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IL-12 Promotes Myeloid-Derived Suppressor Cell Recruitment and Bacterial Persistence during *Staphylococcus aureus* Orthopedic Implant Infection

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*Staphylococcus aureus* is a leading cause of human prosthetic joint infections (PJIs) typified by biofilm formation. We recently identified a critical role for myeloid-derived suppressor cells (MDSCs) in *S. aureus* biofilm persistence. Proinflammatory signals induce MDSC recruitment and activation in tumor models; however, the mechanisms responsible for MDSC homing to sites of biofilm infection are unknown. In this study, we report that several cytokines (IL-12p40, IL-1β, TNF-α, and G-CSF) and chemokines (CXCL2, CCL5) were significantly elevated in a mouse model of *S. aureus* PJI. This coincided with significantly increased MDSC infiltrates concomitant with reduced monocyte, macrophage, and T cell influx compared with uninfected animals. Of the cytokines detected, IL-12 was of particular interest based on its ability to possess either pro- or anti-inflammatory effects mediated through p35-p40 heterodimers or p40 homodimers, respectively. MDSC recruitment was significantly reduced in both p40 and p35 knockout mice, which resulted in enhanced monocyte and neutrophil influx and bacterial clearance. Adoptive transfer of wild-type MDSCs into infected p40 knockout animals worsened disease outcome, as evidenced by the return of *S. aureus* burdens to levels typical of wild-type mice. Tissues obtained from patients undergoing revision surgery for PJI revealed similar patterns of immune cell influx, with increased MDSC-like cells and significantly fewer T cells compared with aseptic revisions. These findings reveal a critical role for IL-12 in shaping the anti-inflammatory biofilm milieu by promoting MDSC recruitment. *The Journal of Immunology*, 2015, 194: 3861–3872.

The number of patients undergoing primary total hip and knee arthroplasties has steadily increased over the past decade, with nearly 800,000 procedures being performed in the United States each year (1). Prosthetic joint infection (PJI) is a serious complication following arthroplasty, with *Staphylococcus aureus* being a common inciting pathogen (1). A recent study using National Inpatient Sample data from 1990 to 2003 projected the infection incidence following total hip revision to increase from 3,400 in 2005 to 46,000 in 2030, and for total knee replacements from 6,400 in 2003 to 175,500 in 2030 based on the increased volume of prosthetic joint replacement procedures with an aging population (2). The majority of PJIs are thought to occur during surgery, most likely originating from skin commensals. This has led to increased screening for *S. aureus* carriage to ensure that patients undergo decolonization regimens prior to their surgical procedure in an attempt to minimize infection risk (3–5). Only a small number of bacteria is required to seed an implanted prosthesis; however, once adherent, they can establish a biofilm, affording protection from conventional antimicrobial agents as well as the host immune system (6–8). In addition to biofilm formation on prostheses, PJIs are often associated with chronic osteomyelitis, reflecting biofilm growth on a native surface (9, 10). Due to the difficulty in treating PJIs, patients are often subjected to a staged protocol requiring two surgeries, the first being removal of the infected prosthetic joint and placement of a temporary spacer impregnated with high doses of antibiotics for several weeks, followed by a second surgery for prosthetic joint reimplantation (11–13). However, patients experiencing a prior PJI are at increased risk for subsequent infections after the placement of a new prosthesis (9, 14). The significant disease burden associated with PJIs and the increasing prevalence of antibiotic-resistant strains, such as methicillin-resistant and vancomycin-intermediate *S. aureus*, highlight the importance of investigating alternative treatment paradigms. Our approach has been to understand how the host innate immune response is altered during biofilm-associated PJI, with the goal of redirecting this response to facilitate bacterial clearance in combination with conventional antibiotic therapy. This would provide an opportunity for either nonsurgical or one-stage reimplantation, where removal of the infected prosthesis and reinsertion of the new implant occur simultaneously.

A number of animal models examining *S. aureus* osteomyelitis have shown an elevation of inflammatory cytokines that have been implicated in bone remodeling as well as pathology (15–18). In contrast, the inflammatory events associated with *S. aureus* PJI remain to be fully elucidated. In particular, the presence of a foreign body may alter the kinetics and/or dynamics of the host immune response to inadvertently facilitate biofilm formation and persistence. Although a recent report described a key role for IL-1β in a mouse model of *S. aureus* postarthroplasty infection (19), the cellular source of IL-1β and a detailed analysis of infiltrating leukocytes

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**Abbreviations used in this article:** CT, computed tomography; iNOS, inducible NO synthase; KO, knockout; MDSC, myeloid-derived suppressor cell; PJI, prosthetic joint infection; qRT-PCR, quantitative RT-PCR; WT, wild-type.

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were not assessed. Other investigations using a mouse *S. aureus* tibial implant model have suggested the involvement of Th2 and T regulatory cells in bacterial clearance (20, 21); however, an in-depth analysis of innate immune mechanisms was not conducted. A detailed evaluation of the inflammatory events during *S. aureus* PJI is warranted to identify mechanisms whereby the organism is able to subvert host innate immunity to establish chronic disease.

Our recent report documented an important role for myeloid-derived suppressor cells (MDSCs) in *S. aureus* persistence during PJI (22). In particular, MDSC depletion facilitated biofilm clearance by augmenting the proinflammatory properties of infiltrating monocytes. The objective of the current report was to identify critical cytokines responsible for MDSC action during biofilm formation in vivo. One candidate was IL-12p40, which was markedly elevated during *S. aureus* biofilm formation. IL-12p40 pairs with the p35 or p19 polypeptides to form the heterodimeric cytokines IL-12 and IL-23, respectively (23, 24). However, it is well known that IL-12p40 is produced in excess of the other IL-12 family subunits and can exert negative regulatory effects as a homodimer by competitively binding the IL-12R (25–27). A recent report demonstrated a role for IL-12p40 in *S. aureus* orthopedic implant infection after treatment with a neutralizing Ab (21). However, the only readout reported was a reduction in the percentage of mice that remained infected after 21 d; no information pertaining to bacterial burdens, inflammatory infiltrates, or the cytokine’s mechanism of action was described. In the current study, we found that both p40 and p35 knockout (KO) mice displayed significant reductions in MDSC infiltrates during *S. aureus* PJI, which coincided with significantly increased monocyte and macrophage infiltrates and improved bacterial clearance. A direct role for MDSCs in this process was revealed by the ability of adoptively transferred wild-type (WT) MDSCs to significantly increase *S. aureus* biofilm burdens in IL-12p40 KO mice, implicating a key role for IL-12 in shaping the local inflammatory milieu to favor MDSC accumulation and biofilm persistence. These effects were not observed in p19 KO mice, demonstrating that IL-23 does not play a role in shaping the biofilm inflammatory milieu. Examination of tissues obtained from patients undergoing revision surgery for PJI revealed increased MDSC-like infiltrates and a paucity of T cells compared with aspecific revisions, along with dramatic increases in a variety of proinflammatory mediators. These findings recapitulate what is observed in our mouse model of orthopedic implant infection, demonstrating its utility for deciphering mechanisms of biofilm evasion of host immunity during PJI and therapeutic interventions.

### Materials and Methods

#### Mice

Male C57BL/6Ncr mice (8 wk old) were obtained from the National Cancer Institute (Charles River Laboratories, Frederick, MD). IL-12p40 and p35 KO mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and 12-3p19 KO animals were provided by Genentech (South San Francisco, CA), with age- and sex-matched C57BL/6Td WT mice purchased from The Jackson Laboratory. This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

#### Mouse model of *S. aureus* postarthroplasty joint infection

To model human PJIs, a mouse postarthroplasty infection model was used, as previously described (22, 28). The clinical *S. aureus* isolate USA300 LAC engineered to express bacterial luciferase (USA300 LAC::lux) was grown overnight in brain heart infusion medium (BD Biosciences, Sparks, MD) at 37°C while shaking at 250 rpm under aerobic conditions, until an OD_{600} of 5 was achieved (12–16 h). A working dilution of *S. aureus* USA300 LAC::lux was prepared by diluting organisms to 5 × 10^{7} CFU/ml in PBS, and actual inoculums were calculated retrospectively by plating serial dilutions of the working stock onto blood agar, which were consistently near the target of 10^{3} CFU/animal. Mice were anesthetized with a ketamine/xylazine mixture (Hospira, Lake Forest, IL, and Akorn, Decatur, IL; 100 and 5 mg/kg, respectively), and the surgical site was shaved and disinfected with povidone-iodine. A medial parapatellar arthrotomy with lateral displacement of the quadriceps-patella was performed to access the distal femur. A burr hole was created in the femoral intercondylar notch extending into the intramedullary canal using a 26-gauge needle, where- upon a precut 0.8-cm–length orthopedic-grade Kirschner (K) wire (0.6 mm diameter, Nitinol [nickel-titanium]; Custom Wire Technologies, Port Washington, WI) was inserted into the intramedullary canal, leaving ~1 mm protruding into the joint space. A total of 10^{3} CFU *S. aureus* was inoculated at the implant tip, with control animals receiving PBS. Buprenex (0.1 mg/kg s.c.; Reckitt Benckiser, Hull, U.K.) was administered immediately postinfection and 24 h later for pain relief. After this interval, all mice exhibited normal ambulation and no discernable pain behaviors.

#### Adoptive transfer of MDSCs into orthopedic implant biofilm infections

MDSCs were generated in vitro from the bone marrow of WT mice, as previously described, with minor modifications (29). Briefly, 10^{3} bone marrow cells were plated into 175-mm dishes and incubated for 4 d in RPMI 1640 medium supplemented with 10% FBS, GM-CSF (40 ng/ml), and IL-12p70 (40 ng/ml) at 37°C. 5% CO_{2}. Ly6G/Ly6C MDSCs were purified from the mixed cell population by FACs, whereupon 2.5 × 10^{5} MDSCs were injected s.c. at the site of implant-associated infection in IL-12p40 KO mice at day 1 postinfection. Tissues were collected at day 7 for FACs analysis and quantification of bacterial burdens, as described below.

#### Polyclonal T cell proliferation assays

To establish the functional activity of MDSCs recovered from *S. aureus* PJIs of WT versus p40 or p35 KO mice, T cell proliferation assays were performed, as previously reported (22). Briefly, MDSCs were collected from the soft tissue surrounding the infected knee joint by FACs, as described below, using Ly6G-PE and Ly6C-PerCP-Cy5.5 Abs. To collect T cells, spleens from naive mice were pressed through a 250-μm Nitex filter (Genesee, San Diego, CA) to generate a single-cell suspension. RBCs were lysed (BD Pharm Lyse), and splenocytes were incubated in Fc Block and subsequently stained with CD4-Pacific Blue (BD Biosciences). CD4^{+} T cells collected by FACs were >95% pure and immediately labeled with eFluor670 cell proliferation dye (eBioscience), according to the manufacturer’s instructions. Labeled CD4^{+} T cells were plated at 1.5 × 10^{5} cells/well in a 96-well round-bottom plate in RPMI 1640 supplemented with 10% FBS and 100 ng/ml IL-2 (Invitrogen, Frederick, MD). FACs-purified Ly6G^{−}/Ly6C^{−} MDSCs from either WT and p40 or p35 KO mice were added at 1:1 ratios to CD4^{+} T cells subjected to polyclonal stimulation with CD3/CD28 Dynabeads (Life Technologies, Oslo, Norway), because TCR immunodominant epitopes for *S. aureus* are not defined. Cells were incubated at 37°C for 72 h, whereupon the extent of T cell proliferation was determined by flow cytometry.

#### Tissues from human PJIs

Excess tissues from patients undergoing joint revisions for PJI or aspecific loosening as controls were procured after informed consent was obtained during the presurgical visit. The protocol was approved by the Institutional Review Board of the University of Nebraska Medical Center. Upon excision, tissues were placed in sterile PBS on ice and dissociated for FACs analysis, as described below.

#### Flow cytometry: mouse tissues

To characterize leukocyte infiltrates associated with soft tissues surrounding the knee joints of aspecific and *S. aureus*-infected implants, tissues were excised, dissociated using the rubber end of a plunger from a 3-cm syringe, and passed through a 70-μm filter (BD Falcon Discovery Labware, Bedford, MA). The resulting tissue was washed twice with PBS and centrifuged (300 × g, 10 min), whereupon RBCs were lysed using BD Pharm Lyse per the manufacturer’s instructions. After lysis, cells were washed and resuspended in PBS containing 2% FBS, followed by incubation in Mouse Fc Block (BD Biosciences, San Diego, CA) to minimize nonspecific Ab binding. Cells were then stained with directly conjugated Abs for multicolor flow cytometry analysis, which included two separate panels to identify innate immune populations or T cells. Abs in the innate immune cell panel included CD45-allophycocyanin, CD4-Pacific Blue, CD8-PerCP-Cy5.5, and F4/80-PE-Cy7 (R&D Systems, Minneapolis, MN). Abs in the T cell panel included CD3ε-allophycocyanin, CD4-Pacific Blue, CD8-PerCP-Cy5.5, and CD147-PE-Cy7 (BD Biosciences, San Diego, CA) to characterize cell populations of interest.
CD8α-FITC, Ly6C-PerCP-Cy5.5, and TCR γδ-PE. All fluorochrome-conjugated Abs were purchased from either BD Biosciences or eBioscience, unless otherwise noted. Controls included cells stained with isotype control Abs to assess the degree of nonspecific staining, as well as fluorescence minus one to identify gating thresholds (30). Analysis was performed using BD FACSDiva software with results presented as the percentage of CD45+ gated cells.

Flow cytometry: human tissues

Immediately postexcision, surgical specimens were placed in isotonic saline on ice and rapidly processed. Briefly, tissues were weighed, placed onto a piece of 250-μm Nitex mesh, and dissociated with a mortar and pestle in HBSS plus 10% FBS. Cells were washed twice with HBSS plus 10% FBS, and after the final wash were layered over a Ficoll-Paque gradient and centrifuged, according to the manufacturer’s instructions (GE Healthcare, Uppsala, Sweden). Leukocytes were collected from the interface, washed, and counted. Cells were then incubated with Human Fc Receptor Binding Inhibitor (eBioscience, San Diego, CA) to minimize nonspecific Ab binding. Cells were then stained with directly conjugated Abs for multicolor flow cytometry analysis, which included anti-human CD66b-FITC (BioLegend, San Diego, CA), CD14-PE, CD16-allophycocyanin, CD33-PE-Cy5, HLA-DR-PE-Cy7, CD45-eFluor450, CD3-allophycocyanin-Cy7, CD11b-BV510, and CD11c-BV605. All fluorochrome-conjugated Abs were purchased from either BD Biosciences or eBioscience, unless otherwise noted. To exclude dead cells from analysis, a Live/Dead Fixable Stain Kit (Life Technologies, Eugene, OR) was used according to the manufacturer’s instructions. Controls included cells stained with isotype control Abs to assess the degree of nonspecific staining. Analysis was performed using BD FACSDiva software with cells gated on the live CD45+ leukocyte population.

Quantitative RT-PCR

CD45+ leukocyte populations (CD33+HLA-DR−, CD33+HLA-DR−CD16+, and CD33+HLA-DR−CD16+) from human PJI tissues were purified by FACS, whereupon total RNA was immediately isolated using the TaqMan gene expression Cells-to-CT kit (Ambion, Austin, TX). Quantitative RT-PCR (qRT-PCR) was performed using TaqMan primer/probe mixes (Applied Biosystems, Foster City, CA) for the following genes: IDO-1, proteinase 3, inducible NO synthase (iNOS), arginase-1, IL-10, CD206, TNF-α, cyclooxygenase-2, and the housekeeping gene GAPDH. Results are reported as genes being expressed (+) or absent (−) in each of the CD45+ leukocyte populations.

Bacterial enumeration

Bacterial burdens associated with orthopedic implants in the mouse model were determined at weekly intervals from days 7 to 28 postinfection. Briefly, the skin overlying the leg was removed, and the s.c. granulation tissue surrounding the joint was excised, weighed, and processed for flow cytometry, as described above. Tissues from animals receiving aseptic implants were treated in an identical manner. After processing, a small aliquot was removed for quantitation of bacterial burdens. The implant was then extracted from the femur and sonicated for 5 min in 1 ml PBS to detach bacteria from the device. The knee joint (including cartilage and ligaments) and femur were homogenized using two sequential procedures due to the resilient nature of these tissues: initially a 30-s dispersal using a hand-held homogenizer, followed by disruption in a Bullet Blender (Next Advance, Averill Park, NY) using stainless steel beads (0.9–2.0-mm stainless steel blend). Serial 10-fold dilutions of tissue, knee, or femur homogenates were plated on trypticase soy agar with 5% sheep blood (Remel Products, Lenexa, KS) with titers expressed as CFU per gram of tissue. Remaining homogenates were centrifuged (20,000 × g, 20 min) and frozen at −80˚C until further analysis by MILLIPLEX bead arrays, as described below.

Multianalyte microbead arrays

To quantitate inflammatory mediator production in tissues associated with infected and aseptic implants in the mouse model, a custom-designed mouse microbead array was used according to the manufacturer’s instructions (MILLIPLEX; Millipore, Billerica, MA) and included the following inflammatory mediators: G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, CCL2, CCL3, CCL5, CXCL1, CXCL2, CXCL9, CXCL10, TNF-α, and vascular endothelial growth factor. For analysis of human PJI tissue specimens, a human 38-plex panel was used, which included the following: epidermal growth factor, fibroblast growth factor-2, Flt-3L, G-CSF, GM-CSF, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, CXCL1, CCL2, CCL3, CCL4, CCL7, CCL11, CCL22, CXCL1, CXCL10, soluble CD40L, TGF-α, TGF-β, TNF-α, and vascular endothelial growth factor. Results were analyzed using a Bio-Plex Workstation (Bio-Rad, Hercules, CA) and normalized based on the amount of total protein to account for differences in tissue sampling size. The level of sensitivity for most analytes in the array was 3.2 pg/ml.

ELISA

IL-12p40 production in tissue homogenates associated with infected and aseptic implants in the mouse model was determined using a sandwich ELISA kit (BD OptEIA; BD Biosciences). Results were normalized to the total amount of protein in each tissue sample. The lower limit of detection for this assay was 15.6 pg/ml.

FIGURE 1. S. aureus persistence during orthopedic implant infection. The femur, knee joint, and surrounding soft tissue associated with S. aureus-infected titanium implants were collected at the indicated intervals for quantitation of bacterial burdens. Animals receiving aseptic implants did not display any bacterial growth and are not shown. Results are expressed as CFU per gram of tissue to normalize for differences in sampling size and are presented from individual animals combined from three independent experiments (n = 12–24 mice/group).
Computed tomography of orthopedic implant infections

Bone integrity after S. aureus PJI or aseptic implant placement was evaluated using live computed tomography (CT) scans. Briefly, mice were anesthetized with 1.5% isoflurane in a 70% nitrous oxide/30% oxygen mixture and imaged using a FLEX Triumph X-ray CT/single photon emission CT system and software (TriFoil Imaging, Northbridge, CA). The 1024 CT projections for each image were acquired at 75 kVp and reconstructed using Triumph X-O 4.1. CT images were generated using the three-dimensional image visualization and analysis software VIVID, which is based on Amira 4.1 (TriFoil Imaging).

Histological evaluation

Implant-associated tissues were fixed in 10% formalin and washed with ddH2O prior to decalcification (Super Decalcification I/Delicate Decalicifier; Polysciences, Warrington, PA), according to the manufacturer’s instructions. Following decalcification, tissues were washed thoroughly in ddH2O and a small incision was made in the quadriceps muscle to facilitate removal of the titanium K-wire. Tissues were embedded in paraffin, and 4-μm sagittal sections were mounted and stained with H&E. Additional sections were subjected to Gram staining (Sigma-Aldrich, St. Louis, MO) to visualize bacteria associated with the infected joint.

Statistics

Significant differences between experimental groups were determined by an unpaired two-tailed Student t test or Wilcoxon rank sum test using GraphPad Prism version 4 (La Jolla, CA). For all analyses, p < 0.05 was considered statistically significant.

Results

S. aureus orthopedic implant infections are typified by immune skewing and chronicity

One complication after joint replacement surgery is persistent biofilm-associated PJI, with S. aureus being a frequent etiological agent (9, 31–33). In this study, we used a mouse model of S. aureus postarthroplasty joint infection that mimics PJI (34, 35). Prior studies from our laboratory and others have demonstrated ultrastructural evidence of biofilm formation on infected implants in this mouse model by SEM (22, 36). Biofilm formation is typified by bacterial persistence, which was demonstrated in the knee joint, surrounding soft tissue, and femur until day 28 postinfection (Fig. 1) with bacterial burdens still evident at 3 mo (data not shown). Fewer bacteria were associated with the implant (10–1000 CFU; data not shown), despite the continued colonization of neighboring sites. This suggests that our model has features of chronic osteomyelitis, which is a common sequela of PJI (9, 10). However, the possibility remains that PJI-associated bacteria were dislodged from the implant upon removal from the medullary cavity because of the constrained space. Bacterial growth was never detected in animals receiving aseptic implants in any of the tissues examined (data not shown).

Histological evaluation revealed dramatic inflammation of the soft tissues surrounding infected joints at day 7, which was increased by day 28 (Supplemental Fig. 1B, 1D). Gram stains revealed bacterial localization along the implant-tissue interface in S. aureus–infected animals suggestive of biofilm formation (Supplemental Fig. 1E). CT analysis revealed substantial bone loss at the distal tip of infected implants near the patella, which was most discernable at later stages of infection (Supplemental Fig. 2). In contrast, animals receiving aseptic implants showed no evidence of bone destruction. Collectively, these features demonstrate good fidelity of the mouse model to pathology that occurs during PJIs in humans.

A better understanding of the inflammatory processes that ensue during persistent PJI is needed to develop more effective treatment paradigms. To this end, we first examined the production of inflammatory mediators in the knee joint, surrounding soft tissue, and femur in the mouse model. Several proinflammatory cytokines, including IL-12p40, IL-1β, TNF-α, and G-CSF, were increased in

![FIGURE 2. S. aureus orthopedic implant infection elicits sustained cytokine and chemokine production.](http://www.jimmunol.org/)

Tissues surrounding the knee joint of mice with S. aureus–infected (n = 6) or aseptic (n = 8) implants were collected at the indicated time points, whereupon IL-12p40, IL-1β, TNF-α, G-CSF, CXCL2, and CCL5 production was measured by multi-analyte bead arrays. Results are normalized to the amount of total protein to correct for differences in tissue sampling size (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, unpaired Student t test), ND, not detected.
all tissue regions from \textit{S. aureus}–infected mice throughout the 28-d time course (Fig. 2). Likewise, numerous chemokines, such as CXCL2 and CCL5, were also markedly elevated in infected tissues (Fig. 2). Aseptic implants elicited transient inflammatory mediator production (Fig. 2), which most likely originated from the trauma generated during the surgical procedure. To evaluate whether enhanced chemokine production in \textit{S. aureus}–infected joints translated to increased leukocyte infiltrates, FACS analysis was performed at weekly intervals for 1 mo postinfection. Of the total CD45+ leukocyte infiltrate, the predominant cell type detected was Ly6G$^{high}$Ly6C$^+$, which we have recently identified as functional MDSCs (Fig. 3A, 3B) (22). Contrary to the significant influx of MDSCs into \textit{S. aureus}–infected tissues, monocyte (Ly6C$^+$Ly6G$^-$) and macrophage (F4/80$^+$) infiltrates were significantly decreased compared with mice receiving aseptic implants (Fig. 3C, 3F), despite the heightened expression of numerous chemokines that target these specific cell populations (Fig. 2). In contrast, neutrophil (Ly6G$^{low}$Ly6C$^{low}$) influx was significantly higher in \textit{S. aureus}–infected animals (Fig. 3D). In addition to these innate leukocyte populations, CD3$^+$ T cells were decreased in infected tissues, particularly during the first 2 wk postsurgery (Fig. 3G). Collectively, these results demonstrate that \textit{S. aureus} PJI actively augment MDSC influx while suppressing the recruitment of numerous leukocyte subsets.

Tissues from human PJIs display increased MDSC-like and reduced T cell infiltrates with elevated proinflammatory mediator expression

To determine whether the patterns of leukocyte infiltration and inflammatory mediator production observed in our mouse model translated to human infection, these parameters were assessed in tissue samples from patients undergoing revision surgeries for PJIs or aseptic loosening as a control (Fig. 4A). Of the infected tissues analyzed in this study, three were confirmed group B \textit{Streptococcus} and two were \textit{Staphylococcus epidermidis}. In this study, we report findings with \textit{S. epidermidis}, because it is most closely related to \textit{S. aureus}, which was used throughout our mouse studies. Compared with mice, human MDSC markers are less well defined, with some reports describing this population as CD33$^+$ HLA-DR$^-$ (37, 38). Similar to our mouse model, a population of MDSC-like cells (CD33$^+$HLA-DR$^-$) was detected in tissues from a patient with confirmed \textit{S. epidermidis} PJI, whereas few of these cells were observed in aseptic samples (Fig. 4C). In contrast, T cell influx was minimal in infected specimens, whereas T cells were the most abundant infiltrate associated with tissues recovered from aseptic orthopedic revisions (Fig. 4B). Additional CD45$^+$ leukocyte populations were also detected in infected patient tissues, including CD3$^+$ T cells as well as CD66$^+$ CD14$^-$ and CD66$^+$CD14$^+$ cells (Fig. 4D). To better define these

FIGURE 3. \textit{S. aureus} orthopedic implant infections are typified by a robust MDSC infiltrate. Soft tissues surrounding the knee joint of mice with \textit{S. aureus}–infected \((n = 6–8)\) or aseptic \((n = 8)\) implants were analyzed by flow cytometry for leukocyte infiltrates at the indicated days postinfection. Results were calculated after gating on the CD45$^+$ population. (A) Representative contour plots of Ly6G- and Ly6C-stained cells and quantitation of (B) Ly6G$^{high}$Ly6C$^+$ MDSCs; (C) Ly6C$^+$Ly6G$^-$ monocytes; and (D) Ly6G$^{low}$Ly6C$^{low}$ neutrophils. (E) Representative contour plots of CD45 and F4/80-stained cells and quantification of (F) F4/80$^+$ macrophages and (G) CD3$^+$ T cells. Results are representative of three independent experiments (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$, unpaired Student \(t\) test).
leukocyte populations and examine their activation status, subsets were purified from infected patient tissues by FACS, whereupon RNA was immediately isolated to examine gene expression by qRT-PCR. The CD33+HLA-DR2 population recovered from the confirmed S. epidermidis infection expressed several genes characteristic of MDSCs, including arginase-1, iNOS, and IDO-1, further strengthening the classification of these cells as MDSC-like (Fig. 4E) (37, 38). Interestingly, none of the genes analyzed were detected in CD332HLA-DR2CD66b+ neutrophil-like cells, whereas CD332HLA-DR+ monocyte/macrophage infiltrates expressed iNOS, IDO-1, and TNF-α (Fig. 4E). Analysis of inflammatory mediator expression revealed elevated levels of G-CSF, IL-1β, IL-6, IL-8, and soluble CD40L in S. epidermidis–infected tissues compared with aseptic specimens (Fig. 4F). These results confirm that similar leukocyte infiltration patterns are observed between our mouse post-arthroplasty infection model and human PJI tissues, namely, increased MDSC-like cells and a paucity of T cells. In addition, human PJI tissues were typified by elevated inflammatory mediator expression, as was seen in the mouse. These findings demonstrate the utility of the mouse model for deciphering mechanisms of biofilm evasion of host immunity during human PJI and therapeutic interventions.

IL-12 is critical for MDSC recruitment and attenuating innate immune cell influx during S. aureus orthopedic implant infection

The IL-12 family of cytokines possesses proinflammatory properties that regulate macrophage and T cell activation (39–42). In addition, proinflammatory cytokines have been reported to recruit and activate MDSCs (43, 44), which are significantly elevated in our S. aureus orthopedic implant infection model (Fig. 3) and inhibit T cell activation (22). Based on the increases in IL-12p40 expression in human PJI tissues (data not shown) and our mouse model (Fig. 2) and its ability to exert either pro- or anti-inflammatory activity mediated by p35-p40 heterodimers or p40 homodimers, respectively, we examined the importance of IL-12 family members during early S. aureus orthopedic infection using IL-12p40 and p35 KO mice. Examination of leukocyte recruitment in S. aureus–infected IL-12p40 and p35 KO animals revealed significant decreases in Ly6Ghigh Ly6C+ MDSCs (Fig. 5A, 5B). To determine whether these residual MDSC infiltrates retained suppressive activity, MDSCs from IL-12p40 and p35 KO mice were purified by FACS at days 7 and 14 postinfection and examined for their ability to inhibit polyclonal T cell activation. MDSCs recovered from both IL-12p40 and p35 KO mice were capable of inhibiting T cell proliferation (Fig. 5C, 5D, respectively). We confirmed that IL-12p40 KO mice do not display any evidence of defective MDSC development as the percentages of bone marrow–derived MDSCs were similar between IL-12p40 KO and WT animals (Supplemental Fig. 3A). In addition, analysis of MDSCs from the spleens of WT and IL-12p40 KO mice revealed similar percentages in both naive animals and following S. aureus PJI (Supplemental Fig. 3B). Collectively, these findings suggest that IL-12 promotes MDSC recruitment rather than functional activity. Our recent report demonstrated that MDSC depletion during S. aureus PJI enhanced monocyte recruitment and intrinsic

**FIGURE 4.** Tissues from human PJIs display increased MDSC-like and reduced T cell infiltrates. (A) Tissues surrounding aseptic and S. epidermidis–infected knee prostheses were collected for flow cytometric analysis, qRT-PCR, and inflammatory mediator production. Representative contour plots of (B) CD3+ T cells; (C) MDSC-like infiltrates (CD33+HLA-DR−); and (D) CD66b+ granulocytes and CD14+ mononuclear cells gated on the CD45+ population. (E) qRT-PCR analysis of genes that were detected (+) versus absent (−) in each sorted cell population and (F) quantitation of G-CSF, IL-1β, IL-6, IL-8, and soluble CD40L expression in tissues shown in (A). Results were normalized to the amount of total protein to correct for differences in tissue sampling size. ND, not detected.
proinflammatory activity (22). Similarly, the nearly 70–80% reduction in MDSC infiltrates observed in IL-12p40 and p35 KO mice translated into significant increases in Ly6G$^{\text{high}}$Ly6C$^+$ monocyte recruitment (Fig. 6) and improved bacterial clearance in both IL-12p40 and p35 KO mice compared with WT animals (Fig. 7). Coincident with reduced bacterial burdens, the production of nu-

**FIGURE 5.** Recruitment of T cell–suppressive MDSCs during *S. aureus* orthopedic infection is regulated by IL-12. Ly6G$^{\text{high}}$Ly6C$^+$ MDSCs associated with the knee joint of *S. aureus*–infected WT and (A) IL-12p40 or (B) IL-12p35 KO mice (*n* = 8/group) were quantified by flow cytometry at the indicated times postinfection. Results were calculated after gating on the CD45$^+$ population and represent the mean ± SEM of three independent experiments. MDSCs were purified from infected knee tissues of WT and (C) IL-12p40 or (D) IL-12p35 KO mice at days 7 and 14 postinfection for T cell proliferation assays. Results are expressed as the percentage of proliferation with T cells alone (−) and CD3/CD28-stimulated T cells (+) as controls. Results represent two independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001, unpaired Student *t* test).

**FIGURE 6.** IL-12 deficiency increases monocyte influx during *S. aureus* orthopedic implant infection. Ly6G$^-$Ly6C$^+$ monocytes associated with the knee joint of *S. aureus*–infected WT and (A) IL-12p40 or (B) IL-12p35 KO mice (*n* = 8/group) were quantified by flow cytometry at the indicated times postinfection. Results were calculated after gating on the CD45$^+$ population and are representative of three independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001, unpaired Student *t* test).
numerous inflammatory mediators, including IL-1β, TNF-α, G-CSF, and CXCL2, was decreased in IL-12p40 and p35 KO mice (Fig. 8). Because IL-23p19 also shares the common p40 subunit (45), we examined S. aureus infection in p19 KO mice, but found no differences in inflammatory indices or bacterial burdens in comparison with WT animals (data not shown).

To further implicate MDSCs as the key cell type responsible for inhibiting innate immune cell influx and promoting S. aureus persistence during PJI, bone marrow–derived MDSCs from WT mice were adoptively transferred into IL-12p40 KO animals 1 d postinfection, whereupon bacterial burdens were assessed 7 and 14 d later (Fig. 9). MDSCs for these experiments were expanded from bone marrow in vitro and confirmed for their ability to inhibit T cell proliferation (Supplemental Fig. 4). The adoptive transfer of WT MDSCs into IL-12p40 KO animals restored bacterial burdens to those typically observed in WT mice and reduced monocyte and neutrophil infiltrates (Fig. 9). These effects are most likely the result of MDSC activity and not IL-12 release per se, because MDSCs are not a major source of IL-12. This is supported by our recent study showing that IL-12p40 expression is minimal in MDSCs isolated from PJIs (22), and we failed to detect IL-12p40 expression in MDSCs recovered from PJIs of WT mice by

**FIGURE 7.** IL-12 is critical for the establishment of S. aureus implant-associated infection. A titanium implant was placed in the femur of WT and IL-12p40 or IL-12p35 KO mice (n = 18–26/group), followed by inoculation with 10^3 CFU S. aureus. The femur, knee joint, and surrounding soft tissue were collected at the indicated intervals postinfection for quantitation of bacterial burdens. Results are expressed as CFU per gram of tissue to normalize for differences in sampling size and are combined from three to five independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001, Wilcoxon rank sum test).
qRT-PCR in the current study (data not shown), indicating that MDSCs are not a major source of IL-12 production. Adoptive transfers were not performed in p35 KO animals, because all of our prior studies demonstrated a concordance in phenotypes between p40 and p35 KO mice (Figs. 5–8). Collectively, these findings demonstrate that IL-12 plays a key role in MDSC recruitment during S. aureus PJI to actively suppress monocyte and neutrophil influx and promote bacterial persistence.

Discussion

Despite extensive antiseptic precautions, most PJIIs are thought to occur by hardware contamination from skin microflora during surgical insertion (9, 46). As such, PJIIs often take several months to years to manifest and most are attributed to S. aureus and S. epidermidis (47–49). The paramount obstacle for effective treatment of PJIIs is their recalcitrance to antibiotic therapy coupled with the ability to skew the immune response toward an anti-inflammatory, profibrotic state (6, 7). Therefore, the current standard of care to treat chronic PJIIs requires removal of all foreign material, i.e. antibiotics for 4–6 wk, and reimplantation of the prosthetic joint. This treatment does carry a high success rate of 80–90%; however, the morbidity and mortality associated with the patient’s infection and its treatment is one of the most catastrophic complications in orthopedics (34, 50, 51). Another important point is that the majority of prosthetic joint replacement surgeries are performed in the elderly, a population known to have waning immunity compared with younger patients (52, 53). Therefore, with the expanding aging population, the frequency of PJIIs is projected to increase. Collectively, these facts highlight the need for a better understanding of the underlying mechanisms involved in immune deviation during PJIIs, which may facilitate the development of novel therapeutics.

Importantly, our mouse model of PJI utilizes a very low infectious inoculum (i.e., 1000 bacteria), which represents a realistic level of bacterial exposure that might occur following the inadvertent transfer of organisms during prosthesis insertion in patients. The ability of the mouse model to recapitulate features of human PJI was substantiated by our analysis of tissues collected from patients undergoing revision surgeries for the treatment of PJI. Namely, cells with markers and gene expression profiles indicative of MDSCs (i.e., CD33+HLA-DR−) were evident with few/no T cell infiltrates detected. Both infected human and mouse tissues were typified by heightened inflammatory mediator expression compared with aseptic specimens; however, this was not sufficient to program infiltrating phagocytes for anti-biofilm activity, because infections persisted. We show that this results from MDSC action, which actively inhibits phagocyte microbicidal activity. Of the tissues collected from patients undergoing revision surgeries for PJI in this study, three were confirmed group B Streptococcus and two were S. epidermidis. In general, immune profiles were similar among these patient samples, suggesting that common immune responses are elicited during chronic PJI regardless of the inciting pathogen. This is important, because it implies that identifying methods to augment anti-biofilm immunity for the treatment of PJI might be efficacious against numerous bacterial species.

In these studies, IL-12 was critical for organizing the local inflammatory milieu, as revealed by the significant reduction in MDSC infiltrates at the site of PJI in both p40 and p35 KO mice. Impaired MDSC recruitment coincided with increased phagocyte influx, including monocytes and neutrophils, which resulted in enhanced biofilm clearance. A direct role for MDSCs in this process was confirmed by the ability of adoptively transferred WT MDSCs to worsen disease outcome in IL-12p40 KO animals, as evidenced by the return of S. aureus burdens to levels typical of WT mice concomitant with significant reductions in monocyte and neutrophil recruitment. The finding that immune phenotypes were similar in p40 and p35 KO mice and not evident in p19 KO animals suggests that IL-12p70 is important for organizing the immune permissive biofilm response. However, we cannot rule out the potential contribution of inhibitory p40 homodimers, because p40 is secreted in large excess of p40-p35 heterodimers and has recently been shown to bind other polypeptides distinct from the IL-12 family that may also impact the local biofilm inflammatory milieu (54). The requirement for IL-12 was unexpected given its prominent role in Th1 polarization, because few T cell infiltrates were detected at the site of infection in both the mouse model and human PJI tissues. Instead, our findings suggest that IL-12 induces MDSC recruitment, as revealed by the 70–80% reduction in MDSC influx into S. aureus–infected IL-12p40 and p35 KO mice. The fact that the few MDSCs that did infiltrate IL-12 KO tissues retained their
suppressive properties argues against a role for IL-12 in MDSC activation. Although seemingly counterintuitive based on the suppressive properties of MDSCs, proinflammatory signals have been reported to induce MDSC recruitment and activation in the two-step model proposed by Gabrilovich et al. (43, 44, 55). Impaired MDSC recruitment in IL-12p35 and p40 KO mice most likely accounted for improved bacterial clearance due to the removal of this suppressive population in conjunction with elevated granulocytic and monocytic infiltrates, which was confirmed by our adoptive transfer studies. Similar to our findings, a recent report demonstrated a role for IL-12p40 in S. aureus orthopedic implant infection after treatment with a neutralizing Ab (21); however, this is likely not the case in our model, because few T cell infiltrates were observed. Instead, we propose that IL-12 induces MDSC recruitment, leading to the diminished influx of professional phagocytes and impaired bacterial clearance. In addition, a few differences in the models used in these studies may account for these distinct findings. For example, Prabhakara et al. (21) inserted implants that were precoated with S. aureus, whereas our devices were infected after surgical placement and the location of implants was distinct (femur versus tibia).

One question that remains is how does IL-12 promote MDSC recruitment to sites of S. aureus PJI? The most plausible explanation is that IL-12 acts indirectly by inducing the expression of a chemokine(s) with actions on MDSCs. Potential candidates include CXCL2 and CCL2 that are elevated at the site of S. aureus PJI, and MDSCs have been reported to express the associated chemokine receptors CXCR2 and CCR2, respectively (56–60). Of note, both CXCL2 and CCL2 expression were significantly reduced in S. aureus–infected IL-12p40 and p35 KO mice in agreement with

![Figure 9](http://www.jimmunol.org/Downloadedfrom/)

**FIGURE 9.** MDSCs are responsible for S. aureus persistence and inhibition of immune cell influx during orthopedic implant infection. WT and IL-12p40 KO mice (n = 5–10/group) were infected with S. aureus, whereupon IL-12p40 KO animals received an adoptive transfer of 2.5 × 10⁶ purified WT MDSCs s.c. at the implant site 1 d postinfection, whereas WT and a separate group of IL-12p40 KO animals received s.c. injections of PBS. (A) The femur, knee joint, and surrounding soft tissue were collected at day 7 postinfection for quantitation of bacterial burdens. (B) Quantitation of Ly6G⁡⁺Ly6C⁡⁺ MDSCs, Ly6G⁻Ly6C⁻ monocytes, and Ly6G⁻Ly6C⁻ neutrophils. Results were calculated after gating on the CD45⁺ population and represent two independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001, unpaired Student t test).
needed to investigate other cell types as a source of these cytokines. Specifically, CCL2, CCL3, and CCL5 were chronically elevated in S. aureus infection, such as augmenting PGE$_2$ expression, which has also been shown to be a potent MDSC attractant (61–64). MDSCs may also undergo expansion at the site of infection by the action of growth factors, such as G-CSF and GM-CSF. Indeed, G-CSF was significantly elevated in S. aureus PJI tissues (Fig. 2), and qRT-PCR analysis of MDSCs and monocytes recovered from infected tissues revealed that monocytes are the primary source of both G-CSF and GM-CSF (data not shown). Despite this high expression of GM-CSF and G-CSF, monocytes remain a relatively small population during S. aureus PJI (Fig. 3). Therefore, additional studies are needed to investigate other cell types as a source of these cytokines as well as their functional role in the evolution of S. aureus PJI.

Another intriguing finding in this study was the apparent disconnect between the prolonged elevation of monocyte/macrophage chemokines and cell recruitment in S. aureus–infected animals. Specifically, CCL2, CCL3, and CCL5 were chronically elevated during S. aureus PJI (Fig. 2 and data not shown), yet monocyte/macrophage influx was significantly reduced compared with animals receiving aseptic implants (Fig. 3). This could be explained by differential MDSC fates based on the local environment. For example, it is known that MDSCs are recruited to sites of injury, where they differentiate into macrophages and neutrophils to protect against potential infection during the wound-healing process (65, 66). However, in the case of tumors or S. aureus infection, as we recently showed, MDSCs remain arrested in their immature state and exert potent immunosuppressive activity (22, 67). It is challenging to definitively assign this suppressive activity to either granulocytic MDSCs or monocytic MDSCs based on the difficulty to accurately identify each population for downstream analysis. However, our findings to date suggest that granulocytic MDSCs may represent the main effector, based on the propensity of infiltrating MDSCs to express higher levels of the granulocytic marker Ly6G compared with the monocytic marker Ly6C. Our results in this work suggest that S. aureus biofilms play an active role in arresting MDSCs in their suppressive state to foster infection persistence. Indeed, MDSCs can thwart monocyte/macrophage proinflammatory activity (68, 69), which may negatively affect their numbers at the infection site, as we observed. Similarly, although neutrophil chemokines were significantly increased throughout the course of infection, little evidence of neutrophil influx (i.e., Ly6G$^+$Ly6C$^-$) was detected. Instead, lesions were dominated by MDSCs, which are responsive to similar chemokines (67).

In conclusion, we have identified that IL-12 is critical for MDSC recruitment to the site of S. aureus PJI, where they impair phagocyte influx and biofilm clearance. Analysis of tissues from patients undergoing revision surgeries for PJIs revealed similar immune profiles as our mouse model, reflecting the utility of the mouse system to evaluate the efficacy of anti-biofilm therapeutics. Elucidating the mechanisms whereby bacterial biofilms thwart protective immunity may lead to the development of novel immune-mediated approaches to facilitate PJI clearance in combination with conventional antibiotics.

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Disclosures

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References

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