Inflamm. Res. (2009) 58:837–844
DOI 10.1007/s00011-009-0051-5

Inflammation Research

ORIGINAL RESEARCH PAPER

Circulating blood monocytes traffic to and participate in the periprosthetic tissue inflammation

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Received: 2 September 2008 / Revised: 27 April 2009 / Accepted: 11 May 2009 / Published online: 30 May 2009

Abstract
Objective To examine the trafficking of human circulating blood monocytes and their influence on the inflammation of periprosthetic tissues using a novel mouse–human chimera model.
Methods Periprosthetic tissue and bone chips from patients with aseptic prosthetic loosening were implanted into the muscles of immune-deficient SCID mice depleted of host macrophages by periodic intraperitoneal injection of anti-asialo GM1 rabbit sera (ASGM1). Autologous patient peripheral blood monocytes (PBMCs) were labeled with PKH2 fluorescent dye and injected intraperitoneally into the implanted animals. Mice were sacrificed 14 days after PBMC transfusion for molecular and histological analyses.
Results Patient periprosthetic tissues were well tolerated in SCID mice and preserved a high level of viability. Cell trafficking studies revealed the accumulation of fluorescent PBMC within the xenografts, with total cell counts in the xenografts significantly increased following the systemic PBMC infusion. PBMC infusion also promoted the expression of IL-1, IL-6, TNFα, and RANK within the periprosthetic tissue.
Conclusion Systemic PBMC migrated to the implanted periprosthetic tissues and contributed to the local inflammation. The data provide evidence that circulating blood monocytes may play a role in pathologic process during aseptic loosening of total joint replacement.

Keywords Cell migration · Inflammatory models · Macrophages

Responsible Editor: A. Bauhofer.

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Introduction

Total joint replacement is an effective procedure for the treatment of end-stage arthritis. It is estimated that approximately 0.5 million procedures are performed each year [1]. However, there is a relatively high incidence of aseptic loosening of the prosthetic joint component within 15–20 years [2], making this the single most common long-term complication of joint arthroplasty. Periprosthetic pseudo-membranous tissues can be retrieved from the interface between the implants and the surrounding bone during revision surgery. Histological evaluations of these tissues has revealed that cellular composition included macrophages, foreign body giant cells, lymphocytes, plasma cells, and fibroblasts [3, 4]. Polymeric and/or metallic particulate wear debris are ubiquitously present in the tissues, frequently engulfed or in close contact with macrophages [4, 5]. Cumulative evidence has indicated that particulate debris generated from mechanical

wear of prosthetic components plays a critical role in the process of aseptic loosening and osteolysis [4]. It is generally accepted that wear debris provokes biological tissue responses, including vascularized granulomatous tissue formation along the implant-to-bone interface, inflammatory cells (macrophages, lymphocytes) influx, bone resorption, osteolysis, and finally loss of prosthesis fixation [6–8]. Studies have shown that the circulating peripheral blood monocytes (PBMC) are among the first cells to colonize the inflammatory site [9, 10]. However, the interaction and molecular mechanisms that attract the circulating blood monocytes to home to the prosthetic joint and form the periprosthetic pseudo-membranous tissue remain unclear.

We recently developed a mouse–human (SCID-Hu) chimera model to study the role of human periprosthetic tissue in aseptic osteolysis [11]. This SCID-Hu chimera utilizes the severe combined immunodeficient (SCID) mouse as a host that may be transplanted with human periprosthetic inflammatory tissues and bone to establish a "live" tissue reservoir [11, 12]. In order to monitor the human peripheral blood monocytes (PBMCs) trafficking in this SCID-Hu model, we adapted a cell labeling technique using PKH dyes (PKH2) [13]. The objective of this study was to test the hypothesis that the systemic hematopoietic cells (PBMC) respond to the wear debris stimulation and traffic to periprosthetic tissue. The successful outcome of this study would establish a preclinical model to investigate the source of function of mononuclear cells in the periprosthetic tissue, and provide insights on the initiation of osteoclastogenesis and osteolysis.

Materials and methods

Animals

Thirty-two female severe combined immunodeficient (SCID) mice (CB17-Prkdc SCID; Jackson Laboratory, Bar Harbor, Maine) aged 3–4 weeks were quarantined in a pathogen-free environment for at least 1 week before experimentation.

Establishment of the mouse–human chimera model

The study was approved by the Institutional Review Board and informed consent was obtained from all patients before retrieval of the patient tissues. The institutional Animal Investigation Committee approved all of the experimental procedures on mice. Human periprosthetic tissue and adjacent bone at obvious osteolytic sites (confirmed by pre-operative radiographs) were obtained from patients undergoing revision surgery due to aseptic knee or hip prosthetic component loosening. The underlying disease was osteoarthritis for all the donor patients and no infectious or auto-immune diseases were diagnosed at the time of revision surgery. The mouse–human chimera model was established as described previously [11], with minor modifications. Briefly, SCID mice were anesthetized with a mixture of 7.5 mg/kg Xylazine (Akorn, Decatur, IL) and 90 mg/kg Ketamine (Bioniche Pharma USA, Lake Forest, IL). Under strict sterile conditions, the periprosthetic tissue was diced into tissue cubes of 3×5 mm^3 and patient cancellous bone chips were cut into $1 \times 1 \times 2$ mm pieces. The human tissues were surgically embedded into the left quadriceps and the paravertebral muscles of SCID mouse. Similar pieces of the patient tissues were snap-frozen and stored at −80°C as pre-implantation controls. It is generally recognized that the inflammatory granulomatous tissues selected for implantation contained numerous wear debris particles and inflammatory cells [11]. Since periprosthetic tissue usually appeared heterogeneous in composition, attention was paid to avoid obvious fibrotic or fatty tissues. The mice were randomly divided into three groups: (1) PBS control ($n = 8$); (2), fluorescent labeled patient PBMC transfusion ($n = 12$); and (3) fluorescent PBMC transfusion with anti-Asialo GM1 pretreatment ($n = 12$). All mice were sacrificed at 17 days following surgery (2 weeks after PBMC transfusion), and the human xenografts (along with adjacent mouse tissues) were harvested for laboratory examinations.

PBMC labeling and transplantation

To examine the patient PMBCs trafficking in the SCID-Hu model, we first labeled the PBMC with a florescent dye ex vivo. Autologous human peripheral blood was obtained during the revision surgery, and the sample layered on Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) and PBS at room temperature followed by centrifugation at 2,600*g* for 20 min. A layer of PBMC was formed and collected. After further purification by Histopaque-1077 gradient centrifugation, PBMC were resuspended in RPMI 1640 medium at 10^7 cells per ml and labeled with 2 μM of PKH2 green fluorescent dye (Cat no. PKH2GL; Sigma-Aldrich). The efficiency of labeling was determined by daily examination using a fluorescent microscope. After 3 days ex vivo cell culture, 5×10^6 cells per mouse were injected intraperitoneally into 24 of SCID-Hu mice (groups 2 and 3).

Anti-asialo GM1 treatment

To evaluate the role of host macrophage contamination within the xenograft in the model, a group of mice were

treated with anti-asialo GM1 rabbit sera (ASGM1; Wako Bioproducts, Richmond, VA). Each SCID mouse in the group was intraperitoneally injected with 20 µl of ASGM1 at 4 h before human tissue transplantation, and the ASGM1 treatment was repeated once 7 days later.

Histological and immunohistochemical (IHC) analyses

Formalin-fixed xenografts (soft and hard tissues) were decalcified with 10% formic acid/sodium citrate for 6 days before paraffin-embedding and cut to yield 6-µm sections. The sections were stained with H&E to examine cellular infiltration, bone erosion, and periprosthetic tissue–bone interactions. Modified Trichrome staining was performed to examine bone collagen changes, as described previously [14]. Immunohistochemical stains were carried out to identify macrophages (CD68), reveal pro-inflammatory cytokines (IL-1, IL-6, and TNFα) and evaluate mediators of osteoclastogenesis (RANK and OPG) in transplanted tissues. Immunohistochemistry staining kits and polyclonal antibodies against human and mouse CD68, RANK, osteoprotegerin (OPG), interleukin-1 (IL1), tumor necrosis factor (TNFα), and interleukin-6 (IL6) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The reagents exhibited no cross-reactivity between the relevant species (mouse and human). Primary antibodies against human CD68, IL-1, TNFα, RANK, and OPG were applied at 2 mg/ml concentration according to the instructions of antibody vendors, using methods for IHC reported previously [15]. Antibody against mouse-origin CD68 was also employed to reveal the mouse macrophage contamination. In negative control sections, the primary antibody was replaced with appropriately diluted irrelevant anti-sera. Digital images of IHC stained sections were captured and analyzed using the Image-Pro Plus software package (Media Cybernetics, Silver Spring, MD). The level of the positive stains and localization was evaluated in six different fields, and expressed as integrated optical density (IOD). The evaluate the PKH2-labeled fluorescent cell trafficking in vivo, implanted periprosthetic tissue and biopsies of host mouse tissues (lungs, liver, spleen, and kidneys) were harvested, frozen, and 10-µm sections prepared. Positive fluorescent cells were immediately observed and photographed under a fluorescent microscope with 100% emission power. The computerized image analysis system was used for quantitive assessment. Image-Pro Plus software package was also used to quantify the round-nucleated inflammatory cells (aspect ratio less than 1.6) within the xenografts on H&E stained sections.

Real-time quantitative polymerase chain reaction for gene expression

Real-time polymerase chain reaction reagents were obtained from PerkinElmer (Norwalk, Connecticut). All other chemicals were analytical grade. The expression of proinflammatory cytokines IL-1β, TNFα, IL-6, RANK, CPK, and CTR was determined by real-time PCR using the ABI Prism 7700 sequence detector (PE-Applied Biosystems, Foster City, CA) as described previously [16]. Briefly, the human xenografts were carefully separated from surrounding mouse muscles and homogenized on ice using a Polytron (PT-MR2100; Kinematica, Lucerne, Switzerland) operated at three bursts of 15 s each. A fraction of the homogenate was processed for RNA extraction with use of a commercial kit (Tel-Test, Friendswood, Texas) in accordance with the manufacturer's instructions. cDNA was reverse transcribed from 0.5 µg of total RNA in 40 ml of a reaction mixture containing 1× polymerase chain reaction buffer, 500 mM each of nucleotide triphosphates (dNTP), 0.5 U/µl of ribonuclease inhibitor, 2.5 mM of random hexamers, 5.5 mM of $MgCl_2$, and 1.25 U/µl of reverse transcriptase (Perkin Elmer). The reaction mixture was incubated in a DNA Thermal Cycler (Perkin Elmer) at 25°C for 10 min, 48°C for 5 min followed by 95°C for 5 min. For real-time PCR, a final mixture of 50 µl including 2× SYBR Green PCR Master Mix (pre-mixed reaction components—PCR buffer, salts, deoxynucleotides, and AmpliTaq Gold polymerase), 100 nM target primer pairs, 4 µl cDNA and RNase-free water was amplified in the ABI Prism 7700 Sequence Detector (PE Biosystems) for 40 cycles at 60°C/60 s plus 95°C/15 s each cycle. The fluorescent signals were recorded dynamically. Normalization and analysis of the reporter signals (ΔRn) at the threshold cycle was carried out using the software provided by the manufacturer, and target gene copies were calculated against the regression of the standard curve. Selected reaction mixtures after real-time polymerase chain reaction were electrophoresed on 1.8% agarose gels containing ethidium bromide to verify the amplification of the correct target gene.

Statistical analysis

The software package "PS Power and Sample Size Calculations" (William D. Dupont and Walton D. Plummer, web page http://www.mc.vanderbilt.edu/prevm ed/ps.htm) was utilized to estimate the number of mouse samples required for the current study. A minimum of eight mice per group was determined. Data were expressed as

mean ± standard error of the mean (SEM). Data were analyzed using SPSS software package (SPSS version 13.0, Chicago, IL). Statistical analysis among groups was performed by the ANOVA test; with the Scheffe formula for post hoc multiple comparisons. A *P* value of less than 0.05 was considered as significant.

Results

Human PBMC labeled with PKH2 green fluorescent dye

At 24 h after ex vivo labeling with PKH2 green fluorescent dye, 100% of human PBMCs in the culture were positive under a fluorescent microscope (Fig. 1). Dye retention was very long-lived, with positive labeling present up to 6 weeks in culture. Cell viability determined by trypan blue exclusion did not show any cytotoxicity due to the PKH2 labeling in comparison with the control cells. However, the cell counts of the human PBMCs declined after 4 weeks in culture, regardless of the fluorescent dye treatment (data not shown). We therefore determined to limit the length of the animal experiment to 2 weeks.

Acceptance of xenografts in SCID mice

The mice tolerated the surgical procedure well and the embedded human periprosthetic tissue survived in SCID mouse hosts. No infection or tissue rejection reactions were appreciable at the time of tissue harvest. The transplanted human periprosthetic tissue attached firmly to surrounding host muscles, with macroscopic morphology very similar to the original tissue before implantation. Histological assessment using H&E stained sections indicated that the hard and soft xenografts were distinguishable from surrounding host tissue, and the implanted bone chips remained adjacent to the patient periprosthetic tissues (Fig. 2). Wear debris particles, mostly polyethylene, were found ubiquitous in implanted tissues, phagocytosed or surrounded by extensive cellular infiltrates.

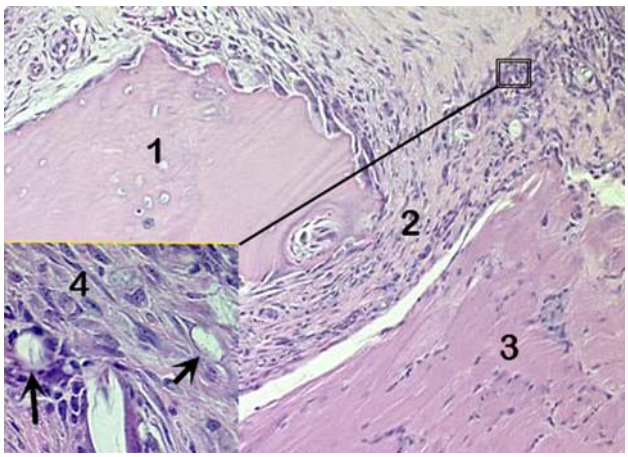

Fig. 2 Typical histological appearance of human periprosthetic tissue and bone chip transplantation in SCID mice for 17 days. Labels on the images indicate: *1* transplanted patient bone tissue, *2* periprosthetic pseudo-membrane, *3* host (mouse) muscle, *4* magnified region showing the cell morphology and particulate polyethylene debris (*arrows*) (×100 magnification, *insert* ×400)

The viability of the cells within the xenograft was confirmed by the intact nature of the cell boundaries, the normal granulation of the cellular cytoplasm, and the size and staining characteristics of the nucleus (Fig. 2). There were only a few cases where necrotic changes were present at the center of the xenografts.

Distribution of transfused PBMCs in the SCID-HU model

Transplanted human periprosthetic tissues and various host tissues were harvested at 2 weeks after the transfusion of PKH2-labeled patient PBMCs into the SCID-HU model. A fluorescent microscope was employed to examine the presence of fluorescent cells. A remarkable number of fluorescent cells were identified in the transplanted human periprosthetic tissues (Figs. 3a, 4). Although sporadic labeled-cells were also detected in host

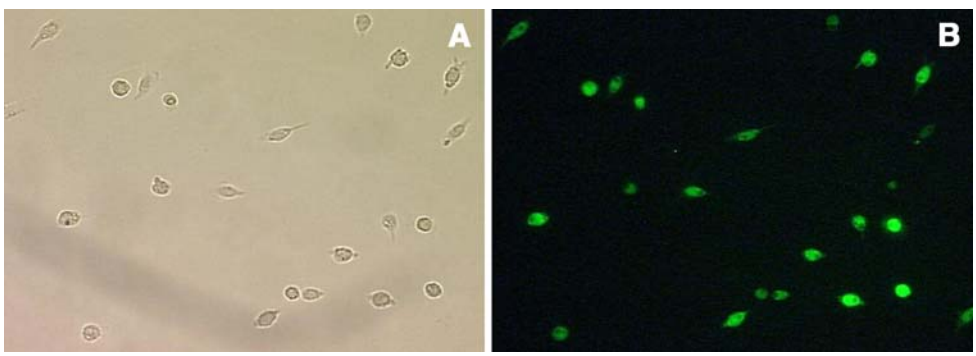

Fig. 1 Representative photomicrographs of PBMC cells labeled with PKH2 green fluorescent dye for 3 days before in vivo transfusion. **a** Bright-field image and **b** dark-field image to show the fluorescent cells (×200 magnification)

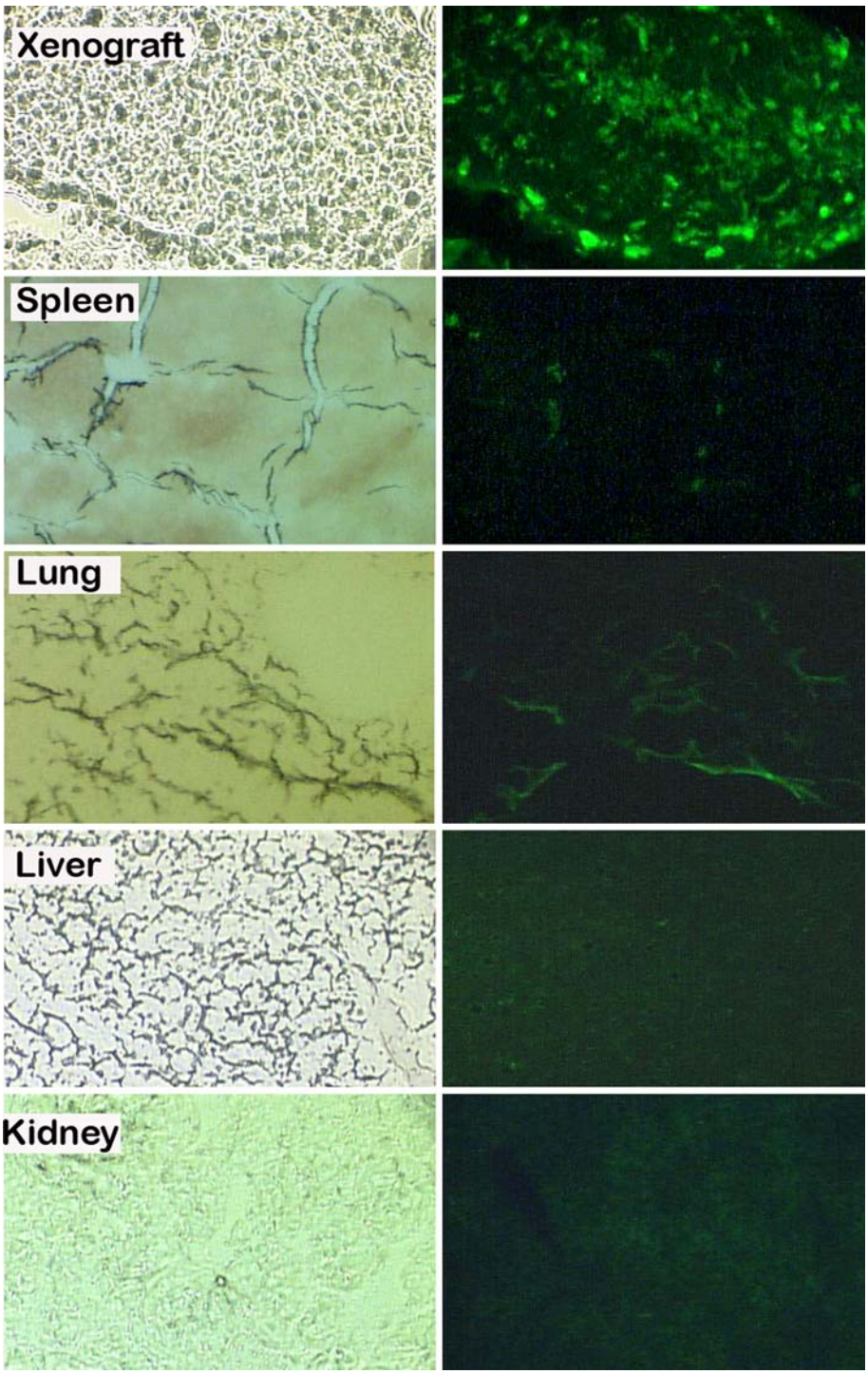

Fig. 3 PKH2-labeled circulating monocytes trafficking in SCID mice: tissues were frozen-sectioned and examined using a fluorescent microscope. The *left column* reveals the bright-field appearance of the tissues and the *right* images represent the dark-field images (×200)

spleen tissue, the examination of kidney, liver, and lung tissues resulted in universally negative staining (Fig. 3). Due to the fast fade-off nature of the fluorescent cells and the heterologous distribution of the monocytes within areas of xenograft, total round-nucleated cell counts per square millimeter in xenografts were also used as a comparison parameter among groups. Round nucleated inflammatory cell numbers in xenograft tissues with PBMC transfusion were statistically higher than that in the tissues without systemic cell transfer ($P < 0.05$; Fig. 4). However, transplanted periprosthetic tissues in mice receiving ASGM1 treatment contained less cell infiltration comparison with the tissue in mice treated with PBS (Fig. 4).

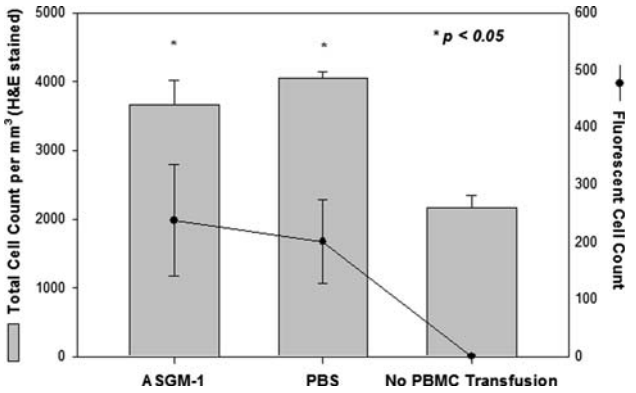

Fig. 4 Total round-nucleated inflammatory cell counts of the xenografts within different treatment groups were quantified using a computerized image analysis system (*bar-graph*, $P < 0.05$). Fluorescent-labeled cells within xenograft are shown as a *line-graph*

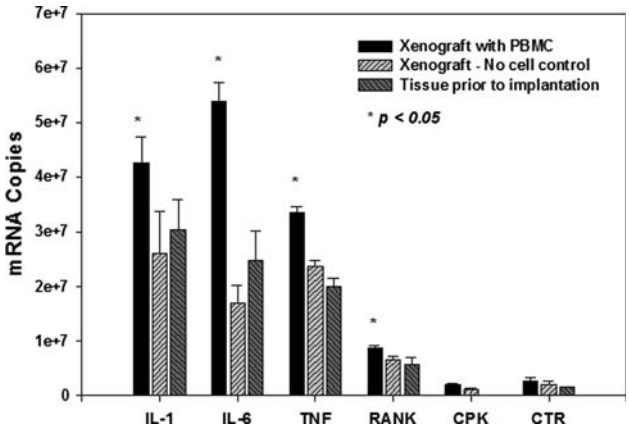

Fig. 6 Real-time PCR to examine mRNA expression changes in patient periprosthetic tissues before and after host implantation, and the transfusion of PBMC cells. The mRNA copies were internally normalized with the expression of house-keeping gene, glyceraldehydes-3-phosphate

Immunohistochemical and gene expression assessments

The xenografts retrieved from the hosts were immunostained with anti-human and anti-mouse CD68, respectively. While human-CD68 positive cells comprised the majority of the cell population within the xenografts from all the groups (Fig. 5a, c), host-originated macrophages (mouse CD68+ cells) did infiltrate the xenografts to a lesser extent (Fig. 5). ASGM1 treatment dramatically decreased the mouse CD68+ cell infiltration within the xenografts (Fig. 5d). Gene expression of pro-inflammatory cytokines and the osteoclast markers in the harvested xenografts were examined by real-time PCR. Notably, there was significantly higher mRNA expression of IL-1, IL-6, TNFα, and RANK in the samples with PBMC transfusion compared to the non-transfusion controls and the periprosthetic tissue prior to transplantation to mouse hosts (Fig. 6). The quantification of the levels of the immunochemical stains against IL-1, TNFα, and IL-6 using image analysis showed a high degree of concordance with the gene expression data (not shown).

Discussion

Aseptic loosening is the single important long-term complication of total joint replacement, a problem identified over 40 years ago but still not fully understood and without

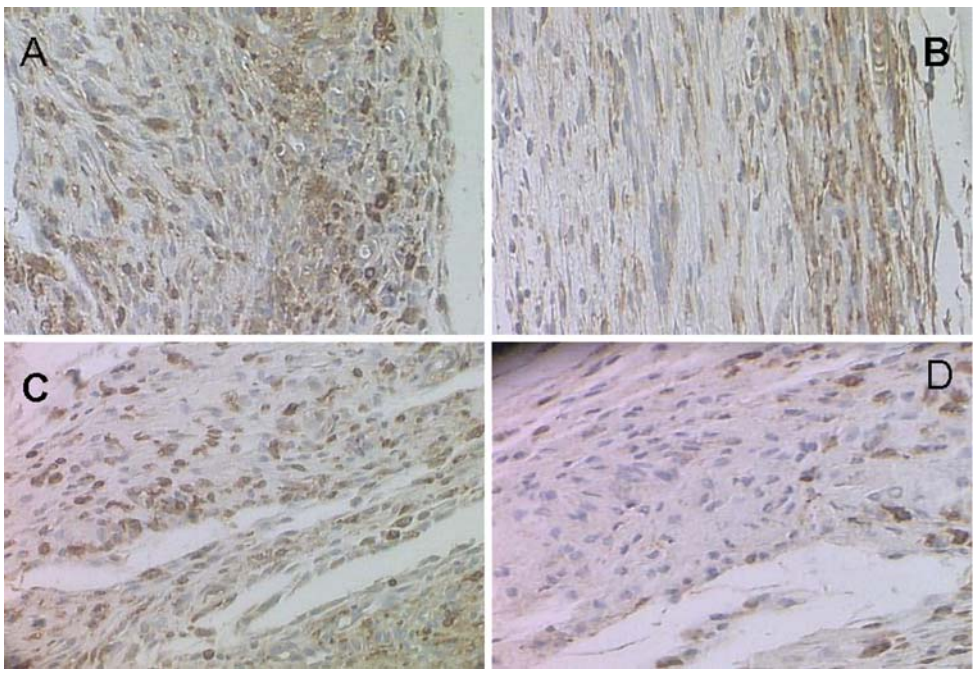

Fig. 5 Xenografts with human PBMC transfusions were stained immunohistochemically with anti-human CD68 (*left column*, micrographs **a** and **c**) and anti-mouse CD68 (*right column*, micrographs **b** and **d**). Hematoxylin counter-stain reveals the cell nuclei. **a**, **b** Tissue sections receiving PBS treatment, while **c**, **d** were from the animals treated with ASGM1

prevention or cure besides surgical revision. Particulate wear debris-induced osteolysis is thought to be the primary cause of aseptic loosening and prosthetic failure. High rates of osteolysis have been reported in association with certain hip implants [17], and osteoclast precursors (monocytes and macrophages) observed in periprosthetic tissues are believed to play a role in the osteolytic process [18]. Particulate wear debris can be phagocytosed by macrophages and a broad series of cytokines are secreted at the periprosthetic site. To study the pathogenesis of the problem and investigate therapeutic approaches, several animal models have been developed. Unfortunately, most current models for aseptic prosthetic loosening involve the use of large animals, or are for short-term study only. More importantly, there are numerous differences between animal tissues and the clinical circumstances seen in the human condition. We recently reported a mouse–human chimera model to study the in vivo human tissue responses during the aseptic loosening process [11]. An immune-deficient strain (SCID) of mice was adopted as a host [19]. Since SCID mice fail to develop mature T and B lymphocytes, they can accept allogeneic and xenogeneic grafts, making them an ideal model for cell and tissue-transfer experiments [12, 20, 21]. We reported that human periprosthetic tissues were successfully transplanted and were well accepted in SCID mice [11]. The current study modified the SCID-human chimera model of aseptic loosening by adding cancellous bone to the periprosthetic tissue in the SCID mouse hosts. Inclusion of cancellous bone chips adds the ability to evaluate osteoclastogenesis and bone resorption on sites. However, the osteolysis data was omitted to focus the study on the correlation of PBMC trafficking and local inflammation. Xenogenous tissues were transplanted intramuscularly into quadriceps and paravertebral muscles to ensure a plentiful blood supply at the implantation site and minimum tissue damage. Concomitantly, peripheral white blood cells were introduced to resemble an in situ setting of a human failing prosthetic joint with a systemic hematopoietic environment.

Although it is known that hematopoietic monocytes are among the first cells to colonize the periprosthetic site during the aseptic loosening process [9, 10], the molecular mechanisms of the circulating monocytes homing to the prosthetic joint and forming the periprosthetic tissue have yet to be fully understood. A recent report [22] has compared the gene expression profiles of periprosthetic tissue from osteolytic aseptic loosening patients with synovium of OA patients receiving primary total joint replacement. Their data indicated a significantly higher expression of IL-8, MIP1α, MMP-9, and CHIT-1 that (in agreement with the finding of Ishiguro et al. [23]) suggests an elevation of chemoattractants for blood monocytes in osteolytic periprosthetic tissues. Our present study investigated the trafficking of systemic blood monocytes (PBMCs) using our mouse–human chimera model. We adapted a cell labeling technique using PKH2 dye to monitor the human PBME cell trafficking and function [13, 24, 25]. This technique uses a membrane labeling technology to stably incorporate fluorescent dyes with long aliphatic tails (PKH2) into the lipid regions of the cell membrane, resulting in fluorescent intensity of labeled cells remaining for 60 days or longer in vivo [13]. Our results clearly showed that a significant number of florescent-labeled PBMCs accumulated within the transplanted human periprosthetic tissue in the model, while only trace numbers of fluorescent cells migrated to other host tissues. Cell quantification in xenograft tissues indicated that peritoneal injection of PBMC significantly increased the total and CD68-positive cell counts. These data suggest a specific migration of systemic hematopoietic monocytes to wear debris containing periprosthetic sites and the subsequent participation of the cells within the periprosthetic membrane. One may validly argue that a control group of non-wear debris-stimulated xenograft should be included for the systemic cell trafficking study; however, it is difficult to obtain "normal" human tissues for our research effort. Recently, a preliminary study in our laboratory has provided evidence that transfused PBMC markedly home to inflammatory periprosthetic tissue compared to the non-inflammatory adipose xenograft (unpublished data).

Although SCID mice have no T or B cell function, the literature indicates that they retain a normal nonspecific immune system [consisting of natural killer (NK) cells and macrophages] which might be a barrier to the long-term survival of xenografts [26]. Thus, host macrophage infiltration within the xenografts might interfere with the evaluation of the transplanted human monocytes trafficking. The immunohistochemical stains with anti-human and anti-mouse CD68 antibodies against retrieved xenografts in this study have confirmed the existence of host macrophage infiltration (Fig. 5). ASGM1 anti-sera treatment has been used to deplete host residue NK cells and macrophages [27–30], and the use of this reagent specifically eliminated mouse monocytes and NK cells and clearly demonstrated that infiltration by mouse inflammatory cells was minimized following ASGM1 treatment.

The current study also examined the gene expression profile of common pro-inflammatory cytokines and osteoclast markers in the xenografts after 14 days implantation. It appeared that the patient periprosthetic tissues maintained a high expression of IL-1, IL-6, and TNFα within the xenograft, as well as the expression of osteoclast markers such as RANK, CPK, and CTR. PBMC transfusion resulted in significantly higher levels of inflammatory cytokine expression, suggesting a role for systemic blood monocytes in the inflammatory aspects of the aseptic loosening

process at the prosthetic site. The data also suggests that transfused PBMC are attracted to the periprosthetic tissue implantation site. Further study is warranted to investigate the properties of wear debris in activation of PBMC to participate the formation of the periprosthetic tissue and local bone resorption.

Acknowledgments We thank Ms. Zheng Song, Bin Wu, and Li Mao for their excellent technical assistance. This work was supported by research grants from Orthopaedic Research and Education Foundation (OREF, S.-Y.Y.), NIH (5R03AR054929-02, S-Y.Y.), and Veteran Administration (Merit Award, P.H.W.).

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