Staphylococcus aureus toxin suppresses antigen-specific T cell responses

Brandon Lee,[1] Reuben Olaniyi,[2] Jakub M. Kwiecinski,[3] and Juliane Bubeck Wardenburg[2]

[1]Committee on Immunology, UChicago Biosciences, University of Chicago, Chicago, Illinois, USA. [2]Department of Pediatrics, Washington University School of Medicine, St. Louis, Missouri, USA. [3]Department of Immunology and Microbiology, University of Colorado School of Medicine, Aurora, Colorado, USA.

***Staphylococcus aureus* remains a leading cause of human infection. These infections frequently recur when the skin is a primary site of infection, especially in infants and children. In contrast, invasive staphylococcal disease is less commonly associated with reinfection, suggesting that tissue-specific mechanisms govern the development of immunity. Knowledge of how *S. aureus* manipulates protective immunity has been hampered by a lack of antigen-specific models to interrogate the T cell response. Using a chicken egg OVA–expressing *S. aureus* strain to analyze OVA-specific T cell responses, we demonstrated that primary skin infection was associated with impaired development of T cell memory. Conversely, invasive infection induced antigen-specific memory and protected against reinfection. This defect in adaptive immunity following skin infection was associated with a loss of DCs, attributable to *S. aureus* α-toxin (Hla) expression. Gene- and immunization-based approaches to protect against Hla during skin infection restored the T cell response. Within the human population, exposure to α-toxin through skin infection may modulate the establishment of T cell–mediated immunity, adversely affecting long-term protection. These studies prompt consideration that vaccination targeting *S. aureus* may be most effective if delivered prior to initial contact with the organism.**

Introduction

Staphylococcus aureus is both a human skin commensal and a leading cause of infection. Skin and soft tissue infection (SSTI) remains the most common form of *S. aureus* disease (1), with an incidence of over 100 cases per 100,000 persons and a cost of more than $4 billion per year in the USA (1, 2). SSTIs can lead to disseminated disease and have exacerbated the health burden of antibiotic resistance (3, 4). Efforts to develop vaccines against *S. aureus* have failed, and correlates of human immunity remain elusive (5).

Recurrence of SSTI can exceed 50% (6, 7) and primarily afflicts those at the extremes of age (2, 8, 9). In contrast, fewer than 20% of patients who recover from invasive disease experience reinfection (7, 10). This dichotomy suggests that staphylococcal immunity depends on the initial infection site and may relate to temporal determinants of exposure. Although the molecular pathogenesis of recurrent infection is poorly understood, host and pathogen factors contribute to susceptibility. Humans harboring defects in neutrophil and T cell function and IL-17 signaling present with recurrent infection (11), an observation corroborated by mouse models that demonstrate

the importance of innate and adaptive immunity (12–16). *S. aureus* thwarts immunity through an array of virulence factors (17) including α-toxin (Hla), a pore-forming cytotoxin that contributes to superficial and invasive disease (18–20) and perturbs host immunity during skin infection (21) and recurrent disease (22). Commensurate with this observation, the anti-Hla antibody response is a correlate of protection against recurrent skin infection (7) and bacteremia (23).

The success of *S. aureus* as a human skin commensal suggests that a tissue-specific host-microbe interaction exists within this niche. We hypothesized that differential analysis of SSTI and invasive disease in recurrent infection models would illuminate skin-specific modulation of the host response that predispose to recurrence. Using a *S. aureus* strain engineered to express chicken egg OVA, we demonstrate that skin infection impaired the antigen-specific CD4+ T cell response dependent on Hla production. Abrogating the effects of Hla restored T cell function, informing approaches to engender immunity against *S. aureus*.

Results and Discussion

To recapitulate the clinical observation that tissue-specific responses to *S. aureus* shape immunity, we challenged mice with *S. aureus* USA300/LAC via intravenous or subcutaneous routes to model bacteremia and skin infection (Figure 1A). Bacteremic mice experienced approximately 15%–20% weight loss, regaining weight over 12 to 14 days (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI130728DS1), whereas mice exposed to skin infection harbored lesions that peaked within 2 days and resolved by day 14 (Supplemental Figure 1B). We challenged mice with *S. aureus* skin

Figure 1. Primary *S. aureus* skin infection blunts the development of immunity. (A) Experimental timeline of primary intravenous (5 × 10⁶ CFU/mouse) and skin infections (1 × 10⁸ CFU/mouse) and skin reinfection (1 × 10⁸ CFU/mouse). **(B)** Representative gross and histopathologic (H&E-stained) images of skin lesions on postinfection day 4. Arrows denote dermonecrosis. Scale bars: 1000 μm. **(C)** Quantitation of dermonecrotic area following secondary skin infection in mice subjected to primary skin or intravenous challenge. **(D)** CFU analysis of lesions from mice in **C**. ****$P < 0.0001$, by parametric 2-tailed Student's *t* test after log₁₀ transformation and confirmation of normality with Shapiro-Wilk and Anderson-Darling tests. Data are representative of 3 (**A**–**C**) and 2 (**D**) independent experiments.

that the reduced antibody response following primary skin infection was not solely attributable to a quantitative B cell defect. To further evaluate the importance of the antibody response, primary intravenous challenge of mice deficient in mature B cells (μMT) revealed poor control of dermonecrosis (Supplemental Figure 1, F and G) and bacterial burden (Supplemental Figure 1H) during secondary skin infection compared with WT mice, a finding that correlated with loss of the anti-Hla response (Supplemental Figure 1I). Delivery of an identical inoculum (5 × 10⁶) for primary skin and bacteremic infection recapitulated these findings (Supplemental Figure 1, J and K). Although the clinical and pathologic endpoints of bacteremia and skin infection differ, these models reflect the observation in humans that *S. aureus* immunity depends on the initial infection site. We therefore viewed these models as comparative tools to interrogate host immunity.

We hypothesized that the αβ-T cell response may underlie tissue-specific features of immunity. Consistent with this, antibody-mediated depletion of CD4⁺ T cells concurrent with primary bacteremia (Supplemental Figure 1L) abrogated the anti-Hla response (Supplemental Figure 1M) and eliminated secondary infection protection (Supplemental Figure 1, N and O). Although multiple studies have analyzed cellular injury during skin infection (18, 21, 22), these studies have not evaluated antigen-specific T cell responses in vivo. To this end, we used the OT-II CD4⁺ T cell system (24) in combination with OVA-expressing *S. aureus* USA300/LAC (USA300_OVA). OVA was not detectable when expressed via the *lgt* promoter (pww412_OVA) (Supplemental Figure 2, A and B; see complete unedited blots in the supplemental material), however, expression was achieved using a modified promoter-enhancer element (pKL_OVA). To track antigen-specific CD4⁺ T cell responses, we transferred CD45.1⁺ OT-II T cells into mice prior to intravenous or skin challenge with USA300_OVA or an empty vector–harboring control strain (USA300_CTL) (Figure 2A). By postinfection day 7, we observed that OT-II T cells accumulated in the skin dLNs of mice following primary infection via both routes (Figure 2B, left), whereas only bacteremia prompted splenic OT-II T cell accumulation (Figure 2B, middle). OVA-specific T cell recovery following skin infection decreased to baseline 14 days after challenge, whereas an approximately 10-fold increase in OT-II T cells persisted after intravenous infection. To assess whether primary infection produced antigen-specific memory T cells, we analyzed the expression of central memory CD44^hiCD62L^hi (CM) and effector memory CD44^hiCD62L^lo (EM) cell markers. Both infection routes induced EM and CM responses on day 7 (Figure 2C), with an EM predominance in the spleen after bacteremia. Only primary bacteremia elicited a detectable memory OT-II T cell response until day 14.

infection on day 40, and assessed bacterial control 4 days later. Mice challenged with intravenous *S. aureus* USA300/LAC exhibited smaller lesions during the secondary skin challenge than did mice exposed to a primary skin infection, as evidenced by tissue pathology (Figure 1B), dermonecrosis (Figure 1C), and bacterial burden (Figure 1D). Given the role of *S. aureus* α-toxin (Hla) in primary and recurrent skin infection (18, 19, 21, 22), we evaluated the anti-Hla response and found that bacteremia elicited higher IgG levels against Hla (Supplemental Figure 1C) and staphylococcal lysates (Supplemental Figure 1D) compared with primary skin infection. Following the secondary infection, initial skin exposure resulted in a limited anti-Hla response, as previously observed (22), relative to the response in mice initially subjected to bacteremia (Supplemental Figure 1C). In contrast, anti-staphylococcal antibody titers were indistinguishable (Supplemental Figure 1D), suggesting specificity of the response. Primary skin infection was associated with increased CD19⁺ B cells in the draining lymph node (dLN) compared with bacteremic infection, whereas splenic B cell recovery was similar (Supplemental Figure 1E), suggesting
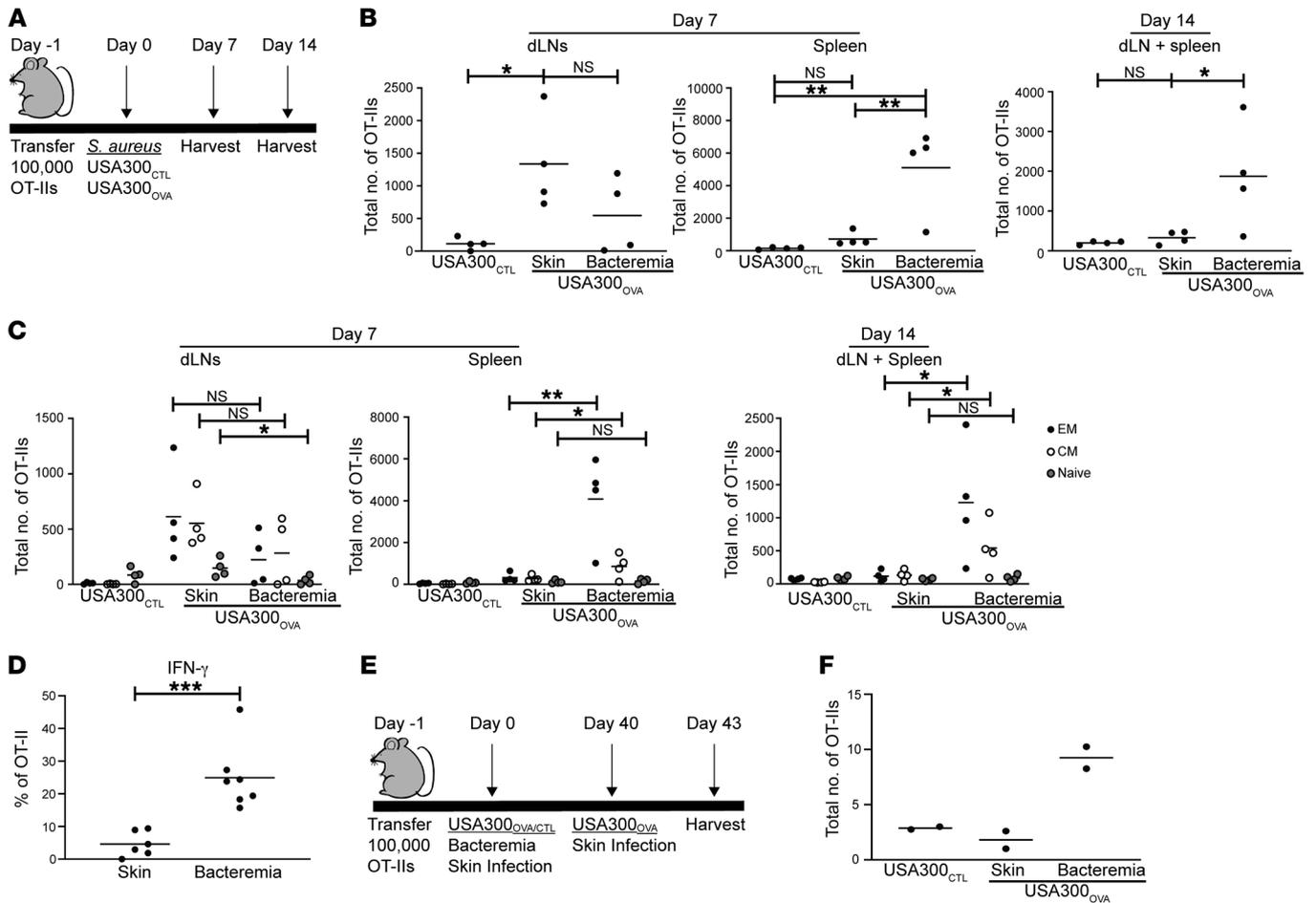
Figure 2. Antigen-specific T cell priming depends on tissue site of infection. (**A**) Experimental timeline of infection with *S. aureus* USA300$_{OVA}$ and cellular analysis. (**B**) Quantification of OT-II T cells harvested from skin dLNs and spleen 7 days after primary infection (1 × 10^8 CFU/mouse for skin infection, and 5 × 10^6 CFU/mouse for bacteremia) and from pooled dLNs and spleen 14 days after infection. (**C**) OT-II T cells as in **B**, classified into EM, CM, and naive phenotypes. (**D**) Analysis of IFN-γ expression by cells harvested from infected mice. (**E**) Timeline of infection and cellular analysis following secondary infection. (**F**) OVA-specific T cell quantification from pooled dLNs and spleens following secondary infection in mice as in **E**. Each dot represents 1 independent group of least 5 mice. *$P < 0.05$ and **$P < 0.01$, by 1-way ANOVA with Sidak's multiple comparisons test (**B** and **C**) or parametric 2-tailed Student's *t* test (**D**). Data are representative of 3 independent experiments (**A**–**D**).

T cell differentiation toward effector and memory cell phenotypes during infection is shaped by local cues from antigen-presenting cells and the cytokine milieu. Although an IL-17–predominant response to *S. aureus* infection correlates with epithelial protection (14, 15, 25), an IFN-γ–dominant T cell response is elicited by systemic infection and required for protection (16). Our model enables assessment of the cytokine response in antigen-specific T cells and quantification of the memory response. Evaluation of OVA-specific T cells elicited by primary bacteremia revealed an increased percentage of IFN-γ–producing cells (Figure 2D); the IL-4, IL-10, and IL-17 responses did not distinguish tissue sites (Supplemental Figure 2C). To assess the recall response, mice received USA300$_{OVA}$ intravenously or intradermally and were then rechallenged on day 40 to generate skin infection (Figure 2E). Bacteremia elicited an increase of approximately 3-fold in OT-II T cell accumulation 3 days after rechallenge relative to that seen with skin infection (Figure 2F), with a divergent trend toward CD44^{hi} memory T cell accumulation (Supplemental Figure 2D).

Primary skin infection with an isogenic Hla mutant (USA300 *hla::erm*) protects against skin rechallenge (22). We hypothesized that Hla may modulate DC–T cell crosstalk during primary infection, as CD11b$^+$ and CD103$^+$ dermal DCs and epidermal Langerhans cells (LCs) are principal skin antigen-presenting cells (26). We subjected mice to USA300 or USA300 *hla::erm* skin infection, and evaluated DC numbers 4 days after infection. Administration of USA300 led to a reduction in the dLN and skin DC populations, which were restored in the absence of Hla (Figure 3A). Analysis of specific cell subpopulations revealed preservation of CD11b$^+$ (Figure 3B) and CD103$^+$ (Figure 3C) DCs in USA300 *hla::erm*–infected mice. LC numbers showed a trend consistent with protection following USA300 *hla::erm* infection, with a significant increase in the dLNs (Figure 3D). Diminution of the DC compartment may be the result of direct cytotoxicity by Hla or other *S. aureus* toxins, or may reflect tissue injury, in which local cellular damage engenders a microenvironment that is unfavorable for DC survival.

To evaluate the impact of Hla on memory T cell induction, OT-II T cell recipients were infected via the subcutaneous route
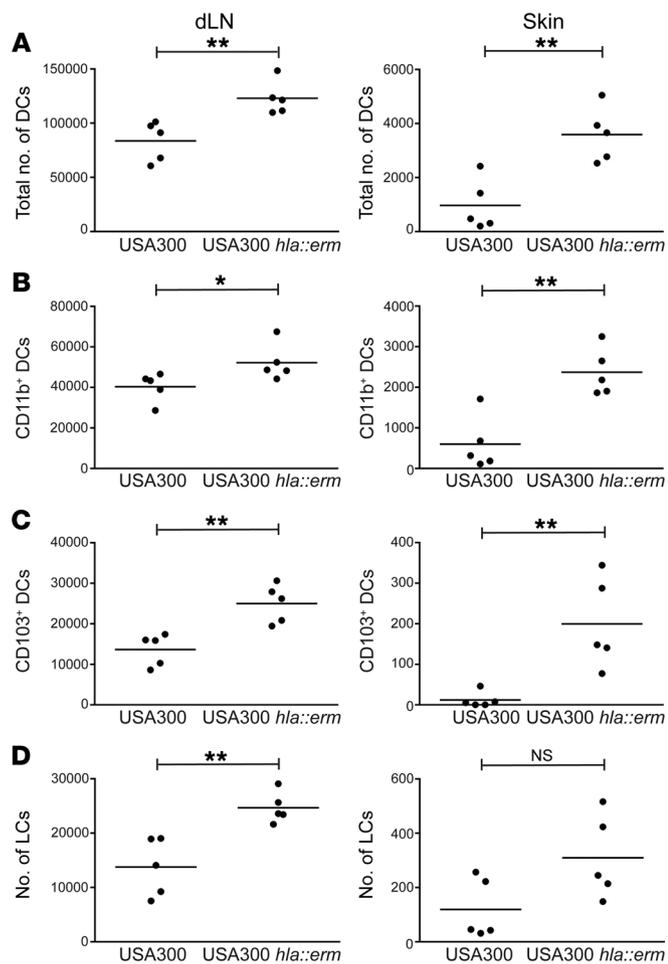
Figure 3. Hla alters the skin DC response. (**A**) Total DC accumulation in skin dLNs and skin in mice subjected to primary skin infection with USA300 or USA300 *hla::erm* (1×10^8 CFU/mouse). CD11b^{+} DC (**B**), CD103^{+} DC (**C**), and LC (**D**) accumulation following infection as in **A**. *$P < 0.05$ and **$P < 0.01$, by parametric 2-tailed Student's t test. Data are representative of 3 independent experiments.

with USA300$_{OVA}$ or USA300 $_{OVA}$ *hla::erm*, and OT-II T cell accumulation was assessed 7 days after infection. Although these conditions only elicited a minimal anti–OVA IgG response (OD$_{450}$: USA300$_{OVA}$, 0.07 ± 0.01; USA300$_{OVA}$ *hla::erm*, 0.06 ± 0.03 vs. OVA-immunized control 0.8 ± 0.01), Hla deletion augmented EM and CM cell numbers in skin dLNs (Figure 4A) and spleen (Figure 4B). OT-II T cells were recovered from the skin only during infection with USA300 $_{OVA}$ *hla::erm* (Figure 4C). Together, these data demonstrate that Hla impairs the memory response, thereby limiting antigen-specific T cell localization.

We sought to examine whether active immunization against Hla would protect the cellular immune response during skin infection. Mice were immunized with adjuvant or inactive Hla (Hla$_{H35L}$) prior to skin challenge. DCs were protected at the dLN and skin sites after infection (Figure 4D), with accumulation of DCs and LCs observed in Hla$_{H35L}$-immunized mice (Supplemental Figure 2, E, F, and G). We found that DC compartment preservation was associated with enhanced generation of antigen-specific T cells (Figure 4E) that exhibited an effector phenotype (Figure

4F). *S. aureus* recovery was reduced in skin lesions of immunized mice (Supplemental Figure 2H), mirroring findings upon infection with an Hla-deficient strain (21). *S. aureus* expresses leukocidins and phenol-soluble modulins that target DCs, inhibiting antigen uptake, presentation, and T cell proliferation (27, 28). As species-specific cellular receptors define the activity of multiple staphylococcal toxins (29), our observations of the role of Hla in modulating the murine antigen–specific T cell response suggest that this response may also be modified in humans through the combined cellular action of toxins. *S. aureus* may thus rely on multiple virulence factors in skin infection to simultaneously cause injury and manipulate host immunity.

S. aureus colonizes up to 50% of infants by 8 weeks of age (30), raising the possibility that immunity is templated early in life. Indeed, studies in a *Staphylococcus epidermidis* neonatal skin colonization model using an antigen-specific reporter T cell system revealed that early antigen exposure promotes immunologic tolerance, characterized by the establishment of commensal-specific Tregs (31). To determine whether Hla neutralization is sufficient to protect the T cell compartment using a strategy suited for early life intervention, we evaluated the impact of maternal immunization. Mice born to Hla$_{H35L}$-immunized dams had protection against skin infection relative to the offspring of control-immunized dams (Figure 4G). Enhanced OT-II T cell recovery (Figure 4H) characterized by an increase in EM cells was seen in the offspring of Hla$_{H35L}$-immunized dams (Figure 4I), suggesting that passive transfer of maternally derived Hla-neutralizing antibodies engenders protection of the antigen-specific T cell response.

These studies underscore the importance of T cell–mediated immunity in protection against *S. aureus* disease. The T cell response will not only reflect the tissue environment during primary infection, but modulate the B cell–derived humoral response. Therefore, a detailed understanding of T cell specificity and effector phenotype will be beneficial to elicit vaccine-derived protective immunity. As our studies assess the T cell response to a single exogenous antigen, further analysis of endogenous T cells elicited by infection will be necessary to discern whether Hla exposure alters T cell receptor diversity and specificity of the host antibody response. If the effects of Hla on T cell–mediated immunity occur during initial skin exposure in humans, the T cell repertoire may be perturbed by colonization or infection in infancy. This consideration has 3 important implications: first, individuals with *S. aureus* exposure harbor a preexisting T cell repertoire influenced by the pathogen. Thus, post-exposure vaccine trials may not be capable of favorably altering the diversity of the T cell response or specific effector functions necessary for protective immunity. Second, strategies such as maternal immunization and/or infant vaccination may be required to generate population-level protective immunity. Third, the strategic design of vaccine adjuvant and tissue delivery systems will be essential to instruct the T cell effector and memory response. By removing the suppressive effects of Hla on host immune function, immunization against Hla may expand antigen-specific T cell diversity and allow natural *S. aureus* exposure to amplify the T cell repertoire rather than elicit tolerogenic or suppressive responses. As potential
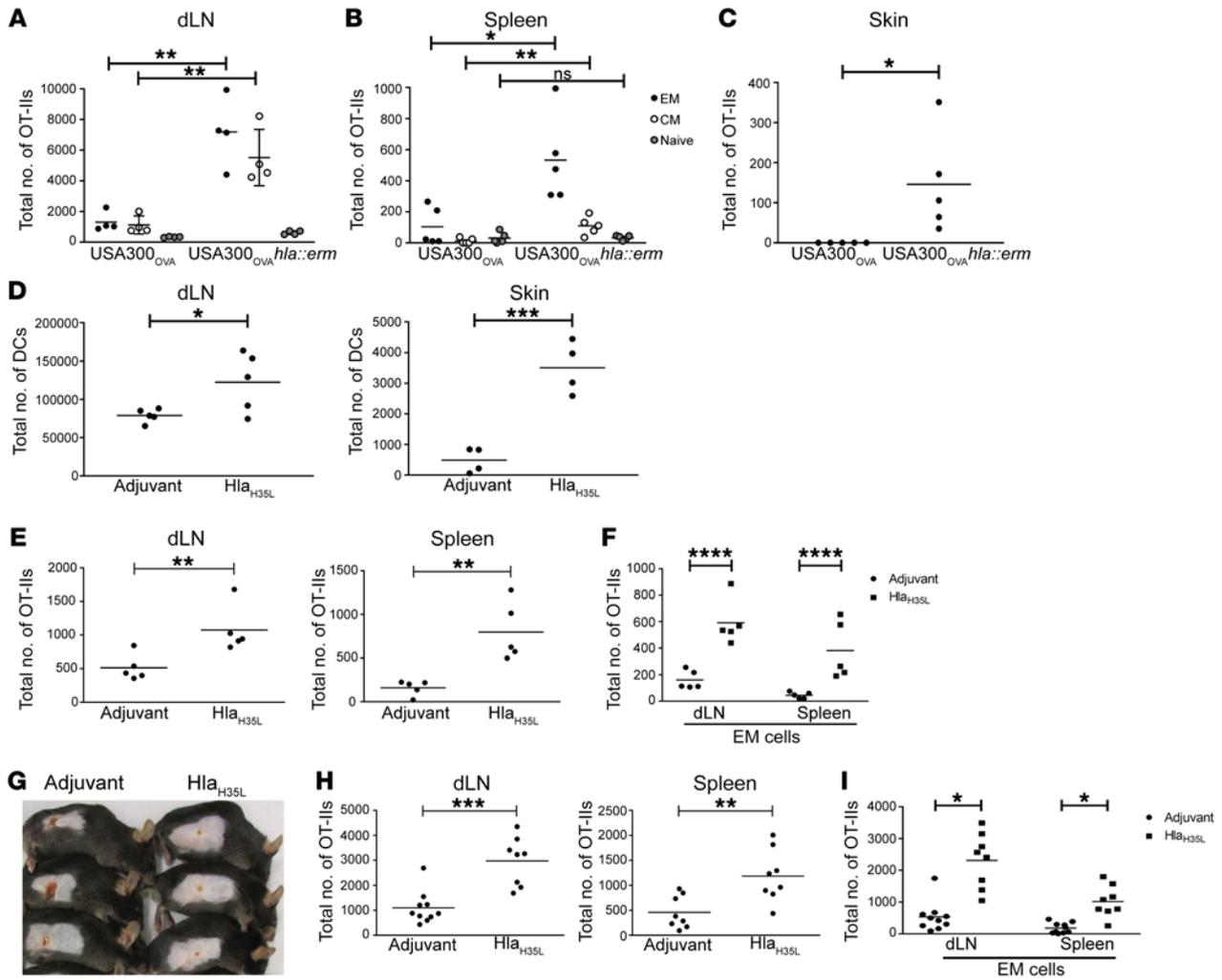
Figure 4. Modulation of the antigen-specific T cell response by Hla. (**A**) OT-II T cell quantification in the dLNs and (**B**) spleen, classified into EM, CM, and naive phenotypes 7 days after infection with USA300$_{OVA}$ or USA300$_{OVA}$ *hla::erm* (1 × 10^8 CFU/mouse). (**C**) Skin OT-II T cell accumulation 7 days after infection as in **A**. (**D**) DCs in the dLNs and skin in mice subjected to adjuvant only or Hla$_{H35L}$ vaccination prior to USA300$_{OVA}$ infection. (**E**) OT-II T cell quantification in the dLNs and spleen in mice subjected to adjuvant only or Hla$_{H35L}$ vaccination prior to infection with USA300$_{OVA}$. (**F**) Quantification of EM cells from mice as in **E**. (**G**) Skin gross pathology 4 days after infection in mice born to adjuvant only or Hla$_{H35L}$-vaccinated dams. (**H**) Quantification of OT-II T cells in the dLNs and spleen following infection of mice with USA300$_{OVA}$ as in **G**. (**I**) EM T cell phenotype analysis of cells harvested as in **H**. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$, by multiple *t* test comparison with Bonferroni-Dunn correction (**A** and **B**), parametric 2-tailed Student's *t* test (**C**–**E** and **H**), or 2-way ANOVA with Sidak's multiple comparisons test (**F** and **I**). Data are representative of 2 independent experiments.

S. aureus vaccine targets exhibit human specificity including the T cell superantigens, leukocidins LukAB, HlgCB, and Panton-Valentine leukocidin (PVL) (29), it will be essential to expand our understanding of the tissue-specific immune response to *S. aureus* infection beyond the constraints of mouse modeling. Further analysis of the early human response to *S. aureus* may thus provide insight on vaccine design.

Methods

Details on the methods used in this study are provided in the Supplemental Methods.

Statistics. Data are presented as the mean ± SD. Group sizes were determined on the basis of the number needed to achieve statistically relevant scientific results, with random assignment of mice to groups. Normality of data was assessed and statistical

significance determined by 2-tailed, unpaired Student's *t* test or 1-way ANOVA, with statistical significance noted when the *P* value was less than 0.05.

Study approval. All animal experiments were approved by the IACUCs of the University of Chicago and Washington University.

Author contributions

BL, RO, and JBW designed the research studies, analyzed data, and wrote the manuscript. BL and RO performed all experiments. JMK and BL generated the pKL plasmid for OVA expression. BL initiated these studies, established the model system, and provided fundamental insight on the antigen-specific T cell response to infection. RO extended these studies to conclusively demonstrate the role of Hla in modulation of the T cell response and to define the ability of immunization strategies targeting Hla to protect the antigen-spe-

cific T cell response. The contributions of BL and RO were equal in significance to the final conclusions of the study; therefore, the authorship order reflects their temporal involvement in the project.

Address correspondence to: Juliane Bubeck Wardenburg, 660 South Euclid Avenue, Box 8208, St. Louis, Missouri 63110, USA. Phone: 314.286.1747; Email: jbubeck@wustl.edu.

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