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O-acetylation of peptidoglycan limits helper T cell priming and permits *Staphylococcus aureus* reinfection

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Summary

Humans do not usually develop effective immunity to *Staphylococcus aureus* reinfection. Using a murine model that mimics human infection, we show that lack of protective immunity to *S. aureus* systemic reinfection is associated with robust IL-10 production and impaired protective Th17 responses. In dendritic-cell co-culture assays, priming with *S. aureus* promotes robust T-cell proliferation, but limits Th cells polarization and production of IL1-β and other cytokines important for Th1 and Th17 differentiation. We show that O-acetylation of peptidoglycan, a mechanism utilized by *S. aureus* to block bacterial cell-wall breakdown, limits the induction of pro-inflammatory signals required for optimal Th17 polarization. IL-10-deficiency in mice restores protective immunity to *S. aureus* infection, and adjuvancy with a staphylococcal peptidoglycan O-acetyltransferase mutant reduces IL-10, increases IL1-β, and promotes development of IL-17-dependent, Th cell-transferable protective immunity. Overall, our study suggests a mechanism whereby *S. aureus* modulates cytokines critical for induction of protective Th17 immunity.

eTOC

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AUTHOR CONTRIBUTIONS

G.A.M. and G.Y.L conceived and directed the project. M. S., S. L. K., S. M., M.A., D.M.U., G. A. M. and G.Y.L. designed experiments, and prepared the manuscript. M. S., S. L. K., S. M., C. R., D.C., R.S., G.A.M. and G.Y.L. conducted most of the experiments and analyzed the data. A.J.W. and C.O. performed some of the in vitro molecular and cellular experiments.

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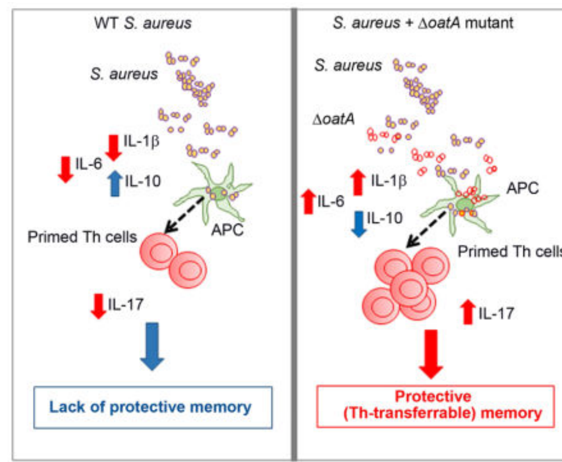
Humans do not develop robust protective immunity to *S. aureus* infection. Sanchez *et al.* show that mice, like humans, do not develop a protective Th17 response after bloodstream *S. aureus* infection. *S. aureus*, via modification of cell wall peptidoglycan, limits priming of Th17 cells and blocks development of protective immunity.



INTRODUCTION

S. aureus is a pathogen that induces significant morbidity and mortality worldwide. With the advent of methicillin-resistant *S. aureus* and the threat of antibiotic resistance, developing a vaccine against *S. aureus* has become a priority. Humans do not generally develop robust protective immunity upon *S. aureus* infection, as indicated by the finding that individuals can be infected with *S. aureus* many times throughout life (Karauzum and Datta, 2016). Although there is evidence that protective immunity could be acquired, for example, in subjects colonized nasally with *S. aureus*, protection is generally modest (Wertheim et al., 2004). The reason behind the lack of robust protection to *S. aureus* reinfection is unclear and of significant interest for the development of strategies to create efficacious vaccines against *S. aureus*.

Studies of the effects of *S. aureus* infection on T and B cells have uncovered a number of mechanisms whereby *S. aureus* interferes with the host adaptive immune response. Evasion factors include *S. aureus* superantigens that induce T cell anergy or deletion (Cantor et al., 1993), and *S. aureus* Protein A, which induces deletion of specific B cell populations (Goodyear and Silverman, 2003) in addition to preferential shifting of host antibody response and blocking of antibody-mediated phagocytosis (Rosenblatt et al., 1977). Additionally, *S. aureus* secretes an MHC-like molecule that is thought to drive Th2 differentiation (Lee et al., 2002) and toxins that induce T cell cytolysis or apoptosis (Alonzo et al., 2013). Although many of these bacterial strategies have been implicated in immune response evasion in *S. aureus* infection models, so far only Protein A has a confirmed role with a defined mechanism of promoting *S. aureus* reinfection (Falugi et al., 2013). Studies have shown a strong link between IL-17 deficiency in patients with hyper-IgE syndrome (defect in STAT3 phosphorylation) and severe *S. aureus* infections (Milner et al., 2008). Coupled with reports of the detrimental effects of IL-17 on *S. aureus* (Cho et al., 2010), the

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findings suggest that minimizing induction of IL-17 would be a logical strategy used by *S. aureus* to evade host immunity, as pathogens routinely adapt elaborate strategies to block host defenses that interfere with their survival. To date, *S. aureus* strategies targeting IL-17 have not been reported.

Numerous studies involving *S. aureus* reinfection have been published in which mice undergo repeat infections with a single strain of *S. aureus* and, surprisingly, develop significant protection from reinfection (Brown et al., 2015; Montgomery et al., 2014; Murphy et al., 2014; Sasaki et al., 2006). These studies have led to important insights on how the host limits *S. aureus* infection by promoting specific T and B cell responses. However, for the purpose of addressing why humans are not robustly protected from reinfection, these models are less informative. In the current study, we investigated potential mechanisms deployed by *S. aureus* to subvert host protective T cell responses.

RESULTS

Mice remain susceptible to *S. aureus* reinfections in a model that mimics human disease

Most humans experience *S. aureus* infections of varying severity throughout life, which are routinely controlled within a few days by a robust immune system or by antibiotic treatment. To mimic human infections, we used an intraperitoneal (i.p.) infection model that leads to spontaneous clearance of *S. aureus* after 3–4 days, and reinfected the mice 21 days after the first infection. For these experiments, we utilized a USA300 MRSA strain (LAC) which is a common cause of human infections. To enable comparison to an infection model that induces protective immunity, we performed parallel infection experiments using Group A streptococcus (GAS, M49 strain), which confers protective immunity against GAS of the same M-protein serotype in humans (Cunningham, 2000). We determined the highest inoculum of *S. aureus* or GAS to induce an infection without causing mortality for *S. aureus* LAC (1×10^7) and GAS M49 (5×10^6–1×10^7) and used those inocula for the remaining experiments. We confirmed that animals infected using those inocula clear the infection fully prior to reinfection on day 21 (usually 3–5 days after infection, data not shown). As shown in Figures 1A, 1B and S1A, GAS induces robust protection from reinfection as measured by CFU, body weight and LD_{50}. *S. aureus* did not induce protective immunity after two or four consecutive infections in C57BL/6 mice (Figure 1A and 1C). Similar results were obtained in BALB/c mice (Figure S1B). Hence, this model mimics the observation in humans that *S. aureus* infection does not lead to the development of robust protective immunity.

S. aureus induces modest production of IFN-γ and IL-17A upon reinfection

We queried if protection (GAS infection) or lack of protection (*S. aureus* infection) in our model correlates to changes in cytokines associated with Th cell polarization in vivo. We observed that GAS reinfection enhanced production of IL-17A and IFN-γ in the spleen when compared to the initial GAS infection (Figures 1D and E). In contrast, *S. aureus* infection induced low levels of IL-17A and IFN-γ, which were not further increased upon reinfection, suggesting defective Th1 and Th17 memory responses. Conversely, *S. aureus* reinfection was associated with increased IL-10 production (Figure 1E). Further increasing the number of *S. aureus* reinfections did not lead to increase in IFN-γ or IL-17A expression

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in the spleen, consistent with impaired induction of protective immunity (Figure S1C). Protective immunity to GAS in our i.p. model was abolished when the infection was performed in IL-17$^{-/-}$ mice, corroborating the importance of IL17 in immunity against GAS reinfection (Figure S1D). Overall, these data suggest that GAS mounts robust protective IL17-dependent immune responses whereas *S. aureus* fails to induce protective immunity.

S. aureus-induced dendritic cell maturation leads to efficient proliferation but limited Th polarization in vitro

The limited Th17 and Th1 responses observed in our *S. aureus* reinfection model suggested the possibility that, in comparison to GAS, *S. aureus* would induce suboptimal priming of T cells by antigen presenting cells. Therefore, we studied initial priming of naïve CD4^{+} T cells isolated from OT-II transgenic Rag2$^{-/-}$ mice, using OVA peptide as a model antigen and bone marrow-derived dendritic cells (BMDC) infected in vitro with either GAS or *S. aureus*.

BMDC were incubated with either *S. aureus* or GAS, pulsed with OVA peptide and then co-cultured with OT-II TCR transgenic CD4^{+} T cells for 7 days prior to analysis. As shown in Figure 2A, OVA peptide-induced proliferation of T cells was not significantly different after DC priming with *S. aureus* or GAS. However, consistent with our in vivo findings, *S. aureus*-infected DC induced significantly lower amounts of IFN-γ and IL-17A than GAS-infected DC (Figure 2B). To determine if *S. aureus* and GAS differentially induce DC maturation, we measured expression of MHC II, CD80 and CD86 in *S. aureus* or GAS-infected DC. Expression of MHC II and CD80 was comparable in DC stimulated with GAS or *S. aureus*, but GAS induced higher expression of CD86 (Figure 2C), consistent with a previous study in human monocytes (Wang et al., 2012). Although CD86 has been show to favor Th17 differentiation in certain circumstances (Huang et al., 2012), at least one recent study (Kang et al., 2016) shows that Th17-inducing DC has low CD86 expression. In our in vitro co-culture system, it is likely that the reduced CD86 expression in *S. aureus*-infected DC did not influence Th differentiation, as we could not detect any differences in Th cell proliferation, which is required for Th cell terminal differentiation. However, this remains to be formally tested.

Differential regulation of DC gene expression by *S. aureus* and GAS

To compare the gene profile of DC after stimulation with GAS or *S. aureus*, we performed transcriptome analysis of DC stimulated with either bacteria or media alone (No stimuli, NS). As shown in Figure 2D, both *S. aureus* and GAS induced significant changes in DC gene expression, however GAS infection altered the expression of substantially more genes (2921 total) than *S. aureus* infection (426 total) (adjusted p 0.05 and fold change 2). GAS-infected DC had 1409 genes up regulated and 1512 genes downregulated whereas *S. aureus*-infected DC showed upregulation of 329 genes and downregulation of 97.

A total of 87 genes were downregulated by both *S. aureus* and GAS. These included important regulators of cytokine signaling such as *SOCS2* (Hu et al., 2009; Jackson et al., 2004), *IRF4* which have been previously associated with the regulation of DC maturation and function (Tussiwand et al., 2015) and *MYC* (Fig. 2D) previously shown to function as a negative regulator of DC activation, (Kim et al., 2016) among others (Table S1 and S2). We

also found that GAS but not *S. aureus* induced the downregulation of genes associated with inflammasome activation (*NLRP1B*), regulation of NF-κB (*CARD11*) and the activation receptor *CLEC9a*, whereas *S. aureus* specifically downregulated expression of few genes (10 total), including genes associated with regulation of cell cycle and proliferation (G1/S-Specific Cyclin-E1 *CCNE1* and the GTPase Ras family member *DIRAS2*) and survival (*RELL1*) (Cusick et al., 2010). Of note, the genes encoding the inflammatory cytokines IL-1β (*IL1B*), TNF-α (TNF) and IL12p35 (*IL12A*) were amongst genes upregulated by both *S. aureus* and GAS, however induction by GAS infection was substantially higher than by *S. aureus* (Figure 2D).

S. aureus also selectively induced genes associated with DC activation (*TNFRSF1B*), negative regulators of DC function (*IRAK3* and *TNIP1*) (Sumpter et al., 2011)(Callahan et al., 2013), in addition to *KCTD1*, a negative regulator of the canonical Wnt signaling pathway (Li et al., 2014) among others. On the other hand, genes selectively induced by GAS included several inflammatory cytokines, such as *Il6*, IL12p40 (*IL12B*), and IL23p35 (*IL23A*) (Figure 2D). In addition, GAS but not *S. aureus* infection leads to upregulation of Caspase1 (*CASP1*) and *CD86* expression.

The preferential induction of select pro-inflammatory cytokines by GAS in comparison to *S. aureus* was confirmed by ELISA assays (Figure 2E). Expression of TGF-β was comparable between GAS and *S. aureus* infected DC, and GAS also induced significant expression of IL23, which was not detected in *S. aureus*-infected DC. Thus, in line with the weak induction of Th1 and Th17 memory responses to *S. aureus* infection, DC infection with *S. aureus* induced lower expression of pro-inflammatory cytokines previously associated with priming of Th1 (IL12) and Th17 (IL1β, IL-6, IL-23) cells. Pro-inflammatory cytokines (IL1α, IL-6 and IL-12, but not IL1β) were also induced at a lower level by a panel of *S. aureus* isolates compared to GAS, suggesting that suboptimal induction of these cytokines by *S. aureus* is not unique to the LAC strain (Figure S2).

S. aureus peptidoglycan O-acetyltransferase represses the induction of select innate immune genes associated with the development of Th17 immunity

The finding that *S. aureus* is a poor inducer of inflammatory cytokines required for development of protective T cell immunity led us to hypothesize that *S. aureus* limits signals required to induce protective Th cell immunity. The cell wall PGN of *S. aureus* is a potent inducer of pro-inflammatory signals via recognition by innate pattern recognition receptors. Degradation of PGN directly promotes the activation of the NLRP3 inflammasome in macrophages (Shimada et al., 2010), and lysis of the bacteria leads to secondary release of PAMPs (Pathogen-associated molecular patterns) that are further recognized by additional receptors (Wolf et al., 2011). O-acetylation of MurNAc on the *S. aureus* PGN backbone has been shown to confer protection against host lysosome degradation (Bera et al., 2005). This actively limits inflammasome activation, IL-1β beta secretion and to a lesser extent the secretion of other cytokines in *S. aureus*-infected macrophages (Shimada et al., 2010; Wolf et al., 2011). Based on these data, we hypothesized that O-acetylation of *S. aureus* PGN is responsible for limiting induction of key cytokines required for Th differentiation and induction of protective immunity from reinfection.

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To address this, we tested if a *S. aureus* O-acetyltransferase mutant (*oatA*) could induce production of four of the main cytokines required for the induction of Th17 differentiation, using a well characterized isogenic WT/ *oatA* mutant pair in the SA113 background (Bera et al., 2005). SA113 induces similar levels of IL-1β, IL6, IL12 and IL-1α compared to LAC (Figure S2), but *oatA* has been more extensively studied in the SA113 background (Shimada et al., 2010; Wolf et al., 2011). As shown in Figure 3A and S3, BMDC stimulated with *oatA* or a 1:1 mixture of WT and *oatA* in vitro secretes higher amounts of IL1-β, IL-6 and TGF-β compared to WT *S. aureus*-infected DC. IL-23 was not induced by either strain (data not shown). We next infected mice with $2{\times}10^7$ WT *S. aureus* alone (SA113), or with 10^7 WT *S. aureus* together with 10^7 *oatA* i.p., three times at 7 day intervals. Adjuvancy with *oatA* led to enhanced production of IL-1β and IL-23 and lower production of IL-10 compared to infection with WT *S. aureus* alone (Figure 3B). Ex-vivo restimulation of total spleen cells with heat-killed *S. aureus* 5 days after the last infection revealed increased production of IL-17A (but not IFN-γ) and lower IL-10 levels in the cultures of WT/ *oatA S. aureus*-infected mice in comparison to cultures from WT *S, aureus*-infected mice (Figure 3C), suggesting enhanced differentiation of Th17 cells in these mice.

Adjuvancy with S. aureus **O-acetyltransferase mutant or absence of IL-10 promotes the development of protective immunity against *S. aureus* reinfection**

To determine if enhanced generation of Th17 cells leads to protection from *S. aureus* reinfection, we infected mice three times with WT SA113 and *oatA* bacteria, and then challenged the mice with WT *S. aureus.* As shown in Figure 4A, mice initially infected with mixed *oatA* and WT *S. aureus* and then challenged 10 days later with WT *S. aureus* showed enhanced *S. aureus* clearance compared to mice infected previously with WT *S. aureus* only. Prior infection with WT and *oatA S. aureus* (once) or *oatA S. aureus* (three times) did not provide significant protection against reinfection (data not shown).

We also found that adoptive transfer of CD4 T cells (5×10^6 cells; purity >95%) from mice infected three times with *oatA*/WT conferred protective immunity to *S. aureus* infection in naïve mice (Figure 4B), indicating that protection induced in this model can be transferred by Th cells. To determine whether, specifically, Th17 cells are important for the induced protection, we evaluated if transfer of purified CD4+ T cells from previously infected IL-17A$^{-/-}$ mice could induce protection in naïve mice. As shown in Figure 4B and S4, protection against *S. aureus* is IL-17A dependent since transfer of T cells from similarly treated IL-17A$^{-/-}$ mice did not confer protection.

The enhanced induction of IL-10 observed upon *S. aureus* reinfection (Fig. 1E) and the reduced IL-10 produced in the context of *oatA* adjuvancy seem particularly noteworthy. Sallusto and colleagues previously described in vitro generation of human *S. aureus*-specific memory Th17 cells that produce IL10 upon reactivation, wherein IL-10 production could be inhibited by IL-1β (Zielinski et al., 2012). IL-10 has a well-described role in inhibition of Th17 cell development (Chaudhry et al., 2011; Frodermann et al., 2011). To address the role of IL-10 in our *S. aureus* reinfection model, we infected WT and IL-10$^{-/-}$ mice with WT *S. aureus* SA113 as before. As shown in Figure 4C–D, *S. aureus*-infected IL-10$^{-/-}$ mice showed enhanced production of IL-1β, IL-6, IL-17A and IFN-γ in comparison to WT mice.

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Infection in IL-$10^{-/-}$ mice also improved clearance of *S. aureus* during reinfection (Figure 4E). Moreover, we find that protection induced by lack of IL-10 can be transferred to naïve mice by CD4 T cells purified from IL-$10^{-/-}$ mice previously infected with WT *S. aureus* (Figure 4F).

Overall, these data suggest that O-acetylation of PGN by *S. aureus* limits pro-inflammatory signals and enhances IL-10 production, and the combined influence of these cytokines repress protective Th17 development and permit reinfection of the host.

DISCUSSION

Our study highlights a mechanism whereby *S. aureus* represses the production of cytokines needed to develop protective Th17 immunity. O-acetylation of PGN by *S. aureus* prevents breakdown of PGN and leads to inhibition of inflammasome activation in bone marrow-derived macrophages (Shimada et al., 2010). The modification of PGN further prevents secondary release of PAMPs trapped within the bacterial cell wall or cytoplasm (Wolf et al., 2011). Our finding was striking in that deletion of *oatA* provided sufficient stimulation for development of protective Th17 immunity, albeit several reinfections were required. Infection with *oatA* alone was not sufficient to induce immunity (data not shown), likely because *oatA* is much more rapidly cleared during infection (Shimada et al., 2010). Our study did not explore and does not exclude other possible mechanisms underlying protection conferred by the *oatA* strain.

At a basic level, our model posits that O-acetylation of PGN by *S. aureus* limits the production of inflammatory cytokines, including IL-1β, IL-6, and TFG-β, and thereby hampers the development of a protective Th17 response. The importance of IL-1β and IL-6 in Th17 development is well-established (Korn et al., 2009). Less well-understood are the characteristics of Th17 cells that confer or do not confer protection to *S. aureus* reinfection. In their landmark study, Sallusto and colleagues highlight the generation of different subtypes of Th17 cells in response to infections (Zielinski et al., 2012). Using human naïve T cells and autologous monocytes pulsed with dead *S. aureus* or Candida, they showed that *S. aureus* preferentially induce development of Th17 cells that co-produce IL-17A and IL-10. In comparison, Candida-pulsed monocytes induced Th17 cells that secrete IL-17 and IFN-γ, but not IL-10. They further demonstrated that addition of exogenous IL-1β can drive *S. aureus*-primed Th17 cells to increase IL-17*A* and IFN-γ and reduce IL-10 produced upon reactivation. What was not clear from that study were the functional implications of those Th17 cell subtypes in vivo. Although our study was not designed to dovetail with that report, our results are consistent with their findings, as we demonstrate that *S. aureus* induces, in vivo, a modest Th17 response associated with enhanced IL-10 production. That Th17 response proves not to be protective against reinfection, but coinfection with a *Oat* mutant that significantly boosted IL-1β and other selective cytokines fostered Th17 responses and reduced IL-10 expression, thus leading to IL-17-dependent Th cell-mediated protective immunity. Using a genetic approach, we showed that deletion of IL10 in vivo increases IL-1β production, enhances Th17 responses and promote protection against reinfection. IL-10 production in response to *S. aureus* has been shown to be associated with *S. aureus* regulation of Th17 cells in vitro (Frodermann et al., 2011). It is not clear if the increase in

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IL-1β or other cytokines, reduction of IL-10 or other possible alterations of the Th17 subtype are the primary drivers of the phenotypic change from a non-protective to a protective immune response in vivo. Our model should prove useful for future studies of the role of specific cytokines and Th17 responses in protective immune memory to *S. aureus* infection.

Our findings further advance the current understanding of how the host responds to *S. aureus* reinfection in mice. Many localized reinfection models (peritoneal or skin) reported the development of protective immunity following *S. aureus* infection (Brown et al., 2015; Montgomery et al., 2014; Murphy et al., 2014). Protective mechanisms identified include the induction of Th1 cells and IL-17-producing γδT cells from the peritoneum and antibody and Th17 responses following subcutaneous infection (Brown et al., 2015; Montgomery et al., 2014; Murphy et al., 2014). We and others (Kim et al., 2011) have observed in contrast that upon systemic bloodstream infection (i.p. or i.v.) protective immunity is not induced. Interestingly, studies of human PBMC have reported that *S. aureus*-specific Th1 responses are more readily demonstrated than Th17 responses following bloodstream infection (Brown et al., 2015). It is likely that mucosal environments, which favor development of Th17 immunity (Korn et al., 2009), contribute significantly to tissue-specific difference in *S. aureus* infection outcome. Different host compartments could have additional effects on virulence gene expression and the involvement of these factors in pathogenesis, and therefore could impact the induction of a protective Th response.

Overall, our study suggests a mechanism by which *S. aureus* interferes with development of an effective Th17 response against reinfection and therefore provides an explanation for the conundrum that both human and mice fail to mount a robust protective response to systemic infection. Our study also suggests that induction of IL-17-producing Th cells may require additional context to predict protection, since the quantity of IL-17A secreted, the subtype of Th17 cells, and other parameters that govern Th17 development are all potentially important. Therefore, future vaccines that aim to induce a protective Th17 response need to consider these subtleties of Th17 biology.

STARS METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, George Liu MD PhD (George.liu@cshs.org)

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Microbes—GAS M49, *S. aureus* LAC, COL, Newman, MW2, SA113, and *oatA* SA113 and complemented *oatA* SA113 (courtesy of Dr. Fritz Goetz) were routinely cultured in Brain Heart Infusion, Todd Hewitt broth or Todd Hewitt broth supplemented with 0.5% Yeast Extract. Mid-log phase bacteria sub-cultured from overnight cultures or overnight cultures were used for experiments. Bacteria were routinely washed twice in PBS prior to use and inocula were confirmed by determination of CFU on agar plates. Heat-killed *S.*

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aureus was prepared by incubating a washed overnight culture of *S. aureus* for 1h at 60°C. Sterility of killed bacteria was confirmed by enumeration of CFU on an agar plate.

Primary cells—DC from 8–12 week old female C57BL/6 mice were derived by incubating murine bone marrow cells with in RPMI 1640 media containing Pen/Strep and supplemented with 10% FBS and 20ng/mL of mGM-CSF (PeproTech). After 9 days, the differentiated cells were used for in vitro stimulation assays. CD4 T cells were isolated from spleen and lymph nodes of 8–12 week old female C57BL/6 mice infected with *S. aureus* using a negative selection kit (STEMCELL). Purity of CD4 T cells was confirmed by flow cytometry (>95%).

Animals—C57BL/6 and BALB/c mice were purchased from Jackson Lab. IL-17A$^{-/-}$ (Courtesy of Dr. Yoichiro Iwakura), IL-10$^{-/-}$ and OT-II transgenic/Rag$^{-/-}$ mice were maintained at Cedars-Sinai. All mice were housed in specific-pathogen free facilities and 8 to 12-week old age-matched female mice were used for *in vitro* and *in vivo* experiments. Because of sex-related differences in susceptibility to *S. aureus* (Yanke et al., 2000), only female mice were used in this study. This study was performed under strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. CSMC is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), and in compliance with NIH guideline of laboratory animal care and use. The protocol was approved by the institutional animal use and care committee of the Cedars-Sinai Medical Center.

METHOD DETAILS

***S. aureus* and GAS reinfection models—**Reinfection (two-infection) model - Mice were inoculated with *S. aureus* or GAS via intra-peritoneal injection. After 21 days, the mice were reinfected i.p. with the same inoculum of *S. aureus* or GAS unless indicated otherwise. Mice were killed 1–2 days after reinfection and spleen and kidneys were harvested, homogenized and plated on blood agar plates. CFU were counted after overnight incubation at 37°C.

Multiple reinfection model (WT SA113 and *oatA* SA113) – Mice were infected with 2×10^7 CFU of WT *S. aureus* (SA113) or with 10^7 SA113 + 10^7 SA113 *oatA* every 7 days for a total of 3 reinfections. Seven or ten days after the last infection, mice were challenged 2×10^7 WT SA113 *S. aureus*. After 1–2 days, the mice were sacrificed and spleen and kidneys were harvested for further analyses. For the multiple reinfection experiment performed in Figure 1 using *S. aureus* LAC, the experiment was performed as described in the figure legend.

Adoptive Transfer of CD4+ T cells - For adoptive transfer experiments, 5×10^6 CD4$^+$ T cells were injected i.v. into recipient mice.

Mice were divided into cages by comparative medicine staff with no involvement in study design, and cages were randomly assigned to experimental groups. The investigators were not blinded to the group allocation during the experiment or when assessing the outcome.

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ELISA and flow cytometry—Enzyme linked immunosorbent assays (ELISA) were performed according to the manufacturers' instructions.

FACS analysis – Cells were incubated in FACS buffer (PBS containing 3% FBS and 0.1% sodium azide) in the presence of purified neutralizing monoclonal antibodies against CD16:CD32 (Fc Block; eBiosciences) for 20 minutes at 4°C prior to staining. Specific antibodies (1:100 dilution) in FACS buffer were then added for 30 min at 4°C in the dark. Samples were then analyzed using a FACS LSRII Cytometer or Fortessa (BDbiosciences) and FlowJo 7.6.1 software (TreeStar, Co).

For intracellular cytokine staining, cell suspensions were incubated in ice-cold PBS supplemented with 3% (v/v) FBS cells containing anti-TCRβ and anti-CD4, and then fixed and resuspended in permeabilization buffer containing anti–IFN-γ or anti-IL-17A antibody. Data were acquired on a LSRII Flow cytometer and analyzed with FlowJo Software (Treestar, Ashland, OR).

Ex vivo stimulation of splenocytes—Splenocytes were isolated at indicated time points after infection. Red blood cells were lysed and leukocytes were resuspended in RPMI 1640 media containing Pen/Strep and supplemented with 10% FBS. For specific restimulation, 5×10^5 cells were cultured with heat-killed *S. aureus* or GAS at an MOI of 10. Supernatants were collected after 48–72 h for analysis of cytokines by ELISA. For intracellular cytokine analysis, the splenocytes were stimulated with phorbol 12-myristate 13-acetate (500 ng/ml), ionomycin (50ng/mL), and GolgiStop for 4 h. Intracellular cytokines were then analyzed by flow cytometry.

BMDC stimulation and DC-TC co-culture assays—BMDC were plated in 48-well plates at a density of 2×10^5/ml and infected with *S. aureus* or GAS at the indicated MOI. Infected DC were incubated for 1 h in antibiotic-free medium, washed twice to remove unbound bacteria and further incubated in RPMI media supplemented with 10% FBS and 100 μg/ml of Gentamicin for 12 hr. After washing, cells were resuspended in fresh media containing 100 μg/ml Gentamicin and further incubated for 6 h with 5μg/mL of OVA peptide. $CD4^+$ T cells were isolated from spleen and lymph nodes of OT-II mice using a negative selection kit (STEMCELL). Cells were labeled with CFSE and added (1×10^6/well) to the DCs and then incubated for up to 7 days at 37°C.

RNA isolation and quantitative Reverse Transcription PCR—BMDC were infected with bacteria at an MOI of 3 for 1hr, and washed with gentamicin containing media to kill the extracellular. Total RNA was isolated using an RNeasy Mini-kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Briefly, at 24 hr, cells were centrifuged and lysed in RLT lysis buffer (Qiagen) with 10μl/mL of 2-ME. RNA was isolated following manufacturer's instructions. The concentration and the RNA integrity number were measured using an ND-1000 spectrophotometer (NanoDrop Technologies) and a Bioanalyzer, and only RNA samples with a concentration >1μg and a 260/280 ratio 2.0 were retained for further analysis. Total RNA was treated with DNase (Life Technologies) and used as template for cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Invitrogen).

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Library Construction and RNA Sequencing—Total RNA was extracted as described for RT-QPCR analysis above. mRNA was purified from one microgram of total RNA using the Ambion Dynabeads® mRNA DIRECT™ Micro Purification Kit (Austin, TX) per manufacturer's recommendations. Purified mRNA was fragmented, adapters ligated, reverse transcribed to make cDNA, and cDNA amplified with Ion Total RNA-Seq Kit v2, Ion Torrent™, per manufacturer's recommendations. Samples were individually barcoded using Ion Xpress™ RNA-Seq Barcode 1–16 Kit, Ion Torrent™, before their cDNA was amplified. RNAseq libraries were assessed for concentration and length using Invitrogen Qubit® dsDNA HS Assay Kit (Carlsbad, CA) and Agilent DNA 1000 Kit respectively (Carlsbad, CA). Samples were multiplexed to obtain 10 million reads each for sequencing. The pooled libraries were amplified onto Ion Sphere™ particles using Ion PI™ Template OT2 200 Kit, Ion Torrent™ per manufacturer's recommendations. Ion Sphere™ particles were purified per manufacturer's recommendations and prepared for sequencing using the Ion PI™ Sequencing 200 Kit, Ion Torrent™ per manufacturer's recommendations. Sequence data was then pre-processed using the Torrent Suite software and the FASTX-toolkit (Hannon laboratory, CSHL). Quality control was performed using FastQC.

Sequencing data analysis—Single-end RNA-seq reads were aligned to the transcriptome of mouse genome GRCm38/mm10 using the Kallisto package with the quant command. An estimated average fragment size of 200 bp was used, with an estimated standard deviation of 20 bp. Sequence based bias correction was also performed during alignment and quantification of transcripts. For each sample, BAM files, containing all the alignments, were obtained along with the estimated counts for each transcript; these were then summed up for each gene. The genic counts for all the samples were assembled, quantile normalized, before getting the counts per million to account for the library sizes. These normalized counts were log2 transformed, then input to the R package limma's lmFit function - and using empirical Bayes methods to borrow information between genes along with correcting for mean-variance trend - to get Differentially Expressed Genes (DEGs) for SA vs NS (*S. aureus* vs Non Stimulated) and GAS vs NS. The p-values obtained using t-tests were adjusted using the Benjamini-Hochberg procedure. Log 2 Fold Changes (FCs) were also returned, with positive values indicating over-expression compared to the Non Stimulated condition, and negative values indicating under-expression with SA or GAS. DEGs were called using adjusted p-value < 0.05 and actual FCs > 2 in either direction.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are expressed as mean ± standard error (SEM). We assumed a Gaussian distribution for our data. Accordingly, statistical significance was determined by two tailed unpaired *t*-test or 1-way ANOVA with Bonferroni correction for multiple comparisons. In vivo experiments frequently follow a non-Gaussian distribution. Therefore, in vivo experiments were analyzed using non-parametric Mann–Whitney U-test or Kruskal-Wallis test in the case of missing normality. Log rank test was used for analysis of mouse survival. With few exceptions, all in vitro studies were done with at least three sets of independent experiments. GraphPad Prism or Excel were used for all analyses. All statistical details of experiments including the number of replicates can be found in the figure legends. p values less than 0.05 are considered significant.

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DATA AVAILABILITY

RNAseq data has been submitted to the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=qfcjueckdzyxnuv&acc=GSE96086).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- Protective T cell memory is not induced after systemic *S. aureus* infections
- *S. aureus* induces proliferation but limits polarization of Th1 and Th17 cells
- O-acetylation of peptidoglycan represses cytokines required for Th17 polarization
- Adjuvancy with *oatA S. aureus* promotes protective memory to reinfection

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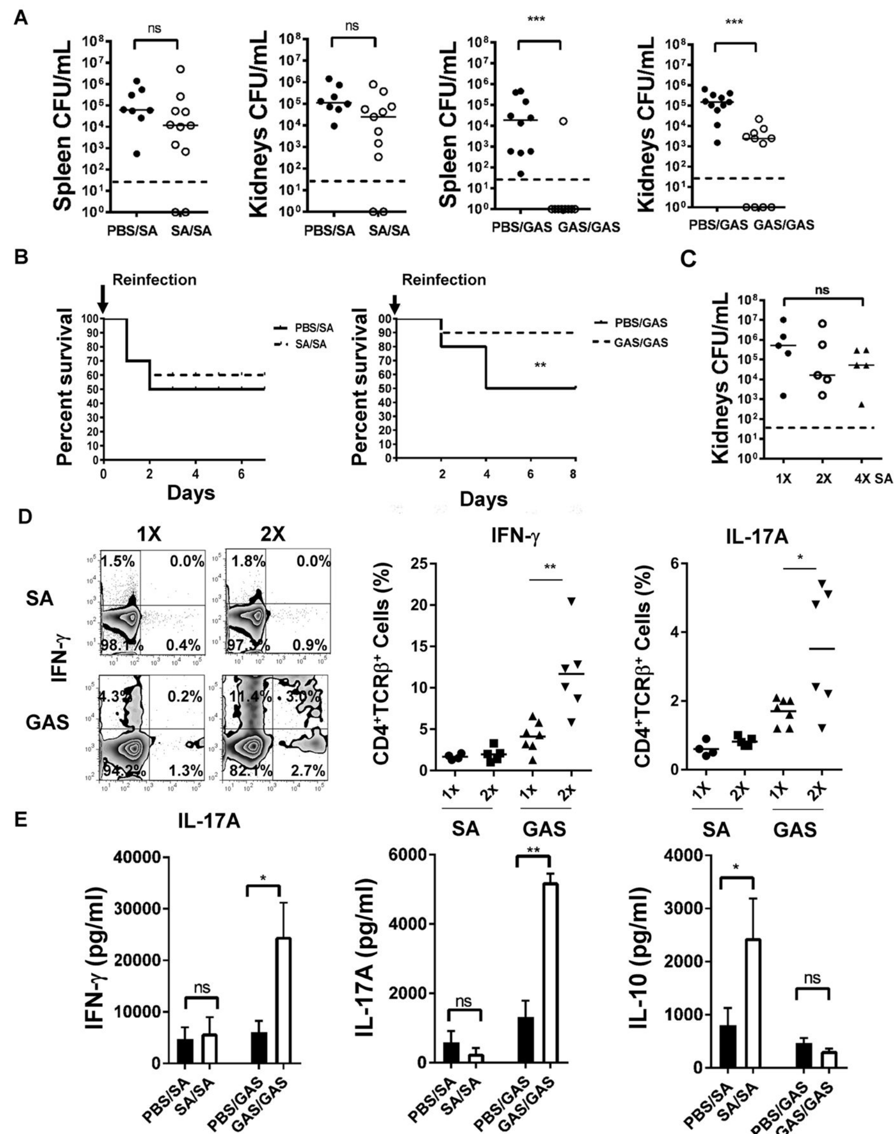

Figure 1. Prior *S. aureus* infection does not induce protective immunity against reinfection

(A) C57BL/6 mice (n=8–11) were injected i.p with 10^7 CFU of *S. aureus* (LAC USA300) GAS (M49) or PBS on d0, and re-injected on d21 with 10^7 CFU of either *S. aureus* or GAS. Bacteria from spleen and kidneys were recovered after 24hr. PBS/SA: injected with PBS on d0 and then injected with SA on d21. Dashed lines indicate limit of detection.

(B) C57BL/6 mice (n=10) were injected i.p with *S. aureus* (10^7), GAS (5×10^6), or PBS. The mice were inoculated after 21 days with *S. aureus* (4×10^7) or GAS (2×10^7) and monitored for survival.

(C) C57BL/6 mice (n=5) were infected i.p. once, twice (d0 and 21), or four times (d0, 21, 28 and 35) with *S. aureus* (10^7). Surviving CFU were enumerated 24hr after the last infection.

(D) Mice (n=5–6) were infected as in (A). Splenocytes were harvested 7 days after the last infection, stimulated with PMA, ionomycin and brefeldin A for 4hr and stained for surface CD4 and TCRβ, and intracellular IFN-γ and IL-17A. Shown are FACS plots and graphs of

IFN-γ and IL-17A expression by $CD4^{+}TCR\beta^{+}$ cells. Results are representative of 2 experiments.

(E) Mice (n=8–11) were infected as in (A) and splenocytes harvested 7 days after the last infection were stimulated with heat-killed *S. aureus* or GAS (MOI=10) for 48hr. Supernatants were analyzed by ELISA. Data are representative of 2 experiments. See also Figure S1. For (A), (C) and (D), each data point represents an individual mouse, bars denote median and dashed lines indicate the limit of detection.

For (E), data are plotted as mean ± SEM. Data analysis was performed using Mann-Whitney U-test for (A) and (D), ANOVA for (C), log rank test for (B) and Student's t-test for (E). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

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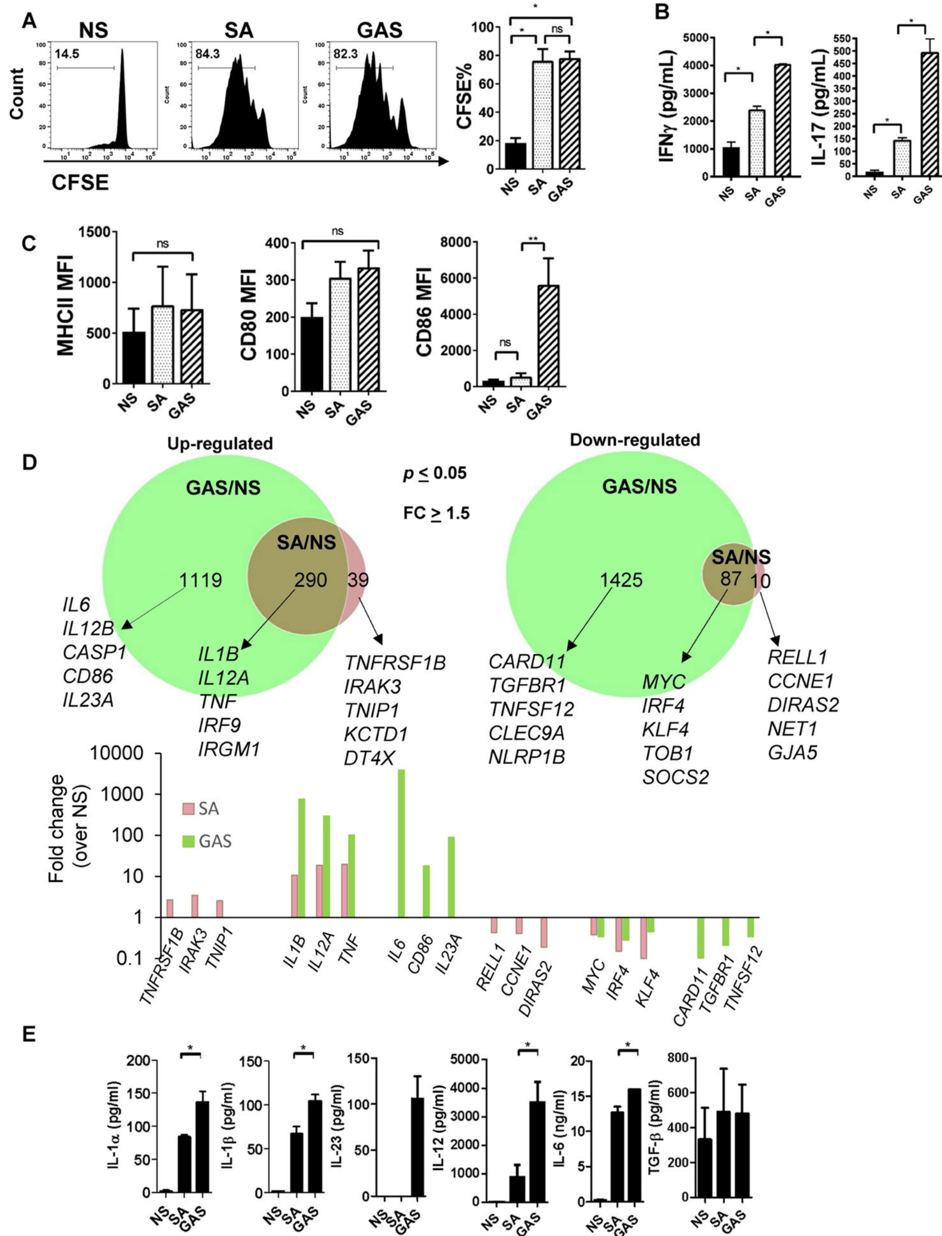

Figure 2. *S. aureus* induces suboptimal polarization of TH1 and TH17 cells associated with differential regulation of gene expression in DC

(A)–(B) BMDC were cultured with medium only (NS – Non stimulated), *S. aureus* (LAC) or GAS (MOI=3) for 1hr, pulsed with OVA peptide (6hr) and then mixed with CFSE-labeled OTII transgenic $CD4^+$ T cells. (A) CFSE dilution in gated $CD4^+$ T cells at day 7 post stimulation and (B) IFN-γ and IL-17A in the culture supernatants after 7 days.

(C) MHCII, CD80 and CD86 expression on BMDC after 24hr of stimulation with GAS or *S. aureus* (MOI=10).

(D) Bone marrow-derived DC were left without any stimulation (NS) or incubated with *S. aureus* or GAS (MOI=3) for 8 hours and total RNA was extracted for RNAseq analysis. Top graph: Venn diagrams showing genes up- (left) or down- (right) regulated by *S. aureus* only

(pink), GAS only (green) or both GAS and *S. aureus* (brown). Selected genes are listed for each group. Bottom graph: Fold change (over NS) for selected genes shown in (D).
(E) Cytokine production as measured by ELISA in the supernatant of DC treated as in (D) for 24 hours. All data are representative of at least 3 experiments except for the IL-23 ELISA which was performed twice. Data are shown as mean ± SEM and data analysis was performed using ANOVA. *p<0.05; **p<0.01. See also Figure S2 and Table S1 and S2.

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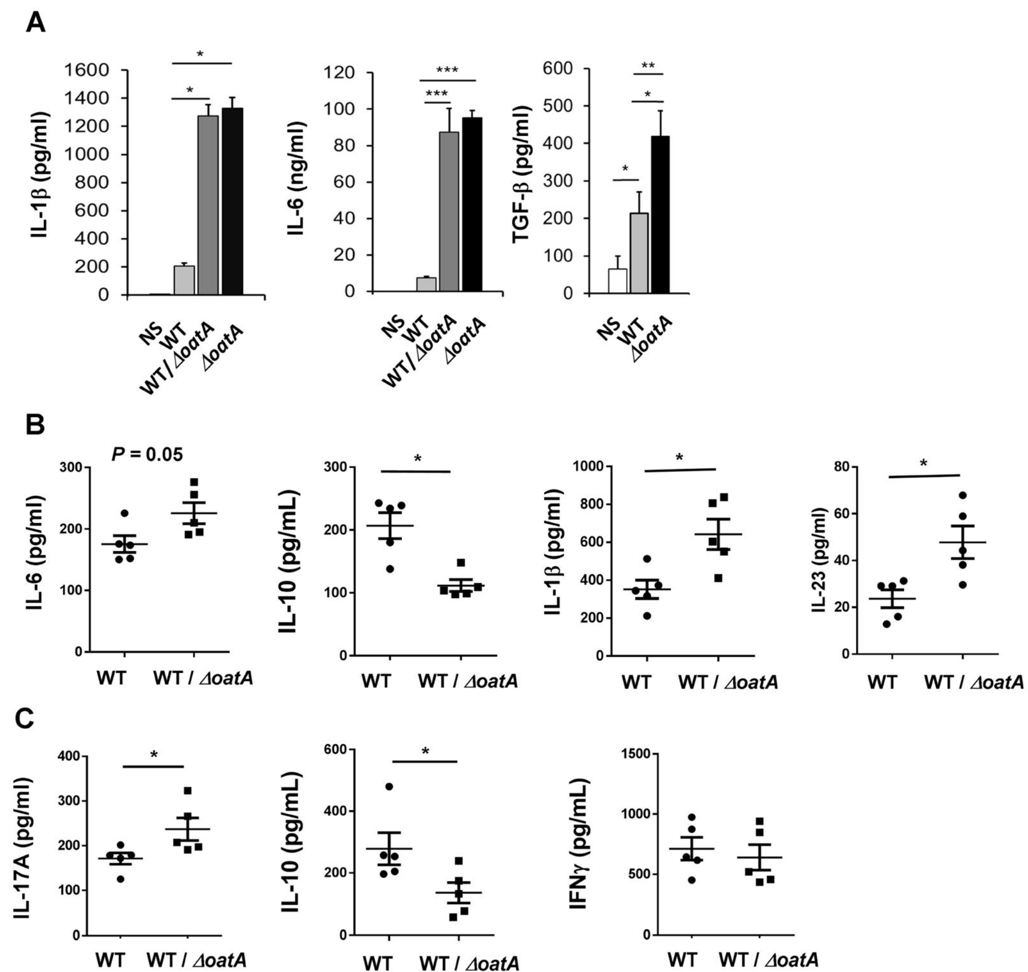

Figure 3. O-acetylation of *S. aureus* PGN limits the induction of pro-inflammatory cytokines critical for protective memory

(A) BMDC were stimulated with WT (*S. aureus* SA113), *oatA* (isogenic SA113 mutant) or 1:1 mixture of WT and *oatA* at MOI of 10. Supernatants were harvested at 24h for cytokine determination. Data are representative of 3 experiments. See also Figure S3.

(B)–(C) Mice (n=5) were infected with WT *S. aureus* (2×10^7) or WT + *oatA* (10^7 CFU of each) at d0, 7 and 14. (B) Two days after the last infection, splenocytes were harvested from the mice for determination of cytokine levels. (C) Seven days after the last infection, splenocytes were harvested and stimulated with heat-killed WT *S. aureus* and cytokines were measured after 3d.

For (B) and (C), each data point represents an individual mouse, and mean ± SEM are shown for all data. Data analysis was performed using Student's t-test (A) and using Mann-Whitney U test for (B) and (C). *$p<0.05$, ***$p<0.001$.

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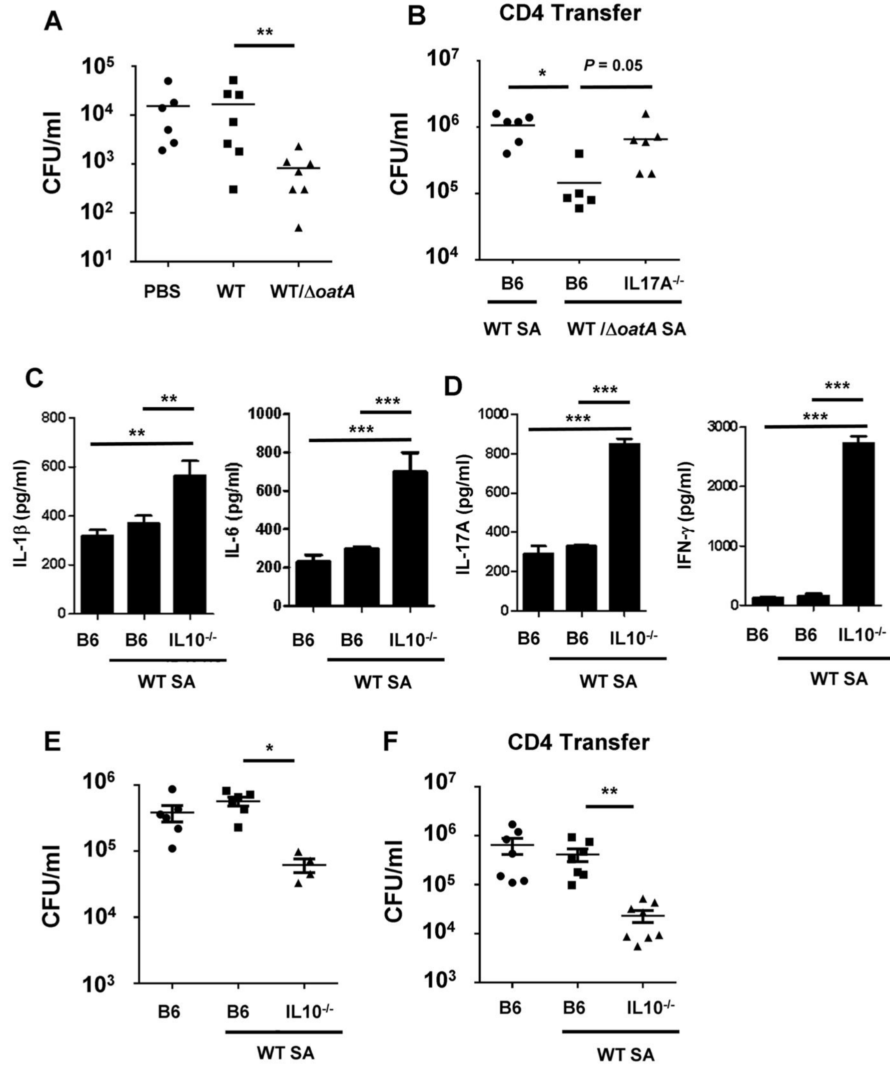

Figure 4. Adjuvancy with oatA or absence of IL-10 promotes development of protective immunity to *S. aureus* reinfection

(A) Mice (n=6–7) were injected with PBS or infected 3 times as in (3B). Ten days after the last injection, all mice were inoculated with WT SA113 *S. aureus* (2×10^7). CFU from the kidneys were enumerated after 24h. The data are representative of two experiments.

(B) WT or IL-17A$^{-/-}$ mice were infected 3 times with *S. aureus* as in (3B). Seven days after the last infection, splenocytes were harvested and 5×10^7 purified total CD4$^+$ T cells were transferred i.v. into naïve mice (n=5–6). The recipient mice were infected with WT *S. aureus* (2×10^7) the next day and kidney CFU were enumerated after 24h. See also Figure S4.

(C)–(E) WT or IL-10$^{-/-}$ mice (n=4–6) were infected with WT *S. aureus* (SA113) three times at 7 day intervals. A non-pretreated group served as a control. Seven days after the last pretreatment, all groups of mice were inoculated with WT *S. aureus* (2×10^7). (C) Spleen cytokine levels 24h after the last infection. (D) Splenocytes harvested 24h after the last infection were stimulated with heat-killed *S. aureus* (MOI=3), and cytokines in the supernatants were assessed after 3d. (E) CFU from splenocytes harvested 24hr after the last infection.

(F) WT or IL-$10^{-/-}$ mice were infected 3 times with *S. aureus* SA113 as in (3B). Seven days after the last infection, splenocytes were harvested and 5×10^7 purified total $CD4^+$ T cells were transferred i.v. into naïve mice (n=5–6). The recipient mice were infected with WT *S. aureus* (2×10^7) the next day and kidney CFU were enumerated after 24h.

For (A), (B), (E) and (F), each data point represents an individual mouse and mean or mean ± SEM are shown. Data analysis was performed using ANOVA. *$p<0.05$, **$p<0.01$.

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