Supplemental Information
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Statins Enhance Formation of Phagocyte Extracellular Traps

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

In Vitro Bactericidal Killing Assays

For in vitro killing assays with lysed cells, neutrophils were sonicated using a 550 Sonic Dismembrator (Fisher Scientific). Neutrophils were cultured in serum-free, antibiotic-free RPMI at 10^6 cells/ml in the presence of 50 μM mevastatin (Sigma) as described above. After 1 hr, cells were stimulated with 156 ng/ml phorbol myristic acetate (PMA, Sigma). For killing assays to determine NET-specificity, micrococcal nuclease was added to a final concentration of 5 U/ml 40 min after addition of PMA. Neutrophils were infected with log-phase *S. aureus* Newman Strain at an moi of 1 at 1 hr after addition of PMA. After centrifugation for 10 min at 1500 rpm, infected PMNs were incubated for 20 min at 37°C in 5% CO_2. After incubation, surviving bacteria were assessed using a DNase killing assay described by Fuchs et al. with some slight modifications. Briefly, EDTA was added to each well to a final concentration of 0.5 mM. The neutrophils where incubated on ice for 15 min. Following this, cells were detached from plates by scraping and mixing. This mixture was diluted into 0.1% Triton X-100. Lysates were diluted and plated on THA plates for enumeration of surviving bacteria. Percent killing by statin-treated leukocytes was determined by dividing the number of cfu recovered from statin-treated neutrophils by the number of cfu from vehicle-treated neutrophils.

Histone Citrullination

To block the PAD-4 mediated histone citrullination, PMNs were additionally treated with Cl-amidine (200 µM) at the same time when treated with mevastatin or simvastatin. After fixation with 4% paraformaldehyde, samples were washed three times with PBS and then blocked for 1 hr with PBST (PBS + 2% BSA + 0.2% Triton X-100). Primary antibody against H3Cit (rabbit anti H3cit) was diluted 1:300 in PBST and cells were stained overnight at 4°C. After washing, cells were stained with the appropriate secondary goat anti rabbit Alexa 488 (1:500 in PBST) for 45 min at room temperature. After washing, cells were mounted in Dapi-Prolongold. Percentage of Net-forming or H3cit-positive cells was determined using a Zeiss Axiolab microscope (Zeiss 40x objective) with an attached Sony Digital Photo Camera DKC-5000 at calibrated magnifications.

Induction of Extracellular Traps in Peritoneal Macrophages

C57BL/6 mice were intraperitoneally treated with 3 ml 3% thioglycolate solution (BD Biosciences). Peritoneal cells were isolated by peritoneal lavage with PBS 4 days later. Peritoneal lavage was treated with 1x RBC lysis buffer (eBioscience) to lyse erythrocytes and washed with PBS. After centrifuagtion, cells were cultured in RPMI + 10%FBS at 1 x 10^5 cells/500 µl/well on 24-well glass-bottom microtiter plates (MatTek Corporations). Next day, cells were further cultured in serum-free RPMI and stimulated with 50 µM mevastatin (Sigma) or appropriate concentrations of vehicle control (DMSO). After 24 hr cells were additionally treated with 156 ng/ml phorbol myristic acetate (PMA, Sigma) for 2 hr before visualization of extracellular traps (as described in Experimental Procedures).

LDH Assay

LDH release was measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer's instructions.

Figure S1, related to Figure 3

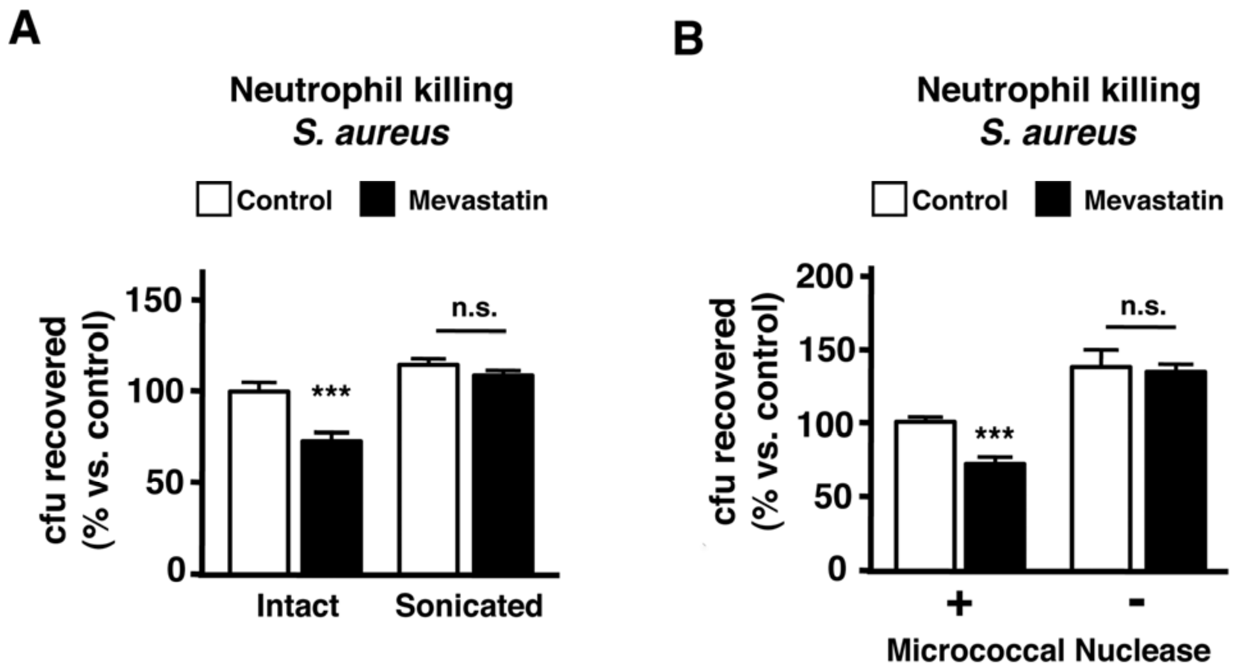

Figure S1. Mevastatin-Induced Killing Can Be Abrogated by DNase Treatment, related to Figure 3

(A) In vitro killing of *S. aureus* by intact or sonicated primary human neutrophils treated with mevastatin or vehicle control.

(B) In vitro killing of *S. aureus* by primary human neutrophils treated with mevastatin or vehicle control. Neutrophils were treated with micrococcal nuclease (or water control) prior to infection to disrupt extracellular traps and to confirm NET-specific killing activity. *$P < 0.5$, ***$P < 0.005$ by one-way ANOVA with Tukey's post-test comparing control versus statin-treated grou.

Figure S2, related to Figure 3, top

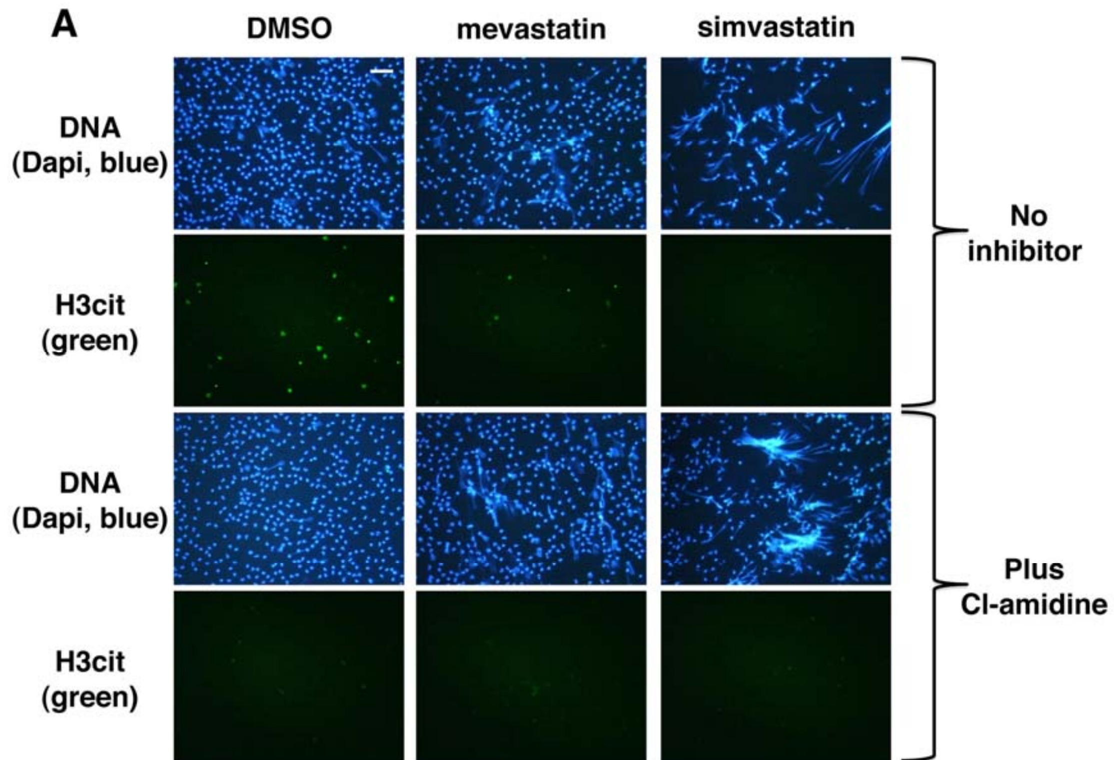

Figure S2, related to Figure 3, bottom

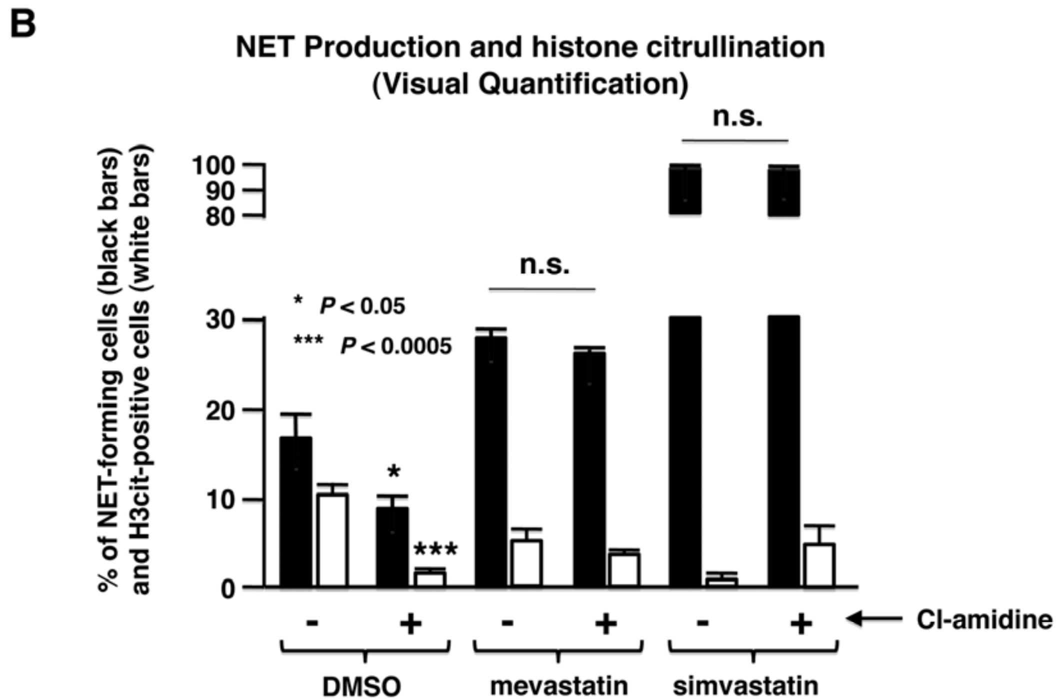

Figure S2. **Statin-Induced NET Induction Independent of PAD4-Mediated Histone Citrullination, related to Figure 3**

(A) Representative fluorescent images of human neutrophils stimulated with mevastatin/simvastatin or vehicle control and PMA to induce NETs. Cl-amidine (200 μM) was used to block the PAD-4 mediated histone citrullination. Net-formation was visualized in blue (DAPI) and histone citrullination (H3Cit) was visualized by Alexa green immunostaining. Bars represent 30 μm.

(B) Quantification of results from above experiment by direct visualization and enumeration of % of NET-forming neutrophils (black bars) or H3Cit-positive neutrophils (white bars), average of 4 high-power fields (HPF) counted containing approximately 125 cells.

Figure S3, related to Figure 4

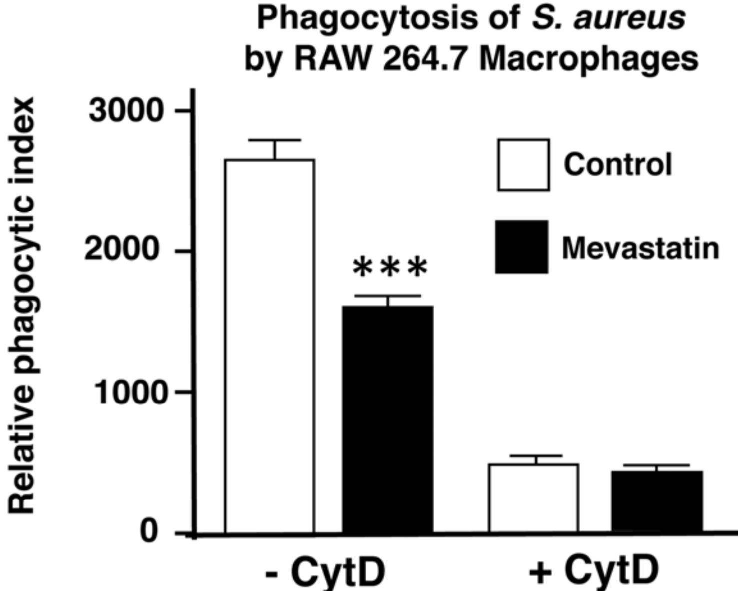

Figure S3. Mevastatin Inhibits Macrophage Phagocytosis of *Staphylococcus aureus*, related to Figure 4

Mean fluorescence intensity was used as parameter for phagocytosis of RAW 264.7 cells after infection with FITC-labelled *S. aureus* Wood strain bioparticles measured by flow cytometry. As control, 10 µg/ml cytochalasin D was added to the samples 10 min prior to infection to prevent phagocytosis. ***$P < 0.005$ by t-test comparing control versus statin-treated group.

Figure S4, related to Figure 4, top

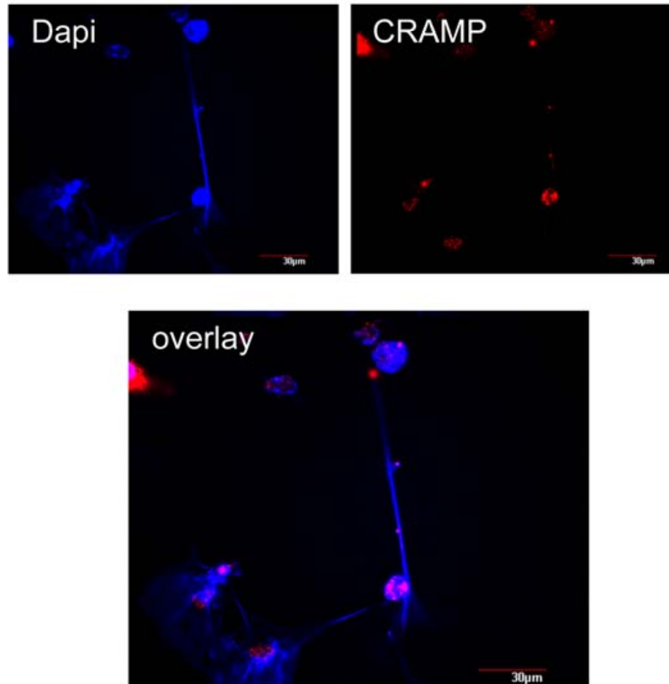

Figure S4, related to Figure 4, bottom

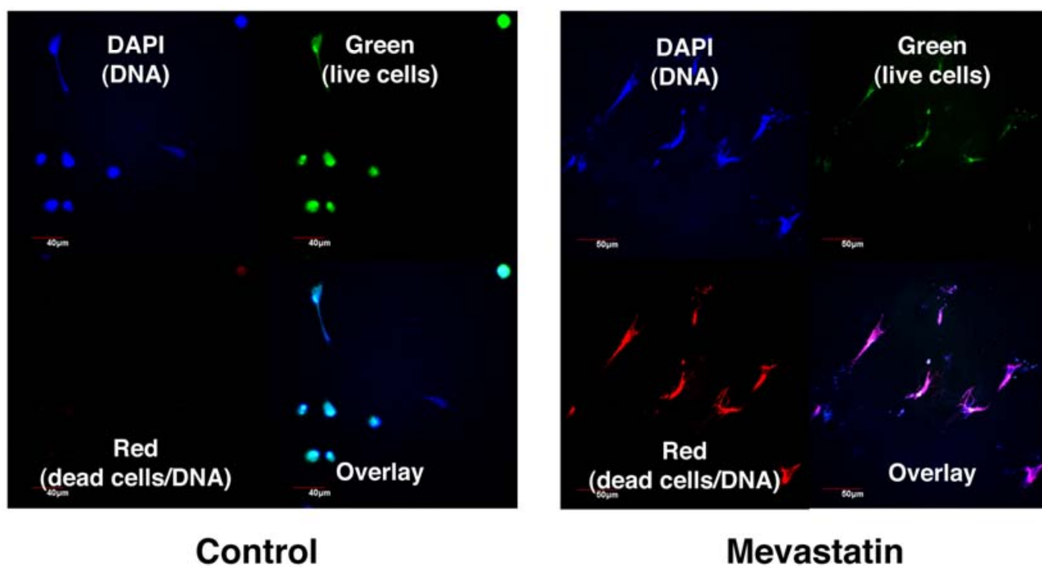

Figure S4. Statins Enhance Formation of Extracellular Traps by Different Macrophage Cell Types In Vitro, related to Figure 4

(A) Representative fluorescent images of RAW 264.7 cells stimulated with simvastatin or vehicle control and PMA to induce METs. MET-formation was visualized in blue (DAPI) and CRAMP-expression (rabbit anti mouse CRAMP, left panel) compared was visualized by Alexa red-immunostaining.

(B) Representative fluorescent images of murine peritoneal macrophage extracellular traps, stained with Live/Dead viability/cytotoxicity kit for mammalian cells to determine viability of trap-forming cells after overnight treatment with mevastatin and subsequent stimulation with PMA. Note that all trap-forming macrophages are dead as shown by the red dye.

Figure S5, related to Figure 5, top

A

In vivo **CRAMP expression as marker for ET release**

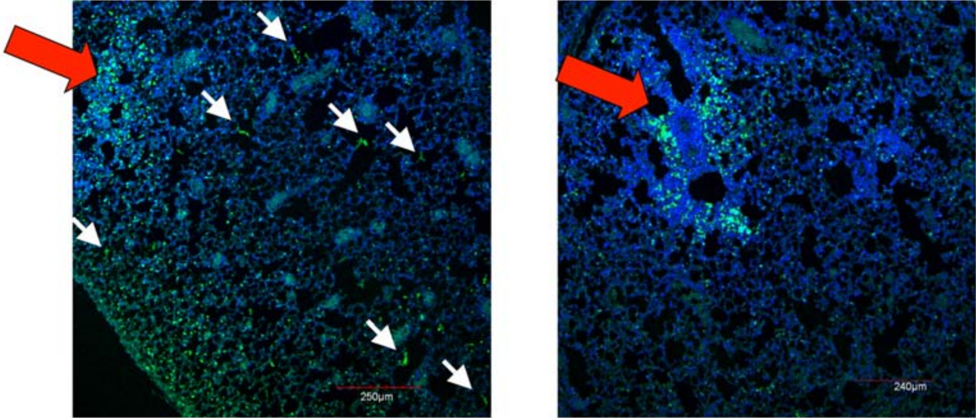

Figure S5, related to Figure 5, bottom

B

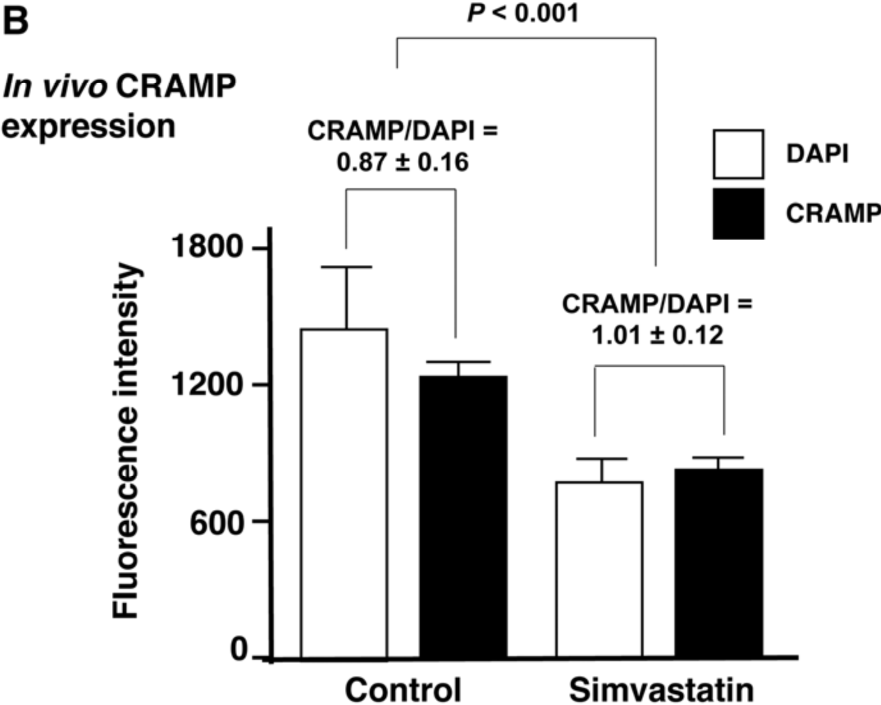

Figure S5. In Vivo Expression of Murine Cathelicidin CRAMP, related to Figure 5

(A) Representative fluorescent images of extracellular trap formation (visualized by Alexa 488 (green)-labeled CRAMP production and counterstained with Dapi) in paraffin-embedded lung sections of mice pre-fed for with standard chow or standard chow supplemented with simvastatin and intranasally infected with 2 x 10^8 cfu of *S. aureus* strain Newman for 48 hr. Note the white arrows indicating extracellular traps released into alveolar space. The red arrows indicate areas of tissue inflammation with intense intracellular staining of CRAMP-positive cells.

(B) Quantification of CRAMP expression compared to total cell amount (DAPI staining) in lung tissue of mice fed with standard chow or standard chow supplemented with simvastatin and infected for 48 hr with *S. aureus* strain Newman. Mean fluorescence intensity was quantified using Image J 1.41 software.

Figure S6, related to Figure 6

RAW 264.7 Mevalonate Rescue

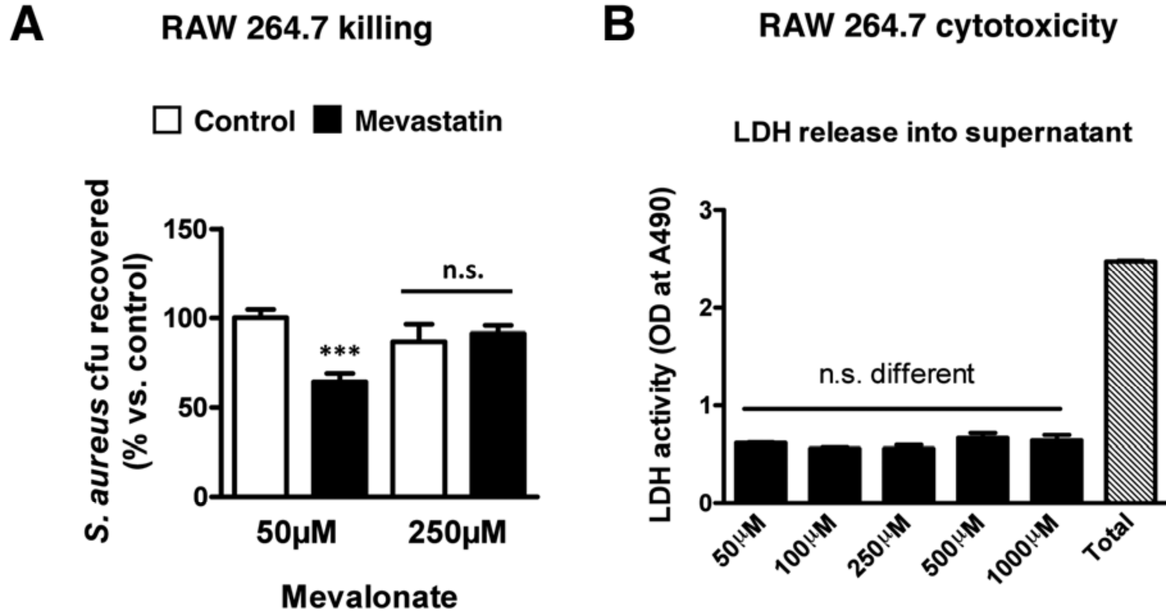

Figure S6. Impact of Mevalonate Rescue on RAW 264.7 Macrophages, related to Figure 6

(A) Killing of *S. aureus* by RAW 264.7 cells following treatment with mevastatin or vehicle control ± mevalonate.

(B) LDH release as marker for cytotoxicity following treatment of RAW 264.7 cells with different concentrations of mevalonate. ***$P < 0.005$, n.s. not significant by two-tailed Student's t-test comparing control versus statin-treated group. Experiments performed 3-4 times with similar results, representative experiment shown ± standard deviation.