Myeloid-Derived Suppressor Cells Contribute to Staphylococcus aureus Orthopedic Biofilm Infection

Cortney E. Heim, Debbie Vidlak, Tyler D. Scherr, Jessica A. Kozel, Melissa Holzapfel, David E. Muirhead and Tammy Kielian

*J Immunol* 2014; 192:3778-3792; Prepublished online 19 March 2014;
doi: 10.4049/jimmunol.1303408
http://www.jimmunol.org/content/192/8/3778

Supplementary Material [http://www.jimmunol.org/content/suppl/2014/03/19/jimmunol.1303408.DCSupplemental](http://www.jimmunol.org/content/suppl/2014/03/19/jimmunol.1303408.DCSupplemental)

References This article cites 71 articles, 30 of which you can access for free at: [http://www.jimmunol.org/content/192/8/3778.full#ref-list-1](http://www.jimmunol.org/content/192/8/3778.full#ref-list-1)

Subscription Information about subscribing to *The Journal of Immunology* is online at: [http://jimmunol.org/subscription](http://jimmunol.org/subscription)

Permissions Submit copyright permission requests at: [http://www.aai.org/About/Publications/JI/copyright.html](http://www.aai.org/About/Publications/JI/copyright.html)

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: [http://jimmunol.org/alerts](http://jimmunol.org/alerts)
Myeloid-Derived Suppressor Cells Contribute to
Staphylococcus aureus Orthopedic Biofilm Infection

Courtney E. Heim, Debbie Vidlak, Tyler D. Scherr, Jessica A. Kozel, Melissa Holzapfel, David E. Muirhead, and Tammy Kielian

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature monocytes and granulocytes that are potent inhibitors of T cell activation. A role for MDSCs in bacterial infections has only recently emerged, and nothing is known about MDSC function in the context of Staphylococcus aureus infection. Because S. aureus biofilms are capable of subverting immune-mediated clearance, we examined whether MDSCs could play a role in this process. CD11b+Gr-1+ MDSCs represented the main cellular infiltrate during S. aureus orthopedic biofilm infection, accounting for >75% of the CD45+ population. Biofilm-associated MDSCs inhibited T cell proliferation and cytokine production, which correlated with a paucity of T cell infiltrates at the infection site. Analysis of FACS-purified MDSCs recovered from S. aureus biofilms revealed increased arginase-1, inducible NO synthase, and IL-10 expression, key mediators of MDSC suppressive activity. Targeted depletion of MDSCs and neutrophils using the mAb 1A8 (anti-Ly6G) improved bacterial clearance by enhancing the intrinsic proinflammatory attributes of infiltrating monocytes and macrophages. Furthermore, the ability of monocytes/macrophages to promote biofilm clearance in the absence of MDSC action was revealed with RB6-C85 (anti–Gr-1 or anti-Ly6G/Ly6C) administration, which resulted in significantly increased macrophages. Furthermore, the ability of monocytes/macrophages to promote biofilm clearance in the absence of MDSC action was revealed with RB6-C85 (anti–Gr-1 or anti-Ly6G/Ly6C) administration, which resulted in significantly increased S. aureus burdens both locally and in the periphery, because effector Ly 6C monocytes and, by extension, mature macrophages were also depleted. Collectively, these results demonstrate that MDSCs are key contributors to the chronicity of S. aureus biofilm infection, as their immunosuppressive function prevents monocyte/macrophage proinflammatory activity, which facilitates biofilm persistence. The Journal of Immunology, 2014, 192: 3778–3792.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature monocytes and granulocytes that are potent inhibitors of T cell activation (1). In mice, MDSCs are characterized by their expression of CD11b and Gr-1, but they can be further subdivided into monocyte- and granulocyte-like subsets based on their differential expression of Ly6C and Ly6G, which are referred to as M-MDSCs and G-MDSCs, respectively (2, 3). CD11b+Gr-1+ cells normally reside in the bone marrow prior to their differentiation into mature granulocytes, macrophages, or dendritic cells. However, MDSCs can be recruited into lymphoid and inflamed tissues during pathologic conditions by the actions of growth factors, such as G-CSF, GM-CSF, and VEGF, where disturbances in cytokine homeostasis block their differentiation into mature myeloid effector cells, resulting in MDSC expansion (3, 4). Several factors influence MDSC activation, including proinflammatory cytokines driven by MyD88-dependent signaling (i.e., IL-6), reactive oxygen species, and cyclooxygenase-2 (COX-2). These proinflammatory mole-

Copyright © 2014 by The American Association of Immunologists, Inc.

www.jimmunol.org/cgi/do1/10.4049/jimmunol.1303408
MDSCs revealed increased expression of typical MDSC molecules, including Arg-1, inducible NO synthase (iNOS), and IL-10. Administration of mAb 1A8 (anti-Ly6G), which specifically depleted the immunosuppressive MDSC population and mature neutrophils, significantly increased monocyte and macrophage proinflammatory activity, which translated into decreased S. aureus burdens in the infected joint. Independent evidence to support the importance of monocytes/macrophages in biofilm containment in the absence of MDSCs was demonstrated by the finding that RB6-C85 (anti-Gr-1 or anti-Ly6G/Ly6C) treatment, which depleted effector monocytes and macrophages in addition to MDSCs and granulocytes, significantly increased S. aureus burdens and proinflammatory mediator expression as well as bacterial dissemination to peripheral organs. These results indicate that MDSCs establish an anti-inflammatory milieu during S. aureus biofilm infection that thwarts monocyte and macrophage proinflammatory activity, leading to persistent colonization. This prominent MDSC infiltrate also explains the paucity of T cells associated with S. aureus biofilms. Collectively, these studies demonstrate a role for MDSCs during staphylococcal biofilm infection, and preventing their immunosuppressive actions may offer novel treatment strategies to thwart these devastating, chronic infections.

Materials and Methods

Mice
Male C57BL/6 mice (8 wk of age) were purchased from the National Cancer Institute (Frederick, MD). These studies were performed in strict accordance with recommendations found in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

Mouse model of S. aureus orthopedic biofilm infection
To simulate infectious complications in patients following surgical device placement, a mouse S. aureus orthopedic implant infection model was used as previously described with minor modifications (20). Animals were anesthetized with ketamine/xylazine (100 mg/kg and 5 mg/kg, respectively) and the surgical site was disinfected with povidone-iodine. A medial parapatellar arthrotomy with lateral displacement of the quadriceps-patella was performed to access the distal femoral intercondylar notch extending into the intramedullary canal using a 26-gauge needle, whereupon a precut 0.8-cm orthopedic-grade Kirschner 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 10^7 CFU of the bioluminescent S. aureus USA300 LAC::lux isolate (16) was inoculated at the implant tip. In some experiments, control mice received sterile implants using an identical procedure. Animals received Buprenex (0.1 mg/kg s.c.; Reckitt Benckiser, Hull, U.K.) immediately after infection and 24 h later for pain relief. After this interval, all mice exhibited normal ambulation and no discernable pain behaviors.

Scanning electron microscopy
Mice were sacrificed at day 45 following S. aureus infection, wherein the whole femur harboring the titanium implant was fixed in 0.5 M Sorensen’s phosphate buffer containing 2% glutaraldehyde and 2% paraformaldehyde for 1 h at room temperature and held in fixative overnight at 4 °C. Fixed specimens were washed three times in TBS followed by three rinses in double distilled (dd)H2O and decalcification in 14% EDTA for 2 d. After rinsing in ddH2O, specimens were dehydrated using a graded series of ethanol washes and critical pointed dried in a Pelco CPD2 critical point dryer (Ted Pella, Redding, CA). Dried specimens were mounted on aluminum stubs with carbon tabs and colloidal silver paste and sputter coated with gold-palladium using a Hummer VI sputter coater (Anatech, Battle Creek, MI). Samples were viewed using a Quanta 200 scanning electron microscope (FEI, Hillsboro, OR) operated at 25 kV.

In vivo depletion studies
To deplete MDSCs in vivo, mice received i.p. injections of either 1A8 (anti-Ly6G) or RB6-C85 (anti-Gr-1) Abs (100 μg/each) 1 d prior to S. aureus infection and every 72 h thereafter until sacrifice. Control mice received equivalent amounts of isotype-matched control Abs (rat IgG2a and IgG2b, respectively) using the same treatment regimen. All Abs were purchased in low endotoxin, azide-free form from BioLegend (San Diego, CA). Animals were euthanized at 7 or 14 d postinfection to determine the impact of cell depletion on S. aureus persistence and tissue-associated leukocyte infiltrates. Bone marrow and splenocytes were also collected to determine the efficiency of Ab-mediated depletion. A separate model of S. aureus catheter-associated biofilm infection was used in some experiments as previously described to confirm the action of RB6-C85 Ab depletion (16, 21).

Computed tomography of S. aureus orthopedic biofilm infections
Bone integrity in the context of Gr-1+ cell depletion during S. aureus orthopedic biofilm infection was monitored 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, Northridge, CA). One thousand twenty-four 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).

Recovery of orthopedic implant and surrounding tissues for S. aureus enumeration
For collecting inflamed soft tissue surrounding the infected knee joint, the skin was removed and the s.c. tissue dorsal to the patellar tendon was excised, weighed, and processed for flow cytometry as described below. Muscle and tendon tissues were excluded from the analysis. After processing, a small aliquot was removed for quantitation of bacterial burdens. Next, the implant was extracted from the femur and sonicated for 5 min in 1 ml PBS to dislodge bacteria from the implant. The knee joint (including cartilage and ligaments) and femur were homogenized using two sequential procedures owing 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 100-μm stainless steel beads (0.9–2.0 mm stainless steel blend). After centrifugation, serial 10-fold dilutions of tissue, knee, or femur homogenates as well as implant beads (0.9–2.0 mm stainless steel blend). After centrifugation, serial 10-fold dilutions of tissue, knee, or femur homogenates as well as implant sonicates were plated on trypticase soy agar with 5% sheep blood (Remel Products, Lenexa, KS). Titers are expressed as CFU per gram of tissue or per milliliter for titanium implants. Remaining homogenates were centrifuged (20,000 × g, 20 min) and frozen at −80°C for further analysis by Milliplex bead arrays as described below.

Morphologic and histologic analysis
To confirm that FACS-purified CD11b+Gr-1+ and Ly6G+Ly6C+ cells recovered from infection sites appeared morphologically similar to MDSCs, cells were adhered to glass slides by cytocentrifugation (Cytop, Wescor, Logan, UT) and stained with StainRITE (Polysciences, Warrington, PA). Images were obtained using a Zeiss Axioskop 40 microscope (Zeiss, Thornwood, NY). For H&E staining, implant-associated tissues were fixed in 10% formalin and washed with ddH2O prior to decalcification (super decalcification I/delicate decalcifier from Polysciences), according to the manufacturer’s instructions. Decalcified tissue was washed thoroughly with ddH2O before an incision was made in the quadriceps muscle and the femur to remove the implant. Tissues were then embedded in paraffin, with 4-μm sections mounted for H&E staining. H&E-stained tissues were evaluated for inflammatory changes by a board certified pathologist (J.A.K.) with the degree of inflammation determined using a scoring scale (0, no observable pathology; 1, minimal pathology; 2, moderate pathology; 3, severe pathology). To evaluate splenic architecture following Ab-mediated cell depletion, spleens were fixed in 10% formalin, paraffin-embedded, and sectioned for H&E staining.

Milliplex multianalyte bead array
To evaluate the effects of Ly6G- versus Gr-1-mediated cell depletion on the inflammatory milieu during S. aureus orthopedic biofilm infection, a custom-designed mouse microbead array was used (Milliplex; Millipore, Billerica, MA), which detects the following cytokines: G-CSF, GM-CSF, IFN-γ, IL-10, 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 VEGF. A Bio-Plex workstation (Bio-Rad, Hercules, CA) was used to analyze results, and values were normalized to the total amount of protein recovered from each sample.
Flow cytometry
To characterize leukocyte infiltrates in infected soft tissues surrounding the knee joint during *S. aureus* biofilm infection, tissues were excised, dissociated using the rubber end of a plunger from a 3-cc syringe, and passed through a 35-μm filter (BD Falcon, Bedford, MA). The resulting filtrate was washed with 1× PBS and cells were collected by centrifugation (300 × g, 10 min) whereupon RBCs were lysed using BD Pharm Lyse (BD Biosciences, San Diego, CA). After lysis, cells were resuspended in PBS containing 2% FBS, followed by incubation in Fc Block (BD Biosciences) to minimize nonspecific Ab binding. Cells were stained with CD45-allophycocyanin, Ly6G-PE, Ly6C-PerCP-Cy5.5, F4/80-PE-Cy7, CCR2-FITC (R&D Systems, Minneapolis, MN), and CD11b-eFluor 450. All fluorochrome-conjugated Abs were purchased from BD Biosciences or eBioscience (San Diego, CA) unless otherwise indicated. An aliquot of cells was stained with isotype-matched control Abs to assess the degree of nonspecific staining and fluorescence minus one was used to identify gating thresholds (22). The number of events analyzed ranged from 20,000 to 100,000 per sample, depending on the experimental setup. Analysis was performed using BD FACSDiva software with cells gated on the total leukocyte population (CD45+).

**MDSC recovery from *S. aureus* orthopedic biofilm infections for T cell proliferation assays**
MDSCs were collected from the soft tissues surrounding infected knee joints as described above, using either Gr-1−PE and CD11b-FITC or Ly6G-PE, Ly6C-PerCP-Cy5.5, and CD11b-eFluor 450 depending on the experimental setup. For comparisons, CD11b−Gr-1− MDSCs were isolated from the spleens of naive and *S. aureus*-infected animals. The purity of MDSC populations was not examined after sorting owing to limiting cell numbers. However, cytopsins and gene expression analysis revealed that sorted MDSCs were highly enriched, as they displayed characteristic markers and nuclear morphologies consistent with those reported for MDSCs in the literature. For CD4+ T cell isolation, 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 using 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 were immediately labeled with eFluor 670 cell proliferation dye (eBioscience) according to the manufacturer’s instructions.

For establishing the functional activity of MDSCs associated with *S. aureus* orthopedic biofilm infections, T cell proliferation assays were performed. Briefly, eFluor 670–labeled CD4+ T cells were plated at 1.5 × 10^5 cells/well in a 96-well round bottom plate in RPMI 1640 with 10% FBS, supplemented with 100 ng/ml recombinant mouse IL-2 (Invitrogen, Frederick, MD). FACS-purified Gr-1−CD11b+, Ly6C^high^Ly6C^low^, Ly6G^high^Ly6C^low^, or Ly6G^low^Ly6C^high^ cells were added at 1:1 or 1:5 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 and supernatants were saved for cytokine evaluation by Milliplex analysis.

**qRT-PCR**
Ly6G^high^Ly6C^high^ and Ly6G^low^Ly6C^low^ inflammatory monocytes from *S. aureus*-infected tissues were purified by FACS, whereupon total RNA was immediately isolated using the TaqMan gene expression cells-to-CT kit (Ambion, Austin, TX). qRT-PCR was performed using TaqMan primer/probe mixes (Applied Biosystems, Foster City, CA) for the following genes: iNOS, Arg-1, COX-2, TNF-α, IL-β, IL-6, IL-10, and IL-12p40. Gene expression levels were normalized to GAPDH expression and are presented as the fold-induction (2^−ΔΔCT) value for Ly6G^high^Ly6C^high^ vs. Ly6G^low^Ly6C^low^ MDSCs relative to the Ly6G^low^ Ly6C^high^ monocyte fraction.

**In vitro macrophage and MDSC experiments**
Ly6G^high^Ly6C^high^ MDSCs were isolated from *S. aureus*-infected tissues or spleens at day 14 postinfection by FACS as described above and bone marrow–derived macrophages were prepared as previously reported (23). Cells were plated at 5 × 10^5 cells/well in a 96-well plate and stimulated with either peptidoglycan (10 μg/ml) or heat-inactivated *S. aureus* (10^5/well) for 24 h. After a 24-h incubation period, supernatants were collected and stored at −80°C until Milliplex analysis.

**Statistical analysis**
Significant differences between experimental groups were determined by an unpaired two-tailed Student *t* test or a one-way ANOVA with Bonferroni’s multiple comparison post hoc analysis using GraphPad Prism version 4 (GraphPad Software, La Jolla, CA). For all analyses, *p* < 0.05 was considered statistically significant.

**Results**
**Accumulation of CD11b^+^Gr-1^+^ cells during *S. aureus* orthopedic biofilm infection**
We recently reported that *S. aureus* biofilms skew infiltrating macrophages toward an alternatively activated M2 state typified by Arg-1 expression (16, 24). However, other Arg-1+ cells distinct from macrophages were also observed, which led us to investigate their identity. A likely candidate was MDSCs based on their robust Arg-1 expression and well-described anti-inflammatory attributes in cancer (2, 4, 5). In the present study, we used a mouse model of orthopedic biofilm infection (20) to demonstrate the presence and functional importance of MDSCs in shaping the anti-inflammatory biofilm milieu in an immunocompetent host. Biofilm formation on the orthopedic implant was confirmed by scanning electron microscopy, which revealed *S. aureus* attachment to a dense matrix deposited on the implant surface and bacterial tower formation (Fig. 1). A prominent CD11b^+^Gr-1^+^ infiltrate was observed, which accounted for ~75% of the total CD45+ leukocyte population by day 14 postinfection (Fig. 2A, 2B). Coexpression of CD11b and Gr-1 is used to define MDSCs, and cytospin preparations of FACS-purified CD11b^+^Gr-1^+^ cells recovered from the site of *S. aureus* biofilm infection confirmed their heterogeneous composition of both granulocytic and monocytic morphologies (Fig. 2C). In particular, cells with ringed nuclei suggested the presence of immature granulocytes, and immature monocytes with large rounded nuclei and little cytoplasm were also observed (Fig. 2C). CD11b^+^Gr-1^+^ cells were also detected in mice receiving sterile implants, which was not unexpected, because MDSCs have been reported in virtually every inflammatory environment and are associated with wound healing responses under normal conditions (19, 25); however, their numbers were significantly lower compared with *S. aureus*-infected animals (Fig. 2B). The abundance of CD11b^+^Gr-1^+^ cells during early *S. aureus* orthopedic infection may be one mechanism that contributes to the establishment of chronic disease.

**CD11b^+^Gr-1^+^ MDSCs recruited to the site of *S. aureus* orthopedic biofilm infection inhibit T cell activation**
A hallmark of MDSCs is their ability to inhibit Ag-specific and polyclonal T cell activation (4, 18). This is a critical attribute based on the promiscuity in surface marker expression between MDSCs and other myeloid lineages (25, 26). To determine whether *S. aureus* biofilm–associated CD11b^+^Gr-1^+^ infiltrates were bona fide MDSCs, we examined their ability to inhibit polyclonal CD4^+^ T cell activation, because *S. aureus* immunodominant TCR epitopes have not yet been identified. MDSCs were recovered from tissues at day 14, which coincided with maximum cell numbers at the infection site (Fig. 2B). CD11b^+^Gr-1^+^ cells from *S. aureus*–infected tissues significantly suppressed T cell proliferation (Fig. 3A), establishing their identity as MDSCs. The inhibitory activity of biofilm-associated MDSCs was further demonstrated by their ability to significantly impair T cell cytokine secretion, including TNF-α, IFN-γ, IL-17, and IL-4 (Fig. 3B–E).

We next determined whether the immunosuppressive nature of MDSCs was restricted to the biofilm infection site or whether they were also suppressive in the periphery, which has been reported for MDSCs in tumor-bearing animals (27, 28). CD11b^+^Gr-1^+^ cells from the spleens of either naive or infected animals were unable to suppress CD4^+^ T cell proliferation (data not shown). It was not unexpected that MDSCs from naive animals failed to inhibit T cell
activation, as pathologic conditions are known to elicit MDSC expansion and activation (2, 4, 29, 30). Several groups have reported that MDSCs only acquire suppressive function after exposure to factors in inflammatory environments (25, 27, 31), and our results suggest that these signals are only present in the local biofilm milieu.

Because the Gr-1 Ab RB6-C85 recognizes both Ly6G and Ly6C epitopes (32), we stained for both markers and identified three distinct populations associated with \textit{S. aureus} orthopedic biofilms, namely Ly6G$^{\text{high}}$Ly6C$^+$, Ly6G$^{\text{low}}$Ly6C$^{\text{low}}$, and Ly6G$^- \text{Ly6C}^+$ (Fig. 4A). Each subset was purified by FACS to determine which was responsible for the observed CD4$^+$ T cell suppression of the original Gr-1$^+$ population (Fig. 3). Ly6G$^{\text{high}}$Ly6C$^+$ cells significantly inhibited CD4$^+$ T cell proliferation in a ratio-dependent manner, confirming their identity as MDSCs (Fig. 4E). Similar to observations with the bulk CD11b$^+$Gr-1$^+$ population (Fig. 3), Ly6G$^{\text{high}}$Ly6C$^+$ cells decreased TNF-$\alpha$ and IL-17 expression (Fig. 4F and 4G, respectively). Cytospins of the Ly6G$^{\text{high}}$Ly6C$^+$ population

**FIGURE 1.** Demonstration of \textit{S. aureus} biofilm formation in vivo on orthopedic implants. Titanium orthopedic implants were isolated from C57BL/6 mice at day 45 following \textit{S. aureus} infection and processed for scanning electron microscopy analysis. \textit{Left}, Biofilm formation is visible on the concave surface of the implant (original magnification $\times 300$) demonstrating the irregular pattern of the biofilm surface with tower structures visible (arrows). \textit{Right}, Higher magnification of the biofilm surface revealing numerous cocci interspersed with matrix material (original magnification $\times 20,000$). The image has been pseudocolored to highlight \textit{S. aureus} (gold).

**FIGURE 2.** Accumulation of CD11b$^+$Gr-1$^+$ cells during \textit{S. aureus} orthopedic biofilm infection. Implant-associated tissues were collected from sterile and infected mice and analyzed by flow cytometry for CD11b$^+$Gr-1$^+$ cells at the indicated time points. (A) Representative contour plots and (B) CD11b$^+$Gr-1$^+$ infiltrates expressed as a percentage of the total CD45$^+$ leukocyte population. (C) Cytospin preparations of FACS-purified CD11b$^+$Gr-1$^+$ cells from infected tissues at day 14 were stained with Wright–Giemsa (original magnification $\times 20$). Arrowheads and arrows indicate cells suggestive of immature granulocytes and monocytes, respectively. Results are representative of three sterile and five infected mice per group. ***$p<0.001$, unpaired two-tailed Student $t$ test.
population revealed an immature granulocytic morphology characterized by numerous ringed nuclei (Fig. 4B), which, when taken together with their suppressive action, is highly suggestive of these cells as granulocytic MDSCs. The Ly6G$^2$ Ly6C$^+$ population was typified by a relatively homogeneous monocyte-like morphology that was unable to suppress CD4$^+$ T cell activation (Fig. 4D, 4E), suggesting that these cells are inflammatory monocytes. Collectively, these results demonstrate the recruitment of a bona fide MDSC population in staphylococcal biofilm infection.

Studies by other groups have reported neutrophil infiltrates in mouse models of *S. aureus* orthopedic infection (33–35). However, these reports used either immunostaining with Ly6G, Ly6G depletion, or LysM-GFP mice to identify neutrophils and, as our results demonstrate, these approaches cannot differentiate between neutrophils and MDSCs (36). It is possible that the Ly6G$^{low}$Ly6C$^+$ cells observed in our model of *S. aureus* orthopedic biofilm infection are neutrophils based on their cytospin morphology, revealing fewer immature cells compared with the MDSC population (Fig. 4C and 4B, respectively) and lack of T cell suppressive activity (Fig. 4E).

**FIGURE 3.** CD11b$^+$Gr-1$^+$ infiltrates from the site of *S. aureus* biofilm infection inhibit T cell proliferation. FACS-purified CD11b$^+$Gr-1$^+$ cells recovered from infected joint tissues at day 14 were immediately cultured ex vivo with eFluor 670-labeled CD4$^+$ T cells at a 1:1 ratio for proliferation assays. (A) Representative histograms of fluorescence intensity, with percentage proliferation reported. (B–E) Supernatants from MDSC/CD4$^+$ T cell cocultures were collected at 72 h to quantitate TNF-α (B), IFN-γ (C), IL-17 (D), and IL-4 (E) by Milliplex. Results are representative of three to nine independent experiments. *p < 0.05, **p < 0.001, unpaired two-tailed Student t test. (−), T cells only; (+), T cells incubated with CD3/CD28 Dynabeads; ND, not detected.

Ly6G$^{high}$Ly6C$^+$ cells recruited to sites of *S. aureus* orthopedic biofilm infection express genes characteristic of MDSCs

Owing to the differential immunosuppressive properties of the Ly6G$^{high}$Ly6C$^+$ and Ly6G$^+$Ly6C$^+$ subsets associated with *S. aureus* orthopedic biofilm infection, we next examined gene expression profiles of FACS-purified populations immediately ex vivo by qRT-PCR as further confirmation of their identity. The Ly6G$^{high}$Ly6C$^+$ MDSC subset displayed increased iNOS, Arg-1, COX-2, and IL-10 concomitant with reduced IL-12p40 expression compared with the Ly6G$^+$Ly6C$^+$ monocytic fraction (Fig. 5), similar to MDSC profiles described in other disease models (1, 37–40). MDSCs play an important role in regulating inflammatory processes through their production of several pro- and anti-inflammatory cytokines (7, 25). To assess the inflammatory status of MDSCs, cells were recovered from the site of biofilm infection or the spleen and immediately stimulated ex vivo with heat-inactivated *S. aureus* or peptidoglycan. We found that regardless of their origin, Ly6G$^{high}$Ly6C$^+$ MDSCs were inherently less proinflammatory than macrophages (Supplemental Fig. 1). Collectively, these results provide further evidence to support the identity of infiltrating Ly6G$^{high}$Ly6C$^+$ cells into *S. aureus* orthopedic biofilm infections as MDSCs.

Depletion of Ly6G$^+$ MDSCs increases monocyte infiltrates and their intrinsic proinflammatory activity, resulting in enhanced *S. aureus* biofilm clearance

To assess the functional role of Ly6G$^{high}$Ly6C$^+$ MDSCs in orchestrating the anti-inflammatory biofilm milieu to facilitate bacterial persistence, mice were treated with the mAb 1A8 to
target Ly6G+ cells (37, 41, 42). This approach would deplete MDSCs, leaving the Ly6C+ monocyte and macrophage populations intact and able to combat S. aureus infection, presumably in the absence of immunosuppression. We confirmed that 1A8 was effective at depleting the Ly6GhighLy6C+ MDSC population, which was more robust at day 7 compared with day 14 (Fig. 6A, 6B). Interestingly, the frequency of Ly6C+ monocytes was significantly increased at day 7 (Fig. 6C), and we predicted that the absence of immunosuppressive Ly6G+ MDSCs would promote the proinflammatory attributes of these Ly6C+ mononuclear phagocytes. To address this possibility, we examined the activation state of FACS-purified Ly6GhighLy6C+ cells from the infection site of 1A8-treated versus isotype control mice by qRT-PCR. In the context of MDSC depletion with 1A8, expression of iNOS, IL-12p40, and IL-6 was increased in Ly6GhighLy6C+ cells at day 7 (Fig. 7). Increased Arg-1 and IL-10 expression was also observed in Ly6G+ Ly6C+ cells (Fig. 7), and although both possess anti-inflammatory properties, they may be important in maintaining a balanced inflammatory environment at the site of infection owing to the absence of normally immunosuppressive MDSCs.

Because Ly6C+ monocyte infiltrates were increased in the context of MDSC depletion and displayed intrinsic proinflammatory activity, we next examined whether this would translate into superior anti-biofilm activity. This prediction was confirmed, because Ly6G+ cell depletion with 1A8 significantly reduced S. aureus burdens in both the tissue and knee joint at days 7 and 14 compared with
isotype control animals (Fig. 8B), which correlated with less gross evidence of exudate formation in MDSC-depleted mice (Fig. 8A). Ly6G+ cell depletion did not cause *S. aureus* dissemination from the primary site of infection (Fig. 8C), and histopathologic analysis of H&E-stained tissues showed no dramatic differences in the degree of joint inflammation or splenic architecture (as a measure of extramedullary hematopoiesis) between 1A8-treated and isotype control animals (data not shown).

To investigate the impact of Ly6G+ cell depletion on the inflammatory milieu during *S. aureus* orthopedic biofilm infection, soft tissues surrounding the knee, knee joint, and femur were analyzed using Milliplex arrays. Several cytokines (G-CSF, IL-1β, and IL-6) and chemokines (CXCL1, CXCL9, and CCL3) were dramatically reduced in 1A8-treated compared with isotype control mice primarily at day 7 (Fig. 9), in agreement with increased bacterial clearance in the former (Fig. 8B). Collectively, these results demonstrate that during *S. aureus* orthopedic biofilm infection, Ly6GhighLy6C+ MDSCs elicit a local microenvironment that restricts monocyte/macrophage proinflammatory activity, facilitating the establishment of an anti-inflammatory milieu that favors bacterial persistence. We propose that these effects were not significantly influenced by neutrophil loss following 1A8 treatment, because most Ly6G+ leukocytes infiltrating infected joints (i.e., ∼75%) were MDSCs.

Gr-1+ cell depletion confirms the inhibitory action of MDSCs on monocyt/macrophages to prevent *S. aureus* biofilm clearance

Our results have established that MDSC depletion with 1A8 facilitated *S. aureus* biofilm clearance, in part due to decreased immunosuppressive effects that promoted the proinflammatory attributes of infiltrating monocytes and macrophages. To further demonstrate that monocytes/macrophages were critical for anti-biofilm activity in the absence of an MDSC infiltrate, we treated mice with the mAb RB6-C85. Similar to 1A8, RB6-C85 depletes Ly6G+ MDSCs and neutrophils, but it also targets monocytes based on its reactivity with Ly6C, which would also impact macrophage numbers by default (29, 37, 41–43). There-
fore, any differences between 1A8 and RB6-C85 depletion would further support a role for monocytes/macrophages in mediating biofilm clearance without the suppressive MDSC population. As previously demonstrated, Ly6G/Ly6C staining detected three cell populations in implant-associated tissues of isotype control animals at days 7 and 14 following infection, namely Ly6G<sup>high</sup> Ly6C<sup>+</sup> MDSCs, a Ly6G<sup>low</sup>Ly6C<sup>low</sup> granulocyte-like population, and a Ly6G<sup>-</sup>Ly6C<sup>+</sup> inflammatory monocyte subset (Fig. 10A). However, at day 7 RB6-C85–treated animals only displayed one cell population that shifted from Ly6G<sup>low</sup>Ly6C<sup>low</sup> to Ly6G<sup>high</sup>Ly6C<sup>+</sup> at day 14 postinfection (Fig. 10B). The percentage of Ly6G<sup>low</sup>Ly6C<sup>low</sup> cells in RB6-C85–treated animals was significantly higher than isotype-treated controls at day 7; however, no differences were apparent at day 14, because the population had shifted to Ly6G<sup>high</sup>Ly6C<sup>+</sup> (Fig. 10B). Although Ly6G<sup>high</sup>Ly6C<sup>+</sup> infiltrates were increased in RB6-C85–treated animals at day 14 postinfection (Fig. 10B), they were unable to inhibit T cell proliferation (Supplemental Fig. 2), and as such they do not represent a true MDSC phenotype. Therefore, we suggest that the presence of Ly6G<sup>low</sup>Ly6C<sup>low</sup> and Ly6G<sup>high</sup>Ly6C<sup>+</sup> populations in the infected joint of RB6-C85–treated mice results from increased demand from the overwhelming infection (Fig. 11), which agrees with the results to follow that demonstrate extensive extramedullary hematopoiesis in the spleens of these animals. This is supported by the finding that Ly6G<sup>+</sup>Ly6C<sup>+</sup> cells were significantly lower in RB6-C85–treated mice receiving sterile implants compared with isotype control Ab (data not shown).

We also examined CCR2 and F4/80 expression as markers for inflammatory monocytes and macrophages, respectively (44–46).
Because RB6-C85 also recognizes the Ly6C epitope, we expected both of these cell populations to be decreased, as CCR2+ inflammatory monocytes express Ly6C and differentiate into F4/80+ macrophages once they have migrated into tissues (45). As expected, the percentage of Ly6C+CCR2+ cells was significantly decreased in RB6-C85–treated animals compared with isotype controls at days 7 and 14 postinfection (Fig. 10C). Likewise, there were significantly fewer F4/80+ macrophages in RB6-C85–depleted mice at day 7 postinfection, and only a very small percentage of cells remained at day 14 (Fig. 10C).

Gr-1+ cell depletion exacerbates S. aureus orthopedic biofilm infection owing to the loss of monocyte/macrophage effectors

Strikingly, S. aureus–infected mice treated with RB6-C85 displayed a grossly visible caseous exudate (Fig. 11A), which was typified by significantly increased bacterial burdens in the knee joint, surrounding soft tissue, and femur at days 7 and 14 postinfection (Fig. 10C). Likewise, there were significantly fewer F4/80+ macrophages in RB6-C85–depleted mice at day 7 postinfection, and only a very small percentage of cells remained at day 14 (Fig. 10C).

Gr-1+ cell depletion exacerbates S. aureus orthopedic biofilm infection owing to the loss of monocyte/macrophage effectors

Gr-1+ cell depletion exacerbated S. aureus orthopedic biofilm infection owing to the loss of monocyte/macrophage effectors

To examine changes in the inflammatory milieu after RB6-C85 treatment, inflammatory mediator expression was assessed. Numerous cytokines (IL-1β, G-CSF, and IL-17) and chemokines (CXCL1, CXCL2, and CCL3) were significantly increased at days 7 and 14 in RB6-C85–treated animals compared with isotype controls (Fig. 11C). Second, RB6-C85–treated animals displayed significant splenomegaly (Fig. 13A, 13B). Histopathology revealed marked expansion of the splenic sinuses and red pulp with extensive extramedullary hematopoiesis, typified by numerous erythroid islands, megakaryocytes, and leukocyte islands in RB6-C85–treated animals, which was not observed in infected isotype control mice (Fig. 13C).

Discussion

An emerging role for MDSCs has been described in several diseases aside from cancer, most recently to include bacterial infections (7–10, 42, 47). Using a mouse model of S. aureus orthopedic biofilm infection, we demonstrate that a population of CD11b+Gr-1+ MDSCs accumulates in the joint tissue and depletion of this population results in improved bacterial clearance by promoting the proinflammatory attributes of infiltrating monocytes and macrophages during S. aureus biofilm infection, which sets the stage for bacterial persistence.
MDSCs are notable for their robust Arg-1 expression, which depletes extracellular arginine, causing T cell dysfunction at multiple levels, including cell cycle arrest, reduced expression of the CD3ζ chain, and a global reduction in several proteins essential for T cell activity (48–51). Limited numbers of CD4+ T cells were detected in implant-associated tissues during *S. aureus* biofilm infection (i.e., 2–5%). We expected T cell infiltrates to be enhanced following Gr-1 and Ly6G depletion originating from the loss of MDSC activity; however, this was not the case. One possibility to explain this finding is that the combined action of MDSCs and regulatory cytokines serve to limit T cell numbers at the site of biofilm infection, which remains to be determined. Besides actions on T cells, arginine depletion via MDSC Arg-1 activity reduces its availability for iNOS, which thwarts M1 classical macrophage activation, as we have previously shown in *S. aureus* biofilms (16). By extension, the significant MDSC infiltrate associated with *S. aureus* biofilms in vivo is likely an important factor in skewing monocytes/macrophages toward a M2 anti-inflammatory phenotype that promotes bacterial persistence, and our studies confirmed that MDSCs recovered from the site of orthopedic biofilm infection express Arg-1 and IL-10. By further extension, we predicted that depletion of the suppressive MDSC population would allow infiltrating monocytes to act as true effector cells. This was confirmed by the finding that Ly6C+ monocytes recovered from MDSC-depleted animals expressed more proinflammatory genes compared with monocytes recovered from IgG-treated mice where the MDSC population remained intact. Additionally, MDSC depletion significantly decreased biofilm burdens, confirming the importance of this population in orchestrating the anti-inflammatory biofilm milieu to facilitate infection persistence. Besides MDSCs, regulatory T cells also possess anti-inflammatory attributes similar to MDSCs (52). However, we did not detect any CD4+CD25+Foxp3+ cells associated with *S. aureus* biofilm infections (data not shown), whereas another group has reported regulatory T cell involvement in biofilm clearance (33). The reasons for these discrepancies are not clear but...
may arise from differences in experimental models and/or *S. aureus* strains tested. Based on our analysis, we propose that MDSCs represent the main immunosuppressive effector cell during *S. aureus* orthopedic biofilm infection. The signals controlling MDSC recruitment, activation, and suppressive activity during *S. aureus* biofilm infection remain ill-defined and are ongoing topics of investigation in our laboratory.

Our RB6-C85 depletion studies revealed significant increases in bacterial dissemination from the orthopedic infection site. As mentioned previously, RB6-C85 recognizes both Ly6G and Ly6C epitopes, effectively depleting MDSCs, neutrophils, monocytes, and, by extension, macrophages. Therefore, although MDSC infiltrates were reduced, effector populations were also targeted, leaving fewer leukocytes either locally or systemically.

**FIGURE 11.** RB6-C85 treatment enhances *S. aureus* biofilm burdens and dissemination. (A) Gross appearance of infected tissues from animals receiving RB6-C85 or an isotype-matched IgG revealed a marked caseous exudate in the former. (B) Bacterial burdens associated with the knee joint, surrounding soft tissue, femur, and orthopedic implant and (C) heart, kidney, and spleen of control IgG– or RB6-C85–treated animals at days 7 and 14 postinfection. Results are expressed as CFU per milliliter for orthopedic implants or CFU per gram of tissue to correct for differences in tissue sampling size. Results are representative of 10 mice per group from two independent experiments. Significant differences between IgG and RB6-C85 animals are denoted as *p* < 0.05, **p** < 0.01, and ***p*** < 0.001 (unpaired two-tailed Student *t* test).
cally to prevent *S. aureus* dissemination to peripheral organs. By extension, we propose that the function of each leukocyte subset differs depending on the local microenvironment; namely, although the inhibitory actions of MDSCs were negated following RB6-C85 treatment, this coincided with a local reduction in inflammatory monocytes/macrophages, such that biofilm growth could not be held in check at the primary infection site (i.e., joint). When biofilm-associated bacteria seeded peripheral sites, the paucity of systemic neutrophils likely accounted for the failure to effectively clear the infection, which is essential because neutrophils are a main effector cell against planktonic *S. aureus* (53–56).

Dissemination was not observed with anti-Ly6G Ab treatment, which was attributed to the local monocyte/macrophage population that remained intact and exhibited heightened proinflammatory activity. MDSC infiltrates were also detected in a *S. aureus* catheter-associated biofilm infection model (Supplemental Fig. 3), and RB6-C85 treatment similarly increased bacterial burdens and dissemination (Supplemental Fig. 4), providing independent confirmation that MDSCs are a hallmark of *S. aureus* biofilm infection.

One notable finding in the present study was the extensive extramedullary hematopoiesis observed in the spleens of RB6-C85-treated animals compared with isotype controls. Extramedullary hematopoiesis is frequently seen during chronic inflammatory diseases and cancer (19), and expansion of CD11b+ Gr-1+ MDSCs has been reported in tumor and polymicrobial sepsis models (7, 57, 58).

When biofilm-associated bacteria seeded peripheral sites, the paucity of systemic neutrophils likely accounted for the failure to effectively clear the infection, which is essential because neutrophils are a main effector cell against planktonic *S. aureus* (53–56).

Dissemination was not observed with anti-Ly6G Ab treatment, which was attributed to the local monocyte/macrophage population that remained intact and exhibited heightened proinflammatory activity. MDSC infiltrates were also detected in a *S. aureus* catheter-associated biofilm infection model (Supplemental Fig. 3), and RB6-C85 treatment similarly increased bacterial burdens and dissemination (Supplemental Fig. 4), providing independent confirmation that MDSCs are a hallmark of *S. aureus* biofilm infection.

One notable finding in the present study was the extensive extramedullary hematopoiesis observed in the spleens of RB6-C85-treated animals compared with isotype controls. Extramedullary hematopoiesis is frequently seen during chronic inflammatory diseases and cancer (19), and expansion of CD11b+ Gr-1+ MDSCs has been reported in tumor and polymicrobial sepsis models (7, 57, 58). During infection, the requirement for myeloid cells dramatically increases in response to an expanding infectious burden, which creates a need for emergency myelopoiesis and the mobilization of immature myeloid cells from the bone marrow and spleen (19). The targeted reduction in Gr-1+ cells coincident with increasing biofilm burdens with RB6-C85 treatment likely explains the extensive extramedullary hematopoiesis observed in the spleens of these animals. Another unexpected finding was that Gr-1+ (Ly6G/Ly6C) infiltrates were increased at the site of orthopedic infection following RB6-C85 administration. However, this was likely a compensatory mechanism in response to elevated bacterial burdens both locally and systemically in Gr-1+–depleted mice, because these newly recruited Ly6G+Ly6C+ cells were unable to suppress CD4+ T cell proliferation, which agrees with reports of polymicrobial sepsis (7). Alternatively, we also observed enhanced levels of G-CSF, IL-6, and VEGF in the serum of RB6-C85–treated mice, all of which contribute to the expansion of immature myeloid cell populations (2, 4). Alternatively, the failure to deplete Ly6G+Ly6C+ infiltrates at later intervals could be explained by the induction of anti-rat IgG Abs that would be expected to impair the efficacy of RB6-C85 treatment (rat anti-mouse Gr-1). However, this appears less likely because RB6-C85 was still capable of significantly reducing inflammatory monocyte and macrophage infiltrates into *S. aureus*–infected joints 2 wk after repeated Ab administration.

Live CT scans revealed significantly more osteolysis in the femurs of RB6-C85–treated animals compared with 1A8 and isotype control mice, which may be attributed to the increased bacterial burdens in the former. The exact mechanisms of osteolysis are still not completely understood, and they differ depending on pathologic conditions (59, 60). However, several studies suggest that proinflammatory mediators, such as IL-1β, could play a role in the initiation and progression of osteolysis (61–63), and numerous proinflammatory mediators were significantly elevated in the joint and surrounding soft tissue following RB6-C85 treatment, including IL-1β, that coincided with increased bone destruction. Additionally, *S. aureus* is not only capable of colonizing the bone matrix, but it can also invade osteoblasts, which could contribute to chronicity (64). Furthermore, *S. aureus* internalization by osteoblasts can lead to apoptosis and disrupt the balance of osteoblast and osteoclast activities, which could facilitate bone destruction (64).

Interestingly, a recent study identified phenol-soluble modulins as a key inducer of osteoblast proliferation in an *S. aureus* osteomyelitis model (65); however, effects on osteoclasts remain to be defined.

Our studies are just beginning to explore the role of MDSCs during *S. aureus* infection. By manipulating these cells with Ab depletion strategies, we demonstrated that their immunosuppressive function prevents monocytes/macrophages from eliminating biofilm-associated bacteria by attenuating their proinflammatory properties. Our findings do not exclude the possibility that the biofilm matrix may also play a role in thwarting immune recognition in vivo; however, this remains an area of debate. Although it is clear that intact biofilms do afford some degree of protection against macrophage phagocytosis as previously shown by our laboratory and others (16, 23, 66–69), it is clear that neutrophils are fully capable of invading and phagocytosing biofilm-associated bacteria (23, 70–72), yet there is no apparent impact on biofilm growth. The fact that staphylococcal biofilms polarize macrophages toward an alternatively activated M2 phenotype does suggest that macrophage surface receptors are triggered to elicit this programing event; however, the identity of these receptors remains unknown. Future studies examining a *S. aureus* biofilm-defective mutant would be valuable for determining...
whether signals from the biofilm itself are responsible for MDSC recruitment and immunosuppressive activities. Preventing the presumed immunosuppressive action of infiltrating MDSCs may offer a novel therapeutic strategy to thwart these devastating, chronic infections.

Acknowledgments
We thank Katherine Estes in the University of Nebraska Medical Center Small Animal Imaging Core for assistance with CT scans, Dr. Charles Kuszynski and Victoria Smith in the University of Nebraska Medical Center Cell Analysis Facility for support with FACS analysis, Roxanne Alter for assistance with cytospin stains, and the University of Nebraska Medical Center Tissue Sciences Facility for imaging of H&E-stained tissues. We also thank John Varrone and Kohei Nishitani at the University of Rochester School of Medicine and Dentistry for providing the protocol for processing infected orthopedic devices for scanning electron microscopy analysis.

Disclosures
The authors have no financial conflicts of interest.


Supplemental Figure 1. MDSCs exhibit less inherent proinflammatory activity than macrophages. Bone marrow-derived macrophages (MΦ) and FACS-purified Ly6G<sup>high</sup>Ly6C<sup>+</sup> MDSCs recovered from the site of *S. aureus* orthopedic biofilm infection (tissue MDSC) or the spleen (spleen MDSC) of infected animals were stimulated with 10<sup>7</sup> heat-inactivated (HI) *S. aureus* or PGN (10 µg/ml). Supernatants were collected at 24 h, whereupon G-CSF (A), CCL5 (B), CCL3 (C) and IL-6 (D) expression was quantitated by MILLIPLEX. Significant differences are denoted by asterisks (*, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001; one-way ANOVA with Bonferroni’s multiple comparison post-hoc analysis) and are representative of three independent replicates.
Supplemental Figure 2. The Ly6G^{high}Ly6C^{+} infiltrate observed during Gr-1 depletion does not suppress T cell proliferation. FACS-purified Ly6G^{high}Ly6C^{+} cells recovered from infected joint tissues of mice treated with RB6-C85 (Gr-1) or isotype-matched control IgG at day 14 were immediately cultured ex vivo with efluor670-labeled CD4^{+} T cells for proliferation assays at either a 1:1 or 5:1 ratio [(−) T cells only; (+) T cells incubated with CD3/CD28 Dynabeads]. Results are representative of two independent replicates with significant differences denoted by asterisks (*, p < 0.05; ***, p < 0.001; one-way ANOVA with Bonferroni’s multiple comparison post-hoc analysis).
Supplemental Figure 3. CD11b^+Gr-1^+ MDSC infiltrates are observed during *S. aureus* catheter-associated biofilm infection. Mice (n = 4 per time point) were infected with 10^3 CFU of USA300 LAC::lux in the lumen of surgically implanted catheters to establish biofilm infection. Catheter-associated tissues were collected from mice at the indicated time points, whereupon CD11b^+Gr-1^+ infiltrates were quantitated by flow cytometry. Results are presented as the percentage of the total CD45^+ infiltrate.
Supplemental Figure 4. RB6-C85 treatment during *S. aureus* catheter-associated biofilm infection results in increased bacterial burdens and dissemination. Mice (n = 4 IgG and 5 RB6-C85 per time point) were infected with $10^3$ CFU of USA300 LAC::lux in the lumen of surgically implanted catheters to establish biofilm infection. Animals received i.v. injections of 100 µg RB6-C85 or IgG isotype control Ab at days -1, 2, and 5 following *S. aureus* exposure, whereupon bacterial burdens associated with infected catheters, surrounding tissue, and dissemination to the kidney were quantitated at day 7 post-infection. Significant differences between groups are denoted by asterisks (*, $p < 0.05$; **, $p < 0.01$; unpaired two-tailed Student *t*-test).