SUPPLEMENTAL MATERIALS AND METHODS

Antibodies and Other Reagents

Abs used were as follows: rat anti-LMIR3 (3-14-11) was obtained from ACTGen Inc. (Nagano-ken, Japan),[1] anti-Flag (M2), and mouse IgG1 (MOPC21) were from Sigma-Aldrich, fluorescein isothiocyanate-conjugated anti-CD11b (M1/70), Gr-1 (RB6-8C5), F4/80 (BM8), CD11c (N418), or FcεRIα (MAR-1), phycoerythrin-conjugated anti-Gr-1 (RB6-8C5), c-kit (2B8), or streptavidin, allophycocyanin-conjugated anti-c-kit (2B8) or streptavidin, phycoerythrin-Cy5-conjugated anti-CD11b (M1/70), or biotin-conjugated anti-c-kit (2B8) were from eBioscience (San Diego, CA), PE- or Alexa Fluor 647-conjugated anti-Siglec-F (E50-2440) was from BD Biosciences (San Jose, CA), PE-conjugated anti-CD63 (NVG-2) or peridinin chlorophyll protein-conjugated anti-CD45 (30-F11) and mouse IgM (MOPC-104E) were from BioLegend (San Diego, CA), anti-CX3CR1 was from R&D Systems (Minneapolis, MN), anti-Mast Cell Trptase (FL-275) were from Santa Cruz Biotechnology (Dallas, TX), anti-ceramide (MID 15B4) was from Enzo Life Sciences (Farmingdale, NY), anti-P2X7 (Hano43) was from AbD Serotec (Raleigh, NC). Anti-LMIR3 and P2X7 Abs were biotinylated according to standard procedures. All cytokines were obtained from R&D Systems. C-24 ceramide was from Toronto Research Chemicals, Inc. (Ontario, Canada); 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine and 1,2-Dipalmitoyl-sn-glycero-3-phosphoserine were from Echelon Biosciences Inc (Salt Lake City, UT). All other reagents were from Sigma-Aldrich unless stated otherwise.

Generation of Liposomes and Fc Fusion Proteins

After 1 mg of dry lipid (C-24 ceramide, PC, or PS) was hydrated with 1 mL of phosphate-buffered saline, liposomes were generated by using an Avanti Mini-Extruder (Avanti Polar Lipids, Inc., Alabaster, AL) according to the manufacturer's instructions, as previously described.[1] The endotoxin levels of the Fc fusion proteins used in these experiments were less than 0.01 ng/μg protein as determined by the limulus amebocyte lysate test (Lonza, Walkersville, MD).[1,2]

Cell Stimulation

Lipids (ceramide, PS, or PC) were diluted to a concentration of 20 μg/mL in methanol. MaxiSorp 96-well plates (catalog no. 430341, Nalge Nunc International Corporation, Rochester, NY) were coated with 50 μL of each solution, air-dried, and washed twice with medium, as described.[1] BMMCs or BMMC transfectants were cultured in the presence of 3 mM ATP (Sigma-Aldrich) for 1 h (to measure β-hexosaminidase release), for 2 h (to measure LTB4 levels), or for 12 h (to measure cytokine/chemokine levels) on lipid or vehicle-coated plates.

Measurement of Cytokines, Chemokines, and LTB4, and Degranulation Assay

ELISA kits for IL-6, IL-17A, TNFα, CXCL1, CXCL2, and LTB4 (R&D Systems) were used. The release of β-hexosaminidase was estimated as described.[1]

Real-time RT-PCR

Real-time RT-PCR was performed as described previously.[2] The reaction conditions were 95℃ for 30 seconds followed by 40 cycles of PCR at 95℃ for 5 seconds, 55℃ for 10 seconds, and 72℃ for 10 seconds. The following primer pairs were used: 5'-GAGGATACCACTCCCAACAGACC-3' (forward) and 5'-AAGTGCATCATCGTTGTTCATACA-3' (reverse) for mouse *Il6*, 5'-ATCAGGACGCGCAAACATGA-3' (forward) and 5'-TTGGACACGCTGAGCTTTGA-3' (reverse) for mouse *I117a*, 5'-TGCCTATGTCTCAGCCTCTTC-3' (forward) and 5'-GAGGCCATTTGGGAACTTCT-3' (reverse) for mouse *Tnfa*, 5'-TGCACCCAAACCGAAGTCAT-3' (forward) and 5'-TTGTCAGAAGCCAGCGTTCA-3' (reverse) for mouse *Cxcl1*, 5'-AGTGAACTGCGCTGTCAATGC-3' (forward) and 5'-AGGCAAACTTTTTGACCGCC-3' (reverse) for mouse *Cxcl2*, 5'-TGCCCCCATGTTTGTGATG-3' (forward) and 5'-TGTGGTCATGAGCCCTTCC-3' (reverse) for mouse *Gapdh*. Product quality was monitored by melting curve analysis via LightCycler software (Roche Diagnostics, Basel, Switzerland). Expression levels were normalized to that of GAPDH.

Flow Cytometry

The percentage of mast cells positive for CD63 (a marker of mast cell degranulation) within the gated mast cells was measured. Flow cytometric analysis was performed with

FACSCalibur (BD Biosciences) equipped with CellQuest software, as previously described.[1,3]

Retrovirus Vectors, Transfection, and Infection

pMXs-IRES-puro^{r} (pMXs-IP), pMXs-Flag-LMIR3 or LMIR3(Y241/289/325F)-IP was previously described.[1] Retroviral transfection and infection were performed as described.[4,5] Cells were infected with retroviruses generated by transient transfection of PLAT-E packaging cells.[5]

BM Transplantation

BM transfer was used to create chimera mice as described.[1] Briefly, one day after lethal γ-irradiation, four-week-old female WT mice were intravenously injected with 2 x 10^6 of WT or *LMIR3*$^{-/-}$ BM cells 8 weeks before experiments.

BMMC reconstitution

Mast cell reconstitution was performed as previously described with minor modifications.[1] In brief, four-week-old female *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice were injected intravenously twice at an interval of two weeks with either 1 x 10^6 WT or *LMIR3*$^{-/-}$ BMMCs 8 weeks before the experiments. Reconstitution of colonic mast cells was confirmed by flow cytometery.

Immunohistochemistry

Immunofluorescence staining was performed as previously described.[1] Briefly, frozen colonic sections were fixed with 4% paraformaldehyde, permeabilized with 0.1% Nonidet P-40, and stained with anti-ceramide Ab and anti-mast cell tryptase Ab followed by Alexa Fluor 488-conjugated goat Ab against mouse IgM (A21042) (Invitrogen, Carlsbad, CA) and Alexa Fluor 546-conjugated goat Ab against rabbit IgG (H+L) (A11035) (Invitrogen), respectively. All sections were counterstained with DAPI (Invitrogen). Fluorescent images were analyzed on a confocal microscope equipped with a CCD camera (Olympus, Tokyo, Japan).

Intra-rectal Administration of Non-Hydrolyzable ATPγS

Mice were intra-rectally administered with 100 μg of non-hydrolyzable ATPγS (Sigma-Aldrich) in 50% ethanol.[3,6]

Measurement of ATP in colonic tissues

The colonic tissues were isolated from mice on day 7 after DSS treatment and were cultured at 100 mg per 100 μL of RPMI-1640 medium for 3 h. ATP concentrations were measured by the CellTiter-Glo Luminescent Cell Viability Assay (Promega, San Luis Obispo, CA).

Measurements of MPO Activity

Neutrophil infiltration in colonic tissues was quantified by measuring MPO activity in lysates of colonic tissues and of BM neutrophils. After equal amounts of colonic tissue

lysates were incubated with o-dianisidine hydrochloride and hydrogen peroxide, the absorbance was measured at 460 nm. Neutrophils in tissue lysates were estimated by a standard curve using BM neutrophils.

Colon Punch Biopsies

Three mm^2 punch biopsies of the colon were incubated for 24 h in RPMI-1640 including 10% fetal calf serum and antibiotics. Supernatants were assessed for cytokine/chemokine production.

SUPPLEMENTAL REFERENCES

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2. Yamanishi Y, Kitaura J, Izawa K, *et al*. TIM1 is an endogenous ligand for LMIR5/CD300b: LMIR5 deficiency ameliorates mouse kidney ischemia/reperfusion injury. *J Exp Med* 2010;207:1501-11.
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4. Kitamura T, Koshino Y, Shibata F, *et al*. Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. *Exp Hematol* 2003;11:1007-14.
5. Morita S, Kojima T, Kitamura T. Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther* 2000;7:1063-6.
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Supplemental Figure 1 2.5% DSS-treated *LMIR3*$^{-/-}$ mice develop more severe colitis than WT counterparts. (A, B) WT or *LMIR3*$^{-/-}$ mice on the C57BL6 background (each, n = 10) were subjected to the 2.5% DSS-induced colitis model. (A) DAI score was monitored daily. (B) Representative H&E-stained colonic sections are shown. Scale bars represent 100 μm. (A) Data are expressed as the mean and ± SEM. *$p < 0.05$, ***$p < 0.001$ (Student's *t*-test). (A, B) The data are from one experiment which is representative of the other one experiment performed.

Supplemental Figure 2 *LMIR3*$^{-/-}$ mice on the BALB/c background are highly susceptible to DSS-induced colitis.

(A-C) WT or *LMIR3*$^{-/-}$ mice on the BALB/c background (n = 10 per genotype) were subjected to the 3% DSS-induced colitis model. (A) Body weight and (B) DAI score were monitored daily. (C) Colon length was measured on day 7 after DSS treatment. (A-C) Data are expressed as the mean ± SEM. *$p < 0.05$, ***$p < 0.001$ (Student's *t*-test). The data are from one experiment which is representative of the other one experiment performed.

Supplemental Figure 3 TNBS-treated *LMIR3*$^{-/-}$ mice develop more severe colitis than WT counterparts. (A, B) WT or *LMIR3*$^{-/-}$ mice on the C57BL/6 background (WT, n = 10; *LMIR3*$^{-/-}$, n = 9) were subjected to the TNBS-induced colitis model. (A) Body weight was monitored daily. (B) Colon length was measured on day 4 after TNBS treatment. Data are expressed as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

(Student's *t*-test). The data are from one experiment which is representative of the other one experiment performed.

Supplemental Figure 4 Percentages of eosinophils in LPMNCs from WT and *LMIR3*$^{-/-}$ mice on the BALB/c background before and after DSS treatment. WT or *LMIR3*$^{-/-}$ mice on the BALB/c background (each, n = 4) were subjected to the 3% DSS-induced colitis model. Percentages of CD11b^{+}Siglec-F^{+} eosinophils in CD45^{+} LPMNCs from the mice on day 0 and 7 after DSS treatment. The data are from one experiment which is representative of the other two experiments performed.

Supplemental Figure 5 Colonic lamina propria mast cells are at comparable levels in WT or *LMIR3*$^{-/-}$ BMMC-transplanted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. Percentages of FcεRI^{+}c-kit^{+} mast cells in CD45^{+} LPMNCs from WT or *LMIR3*$^{-/-}$ BMMC-transplanted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (each, n = 4) before DSS treatment. Data are expressed as the mean ± SEM. The data are from one experiment which is representative of the other one experiment performed.

Supplemental Figure 6 Percentages of neutrophils or eosinophils in LPMNCs from WT or *LMIR3*$^{-/-}$ BMMC-transplanted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice after DSS exposure.

(A, B) Percentages of (A) CD11b^{+}Gr-1^{high} neutrophils or (B) CD11b^{+}Siglec-F^{+} eosinophils in CD45^{+} LPMNCs from WT or *LMIR3*$^{-/-}$ BMMC-transplanted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (each, n = 4) on day 7 after DSS treatment. Data are expressed as the mean ± SEM. *$p < 0.05$ (Student's *t*-test). The data are from one experiment which is representative of

the other one experiment performed.

Supplemental Figure 7 The effect of LMIR3 deficiency on the severity of 2.5% DSS-induced colitis in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. (A, B) *Kit*$^{W\text{-}sh/Wsh}$, *Kit*$^{W\text{-}sh/W\text{-}sh}$*LMIR3*$^{-/-}$, or *LMIR3*$^{-/-}$ mice on the C57BL/6 background (each, n = 4) were subjected to the 2.5% DSS-induced colitis model. (A) Body weight and (B) DAI score were monitored daily. Data are expressed as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's *t*-test). The data are from one experiment which is representative of the other one experiment performed.

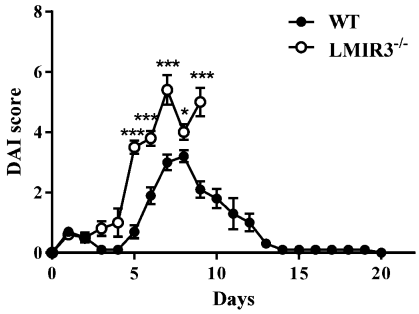
Supplemental Figure 8 The effect of transplantation with WT or *LMIR3*$^{-/-}$ BMMCs on the severity of 2.5% DSS-induced colitis in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. (A-C) *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice transplanted with WT or *LMIR3*$^{-/-}$ BMMCs were subjected to the 2.5% DSS-induced colitis model (*Kit*$^{W\text{-}sh/W\text{-}sh}$, n = 9; *Kit*$^{W\text{-}sh/W\text{-}sh}$ with WT BMMCs, n = 14; *Kit*$^{W\text{-}sh/W\text{-}sh}$ with *LMIR3*$^{-/-}$ BMMCs, n = 14). (A) Body weight and (B) DAI scores were monitored daily. (C) Mice were monitored for survival. (A, B) Data are expressed as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's *t*-test) (C) **$p < 0.01$ (long-rank test). The data are from one experiment which is representative of the other one experiment performed.

Supplemental Figure 9 Surface expression levels of P2X7 as well as FcεRI and c-kit in WT and LMIR3 *LMIR3*$^{-/-}$ BMMCs. Surface expression levels of (A) FcεRI and c-kit,

(B) LMIR3, and (C) P2X7 in WT and *LMIR3*$^{-/-}$ BMMCs were examined. Control staining is shown in gray. The data are from one experiment which is representative of the other two experiments performed.

Supplementary Figure 1.

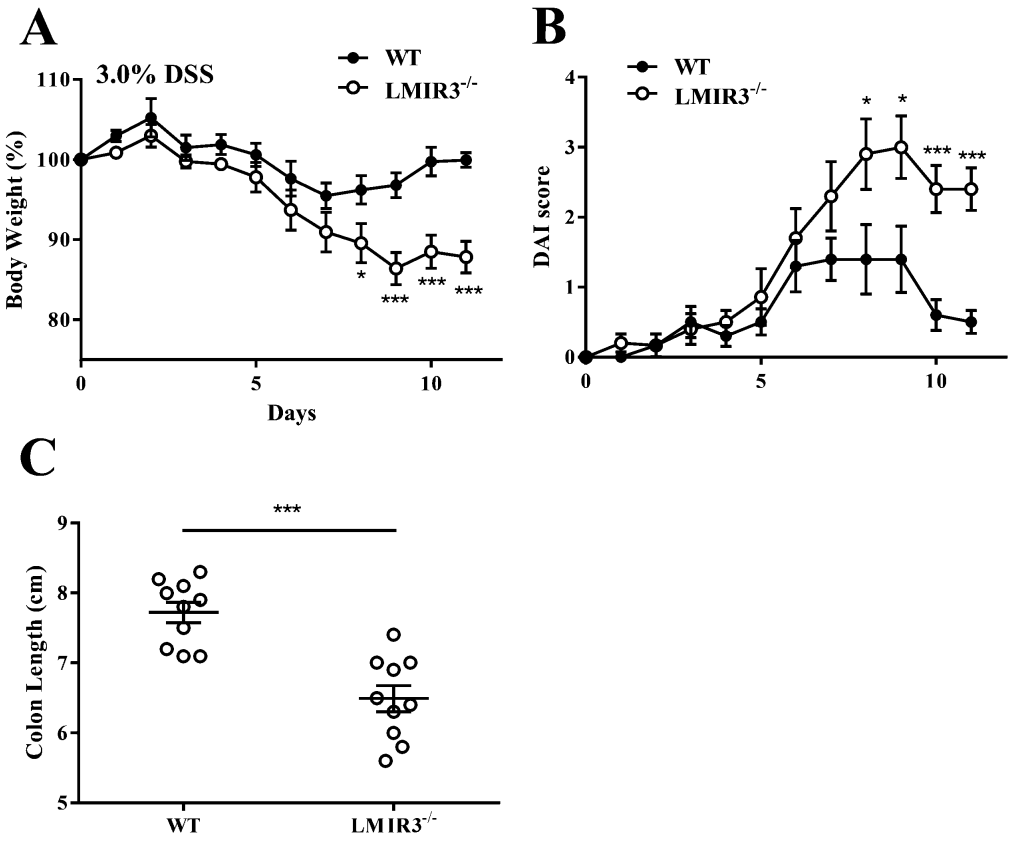
A



B

WT LMIR3$^{-/-}$

Supplementary Figure 2.



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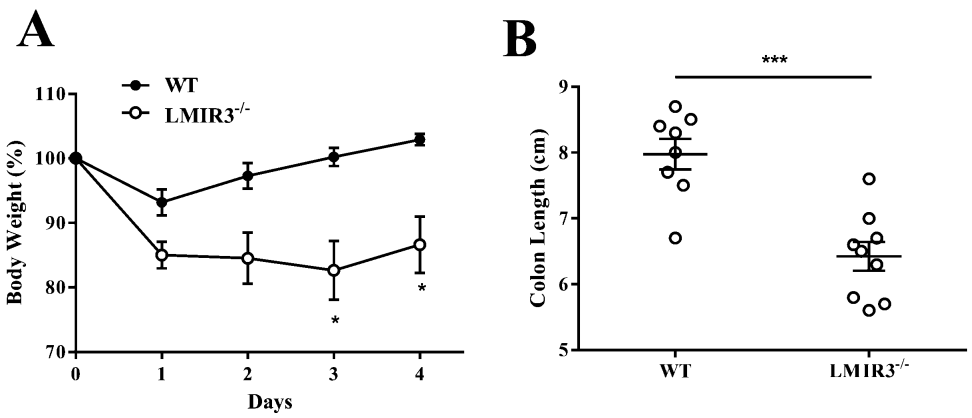

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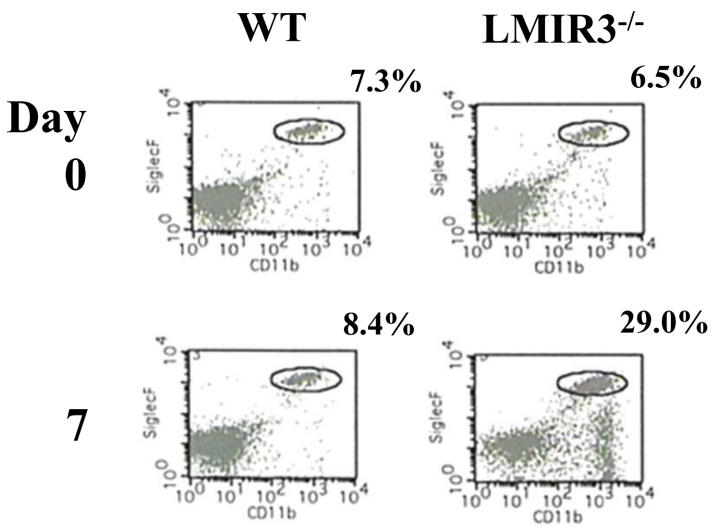

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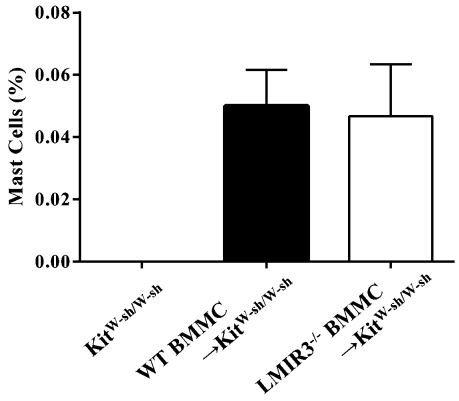

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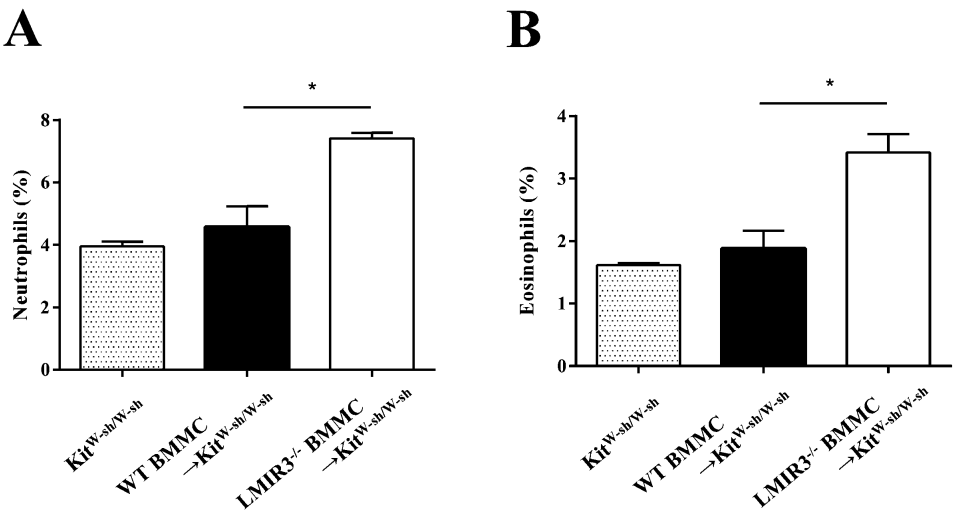

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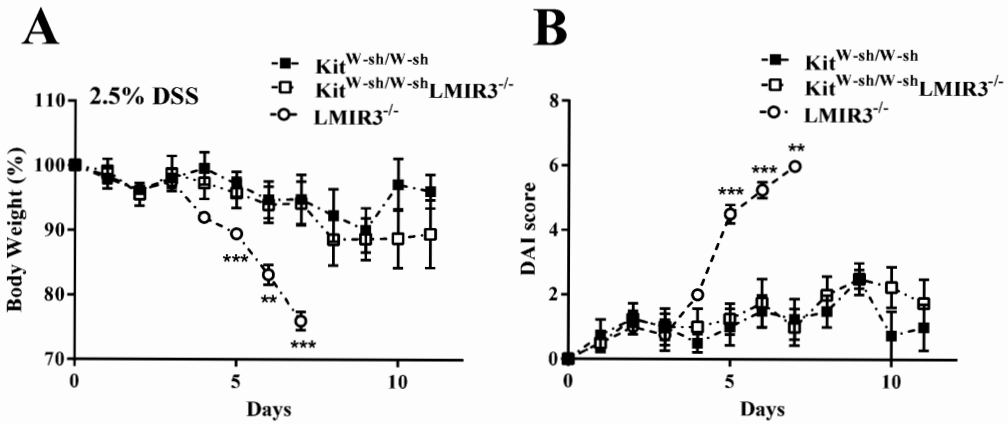
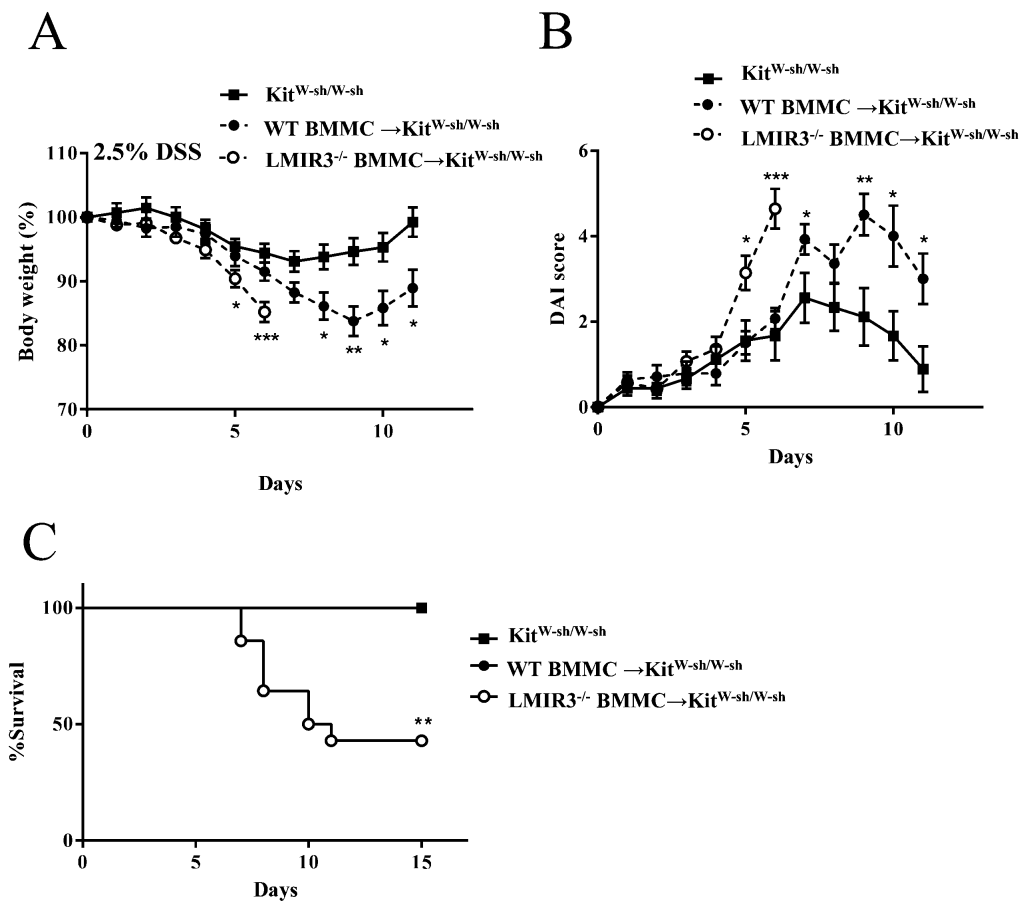


Supplementary Figure 8.



Supplementary Figure 9.