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Supplementary Materials for

The regulatory B cell–mediated peripheral tolerance maintained by mast cell IL-5 suppresses oxazolone-induced contact hypersensitivity

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This PDF file includes:

SUPPLEMENTAL MATERIALS

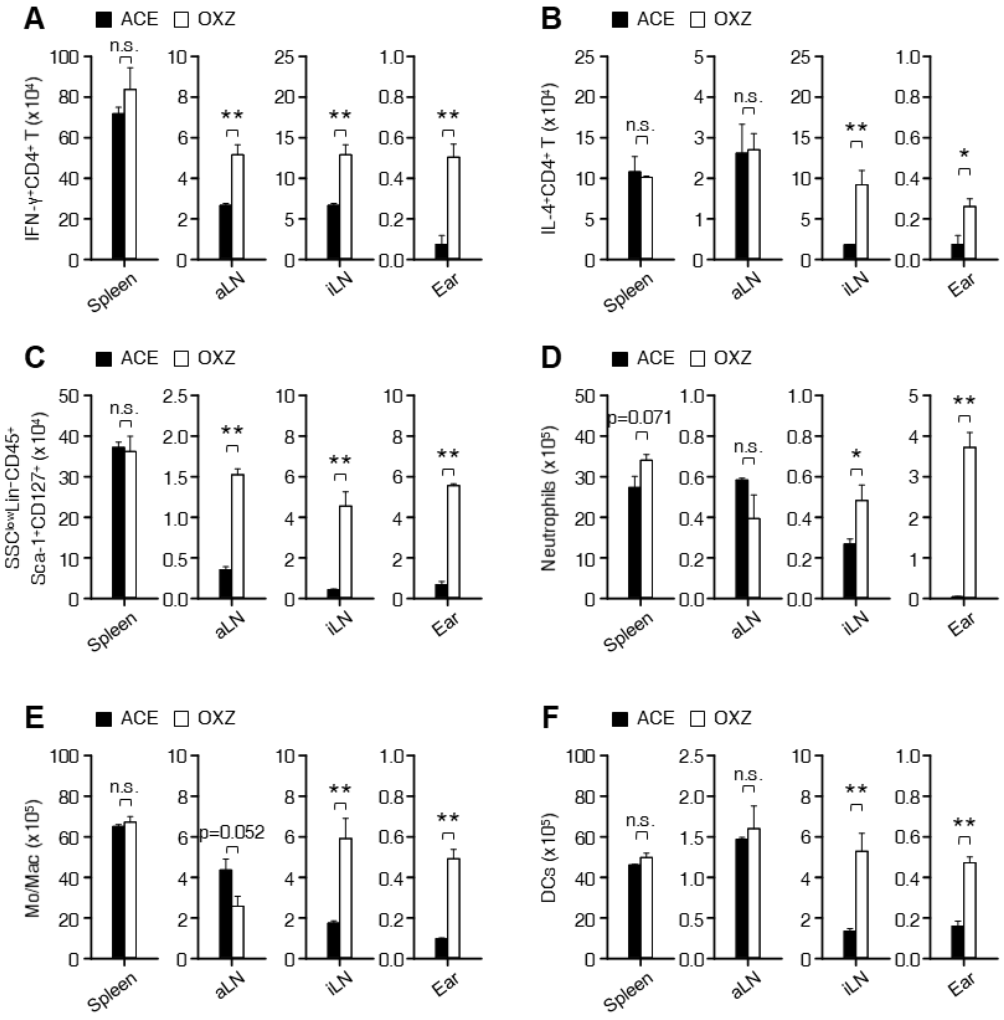

Fig. S1. Leukocyte population changes in mice with OXZ-induced CHS.

Analysis of primary leukocytes in spleen, aLN, iLN, and ear tissue from mice with or without OXZ treatment for 2 days. Histograms show the numbers of IFN-γ$^+$CD4$^+$ T cells (**A**), IL-4$^+$CD4$^+$ T cells (**B**), total innate lymphoid cells (ILCs, Lin$^-$ CD45$^+$CD127$^+$Sca-1$^+$) (**C**), neutrophils (CD11b$^+$Gr-1$^+$) (**D**), monocytes/macrophages (Mo/Mac, CD11b$^+$F4/80$^\pm$) (**E**), and dendritic cells (DCs, Gr-1$^-$CD11c$^+$) (**F**). All data are expressed as the mean ± SEM from two independent experiments (n = 4 for each experiment). *p < 0.05; **p < 0.01; n.s., not significant versus ACE-treated group by Student's t-test.

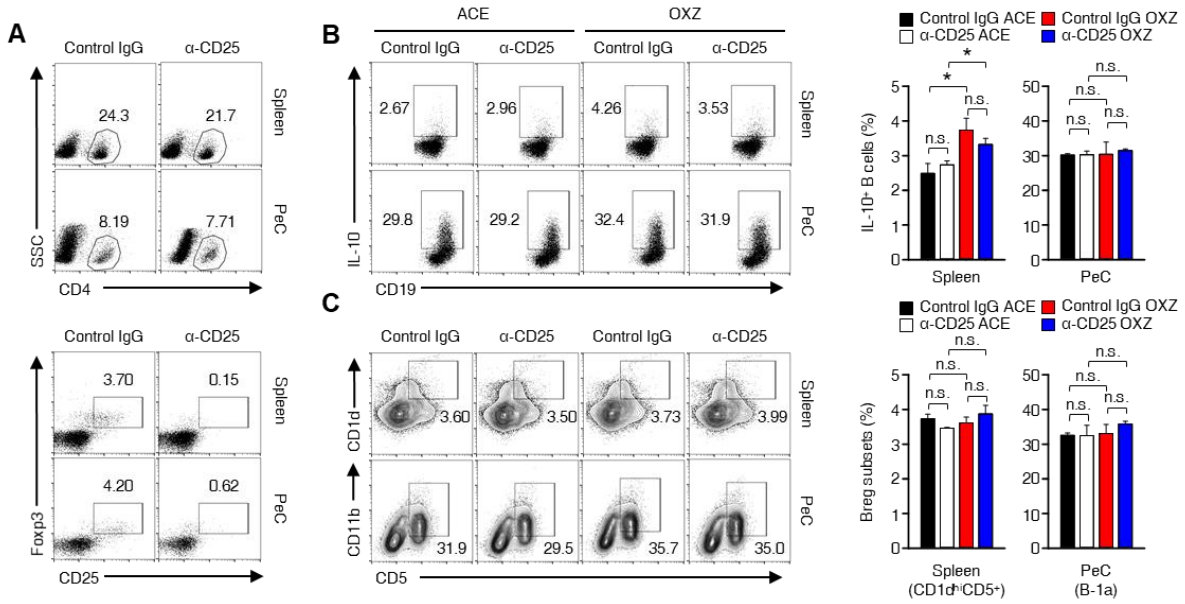

Fig. S2. T_{reg} cells are not essential for the development of IL-10$^+$ B_{reg} cells in peripheral tissues. (**A** to **C**) To deplete T_{reg} cells, the mice were also injected i.p. with 250 μg of an anti-CD25 mAb (PC61, Bio X Cell, West Lebanon, NH) twice on day -5 (sensitization) and day -1 (before challenge). The depletion of the cells was confirmed by flow cytometric analysis. (**A**) Representative flow cytometry images of CD4$^+$ T cells (upper) and Foxp3$^+$CD25$^+$CD4$^+$ T_{reg} cells (lower) in the spleen and peritoneal cavity (PeC) are shown. Representative flow cytometry images (left) and frequencies (right) of IL-10$^+$ B cells (**B**) and CD1d^{hi}CD5$^+$CD19$^+$ B cells (e.g., CD5$^+$CD11b$^+$CD19$^+$ cells in the PeC) (**C**) are shown. All data are expressed as the mean ± SEM from two independent experiments (n = 3 per group for each experiment). *p < 0.05; n.s., not significant by one-way ANOVA with post hoc Tukey's test.

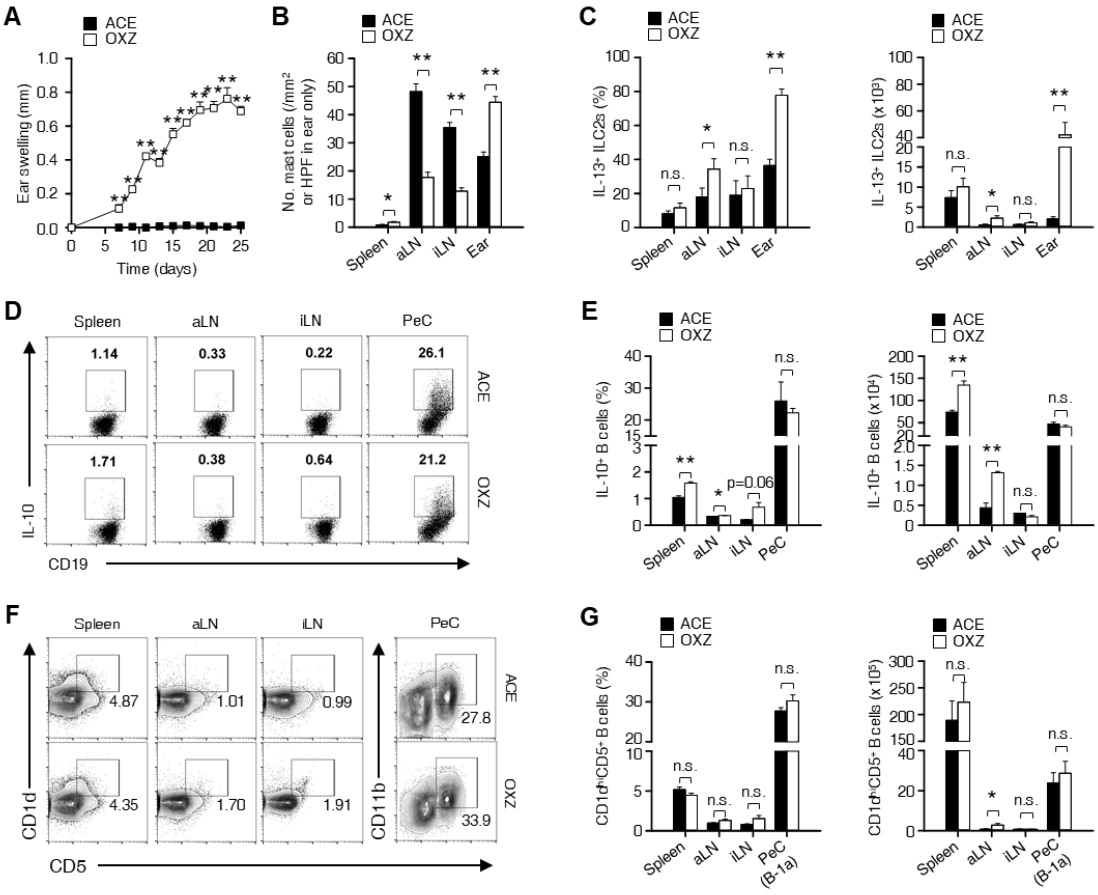

Fig. S3. The population changes in MCs, IL-13⁺ ILC2s, and IL-10⁺ B_{reg} cells during OXZ-induced chronic atopic dermatitis (AD)–like skin inflammation. AD-like skin inflammation was induced by OXZ as previously reported (*15*). (**A**) The ear thickness of AD mice was measured for 25 days after sensitization with OXZ. (**B**) On day 25 after CHS induction by OXZ, the number of MCs by histology with toluidine blue. (**C** to **G**) Cells were analyzed by flow cytometer. (**C**) The frequency and number of IL-13$^+$Lin$^-$CD45$^+$CD127$^+$Sca-1$^+$ (IL-13$^+$ ILC2s) cells are shown. Representative images (D and F) and histograms (E and G) for IL-10$^+$ B cells, or CD1d^{hi}CD5$^+$CD19$^+$ cells (or CD5$^+$CD11b$^+$CD19$^+$ cells in the PeC) were presented as indicated. Data are expressed as the mean ± SEM (**A** to **C**, **E**, and **G**) or representative images (**D** and **F**) from three independent experiments (n ≥ 3 per group for each experiment). *p < 0.05; **p < 0.01; n.s., not significant by one-way ANOVA with post hoc Tukey's test.

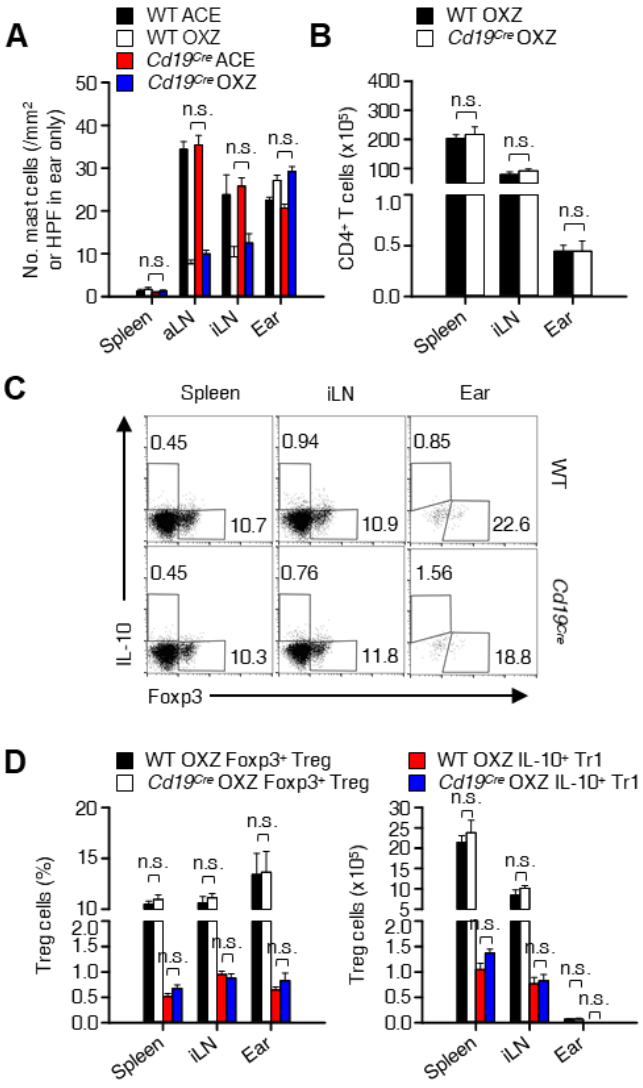

Fig. S4. Comparison of MCs and T_{reg} cells in WT and *Cd19*^{Cre} mice with CHS. (**A**) The histograms show the number of MCs from histology images of ear tissues in WT or *Cd19*^{Cre} mice. Results are shown as the mean ± SEM from three independent experiments (n ≥ 3 per group for each experiment). (**B**) Number of $CD4^+$ T cells in spleen, iLN, and ear tissue from WT or *Cd19*^{Cre} mice. Representative flow cytometry images (**C**) and the histograms for panel C (**D**) show the frequencies and numbers of IL-10^+ or $Foxp3^+CD4^+$ T_{reg} cells. (**A, B,** and **D**) Data are expressed as the mean ± SEM from two independent experiments (n = 3 per group for each experiment). n.s., not significant by Student's *t*-test.

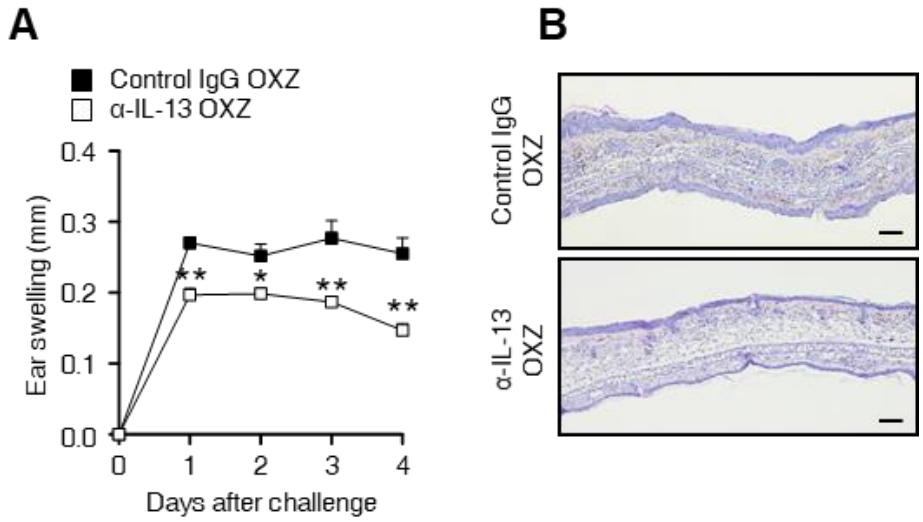

Fig. S5. The treatment of IL-13 mAb suppresses OXZ-induced CHS in mice. (**A**) The mice were injected i.v. with 200 μg of an anti- IL-13 mAb (8H8, Invivogen, San Diego, CA) or control IgG twice on day -1 (pre-challenge) and day 0 (1 hr before challenge). Ear thickness is measured for 4 days after challenge with OXZ. All values are shown as the mean ± SEM. (**B**) Four days after OXZ challenge, representative images of ear tissues stained with H&E are shown (scale bar, 100 μm). (**A** and **B**) All data were obtained from two independent experiments (n = 3 per group for each experiment). *$p < 0.05$; **$p < 0.01$ versus Control IgG by Student's *t*-test.

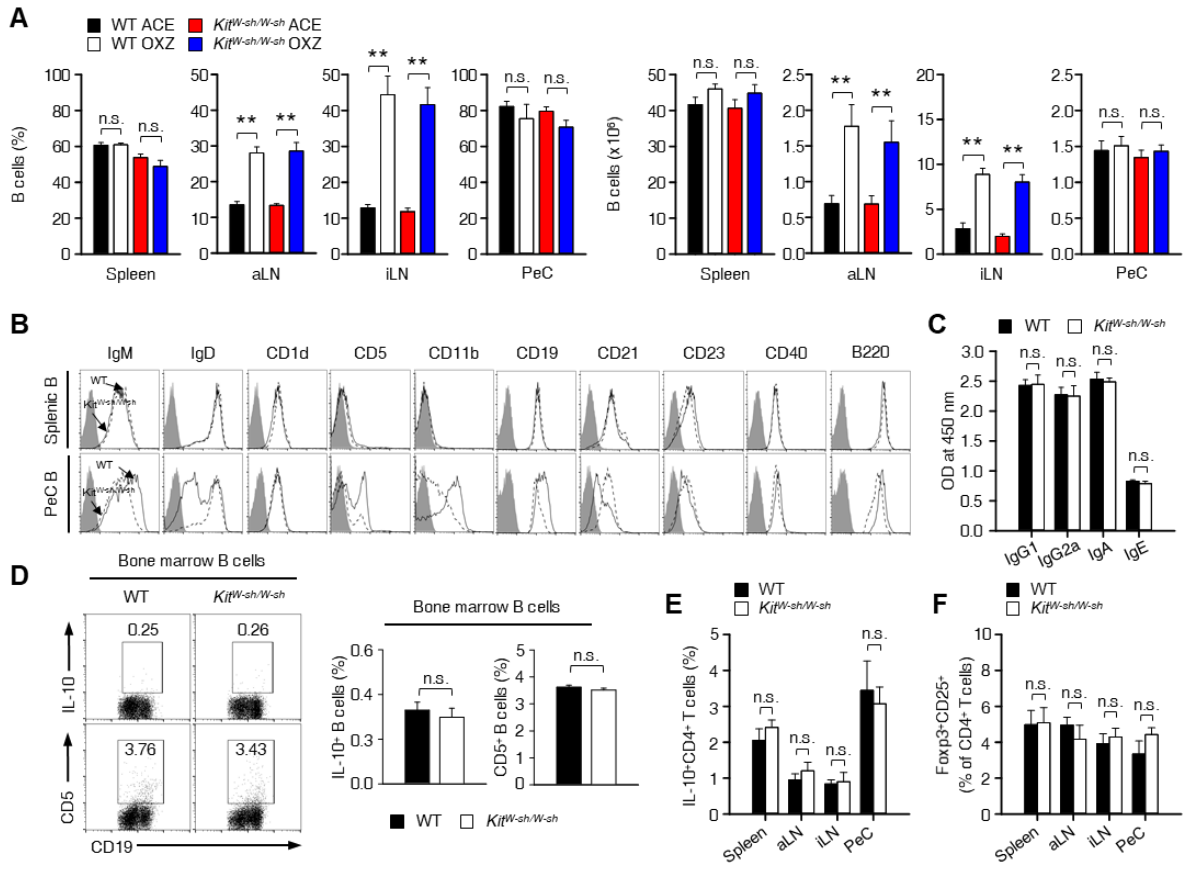

Fig. S6. Analysis of B cells, T_{reg} cells, and serum antibody isotypes from WT or *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. (**A**) Frequencies (left) and numbers (right) of CD19$^+$ B cells in the spleen, aLN, iLN, and PeC with or without CHS were analyzed by flow cytometry. (**B**) Graphs for flow cytometric analysis of cell surface markers of splenic or PeC B cells are shown. (**C**) Serum concentrations of antibody isotypes. (**D**) Flow cytometry images (left) and frequencies (right) of IL-10$^+$ or CD5$^+$ bone marrow B cell subsets. (**E**) Frequency of IL-10$^+$CD4$^+$ T cells and (**E**) Foxp3$^+$CD25$^+$ (in CD4$^+$ T cells) are shown. The results are expressed as the mean ± SEM (**A**, **C**, **D** to **F**) or representative images (**B** and **D**) from two independent experiments (n = 3 per group for each experiment). **p < 0.01; n.s., not significant versus WT by Student's t-test.

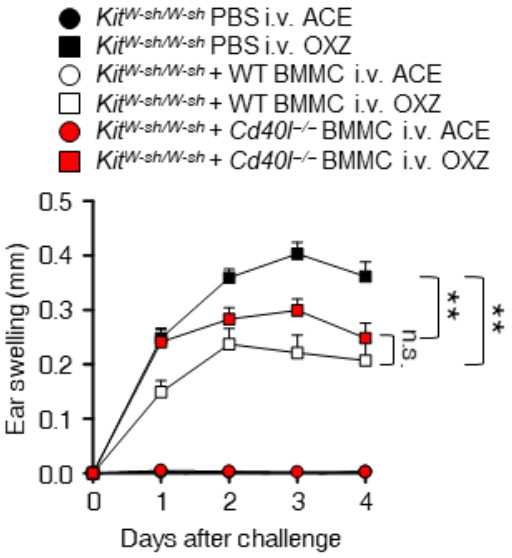

Fig. S7. CD40L on MCs is not critical for the suppression of CHS. Ear thickness of WT or *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice with CHS induced by OXZ with or without the transfer of WT or *Cd40l*$^{-/-}$ BMMCs are measured. The data are expressed as the mean ± SEM from two independent experiments (n ≥ 4 per group for each experiment). ***p* < 0.01; n.s., not significant by one-way ANOVA with post hoc Tukey's test.

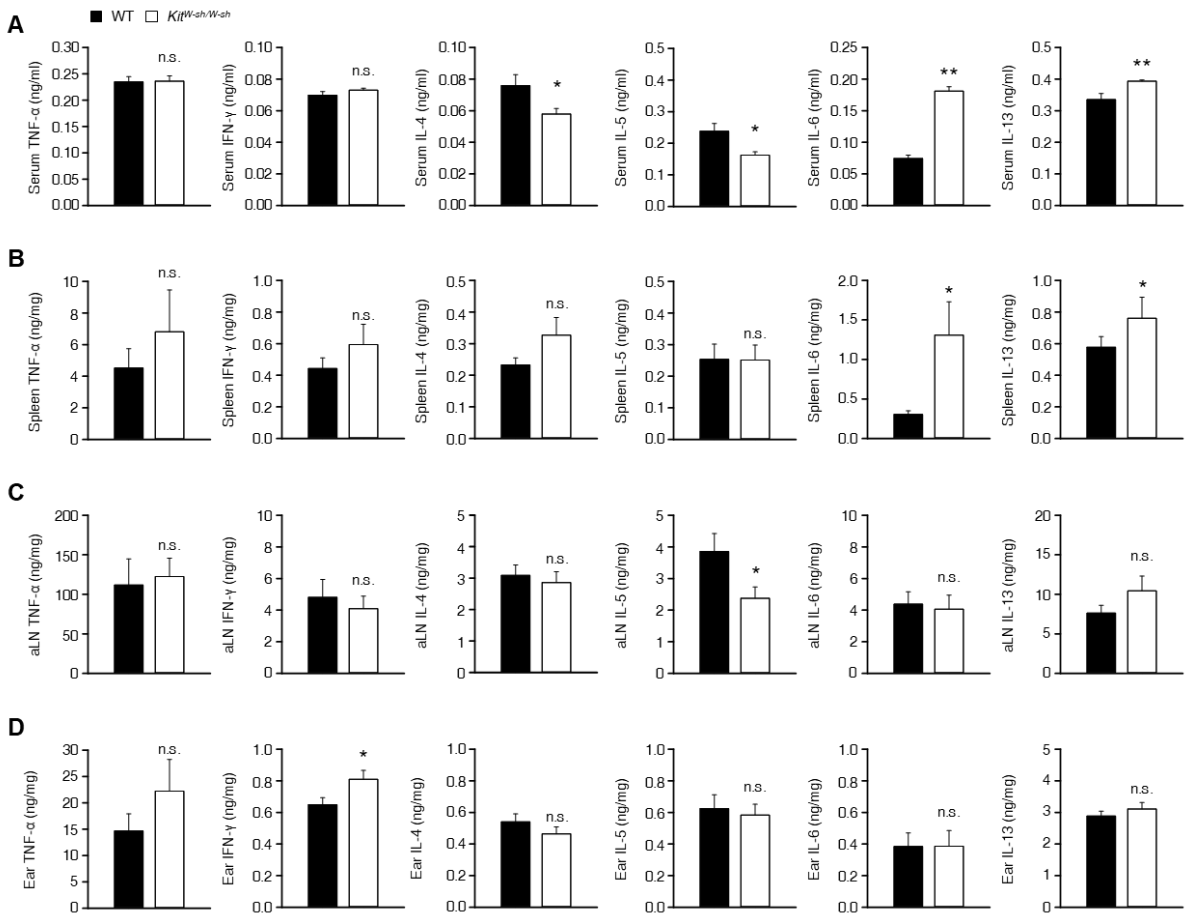

Fig. S8. Amounts of T_H1 and T_H2 cytokines in peripheral tissues from WT or *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. (**A** to **D**) The concentrations of T_H1 and T_H2 cytokines in the serum (**A**), spleen (**B**), aLN (**C**), and ear tissues (**D**) from WT or *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice are measured by ELISA according to the manufacturers' instructions. All ELISA data are expressed as the mean ± SEM from at least two independent experiments (n ≥ 5 per group for each experiment). **p* < 0.05; ***p* < 0.01; n.s., not significant versus WT by Student's *t*-test.

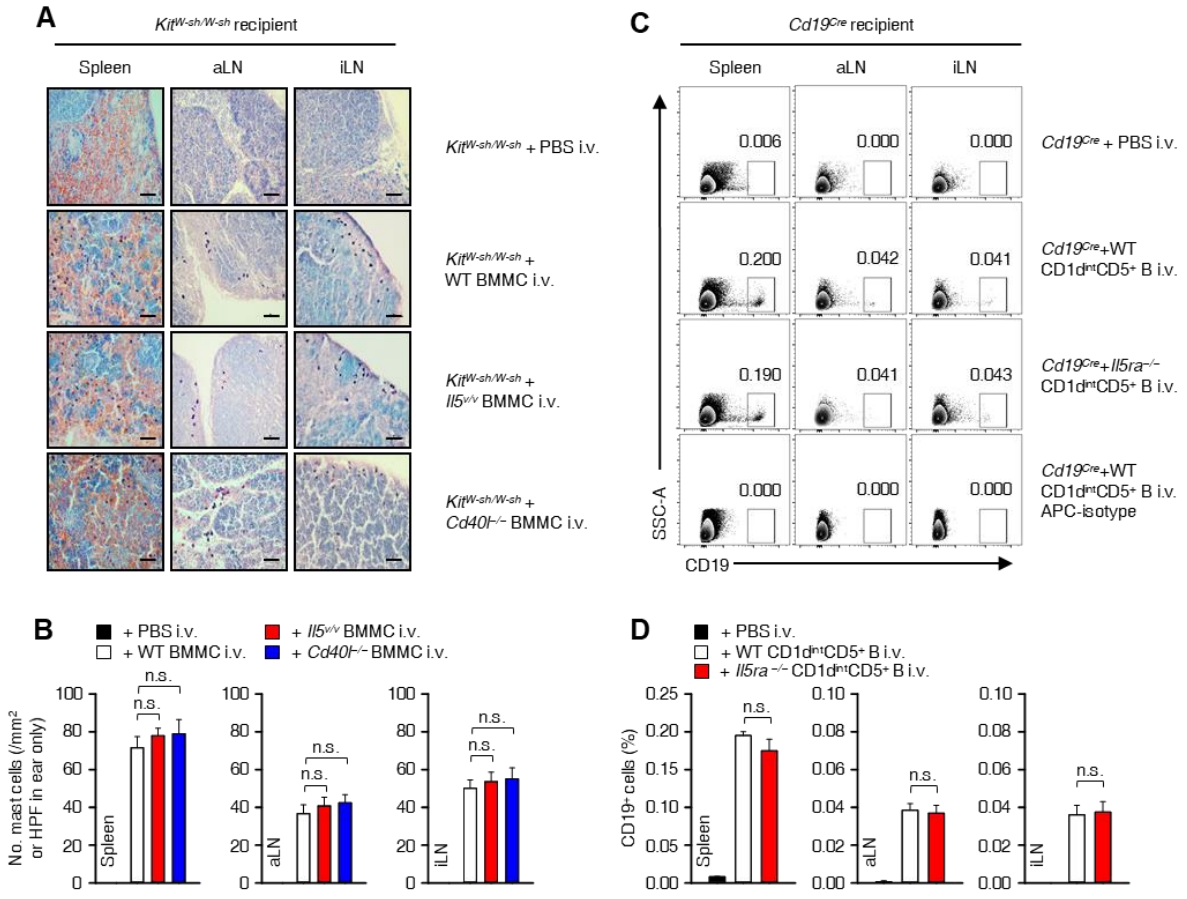

Fig. S9. The tissue distribution of MCs in Kit$^{W\text{-}sh/W\text{-}sh}$ mice and B$_{reg}$ cells in *Cd19^{Cre}* mice after the adoptive transfer of each cell. (**A** and **B**) WT, *Il5$^{v/v}$*, or *Cd40l$^{-/-}$* BMMCs (1 × 10^7 cells/mouse) were i.v. injected into recipient *Kit$^{W\text{-}sh/W\text{-}sh}$* mice for 8 weeks. Representative images for MCs in spleen, aLN, and iLN (scale bar, 50 μm) with toluidine blue are shown. (**B**) The histograms show the number of MCs for panel A. n.s., not significant versus WT BMMC i.v. transferred mice by Student's *t*-test. (**C** and **D**) For the adoptive transfer, each CD1d^{int}CD5$^+$ B cell subset (1.5 × 10^6 cells/0.2 ml of PBS) was transferred i.v. into *Cd19^{Cre}* mice. 4 days after injection, each Breg subset was analyzed by flow cytometry. (**C**) Representative images for spleen, aLN, and iLN are shown. (**D**) The histograms show the frequencies of CD19$^+$ adoptive transferred Breg cells for panel C. The results are expressed as the mean ± SEM (**A** and **C**) or representative images (**B** and **D**) from two independent experiments (n = 3 per group for each experiment). n.s., not significant by Student's *t*-test.

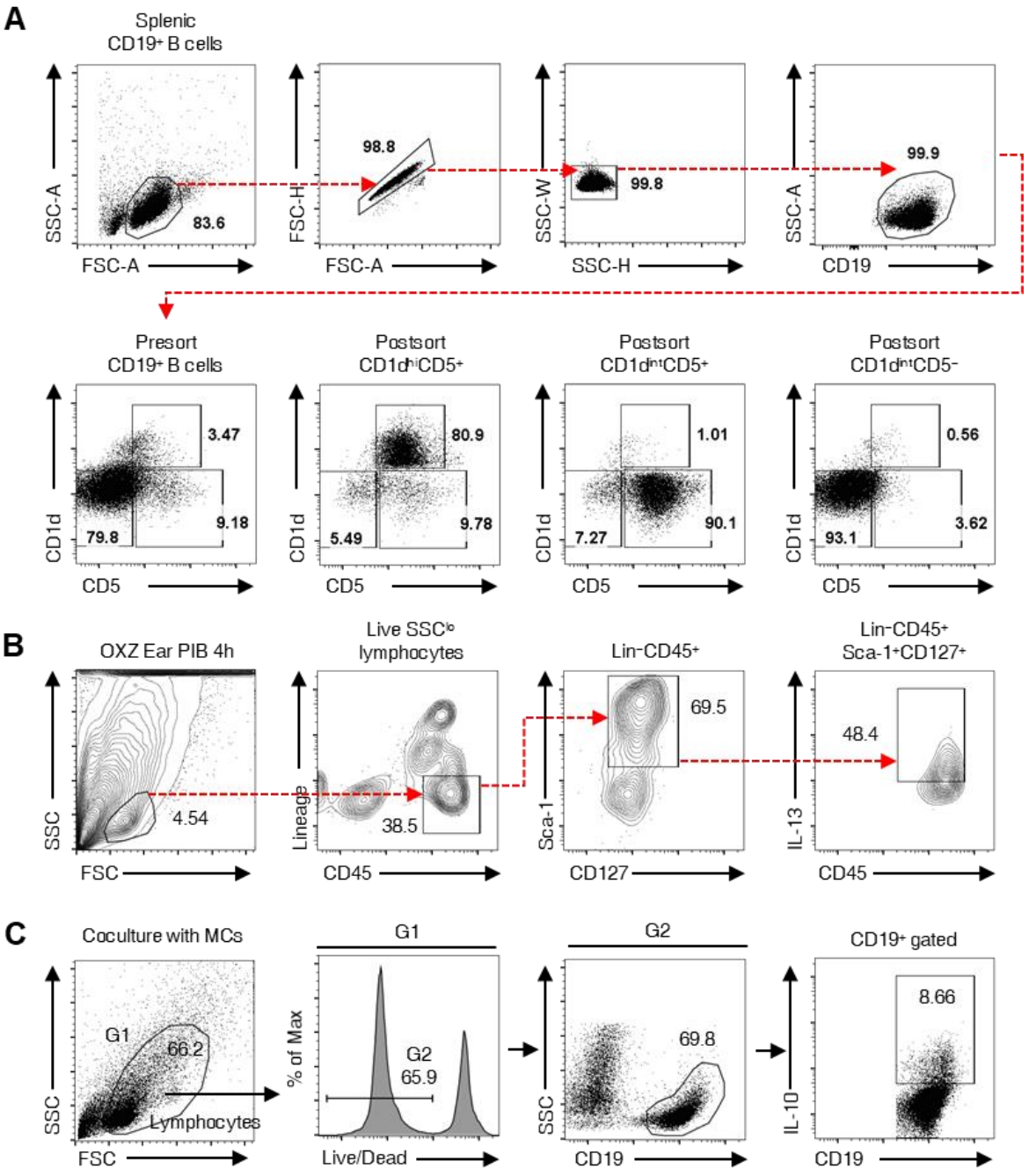
A
Splenic
CD19+ B cells
SSC-A
FSC-A
83.6
FSC-H
98.8
SSC-W
99.8
SSC-H
99.9
CD19
Presort
CD19+ B cells
Postsort
CD1dhiCD5+
Postsort
CD1dintCD5+
Postsort
CD1dintCD5−
CD1d
CD5
3.47
9.18
79.8
80.9
9.78
5.49
1.01
90.1
7.27
0.56
3.62
93.1
B
OXZ Ear PIB 4h
SSC
FSC
4.54
Live SSClo
lymphocytes
Lineage
CD45
38.5
Lin−CD45+
Sca-1
CD127
69.5
Lin−CD45+
Sca-1+CD127+
IL-13
48.4
C
Coculture with MCs
G1
66.2
Lymphocytes
% of Max
G2
65.9
Live/Dead
G2
69.8
CD19+ gated
IL-10
8.66

Fig. S10. The sorting strategies for B cell subsets, IL-13$^+$ ILC2s, and IL-10$^+$ B cells. (**A**) Splenic CD1d^{hi}CD5$^+$, CD1d^{int}CD5$^+$, or CD1d^{int}CD5$^-$ B cell subsets were isolated from mice by sorting with a FACSAria flow cytometer for adoptive transfer. Representative flow cytometry images and purities of the sorted cells are shown. (**B**) Single cells isolated from ear tissue were stimulated with phorbol 12-myristate 13-acetate (50 ng/ml), ionomycin (500 ng/ml), and brefeldin A (3 μg/ml) for 4 hr. Lymphocytes were gated by SSC-A and FSC-A and then by FSC-A and FSC-H. Lin$^-$ CD45$^+$ lymphocytes were further gated by CD127 and Sca-1. Analysis of IL-13$^+$ ILCs was performed with an anti-IL-13 antibody. Representative flow cytometry images are shown. (**C**) Splenic CD19$^+$ B cells (1.5×10^6 cells/well) were cultured with or without an equal number of BMMCs for 43 hr and with LPS (10 μg/ml), phorbol 12-myristate 13-acetate (50 ng/ml), ionomycin (500 ng/ml), and monensin (2 μM) for 5 hr, and the IL-10$^+$ B cells were analyzed by flow cytometry. After gating for lymphocytes (G1), dead cells were excluded with the LIVE/DEAD Dye, and live B cells were detected with an anti-CD19 monoclonal antibody (G2). Analysis of IL-10$^+$ B cells was performed with an anti-IL-10 antibody. Representative images are shown.