell count (×10³/ear)
0 3 6 24 (h)
E
Iso MAR-1
Ear swelling (μm)
1 3 6 24 (h)
Croton oil: 1%
F
Iso MAR-1
Cell count (×10/mm²)
(×10/mm²)
(×10²/mm²)
Eo
Baso
Neu
G
WT Bas TRECK
Ear swelling (μm)
1 3 6 24 (h)
Croton oil: 1%
H
WT Bas TRECK
Cell count (×10/mm²)
(/mm²)
(×10²/mm²)
Eo
Baso
Neu

Figure 4

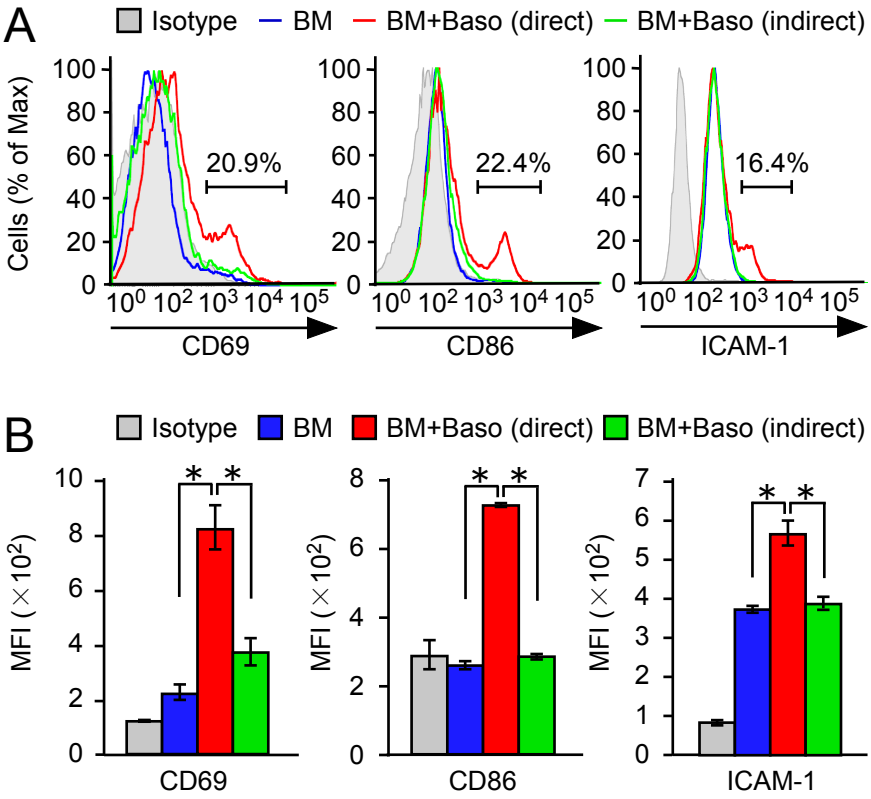
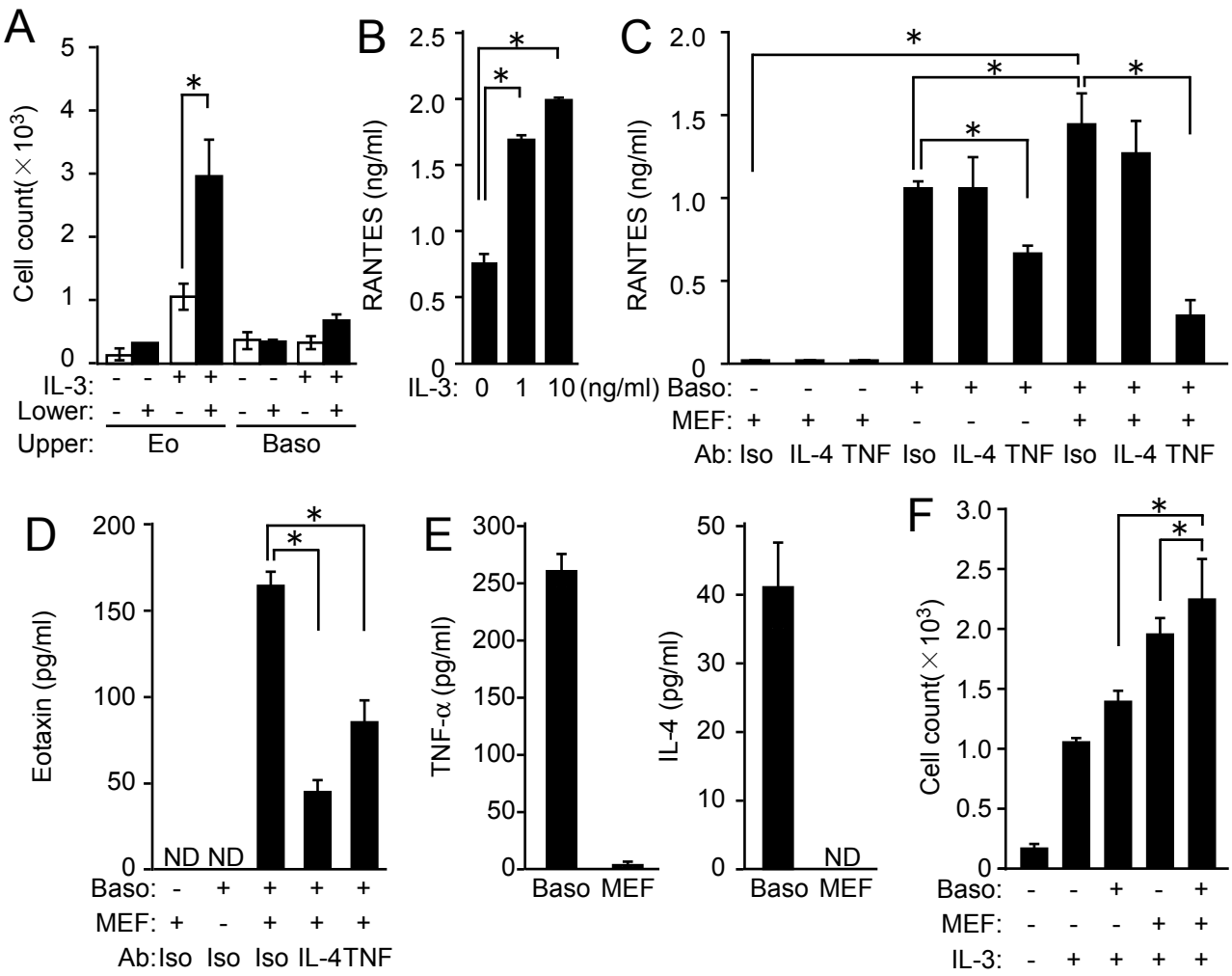

A
Isotype
BM
BM+Baso (direct)
BM+Baso (indirect)
Cells (% of Max)
20.9%
22.4%
16.4%
CD69
CD86
ICAM-1
B
MFI (×10²)
CD69
CD86
ICAM-1

Figure 5


A
Cell count(×10^3)
IL-3:
Lower:
Upper:
Eo
Baso
B
RANTES (ng/ml)
IL-3: 0 1 10 (ng/ml)
C
RANTES (ng/ml)
Baso:
MEF:
Ab: Iso IL-4 TNF Iso IL-4 TNF Iso IL-4 TNF
D
Eotaxin (pg/ml)
ND ND
Baso:
MEF:
Ab: Iso Iso Iso IL-4 TNF
E
TNF-α (pg/ml)
Baso MEF
IL-4 (pg/ml)
ND
Baso MEF
F
Cell count(×10^3)
Baso:
MEF:
IL-3:

SUPPLEMENTAL MATERIALS AND METHODS

Mice

Genetically mast cell-deficient WBB6F$_1$-*Kit*$^{W/Wv}$ mice, and congenic normal WBB6F$_1$-*Kit*$^{+/+}$ mice were purchased from Japan SLC (Shizuoka, Japan).

Reagents, antibodies, and flow cytometry

Monoclonal anti- mouse CCR3 antibody (83103) was purchased from R&D Systems. Purified anti-human basophils (2D7) antibody was purchased from BioLegend.

Immunohistochemistry to detect basophils in human

Human skin samples were collected from ICD patients (n=10). For the identification of basophils by immunohistochemistry, formalin-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated through graded ethanol solutions. To enhance antigen retrieval, the slides were treated with 0.4 mg/ml proteinase K (Dako, Carpinteria, CA, CA) for 5 min at room temperature. Samples were blocked with 10% goat serum for 30 min at room temperature and incubated for 16 h at 4°C with primary antibody (human 2D7, 1:50) followed by an Envision kit (Dako). They were lightly counterstained with hematoxylin and eosin.

ELISA and beads array

The amount of several cytokines in the skin was measured by ELISA and a cytometric bead array (CBA) system (BD Biosciences) according to manufacturer's instructions. For measurement of RANTES and eotaxin in the skin, ear skin was collected 24 h after

applying 1% croton oil and homogenized in 150 μl PBS. The supernatants were collected for ELISA assay and CBA. We also measured the amount of RANTES, eotaxin, IL-4, and TNF-α using organ culture method. Ear skin of WT or Bas TRECK Tg mice was collected 24 h after applying 1% croton oil and split in dorsal and ventral halves. The dorsal (i.e., cartilage-free) halves were cultured in 300 μl PBS at 37°C for 3 h. The culture medium was collected for an ELISA assay and CBA.

Quantitative PCR analysis

Total RNAs were isolated with RNeasy kits and digested with DNase I (Qiagen, Hilden, Germany). cDNA was reverse transcribed from total RNA samples using Prime Script RT Master Mix (Takara Bio, Otsu, Japan). Quantitative RT-PCR was performed by monitoring synthesis of dsDNA during the various PCR cycles using SYBR Green I (Roche, Basel, Switzerland) and lightCycler real-time PCR apparatus (Roche) according to manufacturer's instructions. Primer for eotaxin and RANTES were obtained from Greiner Bio-One (Tokyo, Japan), and the primer sequences were *Eotaxin*,5'-GAA TCA CCA ACA ACA GAT GCA C-3' (forward) and 5'-ATC CTG GAC CCA CTT CTT CTT-3' (reverse); and *RANTES*, 5'-TTT GCC TAC CTC TCC CTC G-3' (forward) and 5'-CGA CTG CAA GAT TGG AGC ACT-3' (reverse). For each sample, triplicate test reactions and a control reaction lacking reverse transcriptase were analyzed for expression of genes, and results were normalized to those of levels. Expression of mRNA (relative) was normalized to the 'housekeeping' glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA by the change in cycling threshold (ΔC_T) method and calculated based on $2^{-\Delta CT}$.

SUPPLEMENTAL FIGURE LEGENDS

Figure E1. Increased levels of RANTES and eotaxin protein in ICD

For measurement of RANTES and eotaxin in the skin, ear skin was collected and homogenized in PBS. RANTES and eotaxin protein levels were measured before (0 h) or 24 h after croton oil application.

Figure E2. Coincidental presence of eosinophils and basophils in inflamed skin of human ICD

Immunohistochemistry with 2D7 and HE staining were performed using inflamed skin of human ICD. $2D7^+$ basophils (black arrowhead) and eosinophils (red arrowhead) were present coincidently. The representative histological findings of two ICD patients were shown. Scale bars, 50 μm.

Figure E3. Intact mast cells in the skin by anti-FcεRIα (MAR-1) antibody treatment

(A) Basophils can be efficiently depleted by anti-FcεRIα (MAR-1) antibody treatment. BALB/c mice were injected twice daily for 3 days with 5 μg isotype-matched control or anti-FcεRIα (MAR-1) antibody. On day 4 the depletion efficiency in blood was examined by FACS. (B) Preservation of mast cell population in the skin detected by toluidine blue staining after MAR-1 treatment.

Figure E4. Basophils were efficiently depleted by DT-treated Bas TRECK Tg mice.

(A) Control BALB/c mice and Bas TRECK Tg mice (BALB/c background) were

injected intraperitoneally with 100 ng of DT per mouse. On day 2 the depletion efficiency in blood was examined by FACS. (B) The number of eosinophils in bone marrow was preserved equivalently in DT-treated Bas TRECK Tg mice.

Figure E5. Impaired ICD in the absence of mast cells

(A) Ear swelling of WBB6F$_1$-*Kit*$^{W/Wv}$ (W/Wv) mice and congenic normal WBB6F$_1$-*Kit*$^{+/+}$ (+/+) mice (n=5 per group). (B) Numbers of infiltrating eosinophils (Eo), basophils (Baso), and neutrophils (Neu) of congenic normal WBB6F$_1$-*Kit*$^{+/+}$ (+/+) mice and WBB6F$_1$-*Kit*$^{W/Wv}$ mice.

Figure E6. Eosinophils were recruited via CCR3 and CCR3 ligands were produced in the skin in a basophil-dependent manner

(A) Administration of anti-CCR3 antibodies significantly suppressed the infiltration of eosinophils in the skin. (B) Production of CCR3 ligands, RANTES and eotaxin, in the skin were reduced in DT-treated Bas TRECK Tg mice using skin organ culture.

Figure E7. RANTES and eotaxin mRNA expression in BMBas and MEFs

Quantitative RT-PCR analysis of RANTES and eotaxin mRNA in BMBas and MEFs.

Figure E8. Depletion of basophils reduced TNF-α and IL-4 protein levels in the skin.

The amounts of TNF-α and IL-4 were reduced in DT-treated Bas TRECK Tg mice using organ culture.

Figure E9. Roles for basophils and eosinophils in ICD

When chemical agents, including irritants, are exposed to the skin, basophils are recruited into the skin as an early phase (Step 1). Activated basophils secrete cytokines including TNF-α and IL-4 (Step 2), which in turn act on dermal fibroblasts and induce to produce chemokines, such as eotaxin (Step 3). In addition, basophil themselves produce RANTES in a TNF-α dependent manner (Step 3). These chemokines recruit eosinophils into the skin (Step 4). Accumulated eosinophils are activated in interaction with basophils and release inflammatory mediators, including ROS and establish skin inflammation (Step 5).

SUPPLEMENTAL TABLE

Table E1. Histological findings of skin 6 h after application of croton oil

Phenotype	ΔdblGATA	WT	IL-5
Inflammation	2.3 ± 0.5	2.4 ± 0.5	3.0 ± 0.0
Neutrophils	1.6 ± 0.5	2.2 ± 0.4	3.0 ± 0.0
Mononuclear cells	1.5 ± 0.5	1.8 ± 0.8	2.8 ± 0.4
Edema	1.6 ± 0.8	2.4 ± 0.9	2.3 ± 0.5
Epithelial hyperplasia	1.0 ± 0.0	2.6 ± 0.5	2.1 ± 0.4

Samples were scored for the severity and character of the inflammatory response using a subjective grading scale.

Table E2. Numbers of eosinophils in the skin lesions

Mouse strain	ΔdblGATA	WT	IL-5 Tg
Eosinophils	0.0 ± 0.0	5.0 ± 1.4	17.6 ± 6.5

Eosinophils in the skin lesions of irritant contact dermatitis of ΔdblGATA, WT, and IL-5 Tg mice (n=5) were counted by microscopic analysis (averaged from 5 high power fields, × 400 magnification) using Papanicolaou staining.

Figure E1

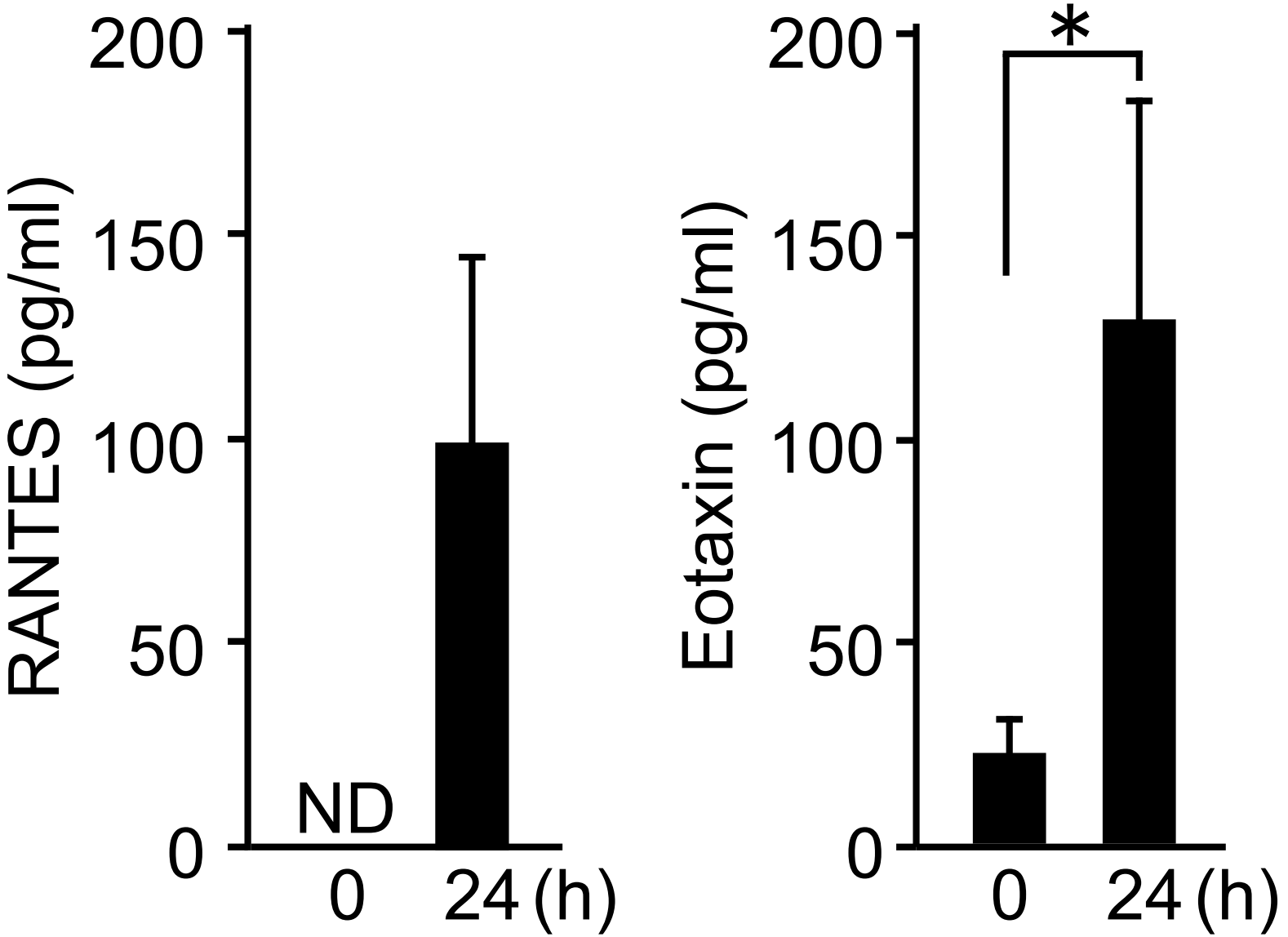

RANTES (pg/ml)
200
150
100
50
0
ND
0
24 (h)
Eotaxin (pg/ml)
200
150
100
50
0
*
0
24 (h)

Figure E2

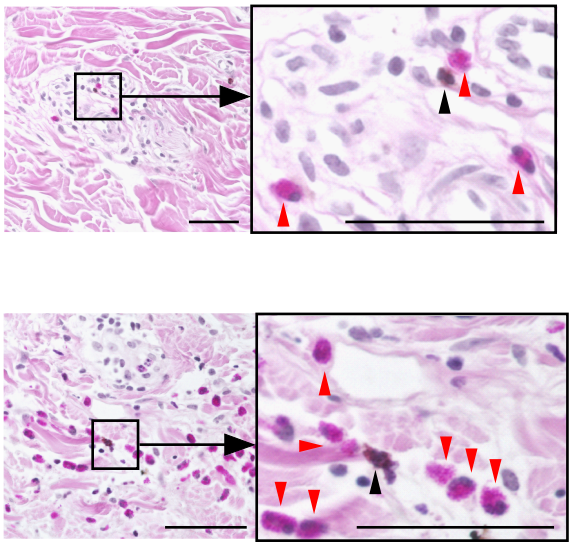


Figure E3

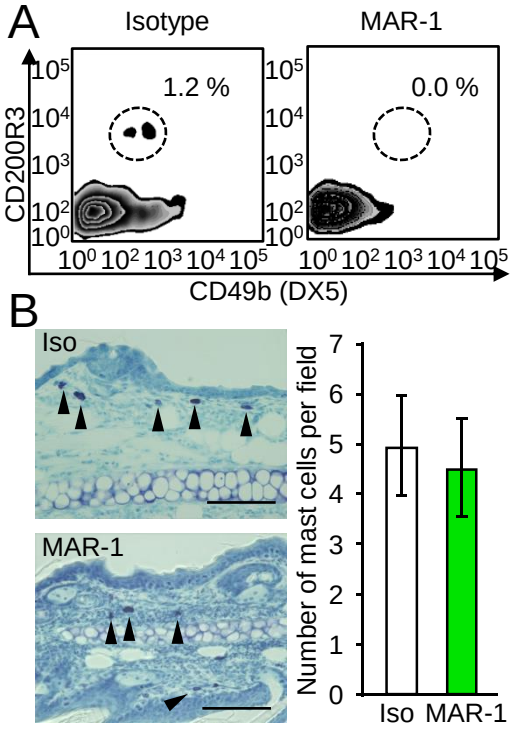

A
Isotype
MAR-1
1.2 %
0.0 %
CD200R3
CD49b (DX5)
B
Iso
MAR-1
Number of mast cells per field
Iso
MAR-1

Figure E4

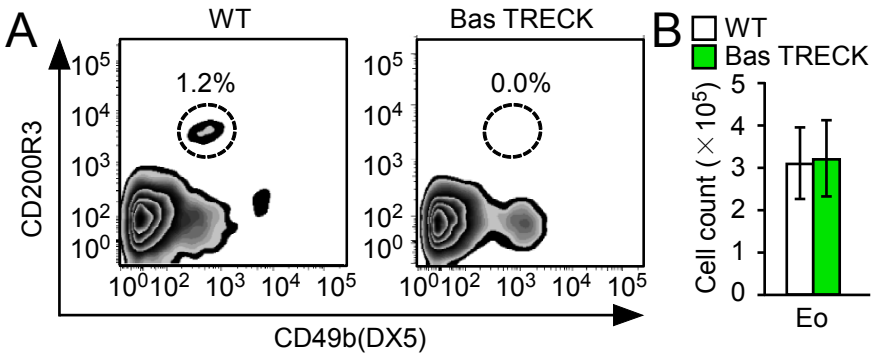

A
WT
Bas TRECK
1.2%
0.0%
CD200R3
CD49b(DX5)
B
WT
Bas TRECK
Cell count (×10^5)
Eo

Figure E5

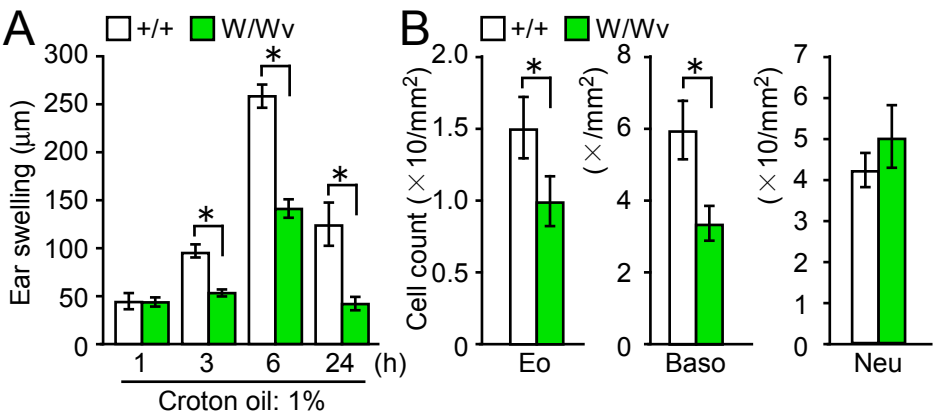

A
+/+
W/Wv
Ear swelling (μm)
300
250
200
150
100
50
0
1
3
6
24
(h)
Croton oil: 1%
*
B
+/+
W/Wv
Cell count (×10/mm²)
2.0
1.5
1.0
0.5
0
Eo
(×/mm²)
8
6
4
2
0
Baso
(×10/mm²)
7
6
5
4
3
2
1
0
Neu

Figure E6

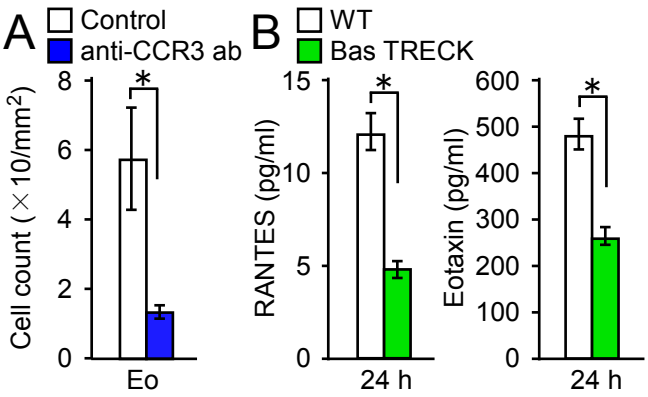

A
Control
anti-CCR3 ab
Cell count (×10/mm²)
8
6
4
2
0
*
Eo
B
WT
Bas TRECK
RANTES (pg/ml)
15
10
5
0
*
24 h
Eotaxin (pg/ml)
600
500
400
300
200
100
0
*
24 h

Figure E7

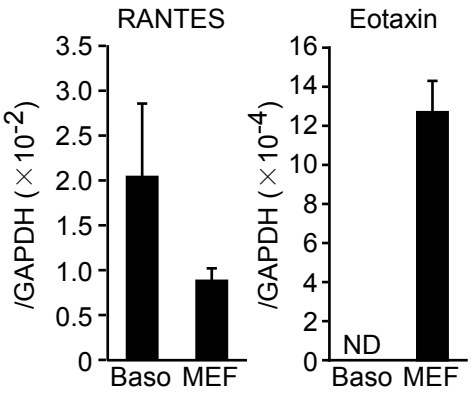

RANTES
Eotaxin
/GAPDH (×10-2)
/GAPDH (×10-4)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
16
14
12
10
8
6
4
2
0
ND
Baso
MEF
Baso
MEF

Figure E8

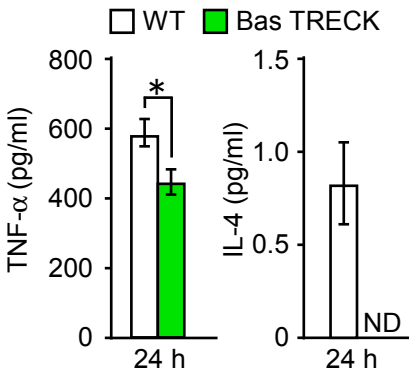
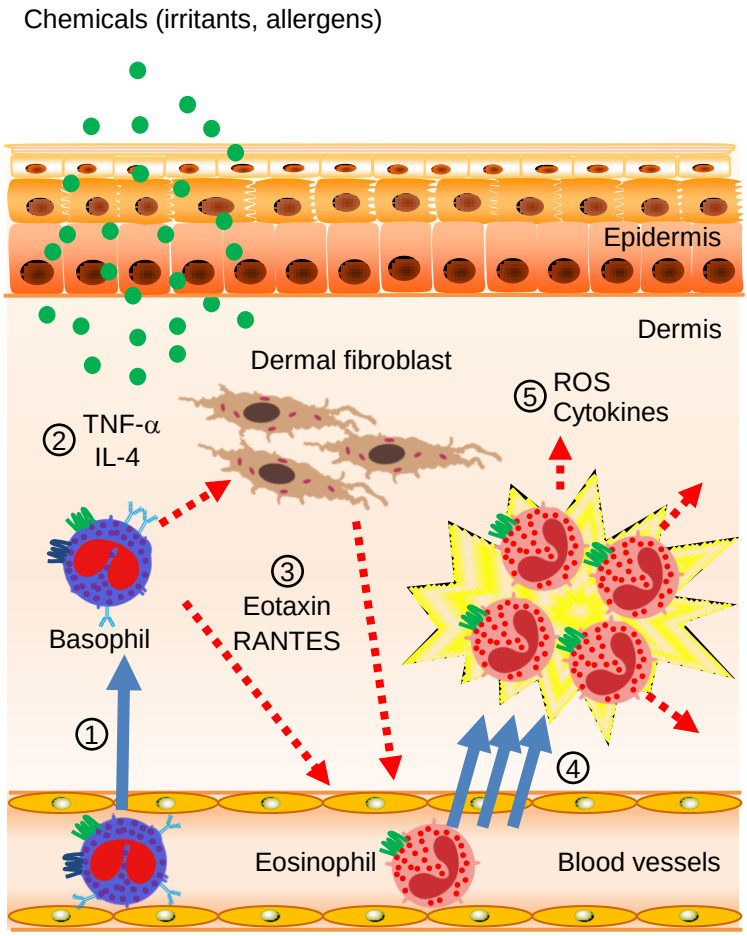

WT
Bas TRECK
TNF-α (pg/ml)
800
600
400
200
0
*
24 h
IL-4 (pg/ml)
1.5
1.0
0.5
0
ND
24 h

Figure E9


Chemicals (irritants, allergens)
Epidermis
Dermis
Dermal fibroblast
⑤ ROS
Cytokines
② TNF-α
IL-4
③
Eotaxin
RANTES
Basophil
①
④
Eosinophil
Blood vessels