SUPPLEMENTAL INFORMATION

Methicillin-Resistant *Staphylococcus aureus* Bacterial Nitric Oxide Synthase Affects Antibiotic Sensitivity and Skin Abscess Development

Nina M. van Sorge[1,§], Federico C. Beasley[1], Ivan Gusarov[4], David J. Gonzalez[1,2], Maren von Köckritz-Blickwede[1], Sabina Anik[1], Andrew Borkowski[1], Pieter C. Dorrestein[2,3], Evgeny Nudler[4], Victor Nizet[1, 3, *]

[1]Department of Pediatrics, [2]Department of Chemistry and Biochemistry and [3]Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, CA 92093

[4]Department of Biochemistry, New York University School of Medicine, New York, NY 10016

[*]To whom correspondence should be addressed: Prof. Victor Nizet, Department of Pediatrics, University of California San Diego, 9500 Gilman Drive, Mail Code 0687, La Jolla, CA, USA 92093 Tel. (858) 534 7408; Fax (858) 534-5611; E-Mail: vnizet@ucsd.edu

Content

Supplemental Experimental Procedures **Page 2**

Figures

Supplemental Figure S1. Daptomycin killing kinetics **Page 4**
Supplemental Figure S2. Effect of iron on bacterial growth **Page 5**
Supplemental Figure S3. SOD activity assay **Page 6**
Supplemental Figure S4. Linezolid killing assay **Page 7**
Supplemental Figure S5. Neutrophil oxidative burst **Page 8**
Supplemental Figure S6, Neutrophil NO production **Page 9**
Supplemental Figure S7, Neutrophil lysis **Page 10**
Supplemental Figure S8, NET induction **Page 11**
Supplemental Figure S9, CD-1 mouse subcutaneous infection **Page 12**

Tables

Supplemental Table S1. Homology of *nos* gene sequences **Page 13**

Supplementary Experimental Procedures

Neutrophil oxidative burst assay

Quantification of the neutrophil oxidative burst was performed using a chemiluminescence assay. Bacteria were grown as described in '*Neutrophil killing assays*' in the Experimental Procedures of the main manuscripts and resuspended in Hanks' balanced saline solution (HBSS) to the desired concentration. Isolated human neutrophils were resuspended at $1x10^6$ cells/ml in HBSS supplemented with 2% 70°C heat-inactivated fetal bovine serum (FBS). Bacteria or buffer were added to 100 ml Luminol (Sigma) in HBSS in a white 96-well plate. After addition of 50 µl neutrophil suspension, luminescence was measured for 0.1 sec every 30 sec over the course of 1 h (Centro LB960, Berthold Technologies). The area under the curve (AUC) was calculated using GraphPad Prism version 5.00.

Neutrophil NO production

Neutrophil NO production was quantified using the NO-specific fluorescent probe DAF-FM diacetate (Molecular Probes). NO production was measured using the same experimental conditions as described in 'Neutrophil killing assay' of the Experimental Procedureswith three modifications. First, neutrophils were loaded with 5 µM DAF-FM diacetate for 20 min at room temperature before addition of PMA. Second, the experiment the assay buffer used RPMI without phenol red to abolish any interference with fluorescence. Third, the experiment was performed in a 96 well plate (flat bottom) using a total volume of 150 µl per well. Fluorescence (excitation 485 nm/emission 520 nm) was measure every 5 min for 2 h at 37°C using a Fluostar Omega plate reader (BMG labtech) for each well. The fluorescence signal from 3 identical wells was averaged and corrected for blank well containing only medium. Total fluorescence at 30 and 90 min is plotted to allow direct comparison with the neutrophil killing experiment. The experiment was performed four times in triplicate using different donors.

Neutrophil lysis

Neutrophil lysis was determined by lactate dehydrogenase release using the CytoTox 96 Non-Radioactive Cytotoxicity kit (Promega) under the experimental conditions described in 'Neutrophil NO production' above with the modification that cells were not labeled with DAF-FM acetate. Absorbance measured at 540 nm reflects amount of LDH released in cell supernatants. Complete lysis of cells served as a positive control, and neutrophil only as a negative control. Lysis was determined at 30 and 90 min after incubation with bacteria to allow direct comparison with the neutrophil killing assay. The experiment was performed three times in triplicate using different donors.

NET quantitation by microscopy and picogreen assay

The quantification of NETs was performed as described (von Köckritz-Blickwede et al., 2010). Briefly, 2×10^5 cells per well (96 well plate) were infected with an MOI of 2 for 90 min at 37°C 5% CO_2. Next, 500 mU/ml micrococcal nuclease was added for 10 min at 37°C. The reaction was stopped with 5mM EDTA and the cells were centrifuged at 200 × g for 8 min. Extracellular DNA in the supernatant was quantified using Picogreen as recommended by the manufacturer. For immunofluorescence microscopic visualisation of NETs, 5×10^5 primary human neutrophils were seeded on poly-L-lysine coated glass slides in a 24-well plate and infected as described above. Then, the cells were fixed with 4% PFA for 15 min at room temperature and subsequently at 4°C. Immunostaining of NETs was done as previously described (von Köckritz-Blickwede et al., 2010) using mouse anti H2A-H2B-DNA complex antibody (#PL2-6 mouse IgG2b stock:2.65 mg/ml 1:3000 diluted (Losman et al., 1992) overnight at 4°C followed by an Alexa 488-conjugated secondary goat anti mouse antibody (1:500-diluted; Invitrogen, Germany) for 1 h at room temperature. Samples were embedded in Dapi-Prolong-Gold (Invitrogen, Germany) to stain all nuclei blue. Mounted samples were examined using an inverted confocal laser-scanning 2-photon microscope Olympus Fluoview FV1000 with Fluoview™ Spectral Scanning technology (Olympus).

Growth curves

Bacteria (containing empty vector control or pNOS) were grown overnight in TSB containing chloramphenicol 20 mg/ml. The next day, bacteria were regrown to $OD_{600\ nm}$ 0.4 in TSB, washed and resuspended in RPMI 1640

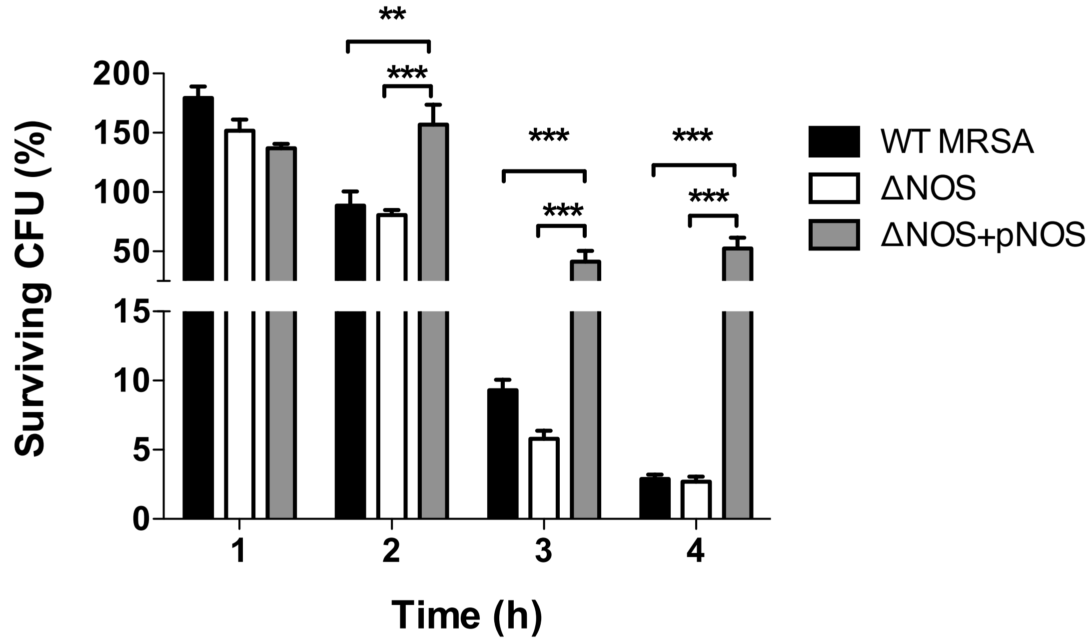
medium supplemented with 1% casamino acids (RPMI-CA). Bacteria ($5x10^5$ CFU) were added to 100 well Bioscreen Honeycomb plates in a total volume of 200 µl RPMI-CA containing with or without $FeSO_4$ concentrations (up to 500 µM). Growth was determined by measuring OD at 600 nm every 15 min for 10 h using Bioscreen C MBR machine.

CD-1 mouse subcutaneous infection
8-10 week-old female CD-1 mice (Charles River) were injected subcutaneously in one flank with USA300 WT and the opposite flank with DNOS bacteria for direct comparison. Bacterial cultures were grown to $OD_{600} = 0.4$ in TSB, washed in PBS and resuspended in PBS mixed 1:1 with 1 mg/ml of Cytodex® microcarrier beads (Sigma Aldrich). One hundred µl containing a final amount of 5×10^6 CFU bacteria was injected subcutaneously. Lesion size, as assessed by measuring length x width (mm^2) of the developing ulcer, was recorded daily for four days. Data are presented as lesion size mm^2 WT - lesion size mm^2 DNOS. Data shown are combined from two independent experiments with a total of 20 mice.

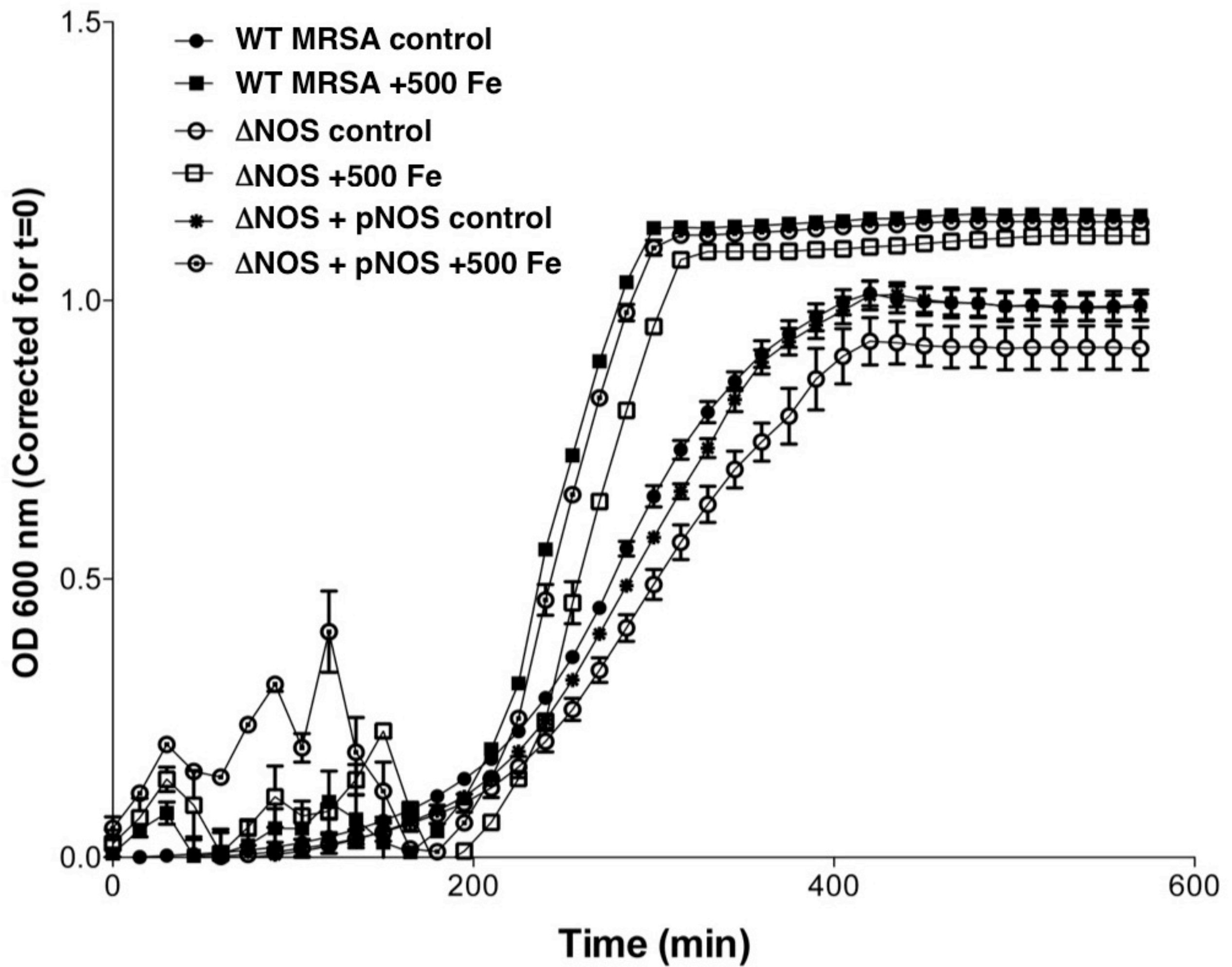
REFERENCES:

Losman MJ, Fasy TM, Novick KE, Monestier M. Monoclonal autoantibodies to subnucleosomes from a MRL/Mp(-)+/+ mouse. Oligoclonality of the antibody response and recognition of a determinant composed of histones H2A, H2B and DNA. J Immunol 1992;148:1561-9.

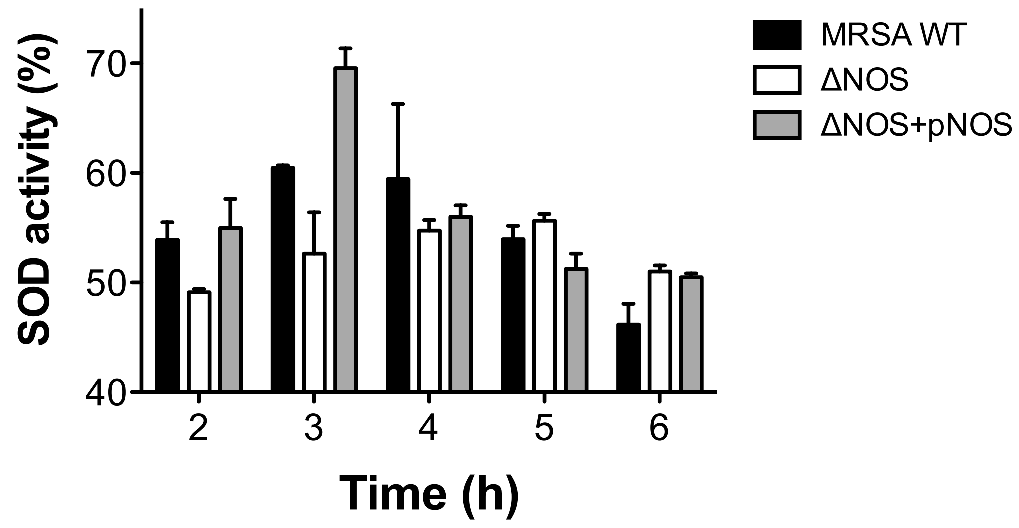
Von Köckritz-Blickwede M, Chow O, Ghochani M, and Nizet V. 2010. Visualization and functional evaluation of phagocyte extracellular traps. In: Immunology of Infection, 3rd edition, edited by Kaufmann S and Kabelitz D. Series: Methods in Microbiology, Academic Press, Elsevier Science.



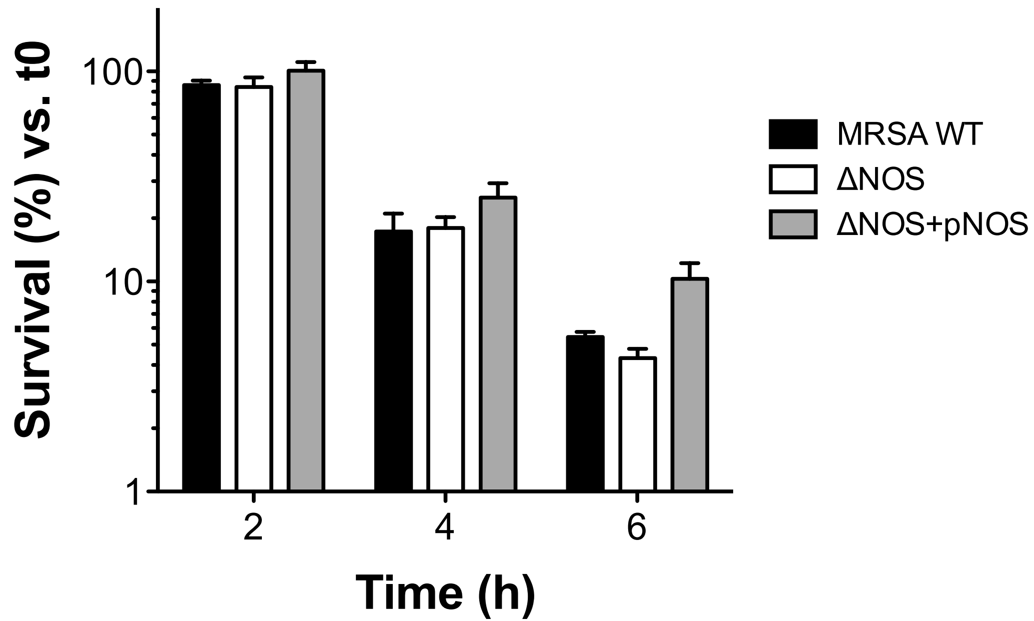
Supplemental Figure S1. Influence of bNOS on kinetics of MRSA killing by daptomycin (4 mg/ml). Overexpression of bNOS diminishes killing of MRSA by daptomycin. Pooled data from three independent experiments are shown (Mean +/- SEM).



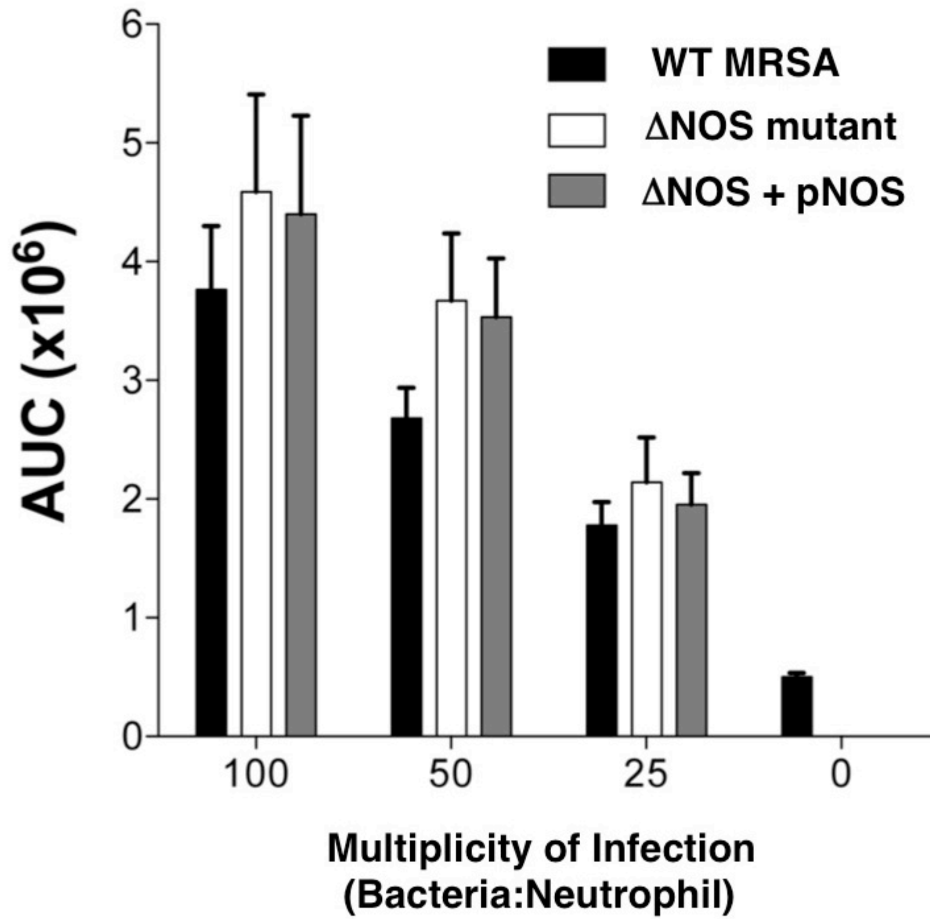
Supplemental Figure S2. bNOS does not contribute to MRSA resistance to high iron concentrations in the absence of oxidative stress. Growth of WT MRSA, ΔNOS mutant and complemented strains in RPMI 1640 media + 1% casamino acids with or without addition of 500 mM $FeSO_4$. No obvious differential toxicity of iron towards MRSA and ΔNOS mutant strain is observed.



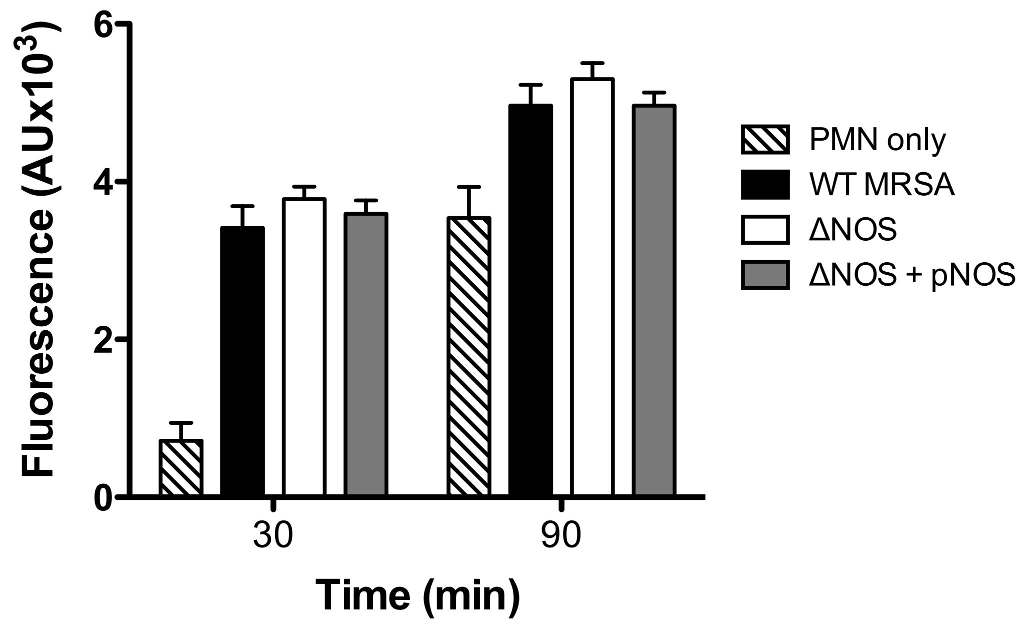
Supplemental Figure S3. SOD activity assay as measured using a specific activity kit.



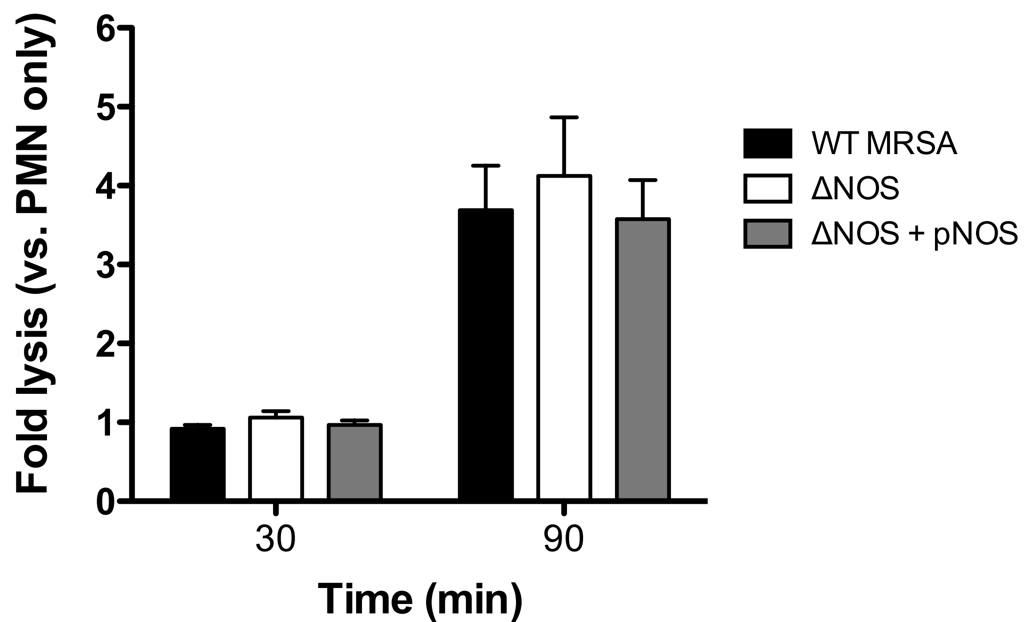
Supplemental Figure S4. bNOS does not influence linezolid susceptibility. Bacterial survival at different time points post linezolid exposure (4 mg/ml). Pooled data from three independent experiments are shown (mean +/- SEM)



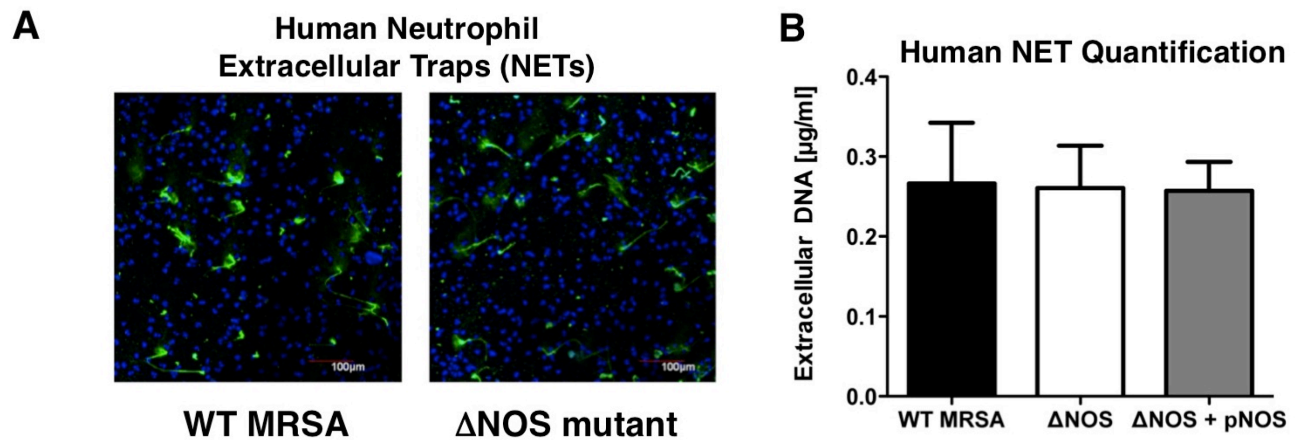
Supplemental Figure S5. bNOS does not affect oxidative burst activity of human neutrophils in response to *Staphylococcus aureus* strains at each indicated multiplicity of infection (MOI). Area-under-the-curve (AUC) as measurement for oxidative burst over 60 min. Pooled data from 4 independent experiments in duplicate (mean +/- SEM)



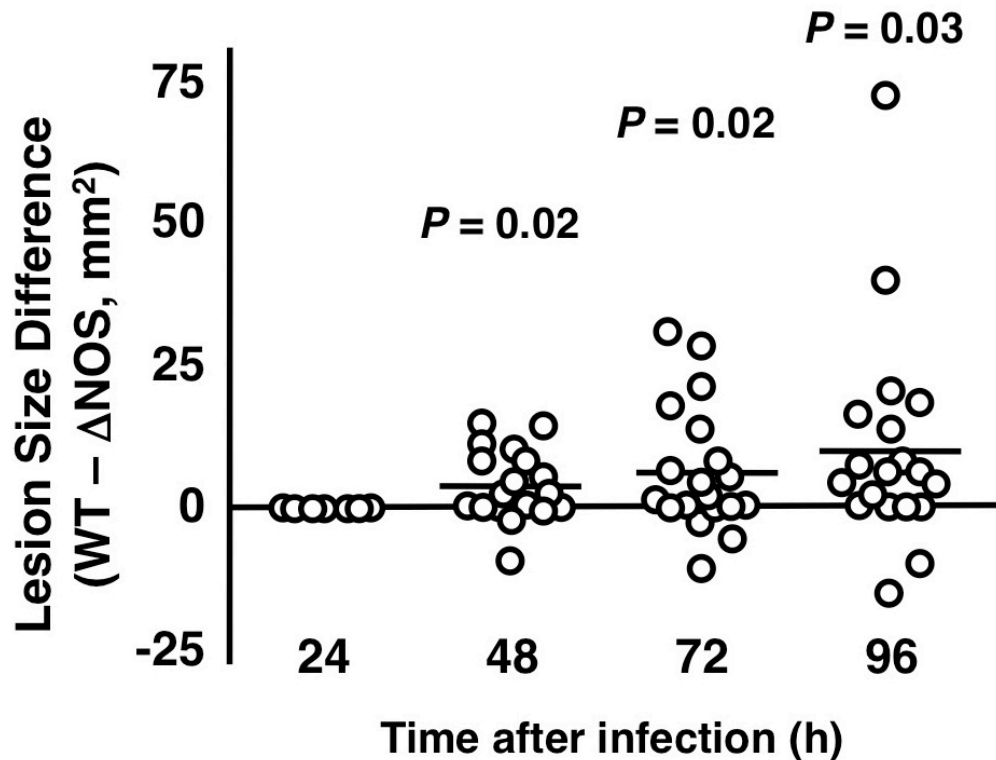
Supplemental Figure S6. bNOS does not affect neutrophil nitric oxide response. NO-specific fluorescence was measured using DAF-FM diacetate under conditions used for neutrophil killing assays. Pooled data from 4 independent experiments in triplicate are shown (Mean +/- SEM).



Supplemental Figure S7. bNOS does not affect neutrophil lysis. Neutrophil lysis was determined by LDH release in neutrophil supernatants under conditions used for neutrophil killing assays. Pooled data from 3 independent experiments in triplicate are shown (Mean +/- SEM).



Supplemental Figure S8. bNOS does not influence the production of neutrophil extracellular traps (NETs). Freshly isolated human neutrophils were incubated with MRSA WT, ΔNOS or complemented strains for 90 min. (A) Representative fluorescent images of NET formation using a mouse antibody against H2A-H2B-DNA-complexes (Alexa488, green). Samples were counterstained with Dapi to stain all nuclei in blue. (B) Quantification of NET formation using picogreen assay. Pooled data from three independent experiments are shown (mean +/- SEM).



Supplemental Figure S9. bNOS contributes to abscess development in a murine subcutaneous infection model. CD-1 mice were injected subcutaneously in opposite flanks with MRSA WT or ΔNOS bacteria. Lesion size (mm^2) was monitored every 24 h for 4 days and plotted as WT-DNOS lesion size. Results from two independent experiments were pooled, total n= 20 mice. Every dot represents the difference in lesion size within one mouse, bar represents median lesion size. * $P < 0.05$, paired Student's *t*-test.

Supplemental Table S1: Homology of *nos* gene sequences from *Staphylococcus aureus* and coagulase-negative *Staphylococcus* genomes

Staphylococcus Strain	Accession Number	Clinical Description of Strain Isolate	% Identity of *nos* Gene
S. aureus MW2	MW1855	CA-MRSA, 16-mo girl septicemia/arthritis (1998)	100
S. aureus N315	SA1730	HA-MRSA, pharyngeal smear (1982)	100
S. aureus Mu50	SAV1914	MRSA/VISA, 4 month old patient, surgical wound pus, sternum	100
S. aureus NCTC 8325	SAOUHSC_02134	Prototypic strain for genetic manipulation	100
S. aureus COL	SACOL1976	MRSA isolated in early 1960s	100
S. aureus JH9	SaurJH9_1970	MRSA/VISA chemotherapy patient on vancomycin therapy	100
S. aureus JH1	SaurJH1_2004	Isogenic to JH9 prior to vancomycin therapy	100
S. aureus Mu3	SAHV_1899	MRSA isolated from sputum, vancomycyin treatment failure	100
S. aureus MSSA476	SA51838	CA-MRSA, severe invasive disease (1998)	100
S. aureus Newman	NWMN_1852	MSSA from human infection (1952)	100
S. aureus USA300-FPR3757	SAUSA300_1895	CA-MRSA, wrist of HIV patient, IV drug user	100
S. aureus USA300-TCH1516	USA300HOU_1916	CA-MRSA, adolescent patient with severe sepsis syndrome	100
S. aureus RF122	SA81851	Bovine isolate, mastitis	99.4
S. aureus MRSA252	SA2007	MRSA acquired postoperatively, adult female	98.6
S. epidermidis ATCC 12228	SE_1598	Non-biofilm forming, non-infection associated strain used in food industry	75.6
S. capitis SK14	STACA0001_1966	Reference genome, Human Microbiome Project	75.4
S. epidermidis P62A	SERP1451	Biofilm producing strain isolated in outbreak of intravascular catheter-associated sepsis	75.4
S. haemolyticus JCSC1435	SH1038	Patient isolate	73.7
S. hominis SK119	STAHO0001_0068	Reference genome, Human Microbiome Project	72.3
S. warneri L37603	STAW0001_0575	Reference genome, Human Microbiome Project	72.0
S. saprophyticus ATCC15305	SSP0877	Type strain isolated from human urine	68.5