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NF κ B Sensing IL-4 Secreting Mesenchymal Stem Cells Mitigate the Proinflammatory Response of Macrophages Exposed to Polyethylene Wear Particles

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Abstract

Total joint replacement (TJR) is a highly effective treatment for patients with end-stage arthritis. Pro-inflammatory macrophages (M1) mediate wear particle-associated inflammation and bone loss. Anti-inflammatory macrophages (M2) help resolve tissue damage and favor bone regeneration. Mesenchymal stem cell (MSC)-based therapy mitigates the M1 dominated inflammatory reaction and favorably modulates the bone remodeling process. In the current study, the immunomodulating ability of 1) unmodified MSCs, 2) MSCs preconditioned by NF κ B stimulating ligands (lipopolysaccharide plus TNF α), and 3) genetically modified MSCs that secrete IL-4 as a response to NF κ B activation (NF κ B-IL4) was compared in a macrophage/MSC co-culture system. Sterile or LPS-contaminated ultra-high molecular weight polyethylene (UHMWPE) particles were used to induce the pro-inflammatory responses in the macrophages. Contaminated particles induced M1 marker expression (TNF α , IL1 β , and iNOS), while NF κ B-IL4 MSCs modulated the macrophages from an M1 phenotype into a more favorable M2 phenotype (Arginase 1/Arg1 and CD206 high). The IL4 secretion by NF κ B-IL4 MSCs was significantly induced by the contaminated particles. The induction of Arg1 and CD206 in macrophages via the preconditioned or naïve MSCs was negligible when compared with NF κ B-IL4 MSC. Our findings indicated that NF κ B-IL4 MSCs have the “on-demand” immunomodulatory ability to mitigate wear particle-associated inflammation with minimal adverse effects.

Keywords

wear particles; inflammation; macrophage polarization; mesenchymal stem cell; IL4; preconditioning

Introduction

Total joint replacement (TJR) is a highly effective treatment for patients with end-stage arthritis¹. Macrophages recognize wear particles generated from the bearing surfaces of implanted devices through pattern-recognition receptors such as toll-like receptors², and induce a pro-inflammatory reaction and subsequent bone loss (periprosthetic osteolysis)³. Revision surgery is required for patients with progressive bone loss, pain, or implant loosening.

The macrophage polarization status is highly relevant in wear particle-associated inflammation and bone loss⁴. Classical activation of macrophages (M1 phenotype) by interferon γ and/or lipopolysaccharide (LPS) induces a pro-inflammatory response that includes increased TNF α , IL1 β , and iNOS production. Alternative activation of macrophages (M2 phenotype) by IL4 or IL13 induces anti-inflammatory and tissue regenerative properties marked by increased arginase 1 (Arg1) and CD206 expression⁵. Wear particles, such as ultra-high molecular weight polyethylene (UHMWPE) and titanium particles, induce pro-inflammatory M1 marker expression in macrophages^{6,7}. Modulation of the pro-inflammatory M1 macrophage reaction into a more favorable anti-inflammatory M2 phenotype has promising potential to mitigate the sequela of particle disease⁸.

Mesenchymal stem cells (MSCs) have direct tissue regenerative and immunomodulatory properties, and have been shown to directly to regulate macrophage function⁹. Due to these properties, MSC-based therapy has been applied in more than 400 clinical trials including bone regeneration and chronic inflammatory diseases¹⁰. The biological responses of MSCs to inflammatory stimulation could further enhance the immunomodulatory and tissue regenerative abilities of MSC-based therapy^{11–13}. Indeed, by simulating the pro-inflammatory environment, we found that MSCs preconditioned with TNF α and LPS synergistically enhanced osteogenic capacity¹³. Expression of Arg1 and CD206 in macrophages co-cultured with the preconditioned MSCs was increased. In addition, we generated genetically modified MSCs that produce IL4 only in response to inflammatory stimulation and NF κ B activation, thus creating an “on-demand” targeted drug delivery system¹⁴. These novel strategies have a potential to mitigate chronic inflammatory diseases including periprosthetic osteolysis.

In the current study, the therapeutic potential of preconditioned or genetically modified (NF κ B-IL4) MSCs in the wear particle-associated inflammatory response was evaluated in an in vitro macrophage/MSC co-culture system (Fig. 1a). The co-culture system was designed to model the clinical application of therapeutic MSCs, with macrophages being first exposed to the particle stimulus followed by introduction of therapeutic MSCs. The pro-inflammatory and anti-inflammatory marker expression in macrophages exposed to sterile or contaminated polyethylene particles was examined at the transcriptional and translational levels.

Materials and Methods

Isolation of mouse bone marrow derived MSCs and macrophages

The method of isolating mouse bone marrow derived MSCs and macrophage has been described previously^{6,15}. In brief, bone marrow was collected from the femurs and tibias of 8–10 weeks old Balb/c male mice. Institutional Animals Care and Use Committee (IACUC) guidelines for the care and use of laboratory animals were observed in all aspects of this project. For MSC isolation, the bone marrow cells were carefully suspended and passed through a 70µm strainer, spun down, and resuspended in α -MEM (Thermo-Scientific, Fremont, CA) supplied with 10% MSC certified (with enhanced clonal expansion efficiency) fetal bovine serum (FBS, Invitrogen, Fremont, CA) and antibiotic antimycotic solution (Thermo-Scientific). The media was replaced the next day with fresh media to remove the unattached cells (passage 1). The isolated MSCs between passage 4–8 were used to conduct the experiments. For macrophage isolation, the bone marrow cells were washed 3 times with culture media (RPMI1640 medium supplemented with 10% heat inactivated FBS, and the antibiotic/antimycotic solution), re-suspended in the culture medium containing 30% of L929 cell conditioned medium and 10ng/ml mouse macrophage colony stimulation factor (M-CSF, R&D, Minneapolis, MN), and re-plated in T-175 culture flasks at a concentration of 4×10^7 cells per flask. Cells were allowed to expand for 5–7 days, with a medium change at the second day to remove non-adherent cells.

UHMWPE Particles

Ceridust 3610 polyethylene particles were washed and resuspended by ethanol, filtered through a 20µm pore membrane to eliminate the larger particles. The filtered particle size ($4.62 \pm 3.76\mu\text{m}$, presented as mean $\pm$ standard deviation) was examined by an electron microscope in the Cell Science Image Facility at Stanford University. (Suppl. Fig.1a). The particles were vacuumed dried for 3 days, weighed, and resuspended in PBS containing 5% bovine serum albumin. The contaminated particles were supplemented with 10ng/ml lipopolysaccharide (LPS, Sigma-Aldrich St. Louis, MO) based on a previous study¹⁶. The sterility of the particles was confirmed by the endpoint chromogenic Limulus Amebocyte Lysate assay (Lonza, Portsmouth, NH)

Generation of NF κ B sensing IL4 secreting MSC

The lentiviral vector preparation was performed as previously described¹⁸. Human embryonic kidney 293T cells (ATCC, Manassas, VA) were used to co-transfect the NF κ B sensing IL4 secreting pCDH-NF κ BRE-mIL4-copGFP expressing lentivirus vector¹⁴ or the control vector pCDH-CMV-MCS-copGFP (System Biosciences, Palo Alto, CA), psPAX2 packaging vector, and pMD2G VSV-G envelope vector using the calcium phosphate transfection kit (Clontech, Mountain View, CA) with 25µM chloroquine. The virus was diluted in MSC culture medium supplemented with 6µg/ml of polybrene (Sigma Aldrich), and infected to murine MSCs at multiplicity of infection (MOI) = 40. The infection efficiency (number of GFP+ cells) was confirmed by a LSRII flow cytometer (Stanford Shared FACS Facility) 4 days post-infection.

Generation of the preconditioned MSC

MSCs were treated with 20ng/ml TNF- α and 20 μ g/ml lipopolysaccharide (LPS) for 3 days¹³. MSC growth medium supplemented with 30 μ g/ml polymyxin B1 was used to wash the preconditioned MSCs to inactivate the LPS.

Macrophage/MSC co-culture system

A summary of the experimental strategy is illustrated in Fig.1a & 1b. Primary macrophages were seeded in the bottom well of the transwell plate (0.4 μ m polycarbonate membrane, Corning). The cells were allowed to attach, and after 2 hours of incubation, the medium was replaced by fresh medium only or contained 0.125% sterile or LPS-contaminated polyethylene particles. The upper chambers were seeded with unmodified MSCs, preconditioned MSCs, NF κ B-IL4 secreting MSCs, or a control group without MSCs. This co-culture was carried out in macrophage growth medium for 24 or 72 hrs. Macrophage viability and polarization status were analyzed as described in the following sections.

Picogreen cell quantification assay

DNase/RNase free water (500 μ l) was added to the bottom chambers containing macrophages. Cellular DNA was released by three freeze-thaw cycles. To quantify the numbers of viable macrophages, 50 μ l of Picogreen dye (Life Technologies, Grand Island, NY) was mixed with 50 μ l samples and fluorescence was read at 480/520nm using a plate reader (Spectramax M2e, Molecular Devices, Sunnyvale, CA).

RNA extraction and quantitative PCR

Cellular RNAs were extracted by using the RNeasy RNA purification kit (Qiagen, Valencia, CA). RNAs were reverse transcribed into complementary DNA (cDNA) using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Probes for 18s rRNA, TNF- α , IL-1 β , iNOS, Arginase 1, and CD206 were purchased from Applied Biosystems. Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed in an ABI 7900HT Sequencing Detection System (Applied Biosystems, Waltham, MA), using the 18s rRNA as the internal control. The 2^{-Ct} relative quantitation method was used to evaluate gene expression level.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) kits for IL4 were purchased from eBioscience. TNF α DuoSet ELISA kits were purchased from R&D Systems. Manufacturers' protocols were followed carefully. The optical densities were determined using a Bio-Rad 3550-UV microplate reader (Bio-Rad, Hercules, CA) set at 450 nm.

Immunofluorescent staining

Macrophages in the bottom well of the transwell plate were fixed in 4% paraformaldehyde for 10 minutes, washed by PBS, and permeabilized by 0.5% Triton-X 100 in PBS for 10 minutes. The fixed cells were washed with PBS and blocked with 1% BSA-PBS for 30 min before staining. Primary antibodies against CD206 (1 μ g/ml, APC-conjugated, monoclonal rat anti-mouse IgG2a κ ; BioLegend, San Diego, CA), Arginase 1 (5 μ g/ml, unconjugated,

polyclonal rabbit anti-mouse IgG; Abcam, Cambridge, United Kingdom) or iNOS (1 μ g/ml, FITC-conjugated monoclonal mouse anti-mouse IgG2a; BD) were diluted in 1% BSA-PBS to the indicated concentrations and then applied to the cells followed by incubation for 1h at room temperature and protected from light. For Arginase 1 staining, the secondary antibody (PE-conjugated; polyclonal Anti-rabbit IgG; Life Technologies) diluted 1/200 in 1% BSA-PBS was applied to the cells and incubated for 1h at room temperature and protected from light, followed by washing with PBS 3 $\times$ for 5 min and mounted with ProLong Gold with DAPI (Life Technologies, Fremont, CA). The images were captured using a fluorescence microscope (Axio Observer 3.1, Zeiss, Oberkochen, Germany) in 3 randomly selected fields of view, and the signals of each marker were quantified by using NIH ImageJ.

Statistical analysis

A one-way ANOVA and Dunnett's posthoc tests were conducted using Prism 7 (GraphPad Software, San Diego, CA). The sterile and contaminated PE particle treated groups were compared to the corresponding controls without particle exposure. The control MSCs, preconditioned MSCs, and NF κ B-IL4 MSCs co-culture groups were compared to the corresponding control groups without MSCs in the transwell system. All the experiments were done in triplicate. Data were reported as mean $\pm$ standard deviation. $P < 0.05$ was chosen as the threshold of significance.

Results

Quantification of macrophage viability in the co-culture system

The macrophage/MSC co-culture system was set up as illustrated in Fig. 1a. Co-culture with MSCs did not change macrophage viability in the untreated groups. Unmodified MSCs increased macrophage viability at 72 hrs in the presence of the sterile particles (Fig. 1c). Unmodified or preconditioned MSCs reduced macrophage viability at 72 hrs in the presence of contaminated particles, compared to the untreated controls; no significant differences were observed in the no MSC or NF κ B-IL4 MSC groups ($p = 0.086$ and $p = 0.48$, respectively, Fig. 1c). No significant differences were found among the treatment groups exposed to the contaminated particles.

NF κ B-sensing IL4-secreting MSCs decreased TNF α , IL1 β , and iNOS expression in macrophages

Pro-inflammatory marker transcription in macrophages (TNF α , IL1 β , and iNOS) was significantly induced by exposure to contaminated particles (Fig. 2). TNF α transcription was only transiently induced at 24 hrs and reduced to basal levels at 72 hrs (Fig. 2a), while the induction of IL1 β and iNOS transcription remained at a higher level until 72 hrs (Fig. 2b & c). The immunomodulating effects of the MSCs in the upper chamber were compared to the control group with no MSCs. Unmodified MSCs increased TNF α , IL1 β , and iNOS transcription in macrophages at 24 hrs in the presence of contaminated particles. The preconditioned MSCs reduced TNF α transcription but increased iNOS transcription at 24 hrs. At 72 hrs, IL1 β transcription was increased, and a trend for reduction in TNF α transcription was observed ($p = 0.059$, Fig. 2a). NF κ B-IL4 MSCs reduced TNF α transcription in macrophages at 24 hrs in the presence of contaminated particles, and at 72 hrs, regardless

of particle exposure. NF κ B-IL4 MSC decreased the transcription of IL1 β at 72 hrs and iNOS at 24 and 72 hrs in the presence of contaminated particles (Fig.2b & c).

We further examined the expression of other pro-inflammatory factors, including IL6 and MCP1, in the co-culture system and found the expression was not affected by NF κ B-IL4 MSC (data not shown). TNF α protein secretion was significantly induced by exposure to the contaminated particles, and no significant differences were observed when macrophages were co-cultured with unmodified, preconditioned, or IL4 secreting MSC at 24 and 72 hrs (Fig.3).

NF κ B sensing IL4 secreting MSCs increased anti-inflammatory marker expression in macrophages in response to inflammatory stimulation

IL4 secretion was only found in NF κ B-IL4 MSC co-culture groups, and the secretion was induced from basal levels (100–150ng/ml) to a significantly higher level (>5,000ng/ml) when exposed to contaminated particles (Fig.4a). Transcription of the anti-inflammatory M2 markers Arg1 and CD206 was increased when exposed to contaminated particles, which is likely regulated by a protective feedback mechanism (Fig.4b & c). The induction of Arg1 and CD206 in NF κ B-IL4 MSC co-culture groups were comparable at 24 hrs in the control, particle, and contaminated particle groups, and significantly higher at 72 hrs in the contaminated particle group. The induction of Arg1 and CD206 in the preconditioned MSC co-culture groups in the control and sterile particle groups was negligible when compared together with NF κ B-IL4 MSC groups (Fig.4b & c).

Protein expression of anti-inflammatory markers was further examined by immunofluorescence staining (Fig.5a). Arg1 (orange) and CD206 (red) expression in macrophages were significantly increased when co-cultured with NF κ B-IL4 MSC at 24 and 72 hrs, and the induction was further increased in the particle (CD206, 24 hrs) and contaminated particle groups (Fig.5b & c). When macrophages were co-cultured with preconditioned MSCs, Arg1 expression was significantly increased at 72 hrs in the control and sterile particle groups (Fig.5c). iNOS expression (green) was too weak to make representative quantifications using ImageJ.

Discussion

We report that genetically modified NF κ B-IL4 MSC modulated pro-inflammatory macrophages (TNF α /IL1 β /iNOS high) induced by contaminated PE particles into an anti-inflammatory phenotype (Arg1/CD206 high) using an in vitro transwell co-culture system. The immunomodulatory ability was more efficient compared to the use of preconditioned MSCs or unmodified MSCs. The results suggest that NF κ B-IL4 MSCs may have great potential to mitigate wear particle-associated inflammation.

We previously demonstrated that UHMWPE particles recovered from joint simulators (particle size $0.43 \pm 0.104 \mu\text{m}$, provided by Dr. Timothy Wright from the Hospital for Special Surgery, New York¹⁷.) induced a pro-inflammatory response and bone loss using several in vitro and in vivo models (Supplementary Fig.1b & c)^{6,19,20}. The manufactured UHMWPE particles (Ceridust 3610) only induced pro-inflammatory marker expression

when contaminated with endotoxin (Fig.2 & 3). The differences in the particle-induced inflammation between the simulator particles and Ceridust could be due to the size, shape, and the surface characteristics of the particles²¹. Undiagnosed low-grade bacterial contamination has recently been described in some revised implants thought previously to be aseptically loose^{22–27}. Furthermore, endotoxin released from intestinal flora or dental procedures to the circulation could bind to wear particles and induce an inflammatory reaction^{28,29}. Therefore, particles contaminated with low levels of LPS might simulate the condition of patients with aseptic loosening without laboratory or clinically diagnosed infection.

Transient induction of pro-inflammatory marker expression (TNF α , IL1 β , and iNOS) was found in macrophages co-cultured with unmodified MSCs exposed to the contaminated particles for 24 hrs compared to the control group without MSCs (Fig.2 & 3). Waterman et al. has demonstrated that MSCs primed with low dose toll-like receptor (TLR) 4 ligands (10ng/ml LPS) exhibited an inflammatory phenotype (MSC1), while MSC primed with TLR3 ligand (1 μ g/ml poly I:C) exhibited an anti-inflammatory phenotype (MSC2)³⁰. Our data showed that MSCs react to different environmental cues in distinct ways. Therefore, strategies such as preconditioning or genetic modification of MSCs for cell-based therapies could be applied to generate specific immunosuppressive phenotypes.

Crosstalk between immune cells and osteoprogenitor-lineage cells could determine the status of bone regeneration^{31–33}. Acute inflammation is essential for the bone regeneration. Depletion of macrophages at an earlier stage suppressed bone repair in a murine model³¹. On the other hand, chronic inflammation negatively regulates bone regeneration and increases bone loss²⁰. We have demonstrated that adding IL4 at day 3 to day 4 enhanced osteogenesis in MSC³⁴ or MC3T3 osteoprogenitors co-cultured with macrophages³². Our data showed that NF κ B-IL4 MSCs inhibited TNF α transcription at 24 (by 67%) and 72 (by 92%) hrs, but the inhibition at the protein secretion level was not significant at 24 (p=0.50) and 72 (p=0.09) hrs (Fig.3). The inhibition of TNF α by NF κ B-IL4 MSCs with persistent NF κ B activation will be clarified in a chronic inflammatory disease model.

Local delivery of IL4 mitigated inflammation-associated bone loss and enhanced bone regeneration in murine periprosthetic osteolysis models^{35,36}. The development of controlled-release mechanisms is crucial for translational applications and may avoid potential adverse effects of IL4^{37–39}. The NF κ B-IL4 MSC-based cell therapy significantly improved immunomodulation and minimized adverse effects. The crosstalk between MSCs and M2 macrophages could potentially further enhance bone regeneration.

The induction of M2 marker expression (Arg1 and CD206) in macrophages by the preconditioned MSCs was negligible when compared with NF κ B-IL4 MSC (Fig.4). Nevertheless, the strategy of MSC preconditioning remains valuable in the application of inflammatory bone diseases. Transient induction of an acute inflammatory signal is crucial for successful bone regeneration and protective immunomodulation by MSCs. However, unresolved chronic inflammation is associated with continuous tissue damage and osteoclast activation. MSCs preconditioned by LPS and TNF α ex vivo simulate environmental stress and induce endogenous protective mechanisms. This simple procedure avoids the potential

risk of genetic modification-mediated cell transformation. In addition, the induction of osteogenesis in the preconditioned MSCs was found *in vitro*¹³, which was not observed with IL4 secreting MSCs¹⁴.

In conclusion, NF κ B-IL4 MSC has shown an “on-demand” immunomodulatory ability to mitigate the pro-inflammatory response and subsequent tissue damage. The therapeutic potential of NF κ B-IL4 MSCs in periprosthetic osteolysis or other chronic inflammatory diseases will be further examined in translational *in vivo* models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

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Fig.1. Endotoxin-contaminated polyethylene particle decreased viable macrophage numbers in the macrophage/MSC co-culture system.
a) Experimental outline of the macrophage/MSC co-culture system. The illustrations of macrophages and MSCs are modified from the Motifolio scientific illustration toolkits. **b)** Summary of the experimental layout. **c)** Quantification of viable macrophage numbers after 24 and 72 hrs in the co-culture system. # $p < 0.05$ compared to the corresponding untreated control. * $p < 0.05$ compared to the corresponding No MSC control.

Fig.2. NF- κ B sensing IL4 secreting MSCs suppressed TNF α , IL1 β , and iNOS expression in macrophages exposed to the contaminated polyethylene particles.

The mRNA expression of a) TNF α , b) IL1 β , and c) iNOS in the macrophages after 24 and 72 hrs in the co-culture system were examined by quantitative PCR. Data was normalized to the untreated macrophage group co-cultured without MSC. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.005$ compared to the corresponding untreated control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ compared to the corresponding No MSC control.

Fig.3. Quantification of TNFα secretion in the macrophage/MSC co-culture system exposed to sterile or contaminated polyethylene particles.

The supernatants of the co-culture system at 24 (**a**) and 72 (**b**) hrs were collected, and the secretion of TNFα was quantified by ELISA. ### p<0.005 compared to the corresponding untreated control.

Fig.4. Modulation of anti-inflammatory M2 marker expression in macrophages via co-cultured with the preconditioned or NFκB sensing IL4 secreting MSC.

a) The supernatants of the co-culture system at 24 and 72 hrs were collected, and the secretion of IL4 was quantified by ELISA. The mRNA expression of **b)** Arginase 1 (Arg1) and **c)** CD206 in the macrophages after 24 and 72 hrs in the co-culture system was examined by quantitative PCR. qPCR data was normalized to the untreated macrophage group co-cultured without MSC. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.005$ compared to the corresponding untreated control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ compared to the corresponding No MSC control.

Fig.5. Immunofluorescent staining of M1/M2 polarization markers in macrophage co-cultured with or without MSCs.

The expression of iNOS (green), Arginase 1 (Arg1, orange), and CD206 (red) in the macrophages co-cultured with MSCs or controls. Cell nuclei were counterstained with DAPI (blue). (a) The staining result of macrophage co-cultured with NFκB-IL4 MSC. (b & c) Quantification of the staining result at 24 (b) and 72 (c) hrs using ImageJ. # $p < 0.05$, ### $p < 0.005$ compared to the corresponding untreated control. * $p < 0.05$, *** $p < 0.005$ compared to the corresponding No MSC control.