ANIMAL MODELS

Macrophage Polarization in IL-10 Treatment of Particle-Induced Inflammation and Osteolysis

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This study investigated the therapeutic influence and potential mechanism of IL-10 in ameliorating orthopedic debris particle-induced inflammation and osteolysis. A murine air pouch with bone implantation and polyethylene particles was also used to evaluate the therapeutic effects of IL-10. The data suggested that the particle challenges significantly promoted macrophage activation and osteoclastogenesis, with dramatically increased macrophage infiltration into the pouch membranes and elevated tartrate-resistant acid phosphatase–positive cell deposition. Immunohistochemical stains revealed a significantly higher ratio of induced nitric oxide synthase–expressing cells in the particle-challenged group; treatment with IL-10 resulted in marked switching to CD163⁺ cells. Also, IL-10 effectively reduced tartrate-resistant acid phosphatase–positive stained cells in the pouch membranes, and minimized the bone mineral density loss compared with untreated samples. Real-time PCR and Western blot examination indicated that IL-10 treatment significantly diminished the particle-induced IL-1 β expression but promoted expression of CD163, transforming growth factor- β 1, and CCR2. Furthermore, IL-10 significantly inhibited the ultra-high-molecular-weight polyethylene particle-elevated phospho-STAT1 and phospho-NF- κ B p65 productions, and promoted phospho-STAT3 expression. Overall, the data indicate the pivotal effects of IL-10 on macrophage polarization. The effects of IL-10 in ameliorating local inflammation and osteolysis may be associated with macrophage polarization through the up-regulation of the Janus activating kinase/STAT3 signaling pathway, and the down-regulation of NF- κ B and Janus activating kinase/STAT1 expression. (*Am J Pathol* 2016, 186: 57–66; <http://dx.doi.org/10.1016/j.ajpath.2015.09.006>)

Total joint replacement is an effective procedure for treatment of end-stage disabling arthritis,¹ although a subgroup of the implant recipients do develop aseptic loosening, which has become the most common long-term complication.² Ultra-high-molecular-weight polyethylene (UHMWPE) has been widely used as a biomaterial in total joint prosthesis, and the UHMWPE components often show wear at revision surgery.³ It has been widely accepted that the production of wear debris may be the predominant causative agent in periprosthetic osteolysis.⁴ In the process of periprosthetic osteolysis, macrophages activated by wear particles through phagocytosis drive an active biological cascade.⁵

In recent decades, the functions of macrophages have been generally recognized. Macrophages, derived from monocyte precursors, are a versatile cell population that commits to a variety of phenotypes in response to different local tissue

environments.⁶ Monocytes from bone marrow are distributed into the blood circulation system and migrate to most tissues within the body, where they can differentiate to mature macrophages.⁷ A subset of macrophages reside in special organs, such as liver Kupffer cells, brain microglia, lung alveolar macrophages, spleen macrophages, intestinal macrophages, peritoneal macrophages, adipose tissue macrophages, atherosclerotic plaque foam cells, and bone osteoclasts.^{6,8,9} Because heterogeneity and plasticity are hallmarks of macrophages, the concept of macrophage polarization has been established.^{10,11} Two major macrophage subsets [classically activated/inflammatory (M1) macrophages and

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alternatively activated/anti-inflammatory (M2) macrophages] have been categorized on the basis of their distinct functionalities.^{12–15} Pathogen- and local environment–derived stimuli, such as interferon- γ , lipopolysaccharide, and tumor necrosis factor- α , often induce the M1 macrophage phenotype, whereas M2 macrophages are induced by IL-4 or IL-13 (M2a subtype), immune complexes (M2b subtype), IL-10, or transforming growth factor (TGF)- β (M2c subtype). This macrophage polarization mirrors the type 1 helper T-cell (TH1)/TH2 polarization and mediates the TH1/TH2 immune responses, respectively.^{16–19} Studies have suggested that the mechanism of M1-associated gene induction is associated with the activation of STAT1, NF- κ B, phosphoinositide 3-kinase, and mitogen-activated protein kinases (MAPKs).^{15,16,20} On the other hand, IL-4 and IL-13 lead to M2 phenotype polarization by activating the STAT6, followed by nuclear translocation and M2-associated gene induction.^{5,15,16} Evidence from *in vitro* and *in vivo* studies has demonstrated that IL-10 activates STAT3 to trigger M2-like macrophage polarization.^{5,16,17} Moreover, macrophages can switch from an activated M1 state back to M2 phenotype or vice versa,^{21,22} which might suggest a novel therapeutic strategy applicable to several diseases.^{19,23–26}

The objective of the current study was to examine the mechanism of IL-10 in alleviating UHMWPE particle-induced macrophage activation, local inflammation, and periprosthetic osteolysis, using a characterized bone-embedding murine air pouch model.

Materials and Methods

Animals and Materials

All animal and surgical procedures were approved by the Institutional Animal Care and Use Committee (Wichita State University, Wichita, KS). Female BALB/c mice, aged 8 to 10 weeks, were obtained from Jackson Laboratories (Bar Harbor, ME) and quarantined in our facility for 2 weeks before experimentation. All mice weighed 20 g or over at the start of the experiment. UHMWPE particles were generated using a hip simulator and generously provided by Dr. John Cuckler (University of Alabama, Birmingham, AL). The size of particles ranges from 0.4 to 10.8 μ m. The mean diameter of particles and the mean aspect ratio of particles were 2.66 μ m and 0.984, respectively. For *in vitro* testing, poly(methyl methacrylate) (PMMA) particles sized from 0.1 to 10 μ m from a commercial source (Polyscience, Warrington, PA) were used to challenge macrophages. Before use, the particles were washed in 70% ethanol to eliminate endotoxin and examined for endotoxin contamination using a Limulus Amoebocyte Lysate assay kit (Endosafe; Charles River, Charleston, SC). Murine IL-10 protein was purchased from Pepro Tech, Inc. (Rocky Hill, NJ). All primers were obtained from Life Technologies Corp. (Grand Island, NY). Rabbit anti-mouse–induced nitric oxide synthase (iNOS or NOS2) antibody and goat anti-mouse CD163 antibody were purchased from Santa Cruz

Biotechnology, Inc. (Dallas, TX). Anti-phospho-STAT1 (Y701; 58D6), anti-STAT1 (D4Y6Z), anti-phospho-STAT3 (Tyr 705; D3A7), anti-STAT3 (79D7), anti-phospho-stress-activated protein kinase/Jun N-terminal kinase (JNK; Thr 183/Tyr 185; 98F2), anti-phospho-NF- κ B p65 (Ser 536; 93H1), anti-NF- κ B p65 (D14E12) rabbit monoclonal antibodies, and anti-stress-activated protein kinase/JNK polyclonal antibody were purchased from Cell Signaling Technology (Danvers, MA). Secondary antibodies were obtained from Vector Laboratories, Inc. (Burlingame, CA). Real-time PCR reagents were purchased from Life Technologies Corp.

In Vitro Study

BMDMs Induced by IL-10

Primary bone marrow–derived macrophages (BMDMs) were obtained from the femurs and tibiae of Sprague-Dawley rat donors. The bone marrow was flushed into a 50-mL centrifuge tube with sterile Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Invitrogen, Grand Island, NY), 100 U/mL penicillin (Invitrogen), and 100 μ g/mL streptomycin (Invitrogen). The cells were filtered with a 100- μ m cell strainer and spun down, followed by addition of the cold red blood cell lysis buffer (Invitrogen). After centrifugation, the cells were cultured with 30% L929 cultural supernatant and 70% Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics in a 12-well plate at the concentration of 5×10^5 cells per well. After 24 hours, cells were divided into three groups and respectively challenged with Dulbecco's modified Eagle's medium (negative control group), 2 mg PMMA particles in 0.2 mL phosphate-buffered saline (PBS; positive group), or PMMA particles plus 20 ng IL-10 (treatment group). After 5 days of treatment, total RNA was extracted and real-time RT-PCR was used to detect the gene expression level of M1 or M2 markers. For immunocytochemical assay, BMDMs were seeded into eight-chamber slides.

Immunocytochemical Staining

BMDMs (5×10^3 cells per well) were challenged with PMMA or PMMA plus IL-10 for 1 week in eight-chamber slides. Paraformaldehyde (4%) was applied for 15 minutes to fix BMDMs, and 0.5% Triton X-100 was used to increase cell membrane permeability. To eliminate the non-specific staining, all slides were incubated with 3% hydrogen peroxide for 15 minutes at room temperature and blocked with 5% serum for 30 minutes at room temperature. Then, the pretreated slides were incubated with primary antibodies (2 μ g/mL) at 4°C overnight and biotinylated secondary antibodies (1 μ g/mL) at room temperature for 30 minutes, followed by biotinylated horseradish peroxidase complex labeling and diaminobenzidine (Sigma Chemicals, St. Louis, MO) visualization. All slides were sealed with mounting medium and coverslipped after counterstaining with hematoxylin and dehydrating with series of ethanol.

Table 1 Primers Used for Real-Time RT-PCR Amplification

Target	Forward primer	Reverse primer	Species
18S	5'-TGCGGAAGGATCATTAACGGA-3'	5'-AGTAGGAGAGGAGCGAGCGACC-3'	Rat
IL-1 β	5'-CCTTGTGCAAGTGTCTGAAGC-3'	5'-CTCCACGGGCAAGACATAGG-3'	Rat
CD80	5'-TTACAGTTGCCAGCTGATGC-3'	5'-GCAATGCCCTTTCTCTCACC-3'	Rat
MHC II	5'-GTGATGCACCTGCTTACGAA-3'	5'-TCCAGGGAGTTCTTGCTCAT-3'	Rat
CD163	5'-GATGTGGCTCTGCACTTCAA-3'	5'-ATGGGATTTCTCTCCAACC-3'	Rat
IL-10	5'-CGACGCTGTCATCGATTTCTC-3'	5'-CAGTAGATGCCGGGTGGTTC-3'	Rat
TGF- β 1	5'-GACCGCAACAACGCAATCTA-3'	5'-TTCCGTCTCCTTGGTTCAGC-3'	Rat
18S	5'-CGGCTACCACATCCAAGGAA-3'	5'-GCTGGAATTACCGCGGCT-3'	Mouse
STAT1	5'-GTGGTTCACCATTTGTTGCAG-3'	5'-AATGAGCTGCTGGAAGAGGA-3'	Mouse
NF- κ B p65	5'-AGAAGTCTGGGCGCACAGCG-3'	5'-TGGTGGACCTCTTCCGGCC-3'	Mouse
STAT3	5'-TCCTTTCCCACTTGACCTTG-3'	5'-TTGCTGAGGGCCTGTAGTCT-3'	Mouse
STAT6	5'-ATGGGACAATGCCTTCTCTG-3'	5'-TGAGGCTGTTGTCGTTGAAG-3'	Mouse
SHIP	5'-AGGTTCTTCACGAAGCTGGA-3'	5'-CCGGCAGACATAGGAATGTT-3'	Mouse
JNK1	5'-AGAAACTGTTCCCGATGTG-3'	5'-TGATGTATGGGTGCTGGAGA-3'	Mouse
PPAR- γ	5'-AAGAGCTGACCAATGGTTG-3'	5'-ACCCTTGCATCCTTCACAAG-3'	Mouse

JNK, Jun N-terminal kinase; MHC, major histocompatibility complex; PPAR, peroxisome proliferator-activated receptor; SHIP, Src homology-2 domain-containing inositol 5-phosphatase; TGF, transforming growth factor.

Murine Air Pouches

Air pouches were established in female BALB/c mice according to a method described previously.²⁷ The dorsal area of BALB/c mouse was cleaned with povidone-iodine/alcohol and shaved to prepare the pouch site, followed by 3 mL of filtered air injection into s.c. tissue with a 25-gauge needle on a 5-mL syringe. Filtered air (1 mL) was given every other day to maintain the air pouch. On the sixth day, a femur section (0.5 cm, from the head to the shaft), or a fragment of calvarium (0.6 $\times$ 0.3 cm), from genetically identical mouse donors, was surgically placed into the pouch. The pouch layer and skin incisions were closed using 5-0 absorbable sutures, and sterile phosphate-buffered saline (0.1 mL) containing 1:100 penicillin/streptomycin (Gibco BRL, Gaithersburg, MD) was injected into the pouch. After 24 hours, mice were divided into three groups (eight mice per group) to respectively receive injections into the pouches of 0.2-mL PBS (negative control group), 2-mg UHMWPE in 0.2-mL PBS (positive control group), or UHMWPE suspension (2 mg/0.2 mL) plus 20 ng IL-10 (treatment group). IL-10 or PBS vehicle was repeatedly injected into an individual pouch every other day. All mice were euthanized 7 days after the first IL-10 injection, and the bone-implanted pouch membranes were harvested. A portion of air pouch membrane with the intact bone tissue was either snap frozen (calvarium implanted) for crystalized division into sections or fixed in 10% buffered formalin (femur implanted) for paraffin embedding. The remainder of the air pouch membrane was stored for molecular analyses at -80°C .

Histological Evaluation

Formalin-fixed pouches with implanted bone were decalcified in 10% EDTA for 7 days before paraffin embedding

and dividing into sections (6 μm thick). The tissue sections were stained with hematoxylin and eosin for morphology analysis. For immunohistochemical examination, unstained slides were deparaffinized and rehydrated, and antigens were retrieved in sodium citrate (pH 6.0). To reduce non-specific staining, all slides were incubated with 3% hydrogen peroxide for 15 minutes at room temperature and blocked with 10% serum for 60 minutes at room temperature. Then, the pretreated slides were incubated with primary antibodies (2 $\mu\text{g/mL}$) at 4°C overnight and biotinylated secondary antibodies (1 $\mu\text{g/mL}$) at room temperature for 1 hour, followed by horseradish peroxidase complex labeling and diaminobenzidine (Sigma Chemicals) visualization.²⁸ Histochemical tartrate-resistant acid phosphatase (TRAP) staining was performed on frozen-sectioned calvarial pouch sections to examine mature osteoclastic cells

Figure 1 Real-time RT-PCR was performed to determine the gene expression profile changes of the bone marrow cells after poly(methyl methacrylate) (PMMA) particle challenges and IL-10 treatment. The data were adjusted by calibrators (particle-free control group) after normalization with the expression of housekeeping gene 18S. ** $P < 0.01$. MHC, major histocompatibility complex; TGF, transforming growth factor.

using a commercial kit (Sigma Chemicals). Cryosections were fixed in cold-buffered acetone for 30 seconds, followed by incubation at 37°C for 1 hour in 100 mmol/L acetate buffer (pH 5.2), containing 0.5 mmol/L of naphthol AS-BI phosphoric acid, 2.2 mmol/L of Fast Garnet GBC base (Sigma Chemicals), and 8 mmol/L sodium tartrate.

Image Analysis

The images of various stained tissue sections were examined under a Nikon Eclipse E800 light microscope (Melville, NY), and digital images were captured and analyzed using Image-Pro Plus software version 7.0 (Media Cybernetics, Silver Spring, MD). Air pouch membrane thickness, cellular infiltration, and macrophage percentage were quantified on hematoxylin and eosin-stained tissue sections, as described previously.²⁹ To evaluate the protein expression of M1 marker (iNOS) and M2 marker (CD163), $\times 100$ magnified images of immunohistochemical-stained pouch membrane encapsulating femur implants were analyzed. Integrated optical densities (IODs) of the areas inside the inflammatory pouch membranes that interacted with particles were quantified and normalized

with IODs obtained from the negative control tissue sections. Each tissue section provided six pairs of IOD reading at different regions, and at least six sections per group were analyzed to ensure normalization. On account of the blurred boundaries of TRAP-positive osteoclasts in frozen sections, and the difficulty in separating individual cells, IODs of TRAP-positive osteoclastic cells in the inflammatory membranes were recorded³⁰ and compared by the image analysis system among groups.

Microcomputerized Tomography Scans

Microcomputerized tomography scanning was conducted at 24 hours after surgery and at sacrifice using a SCANCO microCT system (vivaCT 40; SCANCO Medical, Brüttisellen, Switzerland). The parameters were 30- μ m isotropic voxel size, 400 projections, 200-millisecond exposure time, 70-kW voltage, and 114- μ A current. The image data were analyzed with microCT Evaluation program software version V6.5-1 (SCANCO Medical) to generate three-dimensional images and focal bone mineral density. The bone volume/total volume ratio was also calculated to assess total bone loss.

Figure 2 Immunocytochemical staining was used to assess the protein expression of induced nitric oxide synthase (iNOS; A–C) and CD163 (D–F) in three groups: particle free (control group; A and D), poly(methyl methacrylate) (PMMA) particle challenge (positive group; B and E), and PMMA particles + IL-10 (treatment group; C and F). G and H: Quantitative image analysis data of iNOS and CD163. * $P < 0.05$, *** $P < 0.001$. Original magnification, $\times 200$ (A–F). IOD, integrated optical density.

Gene Expression Profile Assessment

Cells from *in vitro* studies and the bone-pouch membranes from animal studies were homogenized on ice using a glass homogenizer or polytron PT-MR2100 (Brinkmann Instruments, Inc., Westbury, NY), for total RNA isolation. Real-time RT-PCR was then performed to determine mRNA expression profiles among groups, as detailed previously.²⁸ Briefly, cDNA was reverse transcribed from 1 μ g of total RNA in a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA). Gene expression levels of M1 macrophage markers (IL-1 β and CD80), M2 markers (IL-10, CD163, and TGF- β 1), and many associated cell signaling molecules [STAT1, NF- κ B p65, STAT3, STAT6, JNK1, Src homology-2 domain-containing inositol 5-phosphatase (SHIP), and peroxisome proliferator-activated receptor (PPAR)- γ] were determined by real-time RT-PCR using a StepOnePlus Real-Time PCR System (Applied Biosystems). With the Ct value of 18S as an internal control and the mean Ct value of target gene expression from control group (bone implant + PBS) as the calibrator samples (named 0% expression change), the comparative quantification of target gene expression was quantified according to the formula provided by the manufacturer's manual. Primers for all target genes are listed in Table 1.

Western Blot Analysis Assay

Bone-pouch membranes from animal study were homogenized on ice using polytron PT-MR 2100 homogenizer and lysed in 500- μ L lysis buffer containing 50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1% Nonidet P-40, 1 mmol/L EDTA, 1 mmol/L NaF, 1 mmol/L Na₃VO₄, 0.25% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, and complete protease inhibitor cocktail (Roche, Grenzach, Germany). The concentration of total protein was determined using BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). Then, 30 μ g proteins were loaded and 10% SDS-PAGE was performed, and the proteins were transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% bovine serum albumin for 2 hours at room temperature. Primary antibodies were applied overnight at 4°C, followed by horseradish peroxidase—labeled secondary antibody incubation for 2 hours at room temperature. Protein bands were visualized using enhanced chemiluminescence reagent (Sterling Pierce Co, East Rockaway, NY).

Statistical Analysis

All mice were randomly assigned to three experiment groups with eight animals in each group. The data from three

Figure 3 Typical histological appearance of the bone-implanted air pouches with phosphate-buffered saline (negative control; **A**), ultra-high-molecular-weight polyethylene (UHMWPE) particles (positive control; **B**), and UHMWPE particles + IL-10 (treatment; **C**). The average thickness of air pouch membranes (**D**), the mean cell counts within the pouch membranes (**E**), and the macrophage infiltration (**F**) among groups. *** $P < 0.001$. Original magnifications: $\times 40$ (main images, **A–C**); $\times 200$ (insets, **A–C**).

independent experiments were collected for this study. For statistical analysis of data, one-way analysis of variance and *t*-tests were performed using the SPSS 19.0 software package (SPSS, Chicago, IL). $P < 0.05$ was considered statistically significant. Data were presented as the means $\pm$ SEM.

Results

Gene Expression Change of M1 and M2 Markers *in Vitro*

Real-time RT-PCR was performed to detect the gene expression change of M1 and M2 markers after cells were challenged with PMMA or PMMA plus IL-10. IL-10 dramatically provoked the up-regulation of CD163, IL-10, TGF- β , and CCR2 ($P < 0.01$) and the down-regulation of IL-1. There was no significant difference in the gene expression of CD80 and major histocompatibility complex II between groups (Figure 1).

Immunocytochemical Staining Assay

BMDMs were challenged with PMMA particles, followed by the treatment of IL-10. Immunocytochemical staining revealed that the PMMA particles provoked the BMDMs to differentiate to an M1 phenotype, resulting in an increased expression of iNOS ($P < 0.001$) (Figure 2, B and G). Meanwhile, IL-10 treatment weakened the polarization effect of PMMA particles, confirmed by a dramatic decrease in iNOS expression ($P < 0.001$) (Figure 2, C and G). Also, IL-10 treatment facilitated the overexpression of CD163, which implied that BMDMs differentiated into M2 after the IL-10 treatment ($P < 0.001$) (Figure 2H).

Air Pouch Membrane

The histological appearance of air pouch membranes differs among the three groups (Figure 3, A–C). The introduction of UHMWPE particles significantly provoked local inflammation, resulting in dramatic increases in membrane thickness and cellular infiltration compared with control pouch membranes. The mean thickness of control pouch membranes was 125.8 ± 5.58 μ m, whereas UHMWPE particle-challenged air pouch membranes increased to 449.81 ± 10.59 μ m ($P < 0.001$) (Figure 3D). Also, the cell counts in the pouch membranes were elevated from 4749 ± 144 cells/mm² (controls) to 6564 ± 144 cells/mm² (UHMWPE particle-challenged, $P < 0.001$) (Figure 3E). Although the ratio of round-nucleated inflammatory cells/elongated fibrocytes was higher in particle-challenged pouch membranes than in control samples (Figure 3F), it appears that there was a significant decrease of the total cell infiltration in the IL-10 treatment group (5674 ± 139 cells/mm², $P < 0.001$) compared with the UHMWPE group (Figure 3E).

Function of IL-10 in M2 Polarization and Osteoclastogenesis

We selected iNOS (M1 marker) and CD163 (M2 marker) to verify macrophage polarization after the treatment of IL-10,

using an immunohistochemical staining technique. Histological assessment of the femur-bearing pouch membranes among the groups indicated that both iNOS- and CD163-positive macrophages were significantly concentrated in the membranes challenged with UHMWPE particles compared with naïve pouch membranes ($P < 0.01$) (Figure 4). However, it appeared that treatment with IL-10 resulted in fewer iNOS-expressing cells when compared with control or UHMWPE-challenged samples ($P < 0.05$) (Figure 4A). On the other hand, there was a significantly higher portion of CD163⁺ cells in the IL-10-treated air pouch membranes in comparison with the UHMWPE-particle controls ($P < 0.05$) (Figure 4B).

TRAP staining was performed to investigate whether IL-10 mitigated the UHMWPE-induced osteoclastogenesis. Significantly decreased TRAP⁺ cells appeared in the sections from the IL-10-treated group, whereas samples from UHMWPE particle controls exhibited strong TRAP activities, consistent with previous observations²⁷ ($P < 0.001$) (Figure 5, A–C and G). Microcomputerized tomography scanning demonstrated that osteolysis was ameliorated by IL-10 treatment (Figure 5, D–F, H, and I), and bone mineral density in the treatment group was significantly preserved compared with that in the positive group ($P < 0.05$).

Figure 4 A computerized image analysis system with Image Pro+ software version 6.0 was used on immunohistochemistry sections to quantify induced nitric oxide synthase (iNOS)-positive (A) and CD163⁺ (B) cells among experimental groups. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. UHMWPE, ultra-high-molecular-weight polyethylene.

Figure 5 Typical tartrate-resistant acid phosphatase (TRAP) staining images of bone-embedded pouches among groups: phosphate-buffered saline negative control (A), ultra-high-molecular-weight polyethylene (UHMWPE)—positive control (B), and UHMWPE particles with IL-10 treatment (C). D–F: Examples of microcomputerized tomography (MicroCT) isosurface images of the implanted calvarial bone among groups. G and H: Quantification of TRAP⁺ staining (G), bone mineral densities (H), and bone fraction volume/total volume (I). **P* < 0.05, ****P* < 0.001. Original magnification, ×200 (A–C). IOD, integrated optical density.

(Figure 5H). Meanwhile, significantly higher bone volume/total tissue volume was observed after IL-10 treatment (*P* < 0.05) (Figure 5I).

Selected Molecular Signaling Pathways after IL-10 Treatment

Real-time RT-PCR was performed to examine the change in gene expression of STAT1, NF-κB p65, STAT3, STAT6, JNK1, SHIP, and PPAR-γ after IL-10 treatment. Figure 6 summarizes the effects of IL-10 on the gene expression associated with M1 or M2 polarization when compared with the UHMWPE particle controls. The data demonstrated that IL-10 not only diminished the gene expression of STAT1 (*P* < 0.05), NF-κB p65 (*P* < 0.05), and JNK1 (*P* < 0.01), but also increased the STAT3 gene expression (*P* < 0.05). There was no

statistically significant difference in gene expression levels of STAT6, SHIP, and PPAR-γ between the two experimental groups (Figure 6).

Western Blot Analysis Assay

To examine IL-10 involved signaling pathways at the protein level, Western blot analysis was performed for phospho-STAT1, phospho-STAT3, phospho-NF-κB p65, and phospho-stress-activated protein kinase/JNK. Specimens from the IL-10 treatment group exhibited decreased production of phospho-STAT1 and phospho-NF-κB p65 compared with the non-treated UHMWPE-challenged group (Figure 7). Interestingly, besides elevated phospho-STAT3 expression being observed in the IL-10-treated group, the total STAT3 protein expression appeared higher in this group compared with others (Figure 7).

Figure 6 Selected molecular signaling pathways were explored by real-time RT-PCR on ultra-high-molecular-weight polyethylene (UHMWPE) particle-challenged pouch membranes to reveal the gene expression changes after IL-10 treatment. The data were adjusted by calibrators (phosphate-buffered saline control group) after normalization with the expression of housekeeping gene 18S. * $P < 0.05$, ** $P < 0.01$. JNK, Jun N-terminal kinase; PPAR, peroxisome proliferator-activated receptor; SHIP, Src homology-2 domain-containing inositol 5-phosphatase.

Discussion

Macrophages play a pivotal role in innate and adaptive immunity through engulfing bacteria and other microbes and secreting various inflammatory molecules.³¹ Recent studies have found that the functions of macrophages are also involved in anti-inflammation, tissue remodeling/repair, neovascularization, atherosclerosis, insulin resistance, spinal cord injury prognosis, and tumor development.^{26,31–33} Macrophages are broadly categorized into classic M1 macrophages and M2 macrophages according to their different functions. M1 macrophages contribute to killing microorganisms and the production of proinflammatory cytokines, such as tumor necrosis factor- α , IL-6, and IL-12.^{15,34} In contrast, M2 macrophages produce anti-inflammatory cytokines (IL-10, TGF- β , and IL-1 receptor antagonist) that reduce inflammatory and adaptive TH1 responses.

Aseptic loosening of the prosthetic joint component has become the most common long-term complication after total joint replacement surgery.² It is generally agreed that wear debris-associated local inflammation and consequent periprosthetic osteolysis play critical roles in the initiation and pathogenesis of aseptic loosening.^{4,5} Wear debris particles generated at the interface between the implant component and surrounding bone can stimulate macrophages, lymphocytes, and fibrocytes, which drives the biological cascade of local inflammation and bone resorption through production of proinflammatory molecules.⁵ Anti-inflammatory cytokines, such as IL-10 and IL-4, may regulate immune and inflammatory responses and act as negative mediators to control local inflammation. Although

the direct effects of IL-10 or IL-4 on bone remodeling are not fully understood, evidence has shown that treatment with IL-10 or IL-4 *in vitro* or on various animal models has suggested strong inhibition and protective effects in particle-induced inflammation, osteoclastogenesis, and osteolysis.^{27,35} The group of Goodman and colleagues^{1,12,36} has indicated that PMMA particles can polarize macrophages to the M1 phenotype, and the process can be rescued by IL-4. Our previous studies demonstrated that IL-10 alleviated the local inflammation and bone resorption driven by UHMWPE particles in the mouse air pouch models.^{27,37} The current study extends these findings to suggest that IL-10 polarizes the infiltrating macrophage to the M2 phenotype, and the cell then secretes anti-inflammatory cytokines to inhibit the inflammation process. UHMWPE particles were used to provoke the local inflammation and bone resorption on the murine air pouch model, as previously reported.³⁰ However, we used PMMA particles as inflammation initiators in our *in vitro* studies because of the facts that PMMA particles are also common wear debris at the cemented bone-implant interface and the technical difficulty to have UHMWPE particles in contact with cells in culture media (floating nature of polyethylene particles). Nevertheless, both types of particles significantly promoted the cell inflammatory response and osteoclastogenesis, as evidenced herein and in previous studies.^{28,29} Consistent with our previous findings,³⁷ the data showed that IL-10 treatment dramatically reduced the UHMWPE particle-induced pouch membrane thickness, decreased the inflammatory cellular infiltration, and diminished the number of TRAP⁺ osteoclastic cells at the bone-membrane interface. The current study further revealed that there was a significantly diminished iNOS expression and an increase in CD163 expression after IL-10 treatment. Although many types of cells are capable of expressing inducible nitric oxide synthase (iNOS), it is recognized that M1 macrophages up-regulate the transcription of iNOS in response to cytokines, endotoxin, and phagocytosis.³⁸ Overexpression of iNOS in our samples suggested a predominant M1 macrophage population. We selected CD163 as an M2 macrophage marker because of its distinct expression in

Figure 7 Signaling pathways were examined by Western blot analysis to reveal the protein production changes of phospho-STAT1, phospho-NF- κ B p65, phospho-stress-activated protein kinase (SAPK)/Jun N-terminal kinase (JNK), and phospho-STAT3 with or without IL-10 treatment. Protein specimens were isolated from the inflammatory membranes, and 30 μ g of total protein was loaded onto 10% SDS-PAGE gels. UHMWPE, ultra-high-molecular-weight polyethylene.

mouse M2 phenotype macrophages.³⁹ It is apparent that the increased CD163 expression and diminished iNOS after IL-10 treatment support an M1/M2 phenotype shifting. The immune system works as a process of dynamic balance in the body. Although there is dominant presence of M1 macrophages during the particle-induced inflammation, many M2 macrophages may also accumulate at the local inflammation site regulating the cascade. Therefore, relatively elevated CD163⁺ cells were also observed in the UHMWPE group compared with the particle-free control group (Figure 2).

Real-time RT-PCR data indicated that mRNA expression levels of STAT1, NF- κ B p65, and JNK1 were inhibited and STAT3 was promoted by IL-10 in the particle-stimulated murine air pouch models. Western blot analysis experiments further examined some of the native and phosphorylated signal transduction proteins on IL-10—treated specimens, which complemented the PCR results. The transcriptional and translational data were in agreement with previous findings that the macrophage polarizations are likely mediated by the NF- κ B, the Janus activating kinase/STAT, and the MAPK signaling pathways.^{4,5,15} The JNK signaling pathway belongs to the superfamily of MAPKs, which involves many cellular events.^{40,41} Recent *in vitro* and *in vivo* studies demonstrated that PPAR- γ up-regulates STAT6 expression and down-regulates NF- κ B expression.^{15,21} SHIP, an anti-inflammatory protein, can convert phosphatidylinositol 3,4,5-trisphosphate (PIP3) to phosphatidylinositol 3,4-bisphosphate [PI(3,4)P2] to inhibit phosphoinositide 3-kinase—dependent signaling and negatively regulate NF- κ B activity.¹⁵

In addition, TRAP staining revealed a dramatically reduced TRAP⁺ cell population among tissue specimens of the IL-10—treated group compared with the controls, suggesting a potent therapeutic effect of IL-10 in the blockage of osteoclastogenesis.

Limitations of this study include the short experimental time used to examine the levels of bone resorption among groups, and the significance of safety issues using IL-10 intervention on tumorigenesis. This study did not verify the optimal injection time point and dosage of IL-10. The dosage of IL-10 was determined according to the dosage of IL-4 administrated in previous studies.¹² The study focused on the M1/M2 phenotype alteration of systemic macrophages; however, future investigations are required to explore cell trafficking and the molecular pathogenesis of the wear debris—associated aseptic loosening. In the future, extended markers of the M2 phenotype will be used to define different phenotypes of macrophages within the inflammatory tissues.

Conclusions

This study demonstrates key effects of IL-10 on the process of macrophage polarization in the murine air pouch model

challenged by UHMWPE particles. Besides the direct up-regulation of Janus activating kinase/STAT3 signaling, IL-10 may inhibit the activation of NF- κ B p65, Janus activating kinase/STAT1, and MAPK signaling pathways.

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