Allergin-1 inhibits TLR2-mediated mast cell activation and suppresses dermatitis

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Short Communication

Allergin-1 inhibits TLR2-mediated mast cell activation and suppresses dermatitis.

Satoshi Tsurusaki[1], Satoko Tahara-Hanaoka[1, 2, *], Shohei Shibagaki[1], Shota Miyake[1], Masamichi Imai[3], Shiro Shibayama[3], Masato Kubo[4, 5], and Akira Shibuya[1, 2]

[1] Department of Immunology, Faculty of Medicine, [2] Life Science Center of Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan.

[3] Research Center of Immunology, Tsukuba Institute, ONO Pharmaceutical Co., Ltd. 17-2 Wadai, Tsukuba, Ibaraki 300-4247, Japan

[4] Division of Molecular Pathology, Research Institute for Biomedical Science, Tokyo University of Science, 2669 Yamazaki, Noda, Chiba, 278-0022, Japan

[5] Laboratory for Cytokine Regulation, RIKEN Center for Integrative Medical Sciences (IMS), 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan

*Corresponding should be addressed to Satoko Tahara-Hanaoka, PhD, E-mail: tokothr@md.tsukuba.ac.jp

Running title: Allergin-1 suppresses TLR2 signaling.

Key words: Allergin-1, Dermatitis, Mast cell, TLR2

Abstract

Toll-like receptor 2 (TLR2) recognizes cell-wall components of *Staphylococcus aureus*, which colonizes more than 90% of atopic eczematous skin lesions. The regulatory mechanisms of TLR2 signaling in the skin remain unclear. Allergin-1, an inhibitory immunoglobulin-like receptor containing an immunoreceptor tyrosine-based inhibitory motif (ITIM), is expressed on mast cells (MCs) and inhibits IgE-mediated anaphylaxis in mice. Here, we show that Allergin-1 inhibits TLR2-mediated activation of, and inflammatory cytokine production by, MCs in vitro. Compared with wild-type mice, Allergin-1-deficient mice showed enhanced ear swelling with enhanced collagen deposition and greater $Ly6G^+$ neutrophil recruitment after intradermal injection of Pam2CSK4 into pinnae. Using Mas–TRECK mice, which is a MC-deletion system based on *il4* enhancer elements, we also demonstrated that Allergin-1 on MCs is responsible for the Pam2CSK4-induced ear swelling. These results suggest that Allergin-1 on skin MCs suppresses TLR2-induced dermatitis.

Introduction

Mast cells (MCs) play pivotal roles in allergic reactions in barrier tissues, including skin (1). MCs are activated through both immunoglobulin E (IgE)-dependent and -independent pathways to express various Toll-like receptors (TLRs), which recognize diverse components of pathogens and initiate host defense mechanisms (2). Among TLRs, TLR2 is the predominant receptor for *Staphylococcus aureus*, which colonizes more than 90% of atopic eczematous skin lesions (3). Although TLR2 ligand is known to activate MCs (4), the regulation of TLR2 signaling in MCs remains incompletely understood.

Allergin-1, an immunoglobulin-like receptor bearing an immunotyrosine-based inhibitory motif (ITIM) in its cytoplasmic region, is highly expressed on MCs. We previously reported that Allergin-1 recruits the SH2-containing tyrosine phosphatases SHP-1 and SHP-2, which inhibits high-affinity receptors involved in IgE (FcεRI)-mediated signaling and subsequent MC activation, and thus suppresses IgE-mediated systemic and cutaneous anaphylaxis in mice (5). Because SHP-1 and SHP-2 downregulate TLR-mediated production of proinflammatory cytokines (6,7), we hypothesized that Allergin-1 might influence TLR2-mediated signaling. Here, we examined whether Allergin-1 suppresses Pam2CSK4-induced TLR2 signaling in the

MCs of mice.

Methods

Mice

Wild-type (WT) C57BL/6N mice were purchased from Clea Japan (Tokyo, Japan). Allergin-1-deficient (*Allergin-1*$^{-/-}$) mice were generated in our laboratory, as previously described (5). Mas–TRECK mice on a C57BL/6 background have been described previously (8). All experiments used female mice (age, 8 to 13 weeks) that were bred in the specific pathogen-free facilities at the University of Tsukuba, and all procedures followed the guidelines of the animal ethics committee of the University of Tsukuba.

Cells and qualitative PCR analysis

Bone-marrow–derived cultured MCs (BMMCs) were prepared from mice as described (5) and stimulated with 1 μg/ml Pam2CSK4 (InvivoGen, San Diego, CA, USA). Total RNA was isolated from cell pellets (TRIzol, Thermo Fisher Scientific, Waltham, MA, USA) and used as a template for reverse-transcription reactions (High-Capacity cDNA RT Kit, Applied Biosystems, Foster City, CA, USA). Real-time PCR analysis of *Il6*, *Tnfsf2*, *Il1b*, and *Gapdh* (measured as an internal control for normalization of data) was performed by using an ABI7500 sequence detector (Applied Biosystems) and Power SYBR Green PCR Master Mix (Applied Biosystems). The primers were: *Il6* (forward,

5′-gaggataccactcccaacagacc-3′; reverse 5′-aagtgcatcatcgttgttcataca-3′), *Tnfsf2* (5′-gggccaccacgctcttc-3′; 5’-ggtctgggccatagaactgatg-3′), *Il1b* (5′-actcaactgtgaaatgccacc-3′; 5′-tgatactgcctgcctgaagc-3′), and *Gapdh* (5′-tggtgaaggtcggtgtgaac-3′; 5′-atgaaggggtcgttgatggc-3′). mRNA transcript levels were determined relative to that in the unstimulated WT BMMCs. All experiments were performed in triplicate.

Antibodies

Anti-mouse Allergin-1 (TX83; mouse IgG1) was described previously (5). TX83 was biotinylated with Biotinylation Kit (Sulfo-OSu; DOJIN, Tokyo, Japan). Monoclonal antibodies (mAbs) to mouse c-Kit (ACK45), Ly6C (AL-21), CD11c (HL3), Siglec-F (E50-2440), and CD16/32 (2.4G2) were purchased from BD Biosciences (San Jose, CA, USA). Anti-mouse CD45.2 (104), FcεRIα (MAR1), and CD11b (M1/70) were purchased from eBioscience (San Diego, CA, USA) Anti-mouse Ly6G (1A8) was purchased from TONBO Biosciences (San Diego, CA, USA). Anti-Gr-1 (RB6-8C5) hybridoma provided by Dr. Kazumasa Ogasawara (Shiga University of Medical Science) was used for neutrophil depletion.

Cytometric bead array analysis

The concentrations of various inflammatory cytokines and chemokines were measured by using cytometric bead array analysis (BD Biosciences) according to the manufacturer's instructions.

Pam2CSK4-induced dermatitis model

TLR2 ligand-induced dermatitis model was reported previously (9). The left pinnae of WT, *Allergin-1*$^{-/-}$, diphtheria toxin (DT; Sigma-Aldrich, St Louis, MO, USA)-treated Mas-TRECK, and DT-treated Mas-TRECK mice intradermally injected with 1×10^6 WT or *Allergin-1*$^{-/-}$ BMMCs 5 weeks previously were sensitized by intradermal injection of Pam2CSK4 (4 μg). Neutrophil-depleted mice were generated by intraperitoneal injection of anti-Gr-1 (RB6-8C5, 50 μg) mAb on days -2, -1, +1, +3, and +5 and were injected intradermally with Pam2CSK4 (4 μg) on day 0. Ear thickness at 3 sites was measured daily by using a micrometer; these 3 measures were averaged and reported as the average ear thickness after treatment.

Flow cytometry

Ears were treated with enzyme mixture containing collagenase IV and DNase I (300 U/ml and 50 U/ml, respectively; Worthington Biochemical, Lakewood, NJ) for 1 h at 37 ^{o}C with shaking. Cells were treated with anti-CD16/32 mAb to avoid binding to FcγR on ice for 10 minutes prior to incubation with the indicated combination of Abs. Stained cells were acquired on BD LSR Fortessa (BD Biosciences) and analyzed using Flowjo software (Treestar, Inc., San Carlos, CA).

Histology

Ears were fixed in 10% formalin and were embedded in paraffin. Ear sections were stained with hematoxylin-eosin (HE) or with Masson's trichrome (MT). For analysis of epidermal and collagen-deposited dermal thickness, randomly selected regions in the specimen were measured by using a fluorescent microscope (BZ-X710; Keyence) and its associated software. Epidermal and collagen-deposited dermal thicknesses were expressed as mean ± s.e.m μm of ear skin.

Statistics

Statistical analyses were performed with the unpaired Student's *t*-test. *P* values <0.05 were considered statistically significant.

Results and discussion

To investigate the role of Allergin-1 in the TLR2-triggered production of inflammatory cytokines, we used the TLR2/TLR6-binding diacylated lipopeptide Pam2CSK4 to stimulate BMMCs derived from WT or Allergin-1-deficient mice. Quantitative RT-PCR analysis showed that the mRNA expression of *Il1b*, *Tnfsf2*, and *Il6* was significantly higher in Allergin-1-deficient BMMCs than in WT BMMCs at 2 h after stimulation with Pam2CSK4 (Fig 1A). Notably, *Il1b* expression in Allergin-1-deficient BMMCs was approximately 20 times that in WT BMMCs (Fig 1A). Consistent with these results, Allergin-1-deficient BMMCs produced more TNF-α and IL-6 than did WT BMMCs (Fig 1B).

Because Allergin-1 suppressed the Pam2CSK4-induced production of neutrophil chemoattractants, including IL-6, TNF-α, and IL-1β, which promote the immune defense against *S. aureus* infections (10,11), we next tested whether Allergin-1 inhibits TLR2 signaling-mediated dermatitis. Pam2CSK4 was injected intradermally into the left pinnae of WT or Allergin-1-deficient mice, and ear swelling was measured daily thereafter. Ear swelling in WT mice peaked at day 4 and subsequently declined; in contrast, swelling on day 1 was greater in Allergin-1-deficient mice than in WT mice and remained elevated throughout the study (Fig 2A). In addition, the $Ly6G^+$ neutrophil

population in ear tissue 1 day after Pam2CSK4 injection was greater in Allergin-1-deficient mice than in WT mice (Fig 2B). Histological analysis demonstrated that epidermal thickness was comparable between WT and Allergin-1-deficient mice at 8 days after the intradermal injection of Pam2CSK4 (Fig 2C). However, we observed that Allergin-1-deficient mice exhibited significantly greater amount of collagen deposition, as analyzed by staining with Masson's trichrome, than did WT mice (Fig 2D). Although TNF-α, IL-6 and IL-1β are reported to promote fibrosis (12), increased production of these cytokines in response to Pam2CSK4 injection might be involved in augmented ear swelling in Allergin-deficient mice. Further analyses are required to clarify whether this is also the case.

After we had confirmed that Allergin-1 is expressed on skin-resident myeloid cells, including FcεRI^{+}c-Kit^{+} MCs, CD11b^{+} cells, Langerin^{+} cells, and Ly6G^{+} neutrophils (Fig. 3A), we investigated whether Allergin-1 on MCs contributes to Pam2CSK4-induced ear swelling. For this we used Mas–TRECK mice, which carry a DT-inducible MC deletion system that incorporates *Il4* enhancer elements (8). Flow cytometry analysis confirmed the lack of MCs in ear tissue after the injection of DT in Mas–TRECK mice (Fig. 3B and C). Skin MCs in DT-treated Mas–TRECK mice were reconstituted through intradermal injection of either WT or Allergin-1-deficient

BMMCs (Fig. 3B). Pam2CSK4-induced ear swelling in DT-treated Mas–TRECK mice reconstituted with WT BMMCs was similar to that of their non-reconstituted counterparts (Fig. 3D). However, ear swelling was more severe in DT-treated mice reconstituted with Allergin-1-deficient BMMCs than in those reconstituted with WT BMMCs (Fig. 3D). These results indicate that Pam2CSK4-induced MC activation was specifically inhibited by Allergin-1 in skin.

To examine whether neutrophils were involved in the increased Pam2CSK4-induced ear swelling in Allergin-1-deficient mice, WT and Allergin-1-deicient mice were injected with anti-Gr-1 mAb to deplete neutrophils. Allergin-1-deficient mice still showed significantly more severe ear swelling than did WT mice after injection with Pam2CSK4 even after neutrophil depletion (Fig 3E), indicating that neutrophils were not involved in the increased Pam2CSK4-induced ear swelling in Allergin-1-deficient mice. Moreover, both DT-treated and DT-untreated Mas–TRECK mice showed significantly increased neutrophil recruitment after injection with Pam2CSK4 (Fig 3F), suggesting that Allergin-1-expressing cells such as $CD11b^{+}$ cells, rather than MCs, might be responsible for Pam2CSK4-induced neutrophil recruitment in ear tissue. Together, these results suggest that although Allergin-1 on MCs plays an important role in suppression of Pam2CSK4-induced ear swelling,

neutrophils are dispensable for the increased Pam2CSK4-induced ear swelling in Allergin-1-deficient mice.

The importance of ITIM domains in shaping TLR2-mediated immune responses is demonstrated by the finding that the ITIM-bearing receptors PIR-B and CEACAM-1 directly interact with the pathogens *S. aureus* and *Moraxella catarrhalis*, respectively (13,14). Although pathogens frequently utilize ITIM-containing receptors to evade immune detection, the ITIM-bearing receptors that downregulate TLR2 signaling to maintain host homeostasis are largely unknown. We found here that Allergin-1 on MCs specifically inhibited TLR2-ligand–induced dermatitis in mice.

Allergin-1 is also expressed at high level on myeloid cells, including $CD11b^+$ cells and neutrophils (Fig 3A). $CD11b^+$ cells consist of dendritic cell (DC) and monocytes/macrophages lineages that strongly express TLR2 (15). The recruitment of neutrophil in response to Pam2CSK4 might be induced by $CD11b^+$ cells in DT-treated Mas–TRECK mice. Therefore, Allergin-1 may also inhibit TLR2 signaling in $CD11b^+$ cells as well, and Allergin-1 on MC and $CD11b^+$ cells may cooperate to promote TLR2-mediated inflammation in vivo.

The ligand for Allergin-1 remains undetermined. However, since a fusion protein consisting of extracellular domain of mouse Allergin-1 and Fc portion of human IgG1

bound to BMMC (unpublished observation), it is possible that the Allergin-1 ligand might be expressed on MC and cis- or trans-interaction of Allergin-1 to the ligand on MC may transmit inhibitory signal via Allergin-1 in MC.

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Author contributions

S. T. performed most of the experiments and analyzed the data; S.M and S. Shibagaki, did experiments; M. I., S. Shibayama and M. K. contributed to experimental design; S. T. –H. designed and did experiments, analyzed data and wrote the paper; and A. S. supervised the overall project and wrote the paper.

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Figure Legends

Figure 1. Allergin-1 inhibits Toll-like receptor (TLR) 2 signaling in bone-marrow–derived cultured mast cells (BMMCs) from mice. (A) Quantitative reverse transcription–polymerase chain reaction (RT-PCR) analyses of proinflammatory cytokine expression by BMMCs at 2 h after stimulation with Pam2CSK4 (1 μg/ml). (B) Cytometric bead array analyses of proinflammatory cytokine production by BMMCs at 24 h after stimulation with Pam2CSK4 (10 μg/ml). WT, C57BL/6N mice; KO, Allergin-1-deficient mice; GADPH, glyceraldehyde 3-phophate dehydrogenase (used as an internal control for normalization). *, $P < 0.05$; **, $P < 0.01$; ***, $P<0.005$.

Figure 2. Loss of Allergin-1 exacerbates TLR2-ligand–induced dermatitis.

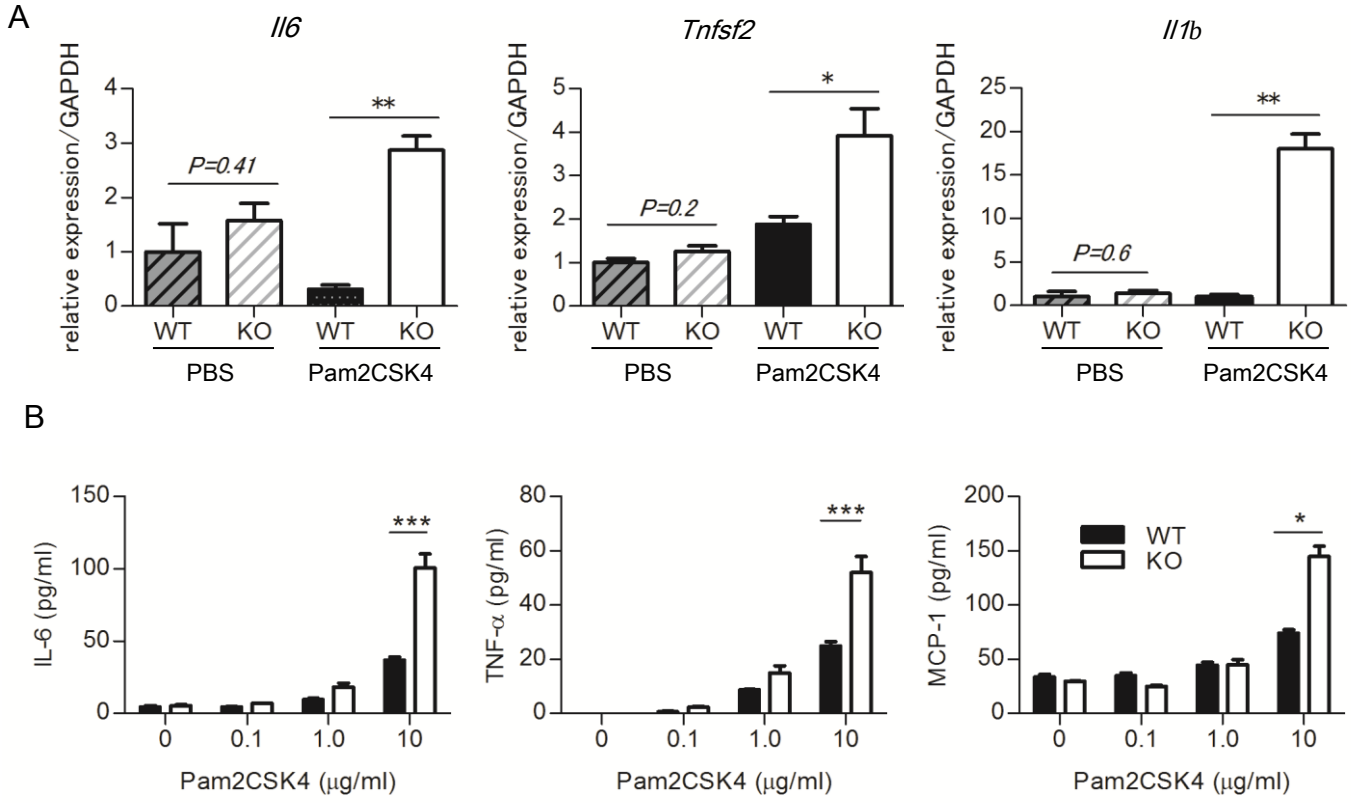
(A) The left pinnae of C57BL/6N wild-type (WT, n=15) and Allergin-1 knockout (KO, n=14) mice were immunized intradermally with Pam2CSK4 (4 μg), after which ear swelling was measured. Data are reported as the means ± SEM of 4 independent experiments. (B) Frequency of $CD45.2^+Ly6G^+CD11b^+$neutrophils in ear skin at 1 day after id injection of Pam2CSK4. Hematoxylin-Eosin (HE) staining (C) and Masson's trichrome staining (MT) (D) of the ear skin at 8 days after id injection of Pam2CSK4. Epidermal thickness (C) and collagen deposition (D) was evaluated as mean + s.e.m.

per mm of ear pinna. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; ****, $P<0.001$.

Figure 3. Reconstitution of skin MCs in diphtheria toxin (DT)-treated Mas–TRECK mice. (A) FACS analysis demonstrated Allergin-1 expression on skin-resident MCs ($CD45.2^+PI^-Fc\varepsilon RI^+c\text{-}Kit^+$), eosinophils ($CD45.2^+PI^-Siglec\text{-}F^+$), neutrophils ($CD45.2^+PI^-CD11b^+Ly6G^+$), $CD11b^+$ cells ($CD45.2^+PI^-CD11b^+Ly6G^-$), and $Langerin^+$ cells ($CD45.2^+PI^-CD11b^+I\text{-}Ab^+Langerin^+$). Black lines indicate WT C57BL/6N mice; gray lines represent Allergin-1 KO mice. (B) The experimental design for reconstitution of skin MCs in DT-treated Mas–TRECK mice. (C) FACS detection of reconstituted MCs in skin at 5 weeks after intradermal injection of BMMCs in DT-treated Mas–TRECK mice. (D) Left ear pinnae of WT or Allergin-1-deficient BMMC-reconstituted DT-treated Mas–TRECK mice were injected with Pam2CSK4, after which ear swelling was measured. (E) Neutrophil-depleted mice were generated by i.p. injection of anti-Gr-1 mAb on days -2, -1, +1, +3, and +5 and were injected intradermally with Pam2CSK4 on day 0, after which ear swelling was measured. (F) DT-treated or -untreated Mas–TRECK mice were i.d. injected with Pam2CSK4. Frequency of $CD45.2^+Ly6G^+CD11b^+$ neutrophils in ear skin at 1 day after injection was determined by flow cytometry analysis. Data are reported as the means ± SEM of 2

independent experiments. *, $P < 0.05$; **, $P < 0.01$; ****, $P<0.001$.

Fig 1


A
Il6
Tnfsf2
Il1b
relative expression/GAPDH
P=0.41
P=0.2
P=0.6
**
*
**
WT
KO
PBS
Pam2CSK4
B
IL-6 (pg/ml)
TNF-α (pg/ml)
MCP-1 (pg/ml)
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*
Pam2CSK4 (μg/ml)
0
0.1
1.0
10

Fig 2

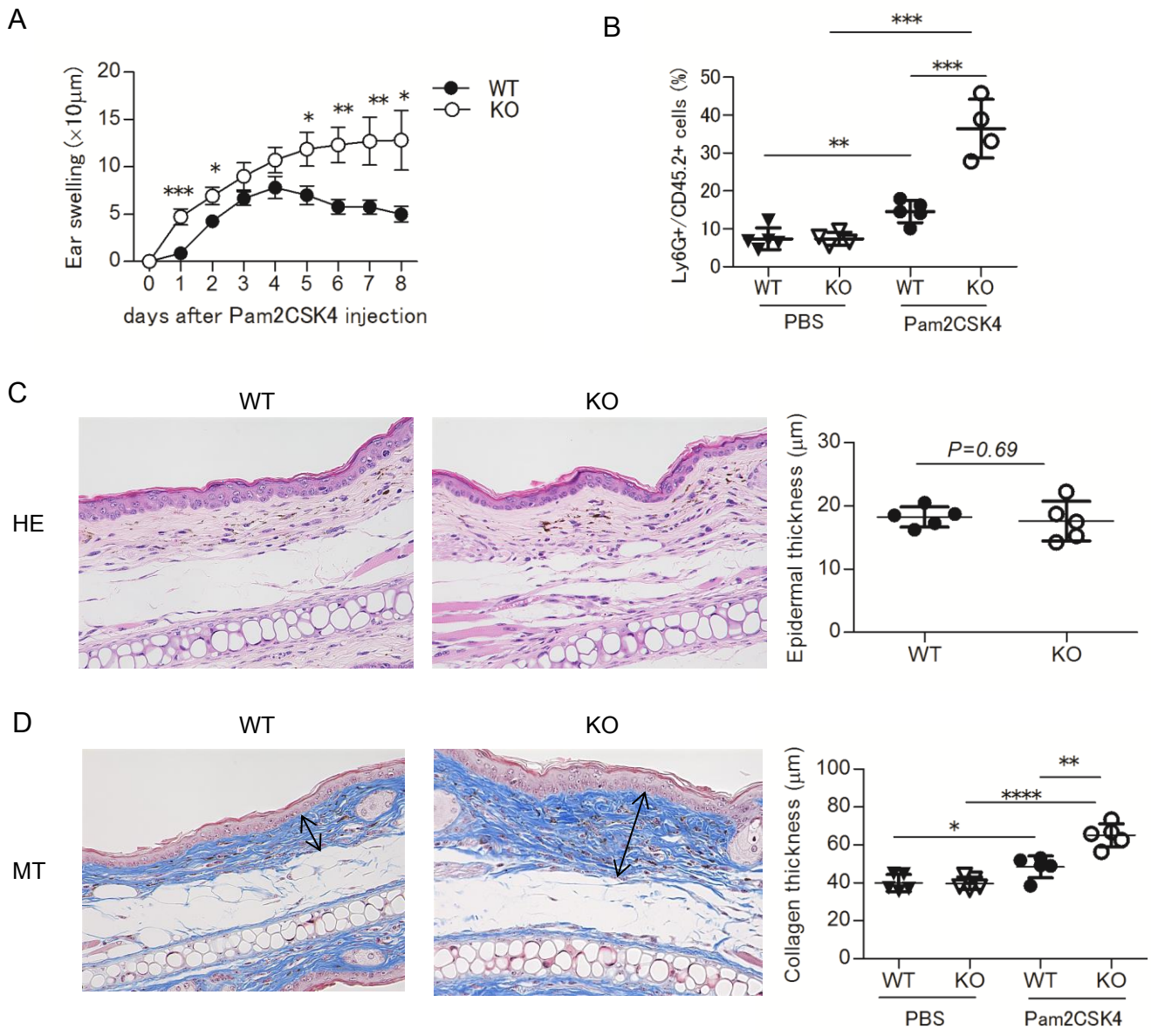
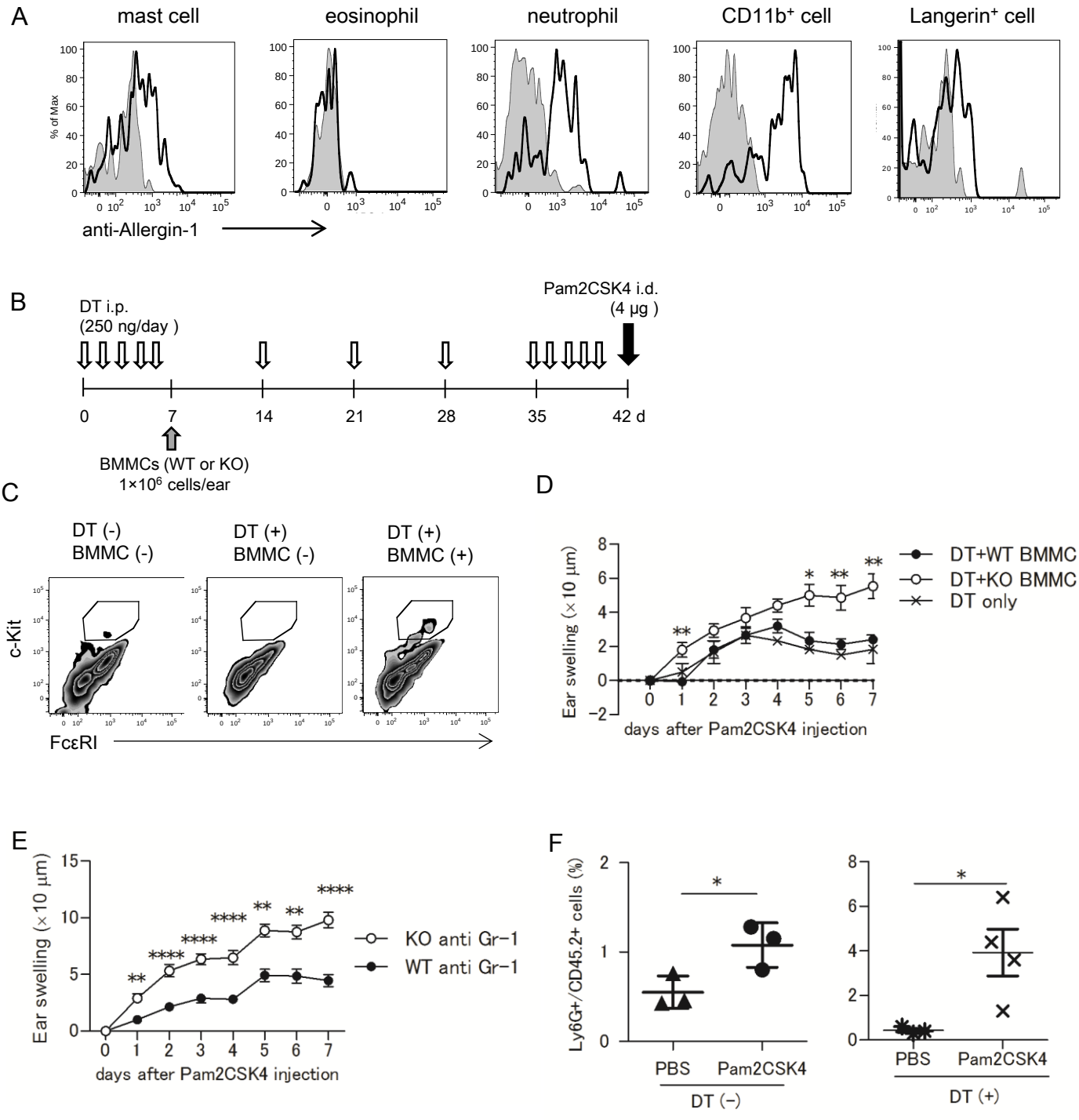

A
Ear swelling (×10μm)
days after Pam2CSK4 injection
WT
KO
B
Ly6G+/CD45.2+ cells (%)
WT
KO
WT
KO
PBS
Pam2CSK4
C
WT
KO
HE
Epidermal thickness (μm)
P=0.69
WT
KO
D
WT
KO
MT
Collagen thickness (μm)
WT
KO
WT
KO
PBS
Pam2CSK4

Fig. 3


A
mast cell
eosinophil
neutrophil
CD11b+ cell
Langerin+ cell
% of Max
anti-Allergin-1
B
DT i.p.
(250 ng/day)
Pam2CSK4 i.d.
(4 μg)
0
7
14
21
28
35
42 d
BMMCs (WT or KO)
1×10^6 cells/ear
C
DT (-)
BMMC (-)
DT (+)
BMMC (-)
DT (+)
BMMC (+)
c-Kit
FcεRI
D
Ear swelling (×10 μm)
days after Pam2CSK4 injection
DT+WT BMMC
DT+KO BMMC
DT only
E
Ear swelling (×10 μm)
days after Pam2CSK4 injection
KO anti Gr-1
WT anti Gr-1
F
Ly6G+/CD45.2+ cells (%)
PBS
Pam2CSK4
DT (-)
DT (+)