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Treating Titanium Particle-Induced Inflammation with Genetically Modified NF- κ B Sensing IL-4 Secreting or Preconditioned Mesenchymal Stem Cells in Vitro

Yusuke Kohno[†], Tzuhua Lin[†], Jukka Pajarinen[†], Eemeli Jämsen[‡], Monica Romero-Lopez[†], Masahiro Maruyama[†], Chi-Wen Lo[†], Masaya Ueno[†], Karthik Nathan[†], Zhenyu Yao[†], Stuart B. Goodman^{*,†,§}

[†]Department of Orthopaedic Surgery, Stanford University, Stanford, California 94063, United States [‡]Department of Medicine, Clinicum, University of Helsinki and Helsinki University Hospital, Helsinki 00029, Finland [§]Department of Bioengineering, Stanford University, Stanford, California 94305, United States

Abstract

Titanium and titanium-based alloys are widely used in orthopaedic implants. Total joint replacement is very successful; however, the foreign body response and chronic inflammation caused by implant-derived biomaterial debris still remain as unsolved issues. Aseptic loosening accompanied by wear debris-induced osteolysis (bone loss) is one of the most frequent causes for late failure and revision surgery. Mesenchymal stem cells (MSCs) and IL-4 may be possible treatment strategies because of their immunomodulatory properties. We investigated the efficacy of novel MSC-based treatments on immunomodulation and osteogenic differentiation in an innovative cell coculture model of titanium particle-induced inflammation in the periprosthetic tissues. MSCs and macrophages were collected from the bone marrow of Balb/c mice. Both MSCs and macrophages (representing endogenous cells at the periprosthetic tissue) were seeded on the bottom wells of the 24-well transwell plates. We generated genetically modified NF- κ B sensing IL-4 secreting MSCs (inflammatory responsive MSCs) and MSCs preconditioned by lipopolysaccharide and TNF- α to further enhance their immunomodulatory function. These modified MSCs (representing exogenous therapeutic cells implanted to the periprosthetic tissue) were seeded on the upper chambers of the transwell plates. These cocultures were then exposed to titanium particles for 7 days. NF- κ B sensing IL-4 secreting MSCs showed strong immunomodulation (significantly reduced TNF- α and induced Arg1 expression) and promoted early osteogenesis (significantly induced Runx2, ALP, and β -catenin as well as reduced Smurf2 expression) at day 7. IL-4 secreting MSCs also decreased TNF- α protein secretion as early as day 3 and increased IL-1ra protein secretion at day 7, suggesting efficacious immunomodulation of particle-induced inflammation. Preconditioned MSCs did not show significant immunomodulation in this short-term experiment, but ALP and β -catenin expression were significantly induced at day 7. Our results suggest that genetically modified IL-4 secreting MSCs and preconditioned MSCs

*Corresponding Author: goodbone@stanford.edu. Phone: +1-650-721-7662. Fax: +1-650-721-3470.

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have the potential to optimize bone regeneration in inflammatory conditions including periprosthetic osteolysis.

Graphical Abstract

Keywords

mesenchymal stem cell; macrophage; titanium; IL-4; immunomodulation; osteogenesis

INTRODUCTION

Long-term biocompatibility of joint replacements depends on initial integration between the bone and implant in an optimal alignment. Another key for success is to avoid subsequent adverse reactions to the byproducts of the implant.¹ Modern implants are generally very durable by facilitating initial osseointegration and functional stability; however, adverse reactions caused by implant-derived biomaterial debris from bearing surfaces and other interfaces still remain unsolved issues.¹ Aseptic loosening accompanied by osteolysis is one of the major reasons for late failure and revision surgery.²

Particles and other byproducts are continuously produced by wear between articulating surfaces. As for titanium (Ti) particles, they are generated from the bone–prosthesis interface or corrosion between modular components.^{3,4} In response to these byproducts, macrophages polarize to a classical inflammatory phenotype (M1 macrophages)⁵ and produce proinflammatory cytokines such as interleukin (IL)-1 β and tumor necrosis factor (TNF)- α ,^{1,6} this leads to further migration of macrophages or monocytes around the interface, which promotes osteoclastogenesis and osteoclast function.^{4,7} Furthermore, these cytokines increase receptor activator of nuclear factor- κ B ligand (RANKL) production, leading to increased differentiation of osteoclasts and osteolysis.^{8,9}

Macrophage polarization is greatly associated with inflammation induced by continuously generated wear particles and subsequent osteolysis.¹⁰ A persistent M1 macrophage-mediated reaction to wear particles is a chronic inflammatory condition, in which the transcription factor nuclear factor- κ B (NF- κ B) is continuously up-regulated.^{11,12} Mesenchymal stem cells (MSCs)¹³ and IL-4^{14,15} have great therapeutic potential because they can polarize proinflammatory M1 macrophages to a tissue-regenerative and anti-inflammatory phenotype (M2 macrophages),⁵ that has been shown to mitigate inflammation and could possibly increase the longevity of the implant. To further enhance the immunomodulatory function of MSCs, we have established NF- κ B sensing and IL-4 expressive lentiviral vectors, which could be transduced into mouse MSCs.¹² We previously reported enhanced immunomodulation of lipopolysaccharide (LPS)-induced inflammation by these genetically modified IL-4 secreting MSCs.¹²

Although chronic inflammation leads to impaired bone formation,¹⁶ acute inflammation is essential for successful osteogenesis.^{17,18} Previous studies have shown that MSC-mediated immunomodulation and osteogenesis could be facilitated by short-term exposure of inflammatory cytokines^{19,20} and bacterial endotoxin.²¹ On the basis of this idea, we treated MSCs with both LPS and TNF- α in expectation of a synergistic effect.²² Indeed, the MSCs preconditioned by LPS and TNF- α for 72 h enhanced immunomodulatory properties and osteogenic differentiation of MSCs.²²

Although possible interventions for the treatment and prevention of osteolysis have been presented, the critical immunomodulatory interactions between these modified MSCs and macrophages exposed to Ti particles, and the consequent osteogenic ability of the MSCs, remain unclear. In this study using an innovative cell coculture model of periprosthetic tissue, we investigated the efficacy of novel MSC-based treatments on immunomodulation and osteogenic differentiation by evaluating (1) M1/M2 macrophage polarization status, (2) secretion of proinflammatory and anti-inflammatory cytokines, and (3) early osteogenic markers.

MATERIALS AND METHODS

Isolation of MSCs and Macrophages from Mouse Bone Marrow.

Institutional guidelines were observed in all aspects of this project. MSCs and macrophages from mouse bone marrow were collected as previously described.^{12,22,23} Briefly, bone marrow cells were isolated from the femurs and tibias of male Balb/c mice aged 8–10 weeks. Regarding isolation of MSCs, the bone marrow cells were suspended in MSC culture medium [α -MEM (Life Technologies, Pleasanton, CA) supplied with 10% MSC certified fetal bovine serum (FBS; Life Technologies), and 1 % antibiotic-antimycotic (Life Technologies)]. It was filtered through a 70 μ m strainer and centrifuged. After aspiration of the supernatant, precipitated cells were resuspended in the MSC culture medium and plated into T-175 flasks. The medium was changed to remove nonadherent cells at day 1 (passage 1). The isolated MSCs (passage 4–8) were used for this study. Regarding isolation of macrophages, the bone marrow cells were suspended in macrophage culture medium [RPMI 1640 medium (Life Technologies) supplied with 30% L929 cell conditioned medium (LCM), 10% heat inactivated FBS (Life Technologies), 1% antibiotic-antimycotic, and 10 ng/mL mouse macrophage colony stimulation factor (M-CSF; R&D Systems, Minneapolis, MN)]. It was filtered through a 70 μ m strainer and centrifuged. After aspiration of the supernatant, precipitated cells were resuspended in the macrophage culture medium and plated into T-175 flasks (4×10^7 cells/flask). The medium was replaced at day 1 to remove unattached cells and every 3–4 days thereafter until the cells reach confluency.

Generating Method of NF- κ B Sensing IL-4 Secreting MSCs.

The lentiviral vector was prepared as described previously.^{23,24} The NF- κ B sensing and IL-4 secreting pCDH-NFKBRE-mIL4-copGFP vector¹² or the control pCDH-CMV-MCS-copGFP vector (System Biosciences, Palo Alto, CA), pMD2G VSV-G envelope vector, and psPAX2 packaging vector were cotransfected to human embryonic kidney 293T cells (ATCC, Manassas, VA) using the calcium phosphate transfection kit (Clontech, Mountain

View, CA) and 25 μM chloroquine. After dilution in the MSC culture medium supplied with 6 $\mu\text{g}/\text{mL}$ of Polybrene (Sigma-Aldrich, St. Louis, MO), the virus was infected into mouse MSCs at multiplicity of infection (MOI) of 40. The infectious efficiency of this method was previously confirmed.^{23,24}

Generating Method of Preconditioned MSCs.

MSCs were processed with the MSC culture medium containing LPS (20 $\mu\text{g}/\text{mL}$) and TNF- α (20 ng/mL) for 3 days.^{22,23} Then, the medium was replaced with MSC culture medium supplied with polymyxin B1 (30 $\mu\text{g}/\text{mL}$) to wash and inactivate the LPS.

Cell Proliferation of the Therapeutic MSCs.

Unmodified MSCs, preconditioned MSCs, and NF- κB sensing IL-4 secreting MSCs were seeded onto the 96-well plate (5×10^3 cells/well) and cultured in the MSC culture medium, which was changed to remove nonadherent cells at day 1. Cell proliferation of the therapeutic MSCs at day 1, 2, and 3 was measured using the MTS assay kit (Colorimetric) (Abcam, Cambridge, UK) following the instructions for use. Optical density (OD) at 490 nm was determined by SpectraMax M2e spectrophotometer (Molecular Devices, San Jose, CA).

Titanium Particles.

Pure Ti particles (Alfa Aesar, Ward Hill, MA) were used in this experiment. Their mean diameter was measured to be $3.7 \pm 1.8 \mu\text{m}$ using scanning electron microscopy.²⁵ The mean surface area was 43 μm^2 assuming that the Ti particles were spherical. Particles were sterilized following Ragab et al.'s protocol.²⁶ In short, the particles were put alternately in 25% nitric acid and 0.1 N NaOH in 95% ethanol for 20 h each at room temperature. Between the processes, Ti particles were washed with pH 7.4 phosphate-buffered saline (PBS; Corning, Corning, NY) twice. After 5 cycles of washes, Ti particles were resuspended in PBS (500 mg/mL) and stored at 4 °C (particle stock). LPS decontamination by this protocol was confirmed previously.⁴ Immediately before use, particle stock was vortexed and then diluted in cell culture medium at a concentration of 1.2 mg/mL. We chose the concentration according to preliminary experiments which evaluated the induction of inflammation and cytotoxicity after Ti particle stimulation using different concentrations.

MSC/Macrophage Coculture System.

The MSC/macrophage coculture is summarized in Figure 1. 1×10^4 unmodified MSCs were seeded on the bottom wells of the 24-well transwell plate (6.5 mm Transwell with 0.4 μm pore polycarbonate membrane insert; Corning) and incubated for 2 h. After attachment of the MSCs was checked, 5×10^4 primary macrophages were seeded on the same bottom wells (representing endogenous cells at the periprosthetic tissue) and incubated for 3 h. Subsequently, the medium was changed by mixed osteogenic culture medium [1:1 ratio of α -MEM and RPMI 1640 with 10% FBS, 5% LCM, 1% antibiotic-antimycotic, 1% L-glutamine (Corning), 10 mM β -glycerophosphate (MP Biomedicals, Santa Ana, CA), 50 μM L-ascorbic acid (Sigma-Aldrich), and 10 nM dexamethasone (Sigma-Aldrich)] containing 1.2 mg/mL Ti particles. Unmodified MSCs (MSC group), preconditioned MSCs (pMSC group), and NF- κB sensing IL-4 secreting MSCs (IL4MSC group) were seeded in the upper

chambers, respectively (representing exogenous therapeutic cells implanted to the periprosthetic tissue, 2×10^4 cells). For a control group, MSCs were not seeded in the upper chambers (no MSC group). This coculture was carried out for 7 days. The medium was changed at days 1 and 3.

Quantitative Real-Time PCR (qRT-PCR).

Cellular RNAs were extracted using RNeasy mini kit (Qiagen, Venlo, Limburg, Netherlands) at day 7. Complementary DNAs (cDNAs) were synthesized from the cellular RNAs using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). A reaction mix containing sample cDNA, one of the TaqMan primer probes [18s, TNF- α , arginase 1 (Arg1), runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), β -catenin, or SMAD-specific E3 ubiquitin protein ligase 2 (Smurf2)], and TaqMan gene expression master mix (Applied Biosystems) was prepared. Analysis was done with ABI 7900HT sequencing detection system (Applied Biosystems). 18s rRNA was used as an internal control. Gene expression level was determined by the $-C_t$ relative quantitation method.

Enzyme-Linked Immunosorbent Assay (ELISA).

The cell culture supernatants at day 1, 3, and 7 were collected and analyzed by ELISA using IL-4, TNF- α , and IL-1ra DuoSet ELISA kits (R&D Systems). The OD was determined using the plate reader set at 450 nm.

Immunofluorescent Staining.

MSCs and macrophages in the bottom wells were fixed at day 7 by 4% paraformaldehyde for 15 min, washed by PBS 3 times for 5 min each, and blocked by 5% normal serum and 0.3% Triton X-100 (Sigma-Aldrich) in PBS for 1 h. Primary antibody against β -catenin (Cell Signaling Technology, Danvers, Massachusetts, MA) was diluted 1/200 in antibody dilution buffer [1% bovine serum albumin (BSA) and 0.3% Triton X-100 in PBS] and added to the fixed cells followed by incubation overnight at 4 °C protected from light. After PBS rinse, fluorochrome-conjugated secondary antibody (Alexa Fluor 488 goat antirabbit IgG; Invitrogen, Carlsbad, CA) was diluted 1/200 in the antibody dilution buffer and added to the cells followed by incubation for 1 h at room temperature in the dark. After another PBS rinse, ProLong Gold antifade reagent with DAPI (Invitrogen) was mounted. The images were captured using a fluorescence microscope (Axio Observer.Z1; Carl Zeiss, Jena, Germany) in 3 randomly chosen fields of view, and the signal of β -catenin was quantified using ImageJ.²⁷

Statistical Analysis.

The no MSC, MSC, pMSC, and IL4MSC groups on the same time point were compared using one-way analysis of variance (ANOVA) with Tukey's posthoc test. All the experiments were performed in triplicate. Data are reported as mean $\pm$ standard deviations. All analyses were conducted using JMP Pro 13.0.0 (SAS Institute Inc., Cary, NC). A p -value <0.05 was selected as the threshold for statistical significance.

RESULTS

Cell Proliferation of Therapeutic MSCs.

Precondition by LPS and TNF- α increased cell proliferation of MSCs (Figure 2). The OD values of the pMSC group (0.43 ± 0.021 at day 1, 0.52 ± 0.043 at day 2, and 0.57 ± 0.015 at day 3, respectively) were significantly higher than those of the MSC (0.20 ± 0.006 , $p < 0.001$; 0.36 ± 0.005 , $p < 0.001$; and 0.44 ± 0.022 , $p = 0.017$; respectively) and IL4MSC groups (0.19 ± 0.009 , $p < 0.001$; 0.34 ± 0.010 , $p < 0.001$; and 0.43 ± 0.060 , $p = 0.009$; respectively).

Macrophage Polarization Status.

Proinflammatory marker transcription in macrophages (TNF- α) was significantly reduced in the IL4MSC group at day 7 compared to the no MSC group ($p = 0.032$) (Figure 3a); anti-inflammatory marker transcription in macrophages (Arg1) was significantly induced in the IL4MSC group at day 7 compared to the no MSC ($p = 0.002$), MSC ($p < 0.001$), and pMSC groups ($p < 0.001$) (Figure 3b). This indicates that macrophages are polarized to an M2 phenotype by NF- κ B sensing IL-4 secreting MSCs in the presence of Ti particles. Preconditioned MSCs did not significantly effect TNF- α expression at day 7 (Figure 3a).

Secretion of Proinflammatory and Anti-inflammatory Cytokines.

IL-4 protein secretion was significantly increased in the IL4MSC group over time (Figure 4a). The secretion levels at day 1, 3, and 7 were 5464 ± 491 , 571 ± 37 , and 37 ± 7 pg/mL, respectively. IL-4 protein secretion in all the other groups was below the detectable range of ELISA.

As expected, Ti particle stimulus induced a production of TNF- α . The IL4MSC group showed decreased TNF- α protein secretion at day 3 compared to the no MSC group ($p = 0.049$) (Figure 4b). All the treatment groups (the MSC, pMSC, and IL4MSC groups) showed significantly decreased TNF- α secretion at day 7 compared to the no MSC group (all $p < 0.001$). TNF- α secretion in the pMSC group was transiently increased at day 1 compared to the no MSC ($p = 0.005$), MSC ($p = 0.002$), and IL4MSC ($p < 0.001$) groups, but it gradually decreased afterward.

Activation of TNF- α induced the production of IL-1ra in all the groups at days 1 and 3, possibly as negative feedback to avoid an overwhelming inflammatory response (Figure 4c). At day 7, IL-1ra protein secretion decreased in the no MSC, MSC, and pMSC groups, but IL-1ra protein in the IL4MSC group was maintained at a significantly higher level than that in the no MSC ($p = 0.024$) and MSC ($p = 0.044$) groups.

Evaluation of Osteogenesis.

Transcription of the Runx2 in the IL4MSC group was significantly induced compared to that of the MSC group ($p = 0.034$), while that in the pMSC group did not show increased transcription (Figure 5a). Transcription of the ALP in the IL4MSC group was significantly induced compared to the no MSC ($p < 0.001$), MSC ($p = 0.002$), and pMSC groups ($p = 0.013$), and that in the pMSC group was also significantly induced compared to the no MSC

group ($p = 0.024$) (Figure 5b). Similarly, β -catenin, which positively regulates Runx2,²⁸ in the IL4MSC group was significantly induced compared to the no MSC ($p < 0.001$), MSC ($p < 0.001$), and pMSC ($p = 0.009$) groups, and that in the pMSC group was also significantly induced compared to that in the MSC group ($p = 0.034$) (Figure 5c). Smurf2, which is associated with β -catenin ubiquitination and degradation,²⁹ in the IL4MSC group was significantly reduced compared to that in the no MSC group ($p = 0.039$) (Figure 5d).

Protein expression of β -catenin was further examined by immunofluorescent staining (Figure 6a). The IL4MSC group showed a trend of increased protein expression of β -catenin compared to the no MSC ($p = 0.086$) and MSC groups ($p = 0.064$) (Figure 6b). The pattern of β -catenin protein expression was consistent with β -catenin gene expression.

DISCUSSION

Titanium and titanium-based alloys are used widely in orthopaedic implants because of their low elastic modulus, resistance to metallic fatigue, and biocompatibility.³⁰ Total joint replacement is very successful;³¹ however, all orthopaedic implants have a risk of wear during usage.³² In fact, “particle disease”³³ caused by polyethylene or Ti particles still remain as unsolved issues of total joint replacement.¹ Modulating the wear particle-induced adverse effects may be one treatment strategy to resolve the issue. In the current in vitro study, we examined the efficacy of novel MSC-based therapies on immunomodulation and osteogenic differentiation against Ti particle-induced inflammation. The results showed stronger immunomodulation and promoted early osteogenesis by NF- κ B sensing IL-4 secreting MSCs. Preconditioned MSCs did not show enhanced immunomodulation under the same conditions, but ALP and β -catenin expression were significantly induced.

Macrophage polarization status greatly affects the response of macrophages to Ti particles. Pajarinen et al.⁴ demonstrated that the inflammatory reaction to Ti particles was increased substantially in M1 macrophages and, contrastingly, inhibited in M2 macrophages compared with nonactivated M0 macrophages. This suggested that the foreign body reaction to Ti particles is mitigated by inducing anti-inflammatory M2 polarization.⁴ Genetically modified MSCs secreted IL-4 in response to inflammatory macrophages exposed to Ti particles; these events led to M2 polarization and mitigated inflammation by anti-inflammatory M2 macrophages. In addition, coordinated cross-talk of MSCs and macrophages via direct cell contact or paracrine regulation is necessary for successful tissue regeneration including bone.³⁴ Previous studies reported that MSC-mediated osteogenesis was promoted by M2 macrophages in vitro.^{35,36} The interaction between MSCs and M2 macrophages polarized by genetically modified MSCs may lead to accelerated osteogenesis.

Although unmodified MSCs alone did not show immunomodulation and early osteogenesis, NF- κ B sensing IL-4 secreting MSCs showed strong immunomodulatory function and subsequent early osteogenic marker expression in this in vitro study. According to previous reports, the direct effects of IL-4 on osteogenesis in vitro may be controversial.^{37–40} Our data did not clarify whether IL-4 exposure directly affects osteogenesis; however, the NF- κ B responsive IL-4, at least, did not have a negative effect on osteogenic marker expression at day 7. Mild inflammation induced by Ti particles was mitigated as early as day 3 by NF- κ B

sensing IL-4 secreting MSCs, which was accompanied by decreased IL-4 secretion over time because the secretion of IL-4 depends in part on NF- κ B up-regulation. This mechanism of regulating IL-4 secretion during only ongoing inflammation is very promising for clinical application, because it has a potential to minimize possible adverse effects of excessive IL-4 production including impairing bone formation,⁴¹ increasing infectious risk,^{42,43} inducing synovial hyperplasia,⁴⁴ and allergic reactions.⁴⁵

The observed IL-1ra differences could be helpful in clarifying the mechanism of the earlier bone regeneration. Ti particle-induced inflammation was associated with higher IL-1ra protein levels at days 1 and 3 in all the groups. However, despite mitigating inflammation at day 7, the IL4MSC group still demonstrated relatively higher amount of IL-1ra; this finding may be due to IL-4 because it is known to induce gene expression and secretion of IL-1ra.⁴⁶ Considering that IL-1ra is a natural inhibitor of IL-1 β , IL-1ra regulates various inflammatory immune responses and pathological processes,⁴⁷ thus contributing to earlier bone regeneration.

Surprisingly, immunomodulation was not found in the pMSC and MSC groups. A previous study demonstrated that MSCs may behave differently depending on specific Toll-like receptor (TLR) stimulus: TLR4 stimulus such as low-dose, short-term LPS applied to MSCs elicited a proinflammatory phenotype, whereas TLR3 elicited an immunosuppressive phenotype.⁴⁸ In our previous MSC/macrophage coculture model (no direct contact between MSCs and macrophages, no wear particles), preconditioned MSCs modulated the interferon- γ -induced proinflammatory response in macrophages and increased Arg1 expression.²² However, in the current model, MSC and macrophage were continuously exposed to the stimulus of Ti particles, which might interfere the immunomodulatory effects of the unmodified or preconditioned MSCs and lead to reduced Arg1 expression in macrophages. Nevertheless, the direct cross-talk between macrophages and MSCs as well as paracrine regulation by preconditioned MSCs may promote osteogenesis and enhanced ALP and β -catenin expression. Furthermore, TLR4 activation by LPS is reported to modulate the biology of MSCs and to protect MSCs from apoptosis induced by oxidative stress, leading to improved cell proliferation as well as prolonged survival.⁴⁹ The idea of MSC preconditioning with LPS and TNF- α ex vivo is to simulate environmental stress that mimics the early stages of fracture healing and thus primes and optimizes the MSCs tissue regenerative properties; this straightforward technique avoids the possible risk for genetic modification-associated cell transformation.²³

Several limitations of this study are acknowledged. First, we were not able to examine osteogenesis at a later stage due to cellular toxicity of prolonged Ti particle exposure in the current in vitro model. Further validation of these markers is warranted to determine the validity of the therapeutic MSCs. Second, the comprehensive in vitro experiments reported herein are “proof-of-principle” preclinical studies. For future translation into clinical use in inflammatory conditions including periprosthetic osteolysis, the therapeutic effects of these modified MSCs or the combined strategy will be further evaluated using translational in vivo models such as our murine continuous wear particle infusion model⁵⁰ in combination with transplantation of therapeutic MSCs.⁵¹

CONCLUSIONS

We performed an innovative cell coculture of Ti particle-induced inflammation and treated this with modified MSCs (genetically modified NF- κ B sensing IL-4 secreting MSCs and preconditioned MSCs), which represented exogenous therapeutic cells. Our results suggest that both strategies, especially NF- κ B sensing IL-4 secreting MSCs, optimize bone regeneration in chronic inflammatory conditions and have a potential clinical application to bone-related conditions including periprosthetic osteolysis.

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Figure 1.

Experimental outline of the MSC/macrophage coculture system. Lower compartment contains MSCs and macrophages (1:5 ratio), which represent endogenous cells, modeling the periprosthetic tissue. Upper compartment contains MSCs, preconditioned MSCs, NF- κ B sensing IL-4 secreting MSCs, or no MSCs, which represent exogenous therapeutic cells that have been implanted to the developing osteolytic lesions. The cells were cultured in mixed osteogenic culture medium contained 1.2 mg/mL titanium particles for 7 days.

Figure 2. Proliferation of the therapeutic MSCs was measured by the MTS assay at days 1, 2, and 3. All the samples were performed in triplicate. *** $p < 0.005$, ** $p < 0.01$, * $p < 0.05$.

Figure 3. Relative expression of the M1 phenotype marker: (a) TNF- α . The M2 phenotype marker: (b) Arg1. Determined by qRT-PCR at day 7. All the samples were performed in triplicate. *** p < 0.005, * p < 0.05.

Figure 4.

Amount of secreted (a) IL-4. Proinflammatory cytokine: (b) TNF- α . Anti-inflammatory cytokine: (c) IL-1ra. Measured by ELISA at days 1, 3, and 7. All the samples were performed in triplicate. *** $p < 0.005$, compared to the other groups on the same day. † $p < 0.05$, compared to the no MSC group at day 3. †† $p < 0.05$, compared to the no MSC and MSC groups at day 7.

Figure 5. Relative expression of osteogenic markers (a) Runx2, (b) ALP, (c) β -catenin, and (d) Smurf2, which is associated with β -catenin degradation and reduced osteogenesis, were determined by qRT-PCR at day 7. All the samples were performed in triplicate. *** $p < 0.005$, ** $p < 0.01$, * $p < 0.05$.

Figure 6.

Immunofluorescent staining of β -catenin (green). Cell nuclei were counterstained with DAPI (blue). (a) The staining of the IL4MSC group at day 7. (b) The staining signal of β -catenin was quantified in 3 randomly selected fields of view using ImageJ.