Supplemental Materials

Staphylococcus aureus α-toxin suppresses antigen-specific T cell responses

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Supplemental Methods

Mice. All mice were housed in specific pathogen-free animal facilities. C57BL/6J mice were purchased from Jackson Laboratories. μMT mice were generously provided by Dr. Michael Diamond. OT-II CD45.1^{+} mice were kindly gifted by Dr. Anne Sperling. For all infections, age-matched 4-6-week old males and females were studied.

Bacterial strains and cultures. *S. aureus* USA300/LAC was engineered to express chicken egg ovalbumin (USA300$_{OVA}$) by cloning OVA$_{139\text{-}386}$ into the pKL plasmid. pKL was generated by modifying pww412 (1) to include an improved translation initiation region with an optimized Shine-Dalgarno sequence (AGGAGG) and translation enhancer (TTAACTTTA). OVA$_{139\text{-}386}$ was cloned from a *S. aureus* codon-optimized chicken egg ovalbumin sequence (Geneart) using 5' primer TCGCATATGAAAACACGTATAGTCAGCTCAGTAACAA CAACACTATTGCTAGGTTCCATATTAATGAATCCTGTCGCTAATGCCGCAGATCAAGCACGTGAATTA which includes the *S. aureus* Hla signal sequence, and 3' primer TCTGGATCCTTATGGTGAAACACAACGACC. The resultant product was ligated into pKL to form pKL$_{OVA}$. Strains of USA300 harboring pKL (USA300$_{CTL}$) or pKL$_{OVA}$ (USA300$_{OVA}$) as well as an Hla-deficient variant of USA300 (USA300$_{OVA}$ *hla::erm*) were generated by electroporation utilizing standard methodologies. For infections, USA300 strains were grown overnight with rotation at 37°C in tryptic soy broth (TSB, Sigma), subcultured and prepared as previously described (2). Media for growth of USA300 *hla::erm* was supplemented with 40 μg/ml erythromycin, while media for strains harboring the pKL plasmid was supplemented with 20 μg/ml chloramphenicol. For all strains, OD$_{600}$ was measured to assess bacterial density, which was verified by serial plating on tryptic soy agar (TSA) plates to quantify colony forming units (CFU) per ml of culture.

Bacterial infections. For subcutaneous infections, mice were infected with 1 x 10^{8} CFU USA300 in 50 μl PBS. For intravenous infections, mice were infected with 5 x 10^{6} CFU USA300 in 100 μl PBS via retroorbital route. For evaluation of infection outcome with identical inocula, mice received 5 x 10^{6} CFU for both skin infection and intravenous infection. In experiments utilizing *S. aureus* strains harboring pKL$_{CTL}$ or pKL$_{OVA}$, 0.5 mg/ml chloramphenicol and 1% sucrose solution drinking water was given to mice one day prior to infection, and maintained for 15 days following primary infection, or throughout the course of secondary infection. For experiments to assess memory T cell responses, water was switched back to normal drinking on day 14 of the

experiment. On designated days post re-challenge, skin lesions were punch biopsied with an 8 mm punch biopsy (Integra Miltex) and placed in 1 ml of 0.1% Triton-X PBS solution then immediately processed using the Bio-Gen Pro200 Homogenizer (Pro Scientific). Homogenates were plated on TSA plates to quantify CFU values.

T cell adoptive transfer and T cell depletion. One day prior to infection, lymph nodes and spleens from OT-II $CD45.1^+$ males were harvested for $CD4^+$ T cell isolation. To extract OT-IIs, the $CD4^+$ T cell Isolation Kit (Miltenyi) was used according to manufacturer instructions. Approximately 1 x 10^5 $CD45.1^+$ OT-II cells were suspended in PBS and then transferred via retro-orbital injection into 5-week old C57BL/6J recipients ($CD45.2^+$). The next day, recipients were infected as described. For CD4+ T cell depletion, 100 μg anti-CD4 monoclonal antibody (GK1.5, University of Chicago Fitch Monoclonal Antibody Facility) or an isotype-matched control antibody was delivered by intraperitoneal injection to each mouse beginning 6 days prior to intravenous infection every 3 days for 7 total doses.

OT-II cytokine assay. Lymph nodes and spleens from mice were harvested, and splenic RBCs were lysed using an ammonium chloride lysis solution. Cells were then F_C CD16/32 blocked on ice for 10 minutes and then incubated with CD45.1 biotinylated antibody (eBioscience) at 1:400 concentration on ice for 30 minutes. Samples were then washed in FACS buffer, resuspended in 300 μl FACS buffer, and 50 μl of streptavidin (Miltenyi) magnetic beads were added per sample for 30 minutes of incubation on ice. Gentle vortexing was performed for a few seconds every 10 minutes to ensure proper bead mixing. Cells were then washed and resuspended in FACS buffer and passed through LS MACS column for positive selection of CD45.1 OT-II cells. Positively selected cells were plated in 96 well tissue culture plates and incubated for 4 hours at 37°C and 5% CO_2 with PMA (50 ng/ml), ionomycin (500 ng/ml), Golgi stop and plug (Biolegend). Cells were then surface stained, fixed, permeabilized and stained per BD manufacturer protocol.

Skin histology. Skin lesions harvested by punch biopsy were extracted from mice after euthanasia, then fixed in 10% formalin (VWR, Avantor). Samples were embedded, sectioned, and stained with hematoxylin and eosin (Nationwide Histology).

Tissue Isolation and Flow Cytometric Staining. Skin lesions were punch biopsied, and blunt removal of fat tissue was performed with forceps. Tissue was minced and digested in RPMI (Gibco) containing 0.25 mg/ml Liberase TL (Roche Diagnostic Corp), 100 μM β-mercaptoethanol, 20 μM HEPES, 100 U/ml penicillin and 100

μg/ml streptomycin, and incubated for 2-3 hours at 37°C and 5% CO_2. For dendritic cell studies, draining axillary, brachial and subinguinal lymph nodes were extracted and digested with 0.1 mg/ml Liberase TL (Roche), 100 μM β-mercaptoethanol, 20 μM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin, and incubated for 30 minutes at 37°C and 5% CO_2. Tissue and lymph nodes were gently strained through a 40 μm cell strainer in single cell suspensions, and then incubated with anti-CD16/32/F_C block (Biolegend) in fluorescence activated cell sorting buffer (FACS, 1% BSA, 0.1% NaN3, 5 mM EDTA) for 15 minutes on ice. Cells were stained in FACS buffer on ice for 30-45 minutes and then analyzed on the cytometer, or fixed in 1% paraformaldehyde solution. For tissue, live-dead staining was performed before blockade of non-specific binding. Briefly, cells were incubated with Live Dead Fixable Aqua (Thermo Fisher) at 1:1000 in PBS for 30 minutes at 4°C in the dark. Cells were washed twice in FACS buffer before incubation with FC block. Flow cytometric analysis was performed on a BD LSR Fortessa II. Murine cell suspensions were incubated with fluorochrome-conjugated antibodies (Biolegend) against the following surface markers: CD45 (30-F11), CD45.1 (A20), CD62L (MEL-14), CD4 (RM4-4, GK1.5), TCRβ(H57-597), CD44 (IM7), CD8b (Ly-3), MHCII I-A/I-E (M5/114.15.2), CD103 (2E7), CD207/Langerin (4C7), CD11c (N418), CD11b (M1/70), anti-CD16/32 (2.4G2), IFNγ (XMG1.2), IL-17A (TC11-18H10.1), IL-10 (JES5-16E3), IL-4 (11B11). Cell counts were enumerated using AccuCount beads (Spherotech) according to manufacturer instructions.

Memory T cell isolation and quantification. OT-IIs were isolated by positive selection as detailed above. Due to the low numbers of OT-IIs isolated per mouse in long-term memory experiments, positively selected cells from 5 mice per group were pooled together, stained for extracellular markers specific to CD45.1 OT-II cells, and analyzed by flow cytometry. For each experimental group, the total number of OT-IIs collected was then divided by the number of mice to obtain the mean, total number of OT-IIs per mouse per group.

Immunization. For active immunization, 4-week old mice received 20 μg Hla_{H35L} protein in complete Freund's adjuvant on day 0 via intramuscular route, followed by a boost with 20 μg Hla_{H35L} protein in incomplete Freund's adjuvant on day 10 prior to infection on day 21. Hla_{H35L} was prepared as previously described (3). Pre-immunization and day 20 sera were collected to assess antibody production. For maternal immunization studies, 8-week old female C57BL/6J mice were immunized on day 0 via intramuscular route with 20 μg of Hla_{H35L} protein in complete Freund's adjuvant followed by a boost with 20 μg Hla_{H35L} protein in incomplete

Freund's adjuvant 14 days post-mating. Pups born to immunized dams were weaned at day 21 of life, then infected between 4-6 weeks of age. For all studies, adjuvant alone was delivered to control immunized mice.

Serum antibody quantification. Mice were bled on day 35 post primary infection by submandibular puncture using 5 mm Goldenrod Animal Lancets. Blood was kept on ice during collection and sera was extracted using (Sarstedt Micro tube 1.1 ml Z-Gel) serum collecting tubes and spun down per manufacturer protocol. Sera was plated as a dilution series in antigen-coated Costar Easy Wash ELISA 96 well plates. Coating was done overnight at 4°C with either purified Hla_{H35L} (1 μg/ml) or staphylococcal lysate (5 μg/ml) in PBS solution. To prepare staphylococcal lysate for coating, a staphylococcal Protein A-deficient *S. aureus* strain was cultivated overnight at 37°C with shaking in Luria-Bertani (LB) medium. $1x10^{10}$ bacteria were harvested, washed with PBS, and resuspended in 200 of PBS buffer containing protease inhibitor cocktail tablets (Complete, Roche Diagnostics, Mannheim, Germany) and 20 μg of lysostaphin (Sigma-Aldrich, Germany). After enzymatic digestion at 37°C for 30min, the cells were disrupted by sonication using a microsonicator (Sonifier 250). Protein concentration was determined using DC protein assay kit (Bio-Rad) and lysate concentration was adjusted to 5 μg/ml in PBS prior to coating of ELISA plates. Blocking was performed with 0.1% BSA in PBS solution for 1 hour at room temperature or overnight at 4°C. After serum incubation, plates were washed 3 times with 0.05% Tween 20/PBS solution and patted dry prior to the addition of HRP-conjugated goat anti-mouse IgG in 0.1% BSA/PBS solution at 1:20,000 dilution for 45 minutes at room temperature. Plates were then washed 5 times with 0.05% Tween 20/PBS solution and developed with TMB substrate following manufacturer (ThermoFisher) recommended protocol. Absorbance (OD_{450}) was measured using a microplate reader (Tecan Infinite M200Pro), and data analysis was performed using PRISM software to determine half-maximal titers of each sample.

References

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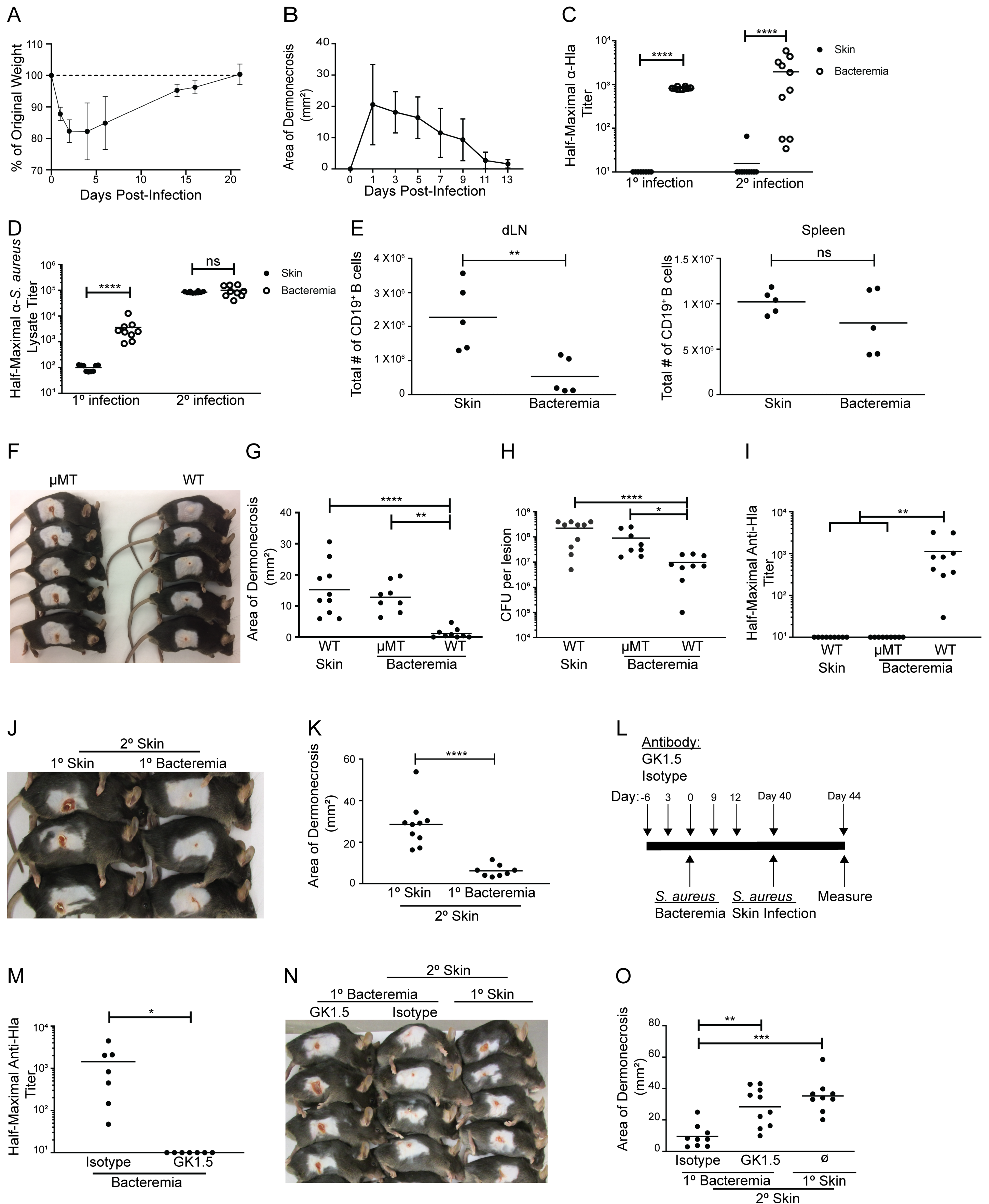
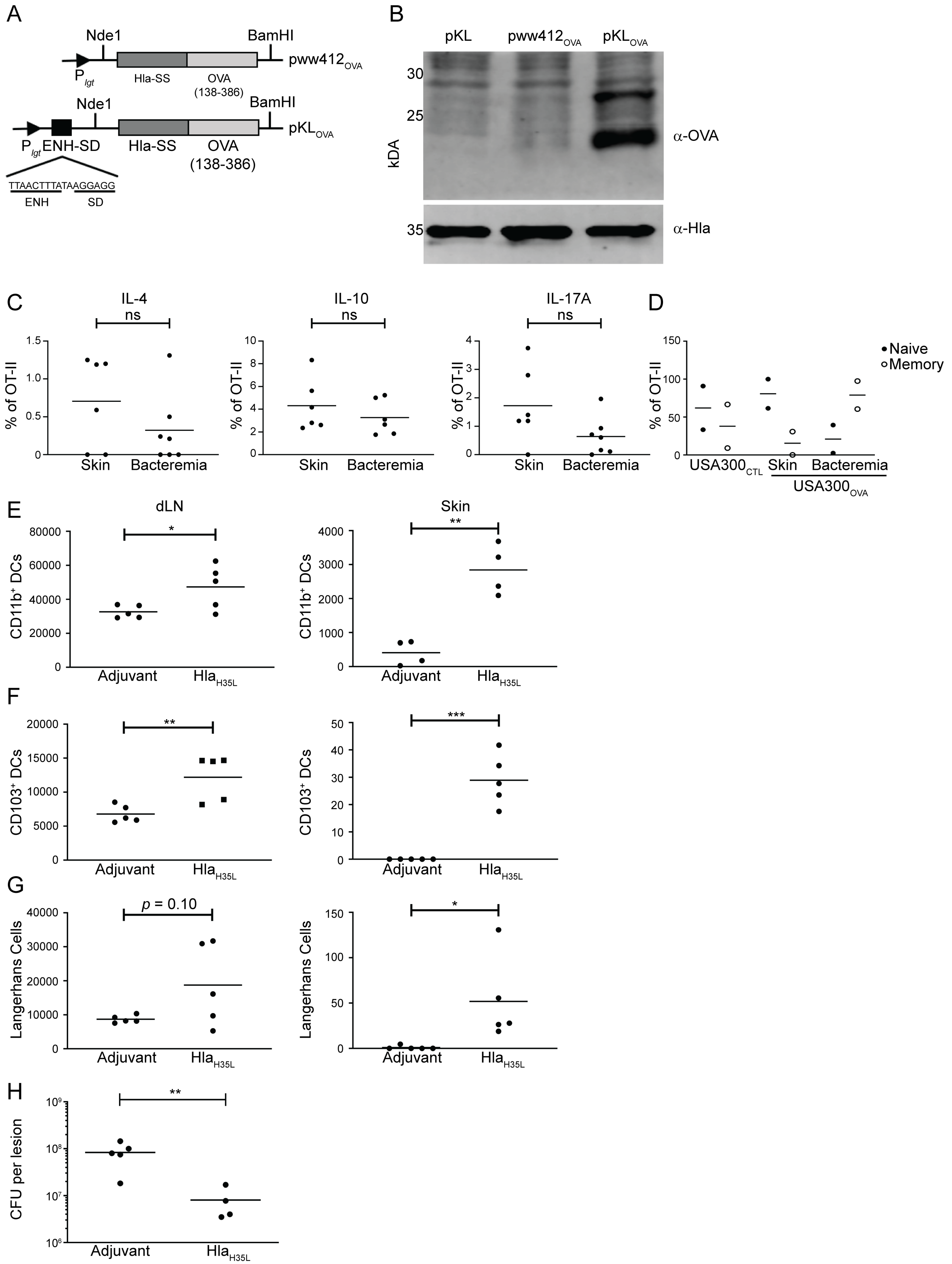
2. Sampedro GR, DeDent AC, Becker RE, Berube BJ, Gebhardt MJ, Cao H, et al. Targeting Staphylococcus aureus alpha-toxin as a novel approach to reduce severity of recurrent skin and soft-tissue infections. *J Infect Dis.* 2014;210(7):1012-8.

3. Kennedy AD, Bubeck Wardenburg J, Gardner DJ, Long D, Whitney AR, Braughton KR, et al. Targeting of alpha-hemolysin by active or passive immunization decreases severity of USA300 skin infection in a mouse model. *J Infect Dis.* 2010;202(7):1050-8.

Supplemental Figures



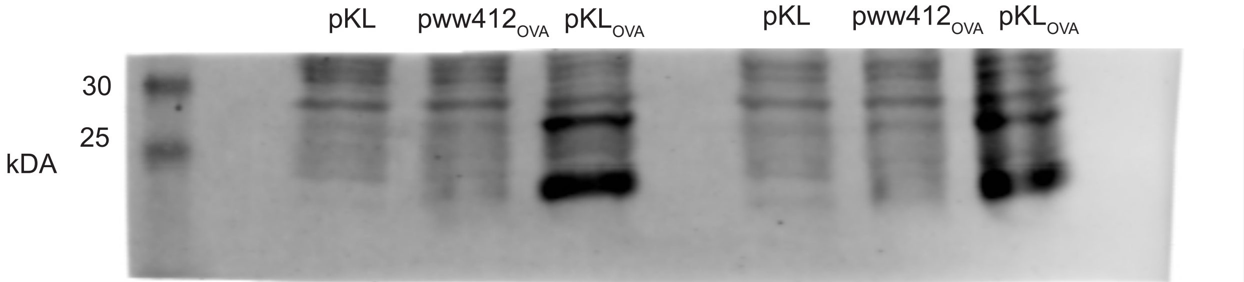
Supplemental Figure 1. Tissue-specific patterning of immunity against secondary *S. aureus* skin infection. A) Weight loss in mice following primary *S. aureus* bacteremia. B) Area of dermonecrosis quantified in mice following *S. aureus* skin infection. For A and B, n=8 mice, error bars represent the standard deviation. C) Quantification of anti-Hla titer in mice following primary and secondary bacteremia or skin infection. D) Quantification of anti-*S. aureus* titer in mice following primary and secondary bacteremia or skin infection. E) Total $CD19^{+}$ B cells in the draining lymph nodes and spleens following primary bacteremia or skin infection. F) Gross pathologic findings in secondary skin infection in mMT and wild-type (WT) mice that were exposed to primary bacteremia. Quantification of dermonecrosis area (G), colony forming unit (CFU) recovery (H), and anti-Hla titer (I). J) Gross pathologic findings and (K) dermonecrosis area in secondary skin infection in mice following primary intravenous or skin infection with 5×10^{6} CFU. L) Experimental timeline of $CD4^{+}$ T cell depletion in mice subjected to secondary skin infection following primary bacteremia infection. M) Quantification of anti-Hla titer 35 days post-T cell depletion. N) Gross pathologic findings and O) dermonecrosis area in mice subjected to secondary skin infection following primary bacteremia infection; mice subjected to secondary skin infection following primary skin infection are included as a control. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$ determined by one-way ANOVA with Sidak's multiple comparison test, or t test where appropriate. Data are representative of (F-H) two or (L-O) three independent experiments.



A
Nde1
BamHI
pww412OVA
Plgt
Hla-SS
OVA (138-386)
Nde1
BamHI
pKLOVA
Plgt ENH-SD
Hla-SS
OVA (138-386)
TTAACTTTATAAGGAGG
ENH
SD
B
pKL
pww412OVA
pKLOVA
30
25
kDA
α-OVA
35
α-Hla
C
IL-4
IL-10
IL-17A
ns
% of OT-II
Skin
Bacteremia
D
Naive
Memory
USA300CTL
Skin
Bacteremia
USA300OVA
E
dLN
Skin
CD11b+ DCs
Adjuvant
HlaH35L
F
CD103+ DCs
G
p = 0.10
Langerhans Cells
H
CFU per lesion

Supplemental Figure 2. A) Depiction of OVA expression constructs $pww412_{OVA}$ and pKL_{OVA} for use in *S. aureus*. pKL_{OVA} includes an improved translation initiation region, containing a translational enhancer (ENH) from gene 10 of phage T7 and optimized Shine-Dalgarno (SD) sequence downstream of the *lgt* promoter. B) Western blot analysis of $OVA_{138\text{-}386}$ (top) and control Hla (bottom) expression in overnight supernatants of *S. aureus* strain $USA300_{OVA}$. C) OVA-specific T cell cytokine analysis following primary skin or bacteremic infection. D) Quantification of OVA-specific T cells from pooled dLNs and spleens following secondary infection in mice infected as in Figure 2E. Naïve ($CD44^{low}$) and memory ($CD44^{high}$) OT-IIs were identified by flow cytometry. Each dot represents one independent experimental group of least 5 mice. Quantification of E) $CD11b^{+}$ DC, F) $CD103^{+}$ DCs, and G) Langerhans cells in dLN and skin following primary skin infection in mice subjected to vaccination with Adjuvant or Hla_{H35L} prior to infection with USA300. H) *S. aureus* CFU recovery from skin lesions in mice vaccinated and infected as in E-G. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; determined by parametric two-tailed t test. Data are representative of (B, C) two or (E-H) three independent experiments.

Unedited gels for Supplemental Figure 2B



Hla Loading Control