This information is current as of April 13, 2017.

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*J Immunol* published online 4 March 2013
http://www.jimmunol.org/content/early/2013/03/03/jimmunol.1202563

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/03/04/jimmunol.1202563.DC1

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Staphylococcus aureus Phenol-Soluble Modulin Peptides Modulate Dendritic Cell Functions and Increase In Vitro Priming of Regulatory T Cells

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The major human pathogen Staphylococcus aureus has very efficient strategies to subvert the human immune system. Virulence of the emerging community-associated methicillin-resistant S. aureus depends on phenol-soluble modulin (PSM) peptide toxins, which are known to attract and lyse neutrophils. However, their influences on other immune cells remain elusive. In this study, we analyzed the impact of PSMs on dendritic cells (DCs) playing an essential role in linking innate and adaptive immunity. In human neutrophils, PSMs exert their function by binding to the formyl peptide receptor (FPR) 2. We show that mouse DCs express the FPR2 homolog mFPR2 as well as its paralog mFPR1 and that PSMs are chemotaxtants for DCs at nontoxic concentrations. PSMs reduced clathrin-mediated endocytosis and inhibited TLR2 ligand-induced secretion of the proinflammatory cytokines TNF, IL-12, and IL-6, while inducing IL-10 secretion by DCs. As a consequence, treatment with PSMs impaired the capacity of DCs to induce activation and proliferation of CD4+ T cells, characterized by reduced Th1 but increased frequency of FOXP3+ regulatory T cells. These regulatory T cells secreted high amounts of IL-10, and their suppression capacity was dependent on IL-10 and TGF-β. Interestingly, the induction of tolerogenic DCs by PSMs appeared to be independent of mFPRs, as shown by experiments with mice lacking mFPR2 (mFPR2−/−) and the cognate G protein (p110γ−/−). Thus, PSMs from highly virulent pathogens affect DC functions, thereby modulating the adaptive immune response and probably increasing the tolerance toward the pathogen. The Journal of Immunology, 2013, 190: 000–000.
known to secrete several immune-modulatory proteins interfering with the innate immune system, such as inhibitors of the complement cascade (9), of leukocyte chemokinesis and extravasation (10), and of the bactericidal activity of defensins (11), to name just a few. However, whereas S. aureus modulation of innate immunity has been explored to some extent, it has remained largely unclear how these bacteria interfere with human adaptive immunity. Recent studies strongly suggest that such mechanisms contribute to S. aureus virulence, thereby limiting the efficacy of Abs, T cells, and vaccines (12, 13). If and how S. aureus modulates the function of APCs has hardly been investigated.

DCs are unique APCs linking the innate and adaptive immunity. In their immature state, DCs are characterized by efficient Ag uptake in the periphery. After stimulation of pattern recognition receptors, for example, TLRs, they undergo maturation, characterized by Ag processing and presentation on MHCII molecules, upregulation of costimulatory molecules, as well as cytokine secretion. Mature DCs enter the lymphatic organs, where they efficiently activate T cells and thereby induce Ag-specific T cell responses (14, 15). Various Th cell subsets play important roles in the immune response against S. aureus infections (16–20). In a mouse infection model, it has been shown that IFN-γ is important for survival of S. aureus–induced sepsis by activating macrophages and neutrophils, leading to enhanced elimination of the pathogen (20). Furthermore, IFN-γ produced by CD4+ T cells induces the secretion of several CXC chemokines, leading to the recruitment of neutrophils to the site of infection (18). In addition, IL-17 secreted by Th17 and γδ T cells further activates the recruitment of neutrophils and induces the secretion of antimicrobial peptides by keratinocytes upon S. aureus infection of the skin (16, 19). Vaccination with the recombinant N terminus of the candidal Als3p adhesin with aluminum hydroxide led to the induction of Th1 and Th17 cells and thereby protected against systemic S. aureus infection (17).

Whereas the influence of PSMs on neutrophils has been well described, their impact on DC functions and the potential consequences for T cell activation are completely unknown. In the current study, we show that PSMs modulate the capacity of DCs to respond to TLR2 ligands, leading to a tolerogenic phenotype. This is characterized by inhibited proinflammatory cytokine, but increased IL-10 secretion and reduced Ag uptake. As a consequence, PSM-treated DCs had a reduced capability to activate T cells and specifically induced regulatory T cells (Tregs) while inhibiting Th1 differentiation. Although PSMs bound to mFPR2-expressing mouse DCs, mFPR2 did not seem to play an important role in the DC-modulatory capacity of PSMs. Thus, we propose that, in addition to their effects on neutrophils, PSM peptides subvert the DC-modulatory capacity of PSMs. Therefore, we propose that, in addition to their effects on neutrophils, PSM peptides subvert the adaptive immunity by modulating DCs, leading to immune evasion.

Materials and Methods

Mice

Female C57BL/6JolaHsd mice were purchased from Janvier (St. Berthevin Cedex, France). OT-II (21), TLR2−/− (22), FPR2−/− (23), and p110α mice (24) with a genetic C57BL/6 background were bred in the animal facilities of the University Clinic of Tübingen and the University of Tübingen. FOXp3−/eGFP mice (25) were generously provided by G. J. Hämmerling (DKFZ, Heidelberg, Germany). All mice held under specific pathogen-free conditions, provided food and water ad libitum, and used for experiments were between 6 and 10 wk of age. Animal experiments were performed in strict accordance with the German regulations of the Society for Laboratory Animal Science and the European Health Law of the Federation of Laboratory Animal Science Associations. The protocol was approved by the Regierungspräsidium Tübingen (Anzeigen 1.12.2010 and 12.9.2011).

Reagents and bacteria

Formylated PSM peptides (PSM1, PSM2, PSM3, PSM4, and δ-toxin) with the recently published sequences (6), a formylated control peptide with the reversed sequence of amino acids from PSM4 (sequence: KAFIDIIAKIKHTGV1IAM), MMKI (sequence: LESIFRSLFIRVM-NH2), and the reversed sequence of amino acids (Biochrom) was used in all cell culture experiments. BM-DCs were prepared using GM-CSF, as previously described (28, 29). Briefly, 2 × 10^6 bone marrow cells, flushed from the femurs and tibias of C57BL/6, TLR2−/−, FPR2−/−, and p110α−/− mice, were seeded in 100-mm dishes in 10 ml medium containing 200 U/ml GM-CSF. After 3 d, an additional 10 ml fresh medium containing 200 U/ml GM-CSF was added to the cultures. On days 6 and 8, half of the culture supernatant was collected and centrifuged, and the resultant cell pellet was resuspended in 10 ml fresh medium containing 200 U/ml GM-CSF and given back to the original plate. At day 7–9, the slightly attached cells were used for the experiments described in this work.

Chemotaxis of BM-DCs

Chemotaxis of BM-DCs toward PSMs was determined by using fluorescently labeled BM-DCs that migrated through a membrane fitted into an insert of a 24-well microtiter plate transwell system (Costar) containing a prewetted 5-μm pore-size polycarbonate filter, as described recently (30, 31). Briefly, 1 × 10^6 BM-DCs per ml were labeled with 1.1 μM acetoxy-ethyl ester of bis-2-carboxyethyl-5- and 6-carboxyfluorescein (Molecular Probes, Invitrogen) for 20 min at room temperature, washed, and resuspended in HBSS containing 0.05% HSA (HBSS-HSA). The upper compartment of the Transwell system was filled with 100 μl labeled BM-DCs and placed into a well containing 600 μl HBSS-HSA with different concentrations of PSMs or diluted S. aureus culture filtrates, as indicated. After incubation at 37°C under 5% CO2 for 100 min, the inserts were removed and the fluorescence of the wells was read in a fluorescence reader with excitation and emission filters of 485 and 530 nm, respectively. The fluorescence measured was used to monitor migration after subtracting the background migration observed when only buffer was added to the lower compartment.

Preparation of splenocytes

Splenocytes were prepared, as previously described (32). Briefly, the spleen was aseptically removed, cut into small pieces, and then digested for 30 min at 37°C in 2 ml modified RPMI 1640 containing collagenase (1 mg/ml; type IV, Sigma-Aldrich) and DNase I (150 μg/ml; Roche). To disrupt DC–T cell complexes, EDTA (0.1 M [pH 7.2]) was added, and mixing continued for 5 min. Single-cell suspensions were made by pipetting the digested organs. Undigested fibrous material was removed by filtration, and erythrocytes were lysed with lysis buffer (150 mM NH4Cl, 10 mM KHCO3, 2 mM NaEDTA).

To enrich DCs and neutrophils prior to cell sorting, CD19-expressing cells were depleted from splenic single-cell suspensions by MACS technology using CD19 magnetic beads (Miltenyi Biotec), following the manufacturer’s protocol. DCs (CD11c<sup>high</sup>MHC II<sup>+</sup>) and neutrophils (Gr-1<sup>+</sup>CD11b<sup>high</sup>) were sorted on a FACs Aria cell sorter (BD Biosciences) and analyzed on a Canto II flow cytometer.

RNA isolation, RT-PCR, and quantitative PCR

Total RNA was isolated from BM-DCs or sorted splenic DCs and neutrophils using the High Pure RNA Tissue Kit (Roche).

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Quantitative PCR was performed with the kit PowerSYBR Green RNA-to-Ct 1-Step Kit (Applied Biosystems). QuantiTect Primer Assay (Qigene) was used for mouse FRP-1, FRP-2, and GAPDH. Quantitative PCR was performed on ABI Prism 7500 7500 Fast Time Real-Time with the following settings: reverse transcription at 48°C for 30 min, polymerase activation at 98°C for 10 min, followed by 40 cycles of primer annealing and DNA extension at 60°C for 1 min and denaturation at 95°C for 15 s. The samples were normalized to GAPDH.

Flow cytometry

FACS buffer (PBS containing 1% BSA [Sigma-Aldrich] and 0.09% NaN₃ [Sigma-Aldrich]) was used for all incubations and washing steps. Cells were stained with FITC, PE, allophycocyanin, PE-Cy7, allophycocyanin–fluoro780, or fluoro450 conjugates of Abs against CD11c (N418), Gr-1 (RB6-8C5), CD4 (RM4-5), CD25 (PC61), CD80 (16-10A1), CD40 (H43–161.1), MHC II (M5/114.15.2), CD11b (M1/70), or TLR4/MD-2 (MT5S101) for 20 min at 4°C. To exclude dead cells, 7-aminoactinomycin D (Sigma-Aldrich) or Aqua Life/Dead (Invitrogen) was used.

To detect intracellular production of IFN-γ and IL-17, cells were fixed with 1% paraformaldehyde (Sigma-Aldrich) in PBS, permeabilized with 0.1% saponin (Sigma-Aldrich) and 0.5% BSA (Sigma-Aldrich) in PBS, and stained for intracellular cytokines with anti-IFN-γ (XMG1.2) and anti-IL-17 (TC11-18H10) for 15 min at 4°C or 10 min with anti-FOXP3 Ab (FJK-16s), the FOXP3 Staining Buffer Set (eBioscience) was used.

Samples were acquired for analysis using a Canto-II flow cytometer (BD Biosciences) with DIVA software (BD Biosciences) and were further analyzed using FlowJo 7.6 software (Tree Star).

For BM-DC surface marker analysis, BM-DCs (5 × 10⁵) were stained in 48-well plates and stimulated with 3 μg/ml S. aureus cell lysate and PSMs (100 μM) for 24 h. For PSMs-binding analysis, 5 × 10⁵ BM-DCs or 2 × 10⁶ splenocytes were incubated with TAMRA-marked PSMs3 (10 μM) for 30 min at 37°C in complete medium. The cells were then stained with fluorescein labeled Abs, as described above, and analyzed by flow cytometry.

Measurement of Ag uptake by flow cytometry

BM-DCs (5 × 10⁶) were stimulated with 3 μg/ml S. aureus cell lysate and PSMs3, as indicated, were incubated in medium with OVA-AlexaFluor647 (10 μg/ml) or lucifer yellow (2 mg/ml; both from Molecular Probes, Invitrogen) for 30 min or 10 min at 37°C, respectively. Unspecific binding was assessed by incubation of the DCs with the markers on ice. For phagocytosis, BM-DCs were incubated with GFP-expressing Yersinia enterocolitica WA314 (serotype 0:8) (33), provided by J. Heesemann (Max von Pettenkofer Institut, Ludwig-Maximilians-Universität München, Germany), at a ratio of 1 bacteria per DC for 30 min at 37°C. Cells were washed three times in ice-cold PBS containing 2% FCS. Subsequently, the BM-DCs were labeled with fluorescein Abs and analyzed by flow cytometry.

For measurement of LPS-induced uptake of TLR4, BM-DCs were incubated with or without PSMs3 1 h prior to stimulation with LPS (100 ng/ml) for 30 min at 37°C. Cells were washed three times in ice-cold PBS containing 2% FCS. Subsequently, the BM-DCs were labeled with fluorescent Abs and analyzed by flow cytometry.

Cytochrome production by BM-DCs

BM-DCs (5 × 10⁵) were seeded in 48-well plates and stimulated with 3 μg/ml S. aureus cell lysate. PSM peptides or culture supernatants of USA300 and its knockout mutant ΔΔβ were added in different concentrations, as indicated, and incubated for 6 h (TNF analysis) or 24 h (IL-6, IL-10). Cytokines in the supernatants were analyzed by bioassay in a Th17-favoring condition, 1 × 10⁷ BM-DCs were cocultured with 5 × 10⁴ CD4⁺ T cells from OT-2 mice in the presence of IL-6 (20 ng/ml; eBioscience), TGF-β (2 ng/ml; eBioscience), anti–IFN-γ (2 ng/ml; XMG1.1; eBioscience), anti–IL-1 (2 ng/ml; 11B11; eBioscience), OVA peptide (0.3 μg/ml), or with or without PSMs3. In both settings, the cells were cocultured for 4 d. After the last 5 h of coculture, T cells were restimulated with OVA peptide (1 μg/ml) and anti-CD3 Ab (1 μg/ml; 145-2C12; eBioscience) together with mornemis (eBioscience). The cells were analyzed for CD25, IFN-γ, and IL-17 expression by flow cytometry.

Treg suppression assay

BM-DCs (5 × 10⁵) were seeded in 96-well U-bottom plates and stimulated with 3 μg/ml S. aureus cell lysate with or without PSMs3 (6 μM) for 24 h. A total of 2 × 10⁵ CD4⁺ T cells from the spleen of FOXP3-eGFP mice was purified, as described above, and added to the BM-DCs. Seventy-two hours after coculture, cells were stained with CD3 and CD4 (145-2C11, BD Biosciences; RM4-5, eBioscience, respectively) Abs, and the CD4⁺FOXP3⁺ Tregs were purified by FACS sorting, according to the expression of CD3, CD4, and eGFP. In addition, CD3⁺CD4⁺FOXP3⁻ from the spleen of FOXP3-eGFP mice were purified by FACS sorting and used as T effector cells (Teffs). The CD3⁺CD4⁺FOXP3⁺ from a naive mouse were used as control Tregs. The Tregs were labeled with 10 μM efluoro670 Cell Proliferation Dye (eBioscience) for 10 min at 37°C. T effs (5 × 10⁵) were cultured for 72 h in U-bottom 96-well plates with irradiated splenocytes from C57BL/6 mice, anti-CD3 Ab (1 μg/ml; 145-2C12; eBioscience), and the indicated numbers of sorted Tregs. Proliferation of T cells was measured by flow cytometry and analyzed with the proliferation platform of Flowjo software 7.6. The division index is depicted in the histograms and is defined as the average number of cell divisions that a cell in the original population has undergone. Neutralizing Abs against IL-10 (10 μg/ml; JESS-16E3; eBioscience) and TGF-β (10 μg/ml; 1D11; R&D Systems) were added, as indicated. TGF-β and IL-10 secretion by Tregs after BM-DC coculture (1 × 10⁵ per well in 250 μl medium) was assessed by ELISA (eBioscience) after further culture for 72 h in an anti-CD3-coated 96-well flat-bottom plate (0.5 μg/ml anti-CD3).

Statistical analysis

Statistical analysis was performed with the GraphPad Prism 5.0 software (GraphPad, San Diego, CA) using one-way ANOVA with Bonferroni posttest or the unpaired two-tailed Student’s t test, as indicated. The differences were considered as statistically significant if p < 0.05 (*), p < 0.005 (**), or p < 0.001 (***)

Results

mFPR expression by mouse DCs

As PSM peptides exert their activity toward human neutrophils by binding with high affinity to FRP-2 and a FRP-2-specific inhibitor blocked their capacity to activate mouse neutrophils (5), the members of the FRP family are also likely targets for PSM peptides on mouse DCs. Analysis of the expression of receptors of the FRP family on mouse BM-DCs revealed that both mFPR-1 and mFPR-2 were expressed on RNA level (Fig. 1A). Quantitative PCR revealed a 500- or 260-fold higher expression of mFPR-1 and mFPR-2 on neutrophils compared with splenic DCs, respectively (Fig. 1A, left). Expression of mFPR-1 and mFPR-2 by BM-DCs was slightly higher than that of splenic DCs. To determine whether the expression of these receptors changes during maturation, quantitative PCR from immature and mature BM-DCs was performed (Fig. 1A, right). Both receptors were slightly upregulated 24 h after DC maturation with S. aureus cell lysate. Furthermore, BM-DCs and splenic DCs bound fluorescently labeled PSMs3 as determined by flow cytometry (Fig. 1B). Splenic neutrophils served as control and bound PSMs3 with similar affinity as DCs, whereas CD4⁺ T cells, which do not express members of the mFPR receptor family (8), revealed no binding of PSMs3. These data show that mouse DCs express mFPR-1 and mFPR-2 and are able to bind PSMs.

PSMs elicit chemotaxis of DCs

S. aureus PSMs have been shown to attract neutrophils by binding to FRP-2 (5). As we could show that mFPR-2 is expressed by DCs,
analyze whether PSMs modulate TLR2-mediated DC activation, as well as the production of proinflammatory cytokines by DCs. To leads to the upregulation of MHCII and costimulatory molecules in infection.

secreting high amounts of PSMs also attract DCs to the site of culture filtrates from a USA300 mutant strain deficient in PSMs induced chemotaxis of BM-DCs, whereas chemotaxis toward 100 nM, indicating that slightly higher concentrations are nec-

we hypothesized that PSMs might also be chemoattractants for DCs and might alter DC functions. PSMo3, one of the most effective PSM peptides regarding receptor-mediated effects on human neutrophils, also induced chemotaxis in mouse BM-DCs at nanomolar concentrations (Fig. 2A). The chemotaxis was char-

BM-DCs were treated with S. aureus cell lysates containing lipopeptides, the major staphylococcal TLR2 ligands. This treatment led to upregulation of MHC class II as well as the costimulatory molecules CD40 and CD80. Treatment of DCs with S. aureus cell lysates in combination with PSMo3 had no influence on the phenotypic DC activation induced by S. aureus cell lysates (Fig. 3A).

The production of the proinflammatory cytokines TNF, IL-6, and IL-12 by DCs was induced by S. aureus cell lysate (Fig. 3B). This induction was TLR2 dependent, as DCs from TLR2−/−mice did not secrete any of these cytokines (Supplemental Fig. 2A) upon stimulation with S. aureus cell lysate. Simultaneous treatment of DCs with S. aureus cell lysate and PSMo3 inhibited the secretion of TNF, IL-6, and IL-12, whereas the secretion of the anti-inflammatory cytokine IL-10 was increased in a dose-dependent manner (Fig. 3B). Interestingly, PSMo3 alone did not induce secretion of any of the measured cytokines. PSMo3 was not cytotoxic toward DCs at concentrations that modulated cytokine secretion and had only little or no cytokine-modulating effects at concentrations <100 nM (Fig. 3B). A reversed scrambled α-PSM congener lacking the α-helical structure of the PSM peptides served as control peptide and had no effects on TLR2 ligand-induced cytokine secretion by DCs (Fig. 3B). Similar results as for PSMo3 regarding cytokine production by DCs were obtained using the other PSMo and δ-toxin, with only PSMo4 having weaker effects (Supplemental Fig. 2B).

Autocrine IL-10 has been shown to modulate IL-12 production in macrophages and DCs (34, 35). Addition of an IL-10–blocking Ab reversed the inhibition of IL-12 and partly of TNF expression by PSMo3 in DCs (Fig. 3C), indicating that the induction of IL-10 expression by PSMo3 in DCs leads to an autocrine inhibition of IL-12 and TNF production.

To further strengthen the results obtained with synthetic PSMs, culture filtrates from S. aureus strain USA300 and a mutant strain deficient in PSMs were used to elucidate the effects of secreted PSMs on DC cytokine production (Fig. 3D). The culture filtrate of the USA300 wild-type strain, harboring the α- and β-PSMs and
δ-toxin, inhibited TNF secretion by DCs, as shown above for the synthetic PSMs, whereas the mutant strain deficient in all PSMs did not. These data demonstrate that PSM peptides are responsible for modulation of DC cytokine secretion by 

**PSMs inhibit clathrin-mediated endocytosis of DCs**

To elucidate possible impacts of PSMs on Ag uptake, a hallmark of DCs and prerequisite for T cell activation, we used fluorescently labeled OVA (OVA-AlexaFluor647) as a model Ag and measured OVA uptake by flow cytometry. DCs were cultured for 24 h with PSMa3 in the presence or absence of *S. aureus* cell lysate and subsequently incubated with OVA-AlexaFluor647 for 30 min. OVA is mainly taken up by clathrin-mediated endocytosis via the macrophage mannose receptor and to a lesser extent by macropinocytosis (36–38). Accordingly, OVA uptake by *S. aureus* cell lysate–treated and PSMa3 in the presence or absence of neutralizing anti–IL-10 Ab or isotype control. 

**Impaired T cell proliferation and Th1 priming by PSM-modulated DCs**

Th1 and Th17 cells play important roles in the immune response against *S. aureus* (16–20). To examine whether the aforementioned effects of PSMs on DC functions affect the ability of DCs to activate and prime CD4+ T cells, BM-DCs were treated with *S. aureus* cell lysate and PSMa3 overnight and subsequently cocultured with splenic CD4+ T cells from congenic mice for 72 h. Incubation with *S. aureus* cell lysate led to strongly increased T cell proliferation as analyzed by [3H]thymidine incorporation (Fig. 5A), which is in agreement with the activating capacity of TLR2 ligands (40). However, T cell proliferation was reduced in a dose-dependent manner when DCs were coincubated with PSMa3 (Fig. 5A). In accordance with this finding, the number of activated CD4+CD25+ T cells was reduced after stimulation with PSMa3-treated DCs (Fig. 5B). 

To analyze the influence of PSMs on T cell priming, DCs were incubated with *S. aureus* cell lysate in the presence or absence of PSMa3, loaded with OVA peptide, and subsequently cocultured with CD4+ T cells from OVA-specific TCR transgenic mice. Flow
cytometry analysis revealed a significantly reduced number of IFN-γ–secreting CD4+CD25+ T cells after coculture of DCs incubated with *S. aureus* cell lysate in the presence of PSMα3 compared with *S. aureus* cell lysate–treated DCs (Fig. 5C). No significant differences could be observed in the percentage of IL-17–secreting CD4+CD25+ T cells (Fig. 5D). These data demonstrate that PSMs not only inhibit T cell activation, but also T cell priming toward Th1 cells.

**Induction of Tregs by PSM-treated DCs**

The reduced T cell proliferation observed with PSM-modulated DCs could be due to a reduced stimulatory capacity of DCs or to the induction of Tregs. The frequency of CD4+CD25+FOXP3+ T cells was significantly increased after 72-h coculture with DCs preincubated with *S. aureus* cell lysate in the presence of PSMα3 compared with DCs pretreated with *S. aureus* cell lysate alone (Fig. 6A). These data suggest that incubation of DCs with PSMα3 induces tolerogenic DCs characterized by IL-10 production and priming of Tregs, leading to the inhibition of Teffs. Next we tested whether there are differences in the suppression capacity of Tregs induced by DCs preincubated with or without PSMα3. Therefore, CD4+ T cells from FOXP3-eGFP mice were cocultured with DCs, as described above. Seventy-two hours later, the FOXP3+ T cells were purified by cell sorting according to their expression of eGFP, and their capacity to suppress Teff proliferation was analyzed. No differences in the suppressive capacity of Tregs, generated by DCs prestimulated either in the presence or absence of PSMα3, could be observed (Fig. 6B). Interestingly, Tregs induced by PSM-treated DCs produced higher amounts of IL-10 compared with control Tregs, whereas no difference in their TGF-β secretion could be observed (Fig. 6C). Thus, PSMα3 modulates DCs to induce IL-10–producing Tregs. To address the mechanism by which the generated Tregs act on Teffs, we performed suppression assays in the presence of function-blocking Abs for IL-10 and TGF-β. Blocking of either IL-10 or TGF-β in coculture experiments increased Teff proliferation (Fig. 6D). Blocking of both cytokines further slightly reduced the suppression capacity of Tregs, but T cell proliferation was still reduced by 27%, indicating that, in addition to TGF-β and IL-10, other yet unknown factors led to suppression capacity of Tregs in this assay. In summary, PSM-modulated DCs show impaired Th1 priming most likely due to the induction of IL-10–producing CD4+CD25+FOXP3+ Tregs.

**PSM-modulated cytokine pattern by DCs is independent of FPRs**

PSMs bind with high affinity to FPR2 on neutrophils (5). To determine whether the induction of tolerogenic DCs by PSMs is mediated via mFPR2, BM-DCs from wild-type and mFPR2−/− mice were analyzed for cytokine production. TLR2 activation led to slightly higher production of TNF and IL-12 by DCs from FPR2−/− mice, whereas IL-10 production was reduced (Fig. 7A). This indicated that PSMα3 modulates DCs to induce IL-10–producing Tregs. The reduced T cell proliferation observed with PSM-modulated DCs could be due to a reduced stimulatory capacity of DCs or to the induction of Tregs. Thus, PSMα3 modulated DCs to induce IL-10–producing Tregs, while IL-12 production was reduced (Fig. 7A). Similar to wild-type DCs, PSMα3 inhibited the production of TNF and IL-12, while inducing IL-10 secretion by DCs from FPR2−/− mice, indicating that PSMα3 modulates DC cytokine production independently of mFPR2.

To analyze whether the changes in cytokine profile by DCs are specific for PSMs or a general DC response to FPR2 ligands, we simultaneously treated DCs with *S. aureus* cell lysates and MMK1, a noncytotoxic FPR2 ligand. MMK1 inhibited TNF production by DCs, but not as efficiently as PSMα3 (Fig. 7A). This inhibition was abrogated using mFPR2−/− DCs, showing that MMK1-mediated inhibition of TNF was FPR2 dependent. Moreover, in contrast to PSMα3, MMK1 did not affect IL-12 and IL-10 secretion, further indicating that DC modulation by PSMα3 is not a general response to FPR2 ligands. Furthermore, by using BM-DCs from p110γ−/− mice, in which the downstream PI3K class Ib signaling of G protein–coupled receptors (GPCRs) is blocked (41), we ruled out the involvement of GPCRs in the in-
production of tolerogenic DCs by PSMs, as p110γ−/− DCs revealed similar cytokine secretion patterns as wild-type DCs (Fig. 7A). In contrast to the modulated cytokine secretion by DCs, the reduction of OVA uptake mediated by PSMα3 and MMK1 was dependent on FPR2 and downstream signaling of GPCRs (Fig. 7B). Taken together, these data point to a FPR2-independent induction of tolerogenic DCs by PSMs produced by CA-MRSA strains.

**Discussion**

PSMs are a group of virulence factors contributing to the virulence of emerging CA-MRSA strains such as USA300 (6). Previous studies have shown the modulatory effects of PSMs on innate immune cells, for example, recruitment and activation of neutrophils and induction of cell lysis (5, 6). However, the impact of PSMs on the adaptive immune response has not yet been addressed. We demonstrate that PSMs produced by CA-MRSA strains modulate the adaptive immune response because they effectively prevent the development of a Th1 response induced by the TLR2 ligands of *S. aureus* (34), IL-10 inhibits IL-12 and also TNF production by DCs in an autocrine manner. It is long known that systemic *S. aureus* infections are associated with the endogenous production of TNF, IL-6, and IFN-γ (43). This proinflammatory response plays a significant role in the clearance of the bacterium as TNF/lympho-toxin-α double-mutant mice show increased mortality upon *S. aureus* i.v. infection (44).

An effective evasion strategy by a pathogen would be to target and instruct DCs to become tolerogenic and prime regulatory IL-10 responses, blocking inflammation and allowing uncontrolled spread of the pathogen (45). Several groups have described the induction of tolerogenic DC by several pathogens (46–49). Thus, we speculate that the anti-inflammatory effects of PSMs on DCs may be beneficial for the spread of the bacterium.

**FIGURE 5.** Impaired T cell proliferation and Th1 priming by PSM-modulated DCs. (A) BM-DCs were incubated with *S. aureus* cell lysate and the indicated concentrations of PSMα3 for 24 h. Splenic CD4+ T cells from C57BL/6 mice were added to the culture, and T cell proliferation was analyzed by [3H]thymidine incorporation 72 h later. (B) The frequency of CD4+CD25+ T cells after DC-T cell coculture with PSMα3 concentration of 10 μM, as described in (A). (C) BM-DCs treated as in (A) with a PSMα3 concentration of 6 μM and cocultured with OVA-specific splenic CD4+ T cells from OT-II mice for 4 d in the presence of OVA23–39 peptide. IFN-γ production by OVA-specific CD4+ T cells was assessed by flow cytometry. (D) PSM-treated BM-DCs were cocultured as in (C) in the presence of IL-6, TGF-β, anti–IFN-γ, and anti–IL-4 for 4 d. IL-17 production by OVA-specific CD4+ T cells was assessed by flow cytometry. The data show one representative of three independent experiments with similar results (A, C, D) or means of four independent experiments (B). The graphs represent means ± SD. *Statistically significant differences [one-way ANOVA with Bonferroni posttest (A) or unpaired Student's t test (B–D)]. *p < 0.05, **p < 0.005.
PSM-treated DCs showed a reduced capability to activate T cells and more specifically blocked Th1 differentiation, whereas Th17 differentiation was unaffected. Such an impaired T cell response might lead to reduced bacterial clearance during infection, as, for example, patients with HIV disease are highly susceptible to *S. aureus* colonization and skin infection due to reduced CD4+ T cell counts (50). Protective CD4+ T cell responses have been described for Th1 and Th17 cells due to their secretion of IFN-γ and IL-17 (16–19, 51). Accordingly, IFN-γ plays a protective role during systemic infection, by activation of macrophages and neutrophils enhancing phagocytosis of the bacteria (51), and wound infection by CXC chemokine-induced neutrophil recruitment (18), whereas it is dispensable in skin infections (16). The findings in the current study point to a reduced capability of the host to clear systemic and invasive infections caused by PSM-producing *S. aureus* infections.

IL-17 produced by Th17 and γδ T cells is important for immunity against skin and other epithelial infections due to neutrophil recruitment and activation of keratinocytes, leading to production of antimicrobial peptides (19). PSMs did not affect priming of Th17 cells, although secretion of the Th17-inducing cytokine IL-6 by DCs was reduced. This is in contrast to data from a noneosinophilic asthma mouse model showing that activation of FPRs by a synthetic W-peptide inhibits both Th17 and Th1 responses by modulating airway DCs (52). This difference might be explained by the different TLR stimuli used or that our in vitro system lacks certain characteristics of an in vivo system, which might influence priming of Th17 cells.

**FIGURE 6.** Induction of IL-10–secreting Tregs by PSM-treated DCs. (A) BM-DCs were incubated with *S. aureus* cell lysate and the indicated concentrations of PSM3a for 24 h and then cocultured with CD4+ T cells for 72 h. The frequency of CD4+CD25+FOXP3+ T cells was analyzed by flow cytometry. (B) Experiment as in (A) with 6 μM PSM3a and coculture with CD4+ T cells from FOXP3-eGFP mice. Splenic CD3+CD4+FOXP3+ T cells from the FOXP3-eGFP mice and the CD4+FOXP3+ Tregs from the coculture were purified by cell sorting. Proliferation of cell proliferation dye efluor670-labeled T cells cultured with or without CD4+FOXP3+ cells was analyzed by flow cytometry. The histogram overlays show proliferation of T cells without (gray dotted line) or with (black line) Tregs. The numbers depicted in the histogram overlays correspond to division indices (the average number of cell divisions that a cell in the original population has undergone) as analyzed by FlowJo software. The graph shows division indices at a T eff:T reg ratio of 2:1. (C) Experiments as in (B). Sorted CD4+FOXP3+ cells were stimulated with anti-CD3 Ab for 72 h, and cell culture supernatants were analyzed for IL-10 and TGF-β by ELISA. (D) Experiment as in (B) (T eff:T reg 2:1) with neutralizing Abs against IL-10 and TGF-β. Division indices are depicted in the histograms. The data represent means of three independent experiments ± SD (A, C) or show one representative of three independent experiments performed in triplicates with similar results (B and D). *Statistically significant differences (one-way ANOVA with Bonferroni posttest (A, B, D) or unpaired Student t test (C). *p < 0.05, **p < 0.005, ***p < 0.001.
During infection, a fine regulation between Tregs and Teffs is needed to control the immune response and contain the infection without inducing immune pathologies (53). Thus, induction of Tregs by the pathogen itself can lead to evasion of protective T cell responses. Inducible populations of Tregs are characterized by high secretion of IL-10, which has been shown to be the key regulator for inhibiting the immune responses against several pathogens (46, 54). In this study, PSMα3-treated DCs induced the differentiation of naïve CD4+ T cells into IL-10–producing Tregs.

However, their suppression capacity was only partly dependent on IL-10 and TGF-β, indicating that other mechanisms such as cell contact–mediated suppression may be involved in Treg-mediated suppression of T cell differentiation. Similar to our findings, filamentous hemagglutinin from *Bordetella pertussis* stimulates IL-10 production, thereby inhibiting IL-12 release by DCs (46). This leads to the induction of IL-10–producing Tregs and the inhibition of a Th1 response. Filamentous hemagglutinin functions as an adhesin and mediates this effect by binding and thereby activating CR3 or CD47 on DCs. It is possible that PSMs induce via modulating DCs mixed populations of activated T cells, thereby probably influencing the outcome of an infection, as we observed less Th1 cells, more Tregs, and unchanged numbers of Th17 cells upon pretreatment of DCs with PSMs. A direct effect of PSMs on T cells can be excluded, as T cells do not express mFPR2 (8) and do not bind PSMα3 (Fig. 1).

We show that PSMs bind to FPRs on mouse DCs and human neutrophils (5). However, the induction of tolerogenic DCs appears to be independent of binding to FPRs, as DCs from mFPR2−/− mice as well as combined inhibition of mFPR1 and mFPR2 did not affect the induction of tolerogenic DCs. Recently, another pore-forming toxin, β hemolysin/cytolysin from group B *Streptococcus*, has been described to induce IL-10 while inhibiting IL-12 and NO synthase 2 production in macrophages (35). The authors of this study tested other pore-forming toxins such as *S. aureus* α-toxin that showed similar abilities to inhibit IL-12 expression but not to increase expression of IL-10. Furthermore, they ruled out the responsibility of membrane damage for the induction of tolerogenic DCs. Together with the fact that noncytotoxic PSM concentrations were used in our study suggests that other mechanisms than pore formation or binding to FPRs mediate induction of tolerogenic DCs by PSMs.

In summary, the net effect of PSMs on the adaptive immune system appears to be the inhibition of Th1 cell activation by the induction of tolerogenic DCs that direct the induction of Tregs. This may represent a novel immune subversion strategy employed, for example, by CA-MRSA strains producing high amounts of PSMs. However, additional experiments are needed to examine whether the findings presented in this work are relevant in in vivo infection models reflecting different diseases of *S. aureus*.

**Acknowledgments**

We thank Nele Nikola, Manina Günther, Daniela Gunst, and Patricia Hrstić for excellent technical support, Sabrina Grimm for operating the cell sorter, and Hans-Georg Rammensee for critical discussion.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


