Protective immunity in recurrent *Staphylococcus aureus* infection reflects localized immune signatures and macrophage-conferred memory

Liana C. Chan^{a,b}, Maura Rossetti^{c,d}, Lloyd S. Miller^e, Scott G. Filler^{a,d,f}, Colin W. Johnson^a, Hong K. Lee^a, Huiyuan Wang^a, David Gjertson^{c,d}, Vance G. Fowler Jr.^{g,h}, Elaine F. Reed^{c,d}, Michael R. Yeaman^{a,b,d,f,1}, and the MRSA Systems Immunobiology Group²

^aLos Angeles Biomedical Research Institute, Harbor–University of California, Los Angeles Medical Center, Torrance, CA 90502; ^bDivision of Molecular Medicine, Department of Medicine, Harbor–University of California, Los Angeles Medical Center, Torrance, CA 90502; ^cDepartment of Pathology and Laboratory Medicine, University of California, Los Angeles Immunogenetics Center, Los Angeles, CA 90095; ^dDepartment of Medicine, David Geffen School of Medicine, University of California, Los Angeles, CA 90095; ^eDepartment of Dermatology, The Johns Hopkins University, Baltimore, MD 21231; ^fDivision of Infectious Diseases, Department of Medicine, Harbor–University of California, Los Angeles Medical Center, Torrance, CA 90502; ^gDivision of Infectious Diseases, Department of Medicine, Duke University, Durham, NC 27704; and ^hDuke Clinical Research Institute, Duke University, Durham, NC 27704

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Staphylococcus aureus is the leading cause of skin and skin structure infection (SSSI), a primary portal of entry for invasive infection. Our prior studies discovered a role for protective innate memory against recurrent methicillin-resistant *S. aureus* (MRSA) SSSI. In the present study, the dynamics and mechanisms of this response were explored in recurrent SSSI in WT mice. Priming by prior infection reduced skin lesion severity and MRSA burden, and protected against dissemination at day 7 but not day 2. Cytokine and cellular signatures in SSSI differed at day 2 versus 7, and were distinct in skin versus blood or spleen. Cytokines associated with protection in skin included increased IL-17, IL-6, monokine inducible by IFN- γ (MIG), and RANTES, while increased IP-10 correlated with protection from dissemination. Cellular signatures of protection included increased Th17, M1 macrophage, and dendritic cell populations in abscesses, and total macrophages in lymph nodes. Priming potentiated *S. aureus*-specific phagocytic killing by bone marrow-derived macrophages in vitro, and their adoptive transfer into naïve skin afforded protective efficacy in vivo. Present findings indicate that protective immunity in recurrent *S. aureus* infection is locally targeted, and involves specific memory conferred by macrophages. These insights provide targets for vaccine and immunotherapeutic development against MRSA.

Staphylococcus aureus | innate immune memory | macrophage | recurrent skin infection | adoptive transfer

The most common cause of skin and skin structure infections (SSSI) is *Staphylococcus aureus* (1–5). Skin infection serves as a primary portal of entry for hematogenous dissemination (6, 7). Despite meritorious attempts, the failure of conventional vaccines targeting *S. aureus* infections has raised key questions regarding the determinants of protective immunity versus this pathogen (8, 9). Reinforcing this concern is the high rate of recurring *S. aureus* infection among otherwise healthy individuals who have no known immune deficiencies or risk factors (10–17). The emergence of *S. aureus* resistant to many gold-standard antibiotics further compounds these issues (18–21). Thus, there is an urgent need to understand the relative contributions of innate versus adaptive immunity in defense against recurrent SSSI and ensuing invasive *S. aureus* infection.

We have previously shown that innate immune memory contributes to host defense against recurrent murine methicillin-resistant *S. aureus* (MRSA) SSSI. Using *rag1*^{−/−} mice, which lack mature T and B cells, we discovered that prior infection (priming) resulted in a protective host response involving innate memory. This protection was highly localized to the skin, and its effectors included macrophages and Langerin⁺ dendritic cells (DC), as well as host defense peptides. In the present study, we analyzed the efficacy and mechanisms of this protective immunity in recurrent

MRSA infection in WT mice, focusing on cytokine signatures and cellular effectors of immune memory.

Results

Immune Protection Differs in Local vs. Disseminated Infection. Priming resulted in localized protection, as evidenced by smaller skin lesions over the course of infection compared with naïve-infected controls, as reported previously (Fig. 1A) (22–24). In primed mice, MRSA burden in skin lesions was similar to naïve mice at day 2, but was significantly reduced by day 7. Importantly, while priming achieved protection in both flanks at day 7, lesions on the flank previously infected had the greatest reduction in severity. Priming initially increased the MRSA burden of the kidney and spleen at day 2, but then reduced it in these organs and the liver by day 7 (Fig. 1B–E). Thus, priming enhances the eventual control of infection in

Significance

Staphylococcus aureus (SA) is the leading cause of skin and skin structure infections (SSSI), which are the main portal of entry for life-threatening invasive infections. Treatment failures are increasingly common due to antibiotic resistance. Importantly, SA SSSI exhibit high 1-year recurrence, despite high antibody levels against staphylococcal antigens. Therefore, critical determinants of immune protection against recurrent SA SSSI are not well understood. The present study offers important insights in this area, including: (i) macrophages can confer protective memory transferrable to naïve hosts; (ii) SA SSSI can induce memory in bone marrow myeloid precursors; and (iii) specific memory against recurring SA SSSI is localized and distinct from disseminated infection. These findings will accelerate novel strategies to prevent and treat SA infections.

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Conflict of interest statement: M.R.Y. and S.G.F. are cofounders of NovaDigm Therapeutics, which is developing novel vaccines and immunotherapeutics for infectious diseases, including methicillin-resistant *Staphylococcus aureus*.

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¹To whom correspondence should be addressed. Email: MRYeam@ucla.edu.

²A complete list of the MRSA Systems Immunobiology Group can be found in the SI Appendix.

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Fig. 1. Priming affords protection during recurrent MRSA SSSI. Lesion sizes of naïve and primed male C57BL/6 mice (A) were measured at days 2 and 7. MRSA burden in abscesses (B), kidney (C), spleen (D), and liver (E) were quantified at days 2 and 7. Bacterial burden in abscess, kidney, and spleen were quantified as CFU per abscess/organ. Burden in the liver was quantified as CFU per milligram liver. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ using Student's t test. At least four mice per group were analyzed.

the target organs. Collectively, these results suggest that priming affords protective immunity that is targeted, and manifests at later time points in the evolution of the infection.

Cytokine Signatures of Protection Differ in Spatial Contexts. Unsupervised proteomic analyses comparing cytokine patterns in skin, plasma, and spleen revealed distinct patterns during MRSA SSSI and invasive infection at day 2 vs. day 7. These patterns clustered into four groups: skin, plasma, spleen, and uninfected control skin (Fig. 2). In this analysis, the primed mice clustered with the naïve mice. As expected, uninfected control mice had minimal variations in the expression of most cytokines or chemokines detected. In supervised analyses, infection in skin resulted in increased proinflammatory cytokines (e.g., IL-1 β , IL-6, TNF- α), chemokines (e.g., MIP-1 α/β , MIP-2, KC), and markers of proliferation or differentiation (e.g., G-CSF, VEGF), compared with spleen or plasma (SI Appendix, Fig. S1). Notably, no specific pattern of IFN- γ response was detected. In comparison with skin, no specific patterns of cytokine responses were observed in the spleen or blood. Taken together, these results suggest that protective cytokine signatures are distinct for specific tissues during MRSA SSSI.

Priming Induces Specific Cytokine Signatures Corresponding to Protective Immunity. Specific cytokine signatures correlated with protective immunity. In skin, priming resulted in increased IL-6 expression at day 2, followed by increased IL-17 expression at day 7 (Fig. 3 A and B). Additionally, priming led to increased MIG (monokine inducible by IFN- γ) and RANTES (regulated upon activation, normal T cell expressed and secreted) in skin at day 7, cytokines that are involved in T cell recruitment and maintenance

(Fig. 3 C and D). During disseminated infection, priming resulted in decreased IL-9 in the spleen and increased IP-10 in the plasma (Fig. 3 E and F). These results support a central role for Th17 responses in protective immunity of the skin, but not in hematogenous dissemination. More broadly, this pattern of findings is consistent with the hypothesis that protective immunity to MRSA involves molecular signals that correspond to specific tissue context and progression of infection.

Cellular Signatures of Protection Differ in Tissue-Specific Contexts.

Cellular responses were analyzed at day 7 during recurrent MRSA infection, as this later time point corresponded with protection observed in primed mice (Fig. 1). Priming caused an increase of M1 macrophage and DC populations in skin abscesses, but a decrease in the total B cell population in skin (Fig. 4A). Concordant with increased IL-17 expression in skin abscesses, priming also increased the relative proportion of Th17 cells in skin, but not that of Th1 or Th2 cells, compared with naïve controls (Fig. 4A). Priming did not significantly change M2 macrophage, neutrophil, or NK cell populations in abscesses (Fig. 4A). Consistent with localized responses in the skin, draining inguinal lymph nodes (iLN) had an increased total macrophage population, but decreased B and NK cell populations (Fig. 4B). No significant changes were found in total T cell, polymorphonuclear leukocyte (PMN), or DC populations in the iLN of primed vs. naïve groups (Fig. 4B). Similar to cytokine results, no significant differences were detected in cell populations in the spleens of primed vs. naïve mice (SI Appendix, Fig. S2).

Macrophages Confer Protective Immunity Against MRSA. The preceding results suggested macrophages present in the skin and iLN of primed mice may be involved in protective immune memory against MRSA. To investigate this hypothesis, the role of bone marrow-derived macrophages (BMDM) from naïve and primed mice were analyzed using in vitro and in vivo models (SI Appendix, Fig. S3).

Priming increases macrophage staphylocidal activity in vitro. First, BMDM from naïve and primed mice were compared for their capacity to phagocytose and kill *S. aureus* with and without recall exposure to *S. aureus*. Phagocytosis of MRSA by BMDM from naïve and primed animals with and without exposure to *S. aureus* was equivalent (SI Appendix, Fig. S4). However, staphylocidal activity was greater in BMDM from primed mice vs. naïve mice as measured by survival of intracellular MRSA, if preexposed to heat-killed *S. aureus* (HKSA) (Fig. 5A). Interestingly, primed and naïve BMDMs preexposed to live *S. aureus* (LSA) were equivalent in their capacity to kill MRSA, as preexposure to LSA induced near maximal MRSA killing in BMDM. Furthermore, preexposure of BMDM to HKSA or LSA resulted in lower intracellular MRSA survival compared with untreated BMDM (Fig. 5A). Next, primed and naïve BMDM were tested for specific killing against comparative staphylococci, Gram-positive, and Gram-negative bacteria. Interestingly, BMDM primed with LAC-USA300 also exerted greater staphylocidal activity against a distinct *S. aureus* strain (SH1000) compared with untreated naïve BMDM (Fig. 5B). This protection was specific to *S. aureus*, as priming with *S. aureus* did not enhance macrophage efficacy against *Staphylococcus epidermidis* (distinct staphylococcal species), *Enterococcus faecalis* (Gram-positive bacterium of different genus), or *Escherichia coli* (Gram-negative bacterium) (Fig. 5 C–E). These in vitro data support the concept that priming of BMDM during in vivo infection affords greater antistaphylococcal activity specifically on recall to *S. aureus*.

Adoptive transfer of primed macrophages confers protective immunity in vivo. Next, we determined the in vivo relevance of primed macrophages in protection against recurring SSSI. BMDM from naïve or primed mice were recalled with either HKSA or LSA and then adoptively transferred into naïve recipient mice before

Fig. 2. MRSA SSSI results in distinct cytokine signatures in specific tissue contexts. Cytokine levels in skin, spleen, and plasma were analyzed using Luminex 32-plex assays. Data from day 2 (A), day 7 (B), and days 2 and 7 combined (C) were arranged via unsupervised clustering. Means of scaled values for each group were color-coded and plotted in heat maps. Unsupervised hierarchical clustering was performed on rows and columns using Euclidean distance as the similarity measure with Ward's linkage. At least four mice per group were analyzed.

MRSA SSSI challenge (*SI Appendix, Fig. S3B*). Transfer of BMDM from primed mice recalled by in vitro preexposure to LSA, but not untreated or HKSA, significantly reduced the abscess dermonecrosis area compared with transfer of BMDM from naïve mice (Fig. 6A). In the primed group, only mice receiving BMDM recalled with LSA achieved reduced abscess severity compared with controls re-

ceiving untreated BMDM (Fig. 6B and C). Consistent with this outcome, only BMDM from primed mice recalled with LSA, but not HKSA, afforded reduced MRSA burden in abscesses compared with control (Fig. 6D and E). Both naïve and primed BMDM recalled by exposure to either HKSA or LSA significantly reduced MRSA burden in kidney, compared with mice that did not receive

Fig. 4. Priming induces distinct changes in localized cell populations during recurrent MRSA SSSI. T cell, macrophage, neutrophil (PMN), DC, B cell, and NK cell populations were analyzed and expressed as percent of total CD45⁺ cells in the skin (A) and draining iLNs (B). T cell subsets (Th1, Th2, and Th17) were expressed as percent of total CD3⁺ T cells. Macrophage subsets (M1 and M2) were expressed as proportion of total macrophages. **P* < 0.05, ***P* < 0.01 using Student's *t* test. At least six samples per group were analyzed.

macrophages, to develop memory that can be specifically recalled and transferred (48–50).

There are limitations to the interpretation of the present work. Mouse models are imperfect surrogates for studying human infection, and further studies are needed to understand protective immune memory in human *S. aureus* SSSI. Moreover, the extensive scope of investigations performed required a focus on one genetic background of mice. Parallel studies in other mouse strains will be informative to further dissect the specific genotypic and phenotypic determinants of protective immunity to *S. aureus*. Finally, we did not exhaust the cell types that may contribute to the observed immune memory. For example, the complete repertoire of cells responsible for production of increased IL-17A at the abscess site was beyond the scope of the present study. Dillen et al. (51) recently demonstrated that $\gamma\delta$ T cells are important producers of IL-17A in protective immune responses against *S. aureus* infection of the skin. Our ongoing studies are further defining the molecular and cellular mechanisms essential to protective immune memory against *S. aureus* infection. This knowledge will enable new anti-infective, vaccine, and immunotherapeutics strategies to meet the challenge of antibiotic-resistant MRSA infections.

In conclusion, current studies support the hypothesis that immune memory is integral to protection against recurrent MRSA SSSI. The mechanisms through which this protection occurs involve early innate and adaptive cytokine responses that shape later Th17 polarization and M1 macrophage intensification in the

skin. Furthermore, protective responses in the skin were localized and distinct from those of disseminated infection. Ultimately, the knowledge gained through this work and investigating epigenetic markers that may be essential to the protective mechanisms of primed BM cells and macrophages should provide important insights and strategies into new, targeted anti-infectives and vaccines to meet the challenge of antibiotic-resistant MRSA infections.

Methods

Bacterial Strains and Preparation. This study utilized MRSA strain LAC, a USA300 strain isolated from an outbreak at the Los Angeles County Jail and the phenotypic and genotypic profile of this strain has been thoroughly characterized previously (52). Studies also included methicillin-sensitive *S. aureus* strain SH1000 [a well-characterized laboratory strain (53)], *S. epidermidis* (ATCC 12228), *E. faecalis* (ATCC 29212), or *E. coli* (ATCC 43889). Bacteria were cultured from virulence-validated master cell banks and grown to log-phase in BHI medium at 37 °C. Resulting cells were harvested, washed, suspended in PBS, sonicated, quantified by spectrophotometry, and diluted to the desired CFU in PBS or RPMI for use in vitro or in vivo. To generate heat-killed LAC, bacteria were prepared as described above and boiled for 30 min.

Mouse of Model of Recurrent SSSI. Animal studies were performed in accordance with approved animal use policies of the Los Angeles Biomedical Research Institute at Harbor–University of California, Los Angeles following NIH guidelines. Male C57BL/6 (WT) mice (20–25 g; Jackson Labs) were studied using a subcutaneous SSSI model as we have previously detailed (22–24). For primary infection, SSSI was established via inoculation with $\sim 1 \times 10^7$ CFU

Fig. 6. Adoptive transfer of primed BMDM recalled with LSA protects naive mice from localized and disseminated MRSA SSSI. BMDM from naive (filled symbols) and primed (open symbols) mice treated with PBS (squares), HKSA (triangles), or LSA (diamonds) was adoptively transferred into the skin of naive mice. Four-hours postadoptive transfer, mice were inoculated with MRSA subcutaneously (*SI Appendix, Fig. S3B*). Dermonecrosis area was measured at days 1, 3, 5, and 7 (A–C). Adoptive transfer of primed BMDM recalled with LSA resulted in smaller lesions compared with naive BMDM recalled with LSA (A). Adoptive transfer of BMDM from primed (B) and naive (C) mice ($\pm$ SA treatment) were compared (red asterisk = LSA vs. no macrophage; black asterisk = LSA vs. untreated macrophage). At day 7, MRSA CFU was quantified from abscesses (D and E), kidneys (F and G), and spleens (H and I). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ using one-way ANOVA corrected for multiple comparison. At least four mice per group were analyzed.

MRSA Tissue Burden. At the 2- or 7-d study endpoint, mice were killed and skin abscesses, kidneys, spleen, and liver were enumerated *ex vivo* via CFU after overnight culture on plates (lower limit of detection = 20 CFU per sample). In parallel, a portion of tissue homogenate was reserved for cytokine analysis as detailed below.

Luminex xMAP Immunoassay. Mouse 32-plex magnetic cytokine/chemokine kits (EMD Millipore) were used per the manufacturer's instructions and fluorescence quantified using Luminex 200 instrument by the University of California, Los Angeles Immune Assessment Core. Cytokine/chemokine concentrations were calculated using Milliplex Analyst software v4.2 (EMD Millipore). Tissue homogenates were normalized by total protein content to 330 μ g/mL by Coomassie Protein Assay Kit (Thermo Scientific).

Cytokine Heat Maps. For hierarchical clustering analysis, analyte abundances irrespective of conditions were normalized using z-scaling. Then, means of scaled values for each group were color-coded and plotted in heat maps. Unsupervised hierarchical clustering was performed on rows and columns using Euclidean distance as the similarity measure with Ward's linkage. R v3.3.1 was used (54).

Immune Cell Subset Profiles. iLNs and spleen of each mouse were mechanically dissociated and cell isolated (23). Skin abscesses were processed and cells isolated per protocol (55). Briefly, skin abscesses were aseptically dissected and placed into skin isolation buffer (HBSS, 10% FBS, 5 mM EDTA, 10 mM Hepes) for 30 min at 150 rpm. Abscesses were placed into fresh media containing collagenase D, chopped, and incubated for 30 min at 150 rpm.

Skin fragments were removed and cells isolated from media. Cells were stained with fluorochrome-conjugated antibodies (*SI Appendix, Table S1*) and analyzed by multicolor flow cytometric analysis using an LSRII cytometer (Becton-Dickinson). Intracellular molecules were accessed for staining using an established protocol for cell permeabilization (Thermo Fisher). Gating strategies can be found in *SI Appendix, Fig. S5*.

BMDM. BMDM were differentiated as previously described (56). Briefly, BM cells were isolated from femurs and tibias of naïve or primed WT mice. Cells were differentiated in BMDM media [DMEM/F12, 10% FBS, 25 mM L-glutamate, penicillin/streptomycin, 500 U/mL recombinant mouse M-CSF (Biolegend)]. On day 10, cells were challenged with heat-killed MRSA or live MRSA [multiplicity of infection (MOI) 1:1] and permitted to acclimate for 4 d. The BMDM were used on day 14. Experimental design for BMDM differentiation and subsequent use in experiments can be found in *SI Appendix, Fig. S3B*.

BMDM Intracellular Survival Assay. The BMDM (5×10^5 cells) were plated on sterile 18-mm glass coverslips and challenged with MRSA. After 30 min, gentamicin (50 $\mu\text{g/mL}$ final concentration) was added to kill extracellular

bacteria. At indicated time points, BMDM were lysed and surviving MRSA CFU were enumerated after overnight culture on plates.

Macrophage Adoptive Transfer in MRSA SSSI. The BMDM ($\sim 5 \times 10^5$ BMDM/100 μL) were adoptively transferred into the right flank of naïve WT mice. Control animals received media alone. Four hours postadoptive transfer, SSSI was established via inoculation with $\sim 1 \times 10^7$ CFU of MRSA by subcutaneous injection into both flanks. Lesions were monitored over 7 d, at which point the mice were killed and the abscesses were excised for quantitative culture. Schematic for experimental design can be found in *SI Appendix, Fig. S3B*.

Statistical Analyses. Differences in experimental results were compared by ANOVA or Student's *t* test as indicated. Data analyzed by GraphPad Prism software and reported as mean $\pm$ SD. *P* values of <0.05 were considered statistically significant.

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