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Supplemental Information

Preserving Immunogenicity of Lethally Irradiated Viral and Bacterial Vaccine Epitopes Using a Radio-Protective Mn^{2+}-Peptide Complex from *Deinococcus*

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Supplemental Table 1, related to Figure 2A. Infectious titers of viral (V3526) samples[a].

Sample	Virus titer ($TCID_{50}$)
V3526, diluted stock , 0 kGy	3.47×10^5
V3526 + Pi, 0 kGy	2.24×10^6
V3526 + Mn-DP-Pi, 0 kGy	4×10^5
V3526 + Pi, 10 kGy	0
V3526 + Mn-DP-Pi, 10 kGy	0
V3526 + Pi, 20 kGy	0
V3526 + Mn-DP-Pi, 20 kGy	0
V3526 + Pi, 30 kGy	0
V3526 + Mn-DP-Pi, 30 kGy	0
V3526 + Pi, 40 kGy	0
V3526 + Mn-DP-Pi, 40 kGy	0

[a]50% tissue culture infectivity dose ($TCID_{50}$) in the 5th passage of Vero cells infected with V3526 irradiated in phosphate buffer (V3526 + Pi) or in Mn-DP-Pi (V3526 + Mn-DP-Pi) was calculated as described previously (Aldovini and Walker, 1990). In cell cultures, the $TCID_{50}$ is the dilution of virus suspension that infects 50% of the cell cultures as measured by visible cytopathic effect (CPE) (See Experimental Procedure, Virus Infectivity Test). For determination of $TCID_{50}$, 10 µl of V3526, irradiated or not, were subjected to tenfold serial dilutions in MEM/NCS media, and 100 µl from each dilution were used for infection of Vero cells (3×10^3 cells). After 72 h of incubation, CPE was evaluated and 50% endpoint dilution was used to calculate the virus titer.

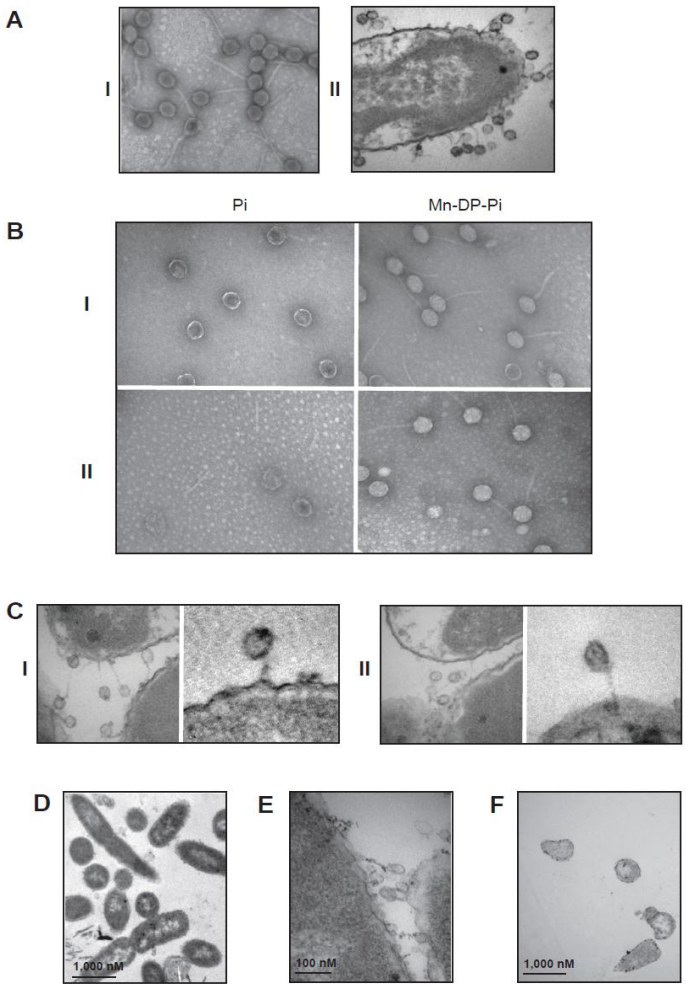

Figure S1, related to Figure 1. Structural integrity and adsorption of λ phage assessed by transmission electron microscopy. (A) Non-irradiated λ phage. I, purified λ phage; II, adsorption to *E. coli* (C600). (B) λ phage exposed to 40 kGy in the absence or presence of Mn-DP-Pi. I, post-irradiation in liquid-holding (4°C); II, frozen (-80°C) after irradiation, then thawed. (C) Binding between λ phage tail and *E. coli* (C600). I, adsorption of non-irradiated λ phage to *E. coli* (left, low magnification) and a single bound λ phage (right, high magnification); II, adsorption of λ phage exposed to 40 kGy in Mn-DP-Pi to *E. coli* (left) and a single bound λ phage (right). (D) TEM of *E. coli* after exposure to 25 kGy in Mn-DP-Pi. (E) TEM of λ phage (non-irradiated) adsorbed to *E. coli* exposed to 25 kGy in Mn-DP-Pi. (F) TEM of *E. coli* exposed to 25 kGy in Pi buffer (pH 7.4). Note, the structural integrity of λ phage tails exposed to 40 kGy in Pi buffer was lost (panel B of this figure and Figure 1C), so their ability to adsorb to *E. coli* was not tested. Similarly, few if any intact *E. coli* cells were visualized by TEM following exposure to 25 kGy in Pi buffer alone (panel F of this figure), and the remnants of irradiated *E. coli* did not adsorb λ phage. This supports that most *E. coli* cells were lysed during irradiation (25 kGy) in Pi buffer lacking Mn-DP-Pi. TEM, transmission electron microscopy of thin sections. Other abbreviations as in Figure 1. λ phage heads are 50 nm in diameter; λ tails are 150 nm long.

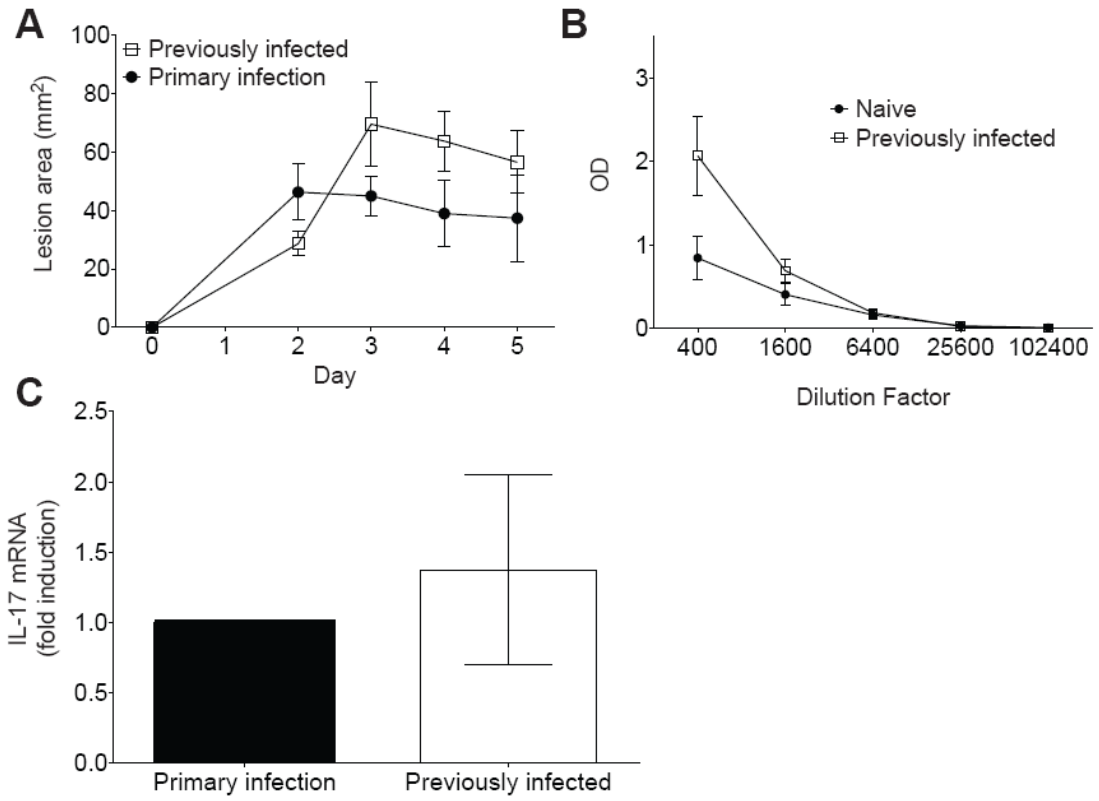

Figure S2, related to Figures 3. Prior MRSA skin infection does not protect against subsequent infection. (A) Lesion size in mice infected with MRSA for the first time or after resolving a previous infection three weeks prior. (B) Anti-*S. aureus* serum antibody in naive mice or mice previously infected 25 days prior. (C) IL-17 mRNA induction in skin lesions five days after primary or secondary (previously infected) MRSA skin infection. n=4-5 mice/group. Data are representative of 2-3 independent experiments and show mean +/- SEM.

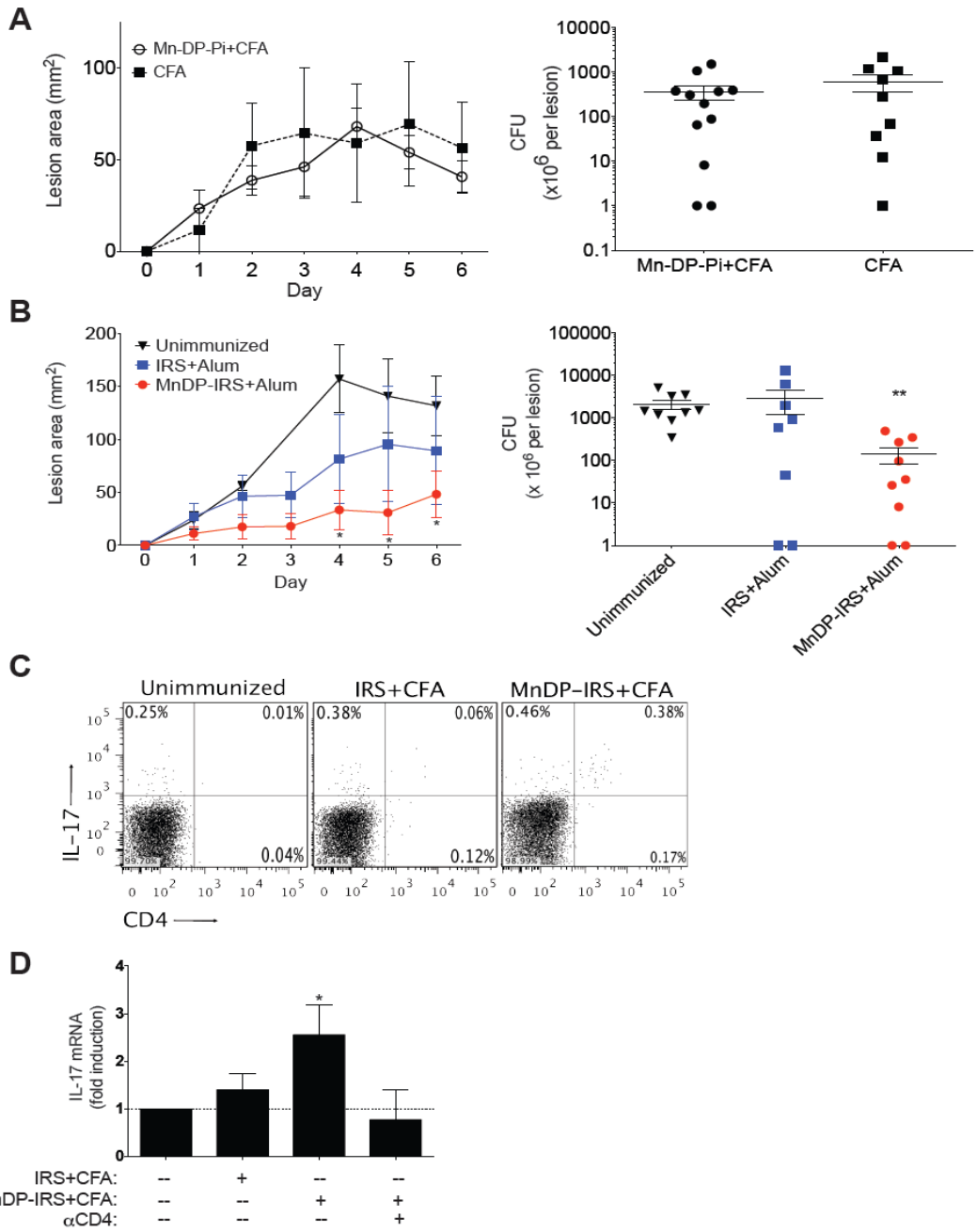

Figure S3, related to Figure 4. MnDP-IRS vaccine elicits protective immune response. (A) Lesion area and skin CFU after MRSA skin challenge in mice previously injected with Mn-DP-Pi+CFA or CFA alone four weeks prior and Mn-DP-Pi+IFA or IFA alone two weeks prior (n=9-12 mice/group). (B) Lesion size and skin CFU in wild-type mice that were challenged after immunization with IRS or MnDP-IRS in alum as described in Figure 4 for CFA (n=8-9 mice/group). (C) CD4 and intracellular IL-17 staining of pooled skin abscess cells (n=4 mice/group) three days after MRSA infection. (D) IL-17 mRNA levels in skin abscess five days after MRSA infection (n=4-10 mice/group). Data shown as mean +/- SEM. Abbreviations as in Figure 4; CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Chemical Reagents. Materials were purchased from Sigma Chemical Company, St. Louis, MO. Bacto-tryptone, yeast extract and bacto-agar were from BD, Franklin Lakes, NJ. Blood agar plates were from Thermo Scientific, Dubuque, IA. Tryptic Soy Broth (TSB) was from General Laboratory Products, Yorkville, IL. Penicillin and streptomycin solutions were from Mediatech, Inc., Manassas, VA.

Strains and Growth Conditions. *Escherichia coli* C600 (ATCC 23724), *E. coli* bacteriophage lambda (λ, ATCC 23724-B2) and Vero cells (ATCC CCL-81) were purchased from the ATCC collection, Manassas, VA**.** Vero cells were grown in 24-well plates (BD Falcon, Franklin Lakes, NJ) at 37^{o}C under 5% CO_2 in 500 µl of 1 × MEM (Minimal Essential Medium from Mediatech, Inc. Manassas, VA) supplemented with 10% NCS (Newborn Calf Serum, HyClone, from Thermo Scientific, Dubuque, IA), and 100 U/ml penicillin and 100 µg/ml streptomycin (MEM/NCS). The USA300 clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA) was a gift from F. DeLeo (Rocky Mountain Laboratories, NIAID, NIH). MRSA was grown in TSB at 37^{o}C with constant agitation and harvested at the mid-exponential growth phase (OD_{600} 0.6-0.8).

Large-Scale Preparation of Bacteriophage Lambda. 0.05 ml of primary lysate (10^9 bacteriophage lambda particles) were mixed with 20 ml of fresh overnight recipient *E. coli* C600 cells (approximately 10^{10} cells) and incubated for adsorption for 10 min at 37^{o}C. 1 L LB-Miller media (10g/L tryptone, 5g/L yeast extract, 10g/L NaCl) containing 10 mM $MgSO_4$ was added and incubated for 7 h at 39^{o}C with shaking. After lysis occurred, DNase and RNase treatments were performed by incubation of lysed culture with 1 µg/ml RNase and 1 µg/ml DNase for 30 min at 37^{o}C. NaCl (58 g/L) and chloroform (0.2 ml/L) were added, and the lysate was clarified by spinning at 10,000 × g for 10 min at 4^{o}C. Lambda particles were precipitated by 10% PEG 6,000 (w/v) for 60 min on ice. Lambda phage pellet was collected by centrifugation at 10,000 × *g* for 10 min at 4^{o}C. The pellet was suspended in 10 ml SM buffer (50 mM Tris-HCl buffer (pH 7.4), 10 mM NaCl, 10 mM $MgSO_4$) and extracted with an equal volume of chloroform. The

aqueous phase containing phage particles was subjected to CsCl step-gradient centrifugation as described previously (Sambrook et al., 1980) using a Discovery 100SE Sorvall ultracentrifuge (Thermo Scientific, Dubuque, IA) with a Beckman rotor VTi50 at 45,000 rpm (167,152 × *g*) for 22 h at 4^{o}C. The phage band was harvested and dialyzed in SnakeSkin Pleated Dialysis Tubing 10,000 MWCO (Pierce Biotechnology, Rockford, IL) for 24 h in 1,000 × volume of SM buffer (with 3 exchanges) and stored at 4^{o}C. The lambda phage titer after purification was 10^{12} pfu/ml.

Southern Blotting of DNA Extracted from Irradiated Lambda Phage. For each irradiation dose, 10 µl of irradiated lambda phage (1.25×10^{9} particles) and 10 µl of the non-irradiated controls (1.25×10^{9} particles) each were mixed with 2.5 µl 5 × tris/proteinase K (50 mM Tris-HCl (pH 7.4), 1 mg/ml proteinase K) and incubated at 65^{o}C for 60 min. After adding loading dye solution (Fermentas International Inc., Glen Burnie, MD), samples were loaded on a 0.8% agarose gel in 1 × TBE (Tris, Borate, EDTA buffer) with 0.5 µg/ml ethidium bromide. Alkaline transfer of lambda DNA to Nytran SuPerCharge membrane (Whatman Inc., Shleicher & Schuel, Florham Park, NJ) was performed in a Turboblotter transfer system according to the manufacturer's protocol. As a probe for hybridization, lambda DNA (New England Biolabs Inc., Ipswich, MA) was labeled using alpha-^{32}P dCTP (MP Biomedicals, Solon, OH) and Ready-to-Go DNA Labelling Beads (-dCTP) (Amersham Biosciences, Piscataway, NJ).

Lambda Phage Protein Gels and Immunoblots. 15 µl of lambda phage suspension after irradiation (1.9×10^{9} particles) were mixed with Laemmli sample buffer (BioRad Laboratories, Hercules, CA) and boiled for 5 min. Samples were separated by standard SDS-PAGE. Two identical 10-20% gradient precast gels (BioRad) were run at 195 V for 50 min. One gel was subjected to staining with Bio-Safe^{TM} Coomassie G-250 (BioRad). Proteins on the second gel were transferred to a nitrocellulose membrane (BioRad). Membranes were blocked with a PBS-Twin-BSA (1 × PBS (pH 7.4), 0.005% Tween 20, 1% bovine serum albumin). The membranes were incubated overnight with primary antibodies at 4ºC followed by incubation for 1 h at room temperature with a peroxidase-conjugated secondary antibody. The membranes were then treated with SuperSignal West

Pico Chemiluminescent Substrate (Pierce, Rockford, IL), exposed on Blue Lite Autorad Film (BioExpress, Kaysville, UT) or imaged by FUJIFILM Luminescent Image Analyzer LAS-1000plus and Image Reader LAS-1000 Life (FUJI Medical Systems USA, Stamford, CT). Primary antibodies were produced in rabbits by Pacific Immunology Corp., Ramona, CA. 4×10^{10} lambda phage were used for each rabbit immunization without adjuvant.

Large-Scale Preparation of V3526 Virus. V3526 was purified from supernatants of infected baby hamster kidney cells (BHK). BHK cells were infected with V3526 working stock virus (BHK p2) at a multiplicity of infection equal to 10. At 48 h post-infection, supernatants were harvested, clarified by low-speed centrifugation (10,000 × *g*, Sorvall RC-5C, GSA rotor) and concentrated by polyethylene glycol (PEG) precipitation (7% PEG, 2.3% NaCl, w/v) at 4°C overnight. Virus was pelleted at 10,000 × *g* for 30 min (Sorvall GSA rotor) and suspended in 10 ml 1 × TNE (10 mM Tris, 0.2 M NaCl, 1 mM EDTA, pH 7.4). Continuous sucrose gradients were generated using the Gradient Master (Biocomp Instruments, Fredericton, NB**,** Canada) as per the manufacturer's protocol. Virus was purified on 20-60% continuous sucrose gradients in 1 × TNE at 100,000 × *g* (Beckman SW 32 Ti) at 4°C for 4 h. To change the buffer, the purified virus was diluted in D-PBS (Invitrogen Corporation, Carlsbad, CA) and pelleted (100,000 × *g* at 4°C for 4 h). The pelleted virus was suspended in D-PBS. Viral titer was determined by standard plaque assay on Vero cells, and protein concentration was determined by BCA Assay (Pierce, Thermo Scientific, Rockford, IL).

Virus Infectivity Test. V3526 virus infectivity post-irradiation was tested by cytopathic effect (CPE). 3×10^3 Vero cells were seeded in each well of a 24-well plate in 500 µl of MEM/NCS. After overnight incubation, cells were infected with the V3526 control and test samples at a multiplicity of infection of 10. Plates were incubated for 72 h at 37°C under 5% CO_2, and CPE was evaluated daily by microscopy. At 72 h post-infection, cell supernatant was collected and cells were fixed and stained simultaneously with 10% neutral buffered formalin containing 0.1% crystal violet. 200 µl of the collected cell supernatant were then used to infect a new batch of Vero cells grown in 300 µl of fresh

MEM/NCS, again for 72 h. A total of five dilution-serial passages in Vero cells were performed followed by CPE evaluation for each round. Any low-level infectious virus particles which remain undetected after the first round can be amplified and detected by 5 rounds of passage. Virus titer was determined in the cell supernatants of the control and test samples by a 50% tissue culture infective dose as described previously (Aldovini and Walker, 1990) (Table S1).

Antibody Binding Assay (V3526). 2 µg of virus protein from control or test samples (equivalent to 9×10^8 pfu) were run on 4-20% Tris-Glycine gradient denaturing gels (Invitrogen Corporation) at 4˚C and 136V for 3 h. Proteins were transferred to nitrocellulose membranes (0.44 µm; Amersham Biosciences UK Limited, Little Chaflon, Buckinghamshire, UK) overnight in a XCell SureLock Mini-Cell System (Invitrogen Corporation) at 4˚C and 125 mA. Membranes were blocked with 3% non-fat dry milk (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in TBST (20 mM Tris-HCL, 150 mM NaCl, 0.1% Tween 20). The membranes were washed with TBST and TBS (20mM Tris-HCL, 150mM NaCl) and incubated for 1 h at room temperature with 0.5 µg/ml of primary monoclonal mouse antibody 13D4-1, which specifically binds to the E3 domain of the PE2 glycoprotein of V3526 (Parker et al., 2010). The membranes were then washed as described above and incubated with the secondary antibody (1:50000 Goat anti-mouse IgG alkaline-phosphatase conjugated, Chemicon International, Temecula, CA) in TBST for 1 h at room temperature. The membranes were then treated with 10 ml of Western Blue stabilized substrate for alkaline phosphatase (Promega Corporation, Madison, WI) for 5 min at room temperature. The reaction was stopped with 10 mM EDTA solution.

TEM Processing for Negative Staining. For negative staining experiments, 5 µl of sample were placed on carbon-stabilized, formvar-coated copper grids for 1-5 min followed by a brief wash in H_2O. Aqueous 2% uranyl acetate was then applied to the grid for 30-60 sec and then wicked off. Grids were allowed to air-dry and were then examined on a Philips CM100 transmission electron microscope (FEI, Hillsboro, OR). Images were

captured on a SPOT Insight 4MP digital camera (Diagnostic Instruments, Sterling Heights, MI).

TEM Processing for Thin Sections. Samples were fixed for 1 h in 2% formaldehyde/2% glutaraldehyde in PBS, washed, and post-fixed in 2% OsO_4 for 1 h. Samples were then dehydrated with ethanol, infiltrated with Spurr's epoxy resin (Electron Microscopy Sciences, Hatfield, PA), and polymerized at 60°C for 48 h. Ultrathin sections (70-80nm) were cut on a Leica UC6 ultramicrotome (Leica Microsystems, Inc, Buffalo Grove, IL) and collected on copper grids. Grids were post-stained in 2% w/v uranyl acetate for 15 min and then Reynold's lead citrate for 5min. Sample examination and imaging was performed as for negative staining.

Mice. Male C57BL/6 and μMT mice were purchased from The Jackson Laboratory, Bar Harbor, ME. Mice in each experiment were 8-14 weeks old during the course of the experiments. All animal experiments were done in compliance with the guidelines of the NIAID Institutional Animal Care and Use Committee.

Immunizations with MRSA. Immediately prior to injection into mice, irradiated MRSA was harvested by centrifugation (5,148 × *g*) for 12 min and the irradiated supernatant containing Mn-DP-Pi was removed. The bacterial pellets were suspended in PBS (Cellgro, Manassas, VA), complete Freund's adjuvant (CFA) (Sigma, St. Louis, MO), or alum (10% w/v Aluminum Potassium Sulfate Dohecahydrate in water, Ricca Chemical, Arlington, TX). Each mouse was injected intradermally 1-2 cm from the base of the tail with 10^8 CFU equivalents per injection (100 μl). The remaining irradiated stocks were left at 4^oC until booster immunizations 2 weeks later. Booster immunization for mice immunized with CFA was done in incomplete Freund's adjuvant (IFA) (Sigma). Two weeks after booster immunization, mice were challenged with live infection as described below. In select experiments, CD4-depleting GK1.5 or isotype control antibodies (BioXCell, West Lebanon, NH) were injected intraperitoneally (1 mg/mouse) 24 h before challenge.

Infections with MRSA. 10^7 CFU of MRSA with Cytodex beads (Sigma, St. Louis, MO) were injected intradermally (100 µl) into the shaved back of each mouse. Areas of the resultant lesions were measured daily using electronic calipers (Mitutoyo America Corporation, Aurora, IL).

MRSA Skin Burden. Skin from a 3 mm punch biopsy at the center of the infected area was homogenized with a TissueLyser II (Qiagen, Valencia, CA) for 7 min at 50 cycles per minute. Serial dilutions in PBS were plated on blood agar and the CFU per lesion was calculated by multiplying the CFU per unit area by the corresponding lesion area.

MRSA Antibody Titers. Irradiated USA300 or Wood 46 bioparticles (Invitrogen Corporation) in PBS (10^6 CFU/100 µl/well) were incubated in a 96-well Immunoplate (Thermo Scientific, Dubuque, IA) at 4°C overnight. Wood 46 bioparticles were used unless otherwise specified. The plate was washed thrice on an Aquamax 2000 (MDS Analytical Technologies (US) Inc., Sunnyvale, CA) with PBS and 0.05% Tween (Acros, Pittsburgh, PA) prior to each subsequent step. 5% Fetal Bovine Serum (FBS; Thermo Scientific) in PBS was added to each well at room temperature for 30 min. Serially diluted serum (100 µl/well) from infected or immunized mice was then added for 1 h. The plate was then incubated for 1 h with biotinylated goat anti-mouse IgG (Bethyl Laboratories, Inc., Montgomery, TX), diluted 1:50,000 in 10% FBS. Tetramethyl-benzidine (100 µl; Sigma, St. Louis, MO) was then added to each well for 14 min in the dark before 2N sulfuric acid (100 µl) was added to stop the reaction. The OD was determined at 450 nm on a Beckman Coulter DTX 880 and reported after subtraction of the background value from wells with no serum added.

Skin mRNA Isolation and Polymerase Chain Reaction (PCR). Abscess tissue was surgically removed, placed in RNAlater (Ambion, Foster City, CA), and stored at -80°C until processed. When ready, the samples were placed in 2 ml Safe-Lock tubes (Eppendorf, Hauppauge, NY) along with one stainless steel ball bearing (Qiagen) and run through the TissueLyser II (Qiagen) for 6 min at 70 cycles per minute in 300 µl of RLT Buffer (Qiagen). Then, the RNA was extracted using the Fibrous Tissue RNAeasy Kit

(Qiagen) per the manufacturer's instructions. RNA yield was measured on a NanoDrop ND-1000. RNA was stored at -80 °C until analyzed by PCR using Taqman One-Step RT-PCR Master Mix per the manufacturer's instructions (Life Technologies, Grand Island, NY). All samples were analyzed on a 7500-fast Real-Time PCR System (Life Technologies). All primers were purchased from Applied Biosciences, Life Technologies. Comparison of signal was performed using the ΔΔCT method.

Opsonophagocytosis-Mediated Killing Assay. 10^6 RAW 264.7 macrophages (ATCC) were added to wells of a 48-well plate and incubated overnight in high-glucose MEM supplemented with 10% FBS and 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM non-essential amino acids (all from Invitrogen Corporation). The next day, serum from immunized, unimmunized, and previously infected mice diluted in PBS was added to achieve the indicated final serum concentrations. 10^6 CFU of MRSA were then added to each well and incubated at 37°C for 1 h. Gentamycin (50 μg/ml) was then added to each well and incubated for an additional 1 h to kill extracellular bacteria. The cells were washed three times in sterile PBS, and lysed with molecular grade water. Serial dilutions were plated on blood agar plates to determine bacterial survival by CFU count.

Splenocyte Restimulation. Bone marrow-derived dendritic cells (DC), generated in a 5-day culture as previously described (Lutz et al, 1999), were incubated overnight with MRSA (5:1 MRSA:DC ratio) in the presence of gentamycin (50 μg/ml). DC were then washed 3 times in PBS. Splenic CD4 T cells from unimmunized mice, mice immunized with IRS, and mice immunized with MnDP-IRS were isolated using the AutoMACS Pro with a CD4 T cell isolation kit II (Miltenyi Biotec, Auburn, CA). The CD4 T cells were then added to the MRSA-pulsed or unpulsed DC at a 4:1 ratio. Culture supernatants were harvested at 72 h and cytokine concentrations determined with the Bio-plex suspension array system (BioRad, Hercules CA).

Skin Flow Cytometry. Single cell skin suspensions from infected abscesses were obtained as previously described (Mendez et al, 2004) and incubated with phorbol 12-myristate 13-acetate (50 ng/ml), ionomycin (1 μM), and brefeldin A (10 μg/ml) for 3.5 h

at 37°C and 5% CO_2 (all reagents from Invitrogen Corporation). Cells were washed and stained with Aqua live/dead stain per the manufacturer's protocol (Invitrogen Corporation). Cells were washed, resuspended in 2% formaldehyde and incubated at room temperature for 20 min. Antibodies for anti-CD45*PerCP5.5, anti-CD4*APC, and anti-IL-17a*PE were purchased from eBioscience (San Diego, CA). Antibody staining was performed in saponin (Sigma, St Louis, MO) with 10% FBS. Cells were analyzed on an LSR Fortessa (BD Bioscience, San Diego, CA). Cells were gated to analyze the CD45-positive live cells, and doublets and fragmented cells were excluded based on forward- and side-scatter.

Statistical Analysis. Means were compared using two-tailed unpaired t test with Prism software (GraphPad, San Diego, CA).

Supplemental References

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