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MyD88 in Macrophages Is Critical for Abscess Resolution in Staphylococcal Skin Infection

Reinhold Feuerstein,*† Maximilian Seidl,*† Marco Prinz,§∥ and Philipp Henneke*‡

When *Staphylococcus aureus* penetrates the epidermis and reaches the dermis, polymorphonuclear leukocytes (PMLs) accumulate and an abscess is formed. However, the molecular mechanisms that orchestrate initiation and termination of inflammation in skin infection are incompletely understood. In human myeloid differentiation primary response gene 88 (MyD88) deficiency, staphylococcal skin and soft tissue infections are a leading and potentially life-threatening problem. In this study, we found that MyD88-dependent sensing of *S. aureus* by dermal macrophages (Mφs) contributes to both timely escalation and termination of PML-mediated inflammation in a mouse model of staphylococcal skin infection. Mφs were key to recruit PML within hours in response to staphylococci, irrespective of bacterial viability. In contrast with bone marrow–derived Mφs, dermal Mφs did not require UNC-93B or TLR2 for activation. Moreover, PMLs, once recruited, were highly activated in an MyD88-independent fashion, yet failed to clear the infection if Mφs were missing or functionally impaired. In normal mice, clearance of the infection and contraction of the PML infiltrate were accompanied by expansion of resident Mφs in a CCR2-dependent fashion. Thus, whereas monocytes were dispensable for the early immune response to staphylococci, they contributed to Mφ renewal after the infection was overcome. Taken together, MyD88-dependent sensing of staphylococci by resident dermal Mφs is key for a rapid and balanced immune response, and PMLs are dependent on intact Mφ for full function. Renewal of resident Mφs requires both local control of bacteria and inflammatory monocytes entering the skin. The Journal of Immunology, 2015, 194: 2735–2745.

*staphylococcus aureus* is part of the resident flora in humans and many other mammalian and nonmammalian species. Up to 30% of humans are asymptomatic mucocutaneous carriers, with the nasal mucosa and moist areas of the skin being preferred colonization sites. In contrast, *S. aureus* is the leading cause of acute bacterial skin and skin structure infections (ABSSSIs), which are associated with significant morbidity and mortality (1). In the last two decades, a surge in hospital contacts of patients with ABSSSI has been documented (2). Increasing resistance against β-lactam antibiotics is further complicating the situation.

Patients with defects in cellular innate immunity, such as chronic granulomatous disease and STAT-3 and NEMO deficiency, typically present with ABSSSI. A striking example for innate immunodeficiency disorders manifesting in ABSSSI as leading complication are human myeloid differentiation primary response gene 88 (MyD88) and IRAK4 deficiencies (3). It seems intriguing that loss of MyD88 and IRAK4 function, which affects the myeloid cell response against virtually all important human pathogens in vitro and in many animal models, leads to a relatively selective susceptibility for staphylococci and streptococci. These bacterial species account for approximately two thirds of all infections in the affected patients (4). The infectious disease–associated lethality in these patients approximates 50%. Accordingly, MyD88 and its interaction partner IRAK4 are dedicated and limiting factors in controlling *S. aureus* at the colonization sites. However, the myeloid cell subset–specific role of MyD88-related signals in sensing staphylococci and instructing the intercellular response at the site of bacterial invasion are incompletely understood.

Abscess formation, that is, the spatial containment of bacteria by a tissue reaction involving both resident and incoming myeloid cells in the dermis and hypodermis, is commonly viewed as the early immune response, which prevents the bacterial spread to deeper tissues and systemic dissemination via the bloodstream (5, 6). Yet abscess formation may already be viewed as failure of the resident immune cells to eliminate staphylococci that spurious penetrate the epidermis and get into contact with the dermis.

Macrophages (Mφs) constitute the major resident myeloid cell type in the dermis. It is well established that they are involved in inflammation in the skin (7, 8). Mφs are furthermore known to largely depend on the TLR system in recognizing staphylococci (9). However, some doubt has been recently shed on TLRs in Mφs as key elements in the cellular skin response to bacteria. First, important Mφ-independent sensing and central networking functions have been assigned to both γδ-T cells and polymorphonuclear leukocytes (PMLs) (10). Next, the NLR system has been found to be essential for staphylococcal sensing (11, 12). Moreover, whereas a significant body of data is available on TLR-sensing of staphylococci in bone marrow–derived Mφs (BMDMs) (13, 14), relatively little is known on *S. aureus* sensing and functional polarization of dermal Mφs, both as resting cells in immediate proximty to staphylococci colonizing the skin and during ABSSSI.
Another area of uncertainty in dermal Mø biology is their cellular origin and renewal. Mø types have been discriminated based on their specific localization in steady-state dermis as perivascular Møs, Møs associated with lymphoid vessels, and Møs within the intervascular space (15). Recent compelling evidence assigns specific functions to these subsets. However, it is unclear whether dermal Møs in staphylococcal infection are exclusively replenished from blood monocytes, or whether they are able to self-renew and differentiate at site without contribution of bone marrow and blood-derived mononuclear cells (16).

Dermal Møs are expressing a wide range of MyD88-dependent TLRs to detect bacterial components like LPS or bacterial nucleic acids and to produce proinflammatory cytokines and chemokines (15, 17–19). Thus, they contribute to a healthy skin and immune cell homeostasis. In this article, we aimed at clarifying the role of dermal Møs in abscess formation and resolution in S. aureus skin infection. We define a very distinct role of MyD88 in Møs in the earliest stages of staphylococcal infection, which determines the fate of the local infection in the coming days. Dermal Møs deliver the initial signal for the recruitment of PML and monocytes after sensing staphylococci in a MyD88-dependent, but TLR2- and endosomal TLR-independent fashion. Although delayed, PMLs are recruited in large numbers and are potently activated in staphylococcal infection in MyD88 deficiency or if Møs are missing. However, these activated PMLs fail to timely clear the infection, which underlines the importance of dermal Møs in the recruitment, activation, and regulation of these first-line effector cells. Once the bacteria are contained, MyD88-independent recruitment of Ly6C<sup>high</sup> inflammatory monocytes entering the skin substantially contributes to local Mø renewal.

Materials and Methods

Animals and cell lines

All mice used are on C57BL/6d or C57BL/6n genetic background. Mice lacking MyD88 were previously described (13). Mice with an UNC-93B-H412R (3D) mutation were kindly provided by Marina Freudenberg (University of Freiburg) (13). CCR<sub>2</sub><sup>−/−</sup> mice and CX3CR<sub>1</sub><sup>−/−</sup> mice were a kind gift of Steffen Jung (Rehovot, Israel), CD11b.DTR mice, and mice lacking MyD88 were a kind gift of Douglas T. Golenbock (Worcester, MA).

Bacterial strains

If not indicated otherwise, S. aureus strain Newman (wild type [wt]) was used. In some experiments, S. aureus SA 113 was used. S. aureus SA 113 and its isogenic lipoprotein diacylglyceroacyltransferase-deficient strain SA 113 (Algt) were kindly provided by Fritz Goetz (Tubingen, Germany). Bacteria were grown in Luria-Bertani broth medium to exponential growth phase, washed with PBS, and resuspended in Dulbecco’s PBS. Bacterial concentrations were determined with an optical spectrophotometer (600 nm, OD<sub>600</sub>). CFU of the inoculum were verified by serial dilutions on blood agar plates (Columbia Agar with 5% Sheep Blood; bioMérieux). In some experiments, bacteria were heat-fixed (hf; 80°C, 45 min) after adjusting the cell number to 10<sup>9</sup> CFU/ml as previously described (13).

Mouse model of skin infection

All procedures were approved by the Regional Council of Baden-Württemberg. Approximately 10<sup>6</sup> CFU S. aureus in 10 µl PBS were intradermally injected (30-gauge needle, U-100 insulin syringe) into the ear pinna of athymectomized mice (i.p. injection of ketamine, 100 µg/g body weight, and xylazine [Rompun], 20 µg/g body weight). In some experiments, hf bacteria were used for intradermal (i.d.) inoculation (~10<sup>9</sup> hf bacteria in 10 µl PBS per ear pinna). Groups of three to four mice were used per experiment, followed by at least one repetition to confirm the results. Lesion morphology was documented by digital photographs (Canon PowerShot A650 IS) of mice ears and analyzed by the software program Adobe Photoshop CS4.

Depletion of dermal Møs in vivo

Resident dermal Møs were depleted with liposomal clodronate (Clodrostone; Encapsula Nanosciences) in the indicated mouse strains. Approximately 10 µl liposomal clodronate or control liposomes were injected i.d. into the ear pinna of anesthetized mice. In addition, CD11b.DTR mice were treated with diphtheria toxin (25 ng/g body weight unnicked, from Corynebacterium diphtheriae; Calbiochem). In both cases, live or hf S. aureus was injected i.d. into the same ear pinna 2 d later. Depletion efficiency was verified by flow cytometric analysis of digested ear skin and histologic analysis of skin cryosections.

Immune cell phenotyping of skin tissue

Mouse ears were subjected to enzymatic digestion by dispase (1 mg/ml; STEMCELL Technologies), collagenase II (2 mg/ml; PAA), and DNase I (0.8 mg/ml; Roche) in PBS for 2 h at 1400 rpm shaking and 37°C. After digestion the samples were filtered with a 40-µm cell strainer (BD), washed with PBS, and stained with the indicated Abs. The following Abs were used: anti-mouse CD45 eFluor450 (eBioscience), anti-mouse CD11b PE-Cy7 (eBioscience), anti-mouse Ly6G FITC (BD Pharmingen), anti-mouse Ly6C PerCP-Cy5.5 (BD Pharmingen), anti-mouse F4/80 allophycocyanin (AbD Serotec), anti-mouse F4/80 PE (eBioscience), anti-mouse CD36 Alexa Fluor 488 (BioLegend), anti-mouse CD169 PE (BioLegend), anti-mouse CD68 allophycocyanin (BioLegend), anti-mouse CD64 PerCP-Cy5.5 (BioLegend), anti-mouse CD115 allophycocyanin (eBioscience), anti-mouse CD207 PE (eBioscience), anti-mouse CD11c FITC (BD Biosciences), and anti-mouse HMC class 2 eFluor450 (eBioscience). Cell samples were analyzed with a 10-laser flow cytometer (Gallio; Beckman Coulter), and data were analyzed with the Kaluza software (version 1.2; Beckman Coulter).

Immune-cell phenotyping of mouse blood

Mouse blood was collected from the submandibular venous plexus of anesthetized mice. After red cell lysis (1× RBC Lysis Buffer Solution; eBioscience), leukocytes were washed with PBS and stained with the indicated Abs. The following Abs were used: anti-mouse CD45 eFluor450 (eBioscience), anti-mouse CD11b PE-Cy7 (eBioscience), anti-mouse Ly6G FITC (BD Pharmingen), anti-mouse Ly6C PerCP-Cy5.5 (BD Pharmingen). Cell samples were analyzed with a 10-laser flow cytometer (Gallio), and data were analyzed with the Kaluza software (version 1.2; Beckman Coulter). Mouse inflammatory monocytes were characterized as CD45<sup>−</sup>CD11b<sup>+</sup>Ly6C<sup>low</sup>Ly6G<sup>high</sup> Mouse PMLs were characterized as CD45<sup>−</sup>CD11b<sup>+</sup>Ly6C<sup>high</sup>Ly6G<sup>high</sup>

Tissue embedding and staining

At various time points ear skin was collected and bisected; ear halves were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek Europe B.V.) and subsequently frozen in liquid nitrogen. Eight-micrometer cryosections were used. Immunohistochemistry was performed using the avidin-biotin complex method detected by an alkaline phosphatase catalyzed red chromogen reaction (Dako REALTM Detection System kit; Dako, Glostrup, Denmark). Photographs were taken on a Zeiss Axioplan 2 (Zeiss, Jena, Germany) with the Olympus LC20 camera (Olympus Germany, Hamburg, Germany) with magnifications as depicted. Immunofluorescence was performed using DAPI, FITC, Alexa 488, PE, and/or allophycocyanin fluorophores. Photographs were taken on the confocal laser microscope Zeiss LSM 710 with a 20× objective lens and analyzed with the Zeiss software Zen 2012. The following Abs were used: anti-mouse CD3e (clone 145-2C11, dilution 1:100; eBioscience), anti-mouse y6 TCR (clone UC7-13D5, dilution 1:100; eBioscience), anti-mouse F4/80 (clone 3/1, dilution 1:300; AbD Serotec; Kidlington, U.K.), and goat anti-rat (secondary Ab, polyclonal; Lot-No 105938, dilution 1:100; Jackson Immunoresearch, Suffolk, U.K.).

ELISA

Skin tissue. Ears were mechanically homogenized by Tissue Lyser (Qiagen). IL-1β concentrations in the homogenized skin tissue were quantified by ELISA kits according to the manufacturer’s instructions (R&D Systems).

HEK cells. IL-8 levels in HEK 293 or HEK TLR2 culture medium after 48-h stimulation with SA 113 WT or SA 113 Algt were quantified by ELISA kits according to the manufacturer’s instructions (R&D Systems).

Quantification of CFU

Mice were sacrificed; then ears were cut off at the hairline and homogenized using a Tissue Lyser (Qiagen). CFU in the homogenized skin tissue were determined by serial dilutions on blood agar plates (Columbia Agar with 5% Sheep Blood; bioMérieux).
In vitro blood cell stimulation and intracellular TNF-α staining

Blood was collected from the retrobulbar venous plexus of anesthetized mice. After red cell lysis (1× RBC Lysis Buffer Solution; eBioscience), leukocytes were washed twice and resuspended in RPMI 1640 medium supplemented with 10% FBS with antibiotics (ciprofloxacin, 10 μg/ml). After washing 200,000 cells/well, 24-well plate; Corning Costar), cells were treated with BD GolgiPlung Protein Transport Inhibitor (BD Biosciences) and stimulated for 5 h at 37°C with hS. aureus (5 × 10⁷/ml). Next, cells were harvested and cell-surface Ags were stained with Abs against CD45, CD11b, Ly6G, and Ly6C. Then cells were fixed, permeabilized (Fixation/Permeabilization Solution; BD Biosciences), and stained for intracellular TNF-α (anti-mouse TNF-α allophycocyanin; BD Biosciences). TNF-α-producing cell populations were calculated by flow cytometric analysis. Blood inflammatory monocytes were characterized as CD45<sup>high</sup>CD11b<sup>high</sup>F4/80<sup>high</sup>, and CD64<sup>high</sup>. Blood PMLs were characterized as CD45<sup>high</sup>CD11b<sup>high</sup>F4/80<sup>high</sup>Ly6G<sup>high</sup>.

Differentiation of BMDMs

Eight- to 10-wk-old donor mice were sacrificed, sprayed with 70% ethanol. Then, both femurs and tibias were rinsed with sterile PBS, separated, and opened. Bone marrow cells were flushed with a 27-gauge needle and passed through a 40-μm cell strainer. Pelleted cells were resuspended in RPMI 1640 medium supplemented with 10% FBS, antibiotics (ciprofloxacin, 10μg/ml), and GM-CSF (50 ng/ml) and plated in T75 cell culture flasks (Greiner Cellstar). BMDMs were used for experiments after 10–12 d of differentiation. After 5 d, the resulting cells are CD45<sup>high</sup>, CD11b<sup>high</sup>Ly6G<sup>high</sup>. CD45<sup>high</sup>CD11b<sup>high</sup>Ly6G<sup>high</sup>. CD64<sup>high</sup>.

In vitro BMDM stimulation and intracellular TNF-α staining

Differentiated BMDMs were plated in 24-well plates (400,000 cells/well; Corning Costar), treated with BD GolgiPlung Protein Transport Inhibitor (BD Biosciences), and stimulated for 5 h at 37°C with hS. aureus (10<sup>⁷</sup> or 10<sup>⁶</sup>/ml). Next, cells were harvested and cell-surface Ags were stained with Abs against CD45 and CD11b. Then cells were fixed, permeabilized (Fixation/Permeabilization Solution; BD Biosciences), and stained for TNF-α (anti-mouse TNF-α allophycocyanin; BD Biosciences) intracellularly. TNF-α–producing cell populations were calculated by flow cytometric analysis.

In vitro BMDM stimulation, RNA preparation, and quantitative real-time PCR

Differentiated BMDMs were plated in 24-well plates (400,000 cells/well; Corning Costar) and stimulated for 3 h at 37°C with hS. aureus (10<sup>⁷</sup> or 10<sup>⁶</sup>/ml). Total RNA was extracted using the RNeasy mini kit according to instructions manual (Qiagen). Quantitative real-time PCR (qRT-PCR) was performed as previously described (20).

Ex vivo stimulation of dermal Mφs, RNA preparation, and qRT-PCR

Skin of wt, MyD88<sup>−/−</sup>, or UNC-93B (3D) mice was sterilized with 70% ethanol and subjected to enzymatic digestion by sterile skin digestion solution as described earlier. After 2 h incubation at 37°C and 1400 rpm, samples were filtered, washed with PBS, and stained with anti-mouse CD45 eFluor450 (eBioscience), anti-mouse CD11b PE-Cy7 (eBioscience), and anti-mouse F4/80 allophycocyanin (AbD Serotec). CD45<sup>high</sup>CD11b<sup>high</sup>F4/80<sup>high</sup> cells were sorted by FACS (MoFlo Astrios), resuspended in RPMI 1640 medium with 10% FBS plus antibiotics (ciprofloxacin, 10 μg/ml), and plated in 48-well plates (30,000 cells/well; Corning Costar). The next day, adherent Mφs were washed once and stained with medium containing hS. aureus (5 × 10⁷/ml) for 2 h at 37°C. Total RNA was extracted using the RNeasy mini kit according to instructions manual (Qiagen). qRT-PCR was performed as previously described (20).

Cytokine production by dermal Mφs and dermal PMLs after stimulation in vitro

Wt and MyD88<sup>−/−</sup> mice were infected i.d. with ~10<sup>⁷</sup> CFU S. aureus. After 24 h, the skin was subjected to enzymatic digestion as described earlier. Then samples were filtered, washed with PBS, and stained with anti-mouse CD45 eFluor450 (eBioscience), anti-mouse CD11b PE-Cy7 (eBioscience), anti-mouse Ly6G FITC (BD Pharmingen), and anti-mouse F4/80 allophycocyanin (AbD Serotec). CD45<sup>high</sup>CD11b<sup>high</sup>Ly6G<sup>high</sup>F4/80<sup>high</sup> dermal Mφs, as well as CD45<sup>high</sup>CD11b<sup>high</sup>Ly6G<sup>high</sup>F4/80<sup>high</sup> dermal PMLs, were sorted by FACS (MoFlo Astrios). Immediately after sorting, total RNA was extracted with the RNeasy mini kit according to instructions manual (Qiagen). qRT-PCR was performed as previously described (20).

Results

MyD88 is essential for the skin immune response to S. aureus

First, we analyzed the phagocyte subset–specific contribution of MyD88 to development and resolution of a defined i.d. staphylococcal infection. Dose-finding experiments revealed 10<sup>⁷</sup> CFU S. aureus to induce a localized inflammation followed by spontaneous healing of the skin in 7–9 d in C57BL/6 mice. Sequential analysis of infiltrating PMLs, as well as staphylococci, revealed lower numbers of PMLs in MyD88<sup>−/−</sup> mice as compared with wt mice at day 2 postinfection (p.i.; Fig. 1A). Yet, whereas PML decreased in wt mice from day 3 onward, MyD88<sup>−/−</sup> mice showed increasing PML numbers until day 6 (Fig. 1A). Notably, despite excessive PML infiltration, MyD88<sup>−/−</sup> mice failed to clear the bacteria (Fig. 1B). Accordingly, we wondered whether MyD88<sup>−/−</sup> PMLs were recruited, but not activated. To this end, we analyzed IL-1β levels of wt and MyD88<sup>−/−</sup> mice at various time points of infection, because IL-1β has been shown to be predominantly formed by PMLs in soft tissue infections in mice (21) (Fig. 1C). Three days p.i., IL-1β concentrations in the wt skin were strongly upregulated and correlated with PML numbers. Surprisingly, 6 d p.i., levels of IL-1β in infected MyD88<sup>−/−</sup> mice exceeded levels found in wt mice. These levels correlated with PML numbers, confirming that PMLs are the primary IL-1β source in the infected skin. The spatial relationship of Mφs, PML, and staphylococci was further studied by immunohistochemistry. One, 2, and 6 d after i.d. infection of wt and MyD88<sup>−/−</sup> mice with 10⁷ CFU S. aureus, the skin was bisected at the infection site, embedded, and the cryosections were stained (Fig. 1D, 1E, and Supplemental Fig. 1). Both wt and MyD88<sup>−/−</sup> mice showed abscesses with granulocytes surrounded by Mφs (Fig. 1D, higher magnifications). Abscesses were generally larger in wt as compared with MyD88<sup>−/−</sup> mice; however, the latter showed a much increased bacterial burden (Fig. 1D, arrowheads highlighting the bacterial front) as compared with wt mice. Immunofluorescence staining showed F4/80<sup>high</sup> Mφs at the abscess margin in both wt and MyD88<sup>−/−</sup> mice (Fig. 1E). In contrast, CD63<sup>high</sup> T cells were found intraepidermally but were not associated with bacteria either in wt or in MyD88<sup>−/−</sup> mice (Fig. 1E).

Accordingly, abundant and highly activated PMLs failed to timely kill staphylococci and to clear the infection in MyD88<sup>−/−</sup> mice. This suggested that MyD88 in other cells than PMLs determines the immune response to S. aureus and that the MyD88-dependent defect in these cells could not be fully compensated for by highly activated PMLs.

Next, we aimed at dissecting bacterial viability and proliferation from PML recruitment by challenging mice i.d. with 10⁸ fixed S. aureus. Notably, at very early stages of infection (4 h p.i.), fixed S. aureus were as potent as viable bacteria in recruiting PMLs to the skin (Supplemental Fig. 2A). As compared with wt mice, MyD88<sup>−/−</sup> mice showed a substantially delayed PML recruitment (Fig. 1F). To confirm that delayed PML recruitment was not a strain-specific characteristic of the Newman strain, we infected wt and MyD88<sup>−/−</sup> mice i.d. with 10⁷ CFU of the distinct serotype 8 S. aureus strain SA 113. We found a similar MyD88 dependence of PMLs infiltrating after 24 h as described with strain Newman (Fig. 1G). Accordingly, MyD88 is important for the early PML recruitment to the site of infection independent of bacterial proliferation and toxin formation. We hypothesized that the skin phenotype in MyD88<sup>−/−</sup> mice, and potentially that in humans, is largely due to defects in resident cells and cannot be
compensated for, or may even be aggravated, by highly active PMLs.

**Ly6C<sup>high</sup> monocytes are involved in the cellular skin immunity to S. aureus**

Next, we wondered whether monocytes were essentially involved in mediating inflammation in staphylococcal skin infection. To address this question, we exploited the fact that the CCR2 is essential for bone marrow emigration of inflammatory monocytes, and thus indispensable for their recruitment and effector function in the periphery (22). CCR2<sup>−/−</sup> mice have significantly reduced numbers of Ly6C<sup>high</sup> inflammatory monocytes (23). Accordingly, wt and CCR2<sup>−/−</sup> mice were i.d. inoculated with 10<sup>7</sup> CFU <i>S. aureus</i> into the left ear pinna and analyzed after 2, 3, and 6 d for (A) skin PML numbers (Ly6G<sup>high</sup> skin cells gated on CD45<sup>high</sup>CD11b<sup>high</sup> cells) by FACS for (B) CFU per lesion (dilution series plated from homogenized skin tissue) and for (C) skin IL-1β levels. (D and E) Eight-micrometer skin cryosections from wt and MyD88<sup>−/−</sup> mice stained with either H&E (D) or immunofluorescence (E) 1 d after i.d. infection with 10<sup>7</sup> CFU <i>S. aureus</i>. (F) wt and MyD88<sup>−/−</sup> mice were i.d. inoculated with 10<sup>3</sup> to 10<sup>6</sup> CFU <i>S. aureus</i> (SA 113) into the left ear pinna and analyzed after 24 h for skin PML numbers (Ly6G<sup>high</sup> skin cells gated on CD45<sup>high</sup>CD11b<sup>high</sup> cells) by FACS. Groups of three to four mice were used per experiment followed by at least one repetition to confirm the results. All the data are mean ± SEM. Original magnification ×20 (D and E). *p < 0.05, **p < 0.01 (two-tailed unpaired t test).

**FIGURE 1.** MyD88 is essential for the skin immune response to <i>S. aureus</i> wt, and MyD88<sup>−/−</sup> mice were i.d. inoculated with 10<sup>7</sup> CFU <i>S. aureus</i> (Newman) into the left ear pinna and analyzed after 2, 3, and 6 d for (A) skin PML numbers (Ly6G<sup>high</sup> skin cells gated on CD45<sup>high</sup>CD11b<sup>high</sup> cells) by FACS for (B) CFU per lesion (dilution series plated from homogenized skin tissue) and for (C) skin IL-1β levels. (D and E) Eight-micrometer skin cryosections from wt and MyD88<sup>−/−</sup> mice stained with either H&E (D) or immunofluorescence (E) 1 d after i.d. infection with 10<sup>7</sup> CFU <i>S. aureus</i>. (F) wt and MyD88<sup>−/−</sup> mice were i.d. inoculated with 10<sup>3</sup> to 10<sup>6</sup> CFU <i>S. aureus</i> (SA 113) into the left ear pinna and analyzed after 24 h for skin PML numbers (Ly6G<sup>high</sup> skin cells gated on CD45<sup>high</sup>CD11b<sup>high</sup> cells) by FACS. Groups of three to four mice were used per experiment followed by at least one repetition to confirm the results. All the data are mean ± SEM. Original magnification ×20 (D and E). *p < 0.05, **p < 0.01 (two-tailed unpaired t test).
bacterial clearance (Fig. 3B), and higher local IL-1β concentrations (Fig. 3C) as compared with skin treated with control liposomes. Moreover, PML recruitment was substantially delayed, when clodronate-treated mice were challenged with fixed bacteria (Fig. 3D). As an alternative approach, dermal Mφs were depleted in transgenic CD11b.DTR mice with diphtheria toxin (25 ng/g body weight) 48 h before S. aureus infection. We found this procedure to deplete F4/80high Mφs by 60–70%, whereas circulating PMLs and monocytes were not affected (Supplemental Fig. 3C). Notably, CD11b.DTR mice developed increased PML numbers in the blood after DT treatment (data not shown). In striking analogy with the situation after clodronate treatment, skin PML numbers were reduced early after staphylococcal challenge (Fig. 3E). Thus, very similar to the situation in MyD882/2 mice, mice devoid of dermal Mφs show a delayed, but then overwhelming PML response that could not control the infection in a timely fashion. The similarity between Mφ-depleted and MyD882/2 mice in S. aureus skin infection suggests that dermal Mφs are recruiting blood PMLs to the site of infection via a MyD88-dependent, and Mφ-specific, signaling pathway. To substantiate this model, we prepared bone marrow chimeric mice (wt → wt and wt → MyD882/2), challenged them with 108 fixed S. aureus per mouse ear, and analyzed skin PML numbers after 4 h (Fig. 3F). MyD882/2 mice transplanted with wt bone marrow mimicked conventional MyD882/2 mice with respect to delayed PML recruitment to the site of infection (p = 0.0566). Of note, only mice with a blood cell chimerism of >90% were used for infection experiments. Taken together, these data strongly suggest that sensing of S. aureus by resident dermal Mφs via the TLR adaptor protein MyD88 determines the course of PML recruitment and bacterial killing over days p.i.

**FIGURE 2.** Ly6C<sup>high</sup> monocytes are involved in the cell immune response to S. aureus. (A) Exemplary FACS blots gated on wt CD45<sup>high</sup>CD11b<sup>high</sup> cells before (0 h) and after (48 h) i.d. infection with 10<sup>7</sup> CFU S. aureus. (B) Proportion of skin and blood Ly6C<sup>high</sup> inflammatory monocytes in the course of skin infection in wt and CCR2<sup>−/−</sup> mice. (C) wt and CCR2<sup>−/−</sup> mice were i.d. inoculated with 10<sup>8</sup> hf S. aureus, and skin PML numbers (Ly6G<sup>high</sup> skin cells gated on CD45<sup>high</sup>CD11b<sup>high</sup> cells) were analyzed after 4 h by FACS. (D) wt and CCR2<sup>−/−</sup> mice were i.d. inoculated with 10<sup>7</sup> CFU S. aureus and analyzed after 2 and 5 d for skin PML numbers. Groups of three to four mice were used per experiment followed by at least one repetition to confirm the results. All data are mean ± SEM. **p < 0.01, ***p < 0.001 (two-tailed unpaired t test).
this, we stimulated primary blood leukocytes from wt and MyD88^{2/2} mice with S. aureus, and TNF-α production was analyzed by FACS (Fig. 4A). Surprisingly, Ly6C^{high} inflammatory monocytes, but not Ly6G^{high} PMLs, showed an MyD88 dependency of the S. aureus–induced TNF-α response. This suggested that inflammatory monocytes, as well as blood PMLs, were important producers of TNF-α in response to S. aureus. With respect to the role of MyD88 in the response to staphylococci, inflammatory monocytes behaved like BMDMs, where MyD88 was absolutely required for the TNF response (Fig. 4B).

Given the variable role of MyD88 in the myeloid subset–specific TNF response to staphylococci, we next analyzed primary dermal MΦs in this context. We purified CD45^{high}CD11b^{high}F4/80^{high} Ly6G^{low} MΦs from wt and MyD88^{2/2} mice by FACS sorting (Fig. 4D). We found dermal MΦs to be CD64^{high}, CD36^{high}, CD68^{high}, CD16^{high}, CD11b^{high}, cx3cr1^{pos}, MHC class II^{high}, CD207^{low}, and CD11c^{low} (Fig. 4E). Stimulation of dermal MΦs with S. aureus ex vivo (Fig. 4C) revealed a strict MyD88 dependency of transcriptional inflammatory cytokine activation (as determined by qRT-PCR for pro–IL-1β and TNF-α). To assess the activation of myeloid cell subsets in vivo, we sorted dermal MΦs and skin-infiltrating PMLs from wt and MyD88^{2/2} mice, which had been challenged for 1 d with S. aureus, and performed qRT-PCR for cytokine transcripts (Supplemental Fig. 2C, 2D). We found that staphylococci induce transcription of both TNF and IL-1β in dermal MΦs and PMLs infiltrating the dermis. In contrast with PMLs purified from mouse blood and challenged in vitro, the in vivo cytokine response of infiltrating PMLs appeared to be MyD88 dependent, although the differences did not reach statistical significance and the role of MyD88 was larger in dermal MΦs (Fig. 4C and Supplemental Fig. 2C, 2D). Thus, resident dermal MΦs are likely to regulate the activity of infiltrating PMLs in an MyD88-dependent fashion in trans. Notably, the important role of MyD88 in the MΦ response to staphylococci was not restricted to the Newman strain, but was similarly apparent when strain SA 113 was used (Supplemental Fig. 4).
Resident dermal Mφs are sensing S. aureus independently of endosomal TLRs

It has been reported previously that ssRNA from S. aureus, as well as many other Gram-positive bacteria, is an essential effector for the activation of inflammatory cytokines in BMDMs (13, 31). Endosomal TLRs are most likely key RNA sensors in Mφs. This assumption was based on the exclusive role of the endoplasmic reticulum membrane protein UNC-93B, which is crucial for endosomal localization of nucleotide-sensing TLRs. In UNC-93B (3D) mice, a single point mutation (H412R) abolishes signaling via TLR3, TLR7, and TLR9 (32). In concordance with these data, S. aureus–induced expression of pro–IL-1β was largely UNC-93B dependent in BMDMs (Fig. 5A). In contrast, inflammatory monocytes and PMLs mounted a robust TNF response to S. aureus in an UNC-93B–independent fashion (Fig. 5B). Moreover, and even more important in the context of this study, dermal Mφs from wt and UNC-93B (3D) mice were indistinguishable in the cytokine response to S. aureus ex vivo (Fig. 5C). This finding suggested that Mφs receive an imprinting by their microenvironment that determines the usage of distinct TLRs when in contact with bacteria. To further address whether sensing of nucleotide acids by endosomal TLRs plays a role in PML recruitment and bacterial killing in S. aureus skin infection, we infected wt and UNC-93B (3D) mice with either fixed S. aureus (Newman or SA 113) or viable S. aureus. We analyzed PML numbers after 4 h (fixed bacteria, Fig. 5D and Supplemental Fig. 4D) or after 2 and 5 d (viable bacteria; Fig. 5E). Moreover, we analyzed the bacterial load and the IL-1β response in these mice (data not shown). UNC-93B–deficient mice showed a slightly faster PML recruitment and the same bacterial killing capacity, as well as IL-1β production, as compared with wt mice. This suggests that sensing of nucleic acids via endosomal TLRs does not play an important role in innate skin immunity to S. aureus. Again, this observation was true for both the Newman strain and the strain SA 113. These data are consistent with the notion that dermal Mφs are sensing S. aureus independently of endosomal TLRs.

TLR2 is dispensable for the early immune response of dermal Mφs against S. aureus

In view of the redundancy of endosomal TLRs in staphylococcal sensing, we wondered whether TLR2 may be important in mediating the early MyD88-dependent response. Thus, we made use of S. aureus with a genetic deletion of the lipoprotein transferase lgt. In these bacteria, the putative TLR2-activating diacylated...
lipoproteins are not formed (33). First, we compared fixed Δlgt
\( S. aureus \) with the isogenic wt strain for its TLR2 activation in
epithelial cells and found deletion of lgt to abrogate the TLR2-
specific IL-8 response (Supplemental Fig. 2B). Next, we ana-
alyzed whether early (4 h) recruitment of PMLs differed between
Δlgt \( S. aureus \) and the isogenic wt strain. Importantly, we used
fixed \( S. aureus \), which induce an early dermal inflammatory re-
sponse that is very similar to that induced by live bacteria
(Supplemental Fig. 2). Thereby we excluded TLR2-independent
effects of the lgt disruption, for example, those related to iron
acquisition and bacterial persistence (34). We found that Δlgt
\( S. aureus \) and isogenic wt were equally potent in cytokine induction
in inflammatory monocytes and dermal M\( \theta \)s (Fig. 5F, 5G). Using
Δlgt \( S. aureus \) offered the opportunity to test whether TLR2 and
dermal TLRs were redundant in their recognition of \( S. aureus \),
thereby compensating for the lacking engagement of the other
receptor group. Accordingly, we abrogated both TLR2 and
dermal TLR activation by infecting UNC-93B–deficient mice with
Δlgt \( S. aureus \) (Fig. 5H). Notably, we did not observe a dif-
cference between wt mice infected with wt \( S. aureus \), UNC-93B–
deficient mice infected with Δlgt \( S. aureus \), and all other combi-
nations. Accordingly, both TLR2 engagement by lipoproteins
and endosomal TLR activation by staphylococcal nucleic acids cannot
explain the important role of MyD88 in the defense against
staphylococci.

**Skin infection leads to expansion of resident dermal M\( \theta \)s**

As outlined earlier, our model of a localized staphylococcal skin
infection is associated with dramatic quantitative and qualitative
changes in cellular innate immunity at site. After the infection
has been controlled, immunity has to contract to allow for re-
establishment of cellular homeostasis. Our model revealed that
the kinetics of this resolution are heavily dependent on MyD88.
dermal Mφs initially decreased but expanded in the later course of infection (Fig. 6A, 6B). Notably, MyD88 deletion did not affect Mφ numbers over the entire course of infection, in contrast with PML and monocyte numbers (Fig. 6C). In sharp contrast, CCR2−/− mice failed to replace and expand Mφs until day 7 of infection (Fig. 6E). Therefore, Ly6C<sup>high</sup> inflammatory monocytes contribute to the expansion of dermal Mφs, but this has no apparent functional consequences for the host response to <i>S. aureus</i>. In contrast, Nr4a1<sup>−/−</sup> mice, which are deficient in circulating Ly6C<sup>low</sup> patrolling monocytes (35), showed normal numbers of Mφs during the course of infection (Fig. 6D). These data indicate that resident dermal Mφs are expanding after staphylococcal infection without contribution of circulating Ly6C<sup>low</sup> patrolling monocytes and the TLR adaptor protein MyD88, but in a clear dependency on Ly6C<sup>high</sup> inflammatory monocytes.

**Discussion**

In staphylococcal skin infection, resident dermal Mφs are sensing skin invading staphylococci in a strictly MyD88-dependent, but TLR2- and endosomal TLR–independent, fashion. Proper Mφ function is essential for activation and regulation of skin-infiltrating PMLs. Re-establishment of myeloid cell homeostasis at the site of staphylococcal infection requires early Mφ-specific, MyD88-dependent sensing, timely containment of bacteria, and Mφ renewal by incoming Ly6C<sup>high</sup> inflammatory monocytes.

The TLR system plays an important role in host resistance against staphylococci. Mice that lack the TLR adaptor protein MyD88 are highly susceptible to systemic infections (29). They develop larger skin lesions with higher bacterial counts and show defective PML recruitment and cytokine production (30). Nevertheless, the cell type and sensing mechanisms involved in the early staphylococcal recognition in the skin remained unclear. Our data uncover resident dermal Mφs as initial sensors of skin-infiltrating <i>S. aureus</i>. Surprisingly, conventional MyD88<sup>−/−</sup> mice and mice with local dermal Mφ deficiency exhibit an almost indistinguishable phenotype in staphylococcal infection. The prominent role of MyD88 in Mφ signaling is furthermore highlighted by the myeloid lineage specificity, that is, PMLs sense staphylococci in a largely MyD88-independent fashion.

**FIGURE 6.** Expansion of resident skin Mφs. Proportion of wt skin CD45<sup>high</sup>CD11b<sup>high</sup>Ly6G<sup>low</sup>F4/80<sup>high</sup> cells (% of total skin cells) before (day 0) and after (days 2, 5, and 7) i.d. injection of 10<sup>7</sup> CFU <i>S. aureus</i>. (A) Exemplary FACS blots gated on CD45<sup>high</sup>CD11b<sup>high</sup> cells. (B) Bar graph demonstrating the proportions of wt myeloid skin cells. (C) Proportion of skin Mφs from wt and MyD88<sup>−/−</sup> mice before (day 0) and after (day 6) i.d. injection of 10<sup>7</sup> CFU <i>S. aureus</i>. Proportion of skin Mφs from wt and Nr4a1<sup>−/−</sup> mice (D) and CCR2<sup>−/−</sup> mice (E) before (day 0) and after (days 2, 5, 6, and 7) i.d. injection with 10<sup>7</sup> CFU <i>S. aureus</i>. All data are mean ± SEM. Groups of three to four mice were used per experiment, followed by at least one repetition to confirm the results. **p < 0.01, ***p < 0.001 (two-tailed unpaired t test). F4/80<sup>high</sup>, resident skin Mφs; Ly6C<sup>high</sup>, inflammatory monocytes.
In contrast with MyD88, the established S. aureus–sensing TLRs were dispensable for a proper dermal Mφ response against staphylococci. This seems noteworthy given the relatively large body of literature on TLR2 and endosomal TLRs in the Mφ response to staphylococci, both in vitro and in vivo (13, 29–31, 36, 37). We made use of staphylococci that lack protein acylation and, therefore, do not activate TLR2. This strain allowed us to analyze lacking TLR2 activation in the context of deficient nucleic acid TLR sensing, which is dependent on the endoplasmic reticulum protein UNC-93B. The experiments revealed that both systems are dispensable without compensating for each other. How can differences to other studies with respect to the engagement of specific TLRs by staphylococci be explained? The answer may lie in the specific animal model that is used. We took greatest care to fine-tune our model in a way that the infection remained localized and self-limiting in 6 d in wt mice. Notably, we did not detect any systemic effects of the infection, such as elevated inflammatory cytokines or bacterial spread, and the mice seemed unaffected by the lesion (with respect to activity and weight gain). It is conceivable that more aggressive skin models like those used by Miller et al. (30) are associated with circulation of TLR2-activating lipopeptides from staphylococci. Furthermore, spreading of staphylococci to other body sites but the skin likely impacts on TLR usage by myeloid cells, which is specific for site of residence or origin. This notion is supported by our observation that BMDMs are strictly dependent on UNC-93B in the recognition of staphylococci, whereas this molecule is dispensable for activation of dermal Mφs. The validity of our model as a reflection of the everyday event in humans, where colonizing bacteria enter the skin through minor lesions in the epidermis, receives support by the fact that patients with MyD88 deficiency, but not those with UNC-93B deficiency or TLR2/TIRAP deficiency, present with staphylococcal skin infections as a leading problem (38). The recent and striking finding that S. aureus stimulates granulopoiesis in skin wounds by engaging TLR2 in hematopoietic stem and progenitor cells (39) further underlines the role of cell lineage-specific preference of TLR2 by staphylococci. In addition, our findings constitute a cautionary note in interpreting data generated in BMDMs, which are the workhorses in many studies addressing cellular innate immunity to bacteria.

This study further demonstrates that Mφ- dependent sensing of staphylococci controls PMLs at the site of infection. PMLs exit the bone marrow in response to inflammatory stimuli and contribute to the fast clearance of bacteria via an elaborate antimicrobial machinery (40, 41). PML-derived IL-1β contributes to abscess formation in S. aureus infection (21). However, we show in this article that, when dermal Mφs fail to immediately recruit PMLs to the site of staphylococcal invasion, the finally arriving and fully activated PMLs fail to timely control the infection. Notably, PMLs respond to S. aureus in a largely MyD88-independent fashion. Thus, although PMLs are clearly essential for the clearance of staphylococcal skin infection, they need to be alerted and directed by resident dermal Mφs for proper function.

Resident dermal Mφs are important components of the skin immune system (7, 8) arising either from the hematopoietic system or the fetal liver and yolk sac (42–44). Although the primary source and replacement of most tissue resident Mφs has been well appreciated, the origin of dermal Mφs, as well as their expansion potential in staphylococcal infection, remains incompletely understood. It is further unclear whether these dermal Mφs constitute a homogenous terminally differentiated cell population, which is arising and replenished exclusively from blood monocytes, or whether they are able to self-renew and differentiate at site without contribution of blood-derived mononuclear cells (16). The CCR2 is essential for bone marrow emigration of Ly6C^{high} monocytes, and thus indispensable for the recruitment and function of these inflammatory myeloid cells (22). However, whereas we found circulating inflammatory monocytes to increase over the entire course of active staphylococcal infection, they did not contribute essentially to initiation or clearance of the infection. It appears that Ly6C^{high} inflammatory monocytes are predominantly responsible for dermal Mφ renewal and expansion in the healing process starting late in infection. Indeed, it seems that the increase in Mφs is not directly linked to the resolution of infection, because CCR2^{−/−} mice do not show an overt defect in the staphylococcal skin model. Accordingly, it appears that the recovery of Mφ numbers rather accompanies than induces termination of the infection. It is further tempting to speculate that a rapid re-establishment of tissue cellularity is critical for the response to a subsequent insult. In contrast with inflammatory Ly6C^{high} monocytes, we did not identify any role for circulating so-called patrolling Ly6C^{low} monocytes, neither in controlling inflammation nor in the replacement of dermal Mφs.

In summary, this study highlights the essential and specific role of early MyD88-dependent sensing by resident dermal Mφs in the timely control and clearing of staphylococcal infection accompanied by the re-establishment of the myeloid cell homeostasis in the skin.

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Disclosures

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