SUPPLEMENTAL INFORMATION

Plasma cells promote osteoclastogenesis and periarticular bone loss in autoimmune arthritis

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Supplemental Materials and Methods

Mice

All animals were maintained under specific pathogen-free conditions, and all experiments were performed with the approval of the Institutional Review Board at The University of Tokyo. C57BL/6 mice were purchased from CLEA Japan. *Tnfsf11* $^{flox/\triangle}$ mice[31], *Col6a1*-Cre mice[32], *Mb1*-Cre mice[25] and *Lck*-Cre mice[31] were backcrossed onto a DBA1/J background for 5th generations. ROSA26-loxP-Stop-loxP-YFP reporter mice were described previously[5]. 8- to 12-week-old sex-matched mice were used for all of the experiments unless otherwise noted.

Generation of RANKL-Cre mice

RANKL-Cre mice were generated by the methods using CRISPR/Cas9 system, as described previously[33]. The Kusabira Orange signal was not detected in RANKL-Cre mice by FACS analysis. Thus, we crossed RANKL-Cre mice with ROSA26-YFP reporter mice and generated RANKL-Cre ROSA-YFP mice, in which YFP expression is turned on in the cells that are expressing RANKL or expressed RANKL. The strategies for

plasmid construction are described below. The CRISPR target sequence (5′ -CCGCGCCATGCGCCGGGCCAGCC -3′) was selected for integration of the *Cre-P2a-Kusabira orange (KO)* sequence just after the start codon of *Tnfsf11*. The *pX330* plasmid, carrying both gRNA and Cas9 expression units, was a gift from Dr. Feng Zhang (Addgene plasmid 42230). *Tnfsf11*-CRISPR-F (5 ′ -caccCGCCATGCGCCGGGCCAGCC-3 ′) and *Tnfsf11*-CRISPR-R (5 ′ -aaacGGCTGGCCCGGCGCATGGCG-3′) were annealed and inserted into the entry site of *pX330,* as described previously[34]. This plasmid was designated as *pX330-Tnfsf11*. Transfection to HEK293T cells and fluorescence observations were performed as described[35]. The donor plasmid *pTnfsf11/Cre-P2a-KuO* contained the *Cre*, *P2A* and *Kusabira Orange* sequence. The 1.0-kb 5′ -arm (from 1.0 kb upstream of exon 1 to just before the start codon of *Tnfsf11*) and the 1.0-kb 3′ -arm (from the start codon to 1.0 kb downstream of exon 1) were cloned into this vector. DNA vectors (*pX330-Tnfsf11* and *pTnfsf11/Cre-P2a-KuO*) were microinjected into the male pronuclei of fertilized oocytes which were harvested from superovulated mated C57BL/6J females. Surviving embryos were transferred into the oviduct of pseudopregnant ICR females.

Collagen-induced arthritis

We induced CIA in 8 week old-male DBA/1J mice. Mice were intradermally immunized with an emulsion which consisted of 50 μl of chicken type II collagen (Sigma-Aldrich, 4 mg ml^{-1}) and 50 μl of adjuvant into the base of the tail at two sites. We added heat-killed *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, 5.0 mg ml^{-1}) in incomplete Freund's adjuvant (IFA)(Difco Laboratories). Three weeks after the primary immunization, mice were challenged with the same emulsion as the primary immunization. We judged the development of arthritis in the joint using the following criteria: 0, no joint swelling; 1, swelling of one paw joint; 2, mild swelling of the wrist or ankle; 3, severe swelling of the wrist or ankle. The scores for all of the jointsof forepaws and hindpaws, wrists and ankles were totaled for each mouse (with a maximum possible score of 12 for each mouse).

Analysis of bone phenotype

Histomorphometric analysis was described previously[5]. For microcomputed tomography analysis, the distal femur of the arthritic mice 3 weeks after the 2nd immunization was subjected to three-dimensional microcomputed tomography. CT

scanning was performed using a ScanXmate-A100S Scanner (Comscantechno). Three-dimensional microstructural image data were reconstructed and structural indices were calculated using TRI/3D-BON software (RATOC).

Quantitative RT-PCR analysis

Real-time quantitative RT-PCR analysis was performed with a LightCycler (Roche) using SYBR Green (Toyobo). The level of mRNA expression was normalized by *Gapdh* expression. The following primers were used: *Gapdh*, 5′-TCCACCACCCTGTTGCTGTA-3′ and 5′-ACCACAGTCCATGCCATCAC-3′; *Tnfsf11*, 5′-AGCCATTTGCACACCTCAC-3′ and 5′-CGTGGTACCAAGAGGACAGAGT-3′; *Prdm1*, 5’ TGCTTATCCCAGCACCCC-3’ and 5’- CTTCAGGTTGGAGAGCTGACC-3’.

Flow cytometry and antibodies

Antibodies conjugated with biotin, FITC, Alexa Fluor 488, phycoerythrin (PE), PerCP-Cy5.5, allophycocyanin (APC) and pacific blue (PB) were used at a 1:100 dilution unless

otherwise mentioned. The following monoclonal antibodies were purchased from BioLegend: anti-mouse CD3 (145-2C11), T cell receptor-β (TCR-β) (H57-597), CD8α(53-6.7), TCR γ/δ (GL3), CD45(30-F11), Podoplanin(8.1.1), CD31 (PECAM-1)(390), CD146(ME-9F1), Ter119(TER-119), CD90.2(53-2.1), CD11b(M1/70), CD11c(N418), F4/80(BM8), Ly-6G/Ly-6C(RB6-8C5), CD45R (B220)(RA3-6B2), CD19(1D3), CD267(8F10), CD138(281-2). Anti-mouse CD4 (RM4-5) and RANKL (IK22/5) were purchased from eBioscience. Flow cytometric analysis was performed using FACSCanto II with Diva software (BD Biosciences).

***In vitro* assay of osteoclast differentiation**

Primary bone marrow cells purified from 8 to 12 week-old untreated DBA1/J mice were cultured in medium (α-MEM containing 10% FBS) supplemented with 10 ng ml^{-1} M-CSF (R&D Systems) for 2 days to obtain osteoclast precursor cells. Bone marrow plasma cells (2 x 10^4 cells $well^{-1}$) ($CD3^-Gr1^-CD138^+B220^-$) or B cells ($CD3^-Gr1^-CD19^+$) were FACSAria-sorted from arthritic mice 3 weeks after the 2nd immunization (the purity is more than 99.5%). Osteoclast precursor cells (2 x 10^4 cells $well^{-1}$) were then cultured with bone marrow plasma cells (2 x 10^4 cells $well^{-1}$) or B cells in the presence of 10 ng

ml^{-1} M-CSF, 5 μg ml^{-1} anti-OPG antibody (AF459, R&D) and 2.5 ng ml^{-1} RANKL for 5 days using a 96 well flat-bottom plate, and $TRAP^+$ multinucleated (more than three nuclei) cells (MNCs) were counted. Bone-resorbing activity was confirmed by analyzing the resorption area after staining the bone slices with 0.5% toluidine blue. Alternatively, osteoclast precursor cells obtained from RANKL-deficient mice were co-cultured with bone marrow plasma cells or B cells in the presence of 10 ng ml^{-1} M-CSF, 5 μg ml^{-1} anti-OPG antibody, 10 ng ml^{-1} TNF, 10 μg ml^{-1} plate-coated anti-IgM antibody (Cat No.115-006-020, Jackson ImmunoResearch), 2 μg ml^{-1} anti-CD40 antibody (1C10, BioLegend), 10 μg ml^{-1} anti-IL-4 antibody (11B11, BioLegend) and 10 μg ml^{-1} anti-IL-10 antibody (JES5-16E3, BioLegend) for 5 days using a 96 well flat-bottom plate, and $TRAP^+$ multinucleated (more than three nuclei) cells (MNCs) were counted.

Statistical analyses

Data were analyzed on GraphPad Prism software version 6.0g. Statistical tests, n values, replicate experiments, and p values are all located in the figures and/or legends. All data are expressed as the mean ± s.e.m. *P* values were calculated using one-way ANOVA with Holm-Sidak's multiple comparisons test or two-way ANOVA with Holm-Sidak's

multiple comparison test or unpaired Student's *t*-test (*$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$; N.S., not significant, throughout the paper).

Supplemental References

31. Nakashima T. et al. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat Med.* 2011;17(10):1231-1234.

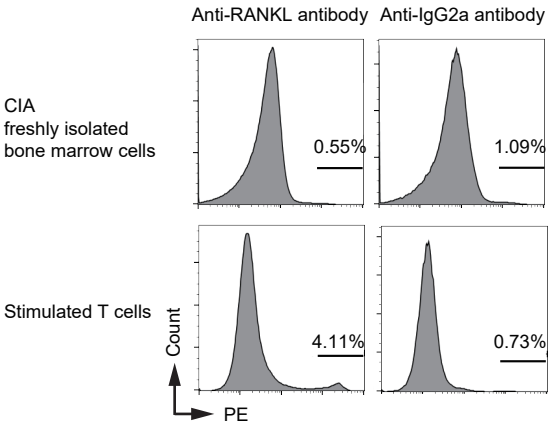
32. Armaka M. et al. Mesenchymal cell targeting by TNF as a common pathogenic principle in chronic inflammatory joint and intestinal diseases. *J Exp Med.* 2008;205(2):331-337.

33. Hasegawa Y. et al. Generation of CRISPR/Cas9-mediated bicistronic knock-in ins1-cre driver mice. *Exp Anim*. 2016;65(3):319-327.

34. Cong L. et al. Multiplex genome engineering using CRISPR/Cas systems. *Science.* 2013;339(6121):819–823.

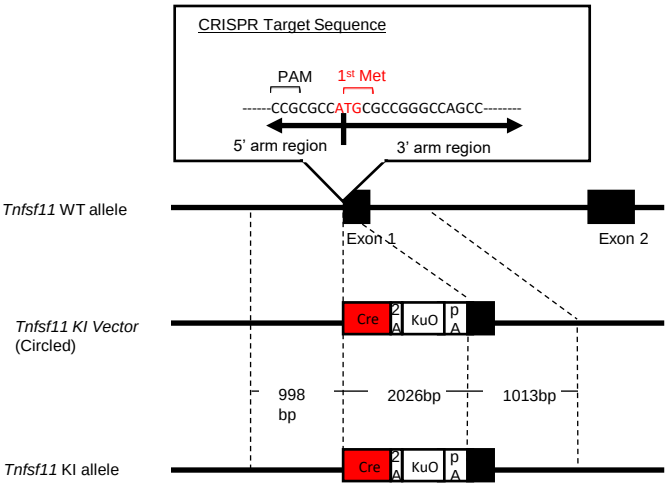
35. Mizuno S. et al. Simple generation of albino C57BL/6J mice with G291T mutation in the tyrosinase gene by the CRISPR/Cas9 system. *Mamm Genome.* 2014;25(7-8):327–334.

Supplementary Figure 1



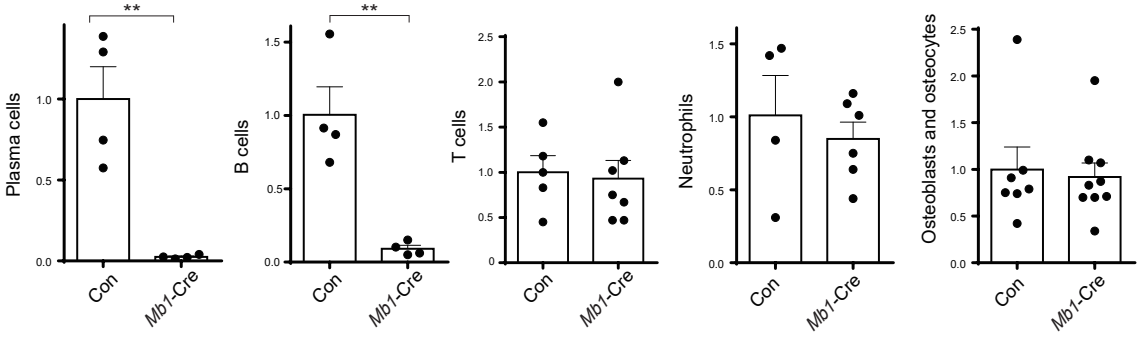
Supplemental Figure 1. An anti-RANKL antibody failed to detect positive signals in freshly isolated bone marrow cells.
Freshly isolated bone marrow cells (upper) and stimulated T cells (lower) were stained for a biotinated anti-RANKL antibody (left) and control biotinated anti-ratIgG2a antibody (right) followed by PE-conjugated Streptavidin. Representative data of three independent experiments are shown.

Supplementary Figure 2



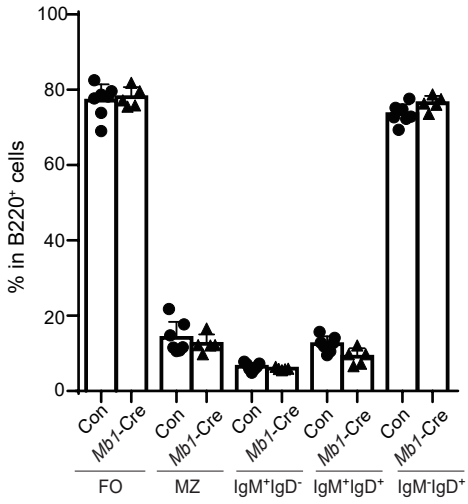
Supplemental Figure 2. Generation of RANKL-Cre mice
The CRISPR target sequence (5′- CCGCGCCATGCGCCGGGCCAGCC -3′) was selected for integration of the *Cre-P2a-Kusabira orange (KO)* sequence just after the start codon of *Tnfsf11*. The *pX330* plasmid, carrying both gRNA and Cas9 expression units, was a gift from Dr. Feng Zhang (Addgene plasmid 42230). *Tnfsf11*-CRISPR-F (5′-caccCGCCATGCGCCGGGCCAGCC-3′) and *Tnfsf11*-CRISPR-R (5′-aaacGGCT GGCCCGGCGCATGGCG-3′) were annealed and inserted into the entry site of *pX330* as described previously(28). This plasmid was designated as *pX330-Tnfsf11*. The cleavage activity of *pX330-Tnfsf11* was confirmed by the traffic reporter system using the *p2color* vector containing the CRISPR target. Transfection to HEK293T cells and fluorescence observations were performed as described29.
The donor plasmid *pTnfsf11/Cre-P2a-KuO* contained the *Cre*, *P2A* and *Kusabira Orange* sequence. The 1.0-kb 5′-arm (from 1.0 kb upstream of exon 1 to just before the start codon of *Tnfsf11*) and the 1.0-kb 3′-arm (from the start codon to 1.0 kb downstream of exon 1) were cloned into this vector.

Supplementary Figure 3



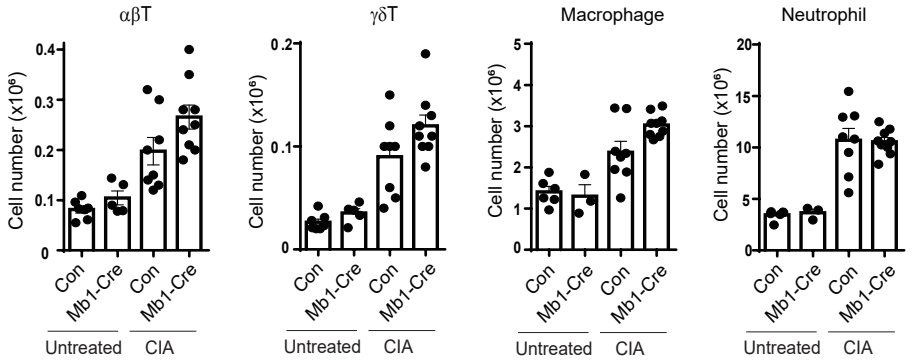
Supplemental Figure 3. Specific deletion of RANKL in B-lineage cells in *Mb1*-Cre *Tnfsf11*$^{flox/\Delta}$ mice
Bone marrow (plasma cells and B cells), spleen (T cells, neutrophils) and tibia (osteoblasts and osteocytes) from *Tnfsf11*$^{flox/\Delta}$ and *Mb1*-Cre *Tnfsf11*$^{flox/\Delta}$ mice were analyzed. Relative *Tnfsf11* mRNA expression was shown (n=4-9).All data are expressed as the mean±s.e.m.

Supplementary Figure 4



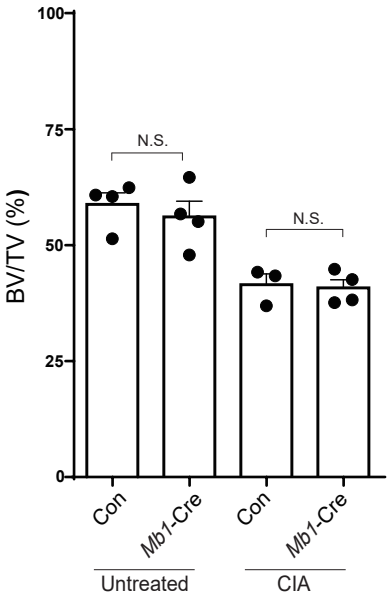
Supplemental Figure 4. Deletion of RANKL in B-lineage cells did not affect the numbers of B cell subsets in the spleen.
Splenocytes from *Tnfsf11*$^{flox/\Delta}$ and *Mb1*-Cre *Tnfsf11*$^{flox/\Delta}$ were analyzed. The frequency of B cell subset (follecular B;FO, marginal B;MZ, IgM^+IgD^-,IgM^+IgD^+, IgM^-IgD^+) in $B220^+$ cells is shown (n=5-7).All data are expressed as the mean±s.e.m.

Supplementary Figure 5



Supplemental Figure 5. Lack of RANKL in B-lineage cells did not affect the numbers of other immune cell subsets in the bone marrow under physiological or pathological conditions. The numbers of αβT cells, γδT cells, macrophages ($F4/80^{+}CD11b^{+}$), neutrophils ($Gr1^{hi}CD11b^{+}$) in the bone marrow under physiological and arthritic conditions (n=3-9). All data are expressed as the mean±s.e.m.

Supplementary Figure 6



Supplemental Figure 6. Lack of RANKL in B-lineage cells did not affect systemic bone loss in autommune arthritis.
Bone volume per tissue volume of the lumber spine (L5) of arthritic mice was analyzed 3 weeks after the 2nd immunization. Con ; *Tnfsf11*$^{flox/\Delta}$, *Mb1*-Cre ; *Mb1*-Cre *Tnfsf11*$^{flox/\Delta}$ mice (n=3-4). Data are expressed as the mean±s.e.m. One-way ANOVA with Holm-Sidak's multiple comparisons test (N.S., not significant).