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A Major Role for Myeloid-Derived Suppressor Cells and a Minor Role for Regulatory T Cells in Immunosuppression during *Staphylococcus aureus* Infection

Christina Tebartz,*⁺¹ Sarah Anita Horst,*⁺¹ Tim Sparwasser,+ Jochen Huehn,‡ Andreas Beineke,§ Georg Peters,¶ and Eva Medina*⁺

*Staphylococcus aureus* can cause difficult-to-treat chronic infections. We recently reported that *S. aureus* chronic infection was associated with a profound inhibition of T cell responses. In this study, we investigated the mechanisms responsible for the suppression of T cell responses during chronic *S. aureus* infection. Using in vitro coculture systems, as well as in vivo adoptive transfer of CFSE-labeled OT-II cells, we demonstrated the presence of immunosuppressive mechanisms in splenocytes of *S. aureus*-infected mice that inhibited the response of OT-II cells to cognate antigenic stimulation. Immunosuppression was IL-10/TGF-β independent but required cell–cell proximity. Using DEREG and Foxp3gfp mice, we demonstrated that CD4⁺ CD25⁺Foxp3⁺ regulatory T cells contributed, but only to a minor degree, to bystander immunosuppression. Neither regulatory B cells nor tolerogenic dendritic cells contributed to immunosuppression. Instead, we found a significant expansion of granulocytic (CD11b⁺Ly6G⁺Ly6C<sub>low</sub>) and monocytic (CD11b⁺Ly6G⁻Ly6C<sub>high</sub>) myeloid-derived suppressor cells (MDSC) in chronically infected mice, which exerted a strong immunosuppressive effect on T cell responses. Splenocytes of *S. aureus*-infected mice lost most of their suppressive activity after the in vivo depletion of MDSC by treatment with gemcitabine. Furthermore, a robust negative correlation was observed between the degree of T cell inhibition and the number of MDSC. An increase in the numbers of MDSC in *S. aureus*-infected mice by adoptive transfer caused a significant exacerbation of infection. In summary, our results indicate that expansion of MDSC and, to a minor degree, of regulatory T cells in *S. aureus*-infected mice may create an immunosuppressive environment that sustains chronic infection.
respond to bacterial Ags with the transition from acute infection to persistence, and they exhibit a dysfunctional state that may explain their failure to promote sterilizing immunity (13). The failure to sustain T cell responsiveness could represent a major driving force for the development of S. aureus chronic infection. This emphasizes the need for a better understanding of the immunosuppressive programs that actively inhibit effector T cell responses.

During infection, the immune response to pathogens must achieve a balance between maximizing clearance of infectious agents and minimizing collateral tissue damage. The immune system achieves this balance by a variety of mechanisms, including regulatory T cells (Treg) (14, 15), regulatory B cells (Breg) (15, 16), tolerogenic dendritic cells (17), and myeloid-derived suppressor cells (MDSC) (18, 19). It is not surprising that pathogens have evolved mechanisms to hijack these immune-regulatory mechanisms to ensure their long-term survival by preventing immune clearance. Indeed, many pathogens, including viruses (20–22), bacteria (23–27), and parasites (28, 29), are capable of sustaining the expression of these immunosuppressive programs to actively suppress the appropriate immune response required for pathogen clearance. In this study, we investigated the immunosuppressive mechanisms arising during persistent S. aureus infection. We found this immunosuppressive program to be multifactorial and mediated by increased numbers of CD4+CD25+Foxp3+ Treg, as well as the expansion of MDSC. Although both Treg and MDSC contributed to immunosuppression, the latter was the major inhibitory mechanism of T cell responses. Probably, these immunosuppressive mechanisms were triggered during S. aureus infection to downregulate the excessive immune responses and to limit inflammation-mediated tissue pathology; in addition, they blocked Ag-specific effector immune responses that are essential for clearing the pathogen. Thus, the resulting immune dysfunction inevitably contributed to chronic infection by enabling bacterial persistence.

Materials and Methods

Bacterial strains

The rsbU+ derivative of S. aureus strain 8325-4 named SH1000 (30) and S. aureus strain 6850, originally isolated from a patient with bacteremia, osteomyelitis, septic arthritis, and multiple systemic abscesses (31), were added to the in vivo–proliferation assay.

In vivo–proliferation assay

At the specified times, spleens and lymph nodes from uninfected or S. aureus–infected OT-I/Thy1.1 or OT-II/Thy1.1 mice were stimulated with a single-cell suspension and seeded into 96-well flat-bottom plates at 0.5–1 × 10^6 cells/well in 100 µl complete RPMI 1640 medium. The cells were stimulated with different concentrations of OVA323–339 or OVASINFEKL peptide (provided by W. Tegge, Helmholtz Centre for Infection Research). After three days of incubation at 37°C and 5% CO2, the cells were pulsed with 1 µCi [3H]thymidine (Amersham) and harvested 16–18 h later on a gamma scintillation counter.

In vitro–immunosuppression

Cells isolated from spleen and lymph nodes of OT-I/Thy1.1 or OT-II/Thy1.1 mice were stimulated with a single-cell suspension and seeded into 96-well flat-bottom plates at 0.5–1 × 10^6 cells/well in 100 µl complete RPMI 1640 medium. The cells were stimulated with 1 µg/ml OVA323–339 or OVA–sens peptide, in the presence of titrated numbers of cells obtained from the spleen and lymph nodes of infected or S. aureus–infected C57BL/6 mice (>21 d of infection) at ratios of 1:4, 1:2, or 1:1 in a total of 200 µl/well and incubated for 72 h at 37°C and 5% CO2. Cells were pulsed with 1 µCi [3H]thymidine and harvested 16–18 h later on Filtermats A using a cell harvester. The amount of [3H]thymidine incorporation was measured in a gamma scintillation counter.

In some experiments, titrated concentrations of anti-IL-10 or anti-TGF-β mAb (eBioscience) were added to the culture. For experiments using the Transwell system, 1.5 × 10^6 cells/well from OT-II–transgenic mice were added to the lower chamber, together with 1 µg/ml OVA323–339 peptide, and incubated with equal numbers of cells obtained from uninfected or S. aureus–infected C57BL/6 mice (>21 d of infection), which were added to the upper chamber of the Transwell system. The chambers were separated by a membrane with a pore size of 0.4 µm (Costar). Cells were incubated and harvested as mentioned above for the proliferation assay.

In vivo immunosuppression

CD4+ T cells were purified from the spleen and lymph nodes of OT-II/Thy1.1–transgenic mice using MicroBeads and MACS MS columns (Miltenyi Biotec), according to the manufacturer’s protocol. Purified CD4+ T cells (>95% purity) were labeled with 2.5 µM CFSE (eBioscience) for 10 min at 37°C. Approximately 2.5 × 10^6 CFSE-labeled purified CD4+ T cells were injected i.v. into recipient C57BL/6 mice that were either left uninfected or infected with S. aureus for 21 d. Recipient mice received 25 µg OVA (#A7641; Sigma-Aldrich) s.c. into each footpad at 24 h post adoptive transfer of TCR-transgenic CFSE-labeled CD4+ T cells. The popliteal lymph nodes were removed 72 h later. T cells stained with anti-CD4–PE and anti-Th1.1–PE–Cy7 Ab (BD Biosciences) were analyzed by flow cytometry using a BD LSR II (BD Biosciences). Immunosuppression was determined by the reduction in proliferation of CD4+ T cells reisolated from S. aureus–infected recipients in comparison with the proliferation of those reisolated from uninfected recipients, as measured by the loss of CFSE fluorescence.

Purification and depletion of specific cell populations

Positive selection of B220+ B cells, CD11b+ cells, and CD4+ T cells was performed using MicroBeads and MACS MS columns (Miltenyi Biotec), according to the manufacturer’s protocol. Positively selected cells were passed over a second selection column to increase their purity. Purity of selected and depleted cells was verified by flow cytometry. The purity of the isolated populations was >95% for B and CD4+ T cells and >90% for CD11b+ cells.

Magnetic depletion of B cells, CD4+ T cells, and CD11c+ cells was performed using MicroBeads and MACS MS columns (Miltenyi Biotec), according to the manufacturer’s protocol. Successful depletion...
was ensured by flow cytometry. Depletion of B cells, CD4+ T cells, and CD11c+ cells was >95%, and depletion of CD11b+ cells was >75%.

Depletion of Foxp3+ Treg was accomplished by treating S. aureus–infected DEREG mice (>21 d of infection) with 1 µg diphtheria toxin (Sigma-Aldrich) delivered i.p. on days 19 and 20 of infection or 1 d before infection and then on days 3, 7, 12, 17, and 20 of infection. Mice were sacrificed on day 21 of infection, and their kidneys were aseptically removed. Bacterial loads were determined by plating serial dilutions of kidney homogenates on blood agar.

**Flow cytometry**

Cells were incubated with purified rat anti-mouse CD16/CD32 (BD Biosciences) for 5 min to block FcR and then stained with Ab against CD11b-PE/Cy7 (BioLegend), Ly6C-allophycocyanin (BioLegend), and Ly6G-PE (Milenyi Biotec) in 2% FCS in PBS for 30 min at 4°C. Cells were washed with PBS and fixed with 2% paraformaldehyde in PBS at 4°C overnight. After additional washing steps with PBS, cells were analyzed using a BD LSR II flow cytometer (BD Biosciences) and analyzed by FlowJo software.

**Immunohistochemistry on paraffin sections**

Kidney tissue sections from uninfected or S. aureus–infected (> 21 d) C57BL/6 mice were placed in 10% neutral-buffered formalin, embedded in paraffin, and cut into 0.5-µm thin sections. Paraffin-embedded tissues were deparaffinized in Roticlear (Carl Roth) and rehydrated through graded alcohols. Endogenous peroxidase activity was blocked with 0.5% H2O2 in methanol, and tissue sections were incubated with rat anti-Foxp3 mAb (eBioscience) overnight at 4°C. Ag-retrieval pretreatment was performed by incubation with citrate buffer (pH 6) for 20 min in a microwave (800 W). Specificity controls included substitution of the respective primary Ab with an isotype-matched rat control Ab. Incubation with primary Ab was followed by incubation with biotinylated secondary Ab for 30 min at room temperature. Subsequently, the avidin–biotin–peroxidase complex (VECTASTAIN Elite ABC Kit; Vector Laboratories) was added and incubated for 30 min at room temperature. Ag–Ab reactions were visualized by incubation with 3,3′-diaminobenzidine tetrahydrochloride with 0.03% H2O2 for 5 min, followed by counterstaining with Mayer’s Hemalun. Slides were examined using an Olympus BX51 microscope.

**Immunohistochemistry on frozen sections**

Frozen kidney tissue sections (5 µm) were cut on a cryostat (Leica CM 1950), mounted on Superfrost Plus slides (Menzel-Gläser), fixed in acetone for 10 min at room temperature, and stored at −70°C. Sections were air-dried and rinsed twice with PBS for 10 min, and endogenous peroxidase was blocked by incubation for 30 min with 0.03% H2O2 in PBS. Tissue sections were incubated with rabbit serum (dilution 1:5) for 30 min to block nonspecific binding sites and then with monoclonal anti-mouse Ly6C (clone HK1.4, dilution 1:500; BioLegend) or anti-mouse CD11b Ab (clone M1/70, 1:500; BioLegend) for 1.5 h at room temperature. Subsequently, sections were treated for 30 min at room temperature with the secondary Ab (biotinylated rabbit anti-rat Ig), followed by incubation with avidin–biotin–peroxidase complex (PK 6100; both from Vector Laboratories) for 30 min at room temperature. After development of the positive Ag–Ab reaction by incubation with 3,3′-diaminobenzidine tetrahydrochloride with 0.03% H2O2 for 5 min, followed by counterstaining with Mayer’s Hematoxylin. Slides were examined using an Olympus BX51 microscope.

**MDSC depletion**

C57BL/6 mice were inoculated i.v. with S. aureus, as described above. Infected mice were injected i.p. with 120 µg genticinib (Sigma-Aldrich) diluted in PBS or with PBS alone (200 µl) on days 14, 17, and 20 of infection. For depletion of MDSC using anti–Gr1 Ab, S. aureus–infected mice received 100 µg anti-mouse Gr-1 Ab, a kind gift from Siegfried Weiss (Helmholtz Centre for Infection Research), i.p. on days 14, 17, and 20 of infection. Control mice received equivalent amounts of isotype control Ab in sterile PBS. The level of MDSC depletion after the different treatments was determined by flow cytometry analysis after staining spleen cells with Ab against CD11b, Ly6C, and Ly6G.

**Adoptive transfer of MDSC in vivo**

For adoptive transfer of MDSC, spleen cells were isolated from S. aureus–infected donor mice at day 14 of infection and incubated with PE–conjugated anti-Ly6G and anti-Ly6C Ab followed by anti-PE MicroBeads, and MDSC were positively selected using LS columns, according to the manufacturer’s instructions (Milenyi Biotec). MDSC were eluted after removing the column from the magnetic field, washed with PBS, and injected (5 × 106 cells/animal) into the tail vein of S. aureus–infected recipient mice at day 7 of infection. The purity of MDSC cells was confirmed by flow cytometry to be >95%. For comparison, an additional group of S. aureus–infected recipient mice was injected i.v. with the same amount of cells from the flow-through fraction, which consisted of MDSC-depleted splenocytes.

**Statistical analyses**

All data were analyzed with Excel 2000 (Microsoft Office; Microsoft) or GraphPad Prism 5.0 (GraphPad). Depending on the distribution of the dataset, comparisons between groups were made using a parametric ANOVA test with Tukey posttest or a t test. The p values < 0.05 were considered significant.

**Results**

**Presence of immunosuppressive mechanisms in S. aureus–infected mice**

In a previous study (13), we reported a profound unresponsiveness of T cells to TCR stimulation during the chronic phase of S. aureus infection in mice. In this study, we investigated whether immunosuppressive mechanisms contributed to the impaired functionality of Ag-specific CD4+ T cell responses observed during S. aureus chronic infection. For this purpose, transgenic OT-II mice carrying CD4+ T cells with a TCR specific for the MHC class II–restricted OVA323–339 peptide were infected i.v. with S. aureus strain SH1000, and the response of CD4+ T cells to OVA323–339 was determined in in vitro proliferation assays. We found that splenocytes isolated from uninfected transgenic OT-II mice proliferated after Ag recognition in a dose-dependent manner (Fig. 1A). In contrast, the proliferative response of splenocytes from S. aureus–infected (day 21 of infection) OT-II mice to OVA323–339 was completely abrogated, independently of the amount of stimulating Ag (Fig. 1A). A similar immunosuppressive effect was observed in experiments using OT-I mice carrying transgenic CD8+ T cells with TCR specific for the MHC class I–restricted OVA80–90 peptide (Fig. 1B). These observations clearly demonstrate the presence of immunosuppressive mechanisms in S. aureus–infected mice that are capable of inhibiting CD4+ and CD8+ T cells bearing TCR specificity unrelated to S. aureus Ags. The induction of suppressive mechanisms during S. aureus infection was further demonstrated in cocultures of OT-II cells with cells isolated from S. aureus–infected (21 d of infection) or uninfected C57BL/6 mice at different ratios. Thus, CD4+ OT-II cells proliferated strongly in the presence of cells from uninfected C57BL/6 mice but were completely repressed when cocultured with cells from S. aureus–infected mice (Fig. 1C). Cells from S. aureus–infected mice also were capable of suppressing the proliferative response of CD8+ T cells from transgenic OT-I mice to OVA80–90 peptide stimulation in coculture assays (Fig. 1D).

To further demonstrate the presence of immunosuppressive mechanisms in an in vivo setting, purified CD4+ T cells from OT-II/Thy1.1 mice were labeled with a proliferation dye (CFSE) and adoptively transferred into either uninfected or S. aureus–infected (21 d of infection) C57BL/6 mice. Recipient mice received a s.c. injection of OVA in the footpad 24 h after cell transfer. Cells were harvested from the popliteal lymph nodes of recipient mice 72 h after OVA injection, and the proliferation of CD4+Thy1.1 OT-II cells was determined by flow cytometry. Consistent with the in vitro results, proliferation of CD4+ responder cells was suppressed more potently in infected mice than in uninfected recipient mice (Fig. 1E).

We next determined whether the induction of immunosuppressive mechanisms was a general phenomenon linked to chronic S. aureus infection or was dependent on the specific strain of S. aureus used for infection. For this purpose, we used an exper-
Antibacterial model of chronic osteomyelitis (bone infection) that closely resembles the pathological features of the chronic human disease (34). In this experimental infection model, osteomyelitis is established after i.v. inoculation of mice with \textit{S. aureus} strain 6850, which was isolated from a patient with a skin abscess that had progressed to \textit{S. aureus} bacteremia, osteomyelitis, septic arthritis, and multiple systemic abscesses (31). Spleen cells isolated from mice infected for 21 d with \textit{S. aureus} strain 6850 completely inhibited the proliferative response of CD4+ OT-II cells in response to OV A323–339 stimulation in mixed cultures (Supplemental Fig. 1). These results indicated that immunosuppressive mechanisms generally were induced in long-term–infected mice, independently of the strains of \textit{S. aureus} used for infection.

Immunosuppressive pathways activated during \textit{S. aureus} infection are independent of IL-10 and TGF-\beta but require cell–cell proximity

We next investigated the potential involvement of immunosuppressive cytokines in the immunosuppressive mechanisms induced during \textit{S. aureus} infection. Because IL-10 and TGF-\beta are the most well-known and best characterized immunosuppressive molecules capable of directly inhibiting T cell function (35, 36), we measured the levels of these cytokines in the culture supernatant of splenocytes from \textit{S. aureus}–infected mice stimulated with anti-CD3/anti-CD28 Ab. Significantly greater levels of both IL-10 (Fig. 2A) and TGF-\beta (Fig. 2B) were released by stimulated cells isolated from \textit{S. aureus}–infected mice in comparison with cells from uninfected mice. However, the addition of increasing amounts of neutralizing Ab against IL-10 or TGF-\beta did not abrogate the immunosuppressive effect exerted by splenocytes from \textit{S. aureus}–infected cells against OT-II cells stimulated with OV A323–339 peptide in vitro cocultures (Fig. 2C). These observations ruled out a role for these mediators in \textit{S. aureus}–induced immunosuppression. We then determined whether immunosuppression required cell–cell proximity. To assess this, we cultured OT-II cells in the lower chamber of a Transwell system, in the presence of OV A323–339 peptide, with cells that were obtained from uninfected or \textit{S. aureus}–infected C57BL/6 mice in the...
FIGURE 2. Immunosuppressive mechanisms generated during S. aureus infection are IL-10/TGF-β independent but require cell–cell proximity. Production of IL-10 (A) or TGF-β (B) by cells from uninfected (day 0) or S. aureus–infected (day 21) mice that were stimulated with immobilized anti-CD3 and anti-CD28 mAb for 96 h. Data represent the mean ± SD of triplicate samples from three independent experiments. (C) Proliferation of OT-II cells in response to OVA_{323–339} peptide stimulation in mixed cultures (1:1 ratio with splenocytes) from either uninfected or S. aureus–infected C57BL/6 mice in the presence of increasing concentrations of either anti–IL-10 (upper panel) or TGF-β (lower panel) mAb. Each symbol represents the mean cpm (± SD) from six replicates. One representative experiment of three performed is shown. (D) Proliferation of OT-II cells stimulated with OVA_{323–339} peptide in cocultures with cells from uninfected or S. aureus–infected C57BL/6 mice (ratio 1:1) that were either in direct contact or separated by a membrane in a Transwell system, which allows bidirectional diffusion of soluble factors but not cell–cell contact. Each symbol represents the mean cpm (± SD) from six replicates. One representative experiment of five performed is shown. ***p < 0.001.

upper chamber. The results of this assay showed that the inhibitory activity of cells from S. aureus–infected mice was abrogated in the Transwell system (Fig. 2D), indicating that their suppressive effect on OT-II cell proliferation required direct cell–cell contact or cell–cell proximity.

CD4^+CD25^+Foxp3^+ Treg were induced during S. aureus infection and contributed to immunosuppression

An important question emerging after the demonstration of the induction of immunosuppressive mechanisms during S. aureus infection was which cell population(s) mediated this effect. Treg are powerful mediators that negatively control immune responses to maintain the balance between anti-inflammatory and proinflammatory responses, thereby protecting the host from exacerbated inflammation and subsequent pathology (37). In the context of chronic infections, these cells can suppress effector T cell responses and promote pathogen persistence (14, 15). Therefore, we investigated whether the number of Treg increased during S. aureus infection using Foxp3^{GFP} BALB/c mice. We found that S. aureus caused a significant increase in the absolute number of CD4^+CD25^+Foxp3^+ Treg, starting at day 14 of infection (Fig. 3A, 3B). We also quantified the numbers of CD4^+CD25^+Foxp3^+ cells in the spleen of S. aureus–infected C56BL/6 mice after bacterial inoculation. A significant expansion of CD4^+CD25^+Foxp3^+ cells was observed in infected mice starting at day 7 of infection, and the number remained significantly higher compared with uninfected mice at later time points (Fig. 3C). Immune staining of kidney tissue isolated from S. aureus–infected mice at day 28 of infection showed that Treg were not only present in lymphoid organs but also infiltrated local sites of infection (Fig. 3D).

To determine the contribution of Treg from S. aureus–infected mice to immunosuppression, we separated the cells isolated from infected mice into CD4^+ and CD4^- fractions and cocultured them with cells from OT-II mice in the presence of OVA_{323–339}. The results show that proliferation of OT-II cells was significantly reduced but not completely abrogated when cocultured with the CD4^+ T cell fraction (Fig. 4A). Intriguingly, the proliferation of OT-II cells was completely abrogated when cocultured with the CD4^- cell fraction (Fig. 4A). These results indicate that, although a population of CD4^+ T cells contributed to the immunosuppressive effect of cells from S. aureus–infected mice, there was an additional cell population(s) with immunosuppressive capacity in the non-CD4 T cell fraction. This was confirmed by the observation that Foxp3-depleted cells from S. aureus–infected DEREG mice retained their capacity to inhibit OT-II cell responses in coculture assays (Fig. 4B). To determine the impact of Treg depletion on the bacterial burden during in vivo infection, infected (day 21 of infection) DEREG mice were either treated with PBS (CTR) or with 1 μg diphtheria toxin (Treg-depleted) i.p. on two consecutive days before being sacrificed. Efficiency of depletion was >95% (Supplemental Fig. 2). Although the amount of S. aureus was lower in the kidneys of Treg-depleted mice, this trend did not reach statistical significance (Fig. 4C). Similar results were obtained when depletion of Treg in DEREG mice started at the very beginning of infection, after treating the mice with diphtheria toxin prior to and at days 3, 7, 12, 17, and 20 of infection (data not shown). Together, these results confirmed the presence of additional immunosuppressive mechanisms in S. aureus–infected mice that were not mediated by Treg.

Neither Breg nor tolerogenic dendritic cells are involved in S. aureus–induced immunosuppression

Our next aim was to identify the S. aureus–induced immunosuppressive mechanism operating in the non-CD4^+ T cell population.
B cells traditionally have been considered to participate in the immune response to pathogens by producing Ab or by their Ag-presentation functions. However, these lymphocytes also have emerged in recent years as key regulatory components of the immune system (15, 16, 38). Although Breg-mediated suppression is important for maintaining peripheral tolerance and inhibiting harmful immune responses, it also can facilitate pathogen persistence (15, 16, 39). To determine a potential participation of Breg in the immunosuppressive effect of cells from *S. aureus*–infected mice, we separated B220+ (purified B cells) and B220- (non-B cells) fractions and cocultured them with OT-II cells in the presence of OVA323–339. OT-II cells fully proliferated when cocultured with purified B220+ cells, whereas their proliferative response was completely suppressed when cultured with the non-B cell fraction (Fig. 5A). These data excluded a potential contribution of B cells to immunosuppression during *S. aureus* infection.

Recently, it was shown that phenol-soluble modulins produced by *S. aureus* can drive in vitro–cultured dendritic cells into a tolerogenic phenotype that inhibits the activation of Th1 cells (40). Therefore, we investigated whether tolerogenic dendritic cells were implicated in the induction of immunosuppression in *S. aureus*–infected mice. Proliferation of OT-II cells was completely inhibited when cocultured with cells from *S. aureus*–infected C57BL/6 mice, irrespective of the presence or absence of CD11c+ cells (Fig. 5B). These observations indicated that tolerogenic dendritic cells were not directly contributing to immunosuppression during *S. aureus* infection.

MDSC significantly expanded during *S. aureus* infection and suppressed OT-II cell responses in cocultures

MDSC are a heterogeneous population of immature myeloid cells that share a common property of suppressing T cell responses (18).
These cells are generally identified in mice by coexpression of CD11b and Gr-1 (Ly6G/Ly6C) surface markers (18). A partial block in the differentiation of immature myeloid cells into mature myeloid cells under pathological conditions, such as cancer, infection, or autoimmunity, can result in an expansion of this population (18). Two main subpopulations can be differentiated within the MDSC subset based on the specific expression of Ly6G and Ly6C: the granulocytic CD11b+Ly6G+Ly6Chigh population and the monocytic CD11b+Ly6G−Ly6Chigh population (41). We asked whether MDSC could be responsible for the suppressive effect observed in the CD4− cells from S. aureus−infected mice. We found that both monocytic and granulocytic MDSC were significantly expanded upon infection with S. aureus (Fig. 6A, 6B). The percentage of monocytic MDSC was ∼4−fold higher in infected mice than in uninfected mice at day 7 of infection, and this persisted for up to 3 wk (Fig. 6A, 6B). The granulocytic MDSC increased significantly in the spleen on day 7 of infection (∼4−fold higher than uninfected mice) and continued to increase with the progression of the infection, reaching numbers that were >8−fold by day 21 compared with uninfected mice (Fig. 6A, 6B).

A similar expansion of monocytic and granulocytic MDSC was observed in the murine model of chronic osteomyelitis (Supplemental Fig. 3). Next, we tested the suppressive capacity of these MDSC by assessing the proliferative response of OT−II cells to OVA323−339 peptide stimulation in coculture with unfractionated cells (C57BL/6) or with either CD4− or CD4+ cell fractions obtained from S. aureus−infected mice at day ≥ 21 of infection. Data are mean cpm (± SD) of six replicates. Similar results were obtained in three independent experiments. (B) Proliferative responses of OT−II cells to OVA323−339 peptide stimulation in coculture with spleen cells isolated from S. aureus−infected (≥21 d of infection) DEREG mice depleted of Treg by injection of diphtheria toxin (1 μg i.p.) on two consecutive days before the terminal sacrifice day (Treg-depl. C57BL/6) or from S. aureus−infected (≥21 d of infection) DEREG mice treated with PBS (C57BL/6). Data are mean cpm (± SD) from three replicates and were obtained from one of three independent experiments. (C) Bacterial loads in the kidneys of S. aureus−infected (21 d of infection) DEREG mice that were depleted of Treg by application of diphtheria toxin (1 μg i.p.) on two consecutive days before the terminal sacrifice day (gray bar) or treated with PBS (white bar). Data are mean ± SD compiled from three independent experiments and three mice/group and experimental set. ***p < 0.001.

The immunosuppressive effect of spleen cells from S. aureus−infected mice was reduced significantly after in vivo depletion of MDSC

The contribution of MDSC to immunosuppression during chronic S. aureus infection also was demonstrated in an in vivo setting. For this purpose, S. aureus−infected mice were depleted of MDSC after treatment with gemcitabine, a drug that selectively targets MDSC (42, 43). Gemcitabine treatment markedly reduced both the granulocytic CD11b+Ly6G−Ly6Chigh population and the monocytic CD11b−Ly6G−Ly6Chigh population in S. aureus−infected mice (Fig. 7A, 7B). To evaluate the impact of in vivo reduction of MDSC in immunosuppression, spleen cells isolated from gemcitabine-treated or from PBS-treated S. aureus−infected mice were added to Ag−stimulated OT−II splenocytes at a 1:1 ratio. As shown in Fig. 7C, the splenocytes from gemcitabine-treated mice lost much of their suppressive activity. Furthermore, a strong negative correlation was observed between the degree of inhibition of T cell proliferation and the amount of monocytic (Fig. 7D, upper panel) and granulocytic (Fig. 7D, lower panel) MDSC in the spleen of infected mice.

Next, we determined the impact of gemcitabine treatment on bacterial loads in the kidneys of S. aureus−infected mice at day 21 of infection. S. aureus−infected mice were treated with gemcitabine starting at day 14 of infection and were sacrificed at day 21. As shown in Fig. 8A, the amount of S. aureus in the kidneys did not differ between gemcitabine-treated or untreated mice. Heim et al. (44) recently reported that depletion of both granulocytic Ly6G−Ly6Chigh and monocytic Ly6G−Ly6Chigh MDSC significantly increased S. aureus burden in an experimental model of orthopedic biofilm infection. However, depletion of only the granulocytic Ly6G−Ly6Chigh population (leaving the monocytic Ly6Chigh population intact) resulted in improved bacterial clearance (44).
The investigators argued that granulocytic MDSC suppressed the antimicrobial effect exerted by monocytic MDSC against \textit{S. aureus} within the biofilm (44). To investigate whether this was also true for the experimental model of \textit{S. aureus} systemic infection used in this study, we treated the \textit{S. aureus}–infected mice with an anti–Gr-1 Ab that targeted only granulocytic (Ly6G\text{high}Ly6C\text{low}) MDSC (Fig. 8B). In contrast to what was observed in the biofilm model, we did not observe improved bacterial clearance in the kidneys of anti–Gr-1–treated mice (Fig. 8A). We then hypothesized that treatment with these agents can successfully eliminate MDSC in the spleen but fail to deplete MDSC in the kidneys. To address this, we performed histological examination of kidney tissue sections from gemcitabine-treated and PBS-treated mice stained with Ab against CD11b. Representative photomicrographs (Fig. 8C) clearly show that treatment with gemcitabine failed to deplete CD11b\textsuperscript{+} cells in the kidneys of \textit{S. aureus}–infected mice. Likewise, CD11b\textsuperscript{+} cells were still present in the kidneys of anti–Gr-1–treated \textit{S. aureus}–infected mice (data not shown).

Adoptive transfer of MDSC increased the severity of \textit{S. aureus} infection

An alternative approach to demonstrate the suppressive function of MDSC in the host immune response to \textit{S. aureus} in vivo was to increase the number of MDSC in \textit{S. aureus}–infected mice in adoptive-transfer experiments. For this purpose, we purified MDSC from the spleen of donor mice by positive selection and adoptively transferred 5 $\times$ 10\textsuperscript{6} MDSC to \textit{S. aureus}–infected mice at day 7 of infection. Control groups included mice adoptively transferred with the flow-through fraction that consisted of MDSC-depleted splenocytes (non-MDSC), as well as untreated mice. Flow cytometry analysis confirmed that the purity of adoptively transferred MDSC was $>95\%$ (Fig. 9A). Bacterial loads were determined in the kidneys of adoptively transferred and control mice at day 3 after cell transfer. As shown in Fig. 9B, transferred MDSC caused a significant exacerbation of infection in recipient mice compared with untreated mice or with mice receiving the non-MDSC fraction. These results demonstrated the capacity of MDSC to negatively regulate the host immune response to \textit{S. aureus} during in vivo infection.
Discussion

In the current study, we investigated the immunosuppressive mechanisms that limit T cell immunity during *S. aureus* infection. Using TCR-transgenic OT-II mice, which enable direct monitoring of cognate Ag-driven clonal expansion of CD4+ T cells, we showed that immunosuppressive mechanisms capable of inhibiting the CD4+ T cell responses to a foreign Ag (OVA) were induced during chronic *S. aureus* infection. In an attempt to identify the cellular bases of bystander immunosuppression, we first focused our attention on the potential contribution of Treg. Generally, Treg are characterized by the expression of CD4 and Foxp3, and they can exert a potent suppressive effect on other immune cells (37). Although these cells are important regulators of the immune response and prevent autoimmune pathology by self-reactive lymphocytes (37), many pathogens have evolved mechanisms to exploit the Treg compartment, thereby compromising host defense by suppressing protective immune components (20–24, 28, 29). Using Foxp3+ mice in which the Treg subset is tagged with GFP, we performed a kinetic analysis of Treg in the spleen during *S. aureus* infection (32). We observed a significant expansion of Treg in the spleen and lymph nodes of *S. aureus*-infected mice, as well as recruitment of these cells into the site of local infection in the kidneys. However, the outcome of our in vitro immunosuppression assays using separated CD4+ and CD4+ cell fractions indicated that CD4+ T cells, which included the Treg subset, contributed only partially to the immunosuppressive effect exerted by cells isolated from *S. aureus*-infected mice. Furthermore, depletion of Treg did not significantly affect the immunosuppressive capacity of cells from *S. aureus*-infected DEREG mice. Altogether, these observations indicated that Treg contributed to *S. aureus*-induced immunosuppression only to a minor degree and that additional and more potent mechanisms must be operating. We also excluded the potential contribution of Breg or tolerogenic dendritic cells, both documented to be able to dampen protective immune responses and facilitate pathogen persistence (25, 26), to immunosuppression during *S. aureus* infection.

In our study, we consistently observed a massive expansion of immature myeloid cells in the spleen of *S. aureus*-infected mice exhibiting phenotypic characteristics similar to those previously described for MDSC (18, 19). The development of MDSC is generally not well understood. Under physiological conditions, immature myeloid cells differentiate from hematopoietic stem cells and migrate into the periphery where they differentiate into mature granulocytes, macrophages, or dendritic cells. In pathological situations, such as cancer, autoimmunity, or chronic infections, abundant production of pathology-associated mediators arrest the differentiation of immature myeloid cells with vigorous immunosuppressive potential (18, 19). Although most studies addressing the role of MDSC have focused on cancer, it is becoming increasingly clear that MDSC also play significant roles in regulating the immune response during infections. In this regard, expansion and activation of MDSC were described to be involved in the establishment of chronic viral infections (45). MDSC also were reported to be...
MDSC are CD11b+Ly6G population, and white arrows indicate the percentage of the CD11b+Ly6G+ tissues obtained from PBS-treated (experiment of three is shown. CD11b. CD11b+ cells are indicated by arrows. Scale bars, 25 µm.

FIGURE 8. Gemcitabine treatment does not impact bacterial loads in the kidneys of S. aureus–infected mice. (A) S. aureus–infected mice were injected i.p. with 120 µg of gemcitabine (white bar) or with 100 µg of anti-Gr-1 Ab (hatched bar) every third day, starting at day 14 of infection. Control mice received PBS alone (black bar). Mice were sacrificed at day 21 of infection, and the amount of bacteria in the kidneys was determined by plating. Data are mean CFU (± SD) from the compilation of three independent experiments. (B) Representative dot plots of spleen cells from PBS-treated (upper panel) or anti-Gr-1–treated (lower panel) S. aureus–infected mice stained with Ab against CD11b, Ly6G, and Ly6C. Black arrows indicate the percentage of the CD11b+Ly6G+Ly6C<sub>high</sub> (monocytic) population, and white arrows indicate the percentage of the CD11b+Ly6G+Ly6C<sub>low</sub> (granulocytic) population of total spleen cells. One representative experiment of three is shown. (C) Immunohistochemical staining of kidney tissues obtained from PBS-treated (left panel) or gemcitabine-treated (right panel) S. aureus–infected mice (day 21 of infection) with Ab against CD11b. CD11b<sup>+</sup> cells are indicated by arrows. Scale bars, 25 µm.

induced in individuals infected with Mycobacterium tuberculosis and are suggested to contribute to the inability of the host to eradicate the pathogen because they impair protective T cell responses (46). MDSC accumulate in cystic fibrosis patients chronically infected with Pseudomonas aeruginosa and correlate with disease activity (27). Immunosuppression of T cells during Trypanosoma cruzi (47) and Toxoplasma gondii (48) infection also was linked to MDSC. A similar immunosuppressive effect of MDSC was demonstrated during infections with helminths (49, 50) and Candida albicans (51), and a very recent publication reported the contribution of MDSC to orthopedic biofilm infection caused by S. aureus (44). In our study, we provide evidence for an expansion of MDSC with immunosuppressive effect during the chronic phase of infection in a murine model of systemic S. aureus infection.

MDSC have been broadly classified into two subsets, depending on the level of expression of Ly6G and Ly6C. Granulocytic MDSC have a CD11b<sup>+</sup>Ly6G<sup>+</sup>Ly6C<sub>low</sub> phenotype, whereas monocytic MDSC are CD11b<sup>+</sup>Ly6G<sup>−</sup>Ly6C<sub>high</sub> (41, 52). MDSC polarization links their phenotypic and functional changes to disease development (52). We found that both MDSC subsets were expanded during chronic S. aureus infection; however, expansion of the granulocytic MDSC population was more pronounced. We also demonstrated that in vivo reduction of MDSC by treatment with gemcitabine markedly decreased the immunosuppressive effect of splenocytes from S. aureus–infected mice on T cell proliferation. However, treatment with gemcitabine did not translate into improved bacterial clearance in the kidneys of infected mice. Furthermore, treatment with anti-Gr-1 Ab that targeted only granulocytic MDSC also did not affect the bacterial loads in the kidneys of S. aureus–infected mice. This contrasts with the improved bacterial clearance within the orthopedic S. aureus biofilm reported by Heim et al. (44) after depletion of only granulocytic MDSC. Given our observation that cell–cell contact or close proximity is required for immunosuppression, we hypothesized that an unsuccessful depletion of MDSC in the kidneys could be responsible for the unaffected bacterial clearance, because immunosuppression still would be exerted locally at the site of infection, even if MDSC have been efficiently eliminated at a distant site, such as the spleen. Indeed, histopathological examination confirmed that myeloid cells were still present in the kidneys of S. aureus–infected mice after gemcitabine or anti–Gr-1 treatment. Although these agents have been used extensively for depletion of MDSC in mice, the efficiency of MDSC depletion generally has been determined in the spleen or peripheral blood (42, 43, 53, 54), and little information is available regarding the capacity of these treatments to remove MDSC in other organs. Nevertheless, it was reported that injection of anti–Gr-1 Ab failed to deplete MDSC in the bone marrow (55) and liver (56). An alternative approach to demonstrate the negative impact of MDSC on the control of S. aureus infection in vivo was to increase the numbers of MDSC in infected mice by adoptively transferring cells from donor mice. Using this experimental setting, we showed a significant exacerbation of S. aureus infection in mice adoptively transferred with.

FIGURE 9. Adoptively transferred MDSC exacerbate infection in S. aureus–infected mice. Splenocytes from S. aureus–infected donor mice taken at day 14 of infection were separated into MDSC (Ly6G<sup>+</sup> + Ly6C<sup>+</sup>) and non-MDSC fractions using MicroBeads. (A) Representative graphs showing the purity of the different fractions. (B) A total of 5 × 10<sup>6</sup> MDSC or non-MDSC cells was injected i.v. into S. aureus–infected recipient mice at day 7 of infection. Control mice (CTR) were left untreated. Mice were sacrificed 3 d after adoptive cell transfer, and the amount of bacteria in the kidneys was determined by plating. Each symbol represents the mean CFU (± SD) from the compilation of three independent experiments (n = 9). ***p < 0.001, CTR versus MDSC or non-MDSC.
MDSC but not in mice receiving the same amount of non-MDSC splenocytes. These observations clearly demonstrate that MDSC negatively regulated the immune response to S. aureus in vivo and suggest that targeting these immunosuppressive cells may constitute a novel therapeutic approach for improving the clinical outcome of chronic S. aureus infections.

Despite the progress made in recent years to understand the molecular mechanisms governing MDSC biology, many specific details regarding the factors involved in the origin and expansion of MDSC, as well as the factors that prevent their terminal differentiation, remain unclear. In the context of tumors, increasing evidence exists for the involvement of IL-1β, IL-6, high concentrations of GM-CSF, M-CSF, vascular endothelial growth factor, IL-10, TGF-β, FLT3 ligand, and stem cell factor (c-kit L) (18, 57).

In the specific context of S. aureus infection, our data indicated that immunosuppression required cell–cell proximity and was not mediated by IL-10 or TGF-β. This suggests that the suppressive mechanisms act either through cell surface receptors or through the release of short-lived soluble mediators, such as NO, reactive oxygen species, or H2O2 (18, 58–60). Further research is needed to unravel the specific pathways driving the expansion of MDSC and, as well as the mechanisms by which MDSC exert their immunosuppressive effect in the context of S. aureus infection.

In summary, the results presented in this article indicate that immunosuppression of T cell immunity during chronic S. aureus infection involves the expansion of MDSC and, to a minor degree, of Treg. We believe that counteracting the effects mediated by these immunosuppressive mechanisms is likely to be an additional important way to treat chronic S. aureus infections.

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