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Staphylococcus aureus Downregulates IP-10 Production and Prevents Th1 Cell Recruitment

Zhigang Li,* Benoît Levast,* and Joaquín Madrenas*,†

Staphylococcal superantigens cause toxic shock syndrome, which is characterized by massive T cell activation and a predominant Th1 profile of cytokine production. However, superantigen-producing *Staphylococcus aureus* strains are often part of the human nasal microbiome, and this carrier state has often been associated with some type 2 immune responses such as chronic sinusitis with polyps and atopic dermatitis. We have previously reported that the *S. aureus* cell wall downregulates the human T cell response to superantigens through a TLR2-dependent, IL-10-mediated mechanism. In this study, we show that *S. aureus* also regulates the profile of superantigen-induced T cell recruitment. The staphylococcal superantigen SEE induced the production of Th1 cell-recruiting chemokines, including IP-10, through an IFN- γ -dependent mechanism. Such an induction was suppressed by the concomitant presence of *S. aureus*. The downregulation of IP-10 by *S. aureus* was mediated by components of its cell wall, but was not due to peptidoglycan-induced IL-10 production. Instead, *S. aureus* triggered activation of MAPKs p38 and ERK, as well as inhibition of STAT1 signaling in monocytes, altogether contributing to the downregulation of IP-10 and other Th1 cell-recruiting chemokines (e.g., CXCL9 and CXCL11). These effects translated into inhibition of superantigen-induced Th1 cell recruitment. Taken together, our data may explain why colonization of superantigen-producing *S. aureus* can induce, under some circumstances, mucosal type 2 immune responses. *The Journal of Immunology*, 2017, 198: 000–000.

The Gram-positive bacterium *Staphylococcus aureus* is a common microorganism of the upper respiratory tract microbiota, being present in at least a quarter of the healthy population without causing any apparent symptoms (1). *S. aureus* is also one of the most frequent microbes linked to morbidity and mortality throughout the world, causing severe infections such as pneumonia, osteomyelitis, abscess, sepsis, and toxic shock syndrome (TSS) (2). In the United States alone, >20% of blood infections diagnosed in hospitals are linked to *S. aureus* (3). With the appearance of antibiotic-resistant strains such as methicillin-resistant *S. aureus*, the morbidity and mortality associated to *S. aureus* has increased significantly (4–7).

In combination with neutrophils and macrophages, CD4⁺ T cells play a pivotal role in the immune response to *S. aureus* (8, 9). Host adaptive immunity to this microbe has been linked to the development of Th1/Th17 cells (10–12). For example, staphylococcal superantigens mainly trigger a Th1/Th17 response characterized by high levels of IL-12 (13), IFN- γ (14), and IL-17 (15), whereas staphylococcal cutaneous infections are

mostly cleared by $\alpha\beta$ Th17 cells and $\gamma\delta$ T cells (16). In contrast to these scenarios, colonization and recurrent infections with *S. aureus* are often found in diseases that have been linked to a predominant Th2 profile of cytokine production such as allergic sinusitis (17, 18) and atopic dermatitis (19, 20), which are characterized by the secretion of IL-4, IL-5, and IL-13 (21). This association raises the question of what are the factors that drive such opposite responses to *S. aureus*.

We have recently reported that the staphylococcal cell wall contains peptidoglycan (PGN)-embedded TLR2 ligands that induce a potent IL-10 response, which downregulates the T cell response to superantigens (14). This led us to hypothesize that the association of type 2 immune responses with *S. aureus* colonization may be due to TLR2-dependent inhibition of Th1/Th17 adaptive immunity to *S. aureus* (10). Among the different targets of such inhibition, we focused initially on chemokine production, as these molecules are important regulators of immune cell recruitment to the site of infection.

Similar to other microbes, *S. aureus* and its exotoxins are potent chemokine stimulators (22–24). Whether *S. aureus* and superantigens induce distinct chemokine profiles, resulting in a preferential recruitment of certain type of immune cells, is still unknown. Given that *S. aureus* induces a strong IL-10 response by monocytes and macrophages, and that this cytokine can modulate the production of many chemokines (25–27), it is plausible to suggest that *S. aureus* may induce a biased chemokine profile. In this study, we report that the cell wall of *S. aureus* downregulates the production of Th1 cell-recruiting chemokines through activation of MAPKs p38 and ERK and inhibition of STAT1 signaling, leading to an abrogation of superantigen-induced Th1 cell recruitment.

Materials and Methods

Immune cells and bacteria

Human PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation from whole blood donated by healthy volunteers. Informed consent was given by all individuals in compliance with the Research Ethics Office at McGill University. Monocytes were purified using the monocyte

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Abbreviations used in this article: ChIP, chromatin immunoprecipitation; CHX, cycloheximide; PGN, peptidoglycan; PRR, pattern recognition receptor; rh, recombinant human; RT-qPCR, quantitative real-time PCR; TSS, toxic shock syndrome.

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isolation kit (Miltenyi Biotec or Stemcell Technologies). PBMCs and monocytes were cultured in RPMI 1640 medium (Thermo Scientific) supplemented with L-glutamine, nonessential amino acids, sodium pyruvate, penicillin-streptomycin, and 10% FBS. The *S. aureus* isolate S8 was prepared as previously described (28). Briefly, bacteria were grown overnight in tryptic soy broth, washed, resuspended in PBS, and heat killed for 1 h at 100°C.

Reagents

Staphylococcal PGN and *Escherichia coli* LPS were purchased from Sigma-Aldrich. *Saccharomyces cerevisiae* zymosan, Pam3CSK4, FSL-1, and staphylococcal lipoteichoic acid were purchased from InvivoGen. Inhibitors for p38 (SB239063), ERK (PD98059), NF- κ B [6-amino-4-(4-phenoxyphenylethylamino)quinazoline], JNK (SP600125), and PI3K (wortmannin) were purchased from Sigma-Aldrich. Neutralizing Abs against IFNGR1 (mouse IgG1 clone 92101), IL-10 (mouse IgG2B clone 25209), IL-10R (mouse IgG1 clone 37607), and their appropriate isotype controls were purchased from R&D Systems.

RNA extraction and quantitative real-time PCR

PBMCs (2×10^6 cells per group) were treated with 1×10^7 CFU of heat-killed *S. aureus* or 10 μ g/ml staphylococcal PGN in the presence or absence of 10 ng/ml SEE for 4 h. Monocytes (1×10^6 cells per group) were preincubated with 1×10^7 CFU of heat-killed *S. aureus* for 1 h and subsequently incubated with 10 ng/ml IFN- γ for 3 h. In some experiments, cycloheximide (CHX) (50 μ g/ml) was added 1 h prior to *S. aureus* incubation. For the mRNA half-life experiments, actinomycin D (5 μ g/ml) was added after 3 h of IFN- γ treatment and cultures were incubated for an additional 0.5, 1, 2, or 4 h. Cells were harvested and RNA was extracted using the RNeasy Plus kit (Qiagen). cDNA was synthesized using a cDNA reverse transcription kit (Applied Biosystems). Quantitative real-time PCR (RT-qPCR) was performed using SYBR Master Mix (Invitrogen) with the following primers: CXCL9, 5'-CCGCTATCATCCAAAGGAG-3' and 5'-CTGGTGGGTGGTAGAAGAAC-3'; CXCL10, 5'-CTAGAACTGTACGCTGTACC-3' and 5'-TTGATGGCCTTCGATTCTGG-3'; CXCL11, 5'-AGG-ACGCTGTCTTTGTCATAG-3' and 5'-GCCTTGCTTGCTTCGATTGG-3'; hypoxanthine phosphoribosyltransferase, 5'-ATTGTAATGACCAGTCAAC-AGGG-3' and 5'-GCATTGTTTGGCAGTGTCAA-3'; and RPL19, 5'-GG-CTCGCCTCTAGTGTCT-3' and 5'-GCGGGCCAAGGTGTTTTTC-3'. Hypoxanthine phosphoribosyltransferase and RPL19 were used as reference genes. Data were collected by Opticon (Bio-Rad Laboratories) and analyzed using CFX Man 3.0 software.

Flow cytometry

To assess IP-10 production by intracellular cytokine staining, 1×10^6 PBMCs were treated with or without 10 ng/ml SEE for 21 h, and 3 μ g/ml brefeldin A (eBioscience) was added 4 h prior to staining. Cells were then stained for surface and intracellular Ags with the following fluorochrome-conjugated Abs: anti-human CD3 allophycocyanin-eFluor 780 (clone UCHT1; eBioscience), anti-human CD14 PerCP-Cy5.5 (clone M ϕ P9; BD Biosciences), anti-human CD19 Alexa Fluor 700 (clone HIB19; BD Biosciences), and anti-human IP-10 PE (clone 6D4/D6/G2; BD Biosciences). A Cytotfix/Cytoperm kit (BD Biosciences) was used for intracellular cytokine staining according to manufacturer's instructions. For IFN- γ staining, anti-human IFNGR1 PE (clone GIR-208; BD Biosciences) and anti-human IFNGR2 allophycocyanin (polyclonal goat IgG; R&D Systems) Abs were used. Fixable viability dye eFluor 520 (eBioscience) was used to gate live cells, and normal human serum was used to block Fc receptors prior to staining. To confirm Th1 cell differentiation, T cells were stained with the following Abs: anti-human CD3 allophycocyanin-eFluor 780 (clone UCHT1; eBioscience), anti-human CD4 Alexa Fluor 700 (clone OKT4; BioLegend), anti-human CXCR3 PE-CF594 (clone 1C6/CXCR3; BD Biosciences), and anti-human T-bet Brilliant Violet 421 (clone 4B10; BioLegend). A transcription factor buffer set (BD Biosciences) was used for T-bet staining as per the manufacturer's instruction. The Zombie Aqua fixable viability dye (BioLegend) was used to exclude dead cells, and the cells were incubated with normal human serum prior to staining. Flow cytometry was performed with an LSRFortessa II (BD Biosciences) and FACSDiva software (BD Biosciences) and data analyzed with FlowJo version 10.x (Tree Star, Ashland, OR).

Functional assays

PBMCs (2×10^5 cells per well) or monocytes (5×10^4 cells per well) were seeded in 96-well plates (200 μ l per well) and stimulated as indicated in the figure legends. For the experiments with inhibitors, cells were incubated in the presence of inhibitors for 1 h prior to stimulation. Pro-

duction of chemokines and cytokines was determined by measuring their accumulation in the supernatants by ELISA (BioLegend and eBioscience) or a multiplex assay kit (Meso Scale Diagnostics).

Western blotting

PBMCs (5×10^6 cells per group in 100 μ l) were stimulated at 37°C with 10 ng/ml SEE and/or 2.5×10^7 CFU of heat-killed *S. aureus* for the indicated times. For experiments with IFN- γ , monocytes (2×10^6 cells per group in 100 μ l) were pretreated with or without 2×10^7 CFU of heat-killed *S. aureus* for 1 h prior to addition of 1 ng/ml IFN- γ for the indicated times. Cell lysates were prepared in RIPA lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 mM Na₃VO₄, 1 mM NaF, 5% protease inhibitor mixture) as described previously (29). To assess phosphorylated STAT1 dimerization, monocytes were lysed in 1% Triton X-100, 150 mM NaCl, 10 mM HEPES (pH 7.6), 5 mM EDTA, 1 mM Na₃VO₄, 1 mM NaF, and 5% protease inhibitor mixture, and total proteins were cross-linked using 5 mM disuccinimidyl suberate (Thermo Scientific Pierce) at 4°C for 1 h; the reaction was then quenched with 50 mM Tris (pH 7.5) at 4°C for 15 min. For nuclear and cytoplasmic fractionation, NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific) were used (30). Phosphorylation of ERK1/2, p38, and STAT1 were analyzed by immunoblotting as described (28). β -Actin was used as a loading control, and GAPDH and histone H3 were used to test the purity of cytoplasmic and nuclear fractions, respectively. Anti-GAPDH Ab was purchased from EMD Millipore and all the other Abs were obtained from Cell Signaling Technology.

Chromatin immunoprecipitation

Monocytes (2×10^6 cells per group in 100 μ l) were pretreated with or without 2×10^7 CFU of heat-killed *S. aureus* for 1 h prior to the addition of 10 ng/ml IFN- γ for an additional 45 min. Cells were then harvested and subjected to chromatin immunoprecipitation (ChIP) assay using a magnetic ChIP kit (Thermo Scientific Pierce). DNA bound to phospho-STAT1 was isolated by immunoprecipitation and subjected to RT-qPCR with primers specific for the STAT1 binding region in the IP-10 promoter (5'-TTTGAAAGT-GAAACCTAATTCA-3' and 5'-AAAACCTGCTGGCTGTTCTTG-3'). Data were normalized to untreated cells.

CD4⁺ T cell polarization

CD4⁺ T cells were purified from PBMCs by negative selection using the CD4⁺ T cell isolation kit (Miltenyi Biotec or Stemcell Technologies). Cells were polarized in vitro with 1 μ g/ml plate-bound anti-CD3 Ab and 0.5 μ g/ml plate-bound anti-CD28 Ab plus Th1-inducing media (10 ng/ml recombinant human [rh]IL-12 and 2 μ g/ml anti-IL-4 Ab) for 8 d. Cells were split and 4 ng/ml rhIL-2 was added on day 5. Polarization of T cells was confirmed by determining IFN- γ production in response to PMA and ionomycin and intracellular staining for T-bet using flow cytometry. On day 8, Th1 cells were used for transwell cell migration assay.

Transwell migration assay

PBMCs (1×10^6 cells per group) were treated with 10 ng/ml SEE, 1 μ g/ml staphylococcal PGN, 5×10^6 CFU of heat-killed *S. aureus*, or a combination of these at a final volume of 1 ml for 24 h. The cell culture supernatants were then collected and stored at -80°C before being used for the migration assay. PBMCs from the same volunteer were used for CD4⁺ T cells isolation and subsequent Th1 cell polarization. On the day of transwell assay, Th1 cells were washed with PBS and resuspended in RPMI 1640 medium with the same supplements described above except for 2% FBS. Cells (2×10^5) in 100 μ l were added to the transwell insert. Supernatants stored at -80°C were diluted 2-fold and 600 μ l was added to wells of a 24-well plate. After 2 h incubation at 37°C, cells that migrated into wells were collected and analyzed by flow cytometry. The absolute numbers of migrated cells were calculated by normalizing the numbers of CD4⁺ cells to 123count eBeads (eBioscience).

Statistical analysis

Statistical analysis was performed using ANOVA and Student *t* test using GraphPad Prism. A *p* value <0.05 was deemed significant.

Results

S. aureus selectively downregulates the expression of IFN- γ -dependent chemokines and prevents Th1 cell recruitment

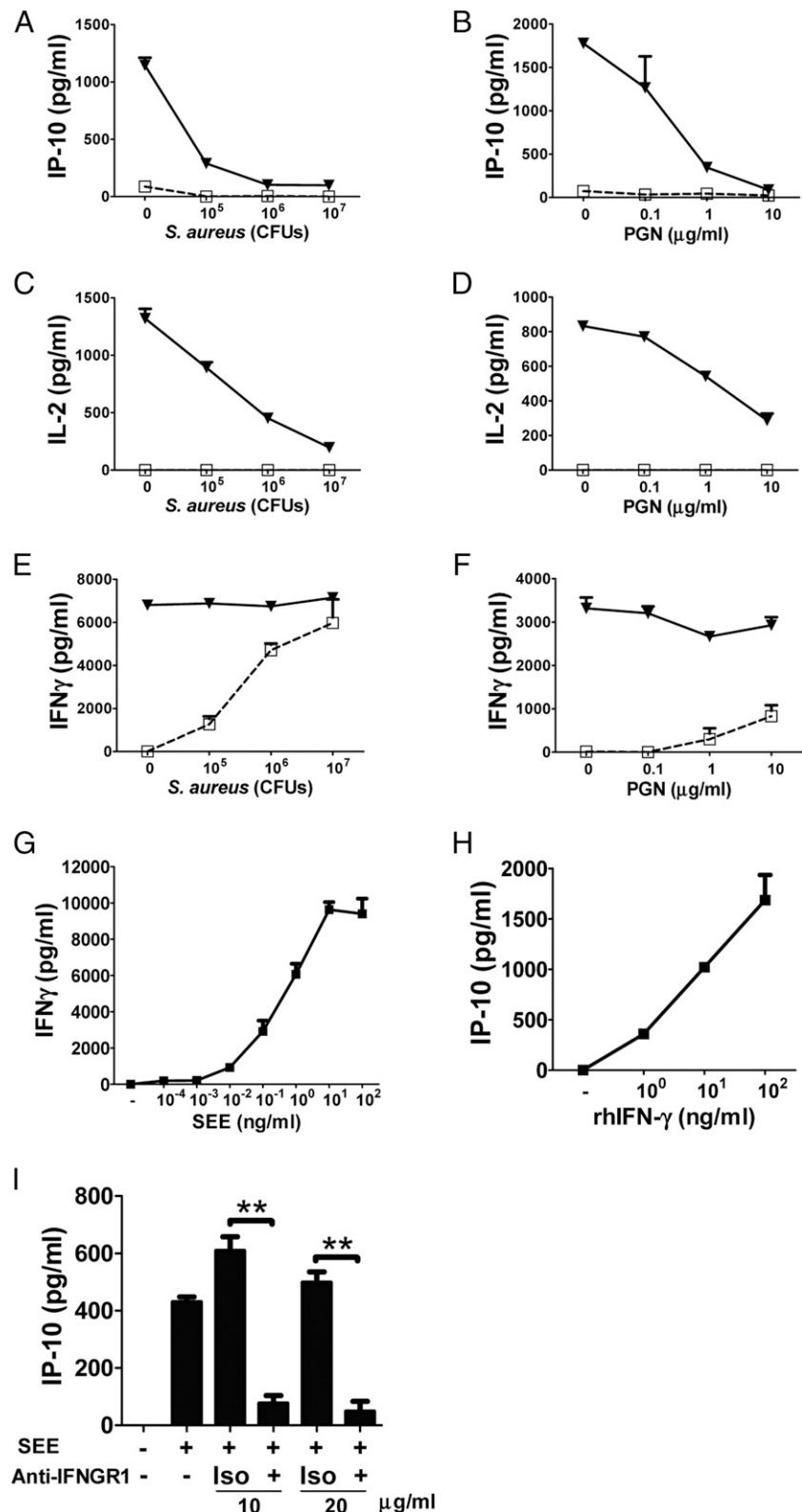
In an initial chemokine-selective microarray, we observed that *S. aureus* inhibited, in a dose-dependent manner, the production of the IFN- γ -dependent chemokine IP-10, also known as CXCL10,

by human PBMCs in response to the staphylococcal superantigen SEE (Fig. 1A). This inhibition of IP-10 production could be recapitulated using crude staphylococcal PGN preparations (Fig. 1B), suggesting that this effect was mediated by the staphylococcal cell wall. As we previously reported (14), superantigen-induced IL-2 production was also inhibited by *S. aureus* and its PGN, whereas IFN- γ was unaffected (Fig. 1C–F). The mechanism of this dissociated effect on IL-2 and IFN- γ is still under inves-

tigation. Similar to SEE (Fig. 1G), rhIFN- γ was able to induce the production of IP-10 in human PBMCs (Fig. 1H). To confirm that the IP-10 production to SEE was dependent on IFN- γ (31), we blocked IFN- γ R signaling using an anti-IFNGR1 Ab and found that this blockade abrogated the IP-10 response to SEE (Fig. 1I).

The inhibitory effect of *S. aureus* on SEE-induced chemokine production was also applicable to the two other IFN- γ -dependent chemokines CXCL9 and CXCL11 (Fig. 2A). To assess whether

FIGURE 1. Superantigen-induced IP-10 production is IFN- γ -dependent, and this induction is downregulated by *S. aureus* and its cell wall. IP-10, IL-2, and IFN- γ accumulation in culture supernatants of human PBMCs stimulated for 18 h with the indicated CFU of heat-killed *S. aureus* (A, C, and E) or concentrations of staphylococcal PGN (B, D, and F) in the presence (▼) or absence (□) of SEE superantigen (10 ng/ml) was determined by ELISA. (G) Quantification of IFN- γ in supernatants from human PBMC cultures stimulated with the indicated concentrations of SEE for 18 h. (H) Quantification of IP-10 in the culture supernatants of human PBMCs stimulated with the indicated concentrations of rhIFN- γ for 18 h. (I) Quantification of IP-10 in supernatants of human PBMC cultures stimulated with SEE (1 ng/ml) in the absence or presence of neutralizing Ab against IFN- γ R (IFNGR1) or its isotype control (Iso) (10 or 20 μ g/ml) for 18 h. Results are plotted as mean $\pm$ SD and are representative of three independent experiments from three different donors (triplicate samples for each experiment). ** p < 0.01.



the downregulation of IFN- γ -dependent chemokines had a functional relevance, we performed an in vitro transwell Th1 cell migration assay using the supernatants of PBMCs stimulated with SEE and/or *S. aureus* or PGN (Fig. 2B). Supernatants from PBMCs stimulated with SEE drastically increased Th1 cell recruitment compared with supernatants from untreated cells. In contrast, the supernatants from *S. aureus*- or staphylococcal PGN-treated PBMCs did not induce Th1 cell recruitment. Importantly, in line with reduced IP-10 production, supernatants from PBMCs treated with SEE and *S. aureus* or SEE and PGN recruited far fewer (65% fewer) Th1 cells than with SEE alone (Fig. 2B), implicating that the modulatory effect of the staphylococcal cell wall is dominant. The possibility that SEE, *S. aureus*, and staphylococcal PGN were themselves chemoattractants was ruled out by the observation that these stimuli did not induce Th1 cell recruitment. Additionally, although supernatants from PBMCs treated with SEE and *S. aureus* or SEE and PGN recruited ~25% fewer Th2 cells (data not shown) than with SEE alone, the reduction did not reach statistical significance. This decrease may be due to some expression of CXCR3 on Th2 cells.

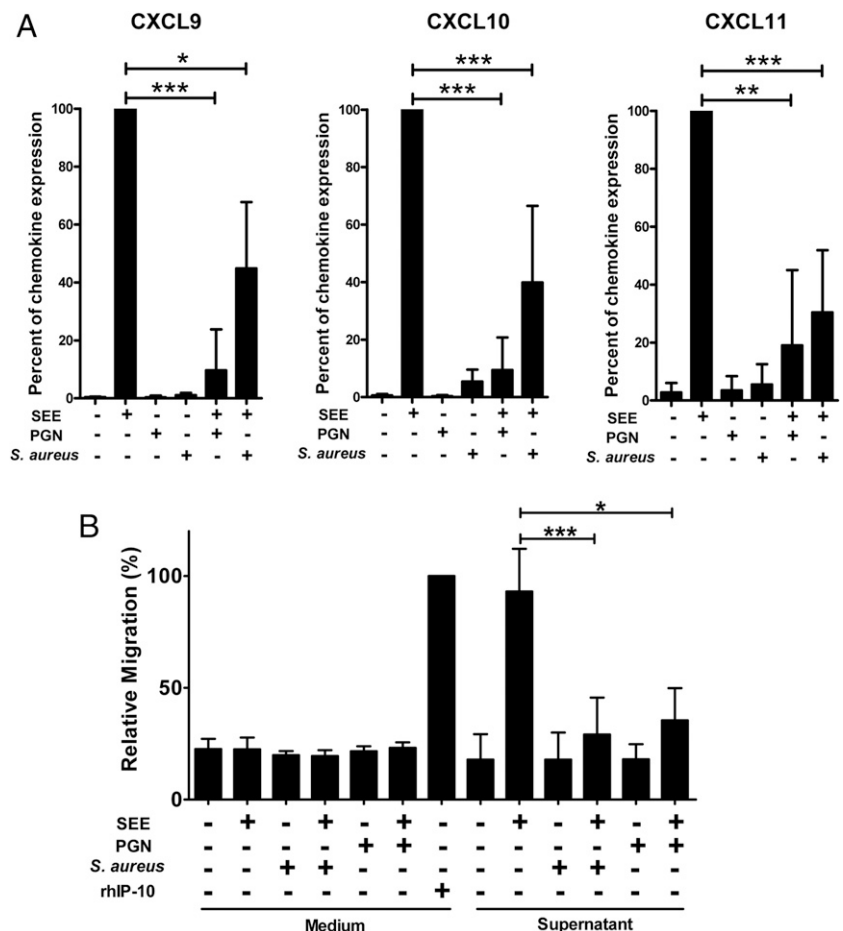
The inhibitory effect of the staphylococcal cell wall on chemokine production was selective for IFN- γ -dependent chemokines, and it was not observed for Th2 cell-recruiting chemokines (e.g., macrophage-derived chemokine and thymus and activation-regulated chemokine), eosinophil-recruiting chemokines (e.g., eotaxin, eotaxin3, and MCP4), neutrophil-recruiting chemokine (e.g., IL-8), or monocyte-recruiting chemokines (e.g., MCP1, MIP1- α , and MIP1- β) (Fig. 3). Furthermore, although *S. aureus* induces a potent IL-10 response by PBMCs (26) (Fig. 4A), such a response did not seem to mediate IP-10 inhibition because

blocking IL-10 R binding with neutralizing Abs against IL-10 or the IL-10 R did not restore IP-10 production in response to SEE (Fig. 4B). The activity of these Abs was confirmed by rescue of the IL-2 response to SEE (Fig. 4C). Collectively, these data show that *S. aureus* and its cell wall prevent the recruitment of Th1-polarized cells by downregulating the production of IFN- γ -dependent chemokines in an IL-10-independent manner.

S. aureus downregulates monocyte IP-10 gene transcription

Next, we investigated the mechanism of inhibition of IP-10 production by *S. aureus*. First, we determined the cellular source of IP-10 in PBMCs using intracellular cytokine staining. We found that the main source of IP-10 in PBMCs was the CD14⁺ monocyte population, with >95% of monocytes producing IP-10 in response to SEE (Fig. 5). Consistent with our previous results, IFN- γ induced IP-10 mRNA in monocytes, and this induction was downregulated by *S. aureus* (Fig. 6A). To test whether de novo protein synthesis is required for this regulation, the protein synthesis inhibitor CHX was used. In the presence of CHX, IP-10 mRNA was superinduced (Fig. 6A), similar to what has been previously reported in glial cells (32). *S. aureus* downregulated IP-10 mRNA in the presence of CHX by 80.5%, implying that de novo protein synthesis is not required for IP-10 downregulation by *S. aureus*. Moreover, the downregulation of IP-10 mRNA was not due to mRNA instability, as the rate of decay of IP-10 mRNA was unaffected by the presence of *S. aureus* (Fig. 6B). Thus, downregulation of IP-10 expression by *S. aureus* was not the result of increased de novo IP-10 mRNA degradation but of decreased IP-10 gene transcription.

FIGURE 2. *S. aureus* downregulates Th1 cell-recruiting chemokine production and prevents Th1 cell recruitment. **(A)** Quantification of CXCL9, CXCL10 (IP-10), and CXCL11 mRNA levels in PBMCs stimulated with SEE (10 ng/ml) in the presence or absence of heat-killed *S. aureus* or PGN for 4 h. Normalized data were plotted as mean $\pm$ SEM of at least three independent experiments from at least three different donors. **(B)** Transwell cell migration assay using culture supernatants of human PBMCs stimulated with SEE and/or *S. aureus* or PGN to chemoattract Th1 cells. Media with the same concentration of SEE and/or *S. aureus* or PGN were used as negative control chemoattractants whereas rhIP-10 in medium was used as a positive control. Migrated cells were phenotyped and counted using flow cytometry. Data were normalized to the positive control and plotted as mean $\pm$ SEM of three independent experiments from three different donors. * p < 0.05, ** p < 0.01, *** p < 0.001.



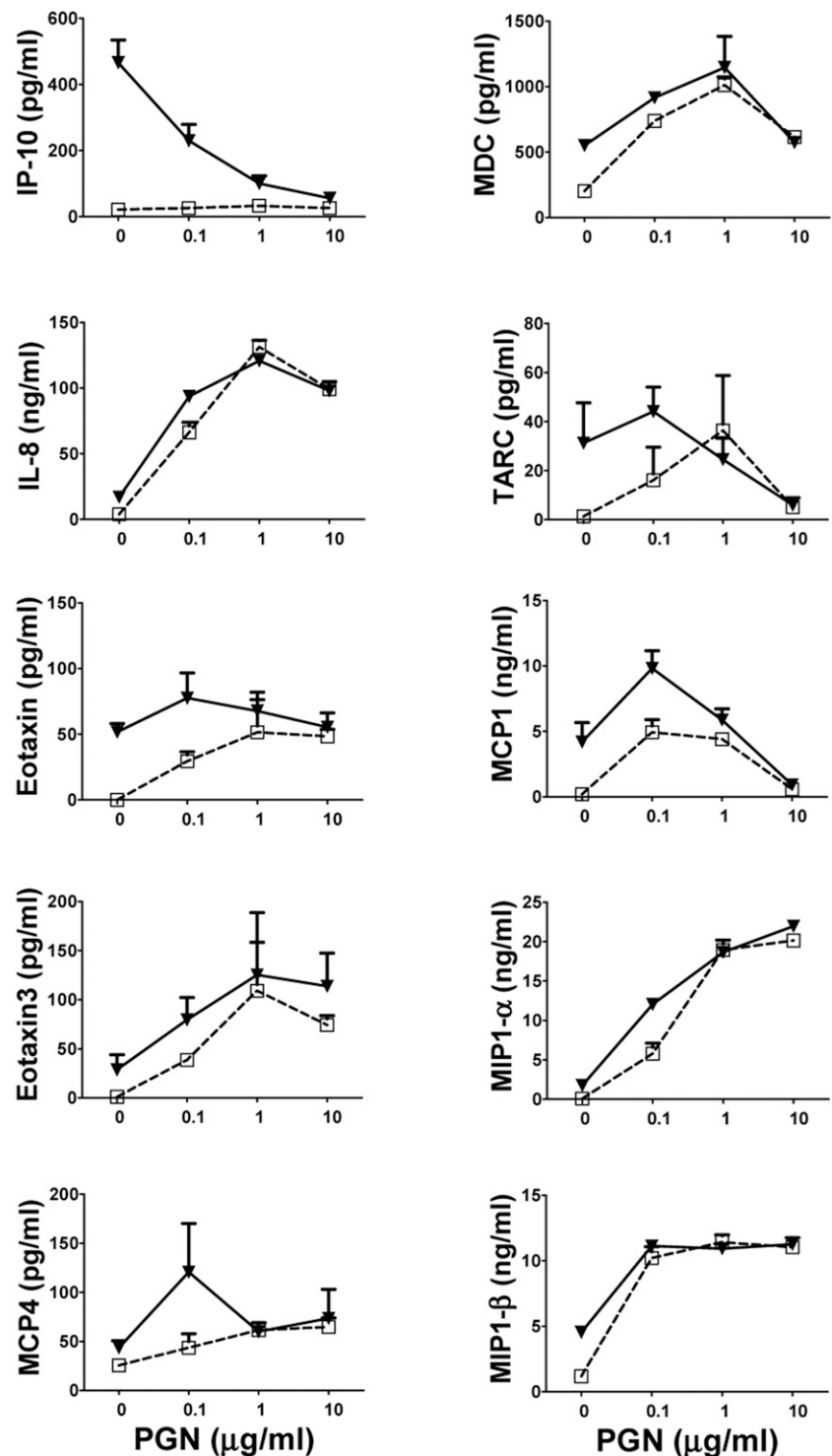


FIGURE 3. *S. aureus* selectively downregulates Th1 cell-recruiting chemokine production by human PBMCs. Human PBMCs were stimulated with the indicated concentrations of PGN in the presence (▲) or absence (□) of SEE (10 ng/ml) for 18 h, and chemokine production in culture supernatants was measured by a Meso Scale Diagnostics human chemokine panel 1 V-PLEX kit. Results are shown as mean $\pm$ SD and are representative of three independent experiments from three different donors (triplicate samples for each experiment).

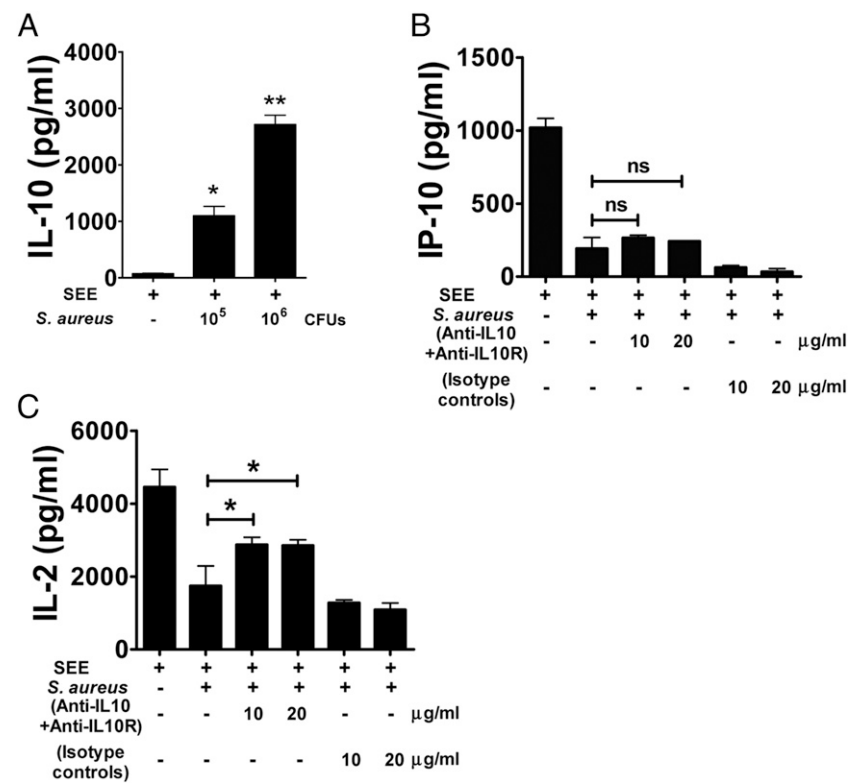
S. aureus modulates IP-10 production through TLR2 and MAPKs p38 and ERK signaling

Next, we examined how the cell wall of *S. aureus* could selectively modulate the production of IP-10 and other IFN- γ -dependent chemokines. Because TLR2 plays a pivotal role in *S. aureus*-triggered immune responses (33), we tested its involvement in the inhibition of IP-10 by *S. aureus*. As we could not obtain a reliable neutralizing Ab against human TLR2, and because it was not feasible to genetically knock down the *Tlr2* gene within the ex vivo half-life of monocytes, we decided to investigate the effect of defined TLR2 ligands on IP-10 production. We found that all

TLR2 ligands tested inhibited SEE-induced IP-10 production, mimicking the effect of *S. aureus* (Fig. 7A). As expected from the data above, such an effect did not correlate with their capacity to induced IL-10 production (Fig. 7B). These findings suggest TLR2 signaling is involved in the *S. aureus* inhibition of IP-10 production.

TLR2 signaling involves activation of MAPKs, NF- κ B, and the PI3K/AKT pathway. To assess the contribution of these signaling cascades on *S. aureus*-induced downregulation of IP-10 production, we used small molecular inhibitors. SB239063, a selective inhibitor of the p38 MAPK pathway, almost completely restored IFN- γ -induced IP-10 production in the presence of *S. aureus*.

FIGURE 4. IL-10 is not required for IP-10 inhibition by *S. aureus*. **(A)** Quantification of IL-10 in the culture supernatants of human PBMCs stimulated with 10 ng/ml SEE plus the indicated amount of heat-killed *S. aureus* for 18 h. **(B)** Quantification of IP-10 in the culture supernatants of human PBMCs stimulated with SEE (1 ng/ml) and heat-killed *S. aureus* (10^5 CFU) in the absence or presence of indicated concentrations of neutralizing Abs against IL-10 and IL-10R or their isotype controls (Iso) (10 or 20 μ g/ml) for 18 h. **(C)** Quantification of IL-10 in the same culture supernatants as in (B), determined as a control for neutralization of anti-IL-10 and anti-IL-10R Abs. Results are shown as mean $\pm$ SD and are representative of at least two independent experiments from two different donors (triplicate samples for each experiment). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns, not significant.



PD98059, a selective inhibitor of the MEK/ERK pathway, had a marginal but significant effect. In contrast, blockade of the JNK, NF- κ B, or PI3K/AKT pathways failed to reverse the effect of *S. aureus* on IP-10 production (Fig. 8A). Taken together, these data indicate that inhibition of the IP-10 production by this microbe is mediated mostly through p38 and ERK MAPK signaling. This conclusion was corroborated by the finding that heat-killed *S. aureus* increased the activation of the MAPKs p38 and ERK, as

detected by their phosphorylation, over the activation of these molecules induced by SEE alone (Fig. 8B).

STAT1 signaling in human monocytes is attenuated by S. aureus

The IP-10 gene is a target of STAT1, which is activated by IFN- γ binding to its receptors IFNGR1 and IFNGR2 (34). Because IFN- γ production to SEE was not affected by *S. aureus* stimulation, we

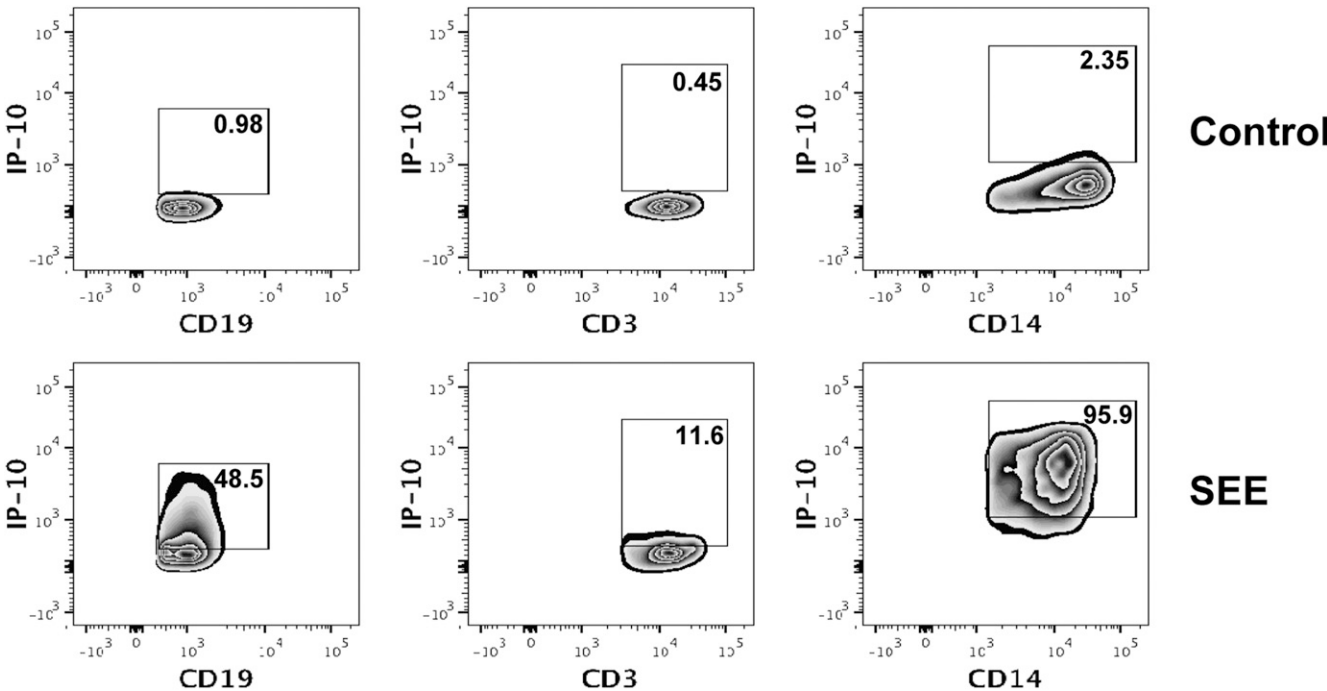


FIGURE 5. Monocytes are the main source of IP-10 in human PBMCs responding to SEE superantigen. Resting PBMCs (control) or PBMCs treated with SEE (10 ng/ml) for 21 h were subjected to intracellular cytokine staining for IP-10. Live singlet CD19⁺ B cells, CD3⁺ T cells, or CD14⁺ monocytes cells were gated. Numbers in the gated square indicate the frequency of IP-10-producing cells. Plots are representative of three independent experiments from three different donors.

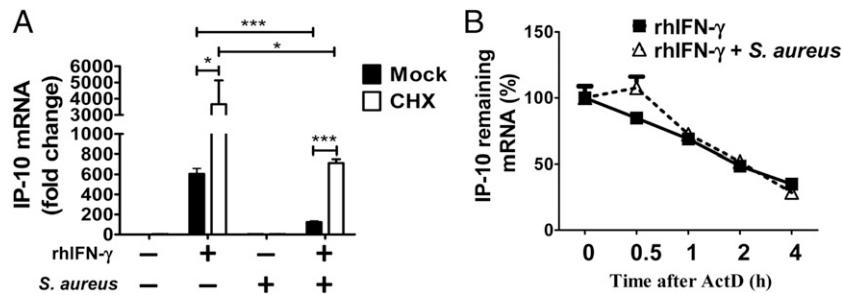


FIGURE 6. Transcriptional downregulation of IFN- γ -induced IP-10 by *S. aureus* in monocytes. **(A)** Quantification of IP-10 mRNA levels in monocytes treated with IFN- γ (10 ng/ml) and/or heat-killed *S. aureus* (10^7 CFU) in the presence or absence of CHX. **(B)** Monocytes treated with IFN- γ (10 ng/ml) in the presence or absence of *S. aureus* (10^7 CFU) for 3 h were further incubated with actinomycin D (ActD; 5 μ M) for indicated times. IP-10 mRNA levels were analyzed by RT-qPCR. Results are reported as mean $\pm$ SD and are representative of at least two independent experiments from at least two different donors (triplicate samples for each experiment). * p < 0.05, *** p < 0.001.

reasoned that *S. aureus* was acting through TLR2 to suppress IFNGR-STAT1 signaling. Thus, we examined the effect of *S. aureus* in this signaling cascade. We observed that cell surface IFNGR1 was slightly downregulated in the presence of *S. aureus*, whereas IFNGR2 was not affected (Fig. 9A). This change was associated with a minor reduction of IFN- γ -induced STAT1 phosphorylation (Fig. 9B). However, the effect of *S. aureus* on STAT1 dimerization, a step associated with STAT1 activation and nuclear import, was more apparent (Fig. 9C).

To assess the nuclear import of STAT1, we prepared cytoplasmic and nuclear fractions of IFN- γ -stimulated monocytes and found that STAT1 in the nuclear fractions was reduced in the presence of *S. aureus* (Fig. 9D). Furthermore, ChIP analysis revealed that the binding of phosphorylated STAT1 to the IP-10 promoter was attenuated by concomitant *S. aureus* stimulation (Fig. 9E). Collectively, these data imply that *S. aureus* downregulates STAT1 signaling leading to the inhibition of IP-10 expression.

Discussion

The development and progression of *S. aureus* infections are mediated by the array of toxins and virulence factors it produces. Among these virulence factors, superantigens stand as the only ones that can fully recapitulate a staphylococcal disease by themselves. These exotoxins trigger massive T cell activation and a subsequent cytokine and chemokine “storm,” predominantly derived from T cells, that characterizes TSS (35). The host immune response to these exotoxins has been characterized as predominantly a Th1 response (36). Additionally, adaptive T cell responses to cutaneous *S. aureus* infections are often characterized by intense Th1 and Th17 manifestations. However, clinical

colonization by superantigen-producing *S. aureus* is often linked to type 2 responses such as allergic sinusitis and atopic dermatitis (37–39). Under these conditions, IL-4-dependent IgE responses to superantigens can be detected (40–42). It is difficult to explain these discrepant observations given that Th1 and Th2 responses negatively cross-regulate each other. One possibility is that *S. aureus* induces an IL-10-dependent anti-inflammatory response that downregulates T cell activation (14) and favors Th2 imprinting of the ensuing response. Indeed, we have previously shown that *S. aureus* inhibits T cell activation by its exotoxins and prevents Th1/Th17 responses (10, 14). In this study, we uncovered

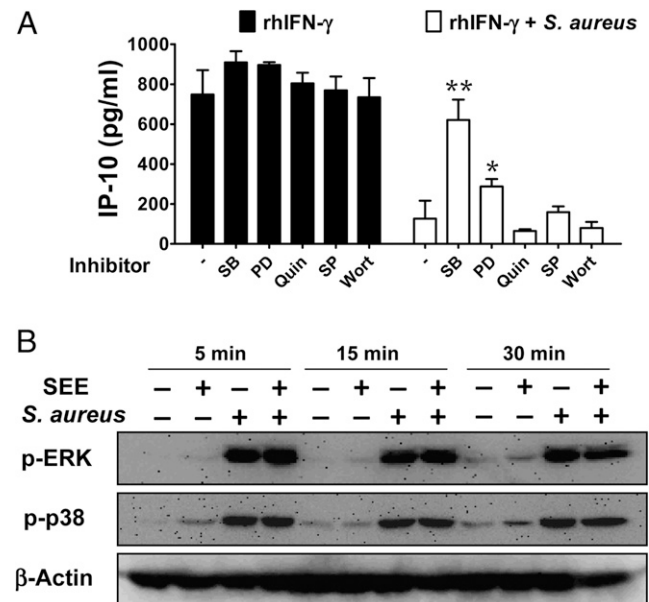


FIGURE 8. *S. aureus* modulates IFN- γ -induced IP-10 production through p38 MAPK and MEK/ERK signaling. **(A)** IP-10 responses of human PBMCs stimulated with 10 ng/ml rhIFN- γ with or without heat-killed *S. aureus* (5×10^5 CFU) in the absence or presence of p38 inhibitor SB239063 (SB; 5 μ M), MEK/ERK inhibitor PD98059 (PD; 5 μ M), NF- κ B inhibitor 6-amino-4-(4-phenoxyphenylethylamino)quinazoline (Quin; 5 μ M), JNK inhibitor SP600125 (SP; 5 μ M) and PI3K inhibitor wortmannin (Wort; 2 nM) for 24 h. **(B)** Whole-cell lysates from PBMCs treated with SEE (10 ng/ml) and/or heat-killed *S. aureus* (10^6 CFU) for indicated times were immunoblotted with anti-phosphorylated p38 and anti-phosphorylated ERK Abs. β -Actin was used as a loading control. Results represent mean $\pm$ SD and are representative of at least three independent experiments from at least three different donors (one donor per experiment, each experiment was done at least twice). * p < 0.05, ** p < 0.01.

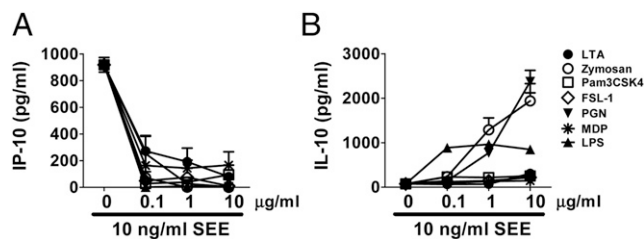


FIGURE 7. *S. aureus*-triggered inhibition of SEE-induced IP-10 production can be recapitulated by TLR2 signaling. Quantification of IP-10 **(A)** and IL-10 **(B)** in culture supernatants of human PBMCs stimulated with the indicated concentrations of TLR ligands in the presence of SEE (10 ng/ml) for 18 h. LPS was used as a control for IL-10 induction by TLR4 signaling. Results are shown as mean $\pm$ SD and are representative of at least two independent experiments from at least two different donors (triplicate samples for each experiment).

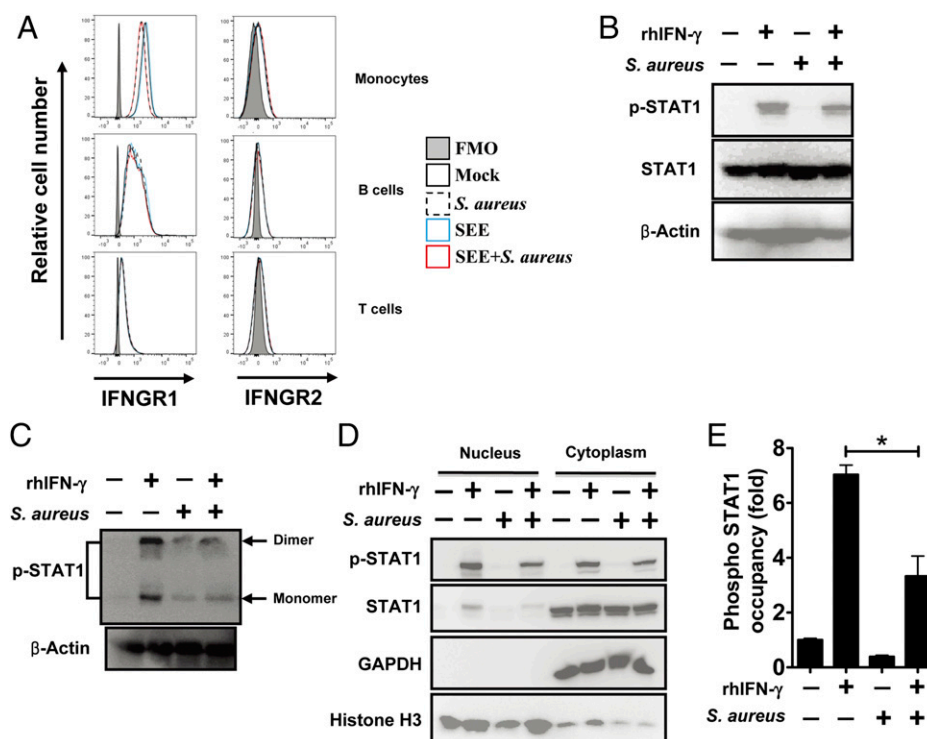


FIGURE 9. *S. aureus* downregulates IFNGR1/STAT1 signaling. (A) PBMCs treated with heat-killed *S. aureus* were stained for IFNGR1 and IFNGR2. Events were gated on live single CD14⁺ monocytes, CD19⁺ B cells, or CD3⁺ T cells. (B) Western blot analysis of monocytes exposed to heat-killed *S. aureus* for 1 h and subsequently treated with rhIFN-γ (1 ng/ml) for 15 min. (C) Whole-cell lysates from monocytes exposed to heat-killed *S. aureus* for 1 h and subsequently treated with rhIFN-γ (1 ng/ml) for 30 min were subjected to disuccinimidyl suberate cross-linking and subsequently Western blot analysis. (D) Monocytes were treated as in (C), and cytoplasmic and nuclear protein were separated and immunoblotted with anti-phosphorylated STAT1 and anti-STAT1 Abs. GAPDH and histone H3 were used to confirm the purity of cytoplasmic and nuclear fractions, respectively. (E) Monocytes exposed to heat-killed *S. aureus* for 1 h and sequentially treated with rhIFN-γ (10 ng/ml) for 45 min were subjected to ChIP assay. Quantification of phospho-STAT1-occupied IP-10 promoter was determined by RT-qPCR. Results are shown as mean ± SD, and are representative of at least two independent experiments from at least two different donors (each experiment was done at least twice). **p* < 0.05. FMO, fluorescence minus one.

an IL-10-independent mechanism of immune modulation by *S. aureus* that contributes to the downregulation of Th1 responses by this microbe. Such a mechanism involves the inhibition of Th1 cell-recruiting chemokine production (e.g., CXCL9, CXCL10, and CXCL11) by a TLR2-mediated interference with STAT1 signaling. This inhibition may lead to a decrease in Th1 cell recruitment to the site of colonization or infection, and thus promote a Th2-biased microenvironment.

In this study, we found that SEE-induced or IFN-γ-induced IP-10 production in human monocytes can be inhibited by components of the cell wall of *S. aureus*. This observation is in line with a previous report of a similar suppression by staphylococcal PGN (43). Importantly, note that this effect on human monocytes is remarkable given that *S. aureus* can induce IP-10 production by itself in other cell types and in our species (e.g., THP-1 cells and mouse splenocytes) (data not shown) (23). Thus, our data point to the existence of an inhibitory pathway that is triggered by *S. aureus* and regulates the production of IP-10 and other Th1 cell-recruiting chemokines.

Differential regulation of Th1 cell-recruiting chemokine expression by *S. aureus* or its virulence factors may contribute to the dual interactions between this microbe and humans, that is, commensalism versus pathogenicity. Production of superantigens is a cardinal pathogenic event in certain staphylococcal infections. We have shown in the present study that the effects of IFN-γ are required for IP-10 production as demonstrated by the inhibitory effect of IFNGR1 blockade. Also, de novo synthesis of IFN-γ is required for the induction of IP-10 because CHX dramatically inhibited SEE-induced IP-10 mRNA in PBMCs (data not shown).

However, unregulated production of IFN-γ and other Th1 cytokines may have lethal effects for the host (e.g., TSS) that ultimately negatively affect the microbe. One can argue that downregulation of Th1 cell-recruiting chemokines has been selected as a mechanism that protects the host by minimizing T cell activation while also benefiting the microbe. This mechanism may be important only during early stages of the response before other mechanisms become operational (e.g., IL-10 production).

The mechanism reported in this study complements the downregulation of superantigen-induced T cell activation by IL-10. Importantly, note that although IL-10 can inhibit IP-10 (44), it is not essential for *S. aureus*-induced inhibition of IP-10, as demonstrated by the observation that neutralizing Abs against IL-10 and the IL-10R did not rescue IP-10 production. Moreover, several TLR2 ligands that lack IL-10-producing capacity (e.g., Pam3CSK4) also downregulated IP-10 production, further demonstrating the dispensability of IL-10 in the regulation of Th1 cell-recruiting chemokine production. However, based on the kinetics of IL-10 production, IL-10-mediated downregulation of T cell activation will likely be predominant at later stages of the response to *S. aureus* and its toxins.

S. aureus downregulates the production of IP-10 and other Th1 cell-recruiting chemokines by interfering with IFN-γ signaling. Other pathogens, such as *Mycobacterium tuberculosis*, *Mycobacterium avium*, and *Brucella abortus*, have also been reported to suppress IFN-γ signaling (44–48). We found that a wide range of pattern recognition receptor (PRR) ligands (LPS, muramyl dipeptide, and depleted zymosan) could downregulate SEE-induced IP-10 production

(data not shown). These observations suggest a crosstalk between PRR signaling and IFN- γ signaling. Given the central role of IFN- γ in host innate and adaptive immunity against pathogens (49), inhibition of IFN- γ signaling by microbial-associated molecular patterns might contribute to commensal colonization and pathogen immune evasion.

Our data indicate that *S. aureus* downregulates IFN- γ signaling by interfering with proximal IFNGR/JAK/STAT1 signaling. Other microbes have been shown to do this in different ways. For example, *Leishmania donovani* and *M. avium* downregulate IFN- γ signaling by inhibiting the expression of the IFNGR and activating tyrosine phosphatase SHP1 and the dominant negative regulator STAT1 β (45, 50, 51). *M. tuberculosis* selectively shuts down some of the IFN- γ -responsive genes without inhibiting STAT1 function. This effect occurs through activation of MAPK pathway and induction of IL-6 expression (48, 52, 53). Based on our experiments, *S. aureus* interferes with IFN- γ signaling by impairing nuclear translocation of activated STAT1, likely in a TLR2-dependent manner.

Downregulation of IFN- γ -responsive genes has been reported upon induction of IL-6, SOCS1, and SOCS3 in different cell types and by different pathogens (53–55). These mechanisms are not essential for *S. aureus*-triggered IP-10 inhibition in human primary monocytes. Rather, *S. aureus*-triggered inhibition is mediated through MAPK p38 and MEK/ERK pathways and direct abrogation of the IFN- γ /STAT1 axis. Inhibition of IFN- γ signaling by MAPK has been previously reported (48, 56). However, the mechanism is still obscure. We show in this study that in the presence of *S. aureus*, downregulation of IFNGR1 and STAT1 activation is subtle but detectable. However, we found a very apparent defect at the level of nuclear import of activated STAT1 and, more importantly, a reduced binding of STAT-1 to the IP-10 promoter. How *S. aureus* interferes with STAT1 subcellular translocation remains unknown. Our data suggest that this may be due to a defect in STAT-1 dimerization, similar to what has been previously reported for type I IFN-induced STAT1 and STAT2 heterodimerization (57). Further experiments are needed to formally test whether *S. aureus* suppresses STAT1 homodimerization and how this downregulation is connected to the MAPK pathway.

IP-10 is a chemokine that recruits CXCR3-expressing cells, such as activated T lymphocytes, macrophages, dendritic cells, and NK cells (58). Aberrant expression of IP-10 has been linked to various diseases, including infections (e.g., *Mycoplasma*, *Helicobacter pylori*, and malaria), autoimmune diseases, marginal periodontitis, and cancer (59). Accordingly, several therapeutic strategies have been developed to target IP-10, including anti-IP-10 mAbs and IP-10 antagonists. The finding in this study that *S. aureus* and other TLR2 ligands inhibit IP-10 production opens up the possibility of using modulatory bacteria or their products as therapeutics. As reported, PRR signaling can trigger both pro- and anti-inflammatory responses and these responses can be uncoupled (28). Therefore, one could envision the generation of less proinflammatory probiotics or TLR2 ligands that interfere with IP-10 production as therapeutic tools for Th1-mediated disease conditions.

In summary, we show in the present study that *S. aureus* downregulates superantigen-induced IP-10 production and prevents Th1 cell recruitment. This regulation is independent of IL-10 production by this microbe, but it is due to activation of MAPK pathways and inhibition of IFN- γ /STAT1 signaling by *S. aureus*. Our results may explain why mucosal colonization by *S. aureus* is often associated with type 2 immune responses despite the fact that staphylococcal superantigens induce type 1 immune responses.

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Disclosures

The authors have no financial conflicts of interest.

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