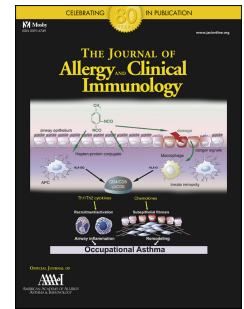


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Opposing functions of TSLP-responsive basophils and dendritic cells in a mouse model of atopic dermatitis

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1 **Opposing functions of TSLP-responsive basophils and dendritic cells in a** 2 **mouse model of atopic dermatitis**

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 15 **Short title:** TSLP-responsive DCs promote AD-like inflammation

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Capsule summary

TSLP promotes inflammation in a mouse model of atopic dermatitis by activating DCs while TSLP-responsive basophils ameliorate pathology. This indicates that DCs rather than basophils should be targeted to improve disease conditions.

Key words: atopic dermatitis, thymic stromal lymphopoietin, basophils, dendritic cells, bone marrow chimeras, calcipotriol

Abbreviations: AD: Atopic dermatitis, DC: dendritic cell, DT: diphtheria toxin, DTR: DT receptor, MBMC: mixed bone marrow chimeras, STAT: signal transducer and activator of transcription, TSLP: thymic stromal lymphopoietin, TSLPR: TSLP receptor

To the Editor

Atopic dermatitis (AD) is a chronic, relapsing inflammatory skin disorder characterized by pruritic rash and scaly skin. AD is further characterized by an inflammatory type 2 immune response accompanied by increased expression of the Th2-associated cytokines IL-4, IL-5 and IL-13 although IFN- γ is also increased in AD lesions. Mutations in genes involved in the maintenance of barrier integrity and expression of alarmins such as IL-25, IL-33, and thymic stroma-derived lymphopoietin (TSLP) are associated with increased risk to develop AD. In mice it has been shown that over-expression of TSLP in keratinocytes or topical treatment with the Vitamin D3 analog MC903 (calcipotriol) causes an AD-like skin inflammation^{1,2}. TSLP can initiate type 2 immune responses through activation of DCs³, but may also directly induce IL-4 expression in CD4 T cells⁴. TSLP has further been shown to elicit basophilia in mice and recruitment of basophils to the skin could be blocked by an anti-TSLP antibody suggesting that basophils respond directly to TSLP and promote inflammation^{5,6}. Basophils are found in skin biopsies of AD patients^{6,7} and human basophils express TSLPR⁵. However, it has not been investigated whether expression of the TSLP receptor (TSLPR) on basophils or DCs is required for MC903-elicited AD-like inflammation. Furthermore, it remains unclear to what extent IL-4, IgE and the adaptive immune system contribute to the pathology in this model.

To gain better insights in the role of TSLP in AD-like inflammation we treated the ear skin of wild-type (WT), TSLP-deficient (TSLP^{-/-}) and TSLPR α -deficient (TSLPR α ^{-/-}) mice for ten days with MC903 and assessed the inflammatory response by measuring the change in ear thickness. As expected, WT mice developed a 3-fold increase in ear thickness of MC903-treated compared to ethanol-treated ears starting at day 5 of treatment (Fig. 1A). In contrast, TSLP^{-/-} and TSLPR α ^{-/-} mice showed a significantly decreased inflammatory ear swelling response, diminished epidermal thickening, lower expression levels of IL-3 and Ccl26

(eotaxin-3), lower serum IgE levels and reduced infiltration of granulocytes to the ear (Fig. 1B-F). Further analysis of eosinophil-deficient Δ dblGata mice revealed an important role for eosinophils in this model of AD-like dermatitis (Fig. 1G).

Next, we analyzed the role of TSLP-responsive basophils and DCs for pathogenesis. TSLP promotes basophil development from bone marrow precursors although at a 100-1000-fold lower efficiency as compared to IL-3 which is consistent with previous data⁵ (Fig. E1). We generated mixed bone marrow chimeric mice (MBMCs) in which only basophils are TSLPR-deficient (Baso-TSLPR $\alpha^{-/-}$) by mixing bone marrow of constitutively basophil-deficient Mcpt8Cre mice and TSLPR $\alpha^{-/-}$ mice in a 4:1 ratio. This creates a situation where all basophils are TSLPR α -deficient whereas 80% of all other hematopoietic cells and 100% of all non-hematopoietic cells remain responsive for TSLP. Unexpectedly, Baso-TSLPR $\alpha^{-/-}$ chimeras developed exaggerated ear swelling as compared to Baso-WT chimeras (Fig. 2A). No difference was observed for the recruitment of neutrophils or eosinophils and basophils were even more abundant in the skin of MC903-treated Baso-TSLPR $\alpha^{-/-}$ chimeras (Fig. 2B). Although TSLP-responsive basophils promoted Th2 polarization in ear-draining lymph nodes (Fig. 2C) this did not result in increased serum IgE levels (Fig. 2D). In addition, increased ear swelling was also observed in basophil-deficient Mcpt8Cre mice (Fig E2).

Thus, TSLP-mediated basophil recruitment occurred without direct TSLP recognition by basophils. Furthermore, basophils did not promote but rather ameliorated the ear swelling response and this effect was dependent on TSLPR expression on basophils.

It was previously demonstrated that DCs can prime Th2 polarization in response to TSLP⁸. Furthermore, it has been shown that DCs can recruit CD4 T cells, which then provide IL-3 to attract basophils that promote Th2 polarization⁹. However, these studies were not performed in mice, in which DCs are deficient for TSLPR α and it remains unclear whether DCs have to respond directly to TSLP. Therefore, we generated MBMCs in which DCs do not express

TSLPR α (DC-TSLPR $\alpha^{-/-}$). When we treated these mice with MC903, the ear-swelling response was significantly reduced in DC-TSLPR $\alpha^{-/-}$ compared to DC-WT chimeras (Fig. 2E). Importantly, DCs were detectable at comparable frequencies in draining lymph nodes of both MBMCs (Fig. E3). A trend to lower numbers of infiltrating granulocytes was observed when DCs could not respond to TSLP (Fig. 2F). Based on our observation that TSLP induces IL-3 and Ccl26 expression in MC903-treated skin of wild-type mice, we determined whether DCs might be the source of these factors. However, in vitro stimulation of bone marrow derived DCs with 100 ng/ml TSLP did not induce IL-3 or Ccl26 mRNA expression indicating that DCs are not a critical source for both molecules (Fig. E4). We further observed reduced production of IL-4 from restimulated T cells in the ear-draining lymph nodes of DC-TSLPR $\alpha^{-/-}$ as compared to DC-WT chimeras (Fig. 2G) which correlated with lower serum IgE levels in these mice (Fig. 2H). In summary, these data indicate that direct activation of DCs by TSLP promotes pathology in this model of AD-like dermatitis.

Having established that TSLP-responsive DCs drive MC903-induced ear swelling and considering the fact that MC903 treatment causes strong Th2 and IgE responses, we further investigated whether the DC-Th2-IgE axis was indeed required for pathogenesis in this model. Therefore we analyzed Rag1-deficient mice which lack B and T cells and observed no difference in the ear swelling response although we noticed a trend to reduced cellular infiltration (Fig. E5). Given the established therapeutic effect of anti-IL-4 treatment during chronic human AD we were surprised to see that both WT and IL-4/IL-13 $^{-/-}$ mice developed MC903-elicited ear swelling response with the same kinetic and same intensity during the initiation and resolution phase of the response (Fig. E6). This result suggests that IL-4 becomes critical only in the chronic phase of AD which is the relevant indication for starting anti-IL-4 treatment in AD patients. However, we currently cannot exclude that species-specific differences in TSLP-induced skin inflammation are responsible for this effect. In

summary, this set of experiments reveals that the adaptive immune system including Th2 cells and IgE are dispensable for acute pathogenesis although both components are strongly induced by TSLP in this model of AD-like dermatitis.

Therefore, we conclude that in the MC903 mouse model of AD TSLP-responsive DCs orchestrate an IL-4/IL-13-independent innate immune response that leads to recruitment of effector cells and massive ear swelling. Further clarification of the innate effector pathways for pathogenesis in this model could help to identify new targets for early therapeutic interventions during development of AD in humans .

Acknowledgment

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Figure legends

Fig 1. TSLP promotes MC903-induced ear swelling and inflammation. **A**, Ear swelling response upon MC903 treatment. **B**, H&E staining of ears from indicated mice. Scale bars = 200 μ m. **C**, Quantitative RT-PCR of total ear tissue. **D**, Dot plots for indicated cell populations of WT mice. **E**, Cell infiltration into the ear skin. **F**, Serum IgE concentrations. **G**, Ear swelling in eosinophil-deficient Δ dblGata mice. n = 4-12 mice per group from one (G) or two (A, C, E, F) experiments. * P <0.05; ** P <0.01.

Fig 2. TSLPR-responsive basophils and DCs have opposite functions in MC903-induced **AD.** Comparison of mice with TSLP-responsive and -unresponsive basophils (A-D) or DCs (E-H). **A** and **E**, Ear swelling response upon MC903 treatment. **B** and **F**, Cell infiltration into the ear skin. **C** and **G**, IL-4 production from T cells in draining lymph nodes. **D** and **H**, Serum IgE concentrations. n = 10 (A), 7 (B), 4 (C, D, H), 12 (E, F) and 11 (G) mice per group from 2-3 experiments. * P <0.05; ** P <0.01; *** P <0.001.

Figure 1

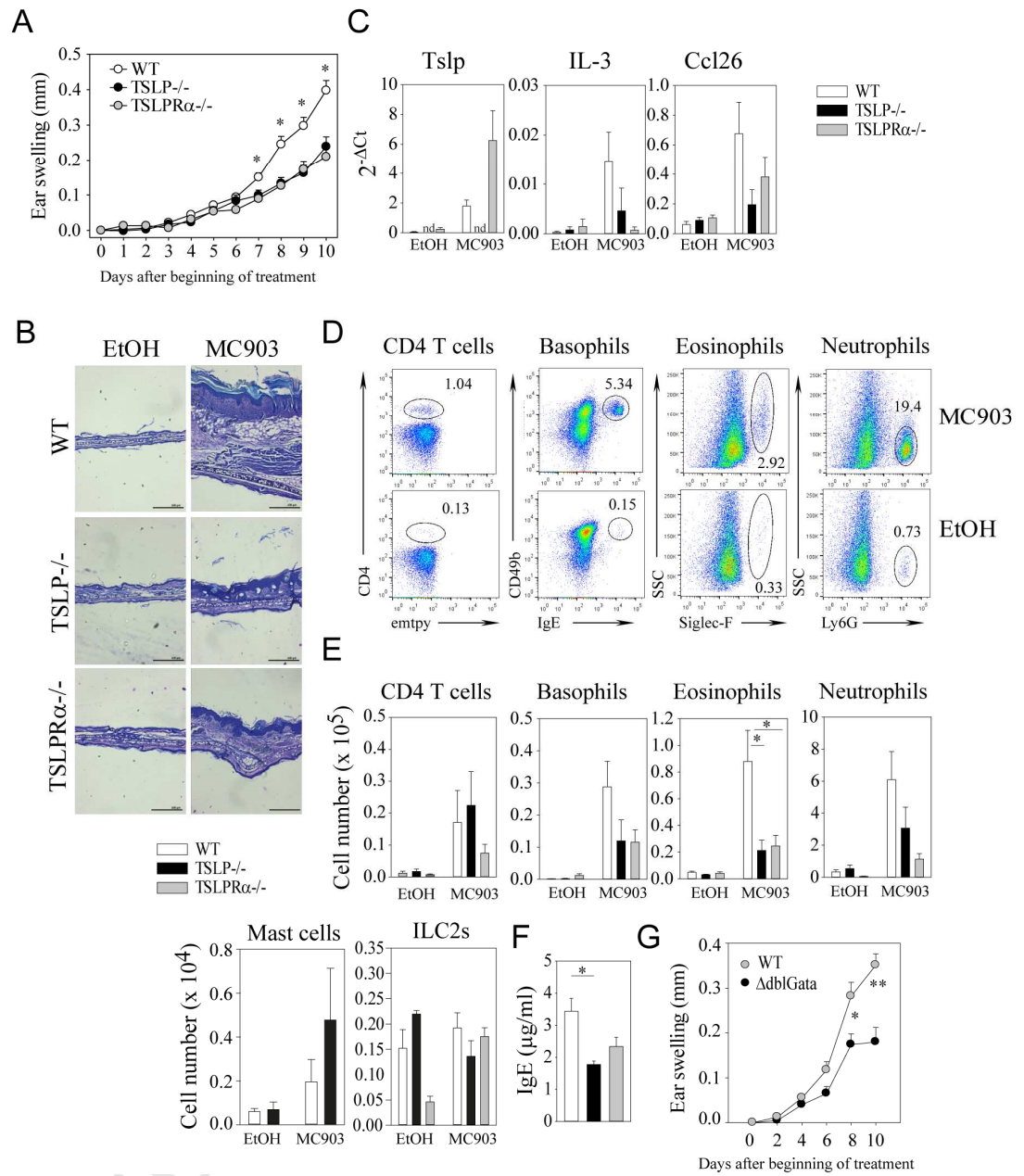
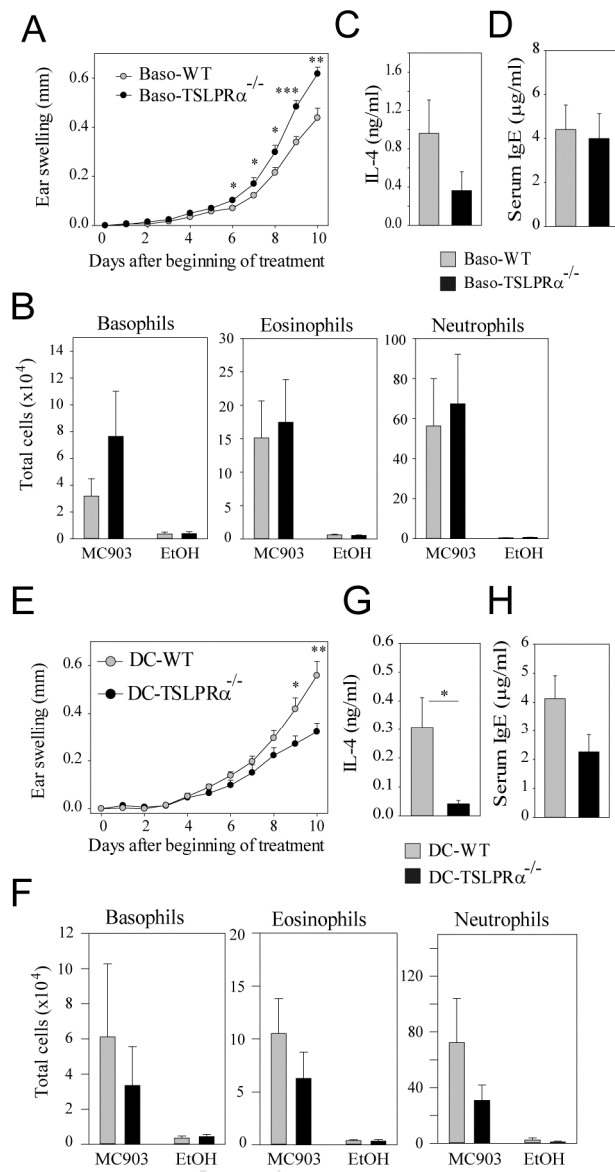


Figure 2



Online Repository

Materials and Methods

Mice

CD45.1 congenic C57BL/6 mice (B6.SJL-Ptprca Pepcb/BoyJ) were obtained from The Jackson Laboratory. TSLP^{-/-} E10, TSLPR α ^{-/-} E11, Basophil-ablated Mcpt8Cre^{E12}, DC-ablated Δ DC^{E13}, Rag1^{-/-} E14, Δ dblGata^{E15} and IL-4/IL-13^{-/-} E16 mice have been described.

Experiments were performed with mice on a C57BL/6 background unless indicated otherwise. All mice were bred and maintained according to institutional guidelines under pathogen free conditions. All experiments were performed in accordance with German animal protection law and European Union guidelines 86/809 and were approved by the Federal Government of Lower Franconia (54-2532.1-4/14).

Generation of mixed bone marrow chimeras (MBMCs)

Bone marrow cells were obtained from tibia and femur of Mcpt8Cre or Δ DC mice and mixed at a 4:1 ratio with bone marrow cells from either WT mice (CD45.1 congenic) or TSLPR α ^{-/-} mice. 2x10⁶ cells were injected into the tail vein of lethally irradiated (9 Gy) recipient C57BL/6 mice to create Baso-WT and Baso-TSLPR α ^{-/-} or DC-WT and DC-TSLPR α ^{-/-} MBMCs. Mice were treated with antibiotic-containing drinking water for 8 weeks after reconstitution.

Skin inflammation

Atopic dermatitis was induced by daily topical application of 4 nmol MC903 (Calcipotriol, Cayman Europe, Tallinn, Estonia) in 10 μ l ethanol onto the right ear. 10 μ l ethanol was applied onto the left ear which served as control. Ear thickness was measured daily with a digital thickness gage and the difference between both ears was calculated.

Histology

Cryosections of ears on day 10 after beginning of MC903 treatment were fixed in 4% paraformaldehyde, dried and stained with a commercial Wright-Giemsa-staining kit (DiffQuik, Merck, Darmstadt) according to the manufacturer's instructions. Pictures were acquired on an AxioVert A1 and analyzed using ZEN software (Zeiss, Jena, Germany).

Quantitative RT-PCR

Quantitative RT-PCR was performed with RNA from total ear tissue on day 10 after beginning of MC903 treatment or with RNA from bone marrow-derived DCs using a CFX connect machine (BioRad, Hercules, CA). The following primers were used: TSLP-fwd 5'-ACGGATGGGGCTAACTTACAA-3', TSLP-rev 5'-AGTCCTCGATTTGCTCGAACT-3', IL-3-fwd 5'-TGCTGCTCCTGCTCCTGATG-3', IL-3-rev 5'-GAGGTTAGCACTGTCTCCAG-3', CCL-26-fwd 5'-TTCTTCGATTTGGGTCTCCTTG-3', CCL-26-rev 5'-GTGCAGCTCTTGTCGGTGAA-3', PBGD-fwd 5'-TGGTTGTTCACTCCCTGAAGG-3', PBGD-rev 5'-AAAGACAACAGCATCACAAGGGT-3'. The PBGD PCR results were used for normalization.

Flow cytometry

Minced ears were digested with Collagenase D (1 mg/ml; Roche Life Science, Mannheim, Germany), liberase (0.25 mg/ml; Sigma-Aldrich, St. Louis, MO) and DNase I (0.2 mg/ml; Sigma-Aldrich) in PBS for 30 min at 37°C before single cell suspensions were prepared by mechanical disruption through a 70 µm cell strainer. Cells were then incubated with anti-CD16/32 for 5 min at room temperature and stained for 20 min on ice with antibodies listed in **Fig E7** in the Online Repository.

In vitro culture of bone marrow cells

Bone marrow cells were prepared from tibia and femur of C57BL/6 mice. 2×10^6 cells were incubated in DMEM (PanBiotech, Aidenbach, Germany) supplemented with 20% fetal calf serum (Gibco, Thermo Fisher Scientific, Waltham, MA), 2 mM L-glutamine, 100 $\mu\text{g/mL}$ penicillin, 100 $\mu\text{g/mL}$ streptomycin and 5×10^{-5} M β -mercaptoethanol stimulated with indicated amounts of IL-3 or TSLP (ImmunoTools, Friesoythe, Germany). Media was exchanged and fresh cytokines were added every other day for eleven days.

Restimulation of draining lymph nodes and ELISA

Ear-draining lymph nodes were excised and single cells suspensions obtained by mechanical disruption through a 70 μm cell strainer. 5×10^5 cells per well were restimulated for 48h in 96-well plates coated with 1 $\mu\text{g/mL}$ anti-CD3 and 5 $\mu\text{g/mL}$ anti-CD28. IL-4 was detected by standard ELISA. Serum IgE levels were measured as described^{E17}.

Statistics

Statistical significance was determined by using two-tailed Student's t-test. Results were considered significant at a *P* value of 0.05 or less.

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Figure Legends

FIG E1. IL-3 is more potent than TSLP for basophil development in vitro. **A**, Bone marrow cells from C57BL/6 mice were cultured for 11 days with indicated concentrations of IL-3 and TSLP. Bars show the frequency and total cell number of basophils (FcεRI⁺c-Kit⁻). n = 6 per condition from 2 experiments. **B**, Bone marrow cells from C57BL/6 or TSLPRα^{-/-} mice were cultured for 11 days with indicated concentrations of IL-3 or TSLP.

FIG E2. Basophil-deficient Mcpt8Cre mice show an enhanced ear swelling response. Ears of wild-type C57BL/6 mice (WT) and Mcpt8Cre mice of the same genetic background were treated daily with MC903 on the right ear and EtOH on the left ear. The ear swelling response was measured by determining the difference in ear thickness between left and right ear. Symbols show the mean+SEM with 9 mice per group from 2 experiments.

FIG E3. Efficient reconstitution of DC compartment in mixed bone marrow chimeras. Mixed bone marrow (BM) chimeras were generated with 80% BM cells from ΔDC mice and 20% BM cells from either wild-type or TSLPRα^{-/-} mice. Dot plots are gated on viable B220-negative cells and show expression of MHC class II (I-A^b) and CD11c to demonstrate comparable reconstitution of the DC compartment in both sets of chimeras.

FIG E4. TSLP does not induce IL-3 or Ccl26 expression in bone marrow-derived DCs. Bone marrow-derived DCs were left untreated (grey bars) or stimulated with 100 ng/ml TSLP for 12 hours (black bars) and subjected to quantitative RT-PCR analysis to determine the expression of IL-3 and Ccl26 (Eotaxin 3). PBGD served as reference gene. Bars show the mean+SEM with 6 samples per group from two experiments.

FIG E5. T and B cells are not required for the ear swelling response and recruitment of effector cells after MC903 treatment. **A**, Ear swelling response in wild-type and Rag1^{-/-} mice. Symbols show the mean+SEM with 14 mice per group from 3 independent experiments. **B**, Infiltration of effector cells in wild-type and Rag1^{-/-} mice. Bars show the mean+SD with 3 mice per group from one experiment.

121 **FIG E6. IL-4 or IL-13 are not required for induction and resolution of the ear swelling response**
122 **after MC903 treatment.** Ear swelling response upon MC903 treatment of indicated mouse
123 strains. Symbols show the mean+SEM with 8 mice per group from two experiments. n.s. =
124 not significant.

125 **FIG E7. Antibodies used in this study.**

Figure E1

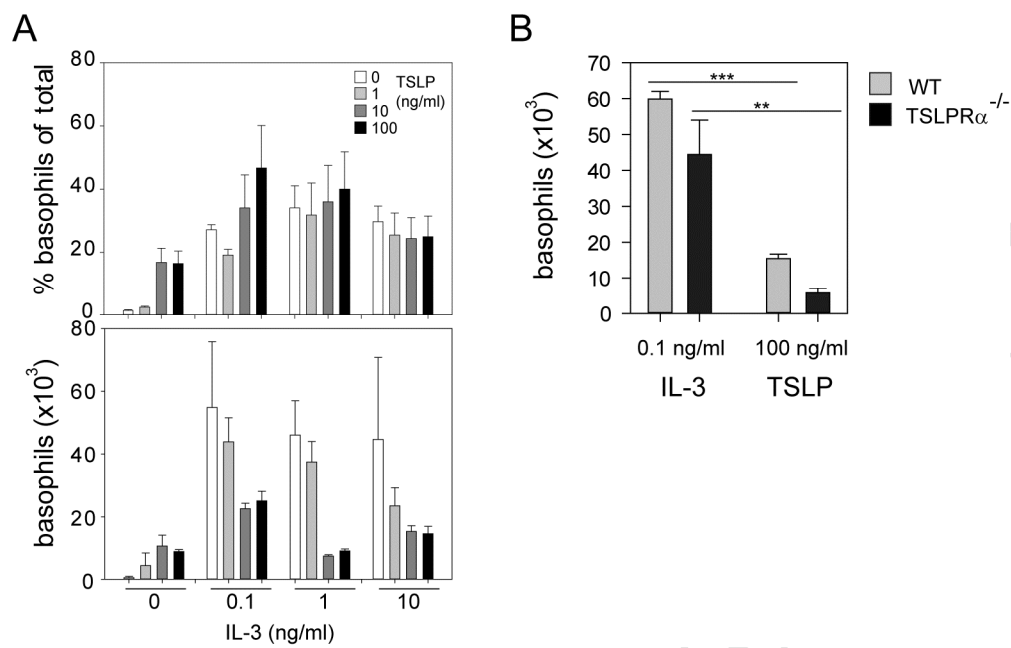


Figure E2

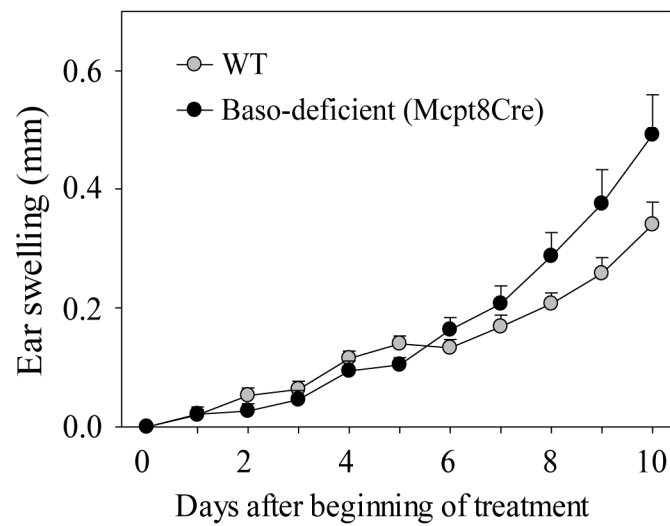


Figure E3

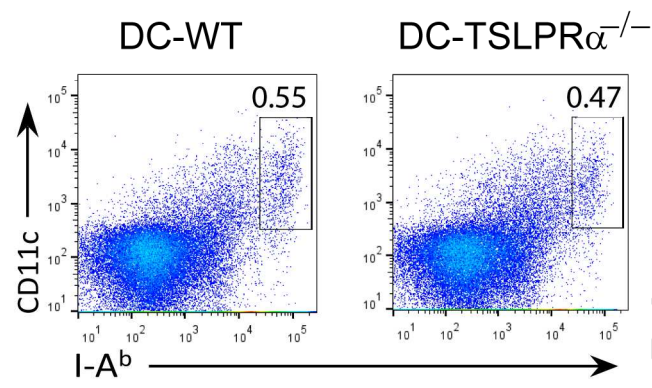


Figure E4

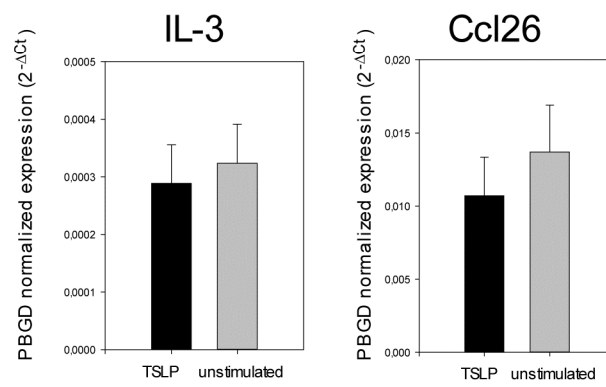
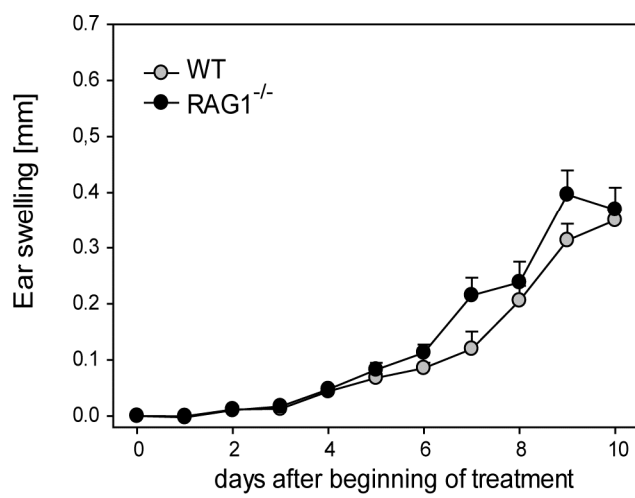


Figure E5

A



B

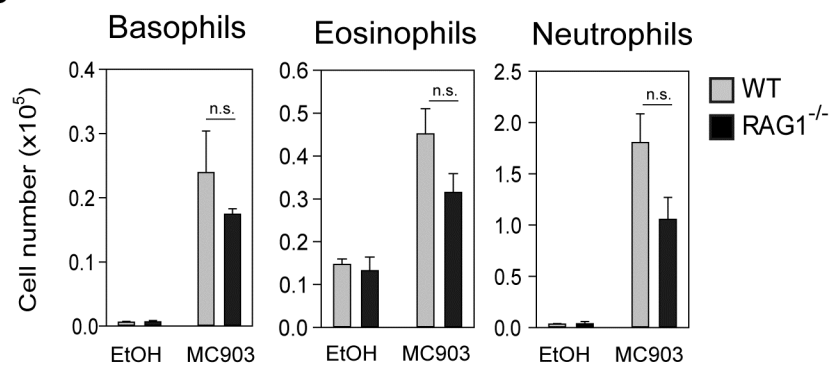


Figure E6

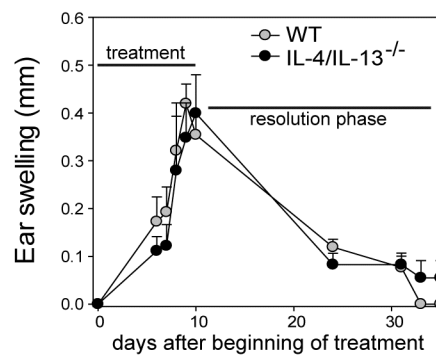


Figure E7

Antigen	Conjugate	clone	Source
CD4	APC	RM4-5	eBioscience
CD4	APC-eFlour780	RM4-5	eBioscience
CD49b	AlexaFlour 647	HMa2	BioLegend
CD200R3	PE, APC	Ba13	BioLegend
IgE	FITC	R35-72	BD
Siglec-F	BV421	E50-2440	BD
CD11b	AlexaFlour488	M1/70	BioLegend
Ly6G	PerCP/Cy5.5	1A8	BioLegend
CD45.1	FITC	A20	Miltenyi
CD45.2	PerCP-Cy5.5	104	eBioscience
MHCII	APC-Cy7	M5/114.15.2	BioLegend
CD11c	PerCP-Cy5.5	N418	eBioscience
CD3	APC	145-2C11	eBioscience
CD8a	APC	53-6.7	eBioscience
CD45R	A647	RA3-6B2	BioLegend
Ter119	biotin	TER-119	eBioscience
CD3	AlexaFlour 647	17A2	eBioscience
FcεRIα	APC, PE	MAR-1	eBioscience
CD11b	APC	M1/70	eBioscience
CD117	PE-Cy7	2B8	eBioscience
GATA3	PE	TWAJ	eBioscience
KLRG1	eFlour 450	2F1	eBioscience
NK1.1	APC	PK136	eBioscience
Streptavidin	APC		eBioscience
CD16/32(Fc block)	pure	2.4G2	BioXCell