

Supplemental Figures

Figure S1

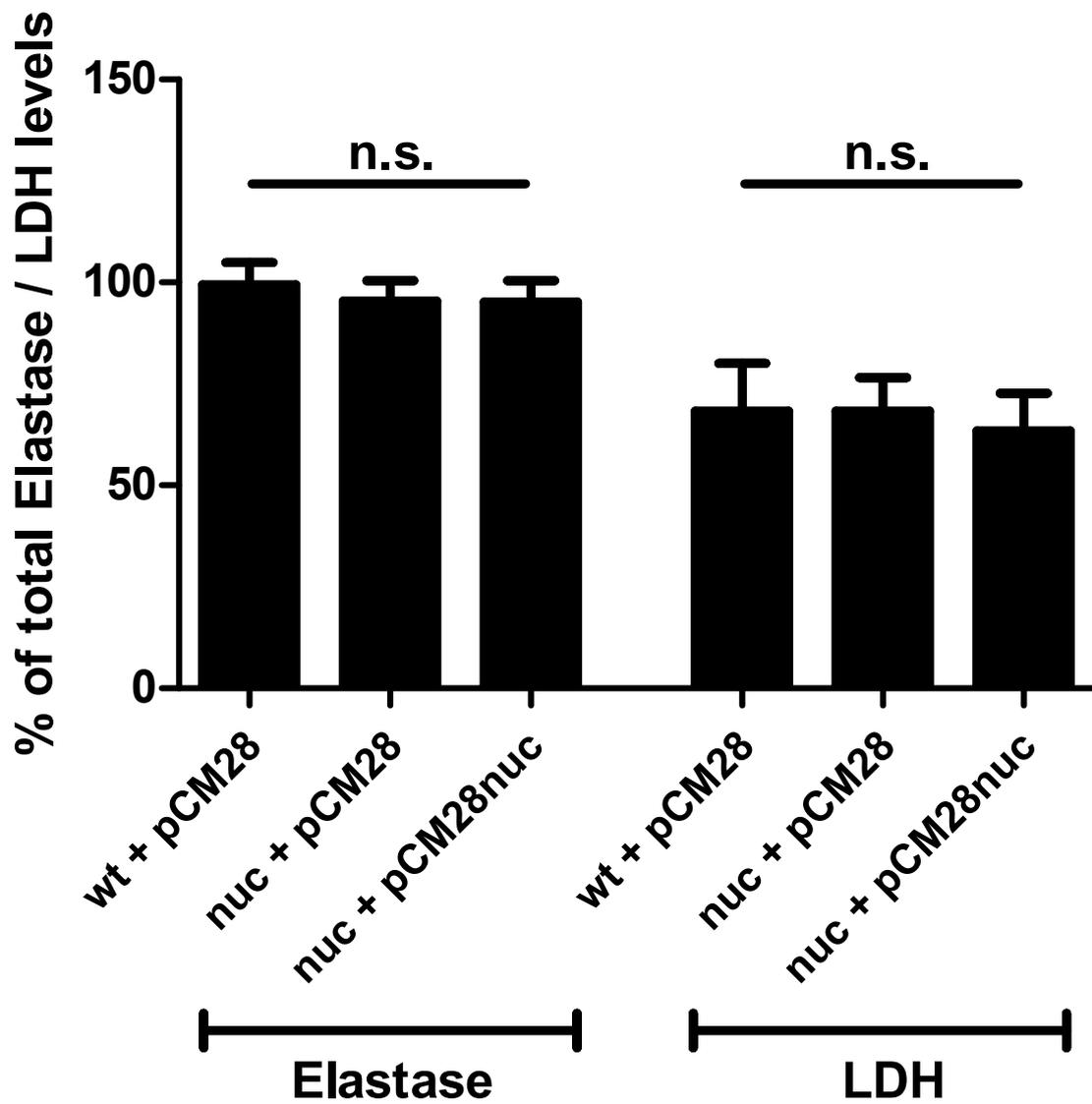


Figure S2

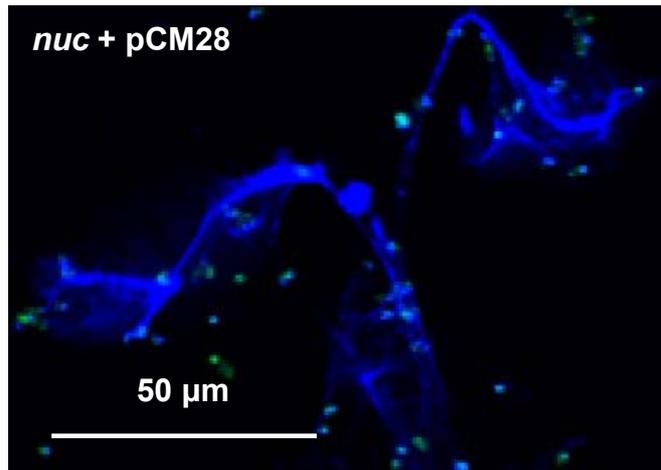
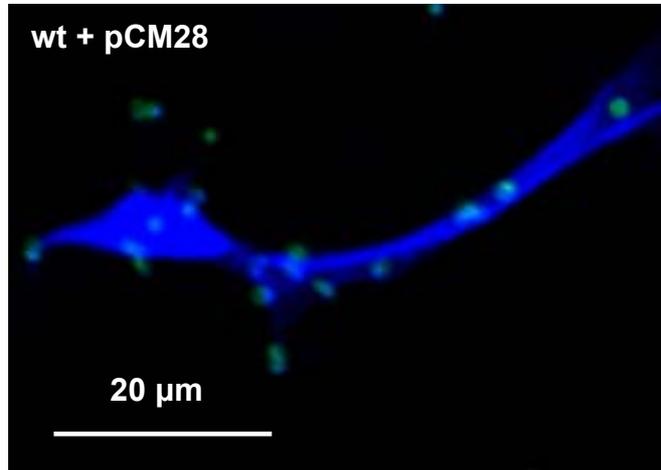


Figure S3

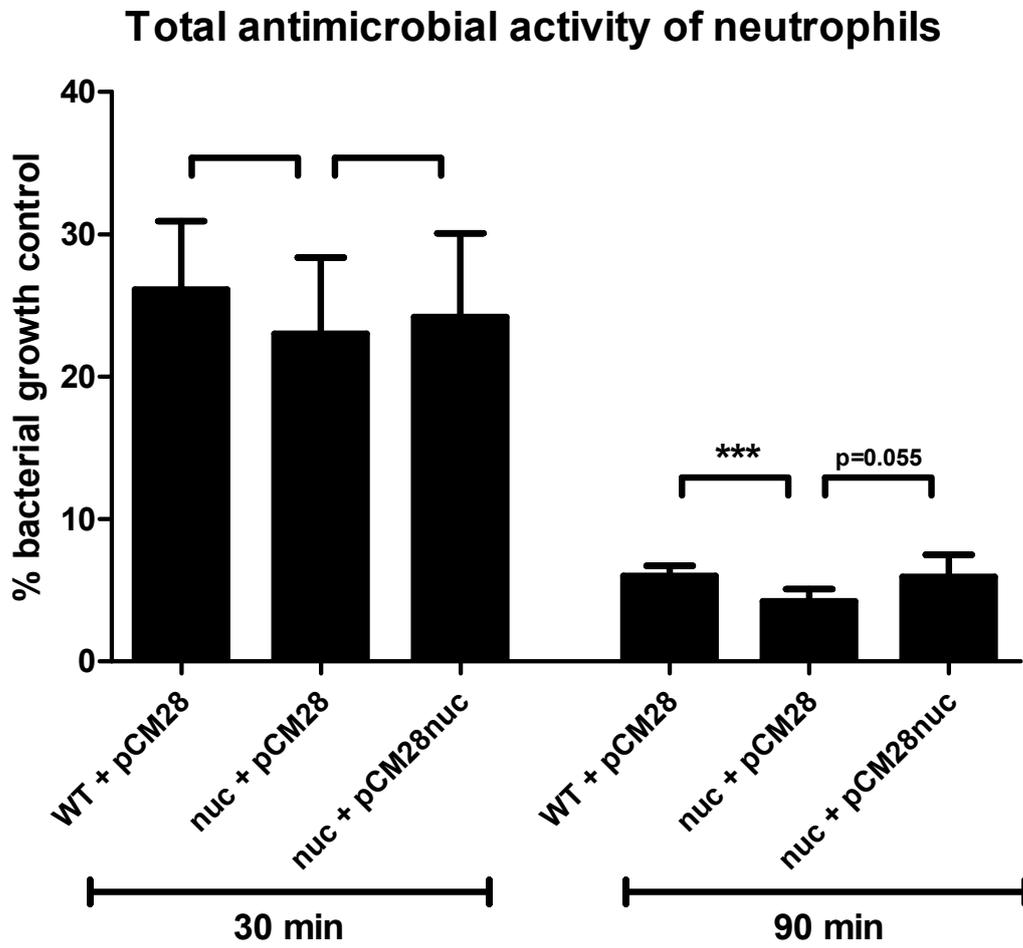
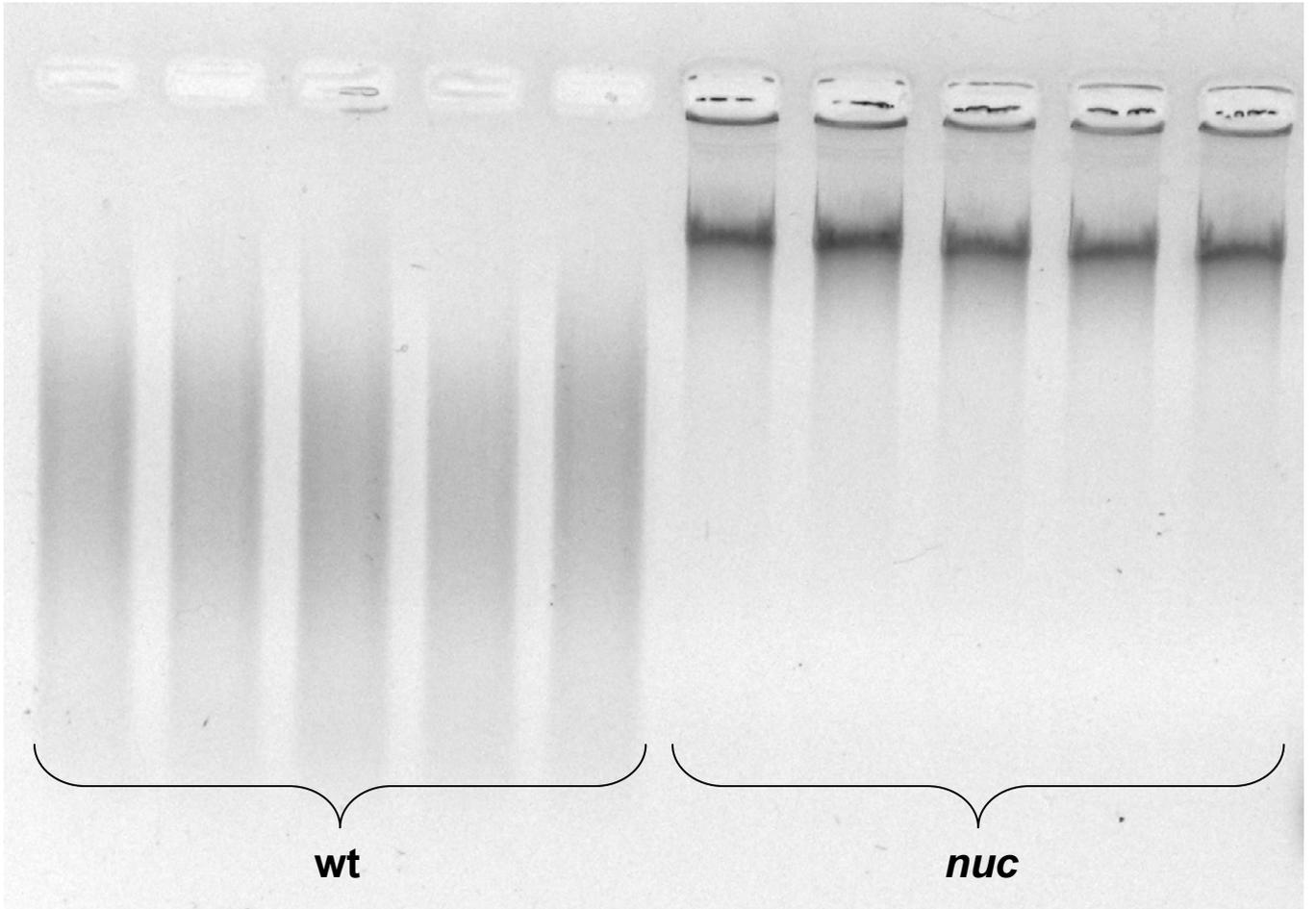


Figure S4



Supplemental Figures

Fig. S1. Nuclease-independent activation and cytotoxicity of neutrophils after co-incubation with *S. aureus*.

Percentage of LDH release (as marker for cytotoxicity) and elastase release (as marker for activation) by PMA-stimulated neutrophils after co-incubation with *S. aureus* LAC wild type empty vector control (wt + pCM28), *nuc*-mutant empty vector control (*nuc* + pCM28) or complemented mutant strain (*nuc* + pCM28*nuc*). The results of n = 3 independent experiments were analyzed using a paired, one-tailed Student's t-test. P values of < 0.05 were considered to be significant. No significant differences were detected between the three different bacterial strains.

Fig. S2. Entrapment of bacteria by NETs.

Representative immuno-fluorescent micrograph showing entrapment of FITC-labelled bacteria within NETs. *S. aureus* LAC wild type empty vector control (wt + pCM28) or *nuc*-mutant empty vector control (*nuc* + pCM28) were co-incubated with PMA-stimulated neutrophils at a MOI of 2 for 90 min at 37°C in 5% CO₂. After washing and fixation, NETs were visualized with DNA-intercalating dye Dapi (blue). Bacteria are shown in green. Note that the remaining NETs that are not eliminated by the wild type strain have the same capability to capture bacteria compared to those NETs co-incubated with the *nuc*-mutant strain.

Fig. S3. Total (extra- and intracellular) antimicrobial activity of neutrophils against *S. aureus* strains.

Bacterial growth inhibition after co-incubation of *S. aureus* LAC wild type empty vector control (wt + pCM28), *nuc*-mutant empty vector control (*nuc* + pCM28) or complemented mutant strain (*nuc* + pCM28*nuc*) with PMA-stimulated neutrophils. Data are presented as percentage surviving bacteria compared to the respective bacterial growth control (100%). The results of n = 4 independent experiments were analyzed using a paired, one-tailed Student's t-test. P values of < 0.05 were considered to be significant (***) p<0.005).

Fig. S4. Nuc-mutation remains stable *in vivo*.

To confirm stability of the *nuc*-mutant (*nuc*) *in vivo*, bacteria were recovered from murine lungs at 24 h after infection. Harvested bacteria were grown overnight in BHI and culture supernatants were tested for nuclease activity in an agarose-gel-based nuclease assay. A representative agarose gel is shown, which demonstrates that bacteria recovered from wild type (wt)-infected mice show nuclease activity (DNA degradation), whereas the bacteria recovered from lungs after infection with the *nuc*-mutant (*nuc*) do not exhibit nuclease activity.

Supplemental Material and Methods

Elastase and LDH assays

According to the literature, elastase-release was used as marker for neutrophil activity and degranulation [i] and LDH-release was used as marker for cytotoxicity [ii]. Human neutrophils were resuspended in RPMI without phenol red containing 2% nuclease-free FCS (70°C heat-inactivated) and plated in non-treated tissue culture plates (Greiner Bio-One, CELLSTAR®) at a concentration of 2×10^6 cells/ml. Neutrophils were stimulated with 25nM PMA for 20 min at 37°C in 5% CO₂. Then, neutrophils were infected with bacteria at MOI of 2, the plates were centrifuged at 1600 rpm for 5 min and incubated for 90 min at 37°C in 5% CO₂. After incubation, micrococcal nuclease (Worthington) was added at a concentration of 500 mU/ml to degrade NETs and to release elastase from NETs. The reaction was stopped with 5 mM EDTA and the plate was centrifuged at 1000 rpm for 8 min. For elastase measurement, 50 µl of the supernatant was incubated with 50 µl of 200 µM elastase substrate (N-(Methoxysuccinyl)-Ala-Ala-Pro-Val 4-nitroanilide, Sigma) for 30 min at room temperature. Optical density was measured at 405nm (VersaMax Tunable Microplate Reader, Molecular Devices). For LDH measurement, the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) was used according to the manufacturer's recommendations. The percentage of LDH or elastase release was calculated compared to 100% cell lysis control (cells lysed with 0.25 % Triton X-100 for 10 min).

Neutrophil killing assays

Human neutrophils were resuspended in RPMI containing 2% nuclease-free FCS and plated in non-treated tissue culture plates at a concentration of 2×10^6 cells/ml. Neutrophils were stimulated with 25 nM PMA for 20 min at 37°C in 5% CO₂. Then, neutrophils were infected with bacteria at MOI of 2, the plates were centrifuged at 1600 rpm for 5 min and incubated for 30 and 90 min at 37°C in 5% CO₂. After incubation, cells were lysed with 0.25 % Triton X-100 by pipetting up and down. Serial dilutions in sterile PBS were plated on THA plates for enumeration of surviving cfu. The percentage of surviving bacteria was calculated in comparison to bacterial growth control grown under the same conditions in the absence of cells.

References

- i Pham CT: Neutrophil serine proteases: specific regulators of inflammation. *Nat Rev Immunol* 2006;6:541-550.
- ii Fotakis G, Timbrell JA: In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicol Lett* 2006;160:171-177.