Supplementary Information

Mesenchymal Stem Cell Transplantation Reverses Multi-Organ Dysfunction in Systemic Lupus Erythematosus Mice and Humans

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MATERIALS AND METHODS

Antibodies. Purified anti-mouse IgG and anti-CD138 antibodies were purchased from R&D Systems. Purified anti-IL17 and anti-Runx2 antibodies were obtained from Santa Cruz and Oncogene, respectively. Purified C3 antibody was form GeneTex. Anti-CD3 antibody was purchased from abcam. Anti-ALP (LF47) and anti-OCN (LF32) were kindly provided by Dr. Larry Fisher (National Institute of Dental and Craniofacial Research, National Institutes of Health). Anti-β-actin antibody was purchased from Sigma. APC-conjugated and PerCP-conjugated anti-CD4, APC-conjugated anti-CD25, PE-conjugated anti-CD138 and PE-conjugated anti-IL17, were from BD Bioscience. PE-conjugated anti-Foxp3 antibody was obtained from Miltenyi Biotech. Antibodies for mouse immunoglobulins IgG, IgA and IgM were purchased from Invitrogen.

MicroCT and pQCT analysis. Distal femoral metaphyses were harvested from 20-week-old mice. Distal femoral metaphyses were analyzed by μCT (ScanXmate-A100S; Comscantecno Co. Ltd., Kanagawa, Japan). Scanning regions were confined to secondary spongiosa and the thickness was approximately 1.0 mm. Using 2-dimensional images, a region of interest was manually drawn near the endocortical surface. Structual indices and bone mineral density (BMD) were calculated using 3-dimensional image analysis system (TRI/3D-Bon software; Ratoc System Enginerring Co. Ltd.). Structural indices are including bone volume/trabecular volume (BV/TV), bone surface area (BS, mm2), trabecular number (Tb.N, 1/mm), and trabecular separation (Tb.Sp, mm).

Mineralized tissue analysis. Femurs and transplant tissue were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), pH 7.2, overnight at 4°C, and decalcified with 5% EDTA (pH 7.4) in PBS, pH 7.2, for 10 days at 4°C. Bone samples were dehydrated with a graduate series of ethanol, cleaned with xylen, and immersed in paraffin. The samples were embedded in paraffin and cut into 8-μm-thick sections. The sections were deparaffinized, rehydrated and used for H&E staining and further histochemical staining.

TRAP staining. Deparaffinized sections were re-fixed with a mixture of 50% ethanol and 50% acetone for 10 min. TRAP-staining solutions were freshly made (1.6% naphthol AS-BI phosphate in N, N-dimethylformamide and 0.14% fast red-violet LB diazonium salt, 0.097% tartaric acid and 0.04% $MgCl_2$ in 0.2 M sodium acetate buffer, pH 5.0) and mixed in 1:10. The sections were incubated in the solution for 10 min at 37°C under shield and counterstained with toluidine blue. All regents for TRAP staining were purchased from Sigma.

Immunohistochemistry. Sections were treated with 0.3% hydrogen peroxide and 0.1% sodium azide in PBS, pH 7.2, for 30 min, and incubated with indicated primary antibodies, overnight, at 4°C. After washing with PBS, the sections were immunostained using SuperPicTure™ Polymer Detection kit (Invitrogen) according to the manufacturer's instructions. Finally, samples were counterstained with hematoxylin.

Mouse BMMSC isolation and culture. Bone marrow cells were flashed out from bone cavity of femurs and tibias with heat-inactivated 3% fetal bovine serum (FBS; Equitech-Bio) in PBS. All nuclear cells (ANCs) were seeded at 10-20 x 10^6 into 100 mm culture dishes (Corning) and initially incubated for 3 hours under 37°C at 5% CO_2 condition. To eliminate the non-adherent cells, the cultures were washed with PBS twice. The attached cells were cultured for 14-16 days. Colonies-forming attached cells were passed once to use for further experiments. The BMMSCs were cultured with α-MEM (Invitrogen) supplemented with 20% FBS, 2 mM L-glutamine (Invitrogen), 55 μM 2-mercaptoethanol (Invitrogen) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Biofluids).

Human BMMSC culture. Human bone marrow aspirated from iliac of eight donors selected from relatives of the patients and two SLE patients (case 5 and 7 in Table 1) were diluted with PBS containing heparin (1,250 U/ml) and separated using Ficoll-Hypaque (density 1.077 g/ml, TBD) by centrifuge at 2000 rpm for 30 minutes to obtain mononuclear cells. The single cell suspensions (5 x 10^6/ml) were cultured in 25 cm^2 flasks (Corning) with DMEM supplemented

with 10% FBS and antibiotics at 37°C with medium change every 24-48 hours until the BMMSCs achieved required numbers at 3-4 passages. The BMMSCs were used for *in vivo* transplantation and *in vitro* osteogenic differentiation. For the allogenic MSCT, human SLE BMMSCs were cultured under non-serum-depleted condition for 12 hours prior to MSCT, and repeatedly washed with 0.25% trypsin and then mixed with 5% human albumin in physiological saline.

CFU-F assay. ANCs ($1.5x10^6$/flask) were seeded on T-25 flasks (Nunc) and incubated at 37°C. After 3 hours, the flasks were washed with PBS and cultured for 16 days. After washing with PBS two times, the flasks were treated with 2% PFA and 1% toluidine blue solution in PBS. Cell clusters containing ≥50 cells were recognized as a colony under light microscopy. Total colony numbers were counted per flask. The CFU-F number was repeated in five or six independent samples per each experimental group.

Cell proliferation assay. Mouse BMMSCs (10×10^3/well) were seeded on 2-well chamber slides (Nunc) and cultured for 2-3 days. The cultures were incubated with BrdU solution (1:100) (Invitrogen) for 20 hours, and stained with a BrdU staining kit (Invitrogen) according to the manufacturer's instructions. The samples were stained with hematoxylin. BrdU-positive and total cell numbers were counted in ten images per subject. The number of BrdU-positive cells was indicated as a percentage to the total cell number. The BrdU assay was repeated in five or six independent samples for each experimental group.

***In vitro* differentiation assay.** BMMSCs were cultured under osteogenic culture condition containing 2 mM β-glycerophosphate (Sigma), 100 μM L-ascorbic acid 2-phosphate (Wako Pure Chemicals) and 10 nM dexamethasone (Sigma). After the osteo-induction, the cultures were stained with alizarin red. For the adipo-induction *in vitro*, 500 nM isobutylmethylxanthin (Sigma), 60 μM indomethacin (Sigma), 500 nM hydrocortisone (Sigma), 10 μg/ml insulin (Sigma), 100 nM L-ascorbic acid phosphate were added into the medium. Two weeks after the adipo-induction, the cultures were stained with Oil Red-O. The mineralized area and Oil Red-O positive cells were quantified by using an NIH Image-J. Total RNA and total protein were isolated from the mouse BMMSC cultures after two weeks inductions. All experiments were repeated in five or six independent samples for each group.

***In vivo* bone formation assay.** Approximately $4.0x10^6$ of BMMSCs were mixed with 40 mg of HA/TCP ceramic powder (Zimmer) as a carrier and subcutaneously transplanted into the dorsal

surface of 8-10 weeks old immunocompromised mice. Eight weeks post-transplantation, the transplants were harvested, fixed in 4% PFA and then decalcified with 10% EDTA (pH 8.0), followed by paraffin embedding. Paraffin sections were deparaffinized and stained with H&E.

Semi quantitative RT-PCR. Total RNA was isolated from the cultures using SV total RNA isolation kit (Promega) and digested with DNase I following the manufacture's protocols. The cDNA was synthesized from 100 ng of total RNA using Superscript III (Invitrogen). And then, PCR was performed using gene specific primers and Platinum PCR supermix (Invitrogen). The amplified PCR products were subjected to 2% agarose gels which contain ethidium bromide and visualized by UV fluorescent. The intensity of bands was measured by using NIH image-J soft ware and normalized to GAPDH. RT-PCR was repeated in five or six independent samples.

Western blot analysis. Samples were lysed in M-PER extraction reagent (Pierce). The protein was quantified using Bio-Rad Protein Assay (Bio-Rad) and V max micro plate reader (Molecular Devices). Ten μg of protein were applied to each lane and separated on Tris-Glycine SDS-PAGE gel (Invitrogen). Proteins were transferred onto Immobilon-P (Millipore). Membranes were incubated with primary antibodies (1: 100-1000 dilution), over night at 4^{o}C, and then incubated with HRP-conjugated secondary antibodies (Santa Cruz) at 1:5000 dilutions for 1 hour at room temperature. The membranes were washed and reacted with Super Signal chemiluminescence HRP substrate (Pierce) and visualized on BIOMAX MR films (Kodak). The intensity of bands was measured using same methods as semi quantitative PCR and normalized to β actin. Western blotting was repeated in five or six times.

ELISA. Peripheral blood were collected from the retro-orbital plexus, and centrifuged to obtain the blood serum. Urine was also collected. Tissue lysates were extracted form mouse spleen. The samples were centrifuged and used for ELISA. Anti-dsDNA IgG and IgM antibodies, ANA, albumin, C3, IL17, IL6, RANKL and C-terminal telopeptides of type I collagen were measured using commercial available kits (anti-dsDNA antibodies, ANA, albumin, C3, alpha diagnostic; IL17, IL6 and RANKL, R&D Systems; C-terminal telopeptides of type I collagen, Nordic Bioscience Diagnostics A/S) according to the manufactures' instructions. To measure total TGFβ, acid-treated samples were analyzed using a kit (Promega) according to the manufacture's instruction. For the analysis of immunoglobulins, the samples were incubated on wells coated with anti-mouse immunoglobulins antibody (10 μg/ml, Invitrogen), followed by the treatment using Mouse Mono-AB ID/SP kit (Invitrogen) according to the manufacture's protocols.

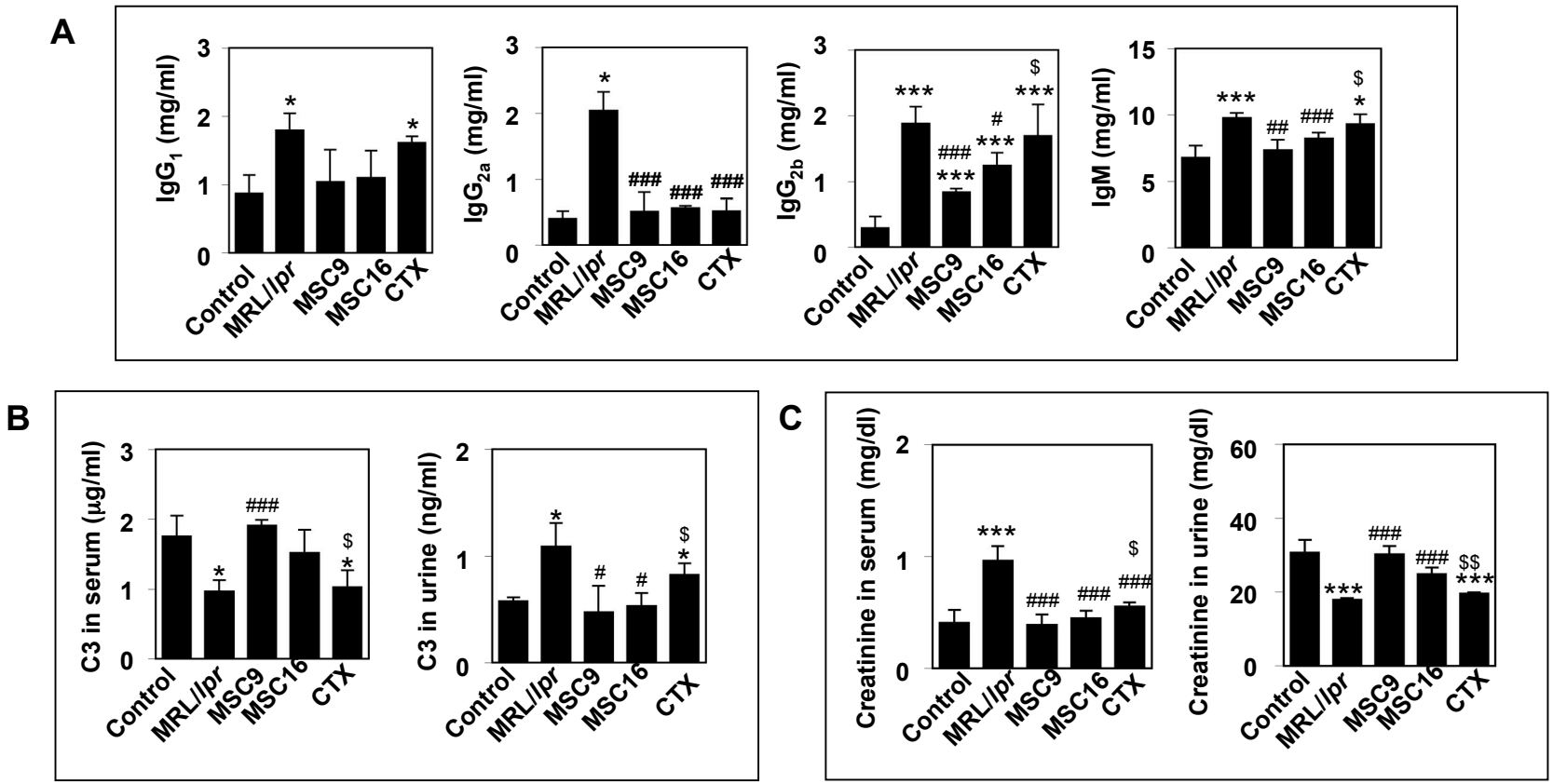
Creatinine levels in serum and urine were assayed using a commercial kit (R&D Systems). The results were averaged in each group. The intra-group differences were calculated between the mean values.

Clinical tests for serum C3 and urine protein. Peripheral blood serum and urine samples were collected from SLE patients and measured C3 levels in the serum and protein level in the urine in the Clinical Laboratory at the Drum Tower Hospital of Nanjing University Medical School.

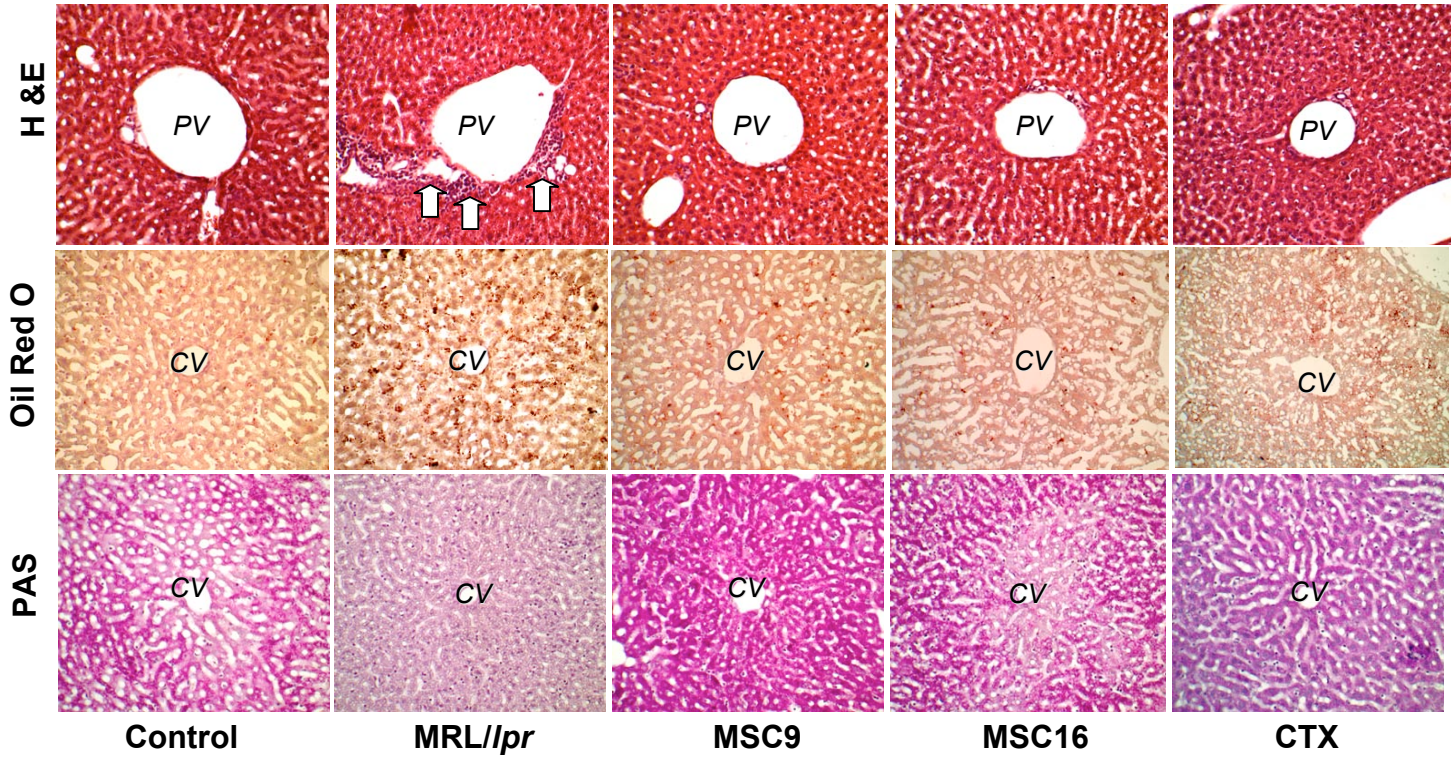
Histological analysis of kidney, liver and spleen. Samples were fixed with 4% PFA for 24 hours at 4^{o}C, and embedded with paraffin or Tissue-Tek O.C.T. compound (Sakura). Paraffin Sections were used for H&E staining, trichrome staining, Periodic Acid Schiff (PAS) staining and immunohistochemistry. Frozen sections were used for immunohistochemistry and Oil Red-O staining.

Histomorphomety. Area of trabecular bone and bone marrow was measured on H&E stained slides. To quantify osteoclast activity in the bones, number of mature osteoclasts was determined by TRAP positive cells that attached on the bone surface. Osteoblastic niche was quantified by the number of osteoblasts lining on the bone surface per bone marrow area with H&E staining. Quantification of newly-formed bone and marrow area was measured on transplant sections with H&E staining. The number of cells and the area were measured from five to seven representative images each sample using an NIH Image-J. The data were average the means in each experimental group. The results were shown as each indicated percentage.

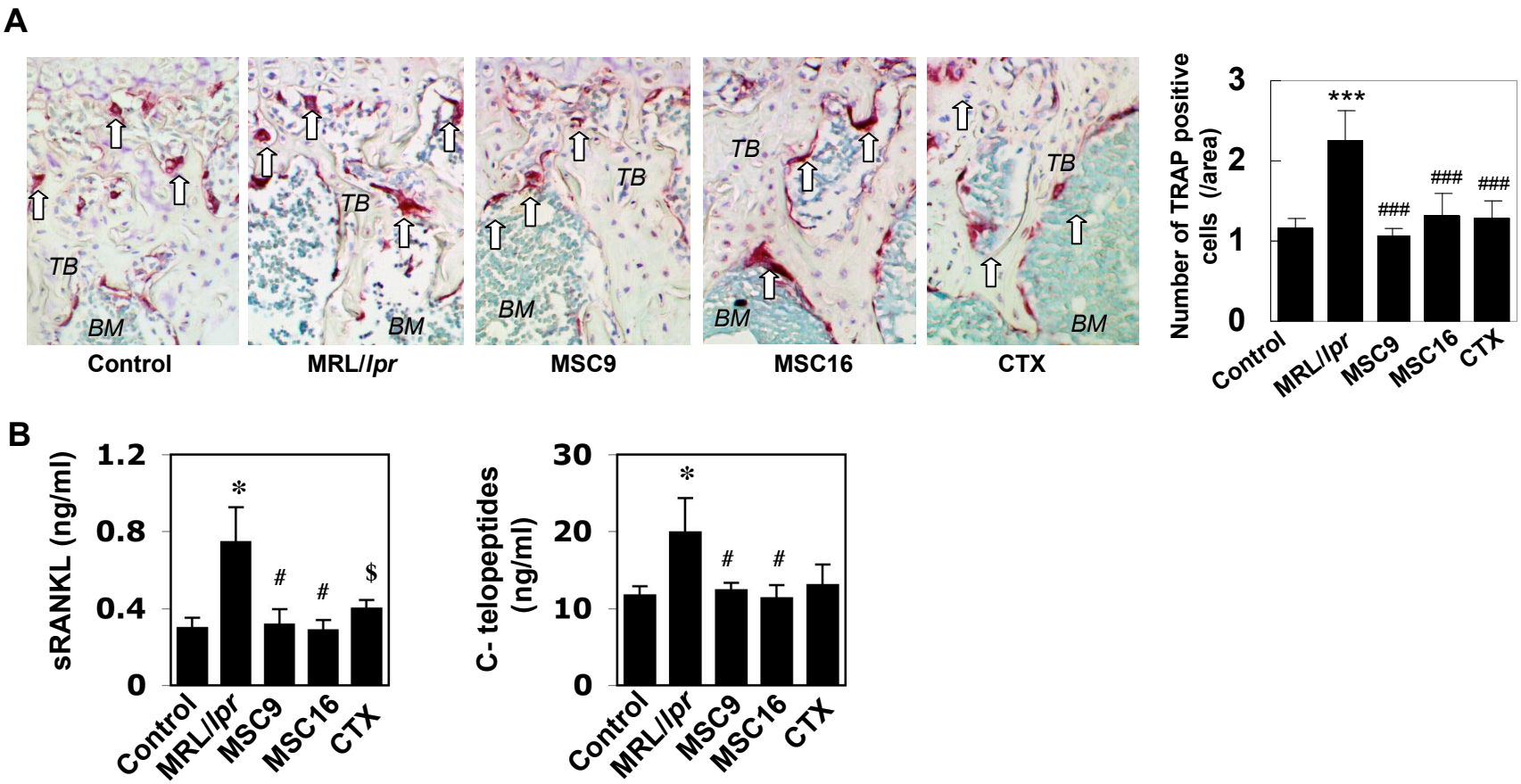
Flow cytometric analysis. Mouse spleen cells and spleen-derived CD4^{+} T lymphocytes were used for cytometric analysis. For analysis in SLE patients (n=4), peripheral blood mononuclear cells were separated using 2 m peripheral blood samples by Ficoll-Hypaque density centrifugation. For Treg staining, cells (1 x 10^{6}/sample or subject) were stained with APC-conjugated anti-CD25 antibody in mouse samples and APC-conjugated anti-CD4 antibody in human samples for 30 minutes under the shield at 4^{o}C, followed by staining with PE-conjugated anti-Foxp3 antibody using Foxp3 Staining Buffer Set (eBioscience) according to the manufacture's protocol for cell fixation and permeabilization. Cells isolated from spleen and bone marrow were stained with PE-conjugated anti-CD138 antibody. The samples were analyzed in a flow cytometer. For Th17 cell staining, cells (1 x 10^{6}/sample) were incubated with PerCP-conjugated anti-CD4 antibody. After cell fixation and permeabilization, cells were stained with PE-conjugated anti-IL17 antibody.



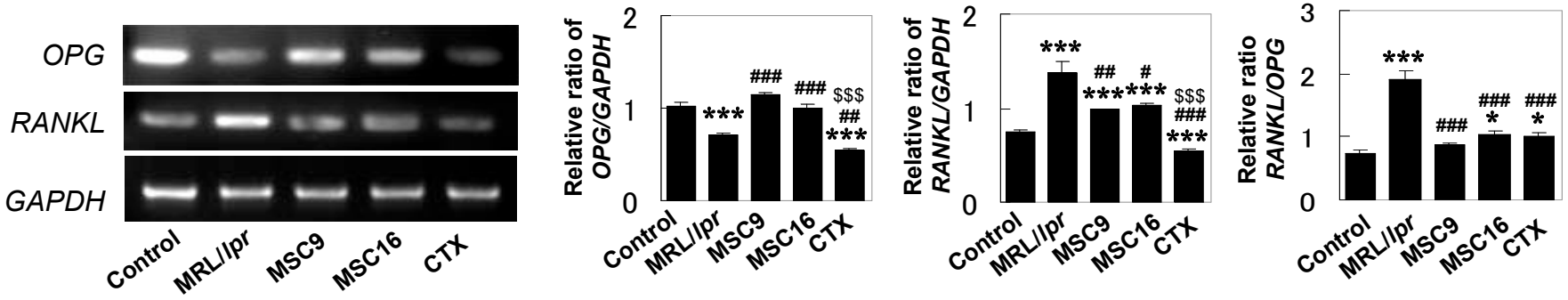
Supplementary Figure 1. **Allogenic MSCT recovered the levels of immnoglobulins, C3 and creatinine in peripheral blood serum and urine** (**A**) ELISA indicated that allogenic MSCT (MSC9, n=6; MSC16, n=6) reduced serum levels of IgG_1, IgG_{2a}, IgG_{2b} and IgM (mean±SD) when compared to *lupus*-like MRL/*lpr* mice (MRL/*lpr*, n=6). CTX treatment (n=6) only significantly reduced IgG_{2a} level. [*** P<0.005 vs. Control (n=6); * P<0.05 vs. Control; $^{###}P$<0.05 vs. MRL/*lpr*; $^{##}P$<0.01 vs. MRL/*lpr*; $^{#}P$<0.05 vs. MRL/*lpr*; $^{\$}P$<0.05 vs. MSC9].(**B**) ELISA revealed that MRL/*lpr* mice (MRL/*lpr*, n=6) showed significantly reduced C3 level (mean±SD) in serum and elevated C3 level in urine compared to control group (n=6). MSCT at 9 weeks of age (MSC9, n=6) significantly recovered C3 levels in serum. MSCT at 16 weeks of age (MSC16, n=6) elevated C3 level in serum was no statistic significance from MRL/*lpr* mice. Both MSC9 and MSC16 were able to significantly reduce urine C3 levels. However, CTX treatment (n=6) failed to recover the C3 level in serum and urine. [* P<0.05 vs. Control; $^{###}P$<0.05 vs. MRL/*lpr*; $^{#}P$<0.05 vs. MRL/*lpr*; $^{\$}P$<0.05 vs. MSC9 (in serum) and MSC16 (in urine)]. (**C**) ELISA revealed that creatinine level (mean±SD) in MRL/*lpr* mice (MRL/*lpr*, n=6*)* showed a significant increase in serum and decrease in urine when compared to control mice (n=6). Allogenic MSCT (MSC9, MSC16) significantly recovered both serum and urine creatinine levels. CTX treatment (n=6) failed to recover the urine creatinine level. [*** P<0.005 vs. Control; $^{###}P$<0.005 vs. MRL/*lpr*; $^{\$\$}P$<0.01, vs. MSCT (MSC9 and MSCT16); $^{\$}P$<0.05, vs. MSC9].



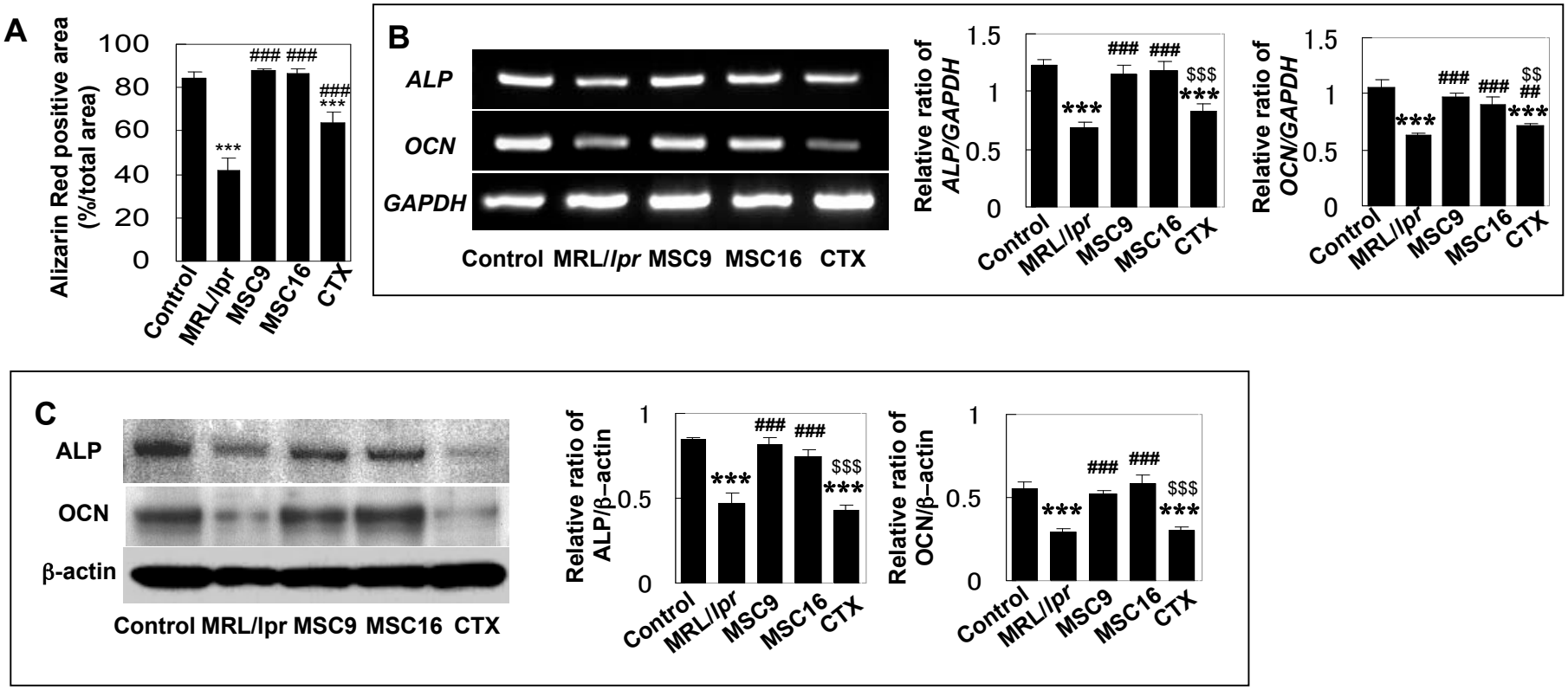
Supplementary Figure 2. Allogenic MSCT improved liver impairment in MRL/*lpr* mice. Inflammatory cells (arrows) presented in the portal triad containing a branches of hepatic portal vein (*PV*), hepatic artery and bile duct in liver of MRL/*lpr* mice (n=6) (upper panels). After MSCT (MSC9, n=6; MSC16, n=6) and CTX treatment (n=6), the inflammatory cells diminished as shown the similar to control liver (n=6). Histochemical staining revealed that all treatments including MSCT and CTX were capable of recovering elevated numbers of Oil red O positive cells (middle panels) and decreased Periodic acid-Schiff positive glycogen cells (lower panels) in MRL/*lpr* mice. *CV*: central vein.



Supplementary Figure 3. Allogenic MSCT reduced osteoclast activity in MRL/*lpr* mice (**A**) TRAP staining showed that MRL/*lpr* mice (n=6) had significantly increased numbers of TRAP positive cells (mean±SD, arrows) in epiphysis of the distal femurs as compared to control mice (n=6). MSCT (MSC9, n=6; MSC16, n=6) and CTX treatment (n=6) resulted in significantly decreased number of TRAP positive cells compared to MRL/*lpr* group**.** (***P<0.001 vs. Control; $^{###}P$<0.001 vs. MRL/*lpr*). (**B**) ELISA revealed that increased levels (mean±SD) of soluble RANKL (sRANKL) (right panel) and type I collagen C-terminal telopeptides of type I collagen (C-telo peptides) (left panel) in serum of MRL/*lpr* mice (n=6) were reduced after allogenic MSCT (MSC9, n=6; MSC16, n=6). CTX treatment (n=6) was capable of decrement of both levels, but not efficiently. [*P<0.05 vs. Control (n=6); $^{#}P$<0.05 vs. MRL/*lpr;* $^{$}P$<0.05 vs. MSC9 and MSC16].



Supplementary Figure 4. Allogenic MSCT reduced OPG level in splenocytes derived from MRL/*lpr* mice. The expression level of Osteoprotegerin (OPG) mRNA in splenocytes of MRL/*lpr* mice was significantly lower than that in the control as assessed by semi-quantitative RT-PCR. MSCT at 9 weeks (MSC9) and 16 weeks (MSC16) elevated expression level of OPG. However, CTX treatment failed to increase the expression level of OPG. The expression level of RANKL mRNA in splenocytes of MRL/lpr mice was significantly higher than that in control assessed by semi-quantitative RT-PCR. MSCT at 9 weeks (MSC9) and 16 weeks (MSC16) partially reduced the expression level of RANKL. CTX treatment was capable of significantly reducing RANKL level. The relative ratio of RANKL/OPG was significantly higher in MRL/*lpr* mice compare to the control. MSCT and CTX treatment could reduce the ratio, especially MSC9 group showed the similar level to the control. Three repeated tests per group showed similar results. Values are mean±SD (***P<0.001 vs. control; *P<0.05 vs. control; ###P<0.001 vs. MRL/lpr; #P<0.05 vs. MRL/lpr; $$$$P$<0.001 vs. MSCT).



Supplementary Figure 5. Osteogenic differentiation of BMMSCs derived from MSCT and CTX treated MRL/*lpr* mice (**A**) BMMSCs derived from MRL/*lpr* mice (n=6) showed significantly decreased calcium accumulation (mean±SD) compared to control mice (n=6) assessed by Alizarin red staining. MSCT (MSC9, n=6; MSC16, n=6) improved calcium accumulation of MRL/*lpr*-derived BMMSCs compared to the control BMMSCs. Also, CTX treatment (n=6) increased calcium accumulation of MRL/*lpr*-derived BMMSCs, but the recovered level was significantly less than the control BMMSCs. (***P<0.001 vs. Control; $^{###}P$<0.001 vs. MRL/*lpr*). (**B, C**) Semi-quantitative RT-PCR (**B**) and Western blot (**C**) analysis indicated that BMMSCs from MRL/*lpr* mice expressed lower levels of ALP and OCN than the controls. MSCT, but not CTX, was capable of recovering expression levels of ALP and OCN compared to the MRL/*lpr* levels. GAPDH and β-actin were used as control for the amount of sample loading in RT-PCR and Western blot, respectively. Six repeated tests per group showed similar results. (***P<0.001 vs. Control; $^{###}P$<0.001 vs. MRL/*lpr*; $^{$$$}P$<0.001 vs. MSCT; $^{$$}P$<0.01 vs. MSCT)

Supplementary Table 1. PCR Primers

Gene Name	Sense	Anti Sense	GenBank Accession Number	Product Size (base pair)
mouse Runx2	5'-CCGCACGACAACCGCACCAT-3'	5'-CGCTCCGGCCCACAAATCTC-3'	NM_009820	289
mouse ALP	5'-GCCCTCTCCAAGACATATA-3'	5'-CCATGATCACGTCGATATCC-3'	NM_007431	372
mouse OCN	5'-AAGCAGGAGGGCAATAAGGT-3'	5'-AGCTGCTGTGACATCCATAC -3'	NM_007541	292
mouse PPARγ2	5'-GCTGTTATGGGTGAAACTCTG-3'	5'-ATAAGGTGGAGATGCAGGTTC-3'	NM_011146	351
mouse LPL	5'-GGGCTCTGCCTGAGTTGTAG-3'	5'-AGAAATTTCGAAGGCCTGGT-3'	NM_008509	198
mouse Foxp3	5'-CCCAGGAAAGACAGCAACCTT-3'	5'-CCTTGCCTTTCTCATCCAGGA-3'	NM_054309	159
mouse IL17	5'-CTCCAGAAGGCCCTCAGACTAC-3'	5'-AGCTTTCCCTCCGCATTGACACAG-3'	NM_010552	141
mouse OPG	5'-ATCAGAGCCTCATCACCTT-3'	5'-CTTAGGTCCAACTACAGAGGAAC-3'	NM_008764	180
mouse RANKL	5'-ATTTGCACACCTCACCATCAA-3'	5'-TAGAGATCTTGGCCCAGCCTC-3'	NM_011613	100
mouse GAPDH	5'-CACCATGGAGAAGGCCGGGGG-3'	5'-GACGGACACATTGGGGGTAG-3'	NM_008084	418
human Runx2	5'-CAGTTCCCAAGCATTTCATCC-3'	5'-TCAATATGGTCGCCAAACAG-3'	L40992	443
human OCN	5'-CATGAGAGCCCTCACA-3'	5'-AGAGCGACACCCTAGAC-3'	X53698	315
human GAPDH	5'-AGCCGCATCTTCTTTTGCGTC-3'	5'-TCATATTTGGCAGGTTTTTCT-3'	M33197	816