Supporting Online Material for

Mesenchymal Stem Cell–Based Tissue Regeneration is Governed by Recipient T Lymphocyte via IFN-γ and TNF-α

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Supplementary Methods

Antibodies and inhibitors. Unconjugated antibodies to TNF-α, Fas, Smad 6 and Smad 7 antibodies were purchased from Santa Cruz Biosciences. Unconjugated antibodies to GFP, OCN, TRAIL, TNFR1, FasL, FLIP, and XIAP were purchased from Abcam Inc. Unconjugated antibodies to Runx2 was purchased from Calbiochem Inc. Unconjugated antibodies to TNFR2, caspase 8, caspase 3 and cleaved caspase 8 were purchased from Cell Signaling Inc. Unconjugated antibody to cleaved caspase 3 was purchased from Millipore. Specific IFN-γ–FITC antibody was purchased from eBioscience. Antibody to β-actin was purchased from Sigma–Aldrich Corporate. LEAF™ Purified mouse specific IFN-γ and TNF-α antibodies were purchased from Biolegend. Recombinant murine IFN-γ, TNF-α, IL-6, IL-17A, IL-4 were purchased from PeproTech. Alexa Fluor® 488 phalloidin was purchased from Invitrogen Corporation. Pan caspase inhibitor I (Z-VAD (OMe)-FMK) was purchased from EMD Chemicals Inc. Inhibitors for caspase 8 (2-IETD-FMK) and caspase 3 (Z-DEVD-FMK) were purchased from R&D System. Inhibitor for internalization (Latrunculin A) was purchased from Sigma–Aldrich Corporate. HyStem™ and Extracel–HP hydrogel were purchased from Glycosan.

Kits. Pan T cell isolation kit II and CD4+CD25+ regulatory T Cell isolation kit were purchased from Miltenyi Biotec. Mouse IFN-γ, TNF-α, IL-6, IL-17A, IL-4 and IL-10 ELISA Ready–SET–GO kits were purchased from eBioscience. SiRNA kits for Fas, Smad 6, TNFR2, IKK, FLIP and XIAP were purchased from Santa Cruz Biosciences.

Isolation of mouse bone marrow mesenchymal stem cells (BMMSCs). Bone marrow cells were flushed out from bone cavity of femurs and tibias with 2% heat–inactivated fetal bovine serum (FBS; Equitech–Bio,) in PBS. Single–cell suspension of all nuclear cells was obtained by passing through 70 µm cell strainer (BD Bioscience). All nuclear cells were seeded at 15 × 10⁶ into 100 mm culture dishes (Corning Corporation) and initially incubated for 48 hours under 37 °C at 5% CO₂ condition. To eliminate the non–adherent cells, the cultures were washed with PBS twice. The attached cells were cultured for 16 days. The BMMSCs were cultured with alpha minimum essential medium (α-MEM, Invitrogen Corporation) supplemented with 20% FBS, 2 mM L-glutamine (Invitrogen Corporation), 55 µM 2-mercaptoethanol (Invitrogen Corporation), 100 U ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin (Invitrogen Corporation).
**Immunofluorescent and immunochemical microscopy.** BMMSC implants were harvested, fixed in 4% paraformaldehyde and then decalcified with 5% ethylenediaminetetraacetic acid (EDTA, pH 7.4), followed by paraffin or optimal cutting temperature compound (OCT, Sakura Finetec Inc.) embedding. The paraffin or frozen sections were blocked with normal serum matched to secondary antibodies, incubated with the specific or isotype-matched mouse antibodies (1:200) overnight at 4 °C. For immunofluorescent staining, the samples were treated with Rhodamin or FITC–conjugated secondary antibodies (1:200, Jackson ImmunoResearch; Southern Biotechnology), and were mounted by means of a vectashield mounting medium containing 4’,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). For immunochemical staining, the samples were stained by using Zymed SuperPicture™ kit (Invitrogen Corporation) according to the manufacturer’s instruction.

**BMMSC–mediated bone formation.** Approximately $4.0 \times 10^6$ of littermate–derived or autologous BMMSCs were mixed with hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic particles (40 mg, Zimmer Inc.) as a carrier and subcutaneously implanted into the dorsal surface of 8–10 weeks old nude mice or C57BL6 mice. When cytokins were used, 250 µl Extracel–HP™ hydrogel (Glycosan Biosystems) containing 200 ng IFN-γ, TNF-α, IL-4, IL-6 or IL-17A were covered on the surface of the implants for slow release of the cytokines. When aspirin was used, BMMSCs were treated with aspirin for 2 days and mixed with HA/TCP particles to be subcutaneously implanted into nude mice, and 250 µl hydrogel containing 100 µg aspirin were covered on the surface of the implants for slow release of aspirin. At eight weeks post–implantation, the implants were harvested, fixed in 4% paraformaldehyde and then decalcified with 5% EDTA (pH 7.4), followed by paraffin embedding. The 6 µm paraffin sections were stained with hematoxylin and eosin (H&E) and analyzed by an NIH Image J. Five fields were selected and newly formed mineralized tissue area in each field was calculated and shown as a percentage to total tissue area.

**Cytokine levels in BMMSC implants.** The cytokine levels in the implants were measured by ELISA kit. Briefly, the implants at different time points were harvested and were put to the tissue grinder immediately. 200 µl lysis buffer was added to the grinder. The implant tissue was grinded with lysis buffer and kept on ice for 1 hour. Then, the
grinded tissue was centrifuged at 10,000 (rpm) for 10 min. After carefully collecting and measuring protein concentration in the supernatant, 100 µg total proteins in 100 µl assay buffer were added to the 96–well plate. The levels of cytokines were measured by using ELISA Ready–SET–GO kit (eBioscience). All experiments were repeated three times.

**Osteogenic differentiation assay.** BMMSCs were cultured under osteogenic culture condition medium containing 2 mM β-glycerophosphate (Sigma–Aldrich), 100 µM L-ascorbic acid 2-phosphate and 10 nM dexamethasone (Sigma–Aldrich). Different doses of IFN-γ (50, 200 ng ml⁻¹) were added to the osteogenic culture condition medium every 3 days. After the osteogenic induction, the cultures were stained with alizarin red. Expressions of Runx2, ALP, and OCN were assayed by western blot analysis.

**siRNA and Inhibitor treatment.** 0.5 × 10⁶ BMMSCs were seeded to 6–well culture plate. siRNAs for Fas, TNFR2, IKK, FLIP and XIAP were used to treat the BMMSCs according to the manufacture’s instruction, and IFN-γ and TNF-α were added for 24 hours. Inhibitors for internalization (Latrunculin A, 0.2 µM), pan caspase (Z–VAD (OMe)-FMK, 50 µM), caspase 8 (2-IETD-FMK, 20 µM) and caspase 3 (Z–DEVD-FMK, 20 µM) were used to treat the BMMSCs at 4 hours before IFN-γ and TNF-α were added. Cell apoptosis were analyzed by immunofluorescence staining and FACS Calibur (Annexin V–PE apoptosis detection kit I), and expressions of caspase 8, cleaved caspase 8, caspase 3 and cleaved caspase 3 were assayed by western blot analysis.

**T lymphocyte isolation.** Pan T, CD4⁺, CD4⁺CD25⁻ T lymphocytes were isolated from mouse total spleen cells using a magnetic sorter, mouse Pan T cell isolation kit II and CD4⁺CD25⁺ regulatory T Cell isolation kit (Miltenyi Biotec) following manufacture’s instruction. Approximately 1.0 × 10⁶ of cells were suspended in 200 µl PBS and injected into the mice via tail vein.

**CD4⁺ T Cell labeling.** CD4⁺ T lymphocytes were isolated from mouse total spleen cells using a magnetic sorter, mouse CD4⁺ T Cell isolation kit (Miltenyi Biotec) following manufacture’s instruction. The PKH26 red Fluorescent Cell Linker Kit (Sigma–Aldrich) was used to label the membrane of CD4⁺ T lymphocytes to red color.

**Co–culture mouse BMMSCs with activated T cells.** Mouse BMMSCs (0.2 × 10⁵ each well) were plated in 24–well flat bottom plates (Corning Costar Co.), and cultured in mouse BMMSC medium for 3 days. Mouse spleen T cells from C57BL6 mice (1 × 10⁶
each well) were cultured with plate–bounded 1 µg ml\(^{-1}\) antibody to CD3ε antibody and 2 µg ml\(^{-1}\) soluble antibody to CD28 (BD Bioscience) in complete DMEM containing 10% FBS for 3 days. The activated T cells were loaded directly on mouse BMMSCs in the cultures 3–5 days. In Aspirin group, BMMSCs were pretreated with different dose of aspirin (25, 100, 200 µg ml\(^{-1}\)) for 3 days and then the same doses of aspirin were added to the co–culture system. The culture supernatant was collected for measuring concentration of IFN-γ and TNF-α by using mouse IFN-γ and TNF-α ELISA Ready–SET–GO kits (eBioscience).

**Culture cells from BMMSC implants.** Thirty days after translation, the implants were harvested and digested in a solution of 3 mg ml\(^{-1}\) collagenase type I (Worthington Biochem) and 4 mg ml\(^{-1}\) dispase (Roche) for 1 hour at 37 °C. Single–cell suspensions were obtained by passing the cells through a 70 µm strainer (BD Bioscience). 1.0–1.5 × 10\(^6\) BMMSCs were seeded into 100 mm culture dishes (Corning) and incubated for 48 hours under 37°C at 5% CO\(_2\) condition. To eliminate the non–adherent cells, the cultures were washed with PBS for twice. The attached cells were cultured for 10 days with alpha minimum essential medium (α-MEM, Invitrogen) supplemented with 20% FBS, 2 mM L-glutamine (Invitrogen), 55 µM 2-mercaptoethanol (Invitrogen), 100 U ml\(^{-1}\) penicillin, and 100 µg ml\(^{-1}\) streptomycin (Invitrogen).

**Western blot analysis.** Total protein was extracted using M-PER mammalian protein extraction reagent (Thermo). Nuclear protein was obtained using NE-PER nuclear and cytoplasmic extraction reagent (Thermo). Protein was applied and separated on 4–12% NuPAGE gel (Invitrogen) and transferred to Immobilon™-P membranes (Millipore). The membranes were blocked with 5% non–fat dry milk and 0.1% tween–20 for 1 hour, followed by incubation with the primary antibodies (1:200–1,000 dilution) at 4°C overnight. Horseradish peroxidase–conjugated IgG (Santa Cruz Biosciences; 1:10,000) was used to treat the membranes for 1 hour, and enhanced with a SuperSignal® West Pico Chemiluminescent Substrate (Thermo). The bands were detected on BIOMAX MR films (Kodak). Each membrane was also stripped using a stripping buffer (Thermo) and reprobed with specific β-actin antibody to quantify the amount of loaded protein.

**IFN-γ and TNF-α synergistically accelerated apoptosis.** 0.5 × 10\(^6\) BMMSCs were seeded to 6–well culture plate and co–cultured with different doses of TNF-α (0, 5, 50,
100 ng ml\(^{-1}\)) with or without IFN-\(\gamma\) (50 ng ml\(^{-1}\)) for 1 day. Then, the BMMSCs were stained by Annexin V-PE apoptosis detection kit I (BD Bioscience) following manufacture’s protocol, and analyzed by FACS\textsuperscript{Calibur} (BD Bioscience).

**Cell survival assay for Trail neutralizing antibody and Fas ligand siRNA treated BMMSCs.** 0.5 \(\times\) 10\(^6\) BMMSCs were seeded to 6–well culture plate and co–cultured with recombined TNF-\(\alpha\) (20 ng ml\(^{-1}\)) and IFN-\(\gamma\) (50 ng ml\(^{-1}\)) for 3 days. Specific Trail neutralizing antibody (Cell Signaling Technology, 1 \(\mu\)g ml\(^{-1}\)) was added to culture medium. \textit{Fas L} siRNA and control siRNA (Santa Cruz Biosciences) were used to treat the BMMSCs according to the manufacture’s instruction. Cell viability was analyzed by using TC10\textsuperscript{TM} automated cell counter (Bio–Rad).

**Calvarial bone defect model in C57BL/6J mice.** The skin of C57BL/6J mice was cut and periosteum was elevated. The surface of mouse cavaorial bone was exposed, and oversize bone defects of 7 \(\times\) 8 mm were established.

**MicroCT analysis**

Calvarial bones were harvested and analyzed by using Inveon microCT system (Siemens AG, Germany). Two-dimensional images were analyzed by NIH Image J.

**Statistics.** SPSS 13.0 was used to do statistical analysis. Significance was assessed by independent two–tailed Student’s \(t\)–test or analysis of variance (ANOVA). The \(P\) values less than 0.05 were considered significant.
Supplementary Figure 1. Autologous BMMSC implantation failed to form bone in C57BL6 mice. (a) When BMMSCs were subcutaneously implanted into nude mice using hydroxyapatite tricalcium phosphate (HA/TCP) as a carrier for 8 weeks, bone (B) and bone marrow (BM) were regenerated around HA/TCP (HA) as shown by H&E staining. (b) When autologous BMMSCs were implanted into C57BL6 subcutaneously, there was no bone formation. Only connective tissue (CT) was found around HA/TCP (HA). Scale bar, 100 µm.
Supplementary Figure 2. CD4$^+$ T cell infusion in nude mice. (a–c) When CD4$^+$ T cells ($1 \times 10^6$) were infused into nude mice, flow cytometric analysis showed that the number of CD4$^+$ T cells were transient increased in bone marrow for 2–5 days (a). However, CD4$^+$ T cells maintained a high level in peripheral blood (b) and spleen (a) for more than one week. (d) Fluorescent immunocytostaining showed that PKH26 red Fluorescent labeled CD4$^+$ T cells (yellow triangles) were localized around BMMSC implant (yellow dot line) at 7 days post infusion. (*$P < 0.05$, **$P < 0.01$, $n = 5$). Scale bar, 50 $\mu$m.
Supplementary Figure 3. Subcutaneous BMMSC implantation in nude mice. Mouse BMMSCs ($4 \times 10^6$) were implanted into nude mice subcutaneously using 40 mg HA/TCP (HA) as a carrier. H&E staining showed connective tissue (CT) around HA/TCP (HA) at 4 and 7 days post implantation. Newly formed bone (B) around HA/TCP (HA) was observed at 14 days post implantation. Significant amount of bone (B) and bone marrow (BM) were generated at 21 days post implantation. Scale bar, 50 µm.
Supplementary Figure 4. Cells produced IFN-γ and TNF-α in BMMSC implants. (a) Littermate BMMSCs (4 × 10^6) were implanted into C57BL6 mice subcutaneously at indicated days. H&E and Giemsa staining showed that neutrophils (arrows) were presented in the implant tissue at 2 days post implantation. From 4 to 7 day post implantation, lymphocytes (arrows) were observed in the implant tissue. The number of inflammation cells were dramatically decreased after 14 days post implantation. (b) Fluorescent immunocytostaining showed that CD4^+ T cell produce IFN-γ (arrow), and both CD4^+ T cells and CD11b^+ macrophages produce TNF-α (arrow) around BMMSC implant (I). Scale bar, 50 µm (a), 25 µm (b).
Supplementary Figure 5. IFN-γ affected BMMSC-mediated bone formation and apoptosis. (a) Infusion of Pan T cells (1 × 10⁶) to nude mice at 2 days prior to BMMSC implantation (4 × 10⁶ with 40 mg HA/TCP), there was no bone formation. H&E staining showed connective tissue (CT) around HA/TCP (HA). (b) Infusion of IFN-γ deficient Pan T cells derived from IFN-γ null mice was not able to block BMMSC-mediated bone formation. H&E staining showed that newly formed bone (B) around HA/TCP (HA). (c) J-image semi-quantitative analysis showed the amount of bone formation. (⁎P < 0.01, n = 5). Scale bar, 100 µm. (d) The Flow cytometric analysis confirmed that the TNF-α at 0, 5, 50, 100 ng ml⁻¹ caused dose–dependent BMMSC apoptosis. Additional IFN-γ (50 ng ml⁻¹) could synergize TNF-α–induced apoptosis. (e) Statistic analysis showed that 50 ng ml⁻¹ IFN-γ significantly enhanced TNF-α–induced BMMSC apoptosis. (⁎P < 0.01, n = 4). (f) Western blot analysis showed that IFN-γ at 20, 50, 100 ng ml⁻¹ induced dose–dependent up–regulated expression of Fas in BMMSCs.
Supplementary Figure 6. IFN-γ and TNF-α treatment induced BMMSC apoptosis via caspase 3 and 8. (a) Fluorescent immunocyto staining showed that TNF-α (20 ng ml⁻¹) and IFN-γ (50 ng ml⁻¹) treatment induced cleaved caspase 8 (upper panel) and cleaved caspase 3 (lower panel) expression in BMMSCs. Scale bar, 50 μm. (b) The neutralizing antibody to Trail (1 μg ml⁻¹) and Fas ligand (Fas l) siRNA failed to block IFN-γ/TNF-α induced BMMSC apoptosis. (c) Western blot analysis showed that IFN-γ/TNF-α treatment did not affect expression levels of Fas L, TNFR1 and Trail. (d) Western blot analysis confirmed efficacy to siRNA in inhibiting TNFR2, IKK, FLIP, and XIAP.
Supplementary Figure 7. Schematic diagram of IFN-γ induced non-apoptotic pathway (a) and IFN-γ/TNF-α induced CD95 apoptotic pathway (b).
Supplementary Figure 8. Aspirin treatment reduced levels of IFN-γ and TNF-α in BMMSC implants. ELISA analysis showed that aspirin reduced levels of IFN-γ and TNF-α, but not for IL-10, in the BMMSC implants (BMMSC/HA + aspirin) when compared to untreated control group (BMMSC/HA) from 2 to 14 days post implantation.
Supplementary Figure 9. Aspirin treatment inhibited IFN-γ and TNF-α production in vitro. (a) Co-culture BMMSCs with activated T cells reduced the levels of IFN-γ when compared to activated T cell group. When aspirin was added to the co-culture system, the levels of IFN-γ were significantly reduced compared to BMMSC/T cell co-culture group. It appeared that 100 µg ml⁻¹ group showed marked reduction of IFN-γ than 25 µg ml⁻¹ and 50 µg ml⁻¹ group. (b) Co-culture BMMSCs with activated T cells reduced the levels of TNF-α when compared to activated T cell group. When aspirin was added to the co-culture system, the levels of TNF-α were significantly reduced compared to BMMSC/T cell co-culture group. It appeared that 100 µg ml⁻¹ and 50 µg ml⁻¹ group showed marked reduction of TNF-α than 25 µg ml⁻¹ group. (*P < 0.05, **P < 0.01, n = 5)
Supplementary Figure 10. Aspirin treatment improved BMMSC survival and bone regeneration in vivo. (a) When GFP positive BMMSCs were subcutaneously implanted for 30 days, the aspirin-treated group (50 µg ml⁻¹ aspirin pre-treated for 2 days, 100 µg aspirin co-implanted) showed significantly increased numbers of survival cells compared to untreated control group from 4 to 30 days post implantation. (b–g) BMMSCs were treated with 50 µg ml⁻¹ aspirin for 2 days and then combined with HA/TCP particles to be implanted into nude mice subcutaneously. The amount of new bone formation was increased in aspirin-treated group compared to untreated control group (b, c). Co-implantation of 200 ng IFN-γ and TNF-α with BMMSCs inhibited subcutaneous bone formation in nude mice (d, e), however, pre-treatment BMMSCs with 50 µg ml⁻¹ aspirin and co-implantation of 100 µg aspirin resulted in a significant rescuing of BMMSC-mediated bone formation (f, g). (h) Image J semi-quantitative analysis showed relative amount of bone formation. (*P < 0.05, **P < 0.01, n = 5). Scale bar, 50 µm (a), 100 µm (b).
Supplementary Figure 11. Schematic diagram of BMMSC/aspirin implantation to repair calvarial defects. (a) BMMSC/hydrogel/aspirin and BMMSC/gelfoam/aspirin were generated for implantation. (b) Calvarial defects were generated along the yellow dots in C57BL6 mice. (c) Implantation of BMMSC/hydrogel/aspirin complex to calvarial bone defects in C57BL6 mice. (d) Covered with BMMSC/gelfoam/aspirin. (e) The calvarial bone defect was sutured.