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Uncoupling of Pro- and Anti-Inflammatory Properties of *Staphylococcus aureus*

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Staphylococcus aureus **is a Gram-positive bacterium that is carried by a quarter of the healthy human population and that can cause severe infections. This pathobiosis has been linked to a balance between Toll-like receptor 2 (TLR2)-dependent pro- and anti-inflammatory responses. The relationship between these two types of responses is unknown. Analysis of 16 nasal isolates of *S. aureus* showed heterogeneity in their capacity to induce pro- and anti-inflammatory responses, suggesting that these two responses are independent of each other. Uncoupling of these responses was corroborated by selective signaling through phosphoinositol 3-kinase (PI3K)-Akt-mTOR and extracellular signal-regulated kinase (ERK) for the anti-inflammatory response and through p38 for the proinflammatory response. Uncoupling was also observed at the level of phagocytosis and phagosomal processing of *S. aureus*, which were required solely for the proinflammatory response. Importantly, the anti-inflammatory properties of an *S. aureus* isolate correlated with its ability to modulate T cell immunity. Our results suggest the presence of anti-inflammatory TLR2 ligands in the staphylococcal cell wall, whose identification may provide templates for novel immunomodulatory drugs.**

Staphylococcus aureus is a Gram-positive bacterium that is frequently associated with localized soft tissue infections (e.g., impetigo and dermatitis) and also systemic complications (e.g., bacteremia, sepsis, and toxic shock syndrome [TSS]) (1–3). It is the most common microbe isolated from intrahospital microbiological samples and the second most common microbe isolated from outpatient samples (4). However, *S. aureus* is also part of the healthy human microbiome of the upper respiratory tract, being chronically carried by more than 25% of the general population with no long-term ill effects (5–7). Therefore, *S. aureus* can be classified as a pathobiont: an organism that is typically safe to its host but that can become pathogenic under certain circumstances other than immunosuppression.

One of the remarkable features of this state of pathobiosis is that commensal *S. aureus* isolates contain many, if not all, of the known virulence factors and microbe-associated molecular patterns (MAMPs) linked to disease (8–10). The pathogenic potential of these isolates is exemplified by the risk of staphylococcal nasal carriers to develop systemic infections caused by the endogenous *S. aureus* strain they carry (7, 11). How these highly pathogenic microbes can behave as commensals and only rarely cause disease remains unknown (12, 13).

Early recognition of *S. aureus* is initiated by pattern recognition receptors (PRRs) on epithelial cells and innate phagocytic cells. Toll-like receptor 2 (TLR2) has emerged as the most important of these PRRs in detecting extracellular *S. aureus* (14). It heterodimerizes with TLR1 or TLR6 to recognize lipopeptides and glycopolymers embedded in the staphylococcal cell envelope, triggering proinflammatory responses. Conventional proinflammatory TLR2 signaling begins with the recruitment of the adaptor proteins TIRAP and MyD88 and the Ser/Thr kinases IRAK-1 and -4. Distal TLR2 signaling activates the NF-κB and mitogen-activated protein kinase (MAPK) pathways to upregulate proinflammatory cytokines (i.e., interleukin 1β [IL-1β], IL-6, tumor necrosis factor alpha [TNF-α], and IL-12p70) and chemokine (i.e., IL-8, CCL2, CCL3, CCL4, and RANTES) production that will then coordinate microbial clearance (15). The importance of this pathway is highlighted by the susceptibility of MyD88/IRAK4-deficient patients to staphylococcal infections (16, 17).

TLR2 also cross talks with other PRRs, including NOD1/2 and TLR9, which recognize fragments of the peptidoglycan (PGN) backbone and CpG DNA, respectively (18). TLR9 activates a similar signaling pathway as TLR2 but without the need for TIRAP bridging, whereas NOD1/2 activate the NF-κB pathway through RIP-2. Signaling from these receptors requires phagocytosis and subsequent endosomal processing of *S. aureus* to liberate typically hidden ligands on the staphylococcal cell wall or in the DNA (19, 20). Digestion of *S. aureus* also releases additional TLR2 ligands that amplify the inflammatory response. Ultimately, cross talk between signaling from these receptors enhances the host's ability to clear infection and avoid disease.

It has been recently shown that in addition to the proinflammatory response described above, *S. aureus* is capable of inducing a robust anti-inflammatory response as measured by production of IL-10 (21–23). We and others have shown that this anti-inflammatory response results from TLR2 signaling upon recognition of staphylococcal PGN-embedded molecules and activation of

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PI3K-Akt signaling to stimulate IL-10 production (21, 24, 25). Moreover, downregulation of the costimulatory molecule CD86 and upregulation of the immunoregulatory molecule PD-L1 may provide complementary effects to limit the development of an adaptive immune response (23). Interestingly, monocytes and macrophages are more potent at activating this response than dendritic cells (22). Together, these studies have shown that anti-inflammatory TLR2 signaling may promote an environment of disease tolerance to *S. aureus* and support commensalism by this microbe (26).

It has been assumed that both pro- and anti-inflammatory responses to TLR2 engagement emanate coordinately and simultaneously from this receptor. If this paradigm is correct, then one would expect that both types of responses result at the same ratio upon receptor engagement. In contrast to this paradigm, we report here that the pro- and anti-inflammatory responses to *S. aureus* are uncoupled, i.e., independent of one another. Such an uncoupling can be observed in the analysis of responses to nasal isolates of *S. aureus* from community carriers of this microbe, suggesting an ongoing selective process for these properties. We show that the human anti-inflammatory response to these *S. aureus* isolates is mediated by the phosphoinositol 3-kinase (PI3K)-Akt-mTOR and extracellular signal-regulated kinase (ERK) pathways and does not require internalization of *S. aureus*, whereas the proinflammatory response utilizes the p38 pathway and is dependent on phagocytosis of this microbe. Moreover, the magnitude of the IL-10-inducing response translates into different regulation of adaptive T cell responses to *S. aureus*. Based on these data, we propose that the cell wall of *S. aureus* contains two sets of TLR2 ligands: one that induces predominantly proinflammatory responses and a second set that induces predominantly anti-inflammatory responses.

MATERIALS AND METHODS

Cells. Human peripheral blood mononuclear cells (PBMCs) were isolated from venous blood of healthy volunteers by Ficoll-Hypaque density gradient centrifugation. Volunteers gave their informed consent in compliance with the Research Ethics Office at McGill University. PBMCs were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin, L-glutamine, nonessential amino acids, and sodium pyruvate. For experiments comparing clindamycin-treated and heat-killed *S. aureus*, clindamycin (1 μg ml^{-1}) was substituted for penicillin-streptomycin in supplemented RPMI 1640. Peripheral blood neutrophils were isolated by red blood cell lysis of the pellet following Ficoll-Hypaque centrifugation.

Bacteria. *S. aureus* isolates were obtained from the nostrils of individuals attending an ear, nose, and throat clinic. All the isolates were confirmed as *S. aureus* using PCR with primers specific for the 16S rRNA gene common to all bacteria (5′-AGAGTTTGATCATGGCTCAG-3′ and 5′-GGACTACCAGGGTATCTAAT-3′) and the *S. aureus nuc* gene (5′-GCGATTGATGGTGATACGGTT-3′ and 5′-ACGCAAGCCTTGACGAACTAAAGC-3′) (see Fig. S1 in the supplemental material). In addition, full-genome sequencing and multilocus sequence typing (MLST) of isolates further confirmed that they were *S. aureus* (27). The clonality of these isolates is representative of *S. aureus* strains in the community (see Table S1 in the supplemental material). Bacteria were grown overnight to stationary phase in tryptic soy broth (TSB), washed, and resuspended in sterile phosphate-buffered saline (PBS). Culture supernatants were collected, filtered through a 0.2-μm filter, and stored at −20°C. Bacteria (~10^{10} CFU) were plate counted and heat killed for 1 h at 100°C in a heating block. Culture supernatants were diluted to 1% in RPMI complete media. For bacterial internalization experiments, *S. aureus* (10^9 CFU) was stained with 5 μg/ml of 6-carboxytetramethylrhodamine (6-TAMRA; Sigma-Aldrich) in 50 μl of PBS for 1 h at room temperature and washed and resuspended in sterile PBS. For bacterial fractionation experiments, *S. aureus* isolates were digested with 1 mg/ml of lysozyme and 0.1 mg/ml of mutanolysin for 1 h in TES buffer (10 mM Tris, 1 mM EDTA, 25% sucrose [pH 8]). Cell wall fractions were separated from protoplast fractions by differential centrifugation at 2,500 × *g* for 10 min, precipitated with 10% trichloroacetic acid, and resuspended in PBS. Protoplast fractions were resuspended in TE buffer (10 mM Tris, 1 mM EDTA, 2% SDS) and boiled for 5 min to reduce viscosity. The protein content of these subcellular fractions was determined by bicinchoninic acid (BCA) assay. For clindamycin treatment experiments, *S. aureus* was grown overnight in TSB, followed by culture in TSB-containing clindamycin (1 μg ml^{-1}) for an additional 6 h.

Reagents. Clindamycin, cytochalasin D, dynasore, PD-98059, rapamycin, SB-203580, staphylococcal peptidoglycan, and wortmannin were purchased from Sigma-Aldrich. The PI3K p110 isoform-specific inhibitors PIK-75, TGX-221, AS-604850, and IC-87114 were purchased from EMB-Millipore. BIRB-0796 was purchased from Cayman Chemicals. Antibodies to phosphorylated Akt at Ser473 (clone 193H12), pan-Akt (clone 11E7), phosphorylated p38 (Thr180/Tyr182; clone 12F8), pan-p38 (9212), phosphorylated ERK1/2 (Thr202/Tyr204; clone 197G2), and pan-ERK1/2 (clone 137F5) were purchased from Cell Signaling Technology. Conjugated antibodies used for flow cytometry were CD3-allophycocyanin (APC)-eF780 from eBioscience and CD14-peridinin chlorophyll protein (PerCP)-Cy5.5, CD19-APC, IL-10–phycoerythrin (PE), TNF-α–Alexa Fluor 700, phospho-Akt(S473)–Alexa Fluor 488, and pan-Akt-BV421 from BD Biosciences.

Functional assays. PBMCs were seeded in 96-well plates (200,000 cells in a volume of 200 μl per well) and stimulated under the conditions indicated in the relevant figure legends. When inhibitors were used, cells were incubated for 1 h prior to stimulation, using 0.1% dimethyl sulfoxide (DMSO) as a control. Cell-free supernatants were collected and stored at −20°C until analyzed for accumulation of cytokines by enzyme-linked immunosorbent assay (ELISA) (eBioscience).

Flow cytometry. PBMCs (1 × 10^6 cells per group) were stimulated with *S. aureus* isolates under the conditions indicated in the relevant figure legends. For intracellular cytokine staining, 3 μg/ml of brefeldin A (eBioscience) was added after 6 h of stimulation, and the stimulation was continued for an additional 12 h. Dead cells were excluded from the analysis using a Zombie Aqua fixable viability kit (BioLegend). Cells were washed in PBS containing 2% FBS and 2 mM EDTA, blocked with 10% normal human serum, and stained for CD3, CD14, and CD19. Cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences) and stained for IL-10 and TNF-α. For phospho-flow, PBMCs were stimulated for 30 min, fixed with Fix Buffer I (BD Biosciences), stained for extracellular markers, permeabilized using Perm/Wash Buffer I (BD Biosciences), and stained for the intracellular molecules of interest. Events were acquired on a LSRII Fortessa (BD), and doublets were excluded based on forward side scatter-area (FSC-A)/forward side scatter-height (FSC-H). Data analysis was performed using FlowJo version 10.x (Tree Star Inc.).

Western blotting. PBMCs (5 × 10^6 cells per group) were resuspended in 100 μl of media and rested for 5 min at 37°C. When used, inhibitors were added at a 1:1 (vol/vol) ratio to the cells at twice the concentration indicated in the figure legends for 1 h. Next, the stimulants were added for 30 min, and cell lysates were prepared, run on 10% acrylamide gels, and immunoblotted as described previously (21, 28).

Statistics. Statistical analysis of intragroup differences was performed using the Student *t* test or analysis of variance (ANOVA) with *post hoc* Bonferroni test on Prism GraphPad. A P value of <0.05 was deemed significant.

RESULTS

Uncoupling of pro- and anti-inflammatory responses to nasal isolates of *S. aureus*. *S. aureus* recognition by host TLR2 induces

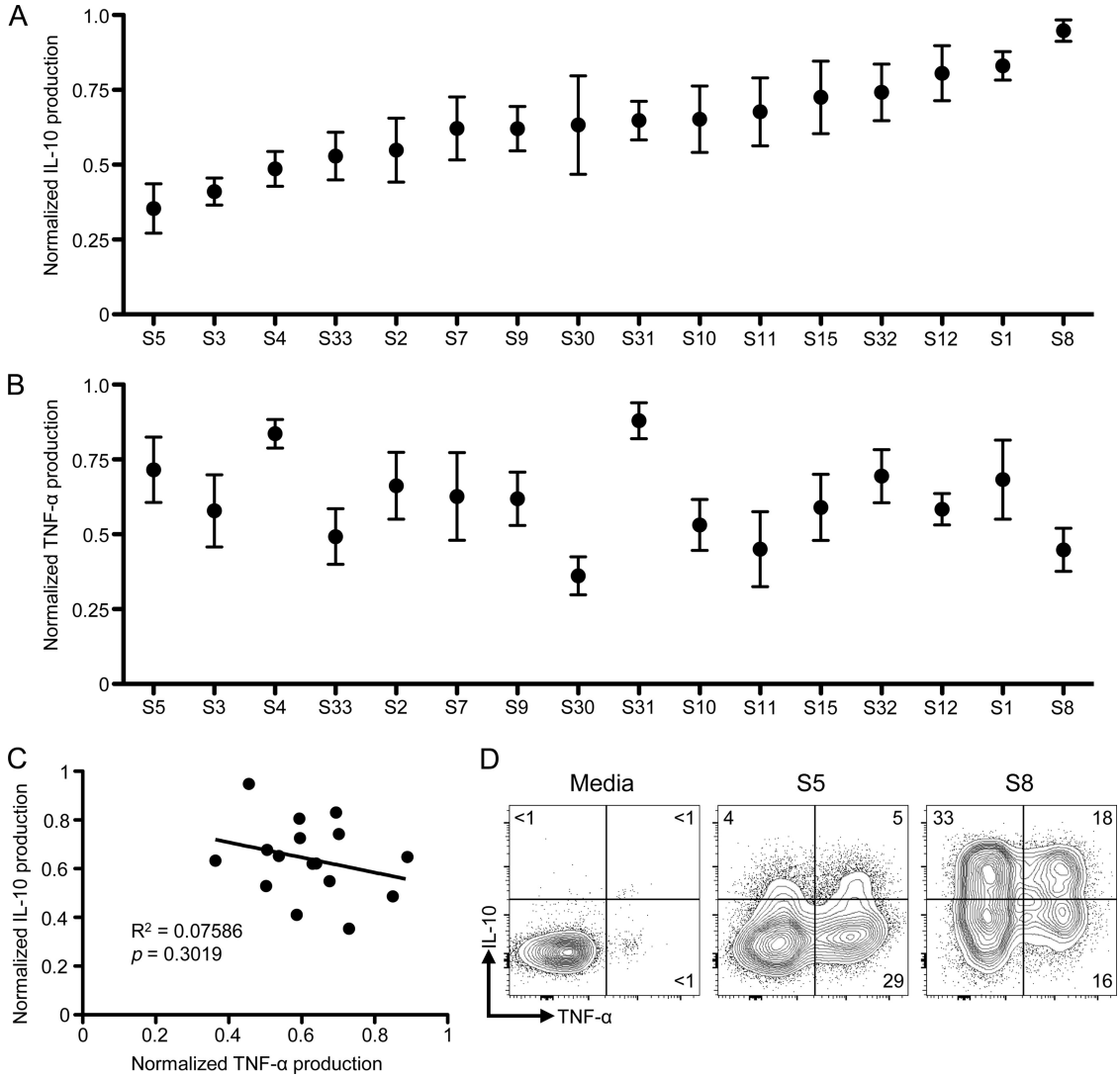

FIG 1 Uncoupling of pro- and anti-inflammatory PBMC responses to nasal *S. aureus* isolates. (A and B) Human PBMCs were stimulated with 16 nasal *S. aureus* isolates for 18 h, and accumulation of IL-10 (A) and TNF-α (B) in the supernatants was measured by ELISA. Normalized data are plotted as means ± SEMs of triplicates of 4 or 5 independent experiments involving 5 different donors. (C) Scattered plot of IL-10 versus TNF-α production by human PBMCs in response to nasal *S. aureus* isolates. (D) Intracellular cytokine staining of IL-10 and TNF-α in human monocytes stimulated with two representative *S. aureus* isolates inducing low-level (S5) or high-level (S8) IL-10 responses (MOI = 5) for 18 h. Stained PBMCs were gated on single, live $CD14^+$ cells. Plots are representative of three independent experiments involving three different donors. Intracellular cytokine staining of T cells, B cells, and neutrophils is shown in Fig. S2 in the supplemental material.

both proinflammatory and anti-inflammatory cytokine production (14). However, the mechanisms governing these responses have been studied only in the context of laboratory isolates, crude staphylococcal preparations, or synthetic TLR2 ligands (21, 22). These experimental systems may not account for the diversity of interactions that the human immune system has with *S. aureus* and the subsequent heterogeneity of responses to this microbe. Indeed, *S. aureus* is capable of causing a spectrum of diseases, in addition to being a part of the human commensal flora. To determine the variation of the host responses to *S. aureus*, we stimulated human PBMCs with 16 *S. aureus* isolates obtained from the nostrils of human carriers. These isolates were representative of a cross-section of *S. aureus* found in the community as indicated by MLST (see Table S1 in the supplemental material) (29). We then measured TNF-α and IL-10 production to assess their pro- and anti-inflammatory properties, respectively. We found up to a 3-fold difference in the IL-10 production in response to these nasal *S. aureus* isolates by PBMCs (Fig. 1A). This response was reproducible and consistent for multiple PBMC donors and was largely determined by the bacterial isolate. A similar heterogeneity was seen in the capacity of these isolates to induce a proinflammatory TNF-α response (Fig. 1B). However, when we compared the capacities for an isolate of *S. aureus* to induce both IL-10 and TNF-α, we observed no correlation between these two responses ($r^2 = 0.07586$; $P = 0.3019$ [Fig. 1C]). From these results, we postulated that the pro- and anti-inflammatory responses to *S. aureus* can be uncoupled.

Human PBMCs are a heterogeneous population consisting mostly of T cells, B cells, and monocytes, which can all produce IL-10 and TNF-α under different conditions (30). One possible

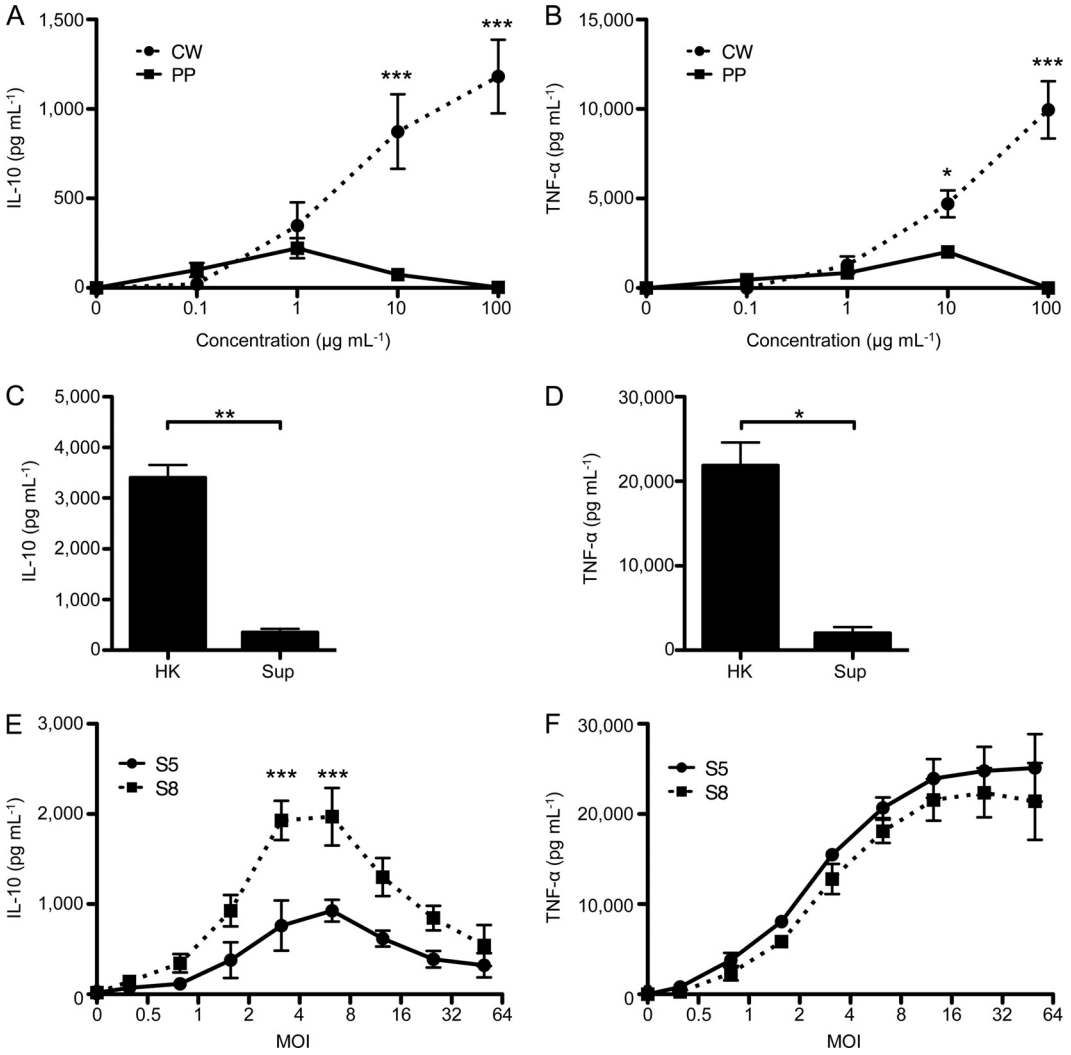

FIG 2 Nasal *S. aureus* isolates have qualitatively different IL-10-inducing ligands embedded in their cell walls. (A and B) Human PBMCs were stimulated with protoplasm (PP) or cell wall (CW) fractions of the S8 *S. aureus* isolate for 18 h. (C and D) PBMC response to heat-killed bacteria (HK) (MOI = 5) or supernatants (Sup) (1% of overnight culture). (E and F) Human PBMCs were stimulated with increasing amounts of two representative *S. aureus* isolates inducing low-level (S5; circles and solid line) or high-level (S8; squares and dashed line) IL-10 responses for 18 h. Quantification of IL-10 (A, C, and E) and TNF-α (B, D, and F) in the supernatants was performed by ELISA. Data are plotted as means ± SEMs for 3 to 5 different donors in experiments performed in triplicate. Statistical analysis was performed using ANOVA with *post hoc* Bonferroni test (A, B, E, and F) or Student *t* test (C and D). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

explanation for uncoupling of the pro- and anti-inflammatory responses is a different cellular source of these cytokines. To address which population(s) was producing IL-10 and TNF-α, we stimulated human PBMCs with isolates that induced a high- or low-level IL-10 response and used intracellular flow cytometry to identify the PBMC population producing these cytokines. For simplicity and from here onward, we show the results obtained with isolates S8 and S5 as representative of the results obtained of high- and low-level IL-10 inducers. Monocytes mounted a robust TNF-α and IL-10 response to both *S. aureus* isolates (Fig. 1D). We detected monocytes producing only TNF-α or IL-10 or both TNF-α and IL-10. More monocytes responded to the S8 *S. aureus* isolate and produced more IL-10 on a per-cell basis than with the S5 *S. aureus* isolate, whereas levels of TNF-α production in response to each isolate were similar. We observed little to no contribution of T cells or B cells to either of these responses (see Fig. S2A and B in the supplemental material). In addition, human peripheral blood neutrophils did not produce IL-10 or TNF-α in response to *S. aureus* when mixed with autologous PBMCs (see Fig. S2C in the supplemental material). Thus, nasal *S. aureus* isolates have differential capacities to independently induce pro- and anti-inflammatory cytokine production by human monocytes.

Nasal *S. aureus* isolates contain qualitatively different IL-10-inducing TLR2 ligands in their cell walls. We have previously shown that the IL-10 response to *S. aureus* is primarily initiated by TLR2 engagement (21). These ligands could be membrane-bound, cell wall-anchored, or secreted molecules. To determine where the IL-10-inducing ligand(s) is located in nasal *S. aureus* isolates, we fractionated a high-level IL-10-inducing *S. aureus* isolate (S8) into its cell wall and protoplasm and tested the IL-10-inducing capacities of these fractions. We observed that the IL-10 and TNF-α responses were almost exclusively induced by the staphylococcal cell wall (Fig. 2A and B). Moreover, culture supernatants of *S. aureus*, which contain the secreted toxins and shed-

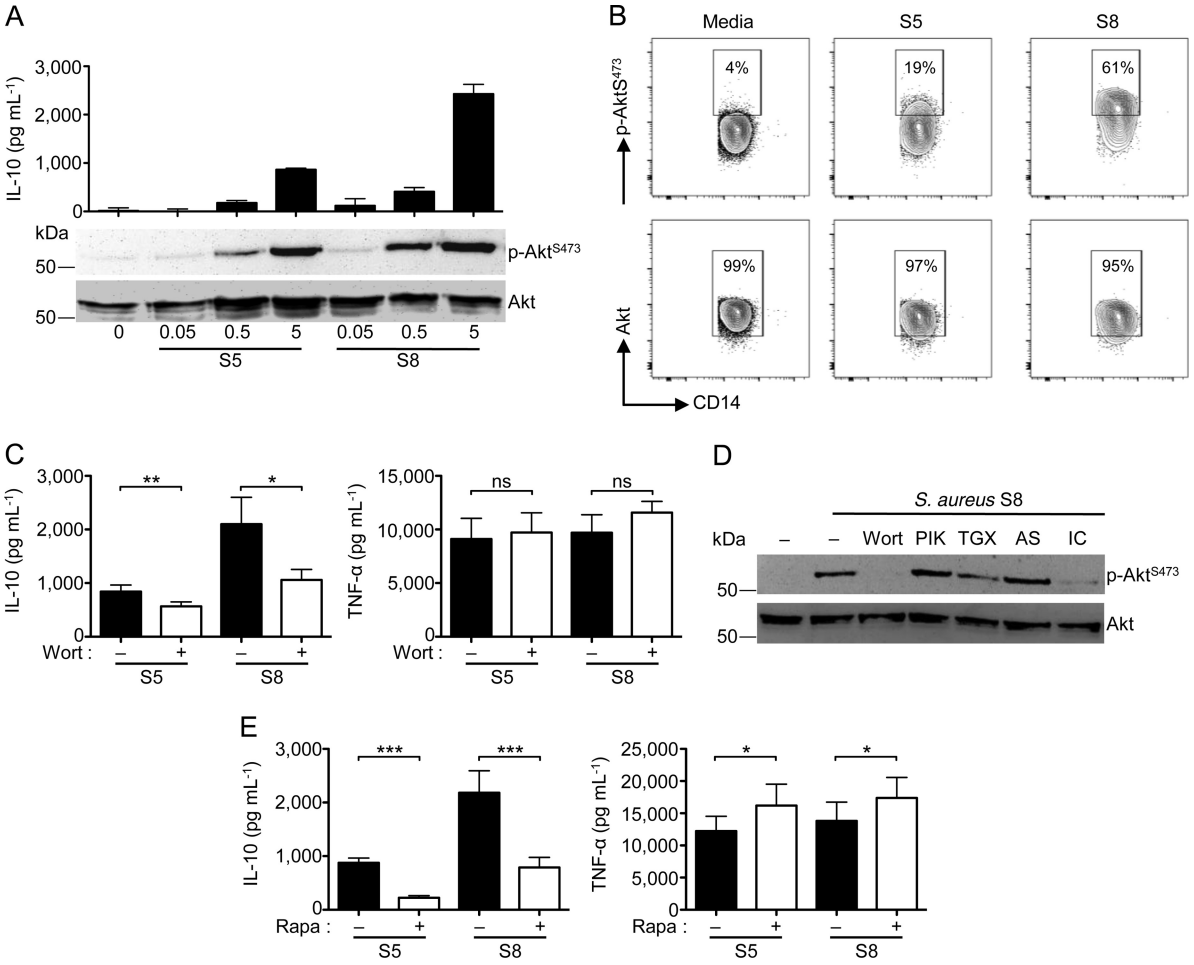

FIG 3 The PI3K/Akt/mTOR pathway mediates the IL-10 response to nasal *S. aureus* isolates. (A) PBMCs were stimulated with two *S. aureus* isolates at the indicated MOIs for 30 min for Western blot experiments or for 18 h in experiments looking at IL-10 accumulation by ELISA. (B) Flow cytometric analysis of Akt phosphorylation (S473) in $CD14^+$ gated human PBMCs stimulated with two representative *S. aureus* isolates inducing low-level (S5) or high-level (S8) IL-10 responses. (C) PBMCs were pretreated with wortmannin (1 μM) and then stimulated with *S. aureus* (MOI = 5) for 18 h. Quantification of IL-10 and TNF-α accumulation in the supernatants was done by ELISA. (D) Western blot of PBMCs pretreated with the pan-PI3K-p110 inhibitor wortmannin (Wort) or p110 isoform inhibitors PIK-75 (PIK; p110α inhibitor; 100 nM), TGX-221 (TGX; p110β inhibitor; 500 nM), AS-604580 (AS; p110γ inhibitor; 10 μM), and IC-87114 (IC; p110δ inhibitor; 5 μM) for 1 h and then stimulated with *S. aureus* S8 (MOI = 5) for 30 min. (E) PBMCs were pretreated with rapamycin (10 nM) and then stimulated with *S. aureus* (MOI = 5) for 18 h. Quantification of IL-10 and TNF-α accumulation in the supernatants was performed by ELISA. Data in panels A, B, and D are representative of three independent experiments from three different donors. Data in panels C and E are plotted as means ± SEMs from eight individual donors in experiments performed in triplicate. Statistical analysis was performed using theStudent *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. ns, not significant.

ded components of the staphylococcal cell wall, minimally induced IL-10 and TNF-α production by PBMCs (<10% of the response to heat-killed *S. aureus*) (Fig. 2C and D). Together, these findings show that the pro- and anti-inflammatory TLR2 ligands are largely restricted to the staphylococcal cell wall. We therefore focused subsequent experiments on the pro- and anti-inflammatory properties of heat-killed *S. aureus*.

Next, we wanted to determine if high-level IL-10-inducing *S. aureus* isolates contain quantitatively more IL-10-inducing ligands or qualitatively different ligands than the low-level IL-10-inducing counterparts. To test this, we performed an extensive titration of *S. aureus* isolates representative of high and low IL-10 induction capacities (shown here for S8 and S5 isolates) to determine the multiplicity of infection (MOI) that induced maximal pro- and anti-inflammatory responses. IL-10 was already detectable at an MOI of 1 and peaked at an MOI of 6 for both isolates (Fig. 2E). At all MOIs tested, the IL-10 production in response to *S. aureus* S8 was at least 2-fold greater than the response to S5. Importantly, the levels of TNF-α production in response to the isolates did not differ (Fig. 2F). These results suggest that the anti-inflammatory response is due to a qualitatively different ligand(s) than those responsible for the proinflammatory response, and therefore, the anti-inflammatory response to *S. aureus* can be uncoupled from the proinflammatory response.

Pro- and anti-inflammatory responses to *S. aureus* are uncoupled at the signaling level. Given that *S. aureus* may contain multiple IL-10-inducing ligands that act on TLR2, we next asked if these ligands activated the same pathway(s). The PI3K-Akt pathway has previously been shown to be essential for the anti-inflammatory response to TLR2 (22) and TLR4 (31) ligands. Thus, we examined activation of this pathway in response to several nasal *S. aureus* isolates. As shown in Fig. 3A, for two representative iso-

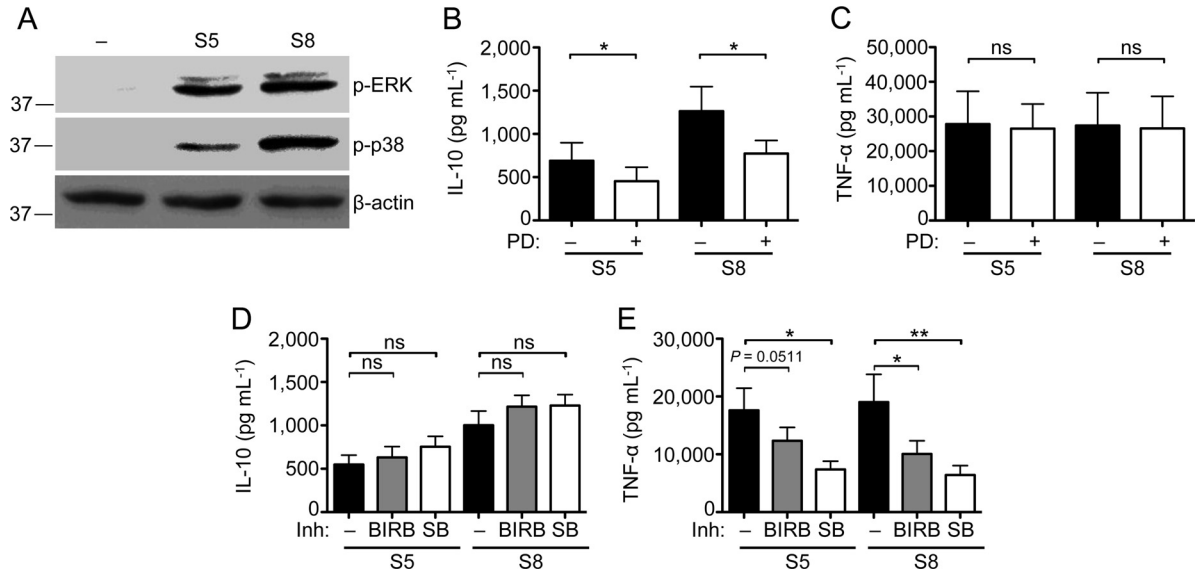

FIG 4 The MAPK p38 mediates the proinflammatory response to nasal *S. aureus* isolates. (A) Western blot of phospho-ERK and -p38 from human PBMCs stimulated with two representative *S. aureus* isolates inducing low-level (S5) or high-level (S8) IL-10 responses (MOI = 5) for 30 min. Blots are representative of three independent experiments involving three different donors. (B to E) PBMCs were pretreated with the ERK inhibitor PD-98059 (B and C) or either of the p38 inhibitors SB-203580 and BIRB-0796 (D and E) for 1 h and then stimulated with two representative *S. aureus* isolates inducing low-level (S5) or high-level (S8) IL-10 responses (MOI = 5) for 18 h. Quantification of accumulation of IL-10 (B and D) and TNF-α (C and E) in the supernatants was done by ELISA. Bar graphs show means ± SEMs of data from five different donors in experiments performed in triplicate. Statistical analysis was performed using the Student *t* test. *, $P < 0.05$; **, $P < 0.01$.

lates, we found that phosphorylation of Akt at S473 correlated with the IL-10-inducing capacity of nasal *S. aureus* isolates. Similar results were obtained using intracellular staining of phospho-Akt^{S473} in monocytes (Fig. 3B). Interestingly, we also observed Akt phosphorylation in a subset of B cells (~ 20%), but this did not differ between isolates inducing IL-10 at high and low levels (see Fig. S2 in the supplemental material). Inhibition of signaling through this pathway using the pan-PI3K inhibitor wortmannin significantly reduced IL-10 production in response to both *S. aureus* S5 and S8 but did not significantly affect the TNF-α response (Fig. 3C). Furthermore, using PI3K p110 isoform-specific inhibitors, we observed that p110δ was the dominant isoform mediating the IL-10 response, with a minor contribution from p110β (Fig. 3D).

To further corroborate the selective participation of the PI3K-Akt pathway in the anti-inflammatory response, we examined signaling steps further downstream in this cascade. We found that the mTOR inhibitor rapamycin significantly decreased the IL-10 response to *S. aureus* S5 and S8 (Fig. 3E). Interestingly, we observed a small but significant increase in TNF-α, which is most likely a reflection of the antagonistic properties IL-10 has on TNF-α production (32). Together, these results show that the PI3K-Akt-mTOR pathway is differentially activated by nasal *S. aureus* isolates, regulating the anti-inflammatory but not the proinflammatory response to this microbe.

The importance of MAPK signaling in the cytokine response to MAMPs is well documented (33). Moreover, p38 and ERK have both been documented to regulate IL-10 production in response to various stimuli (30). Consistent with this, we observed that both p38 and ERK were activated in response to the nasal *S. aureus* isolates (Fig. 4A). To elucidate the specific roles of these members of the MAPK family in the response to *S. aureus*, we used the selective inhibitors PD-98059 for ERK1/2 and SB-203580 and BIRB-0796 for p38. PD-98059 slightly but significantly decreased the IL-10 response to *S. aureus* (Fig. 4B) and had no effect on the TNF-α response (Fig. 4C). In contrast, the p38 inhibitors SB-203580 and BIRB-0796 significantly decreased the TNF-α response without affecting the IL-10 response (Fig. 4D and E). These results further document the uncoupling of pro- and anti-inflammatory responses to *S. aureus* at the level of MAPK signaling by showing the selective dependence of the proinflammatory response on the p38 MAPK signaling pathway.

Pro- and anti-inflammatory responses to *S. aureus* have differential requirements for microbial internalization and phagosome maturation. It has been recently shown that the anti-inflammatory IL-10 response to Gram-negative *Escherichia coli* lipopolysaccharide (LPS) requires PI3K-dependent internalization of TLR4 (31). Thus, we examined whether a similar mechanism could be involved in the anti-inflammatory response to *S. aureus* through TLR2. First, we tested if inhibition of PI3K could prevent internalization of *S. aureus*. As shown in Fig. 5A, this was not the case, as *S. aureus* was still internalized in the presence of wortmannin, suggesting that PI3K signaling is not required for *S. aureus* phagocytosis. Inhibition of phagocytosis with either the actin inhibitor cytochalasin D or the dynamin inhibitor dynasore did not prevent *S. aureus*-induced phospho-Akt (Fig. 5B), which is required for the anti-inflammatory response to *S. aureus*, even though inhibition of phagocytosis was confirmed by flow cytometry (data not shown). Unfortunately, due to cytotoxicity of these inhibitors, we were unable to examine their effects on cytokine production (data not shown). Together, these findings implied that the IL-10-induced signaling from *S. aureus* occurred at the cell surface and did not require *S. aureus* internalization. Consistent with this claim, we saw that the IL-10 response to *S. aureus* was not affected by inhibition of endophagosome acidification, whereas the proinflammatory response was significantly inhibited

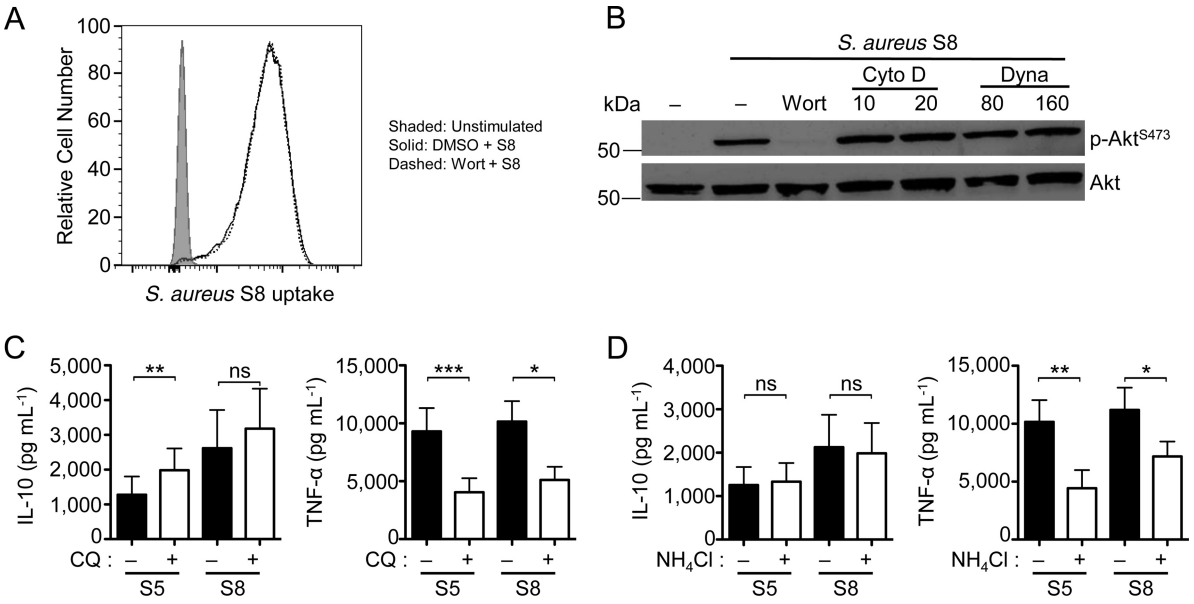

FIG 5 Pro- and anti-inflammatory responses to *S. aureus* have differential requirements for microbial internalization and processing. (A) Human PBMCs were pretreated with DMSO or wortmannin (1 μM) and cultured with TAMRA-labeled *S. aureus* (MOI = 5) for 30 min. Uptake of *S. aureus* by $CD14^+$ monocytes was determined by flow cytometry. The plot is representative of three independent experiments. (B) Phospho-Akt Western blot of human PBMCs stimulated with *S. aureus* S8 for 30 min with or without pretreatment with wortmannin or either of the internalization inhibitors cytochalasin D and dynasore. Blots are representative of three independent experiments from three different donors. (C and D) PBMCs were pretreated for 1 h with chloroquine (CQ) (C) or NH_4Cl (D) and then stimulated with two representative *S. aureus* isolates inducing low-level (S5) or high-level (S8) IL-10 responses (MOI = 5) for 18 h. Accumulation of IL-10 or TNF-α was quantified by ELISA. Bar graphs show means ± SEMs from five donors in experiments performed in triplicate. Statistical analysis was performed using the Student *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

by chloroquine and NH_4Cl (Fig. 5C and D). These results demonstrate a differential requirement for microbe internalization on pro- and anti-inflammatory responses, further documenting the spontaneous uncoupling of these properties in *S. aureus*.

Uncoupling of pro- and anti-inflammatory properties of *S. aureus* translates into differential modulation of adaptive immunity to staphylococcal SAgs. We have previously reported that the IL-10 response to *S. aureus* suppresses superantigen (SAg)-induced T cell activation and may be protective against staphylococcal TSS (21). We therefore predicted that those isolates able to induce a high-level IL-10 response are better at suppressing this T cell activation. We found that this was the case: even though all *S. aureus* isolates induced enough IL-10 to decrease the SAg-induced T cell activation as measured by IL-2 production (Fig. 6A), the high-level IL-10-inducing *S. aureus* isolates (e.g., S8) were significantly better suppressors than the low-level IL-10-inducing isolates (e.g., S3).

Bacteriostatic antibiotics, such as clindamycin, have been recommended for the management of staphylococcal TSS (34). The effectiveness of clindamycin to treat staphylococcal TSS is attributed to blocking the translation of staphylococcal SAg (35). We postulated that in addition to this mechanism, clindamycin may also maintain the integrity of the staphylococcal cell wall to induce IL-10 production. To test this hypothesis, we cultured *S. aureus* in TSB containing clindamycin for 6 h and compared its immunostimulatory capacity to that of heat-killed *S. aureus*. We observed that clindamycin-treated *S. aureus* induced significantly more IL-10 than heat-killed *S. aureus* but did not significantly change TNF-α production (Fig. 6B). This result reveals a potential biological implication of the uncoupling of proinflammatory and anti-inflammatory properties and suggests that clindamycin may be effective for treating staphylococcal TSS by promoting an anti-inflammatory response to *S. aureus*, in addition to inhibiting SAg production.

To further characterize the modulatory effect of the community isolates of *S. aureus* on the adaptive immune response to staphylococcal superantigens, we performed a multiplex analysis of the cytokine response to high- and low-level IL-10-inducing nasal *S. aureus* isolates (Fig. 6C). We observed no difference in the production of proinflammatory IL-1β, IL-6, or TNF-α cytokines among these isolates (Fig. 6D), suggesting that these cytokines are similarly regulated in response to *S. aureus*. However, the Th1 cytokines IL-12p70 and gamma interferon (IFN-γ) were induced to a greater extent by *S. aureus* isolates that had less anti-inflammatory properties (Fig. 6E). Altogether, these results imply that the low-level IL-10-inducing capacity of an isolate of *S. aureus* imprints adaptive immunity to a Th1 profile and thus influences the development of protective proinflammatory responses in the context of staphylococcal diseases.

DISCUSSION

TLR signaling leading to the production of IL-10 and other anti-inflammatory mediators in response to MAMPs has been previously reported (21, 22, 31, 36). However, its qualitative and quantitative relationship to the proinflammatory cytokine response (i.e., IL-1β, IL-6, TNF-α, etc.) triggered by PRR signaling has not yet been studied. Specifically, it is not known whether the two types of responses occur in parallel and to similar extents or whether pro- and anti-inflammatory responses are the result of qualitatively different MAMP:PRR recognition events that can be uncoupled. On the basis of results obtained using multiple nasal isolates of *S. aureus* from chronic human carriers of this microbe,

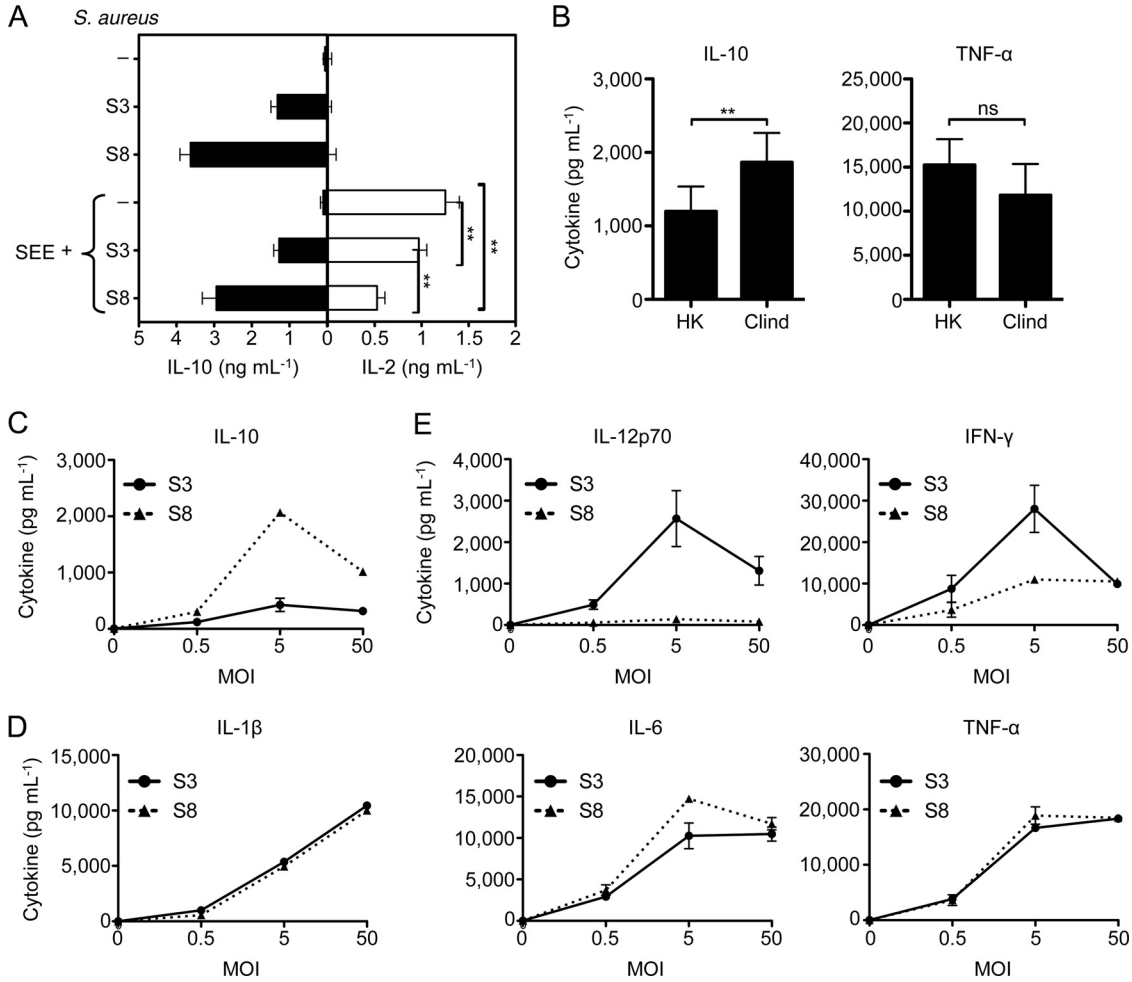

FIG 6 The IL-10-inducing capacity of nasal *S. aureus* isolates correlates with the modulation of adaptive immunity. (A) PBMCs were stimulated with staphylococcal enterotoxin E (SEE) (10 ng ml^{-1}) for 18 h in the presence or absence of representative *S. aureus* isolates inducing low-level (S3) or high-level (S8) IL-10 responses (MOI = 5). Quantification of IL-2 and IL-10 in the supernatants was done by ELISA. Data are plotted as means ± SDs and are representative of three independent experiments involving three different donors. (B) IL-10 and TNF-α responses by PBMCs to heat-killed (HK) or clindamycin-treated (Clind) *S. aureus* S8 (MOI = 5). Data are plotted as means ± SEMs from 5 donors in experiments performed in triplicate. Statistical analysis was performed using the Student *t* test. (C to E) Profiles of cytokine responses of PBMCs to *S. aureus*. PBMCs were stimulated for 18 h with *S. aureus* S3 or S8, and IL-10 (C), the proinflammatory cytokines IL-1β, IL-6, and TNF-α (D), and the Th1 cytokines IL-12p70 and IFN-γ (E) were measured. Data are plotted as means ± SDs and are representative of two independent experiments involving two different donors. **, $P < 0.01$.

we report here that for TLR2, these responses are mechanistically distinct and are naturally uncoupled.

Such an uncoupling was seen in all the isolates of a representative sample of community *S. aureus* independently of the magnitude of IL-10 response they induced. Our findings support the idea that the pro- and anti-inflammatory properties of a given *S. aureus* isolate are the result of qualitatively different responses to recognition of MAMPs on the staphylococcal cell wall. In support of this conclusion, we found that IL-10 production in response to *S. aureus* was dependent on PI3K-Akt-mTOR and ERK signaling, whereas the TNF-α response was dependent on p38. Furthermore, internalization and phagosome maturation were required only for the proinflammatory response and not the anti-inflammatory response. Lastly, natural differences in the capacity of nasal *S. aureus* isolates to induce an IL-10 response in the host, independently of the proinflammatory response they can induce, differentially imprints the adaptive immune response to *S. aureus*.

Whether the uncoupling of pro- and anti-inflammatory properties of a given *S. aureus* isolate is the result of multiple ligands on the staphylococcal cell wall recognized by one PRR or by different PRRs is still unclear. Our previous data suggested that the proinflammatory response occurs from multiple PRRs, including TLR2, TLR9, and NOD1/2, whereas the anti-inflammatory response to *S. aureus* is predominantly secondary to TLR2 signaling (21). NOD1/2 were ruled out as players in the anti-inflammatory IL-10 response because ultrapure staphylococcal PGN (PGN-Sandi), which lacks TLR2-stimulating capacity and thus can only activate NOD1/2, did not induce IL-10 production, despite giving the same proinflammatory profile as crude staphylococcal PGN (22). Both NODs and TLR9 can also be excluded by the fact that IL-10 production in response to *S. aureus* is independent of phagocytosis. Thus, our data support the idea that the uncoupling occurs, in part, by several ligands acting on TLR2. Indeed, previous work from our laboratory has shown that the TLR2 accessory

molecules CD14 and CD36 are only required for the proinflammatory response to staphylococcal PGN preparations (22). Moreover, CD36 is required for *S. aureus* internalization (37), and this may explain the dependency of this molecule only for the proinflammatory response (22). Together, these findings point to structural differences in pro- and anti-inflammatory TLR2 ligands. The molecular nature of these ligands is unknown at the moment, although it may be diverse and present in other microbial species (e.g., polysaccharide A in *Bacillus fragilis* [25]).

TLR2-based uncoupling of pro- and anti-inflammatory responses may stem from differential TLR2 oligomer formation. For example, TLR2 dimerization with TLR6 has been linked to anti-inflammatory responses, whereas TLR2/1 complexes promote proinflammatory responses (38–40). The molecular definition of the resulting signalosomes is uncertain (41, 42). It is plausible to suggest that in response to *S. aureus*, the availability of TLR2 on the cell surface is a limiting factor of the response. If so, the relative abundance of the pro- versus anti-inflammatory ligands would dictate the outcome of the response. We cannot rule out that under some circumstances (e.g., *S. aureus* isolates lacking TLR2-stimulatory capacity [43]) an IL-10 response may occur through an alternative, less efficient mechanism.

Phagocytosis is an important defense mechanism to mount an effective inflammatory response against *S. aureus* (19, 20). We have corroborated this fact using nasal *S. aureus* isolates. Interestingly, though, the anti-inflammatory response did not require phagocytosis by monocytes/macrophages. Since we used heat-killed bacteria as well as cell wall preparations, our findings indicate that the anti-inflammatory ligands are present in the staphylococcal cell wall in a recognizable conformation, unlike their proinflammatory counterparts. Such an arrangement may ensure an anti-inflammatory response that downplays the Th1 response to *S. aureus* and promotes a state of disease tolerance to this microbe (44).

The mechanism of TLR2-dependent anti-inflammatory response is different from that reported recently for the TLR4-dependent anti-inflammatory response. The anti-inflammatory response to TLR4 signaling by LPS requires activation of PI3K p110δ for endosomal translocation and a switch from the TIRAP-MyD88 pathway to TRAM-TRIF pathway for anti-inflammatory signaling to occur (31). In contrast, for TLR2 signaling, PI3K p110δ, although required for the IL-10 response, is not involved in endosomal trafficking of TLR2. We found that inhibition of PI3K did not affect *S. aureus* internalization by monocytes and the IL-10 response was independent of phagocytosis and phagosome maturation. Furthermore, although the switch to anti-inflammatory TLR4 signaling resulted in a diminished proinflammatory response (31), we did not observe such an effect with nasal *S. aureus* isolates. Together, our findings are consistent with those showing that the PI3K-Akt-mTOR pathway directly leads to IL-10 production (36, 45, 46).

We have previously shown that the IL-10 response to *S. aureus* is predominantly mounted by monocytes/macrophages. This is in contrast to the IL-10 response to other MAMPs (25, 47–49). Monocytes/macrophages are early responders to *S. aureus*, and their phenotype would be highly influential in establishing the microenvironment that sets up subsequent adaptive responses. Previous work from our laboratory has shown that the IL-10-producing monocytes/macrophages are classically activated and not alternatively activated macrophages, because of the robust inflammatory response simultaneously observed and the inability to show IL-4/IL-13 during the generation of this response (22). Moreover, a high proportion of these monocytes acquire a dual phenotype (i.e., IL-10$^+$ TNF-α$^+$), a phenotype not reported for M2 macrophages (reviewed in reference 50). The characterization of the different macrophage subsets responding to nasal *S. aureus* isolates based on their cytokine production profiles is a matter for future studies. It is likely that these cells show a phenotype similar to that of inflammatory monocytes seen in the gut during *Toxoplasma gondii* infection (51), in which simultaneous expression of proinflammatory and anti-inflammatory mediators is observed. Proper balance of this expression may determine pathogen elimination while limiting tissue damage and deleterious effects on commensals.

The findings reported here may have clinical implications. The balance between pro- and anti-inflammatory responses to a given *S. aureus* isolate may determine the outcome of the encounter, i.e., commensalism versus disease. *S. aureus* isolates with a high capacity to induce IL-10 would be better at colonizing the upper respiratory tract, as the heightened IL-10 levels may provide a tolerogenic environment and be less likely to cause staphylococcal TSS. Alternatively, a predominance of inflammatory cytokine production may exacerbate mucosal injury (52). Lastly, high-level IL-10 induction by a given isolate may be detrimental during staphylococcal bacteremia by dampening adaptive immunity (53). Formal proof of concept for this model will require *in vivo* testing.

In conclusion, our findings have revealed that the pro- and anti-inflammatory properties of nasal *S. aureus* isolates are independent of each other; i.e., they can be uncoupled. Such an uncoupling obeys different mechanistic requirements. The ability to naturally uncouple these two types of responses suggests that *S. aureus* contains two sets of MAMPs: one that preferentially induces a proinflammatory response and minimal anti-inflammatory mediators and a second set that induces a robust anti-inflammatory response, with few proinflammatory cytokines. Balance between the ensuing responses may determine colonization and disease tolerance versus pathogenicity and disease by *S. aureus*.

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