Suppl. Figure 1

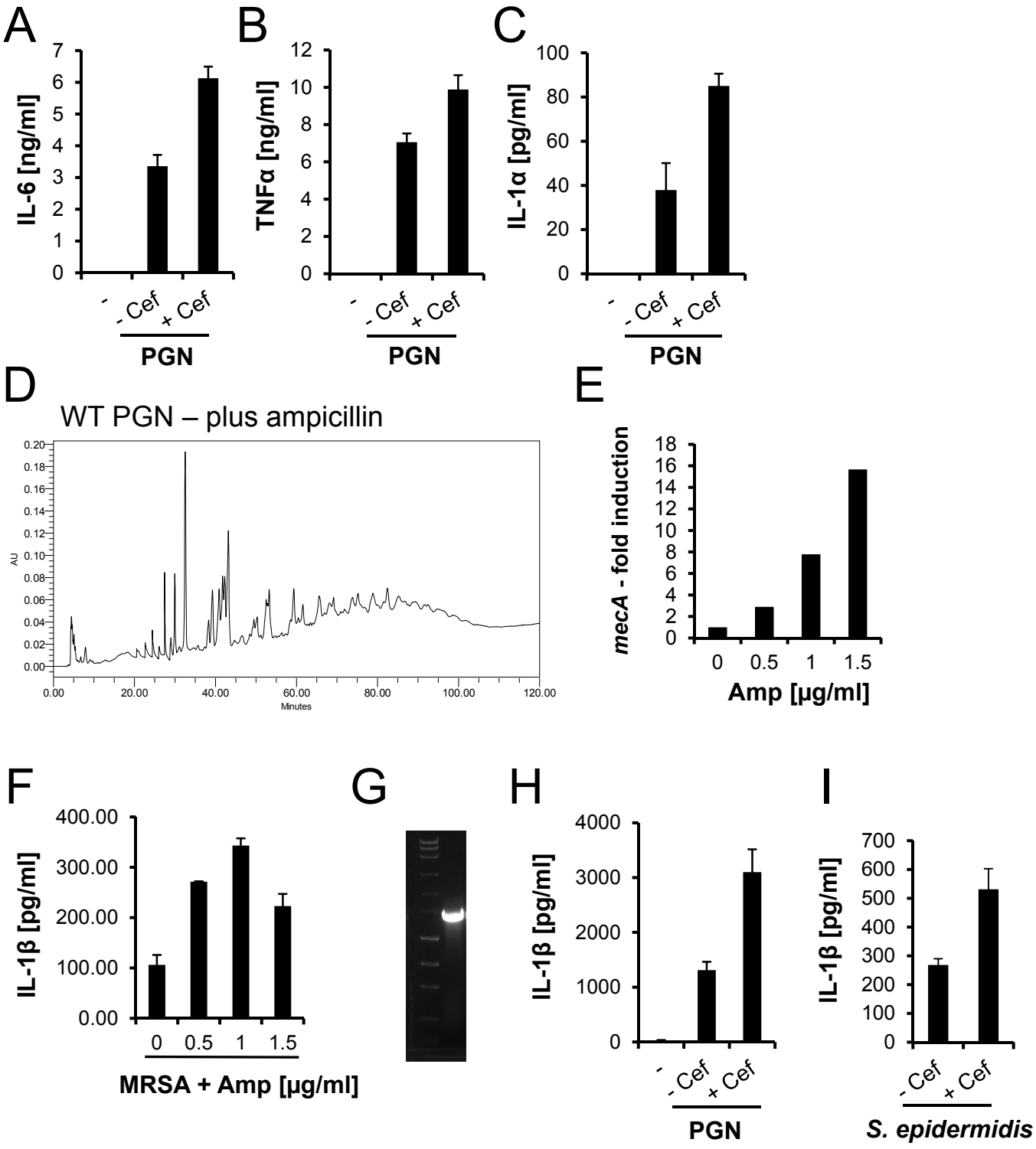

Fig. S1, related to Fig. 1: Cytokine induction and *mecA* expression by β-lactam-challenged MRSA and a clinical *S. epidermidis* strain.

(A-C) BMDM were stimulated with PGN (80 μg/ml) derived from β-lactam-challenged and unchallenged MRSA for 18 hours, and **(A)** IL-6, **(B)** TNFα, and **(C)** IL-1α in the culture supernatants were determined (rep. data of at least 5 exp.). **(D)** PGN was purified from MRSA (USA300 JE2) grown overnight in the absence or presence of 2 μg/ml ampicillin. Reverse-phase HPLC profiles of muropeptides derived from the bacterial PGN are shown. **(E)** MRSA was untreated or treated with ampicillin, and qRT-PCR was performed after 4 hours to assess the induction of *mecA*. *MecA* mRNA was standardized against 16S rRNA level. **(F)** MRSA, untreated or treated with ampicillin for 4 hours, was used to stimulate BMDM for 18 hours. IL-1β levels in culture supernatants were measured (rep. data of 2 exp.). **(G)** Detection of *mecA* in a clinical strain of *S. epidermidis* by PCR. **(H)** LPS-primed BMDM were stimulated for 6 hours with PGN (80 μg/ml) derived from cefoxitin-challenged or unchallenged *S. epidermidis* (rep. data of 3 exp.). **(I)** BMDM were infected for 18 hours with cefoxitin-challenged or unchallenged live *S.epidermidis* (MOI 20). IL-1β was measured in culture supernatants.

Suppl. Figure 2

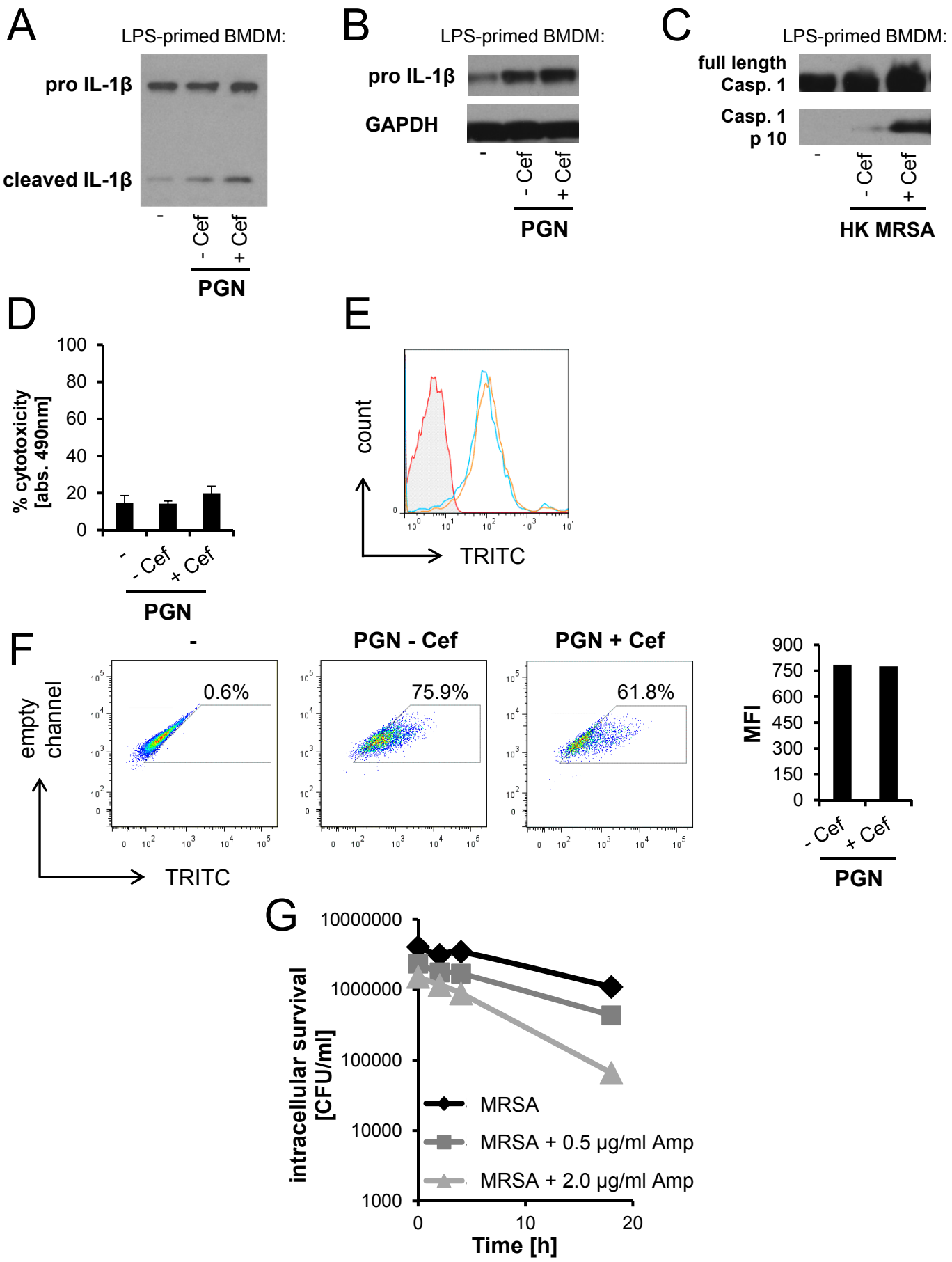

Fig. S2, related to Fig. 2: β-lactam treatment of MRSA increases release of mature IL-1β and enhances MRSA clearance by macrophages without inducing pyroptosis.

(A-C) BMDM were primed with LPS for 4 to 6 hours and then stimulated for 6 hours with PGN (40 or 80 μg/ml), or HK MRSA (MOI 40) prepared from MRSA grown overnight in the absence or presence of cefoxitin (2 μg/ml). Immunoblotting was performed to visualize **(A)** cleaved IL-1β in the supernatant, **(B)** pro-IL-1β levels in cell lysates (GAPDH is shown as loading control), and **(C)** cleaved caspase-1 in supernatant (rep. data of 2-3 exp.). **(D)** LDH release was determined as a measure of cytotoxicity of PGN (80 μg/ml) from cefoxitin-challenged and unchallenged MRSA upon overnight stimulation of BMDM (n=3). **(E)** Fluorescence intensity of TRITC-labeled PGN from unchallenged (blue) and cefoxitin-challenged (orange) MRSA compared to unlabeled PGN (red). **(F)** TRITC-labeled PGN (80 μg/ml) was fed to BMDM for 45 min and uptake was assessed by flow cytometry. Percentage of TRITC-positive BMDM is shown in the left three panels, and MFI including all cells is shown in the right panel. **(G)** Effect of ampicillin treatment on MRSA survival within BMDM (rep. data of 2 exp.).

Suppl. Figure 3

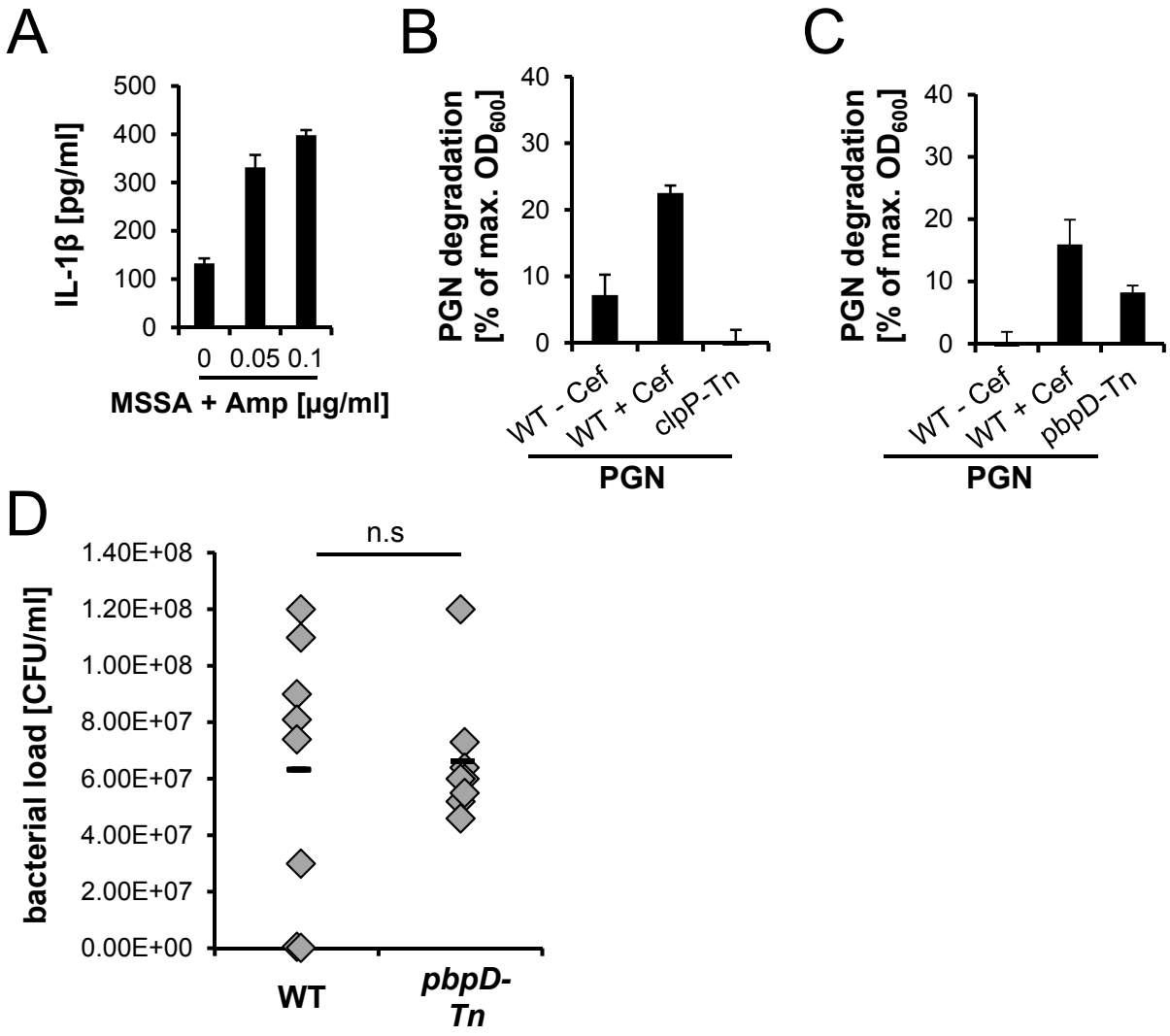

Fig. S3, related to Fig. 3: Induction of IL-1β by MSSA treated with low dose ampicillin, and degradation of PGN derived from mutant MRSA by lysosomal enzymes.

(A) JE2 *mecA-Tn* mutant was grown overnight in the presence or absence of ampicillin, and used to stimulate BMDM. IL-1β in the supernatants was measured after 18 hours (rep. data of 2 exp.). **(B-C)** Degradation of PGN (150 μg) from JE2 WT MRSA, and **(B)** *clpP-Tn* mutant, or **(C)** *pbpD-Tn* mutant by purified lysosomal extract (50 μg) for 24 hours. Degradation was measured by decrease in optical density at 600 nm. Data in **(B)** are representative of 3 independent experiments. **(D)** C57BL/6 mice were infected on the right flank with 10^7 CFUs of WT JE2, and on the left flank with 10^7 CFUs of JE2 *pbpD-Tn* mutant. Bacterial burden in skin lesions was determined at day 5. Statistical analysis was performed using Mann Whitney U-test.

Suppl. Figure 4

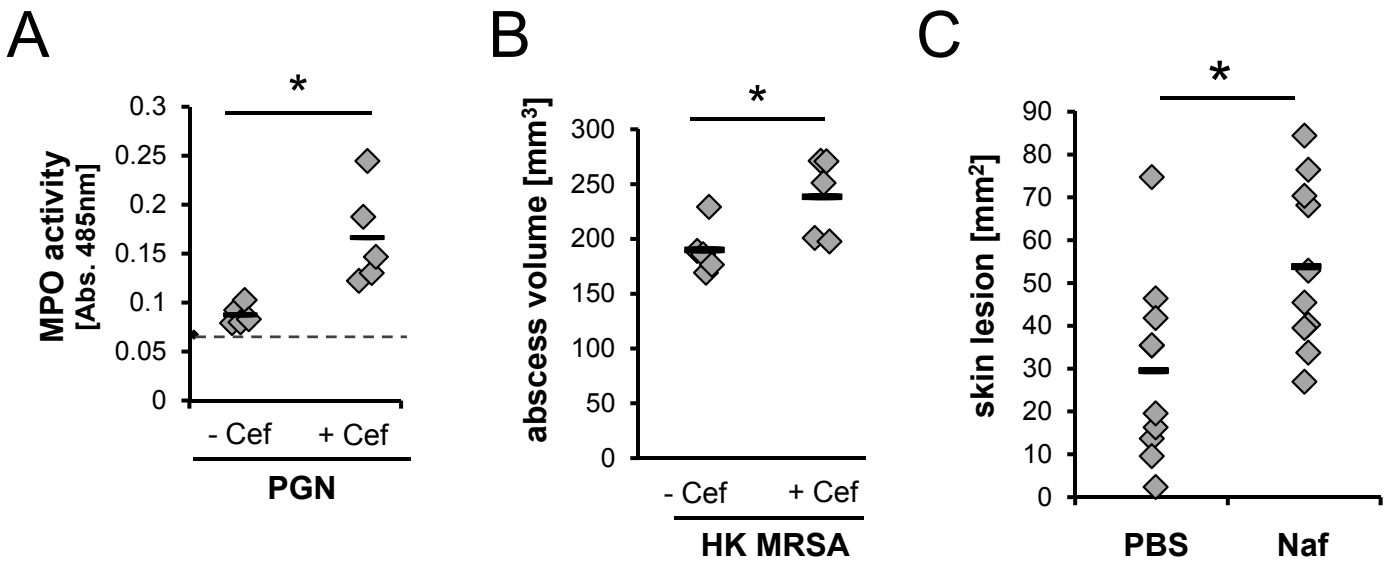

Fig. S4, related to Fig. 4: Neutrophil recruitment by PGN from cefoxitin-treated MRSA and immunopathology caused by β-lactam-challenged MRSA.

(A) C57BL/6 WT mice were injected s.c. with PGN (200 µg) derived from untreated or cefoxitin-treated MRSA. On day 3, the mice were sacrificed and MPO activity from skin homogenates was measured. **(B)** Heat-killed MRSA ($5x10^8$ CFUs), initially grown in the presence or absence of cefoxitin (2 µg/ml), were injected s.c. into C57BL/6 mice. After 3 days, the mice were sacrificed and abscess volume was measured. **(C)** MRSA ($3x10^7$ CFUs) were injected s.c. into C57BL/6 mice. Beginning on day 1 and for the duration of the experiment the mice were treated twice a day s.c. with 100 µl of PBS or 5 mg/ml nafcillin at the site of infection. Distribution of lesion sizes on day 3 is shown. **(A-C)** Statistical analysis was performed using Mann Whitney U-test. * $p<0.05$.

Supplemental Experimental Procedures

Bacterial culture

Frozen bacterial stocks were streaked out on blood-agar plates and incubated overnight at 37°C. For overnight culture, a β-hemolytic colony was picked from the plate, inoculated in 5 ml Tryptic Soy Broth (TSB) and shaken at 250 rpm at 37°C. For late log phase culture, an overnight MRSA culture was diluted 1:500 in fresh TSB and was shaken for 4 hours at 250 rpm at 37°C. Unless otherwise indicated, antibiotics such as cefoxitin (Fluka), ampicillin (Sigma Aldrich), and nafcillin (Sigma Aldrich) were used at a concentration of 2 μg/ml. For experiments, bacteria were adjusted to an OD_{600} of 0.7, which is equivalent to approximately $2x10^8$ CFU/ml, and washed twice in PBS (Corning). CFU numbers used in each experiment were confirmed by plating. HK MRSA were prepared from overnight cultures of MRSA that have been washed twice with PBS and heated to 75°C for 2 hours. Killing of bacteria was confirmed by plating on agar.

Cell culture experiments

Macrophages were plated at least 4 hours prior to stimulation or infection. For most experiments 80,000 BMDM were added to each well in a tissue culture-treated 96 well plate, and experiments were performed at least in triplicates. PGN stimulation was performed by incubating PGN with unprimed BMDM for 18 hours or with LPS-primed BMDM for 6 hours. BMDM were primed with 100 ng/ml LPS for 3-4 hours. When live bacteria were used, media without antibiotic were utilized. Bacteria were added to the macrophages (MOI 20), centrifuged at 450 *g* for 2 min, and incubated for 45 min. The antibiotic-free media were then replaced by media containing 100 μg/ml gentamicin. When applicable, bafilomycin A (Tocris Bioscience), cytochalasin D (Sigma

Aldrich), or E64d (Enzo Life Sciences) was added to the macrophage cultures 30 min prior to the start of the experiment and remained in the culture media until the end of the experiment.

PGN isolation

Bacteria were grown overnight in TSB with or without antibiotics. The cultures were put on ice, spun down at 2000 *g* for 10 min at 4°C, washed with ice cold 100 mM Tris-HCL pH 6.8, and resuspended in 5 ml 100 mM Tris-HCL pH 6.8. Bacteria were added drop-wise to 15 ml of boiling 5 % SDS (100 mM Tris-HCL pH 6.8) and boiled for 30 min, and then centrifuged at 3,200 *g* for 15 min, and washed three times with ddH_2O. The pellet was resuspended in 6 ml of ice cold ddH_2O, and 1 ml of bacteria was mixed with 300 µl of 0.1 mm glass beads and beaten for 20 min at 4°C. Sediment, beads, and unbroken cells were spun down at 100 *g* for 5 min at 4°C. Supernatants were transferred to new tubes, spun down for 30 min at 6,500 *g*, and washed twice with ddH_2O. Pellets were resuspended in 1 ml each of 100 mM Tris-HCL pH 7.5 containing 20 mM $MgCl_2$ and 100 µg/ml RNase A (USB Corp.) plus 100 µg/ml DNase (Qiagen), and were incubated at 37°C overnight with rotation. Afterwards, 20 mM $CaCl_2$ and 200 µg/ml trypsin (Gibco) were added and incubated overnight at 37°C with rotation. After centrifugation for 10 min at 6,500 g and two washes in ddH_2O, the pellets were resuspended in 1.5 ml of 10% TCA in ddH_2O and incubated for 5 h at 4°C on a rotator. Pellets were washed twice with PBS, twice with ddH_2O, and twice with acetone. The samples were either frozen at -20°C or immediately dried in a Speedvac Concentrator for 20 min with heating function switched on. The pellets were weighed and resuspended in PBS at 10 mg/ml, sterilized by boiling for 15 min, and stored at -20°C.

HPLC analysis

For HPLC analysis of muropeptide fragments, samples were prepared as previously described (Corrigan et al., 2011, Glauner et al., 1988), and separated by HPLC (Waters) using a 5 µm particle size, 100 Å pore size, C18 silica gel (spherical) octadecyl reversed-phase column (25 cm x 10 mm, Supelco Ascentis, Sigma Aldrich) with an upstream guard C18 column (Supelguard Ascentis, Sigma Aldrich). Separation was performed at room temperature, and muropeptides were eluded at a flow rate of 2.0 ml/min for 120 min on a linear methanol gradient consisting of 50 mM sodium phosphate (pH 4.75) and 0 to 33% methanol. Eluted muropeptides were detected by absorption at 206 nm.

ELISA and MPO assays

IL-1β in macrophage culture supernatants and tissue homogenates was assessed using an IL-1β ELISA kit (BioLegend) following the manufacturer's protocol. MPO in skin samples was determined as previously described (Tseng et al., 2012).

qRT-PCR

Macrophages or MRSA were lysed with RLT buffer (RNeasy Mini Kit, Qiagen), and RNA was isolated according to the manufacturer's protocol. RNA was reverse transcribed into cDNA (iScript cDNA Synthesis Kit, BioRad), and qRT-PCR was performed using SYBR Green (FastStart Universal SYBR Master (ROX), Roche). mRNA expression levels in macrophages were normalized to β-actin and mRNA expression levels in MRSA were normalized to staphylococcal 16S rRNA. The following primers with were used in the study: β-actin: fwd 5'-ACC CAG GCA TTG CTG ACA GG-3', rev 5'-GGA CAG TGA GGC CAG GAT GG-3', IL-1β: fwd 5'- CAG GCA GGC AGT ATC ACT CA-3', rev 5'-TGT CCT CAT CCT GGA AGG

TC-3', 16S rRNA: fwd 5'-CAT GCT GAT CTA CGA TTA CT, rev 5'-CCA TAA AGT TGT TCT CAG TT, and mecA: fwd 5'-GGA ACT TGT TGA GCA GAG GTT CTT, rev 5'-AGG TAC TGC TAT CCA CCC TCA AA.

Western Blot analysis

Macrophages ($1.5x10^6$) were added to wells of a 6-well plate, or distributed to wells of a 96-well plate at $1x10^5$ cells/well. The next day the cells were primed with 100 ng/ml LPS for 4 to 6 h, washed once with PBS, and then incubated in serum-free Optimem medium (Life Technologies). The macrophages were stimulated for 6 hours or overnight with 40 μg/ml or 80 μg/ml PGN derived from untreated MRSA or MRSA treated with cefoxitin. Supernatants or cell lysates were collected, and proteins in supernatants were precipitated using StrataClean (Agilent Technologies), agitated for 20 min, spun down, and resuspended in Laemmli loading buffer, and boiled for 10 min. Proteins were separated on a 12% Bis-Tris gel. After transfer and blocking of PVDF membrane, mIL-1β was detected using a 1:1000 dilution of a polyclonal goat anti-mIL-1β antibody (R&D systems), and caspase-1 was detected using a 1:500 dilution of a polyclonal rabbit anti-mouse p10 antibody (Santa Cruz).

FACS analysis of skin samples

Single cell suspensions were prepared by resuspending tissues in culture media containing an enzyme cocktail (0.5 mg/ml collagenase type VIII, 5 U/ml DNase) and incubating at room temperature for 30 min with shaking. Cells were then passed through a 70 μm cell strainer, centrifuged at 400 *g* for 10 min, and resuspended in PBS containing 2% FCS and Fc block. The cells were then stained with antibodies against CD11b (APC), Ly6G (PE), and CD45 (Pac Blue)

(BioLegend), fixed with 4% paraformaldehyde, and analyzed by FACS. Gates were set based on isotype controls (BioLegend).

Phagocytosis assay

PGN (1 mg) from antibiotic-challenged or unchallenged MRSA was labeled with TRITC (20 µg/ml) for 60 min at 37°C on a rotator. Labeled PGN was washed three times with 1x PBS + 10% FCS before being resuspended in 1x PBS for experiments.

PGN (80 µg/ml) was added to $3*10^5$ cells in a 24-well plate and spun down at 450 *g* for 2 min. After 45 min cells were lifted for 15 min with 1x PBS + EDTA (5mM) + proteinase K (500U/ml), and cells were fixed with 1x PBS + 2.5% formalin at 4°C overnight. Fluorescence was measured by flow cytometry.

LDH assay

BMDM were stimulated with PGN overnight as described above. Afterwards supernatants were collected and an LDH assay was performed according to manufacturer's protocol (CytoTox 96, Promega)

Lysosome isolation and degradation assay

BMDM were harvested and resuspended in 3 ml of sucrose (0.25 M) + HEPES (5 mM) + EDTA (1 mM). Cells were gently lysed by nitrogen cavitation and lysate was spun down at 6,000 *g* for 5 min at 4°C to remove nuclei and mitochondria. Supernatants were then transferred to an ultracentrifuge, and lysosomes were spun down at 100,000 *g* for 1 hour at 4°C. The pellet was resuspended in 300 µl of an acidic buffer (50 mM sodium citrate buffer pH 5.5 + 2 mM DTT + 0.5% Triton X-100) and extract was kept on ice for 15 min before being spun down at 13,400 *g* for 5 min at 4°C. Protein content was assessed by BCA assay.

Then PGN (150 µg) was mixed with 50 µg of lysosomal extract in a total volume of 200 µl of sodium citrate buffer. Optical density was determined at 600 nm using a spectrophotometer Reaction was incubated at 37°C on a rotator for 24 h, after which OD_{600} was determined again.

PCR

PCR was performed using the Platinum PCR SuperMix (Invitrogen) according to manufacturer's protocol. *MecA* primer sequence: fwd 5'- TCC AGG AAT GCA GAA AGA CCA -3', rev 5'- AAC CAC CCA ATT TGT CTG CC -3'.

Supplemental References

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