Supporting Information

Title

Membrane-Active Amphipathic Peptide WRL3 with *in vitro* Antibiofilm Capability and *in vivo* Efficacy in Treating MRSA Burn Wound Infections

Authors

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TEXT S1

METHODS

Bacterial Strains and Growth Conditions. Methicillin-resistant *S. aureus* (MRSA) ATCC 43300 were purchased from the American Type Culture Collection (ATCC, USA). *S. aureus* GIM 1.142, GIM 1.55, GIM 1.174, and GIM 1.221 were acquired from Guangdong Microbiology culture center (Guangdong, China). Clinically isolated *S. aureus* strains CICC 10786, CICC 10788, CICC 10790, CICC 10201, and CICC 20235 were obtained from the China center of industrial culture collection (CICC) (Beijing, China). The strains were stored at −20°C in 15% glycerol and cultured at 37°C in Mueller-Hinton broth (MHB) with rotary shaking at 180 rpm prior to use.

In Vitro Antimicrobial Testing. The Minimal inhibitory concentrations (MICs) of the peptide and antibiotics were determined using the broth microdilution method[1]. Briefly, the peptides were dissolved and diluted in 0.01% acetic acid and 0.2% bovine serum albumin (BSA). A 50-μL volume of bacteria (10^6 CFU/ml) in MHB was incubated with 50-μL of serially diluted aliquots of the peptides in sterile 96-well plates. The microtiter plates were then incubated statically at 37 °C overnight. MICs were determined as the minimal concentration at which no visible bacterial growth was present. All experiments were conducted in triplicate.

To further evaluate the bactericidal potency of the peptide and antibiotics against MRSA, the lethal concentration (LC) was determined, as described previously[2]. Briefly, the bacterial cells were washed three times with 10 mM sterile phosphate-buffered saline (PBS, pH 7.4) and re-suspended in the same buffer. The bacterial suspensions (10^5 CFU/ml) were incubated with various concentrations (0-256 μg/ml) of the samples tested for 2 h at 37°C as described before. After that, the bacterial samples were collected at fixed intervals and plated on nutrient agar plates. The

surviving colonies were counted after incubation at 37 °C for 18 h. The lowest concentration of peptides at which there was complete killing was taken as the LC. The results were expressed as an average of the data from three independent assays.

Time Kinetics of Bacterial Killing. MRSA cells (2×10^6 CFU/ml) were treated with the peptide and antibiotics at 0 ×, 1 ×, 2 ×, or 4 × MIC in PBS at 37 °C. Aliquots of the peptide-treated bacterial suspensions were withdrawn at different times (0, 5, 10, 20, 40, 60, 80, 100 and 120 min), serially diluted, and plated on MH agar to determine the viable bacterial counts, and each test was reproduced at least three times.

Salt and Serum Sensitivity. To determine whether the antimicrobial activity of the peptide and antibiotics was affected by the presence of salts or serum, we measured the MICs in MHB supplemented with various salt ions (150 mM NaCl, 4.5 mM KCl, 6 mM NH_4Cl, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 4 mM $FeCl_3$) or inactivated mouse serum (25% and 50%), as described above. The results shown were from three independent assays.

Synergistic Effect. The effects of AMPs in combination with antibiotics were assessed by using the broth microdilution checkerboard technique[3]. Briefly, the antibiotics and peptides were serially diluted, 50 μL of antibiotics and 50 μL of peptides were mixed in each microtiter well and inoculated with 100 μL of bacterial solution (approximately 4×10^5 CFU/ml). After incubation at 37 °C for 18 to 24 h, the fractional inhibitory concentration index (FICI) was determined as the inhibitory concentration of the combination divided by that of the single sample. The combination index was derived from the highest dilution of antibiotic combination permitting no visible growth. With this

method, FICI < 0.5 is considered synergy; 0.5 < FICI < 1.0 is considered additivity; 1.0 < FICI < 4.0 is considered indifference; and FICI > 4.0 is considered antagonism.

Hemolysis and Cytotoxicity Assays. Hemolysis assay was measured according to the published methods[4]. Serial dilutions of testing samples were incubated with human red cells (4 x 10^6 cells/ml) at 37 °C for 60 min, the cells were then centrifuged and the absorbance of the supernatant was measured at 540 nm. Maximum hemolysis was determined by adding 1% Triton X-100 to a sample of cells. Hemolysis of testing samples was calculated as the percentage of Triton X-100. The minimal hemolysis concentration (MHC) were defined as the peptide concentration causing 10% hemolysis on human erythrocytes.

The normal human liver cell line L-O2 was obtained from School Of Life Science and Technology, China Pharmaceutical University (Nanjing, China) and cultured in a 96-well plate (1 × 10^5 cells/well) with Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 mg/ml) and 2 mM L-glutamine at 37 °C in a humidified 5% CO2 atmosphere. Cell viability was evaluated in the presence of various concentrations (0-128 μM) of the test samples by 3-(4, 5-dimethylthiozol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma) reduction assay[5]. After incubation for 24 h at 37 °C, MTT solution (50 μL, 0.5 mg/ml) was added in every well and incubated for 4 hours under the same condition. After that, MTT-containing medium was removed gently and replaced with DMSO (150 μL per well) to mix the formazan crystals until dissolved. The plates were read on microtiter plate reader at 570 nm. The experiments were performed in triplicate.

Cell Selectivity Assay in the Presence of Bacteria. To determine the selectivity of peptide and antibiotics towards the bacteria and host cells, the activities of peptide and antibiotics were assayed in a co-culture of L-O2 cells with MRSA [2]. The L-O2 cells (approximately 2×10^5 cells/well in DMEM medium) were placed into 12-well plates and then incubated at 37 °C and 5% CO_2 until a monolayer was formed. The medium was aspirated, and a 100-μl of MRSA suspension (approximately 3×10^7 cells/ml) was added to each well, and 900-μl of medium containing methicillin, vancomycin or WRL3 (at a final concentration of 1×MIC) was then added. The co-culture was then incubated at 37 °C for 0, 30, 60, 90, 120 or 150 min. To determine bacterial survival, 100-μL of the co-culture supernatant was withdrawn and serially diluted and plated on MH agar. After incubation overnight at 37°C, the number of bacterial cells was counted. Subsequently, L-O2 cells were washed with sterile PBS thrice and cell viability was measured as described above. As controls, cells were treated with DMEM medium in the absence (0% cytotoxicity) or presence of MRSA or with 0.1% Triton X-100 (100% cytotoxicity). The results were expressed as an average of the data from three independent assays.

Flow Cytometry. MRSA cells (approximately 2×10^7 CFU/ml) were incubated with the peptide and antibiotics at their 0.5 × MIC, 1 × MIC, or 2 × MIC at 37°C for 30 min. Then a final concentration of 10 μg/ml of propidium iodide (PI) was added to the bacterial suspension and incubated for 30 min. The bacterial cells were centrifuged, washed thrice and re-suspended in PBS. Flow cytometry was performed using a FACScan (Becton-Dickinson, USA) at a laser excitation wavelength of 488 nm[6].

Confocal Microscopy. MRSA cells (approximately 3×10^6 CFU/ml) were exposed to the fluorescein isothiocyanate (FITC)-labeled WRL3 at 1 × MIC for different amounts of time (0, 15, and 30 min), and the bacterial cells were then washed thrice with PBS with centrifugation at 2,000 × *g* for

10 min. The locations of the FITC-labelled peptides in the live bacterial cells were imaged using a confocal microscope (Leica SP2, GER) with a 488-nm band-pass filter for the FITC excitation.

Atomic Force Microscopy. The effects of peptide and antibiotics on the bacterial cell surface were examined by AFM [7]. MRSA cells were cultured to the exponential phase in MHB at 37 °C under constant shaking at 180 rpm. After centrifugation at 2000 × *g* for 10 min, the cell pellets were harvested, washed thrice with PBS, and re-suspended to an OD_{600} of 0.2. The cells were incubated with the peptide and antibiotics at their 1 × MICs at 37 °C for 30 or 60 min, with no peptide added to the control. After incubation, the cells were harvested *via* centrifugation at 5000 × *g* for 10 min, washed thrice and re-suspended in deionized water. 10-20 μL of bacterial suspension was plated on mica, followed by air-drying. The images were obtained using a Dimension Icon atomic force microscope (Bruker, USA).

TEM Observations. Bacterial sample preparation for TEM was conducted using the same protocol as that for AFM. The bacterial cells were pre-fixed with 2.5% glutaraldehyde at 4 °C overnight, washed twice with PBS, and post-fixed with 2% osmium tetroxide for 70 min. After washing twice with PBS, the bacterial samples were dehydrated for 8 min in a graded ethanol series (50, 70, 90, and 100%), followed by 10 min in 100% ethanol, a mixture (1:1) of 100% ethanol and acetone, and absolute acetone. The specimens were then transferred to 1:1 mixtures of absolute acetone and epoxy resin for 30 min and then to pure epoxy resin and incubated overnight at a constant temperature. Specimens were then sectioned using an ultramicrotome, stained with uranyl acetate and lead citrate, and examined using a HITACHI H-7650 TEM[7].

Liposome Preparation. Phosphatidylglycerol (PG), cardiolipin (CL), phosphatidylcholine (PC) and cholesterol (CHO) were obtained from Sigma-Aldrich (USA). Two types of liposomes were prepared as follows: PG/CL with a mass ratio of 3:1 to mimic the *S. aureus* membrane and PC/CHO with a mass ratio of 10:1 to mimic the human erythrocyte cell membrane[8].

For large unilamellar vesicle (SUV) preparations, appropriate amounts of lipids were dissolved in chloroform. The solution was dried by rotary evaporation of the organic solvent to a thin film on the wall of a 50 ml round bottomed flask, and traces of solvent were removed under a stream of nitrogen 6-8 h while connected to a vacuum pump. The lipid thin film was hydrated by vortex agitation with 2 ml of TES buffer (10 Mm Tris-HCl, 1 mM EDTA, 3.5 mM SDS, pH 7.4). The liposome suspension was then sonicated using a vibra cell sonicator for at least two 5-min cycles. The liposomes were diluted properly and incubated with FITC-labeled peptide at 4 μg/ml for 15, 30 or 60 min, the images were captured using a confocal microscopy[9].

Peptide-induced Dye Leakage Assay. Calcein-loaded liposomes were prepared as described previously[10]. Phospholipids were dissolved in chloroform at each of the aforementioned ratios. After vacuum evaporation and drying overnight, a dye solution (60 mM calcein, 50 mM TES, 100 mM NaCl, pH 7.4) was added to each dried sample. Each mixture was ultrasonicated and subjected to 8-10 cycles of freezing and thawing in liquid nitrogen until homogeneous, then extruded 10-15 times through a 0.22 μm polycarbonate membrane with a LiposoFast extruder (Avestin, Inc., Canada). The untrapped calcein was removed by gel filtration through a Sephadex G-50 column and eluted with 10 mM PBS. Lipid concentration was determined by quantitative phosphorus analysis. The calcein-loaded liposomes were diluted to a final lipid concentration of 100 μM prior to use. After

adding the peptide and antibiotics to final concentrations of 2, 4 and 8 μg/ml, the percentage of calcein released from the liposomes was measured by monitoring fluorescence intensity at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The 100% dye leakage release was obtained using 0.1% Triton X-100. The percentage of the calcein release caused by the samples was calculated using the equation: Dye release (%) = $(F_{obs} - F_0)/(F_{100} - F_0) \times 100$ %, where F_0 is the fluorescence intensity of liposomes (background), F_{obs} and F_{100} are the fluorescence intensities achieved by samples and Triton X-100, respectively.

Biofilm Growth Inhibition. Overnight cultures of MRSA cells diluted to 2×10^5 CFU/ml were added into each well of a 96-well plate at a volume of 100 μL/well and allowed to adhere overnight at 37 °C with gentle shaking at 80 rpm. The wells were then rinsed twice with 120 μL of PBS to remove planktonic cells and loosely attached cells, and replenished with 100 μL of fresh broth. Biofilm formation was allowed to proceed up to 6-7 days with daily rinsing and media changes before use in experiments. To determine the effects of peptide or antibiotic treatment on viability of MRSA in biofilms, 100 μL of peptide or antibiotic solutions at their 1 × MIC, 4 × MIC and 8 × MIC levels were added into each well and allowed to incubate for 24 h. Subsequently, the supernatants were removed, rinsed once with 120 μL of PBS before 120 μL of activated XTT solution was added into each well. After 4 h incubation, 60-μL aliquot from each well was transferred to a new 96-well plate for the absorbance determination using a microplate reader (TECAN, Switzerland) at measurement and reference wavelengths of 490 nm and 660 nm, respectively. Relative cell viability was expressed as $[(A_{490\ nm}-A_{660\ nm})$ sample / $(A_{490\ nm}-A_{660\ nm})$ control$] \times 100\%$. Data are expressed as mean ± standard deviations of three replicates per concentration[11].

Biomass Assay and Biofilm Imaging. The biomass of the biofilms was estimated by a crystal violet staining assay[11-12]. Briefly, the formed biofilms were first treated with the peptide or antibiotics for 24 h as described above. After aspirating the culture medium, the biofilms were rinsed once with PBS, fixed with methanol for 15 min at room temperature and stained with 100 μL of 0.1% (weight by volume) crystal violet for 10 min. The excess crystal violet dye was removed by rinsing the wells with deionized water for five times. The dye that was associated with the biofilm was extracted using 100 μL of 33% glacial acetic acid per well and 60 μL aliquot from each well was transferred to a new 96-well plate for quantification of absorbance at a wavelength of 570 nm using a microplate reader. The relative amount of biomass remaining after peptide treatment was expressed as a percentage of the control treated with broth containing 10% (by volume) of water. Data represent mean ± standard deviations of three or five replicates per concentration.

For image observation, Overnight cultures of MRSA cells diluted to 2×10^5 CFU/ml were added into a 6-well plate with a circular coverslip in the bottom at a volume of 2 mL/well and allowed to adhere overnight at 37 °C with gentle shaking at 80 rpm. The wells were then rinsed three to five times with 3 mL of sterile PBS to remove planktonic cells, and replenished with 2 mL of fresh broth. The biofilm formation was allowed to proceed up to 6-7 days with daily rinsing and media changes before use. Subsequently, the formed biofilms were treated with the peptide at 1 × MIC, 4 × MIC and 8 × MIC for 24 h, the residual biofilms on the coverslip were rinsed with PBS, fixed with 4% formaldehyde and then subjected to dehydration using a series of graded ethanol solutions, then dried and coated with gold before imaging using a using a HITACHI S-4800 scanning electron microscope (SEM).

Animals. Male, 6-8 weeks old ICR mice were used in this assay. All mice were housed individually under specific pathogen-free (SPF) laboratory condition at 25 °C and a relative humidity of 55%, and maintained on a 12 h/12 h light/dark cycle with *ad libitum* access to food and aseptic water. All treatments attempted to minimize suffering. All animal care and experimental procedures were in accordance with Nanjing Agricultural University (NAU) guidelines and were approved by the Animal Ethical and Experimental Committee (AEEC) of NAU.

MRSA-infected Burn Wound Mouse Model and Treatment. Eighty mice were randomly divided into eight groups of ten mice each. Each mouse was housed in an individual cage, and the different treatments were caged separately. All mice were allowed to acclimatize to the new environment for one week before treatment initiation. As described previously[13], mice were not anaesthetized when burn injury was induced. The hair on the backs of the mice was shaved using a razor to expose an area of approximately 2 cm × 2 cm. The bare skin of all mice except those in the blank control group (Group 1) was burned with a constant-temperature electric iron (Atten, China) at 75°C for 30 seconds, creating a burned area approximately 1 cm × 1 cm × 0.2 cm. At 1 h after burning, the mice of Group 2 were inoculated with 50 μL of the sterile PBS (pH 7.4), the mice of Group 3 to Group 8 were infected with 50 μL of bacterial suspension (4×10^8 CFU/ml) of MRSA ATCC 43300 in the burned area. After infecting for 3 h, 50 μL of peptide or antibiotic solutions were dropped on the burned skin by the disposable syringes at their 5 × MICs for 60 s, and then wounds were covered with Tegaderm to maintain uniformity and to prevent the loss of applied materials. All mice except those in Group 1, 2 and 3 were treated for two weeks twice daily, as follows: Group 4, Group 5, Group 6, Group 7 and Group 8 received methicillin (MET, 320 μg/ml), vancomycin (VAN,

2.5 μg/ml), WRL3 (10 μg/ml), ceftriaxone (CRO, 40 μg/ml) and a combination of WRL3 (5 μg/ml) and CRO (20 μg/ml), respectively. Group 1 functioned as the blank control group; Group 2 was the burn control group; and Group 3 was the MRSA-infected model group. The wounds at 3, 7 and 14 days were examined to assess the transitions from inflammatory to regenerative and regenerative to resolving phases of wound healing. The eschar of the wounds was observed daily, and the times at which it shed and healed were recorded. At the end of the examination period, animals were euthanized by CO2 inhalation.

Assessment of Wound Infection. On days 1, 3, 5, 7, 10, 14, 17, and 21 after infection, the area of the burned wounds on the mice was measured with a ruler. The serums collected on days 1, 2, and 3 were used to assess the pro-inflammatory cytokine expression by ELISA kit (Invitrogen, USA). The wound of each mouse was individually scraped 7 times with a sterile cotton bud. The cotton buds were then immersed into centrifuge tubes containing 1 ml of sterile PBS, and then the tubes were rolled to drop the bacteria into the solution. The suspensions were diluted serially with sterile PBS, and 20-μL of each dilution was added to the MH agar plates and incubated for 18-24 hours at 37°C. After the incubation period, colony forming units (CFU) were counted, averaged, and expressed as CFU/ml.

Histological Examination. Tissue samples were harvested for microbial and histological analysis, and processed as described previously[13-15]. Samples from each experiment were fixed in 4% buffered paraformaldehyde. Tissue samples were subjected to Gram staining, hematoxylin/eosin (HE) and immunohistochemistry (IHC) was analyzed by three independent investigators. Images were obtained using a light microscope (Eclipse E200, Nikon, Japan).

Data Analysis. The experimental results were indexed by their mean values and standard deviations. All tests, with the exception of the burn wound skin infection model, were carried out three times. All data were examined using the ANOVA (*t*-test) with SAS 9.2 software. Statistical significance is indicated on the graphs by asterisks (*, **, or ***) for *P* values of 0.05, 0.01, and 0.001, respectively.

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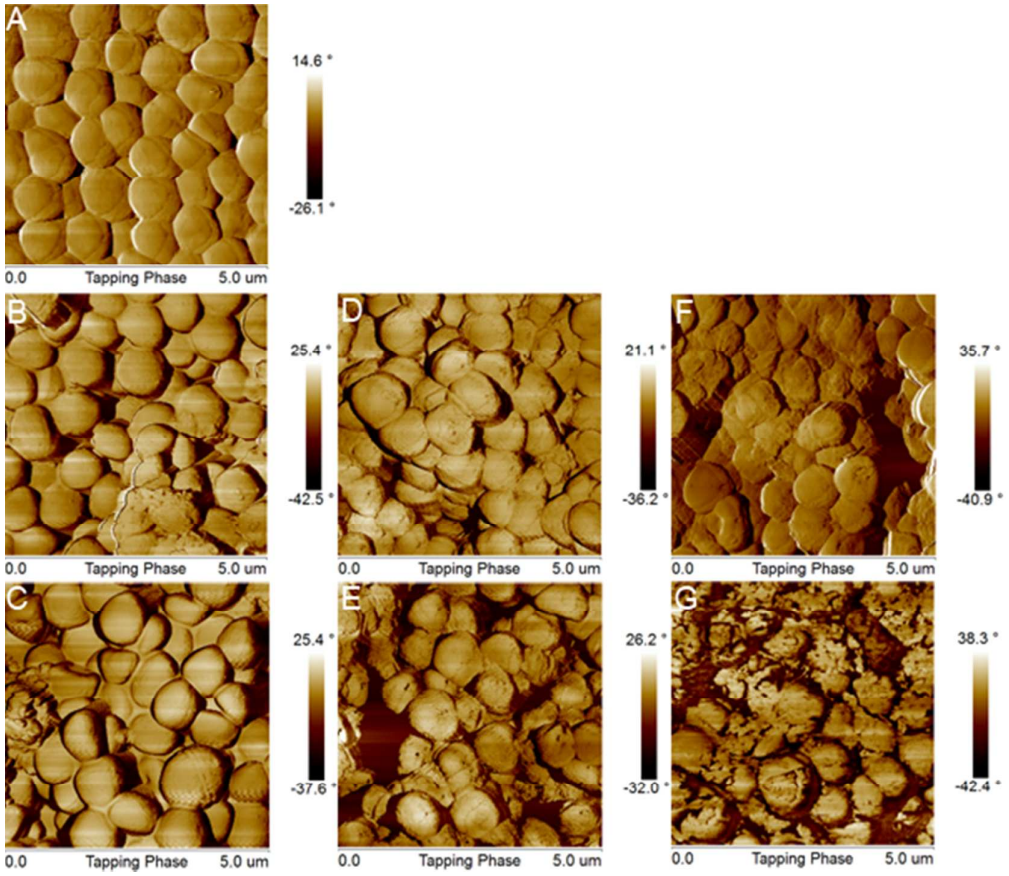
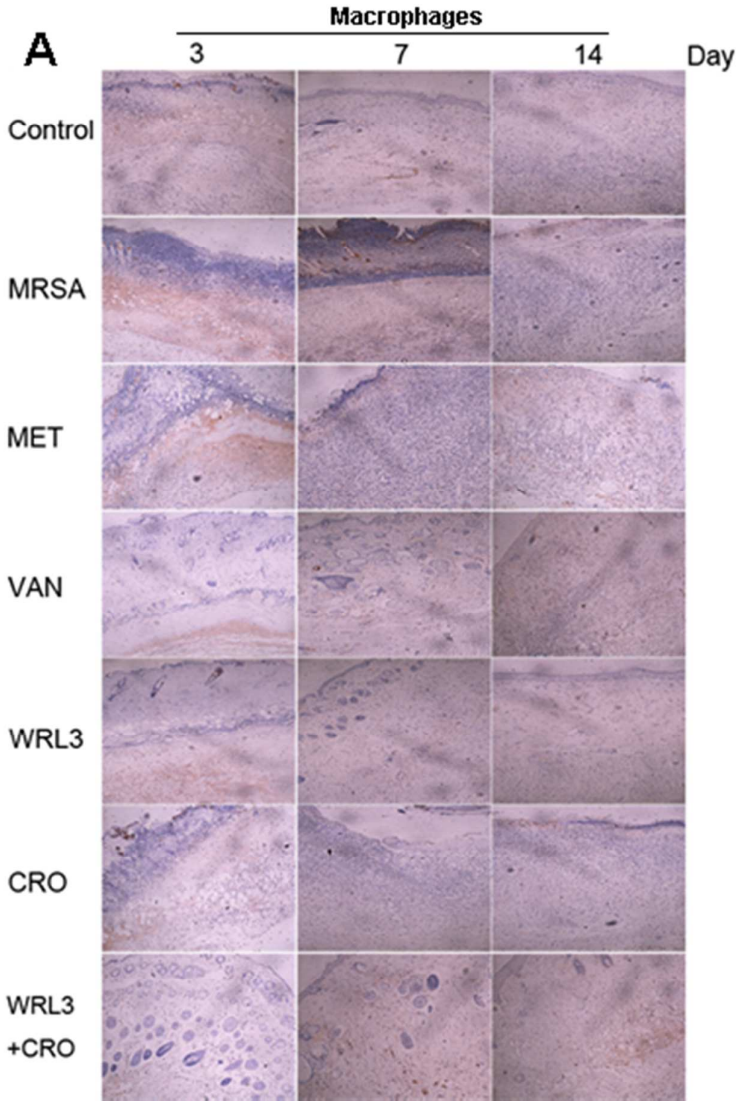


Supplemental Figure S1. AFM images of MRSA. The bacteria cells treated with peptide and antibiotics at their 1 × MICs. (A) Control; (B) methicillin for 30 min; (C) methicillin for 60 min; (D) vancomycin for 30 min; (E) vancomycin for 60 min; (F) WRL3 for 30 min; (G) WRL3 for 60 min; The control was processed without peptides.



Supplemental Figure S2. Immunohistochemical staining. WRL3 regulates the recruitment of (A) macrophages, (B) monocytes and (C) VEGF production in MRSA-infected wounds. A scalded skin region of approximately 1 cm^2 on the back of the mice was infected with 50 µL of broth mix containing 10^8 cfu of MRSA alone, or together with MET, VAN, WRL3, CRO or WRL3 and CRO. Skin samples from the injured area were fixed and subjected to staining with antibodies against macrophages, monocytes, and VEGF at 3, 7, and 14 days post-treatment. Presented figures are representative of three sections examined from 3 mice per group at each time point.

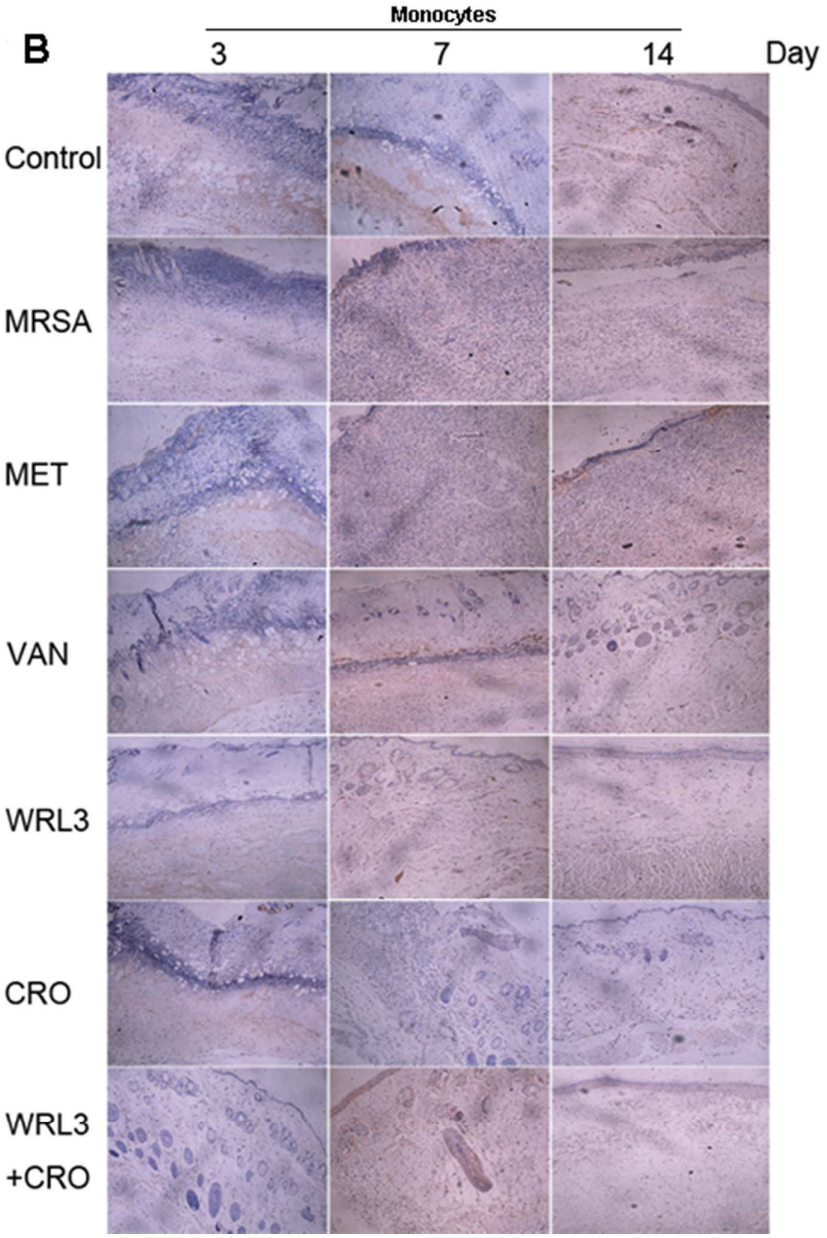


Figure S2 (*continued*).

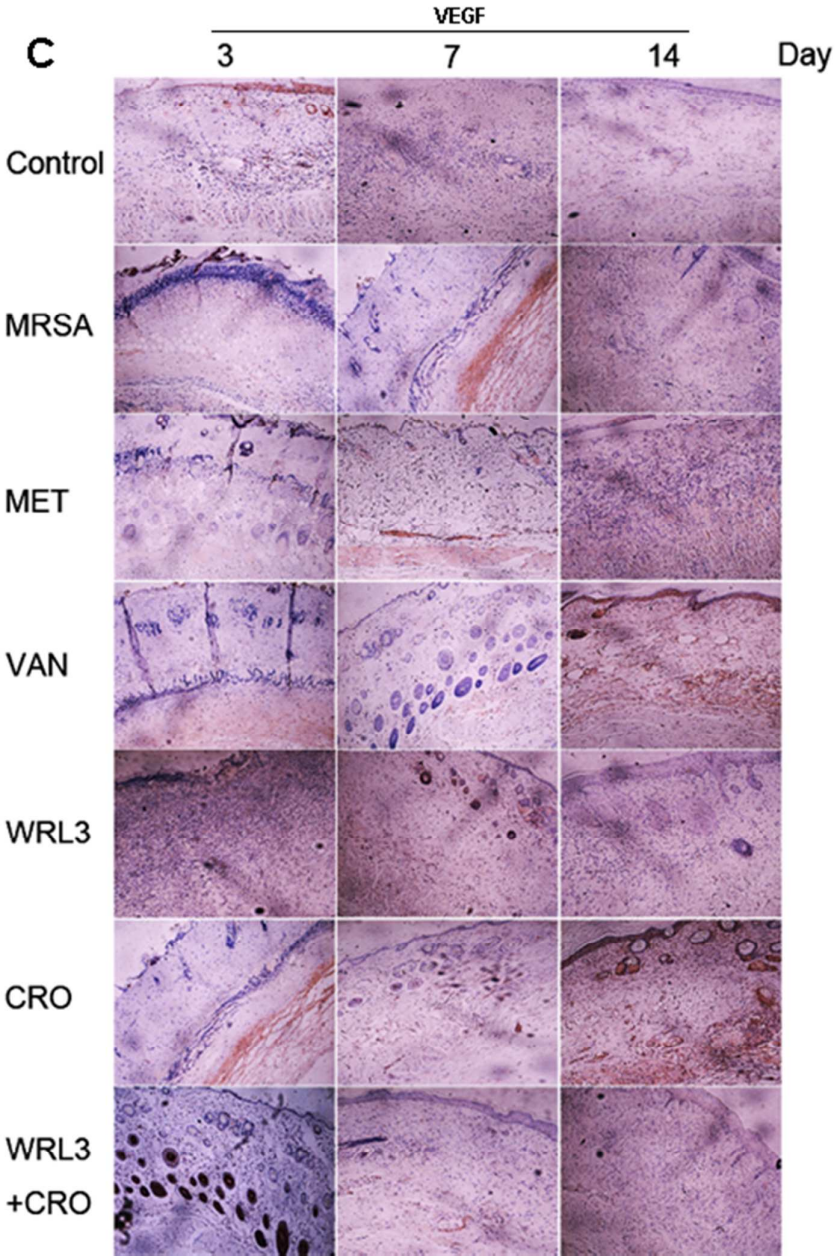


Figure S2 (*continued*).