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# T and B Cells Are Not Required for Clearing *Staphylococcus aureus* in Systemic Infection Despite a Strong TLR2–MyD88-Dependent T Cell Activation

Mathias Schmalzer, Naja J. Jann, Fabrizia Ferracin, and Regine Landmann

*Staphylococcus aureus* infection elicits through its mature lipoproteins an innate immune response by TLR2–MyD88 signaling, which improves bacterial clearing and disease outcome. The role of dendritic cells (DCs) and T cells in this immune activation and the function of T and B cells in defense against *S. aureus* infection remain unclear. Therefore, we first evaluated DC and T cell activation after infection with *S. aureus* wild type (WT) and its isogenic mutant, which is deficient in lipoprotein maturation, in vitro. Lipoproteins in viable *S. aureus* contributed via TLR2–MyD88 to activation of DCs, which promoted the release of IFN- $\gamma$  and IL-17 in CD4<sup>+</sup> T cells. This strong effect was independent of superantigens and MHC class II. We next evaluated the function of T cells and their cytokines IFN- $\gamma$  and IL-17 in infection in vivo. Six days after systemic murine infection IFN- $\gamma$ , IL-17, and IL-10 production in total spleen cells were MyD88-dependent and their levels increased until day 21. The comparison of CD3<sup>-/-</sup>, Rag2<sup>-/-</sup>, and C57BL/6 mice after infection revealed that IFN- $\gamma$  and IL-17 originated from T cells and IL-10 originated from innate immune cells. Furthermore, vaccination of mice to activate T and B cells did not improve eradication of *S. aureus* from organs. In conclusion, *S. aureus* enhances DC activation via TLR2–MyD88 and thereby promotes T<sub>H</sub>1 and T<sub>H</sub>17 cell differentiation. However, neither T cells and their MyD88-regulated products, IFN- $\gamma$  and IL-17, nor B cells affected bacterial clearing from organs and disease outcome. *The Journal of Immunology*, 2011, 186: 443–452.

Successful immune defenses against pathogens results from an immediate innate and a long-lasting specific response, both processes are tightly connected. Dendritic cells (DCs) are indispensable for the initiation and orchestration of adaptive immunity (1, 2). In peripheral organs, immature DCs phagocytose invading pathogens and become concurrently activated through pattern recognition receptors (PRRs) that sense conserved patterns on the microorganism. These pathogen-associated molecular patterns induce maturation of DCs, including upregulation of MHC class II and costimulatory surface molecules, switching of chemokine receptors, and production of inflammatory and anti-inflammatory cytokines (3). Mature DCs activate T cells in lymphoid organs (4) and promote the differentiation of CD4<sup>+</sup> T cells into T<sub>H</sub>1, T<sub>H</sub>2, and T<sub>H</sub>17 cells.

PRR expression in myeloid DCs includes all surface and intracellular TLRs (5–7). TLRs elicit signaling through MyD88 or TRIF proteins, leading to activation of NF- $\kappa$ B and other transcription factors (8) with subsequent upregulation of surface molecules and production of mediators. TLRs have a potent influence on the quality of T<sub>H</sub> responses, but mainly the cytokines secreted by activated DCs program the differentiation of newly primed CD4<sup>+</sup> T cells into T<sub>H</sub>1, T<sub>H</sub>2, or T<sub>H</sub>17 and regulatory T cells.

*Staphylococcus aureus* is one of the most important pathogens causing severe systemic infections such as endocarditis or sepsis. Several staphylococcal molecules are known to act as pathogen-associated molecular patterns for TLRs. Infections of cells and mice with *S. aureus* revealed that lipoproteins trigger the TLR2 signaling cascade, which is required for early activation of the innate immune system (9–12). TLR2 signals exclusively through MyD88, and cells lacking MyD88 produce no or low levels of cytokines after recognition of *S. aureus* (10, 13, 14). The failure of inflammatory defense appears responsible for the increased susceptibility of these mice to staphylococcal infections (10, 13–16).

Besides the unknown spectrum of *S. aureus*-activated PRRs in DCs, data on the skewing of an *S. aureus*-induced T<sub>H</sub> cell response are controversial. One group found a T<sub>H</sub>2 response after stimulation with staphylococcal enterotoxin B through a TLR2-dependent recognition (17), but other studies reported a beneficial effect of T<sub>H</sub>1 responses by depleting CD4<sup>+</sup> T cells or using mice deficient in IFN- $\gamma$ R, IL-4, and IL-10 (18–20). In addition, DCs activated by *S. aureus* peptidoglycan (PGN) promote IL-17 production in memory T<sub>H</sub> cells, possibly by amplification of TLR2-induced IL-23 and IL-1 by NOD2 (21). In the absence of IL-17, mice were more often colonized with *S. aureus* (22) and showed impaired clearance of cutaneous *S. aureus* infection (23). Nevertheless, IL-17 might also be detrimental to the host, because it is known to recruit neutrophils (22), which were found to be attracted by *S. aureus* that were able to reside in infected organs and survive, although the adaptive immune response with IFN- $\gamma$  production was elicited (24).

In this study, we examined the pathway of *S. aureus*-induced DC activation and of the subsequent T cell differentiation. We used *S. aureus* strain Newman and an isogenic mutant that was deficient in mature lipoproteins to evaluate their contribution to TLR2 activation. Our results demonstrate that lipoprotein–TLR2–MyD88 signaling in DCs was required to initiate the production of IFN- $\gamma$  and IL-17 in naive T cells in vitro, whereas only MyD88 was

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Abbreviations used in this paper: DC, dendritic cell; MFI, mean fluorescence intensity; MHC II, MHC class II; MOI, multiplicity of infection; Pen/Strep, penicillin/streptomycin; PGN, peptidoglycan; PRR, pattern recognition receptor; TSB, tryptic soy broth; WT, wild type.

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required for cytokine production during systemic infection. We further demonstrate that innate immune cells produce IL-10 *in vivo*, whereas T cells are the major producers of IFN- $\gamma$  and IL-17. However, the absence of T cells, B cells, or both did not exacerbate either the severity of the disease or the bacterial burden of *S. aureus*-infected mice. We propose that lipoproteins from *S. aureus* activate innate immunity in a TLR2–MyD88-modulated manner, which in turn promotes strong but inefficient T<sub>H</sub>1 and T<sub>H</sub>17 responses *in vivo*.

## Materials and Methods

### Mice

Wild type (WT) inbred C57BL/6, TLR2<sup>-/-</sup>, MyD88<sup>-/-</sup>, Rag2<sup>-/-</sup>, CD3<sup>-/-</sup>, and OT-1 mice were bred in the Animal House of the Department of Biomedicine, University Hospital of Basel, according to the regulations of Swiss veterinary law. MyD88<sup>-/-</sup> mice were a gift from W.D. Hardt (Institute of Microbiology, Swiss Federal Institute of Technology, Zürich, Switzerland), OT-1 mice were from E. Palmer (Department of Biomedicine, University Hospital of Basel), CD3<sup>-/-</sup> mice were from A. Rolink (Department of Biomedicine, University Hospital of Basel) and F. Sallusto (Institute for Research in Biomedicine, Bellinzona, Switzerland); all were backcrossed on C57BL/6 background for 10 generations.

### Abs and reagents

mAbs recognizing CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), CD11b (M1/70), CD11c (N418), CD16/32 (93), CD19 (6D5), CD40 (1C10), CD44 (IM7), CD45R/B220 (RA3-6B2), CD49/Pan-NK (DX5), CD62L (MEL-14), CD69 (H1.2F3), CD80 (16-10A1), CD86 (GL-1), F4/80 (BM8), Gr-1 (RB66-8C5), IFN- $\gamma$  (XMG1.2), NK1.1 (PK136), MHCII (M5/114.15.2),  $\gamma\delta$  TCR (GL-3), and the fixation and permeabilization kit with BD GolgiStop were purchased from Biolegend (San Diego, CA), eBioscience (San Diego, CA), or BD Pharmingen (San Diego, CA). Fluorochrome-conjugated streptavidins were purchased from CALTAG laboratories. Normal rabbit serum was provided by the Animal Care Unit at the University Hospital of Basel. Specificity of staining was confirmed with isotype-matched control Abs.

Pam<sub>3</sub>CSK<sub>4</sub> were purchased from EMC Microcollections (Tübingen, Germany), and smooth LPS from *Streptococcus equi* abortus was provided by Marina Freudenberg (Max-Planck Institute for Immunobiology, Freiburg, Germany). Sandwich ELISAs were purchased from BD Biosciences and R&D Systems (Minneapolis, MN). Phorbol 12-myristate 13-acetate and ionomycin were purchased from Sigma-Aldrich (St. Louis, MO).

### Bacterial strains and growth conditions

In this study, we used Newman WT (25) and SA113 WT (ATCC 35558) strains and their isogenic *lgt:ermB* ( $\Delta$ *lgt*) mutants (10, 26). Bacteria were grown in tryptic soy broth (TSB) for 7 h and subcultured overnight under the same conditions. Overnight cultures were washed in 0.9% NaCl (Bichsel, Interlaken, Switzerland) and used for *i.v.* or heat-killed for 1 h at 90°C for *i.p.* injection. Each inoculum was assessed by CFUs per milliliter counting on Mueller-Hinton agar plates. For infection of DCs, bacteria were subcultured further in TSB to the end-log phase. When appropriate, TSB was supplemented with 10  $\mu$ g/ml erythromycin.

### Generation of DCs

DCs were generated as described previously (27, 28). Bone marrow cells from tibia and femur were flushed with RPMI 1640 medium. RBCs were lysed and CD11b<sup>+</sup> bone marrow DCs were generated by plating of bone marrow progenitors in RPMI 1640 supplemented with gentamicin, 2-ME (Invitrogen, Basel, Switzerland), 10% (v/v) heat-inactivated FBS (Life Technologies, Carlsbad, CA) and 10% conditioned medium from GM-CSF transduced X63 (29). After 3 d, nonadherent cells were removed, and attached cells were cultured further in supplemented RPMI 1640 medium. On days 7 and 8, cells were harvested and analyzed for CD11c<sup>+</sup> expression, which was routinely 80–90% positive.

### Stimulation of DCs

For analysis of cytokine production in supernatants and surface marker expression, 1  $\times$  10<sup>6</sup> DCs per well were cultured in 24-well plates in 1 ml RPMI 1640 containing GM-CSF. DCs were stimulated with smooth LPS (LPS, 1  $\mu$ g/ml) or Pam<sub>3</sub>CSK<sub>4</sub> (10  $\mu$ g/ml). When stimulated with *S. aureus*, DCs were infected with viable *S. aureus* strains at a multiplicity of

infection (MOI) of 10 bacteria per DC for 1 h. After phagocytosis, supernatants were replaced with RPMI 1640 containing 100  $\mu$ g/ml gentamicin, and cells were incubated further. Supernatants were collected 18 h postinfection or incubation with various stimuli, and cells were analyzed for the expression of surface markers by flow cytometry. Cytokines in the supernatants were analyzed by sandwich ELISA per the manufacturer's instructions (BD Biosciences).

### Phagocytosis of *S. aureus* by DCs

DCs (1  $\times$  10<sup>6</sup> cells) were infected for 1 h with *S. aureus* at an MOI of 10. For phagocytosis, extracellular bacteria were killed with 50 U lysostaphin, and cells were lysed with water (pH 11) to assess intracellular bacteria by plating serial dilutions on Mueller-Hinton agar plates.

### *In vitro* T cell activation and differentiation

CD4<sup>+</sup> or CD8<sup>+</sup> T cells from spleen of C57BL/6 background were purified by negative selection by MACS beads (Miltenyi Biotec, Auburn, CA). DCs (1  $\times$  10<sup>4</sup>) were infected with *S. aureus* at an MOI of 10, and bacteria were killed by penicillin/streptomycin (Pen/Strep) and gentamicin 1 h postinfection. DCs were cocultured further with purified T cells (5  $\times$  10<sup>4</sup>) for 72 h in RPMI 1640 without GM-CSF. Supernatants were analyzed by sandwich ELISA and T cells were analyzed by flow cytometry for expression of T cell markers, CD69, or proliferation after CFSE staining.

### *In vitro* stimulation of splenocytes

Spleen cells from infected mice were isolated at indicated time points after injection and mechanically disrupted (70- $\mu$ m meshes) followed by lysis of erythrocytes and resuspension in RPMI 1640. In 96-well plates, 2  $\times$  10<sup>5</sup> cells were infected for 1 h with the same viable bacteria as was used for *in vivo* infection at an MOI of 1. Bacteria were killed with Pen/Strep and gentamicin to prevent bacterial growth. Supernatants were collected after 48 h, and cytokines were analyzed by sandwich ELISA. In some experiments, spleen cells were restimulated with heat-killed *S. aureus* (MOI 1) for 12 h following treatment with phorbol 12-myristate 13-acetate (20 ng/ml), ionomycin (10  $\mu$ M), and GolgiStop for 4 h; intracellular cytokines were analyzed by flow cytometry.

### Flow cytometry

Cell suspensions were stained in ice-cold PBS supplemented with 0.04% (v/v) FCS and 25 mM sodium azide for surface staining. For intracellular cytokine staining, cells were stained with anti-CD3, anti-CD4, anti-CD44, and anti-CD69, and then fixed and resuspended in permeabilization buffer (BD Biosciences) containing anti-IFN- $\gamma$ . Data were acquired on a CyAN Flow cytometer (Dako, Glostrup, Denmark) or FACS Canto II (BD Biosciences) and analyzed with FlowJo Software (Treestar, Ashland, OR).

### Infection of mice with *S. aureus*

Mice were infected *i.v.* with 1  $\times$  10<sup>5</sup> to 1  $\times$  10<sup>6</sup> CFU of *S. aureus* Newman and SA113. In some experiments, mice were vaccinated *i.p.* with 5  $\times$  10<sup>7</sup> CFU of heat-killed *S. aureus* Newman 7 d before *i.v.* challenge with viable bacteria. Weight loss in mice was monitored after infection, and on indicated days mice were killed and organs were collected for determining bacterial load and isolation of cells. Cytokines in plasma were analyzed by ELISA.

### Statistical analysis

Statistical analyses of data were performed using Prism (GraphPad, San Diego, CA). Data for bacterial load, cytokines, or surface molecules were analyzed using either one-way ANOVA and post hoc Bonferroni, and Kruskal Wallis with post hoc Dunn tests, or Mann-Whitney U test. Weight loss was analyzed with two-way ANOVA. The *p* values <0.05 are shown when differences between the compared data sets are significant.

## Results

### *S. aureus* activates TLR2–MyD88 to enhance maturation of myeloid DC

We first investigated whether *S. aureus* induces DC activation *in vivo*. To that aim, we quantified and characterized DCs recruited to the peritoneum after *i.p.* injection of heat-killed *S. aureus* Newman into C57BL/6 mice. Seven days after administration we found 47.67  $\pm$  1.45% CD11b<sup>+</sup>CD11c<sup>+</sup> DC, 14.97  $\pm$  3.68%

CD11b<sup>+</sup>CD11c<sup>-</sup> monocytic cells,  $6.81 \pm 1.3\%$  CD3<sup>+</sup>CD4<sup>+</sup> T cells,  $7.62 \pm 0.56\%$  CD3<sup>+</sup>CD8<sup>+</sup> T cells,  $10.77 \pm 0.9\%$  CD3<sup>-</sup>NK1.1<sup>+</sup> cells and  $17.57 \pm 0.67\%$  other cells in the peritoneal lavage. DCs expressed high levels of costimulatory surface molecules including CD86 ( $19.37 \pm 3.97$ ) and CD40 ( $37.3 \pm 6.58$ ). Subsequently, we addressed the question whether *S. aureus* supports activation of myeloid DCs in vitro and whether this activation requires TLR2 and MyD88 signaling as shown for staphylococcal PGN (30). Therefore, we generated DCs by GM-CSF stimulation of bone marrow cells from C57BL/6, TLR2<sup>-/-</sup>, and MyD88<sup>-/-</sup> mice; 80–90% of the cells were immature and positive for CD11b<sup>+</sup> and CD11c<sup>+</sup> (DCs; data not shown).

After *S. aureus* Newman infection of C57BL/6 DCs, surface expression of MHC class II, CD40, CD80, and CD86 were significantly increased in comparison with unstimulated cells ( $p < 0.01$ ; Fig. 1A). TLR2 and MyD88 were not required for changes of MHC class II expression, but were indispensable for surface CD40, CD80, and CD86 upregulation (Fig. 1A). Treatment with LPS enhanced surface molecules in a TLR2–MyD88-independent manner (Fig. 1A) as reported previously (31).

Complete activation of DCs includes the release of cytokines after pathogen recognition (32). *S. aureus* Newman infection of C57BL/6 DCs induced TNF, IL-1 $\beta$ , IL-6, IL-10, and IL-12 release in comparison with unstimulated cells (black bars; Fig. 1B). Stimulation of TLR2<sup>-/-</sup> and MyD88<sup>-/-</sup> DCs with *S. aureus* Newman revealed that TLR2–MyD88 signaling is required for the induction of cytokines (Fig. 1B). As expected C57BL/6 and TLR2<sup>-/-</sup> DCs, but not MyD88<sup>-/-</sup> DCs, released cytokines after stimulation with LPS (Fig. 1B). Cytokine release by DCs was related to signaling and not to numbers of intracellular *S. aureus* Newman, because immature DCs from all mice phagocytosed viable *S. aureus* Newman to similar numbers (Fig. 1C). Our data indicate that DCs

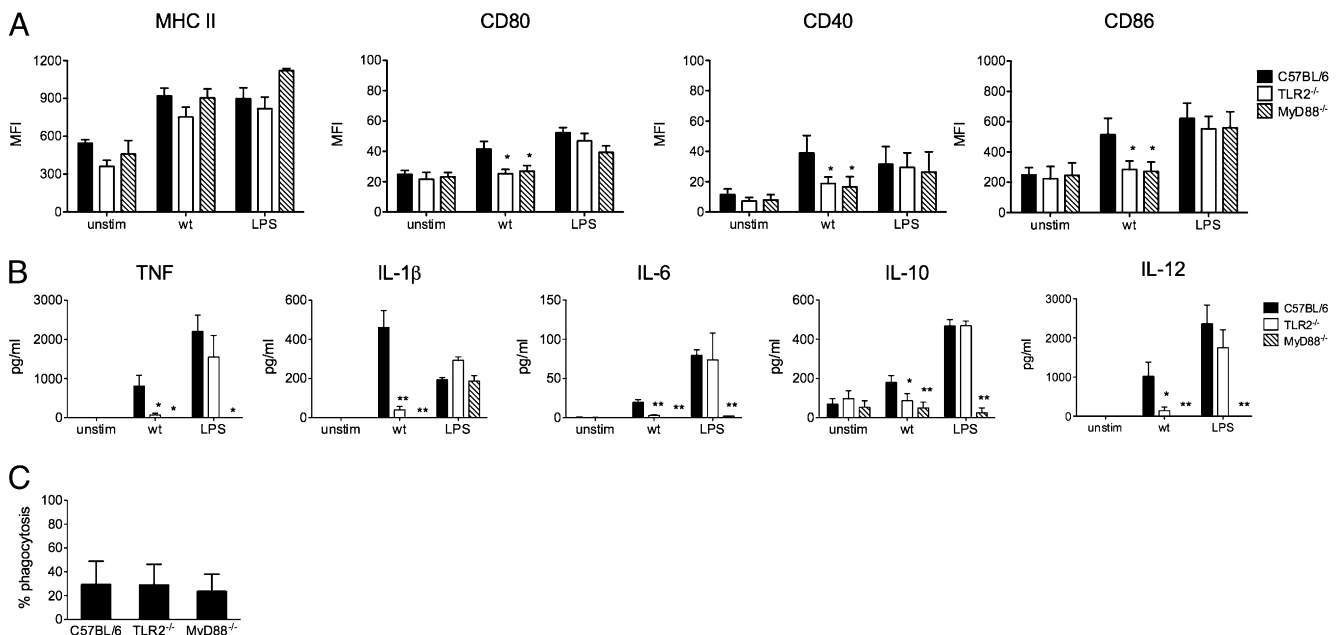
are activated mainly via TLR2–MyD88 signaling during *S. aureus* infection.

#### Lipoproteins trigger TLR2–MyD88 signaling

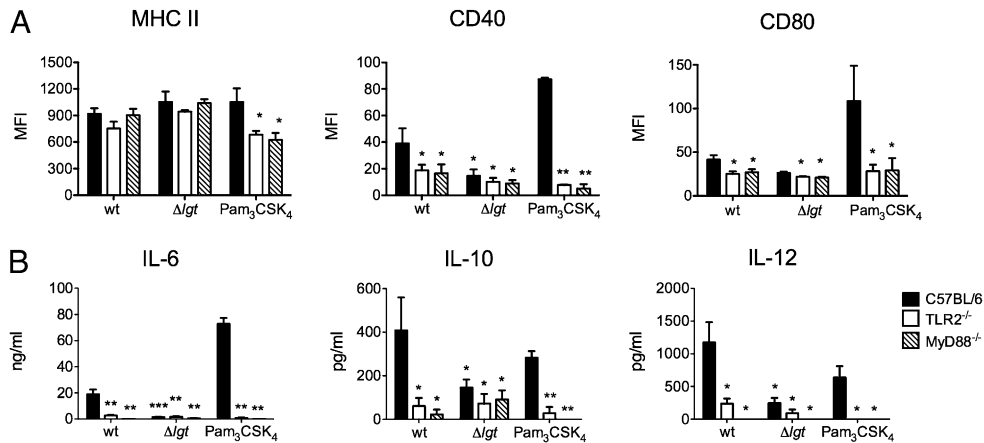
TLR2 in conjunction with CD14 and CD36 recognizes different ligands from *S. aureus*, including lipoproteins, lipoteichoic acid, and PGN or contaminants in it (33–37). We hypothesize that lipoproteins are the major stimuli of TLR2 in DCs, as reported previously for macrophages (10). Therefore, DCs were infected with *S. aureus* Newman WT and with a mutant deficient in lipoprotein maturation ( $\Delta lgt$ ). We found that staphylococcal lipoproteins in viable *S. aureus* Newman or the TLR2–TLR1 ligand Pam<sub>3</sub>CSK<sub>4</sub> enhanced expression of surface comolecules, as shown for CD40 and CD80 (Fig. 2A), and enhanced IL-6, IL-10, and IL-12 levels after *S. aureus* Newman WT infection compared with  $\Delta lgt$  infection (Fig. 2B). Accordingly, comolecule and cytokine induction after *S. aureus*  $\Delta lgt$  infection was similarly low or absent in DCs from C57BL/6, TLR2<sup>-/-</sup>, and MyD88<sup>-/-</sup> mice (Fig. 2A, 2B). Expression of MHC class II was independent of mature lipoproteins in *S. aureus* Newman, but was significantly enhanced by Pam<sub>3</sub>CSK<sub>4</sub> in C57BL/6 cells only (Fig. 2A). *S. aureus* lipoproteins are necessary for the activation of DCs through TLR2–MyD88 signaling.

#### Lipoprotein–TLR2–MyD88 signaling programs DCs to enhance IFN- $\gamma$ and IL-17 production in naive CD4<sup>+</sup> T cells

*S. aureus* primes DCs to promote production of IFN- $\gamma$  and IL-17 in T<sub>H</sub> cells (21). We investigated to what extent lipoprotein–TLR2–MyD88 signaling is required for activating T cells. *S. aureus* Newman WT-infected C57BL/6 DCs induced more IFN- $\gamma$  and IL-17 in naive CD4<sup>+</sup> T cells than did  $\Delta lgt$ -infected DCs (Fig. 3A). Experiments with DCs from TLR2<sup>-/-</sup> and MyD88<sup>-/-</sup> mice



**FIGURE 1.** TLR2–MyD88 signaling enhances upregulation of comolecules and cytokines. DCs from C57BL/6 (black), TLR2<sup>-/-</sup> (white), and MyD88<sup>-/-</sup> mice (striped) were incubated with medium (unstim) or *S. aureus* Newman WT (wt) at an MOI of 10 for 1 h. Fresh medium with gentamicin was added and DCs were incubated for an additional 18 h. As a positive control, DCs were stimulated with 1  $\mu$ g/ml of smooth LPS for 18 h. *A*, Expression of MHC class II, CD40, CD80, and CD86 on the surface of CD11c<sup>+</sup> DCs was determined by flow cytometry. Data show mean  $\pm$  SD of mean fluorescence intensity (MFI) of at least three independent experiments. *B*, TNF, IL-1 $\beta$ , IL-6, IL-10, and IL-12 levels in the supernatants of DCs were measured by ELISA. *C*, Percentage of intracellular *S. aureus* Newman after 1 h of infection. Data are shown as mean  $\pm$  SEM of three to five independent experiments. C57BL/6-DC infected with *S. aureus* WT versus all other groups: \* $p < 0.05$ ; \*\* $p < 0.01$ . No significant differences were detectable among  $\Delta lgt$ -infected DCs from different mouse strains (not shown). MHC II, MHC class II.



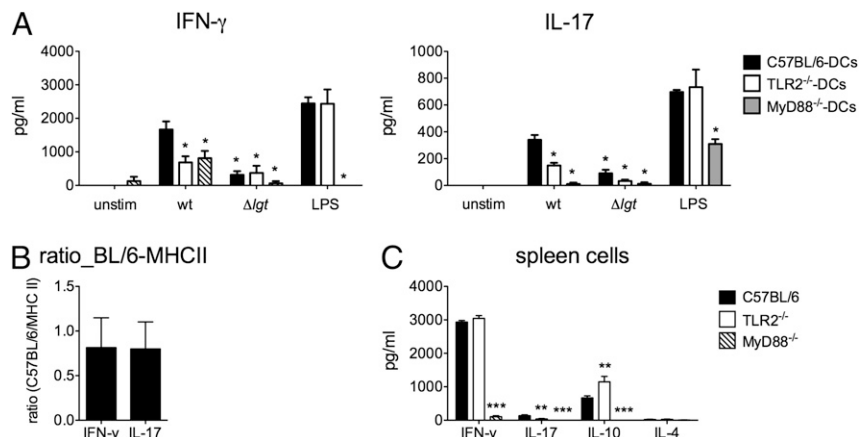
**FIGURE 2.** Staphylococcal lipoproteins activate DCs by TLR2-MyD88 signaling. DCs from C57BL/6 (black), TLR2<sup>-/-</sup> (white), and MyD88<sup>-/-</sup> mice (striped) were incubated with medium (unstim), *S. aureus* Newman WT (wt),  $\Delta lgt$  at an MOI of 10, or 10  $\mu\text{g/ml}$  Pam<sub>3</sub>CSK<sub>4</sub> for 1 h. Fresh medium with gentamicin was added, and DCs were incubated for an additional 18 h. **A**, Expression of MHC class II (MHC II), CD40, and CD80 on the surface of CD11c<sup>+</sup> DCs was determined by flow cytometry. Data show mean  $\pm$  SD of mean fluorescence intensity (MFI) of at least three independent experiments. **B**, IL-6, IL-10, and IL-12 levels in the supernatants of DCs were measured by ELISA. Data are shown as mean  $\pm$  SEM of three independent experiments. C57BL/6-DC infected with *S. aureus* WT versus all other groups: \* $p < 0.05$ ; \*\* $p < 0.01$ . No differences were detectable among  $\Delta lgt$ -infected DCs from different mouse strains.

revealed that the capacity of DCs to enhance IFN- $\gamma$  and IL-17 production in naive CD4<sup>+</sup> T cells resulted from DC activation by lipoproteins, TLR2, and MyD88. After  $\Delta lgt$  infection, T cells from all three mouse strains produced similarly low IFN- $\gamma$  and IL-17 (Fig. 3A). Infected DCs from all tested mice did not induce IL-4 and IL-10 production in naive CD4<sup>+</sup> T cells (data not shown), underscoring the importance of T<sub>H</sub>1 and T<sub>H</sub>17 cells in staphylococcal infection. LPS stimulated IFN- $\gamma$  and IL-17 in a MyD88-dependent manner, but not a TLR2-dependent manner (Fig. 3A). Infected DCs alone or T cells alone did not produce any cytokines after *S. aureus* infection (data not shown).

To identify exactly the proportion of cells producing IFN- $\gamma$  and IL-17, we stained cells for intracellular cytokines at different time points after *S. aureus* Newman infection. Cocultures of OVA-peptide stimulated DCs with CD8<sup>+</sup> T cells from OT-1 mice showed IFN- $\gamma$  positive T cells, whereas IFN- $\gamma$ - and IL-17-positive

CD4<sup>+</sup> T cells were present, but under the detection limit after *S. aureus* Newman infection (data not shown). Furthermore, proliferation of T cells was not found at all (data not shown). Nevertheless, the high purity of isolated cells let us conclude that *S. aureus* Newman-infected DCs prime T cells by TLR2-MyD88 signaling. Surprisingly, *S. aureus* Newman stimulation of MHC class II-deficient DCs did not abolish the release of IFN- $\gamma$  and IL-17 by CD4<sup>+</sup> T cells, suggesting stimulation independent of MHC class II Ag presentation (Fig. 3B).

To assess whether *S. aureus*-infected DCs are able to induce T cell activation in vivo, we systemically infected C57BL/6, TLR2<sup>-/-</sup>, and MyD88<sup>-/-</sup> mice with *S. aureus*. Six days post-infection, spleen cells were isolated, restimulated with *S. aureus* Newman WT in vitro, and compared with unstimulated cells. *S. aureus* Newman WT induced similar levels of IFN- $\gamma$  in stimulated spleen cells of C57BL/6 and TLR2<sup>-/-</sup> mice, but only low levels in



**FIGURE 3.** DCs infected with *S. aureus* promote differentiation of naive T cells to IFN- $\gamma$ - and IL-17-producing T cells by MyD88 signaling. **A**, DCs from C57BL/6 (black), TLR2<sup>-/-</sup> (white), and MyD88<sup>-/-</sup> mice (striped) were infected with *S. aureus* Newman WT and  $\Delta lgt$  for 1 h. Extracellular bacteria were killed with gentamicin and Pen/Strep, and negatively selected CD4<sup>+</sup> T cells were added. Noninfected DCs (unstim) and T cells only (data not shown) were used as negative controls. LPS was added to noninfected DCs as a positive control. Cytokines in the supernatants of cocultures were determined by ELISA after 72 h of coculture. **B**, Ratio of T cell-derived IFN- $\gamma$  and IL-17 promoted by *S. aureus* Newman-infected C57BL/6 and MHC class II<sup>-/-</sup> DCs. **C**, Total spleen cells harvested 6 d after i.v. infection from C57BL/6 (black), TLR2<sup>-/-</sup> (white), and MyD88<sup>-/-</sup> mice (striped) were restimulated with *S. aureus* Newman WT in vitro, and cytokines were determined after 48 h. Data represent mean  $\pm$  SEM of quadruplicates of one out of two independent experiments. C57BL/6-DC infected with *S. aureus* WT versus all other groups: \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

MyD88<sup>-/-</sup> mice (Fig. 3C). Interestingly, IL-17 was partially dependent on TLR2 and almost completely on MyD88, whereas IL-10 was apparently suppressed by TLR2, but dependent on MyD88 (Fig. 3C). IL-4 production was not detectable in either group (Fig. 3C). A similar cytokine pattern was obtained with the less virulent strain SA113 (data not shown). The lacking TLR2-dependence of IFN- $\gamma$  production in vivo was not attributable to changes of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells,  $\gamma\delta$  T cells, NK/NKT cells, B cells, or myeloid cells in the spleens of infected TLR2<sup>-/-</sup> compared with C57BL/6 mice (data not shown). In conclusion, DCs are mainly activated by *S. aureus* lipoproteins through TLR2–MyD88 signaling, which enhances IFN- $\gamma$  and IL-17 in CD4<sup>+</sup> T cells.

#### Adaptive immunity is activated early in *S. aureus* infection

We next asked whether the IFN- $\gamma$  and IL-17 response has an effect on the outcome of *S. aureus* infection. Systemically infected C57BL/6 mice were not able to clear the infection within 9 d (10), which might be a consequence of an insufficient adaptive immune response. Six days postinfection, *S. aureus* Newman-stimulated spleen cells produced IFN- $\gamma$ , IL-17, and IL-10 (Fig. 4A), but no IL-4 (data not shown), indicating early activation of adaptive immune cells. We then investigated the cytokine response 21 d postinfection and found that *S. aureus* Newman-restimulated spleen cells produced higher levels of IFN- $\gamma$ , IL-17, and IL-10 than after 6 d (Fig. 4A). Again, IL-4 was not detectable (data not shown). No IFN- $\gamma$ , IL-4, IL-17, or IL-10 were detectable in the plasma of infected mice (data not shown). When infecting with the low-virulence *S. aureus* SA113 strain, a similar cytokine pattern was observed after stimulation of spleen cells (data not shown). In the above studies, we selected a sublethal inoculum and monitored sepsis over 21 d. In the acute phase, both *S. aureus* strains (Newman and SA113) caused weight loss, but nearly all infected C57BL/6 mice regained weight after 7 d (Fig. 4B; data not shown for SA113). Bacteria were still detectable in kidneys and knees after 3 wk of infection, whereas almost all bacteria were cleared from the blood, spleen, and liver (Fig. 4C; data not shown for SA113). These results indicate that the host responds to *S. aureus* infection with early release of T cell-derived cytokines, which were later enhanced but were not found to be associated with clearing *S. aureus* infection in the organs.

#### Adaptive immune cells do not improve killing of *S. aureus* in early sepsis

Our data thus far demonstrate that T cell-derived cytokines were released, but did not result in better control and eradication of *S. aureus* Newman from the organs. We next sought to examine the origin of the cytokines and the weak role of T cells against

*S. aureus* infection. We infected CD3<sup>-/-</sup> and Rag2<sup>-/-</sup> mice with *S. aureus* Newman WT and assessed the contribution of T cells to cytokines on day 6 of infection. Spleen cells from infected CD3<sup>-/-</sup> and Rag2<sup>-/-</sup> mice restimulated with *S. aureus* Newman produced little or no IFN- $\gamma$ , respectively (Fig. 5A). IL-17 was not detectable in these mice. Spleen cells of all three mouse strains produced equal levels of IL-10 (Fig. 5A), but no IL-4 (data not shown). The data suggest that primarily T cells are required to mount a strong T<sub>H</sub>1 and T<sub>H</sub>17-specific cytokine response against *S. aureus*, whereas IL-10 was derived from innate immune cells.

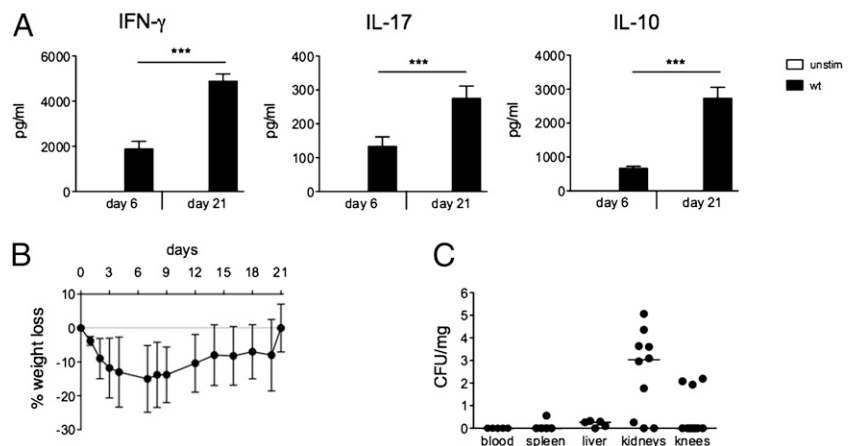
We next aimed to elucidate whether T and B cells contribute to the outcome and clearance of *S. aureus* infection. Weight loss during 6 d was similar in CD3<sup>-/-</sup>, Rag2<sup>-/-</sup>, and C57BL/6 mice (Fig. 5B). Bacterial load was comparable in the kidneys and knees of CD3<sup>-/-</sup>, Rag2<sup>-/-</sup>, and C57BL/6 mice (Fig. 5C). CD3<sup>-/-</sup> and Rag2<sup>-/-</sup> mice showed slightly, but not significantly, higher bacterial numbers in the liver than did C57BL/6 mice. Plasma IL-6 levels were significantly lower on day 6 (Fig. 5D) and kidney abscesses were less organized, containing no necrotic zones with bacteria in CD3<sup>-/-</sup> and Rag2<sup>-/-</sup> mice compared with C57BL/6 mice (Fig. 5E). These results indicate that early in sepsis, T and B cells do not have a strong protective function except for enhancing bacterial clearance in the liver.

#### Adaptive immune cells do not improve killing of *S. aureus* in late sepsis

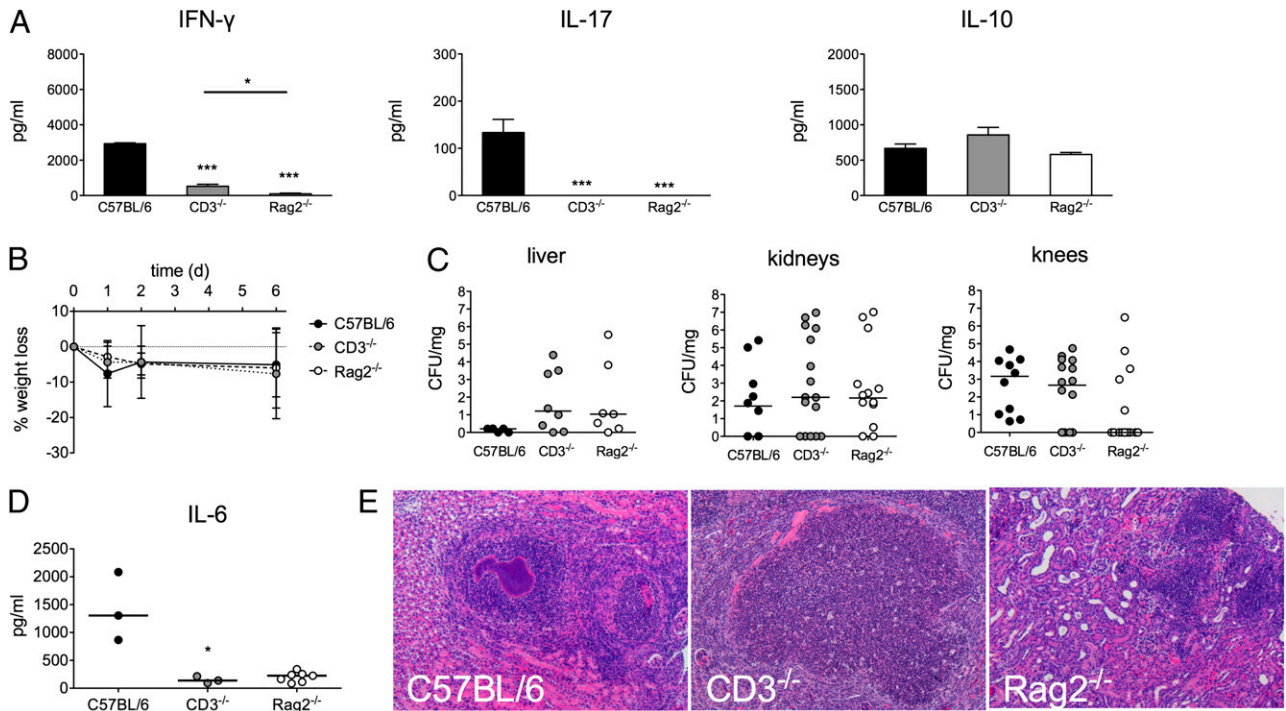
Finally, we were interested in the role of B and T cells in persistence of *S. aureus*. To that aim, we monitored C57BL/6 and Rag2<sup>-/-</sup> mice for 21 d postinfection with *S. aureus* Newman. Restimulation of spleen cells confirmed that T and B cells are the major source of IFN- $\gamma$  and IL-17, whereas levels of IL-10 in Rag2<sup>-/-</sup> mice exceeded those of C57BL/6 cells (Fig. 6A) and were also 10-fold higher than on day 6 (Fig. 5A). IL-4 was absent (data not shown). Notably, although infected C57BL/6 and Rag2<sup>-/-</sup> mice regained weight (Fig. 6B), the majority of mice from both strains were able to clear *S. aureus* Newman from the liver and knees, but not the kidneys (Fig. 6C). The comparison of C57BL/6 mice and Rag2<sup>-/-</sup> mice revealed that the presence of T cells, B cells, and NKT cells is required to induce IFN- $\gamma$  and IL-17, but not for clearance of *S. aureus* Newman from the liver, kidneys, and knees. These data demonstrate that adaptive immune responses mediated by T cells and B cells are of minor importance in the control of *S. aureus* in early and persistent murine sepsis.

#### Vaccination before *S. aureus* infection does not affect outcome

Because we found that T cell-derived cytokine levels increased over time and bacteria were persistent in organs, we examined



**FIGURE 4.** IFN- $\gamma$ , IL-17, and IL-10 are released after *S. aureus* infection. C57BL/6 mice were infected i.v. with *S. aureus* Newman for 6 and 21 d. **A**, Cytokines in the supernatant of spleen cells 48 h after restimulation with *S. aureus* Newman in vitro. **B**, Weight loss of mice during infection. **C**, Bacterial load in the liver, both kidneys, and both knees on day 21. Cytokine data are represented as mean  $\pm$  SEM of quadruplicates of five mice per group. Data for mice are shown as mean  $\pm$  SEM for cytokines, mean  $\pm$  SD for weight loss, and median for CFUs per milligram in five mice per group. Significant differences between mice: \*\*\* $p$  < 0.001.

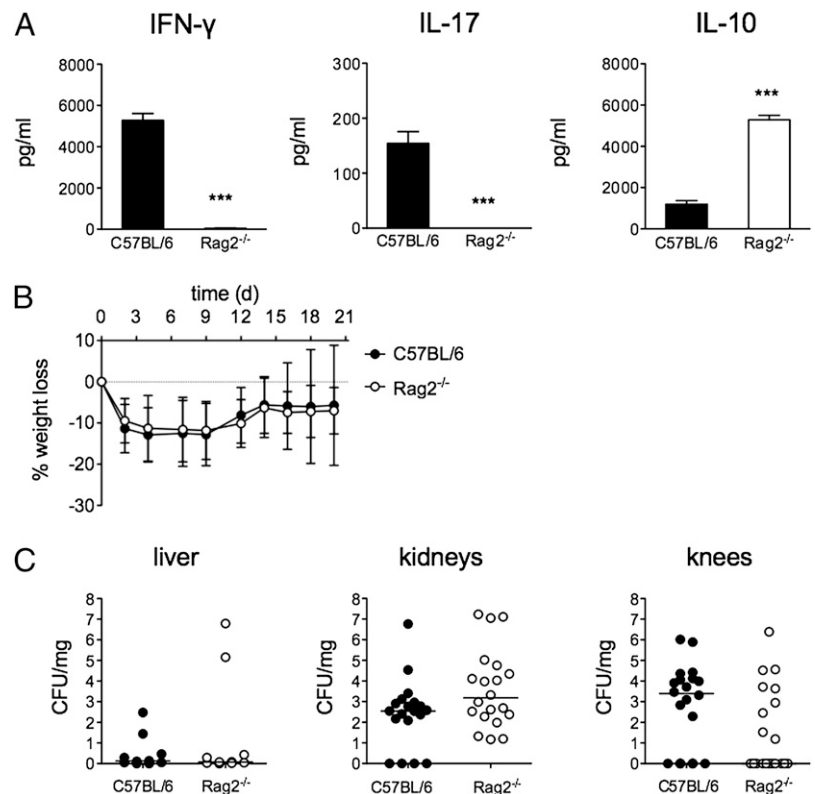


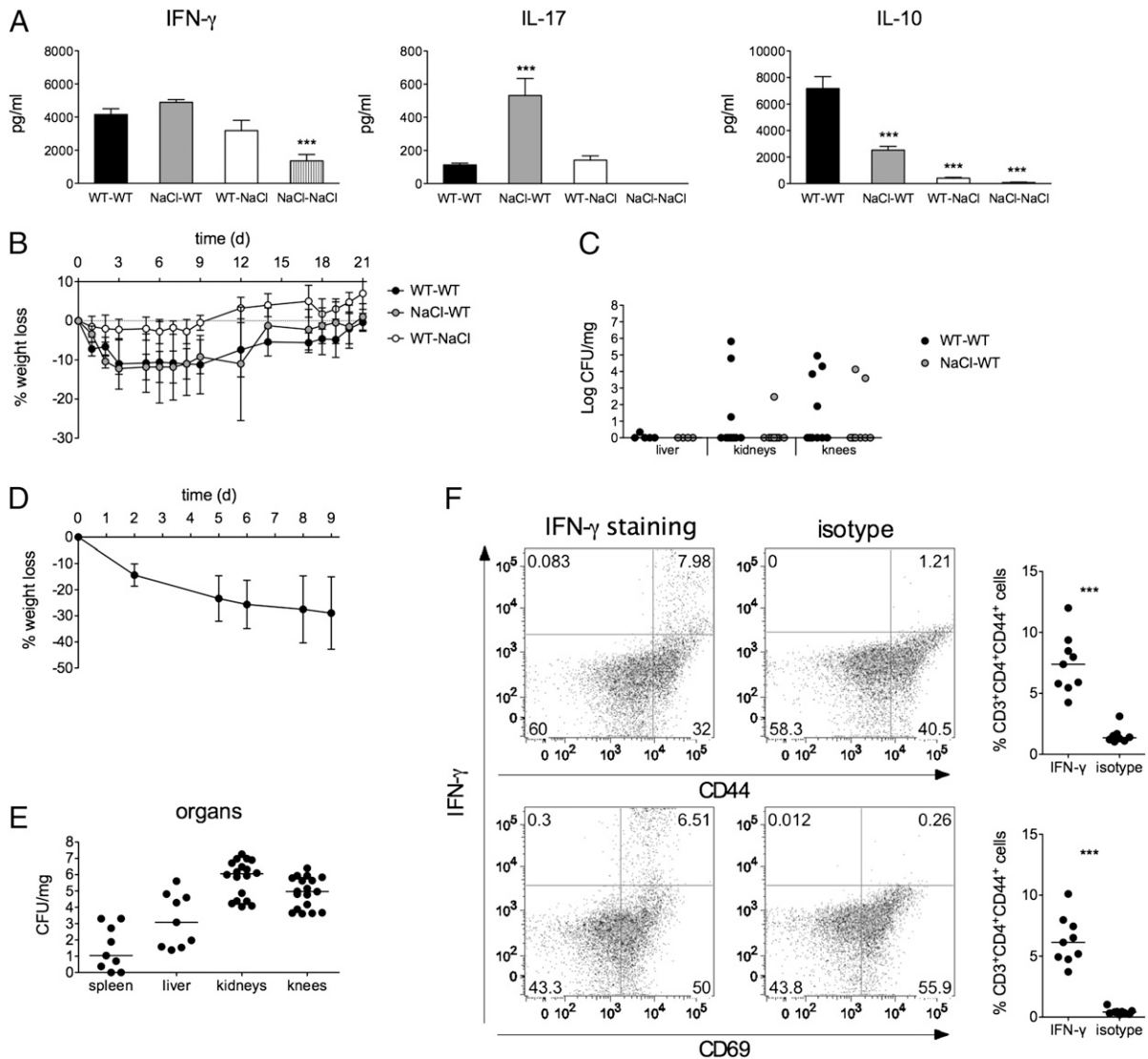
**FIGURE 5.** Absence of adaptive immune cells does not affect *S. aureus* clearing in the organs of mice. C57BL/6 (black, solid line), CD3<sup>-/-</sup> (gray, dotted line), and Rag2<sup>-/-</sup> mice (white, dashed line) were infected with *S. aureus* Newman for 6 d. **A**, Cytokines in the supernatant of spleen cells stimulated with *S. aureus* Newman in vitro. **B**, Weight loss of mice during infection. **C**, Bacterial load in the liver, both kidneys, and both knees. **D**, Plasma IL-6 in mice on day 6. **E**, Histologic analysis of H&E-stained kidneys of infected mice on day 6. Original magnification  $\times 10$ . Data for mice are shown as mean  $\pm$  SEM for cytokines, mean  $\pm$  SD for weight loss, and median for CFUs per milligram in five to eight mice per group. Significant differences between mice: \* $p < 0.05$ ; \*\*\* $p < 0.001$ .

whether preactivation of the host with an i.p. injection of different doses of heat-killed *S. aureus* Newman before infection with viable staphylococci improves the outcome of sepsis after 21 d.

We found a similar IFN- $\gamma$  release from spleen cells after a single preactivation alone (WT-NaCl) and after systemic infection of preactivated (WT-WT) and non-preactivated (NaCl-WT) mice (Fig. 7A). Non-preactivated NaCl-treated mice (NaCl-NaCl)

**FIGURE 6.** Adaptive immune cells do not eliminate *S. aureus* in mice. C57BL/6 (black) and Rag2<sup>-/-</sup> mice (white) were infected with *S. aureus* Newman for 21 d. **A**, Cytokines in the supernatant of spleen cells stimulated with *S. aureus* Newman in vitro. **B**, Weight loss of mice during infection. **C**, Bacterial load in the liver, both kidneys, and both knees on day 21. Data for mice are shown as mean  $\pm$  SEM for cytokines, mean  $\pm$  SD for weight loss, and median for CFUs per milligram in 10 mice per group. \* $p < 0.05$ ; \*\*\* $p < 0.001$ .





**FIGURE 7.** Vaccination alters the cytokine pattern but does not improve *S. aureus* clearing. *A–C*, C57BL/6 mice were preactivated with heat-killed *S. aureus* Newman (black and white) or injected with NaCl (gray and striped) 7 d before i.v. challenge with viable *S. aureus* Newman (black and gray) or NaCl (white and striped). Mice were followed for 21 d. *A*, Cytokines in the supernatant of spleen cells restimulated with *S. aureus* Newman in vitro. *B*, Weight loss of mice during infection. *C*, Bacterial load in the liver, both kidneys, and both knees on day 20. Data for mice are shown as mean  $\pm$  SEM for cytokines, mean  $\pm$  SD for weight loss, and median for CFUs per milligram in three to seven mice per group. Significant differences between WT–WT versus all other groups are indicated. *D–F*, C57BL/6 mice were vaccinated with heat-killed *S. aureus* Newman 14 and 7 d before i.v. challenge with viable *S. aureus* Newman. *D*, Weight loss of mice during infection until sacrifice. *E*, Bacterial load in the liver, both kidneys, and both knees. *F*, Flow cytometry of intracellular IFN- $\gamma$  production versus CD44 (upper panel) or CD69 (lower panel) by CD3<sup>+</sup>CD4<sup>+</sup> T cells from restimulated splenocytes for 24 h with heat-killed *S. aureus* Newman. Data for mice are shown as mean  $\pm$  SD for weight loss and as median for CFUs per milligram and intracellular IFN- $\gamma$  staining of nine mice per group. Significant differences between anti-IFN- $\gamma$  and isotype: \*\*\* $p$  < 0.001.

showed less IFN- $\gamma$  than all other groups. This finding indicates that vaccination did not affect IFN- $\gamma$  production during infection and was sufficient alone for maximal release. Different patterns were measured for IL-17 and IL-10. Whereas IL-17 was highest in the NaCl–WT group, IL-10 was highest in the WT–WT group (Fig. 7A). These data point to a suppression of IL-17 and a stimulation of IL-10 by preactivation with heat-killed *S. aureus* Newman before infection. Preactivation did not confer protection of mice because severity of disease, as assessed by weight loss (Fig. 7B) and clearance of *S. aureus* Newman from the organs (Fig. 7C), was similar in preactivated and non-preactivated mice.

Next, we investigated whether a vaccination approach with two i.p. injections of heat-killed *S. aureus*, 14 and 7 d before infection, would confer better protection against *S. aureus* infection. Unexpectedly, *S. aureus* infection caused high weight loss (Fig. 7D)

and severe symptoms of sepsis, which made sacrificing the mice necessary on day 9. The severe state of disease was associated with a high bacterial burden in the spleen, liver, kidneys, and knees (Fig. 7E). After in vitro restimulation of splenocytes, CD4<sup>+</sup> T cells were partially activated, as assessed by CD44 and CD69 staining, and intracellular IFN- $\gamma$  was detected in CD44<sup>+</sup> and CD69<sup>+</sup> T cells (Fig. 7F). Thus, a repeated strong preactivation of the adaptive immune system, and in particular the IFN- $\gamma$  induction by vaccination, did not improve bacterial clearing.

These results suggest that preactivation or repeated vaccination alters the cytokine pattern toward immunosuppression, with decreased IL-17 and increased IL-10 production. This alteration does not improve bacterial killing during persistent *S. aureus* infection, and it indicates that preactivation of the adaptive immune system may be harmful in defense against *S. aureus*.

## Discussion

In this study, to our knowledge, we show for the first time that staphylococcal lipoproteins expressed in viable *S. aureus* enhance the activation of DCs by TLR2 and thereby promote the differentiation of naive CD4<sup>+</sup> T cells into T<sub>H</sub>1 and T<sub>H</sub>17 cells in vitro in a manner independent of MHC class II. We further demonstrate that in vivo the contribution of lipoproteins and TLR2 in whole spleen cells to IFN- $\gamma$  production is mitigated by other MyD88-signaling receptors, whereas lipoprotein-TLR2 dependence remains detectable for IL-17. Finally, we identify T cells as the main source of IFN- $\gamma$  and IL-17 after systemic *S. aureus* infection, whereas innate immune cells produce IL-10. Surprisingly, T and B cells, or their early activation through vaccination, do not improve disease outcome or killing of *S. aureus* from the organs.

The innate immune response to *S. aureus* has a key function during acute invasive infections. However, it is well known that *S. aureus* also activates the adaptive immune response with its superantigens (38). Yet the incidence of superantigen-related toxic shock syndrome is low, possibly because *S. aureus* downmodulates the T cell response to these superantigens (39); this may be related to the release of cell wall components that preferentially activate the anti-inflammatory axis of the adaptive immune system (40). In addition, *S. aureus* carries many different superantigens, which provoke a strong neutralizing Ab response, and there is abundant evidence of a protective role of these Abs. Furthermore, despite the MHC class II peptide-independent immune activation by superantigens, host susceptibility to superantigens varies due because of MHC class II polymorphisms, which affect the extent of T cell responses. Whereas a synergistic innate and superantigen-mediated T cell stimulus can lead to lethal septic shock in mice, there is no experimental evidence for this two-hit model in human sepsis (41).

However, regulations of the Ag-specific T cell polarization and B cell responses, as well as the effect of T and B cells on outcome in acute *S. aureus* sepsis, are not clear yet. Previous studies on the activation of T cells have used purified staphylococcal molecules, but not viable *S. aureus*. To address this shortcoming, we evaluated the requirements for *S. aureus* and for the host to promote the activation of T cells and, more importantly, the differentiation of T cells into T<sub>H</sub>1, T<sub>H</sub>2, or T<sub>H</sub>17 lineages.

The expression of surface markers (e.g., CD40) is required for contact of DCs with T cells to induce inflammatory cytokines. It has been shown that TLR2 on DCs increased expression of MHC class II and CD80 after stimulation with staphylococcal PGN (30). We now demonstrate that lipoproteins present in viable *S. aureus* contribute modestly through the activation of the TLR2 pathway to induce the upregulation of the surface markers CD40, CD80, and CD86. In contrast, the MHC class II molecule was strongly upregulated after infection with *S. aureus*, but independent of MyD88 signaling as demonstrated repeatedly for LPS [shown by our data and Kaisho et al. (42)].

Aside from upregulating costimulatory molecules, cytokines are released from activated DCs to efficiently induce adaptive immune responses (32). DCs infected with viable *S. aureus* released TNF, IL-6, IL-10, IL-1 $\beta$ , and IL-12. Surprisingly, although viable *S. aureus* was recognized by various PRRs in our in vitro experiments, the cytokine production of DCs was mostly dependent on lipoprotein-TLR2 signaling and not other MyD88-dependent receptors. A role of lipoteichoic acid, another ligand for TLR2, which was reported to induce activation of DCs (30), had a minor effect in our experiments with viable *S. aureus*, because only low amounts of cytokines were released in the absence of mature lipoproteins. IL-10 was induced by *S. aureus* in TLR2<sup>-/-</sup>, but not in MyD88<sup>-/-</sup> cells. In our in vitro study, the recognition of staphylo-

coccal DNA by TLR9 and the sensing of IL-1 by its receptor IL-1R had no effect on cytokines released by DCs (M. Schmalzer, unpublished data). Therefore, it remains to be elucidated whether other MyD88-dependent signaling receptors—such as the bacterial RNA-sensing TLR7 (5) or TLR4, which activates DCs after exposure to staphylococcal leukocidin (43)—are involved in the release of IL-10.

TLR2-MyD88 stimulation of DCs caused their maturation and rendered them able to induce T<sub>H</sub>1, T<sub>H</sub>2, or T<sub>H</sub>17 effector cell differentiation. In this process, IL-12 is known to mediate the differentiation of naive T cells into T<sub>H</sub>1 cells that produce IFN- $\gamma$  (44). Indeed, the high levels of IL-12 released in a lipoprotein-TLR2-dependent fashion in DCs may have contributed, together with the modest IL-10 levels, to IFN- $\gamma$  production by naive T cells (45). The suppressive effect of IFN- $\gamma$  on T<sub>H</sub>2 development (46) might be the reason for undetectable IL-4, and the IFN- $\gamma$ -mediated inhibition of T<sub>H</sub>17 cell differentiation might explain the low levels of IL-17 cells (47–49). In line with this hypothesis, the TLR2-pathway activated by *S. aureus* was also associated with reduced proportions of IL-17-producing CD4<sup>+</sup> T cells in immune cells surrounding brain abscesses (50). However, in the current study, production of IL-17 may have been supported by high quantities of the proinflammatory cytokine IL-6, possibly together with TGF- $\beta$  and amplified by high levels of TNF and IL-1 $\beta$  in *S. aureus*-infected DCs (51).

Stimulation of TLR2, expressed on the surface of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, can induce IFN- $\gamma$  expression (52–55). In this study, we found that *S. aureus* did not elicit IFN- $\gamma$  or IL-17 production directly in naive purified T cells after infection. Therefore, we can exclude a lipoprotein stimulation of TLR2 on T cells. In addition, superantigens were unlikely to contribute to IFN- $\gamma$  and IL-17 in T cells, because infection of TLR2<sup>-/-</sup> and MyD88<sup>-/-</sup> cells with the *S. aureus* strain expressing the same superantigens resulted in a reduced IFN- $\gamma$  response. However, it remains to be documented that the  $\beta$  chain of the TCR is unaltered in TLR2<sup>-/-</sup> and MyD88<sup>-/-</sup> cells before the participation of superantigens in IFN- $\gamma$  and IL-17 production can be ruled out.

After *S. aureus* infection of mice, TLR2-dependency was no longer detectable for IFN- $\gamma$  in restimulated spleen cells, but it remained for IL-17. This finding may be related to the previously described pronounced TLR2-dependent IL-6 and IL-1 $\beta$  production in restimulated spleen cells of mice infected with *S. aureus* Newman (10). However, all cytokines were dependent of MyD88, and this effect was not related to a MyD88 regulation of T cell numbers or spleen size in response to *S. aureus*. This strong MyD88 dependence is in line with the highly impaired cytokine induction in MyD88-deficient macrophages (10). We and others found this lack of cytokines to be associated with a higher susceptibility of MyD88<sup>-/-</sup> mice to sepsis (10, 13, 15, 16). This study extends the findings to DCs, which apparently also contribute to immune defense through T cell activation. This hypothesis is further supported by studies with other bacteria that mainly activate DCs in a MyD88-dependent manner to promote T cell differentiation (56, 57). One possibility for the essential role of MyD88 is that efficient presentation of Ags requires MyD88-dependent signals. For example, TLR2<sup>-/-</sup> DCs showed impaired Ag presentation in response to *S. aureus* (30).

We could assign *S. aureus*-induced IFN- $\gamma$  and IL-17 production to T cells, because we detected significantly less IFN- $\gamma$  in spleens from *S. aureus*-infected CD3<sup>-/-</sup> mice and hardly any IFN- $\gamma$  in those from Rag2<sup>-/-</sup> mice. These results also exclude a role of NK cells that were shown to release IFN- $\gamma$  after activation by *S. aureus*-like lipopeptides (58). Furthermore, the absence of B cells during *S. aureus* infection did not reduce IFN- $\gamma$  release from splenocytes of muMT<sup>-/-</sup> mice (M. Schmalzer, unpublished results).

In contrast, IL-10 was derived from innate immune cells and not natural regulatory T cells and IL-10-producing T<sub>H</sub>1 cells (59), because levels were maintained in CD3<sup>-/-</sup> and Rag2<sup>-/-</sup> mice and even strongly increased during persistent infection in Rag2<sup>-/-</sup> mice. The release of IL-10 by *S. aureus*-infected macrophages (10, 60) and DCs might protect the infected host; however, it remains unclear whether excessive IL-10 is beneficial for the host in the absence of T and B cells.

Several studies using the septic arthritis model in *S. aureus* infection revealed that the IFN- $\gamma$ -dominated immune responses are beneficial for outcomes in mice (18–20). We found no relationship between IFN- $\gamma$  levels and bacterial load, because CD3<sup>-/-</sup> and Rag2<sup>-/-</sup> mice with low or no IFN- $\gamma$  showed a similar bacterial organ load early as late in infection as C57BL/6 mice. This lack of a T or B cell effect on *S. aureus* infection was also reflected in the similar clinical course and bacterial numbers in organs in knockout and C57BL/6 mice. Neither IFN- $\gamma$ -mediated myeloid cell activation nor Abs allowing opsonization appear to be primary. This finding is in strong contrast with the prominent defense mediated by MyD88; this molecule allows chemokine release, phagocyte recruitment, and better clearing in most organs (10). It can now be concluded that, among the MyD88-induced effects, the innate mediators and not the T cell response appear to be crucial. This hypothesis is further supported by the preactivation of mice before challenge with viable *S. aureus*, which did not result in a better killing of bacteria from infected organs because it suppressed IL-17, which promotes granulocyte recruitment (61), and enhanced IL-10, which may deactivate myeloid cells (62). In addition, a 2-fold vaccination allowed even more bacterial growth despite a strong intracellular IFN- $\gamma$  induction. These results clearly show that a well-balanced immune response of the innate and the adaptive immune cells protects the host from severe complications during systemic *S. aureus* infection. Therefore, it remains questionable whether vaccination protects against *S. aureus* infections.

In summary, the data demonstrate that adaptive immune responses mediated by T cells and B cells are of minor importance in the control of *S. aureus* in early and persistent murine sepsis, whereas MyD88-mediated immune responses favor the outcome after *S. aureus* infection (10).

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## Disclosures

The authors have no financial conflicts of interest.

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