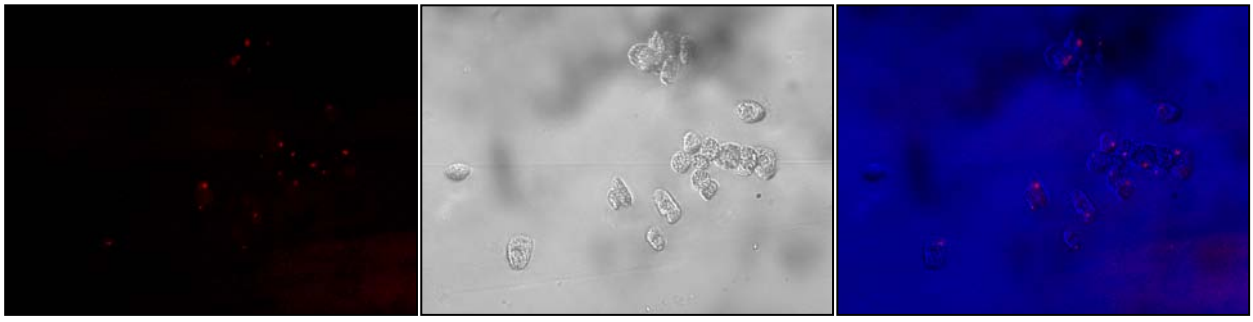
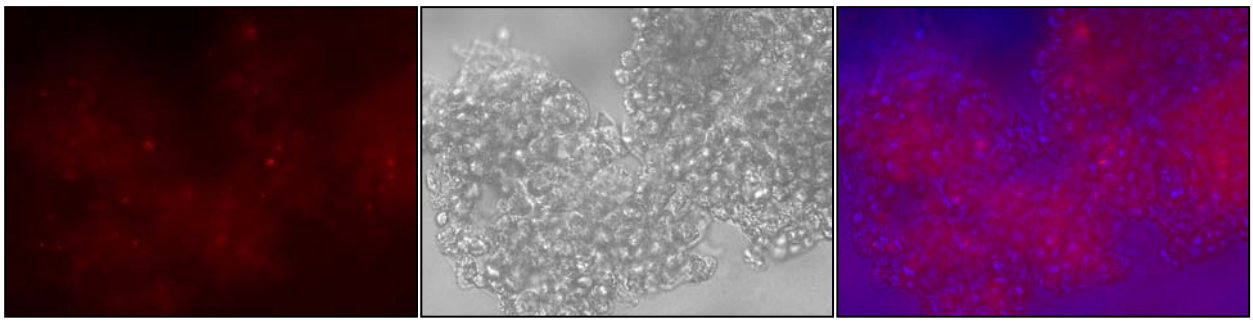
Supplementary Fig. 5. Phagocytosis of *S. aureus* by the hemocytes of horseshoe crab *in vivo* and *in vitro*. Mid-log phase *S. aureus* cells were stained with fluorescent *Bac*Light™ Red (Molecular Probes, Inc., Eugene, OR, USA) following the protocol provided. For *in vivo* analysis, ~ 1×10^7 cells were injected into the heart of a medium-sized horseshoe crab. Blood samples were collected 1 h later into tubes containing a fixative (10 % formalin, 0.5 M NaCl), at the final ratio (v/v) of ~ 1:5. After ~30 min of fixation in an ice bath, the samples were centrifuged at 150 × g for 10 min. The supernatant was removed, and the cells were re-suspended in fresh fixative and stored at 4 °C. For *in vitro* experiments, horseshoe crab hemolymph was bled into sterile falcon tubes and immediately used. Fluorescent labeled *S. aureus* cells were added at the final density of ~ 10^7 cells/ml into the fresh blood, or the blood supplemented with 1/10 (v/v) of 0.5 M EDTA or the complete Protease Inhibitor Cocktail (Roche). It should be noted that the horseshoe crab blood cell density is ~ 2×10^6 cells/ml (Ornberg & Reese, 1981, *J Cell Biol* **90**: 40-54). The tubes were incubated at room temperature with very gentle shaking for 1 h. Thereafter, the blood samples were fixed as above. The fixed cells were analyzed by fluorescence microscopy at 400× magnification. Both bright field image and fluorescence image were taken for each view. **(A)** A typical view of hemocytes taken from the blood of a horseshoe crab injected with *S. aureus*. The internalization of red fluorescent bacterial cell(s) represents *in vivo* phagocytosis. In the pilot experiments, phagocytosis appeared to occur from 30 min after infection (not shown). **(B)** The typical views of *in vitro* experiment, which show that both EDTA and protease inhibitors appear to suppress the phagocytosis. It is noted that under the *in vitro* conditions, the labile horseshoe crab hemocytes tend to aggregate at prolonged incubation, thus posing a possibility that the bacteria may either be engulfed or associated or co-aggregated with the hemocytes. However, theoretically protease inhibitors should not inhibit the non-specifc association of bacteria with hemocytes, thus favoring the process of engulfment/phagocytosis, which, in the absence of EDTA or protease inhibitor is possibly mediated by the protease-activated CrC3 opsonization.

A) *In vivo* phagocytosis

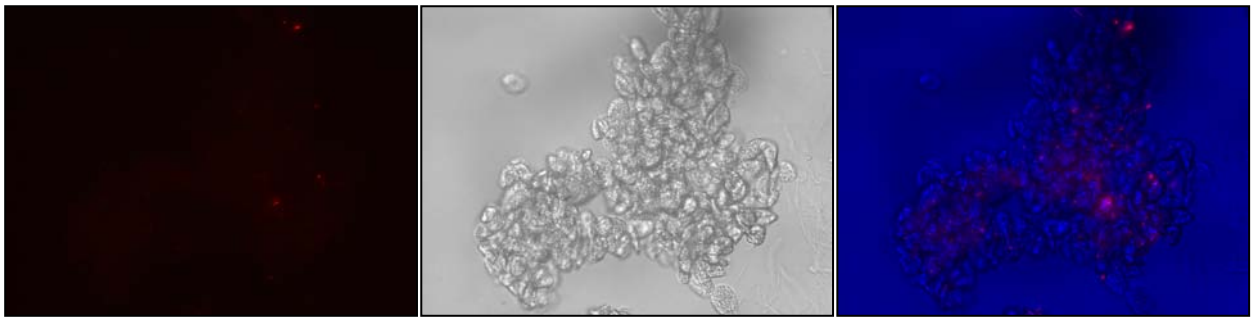


B) *In vitro* phagocytosis

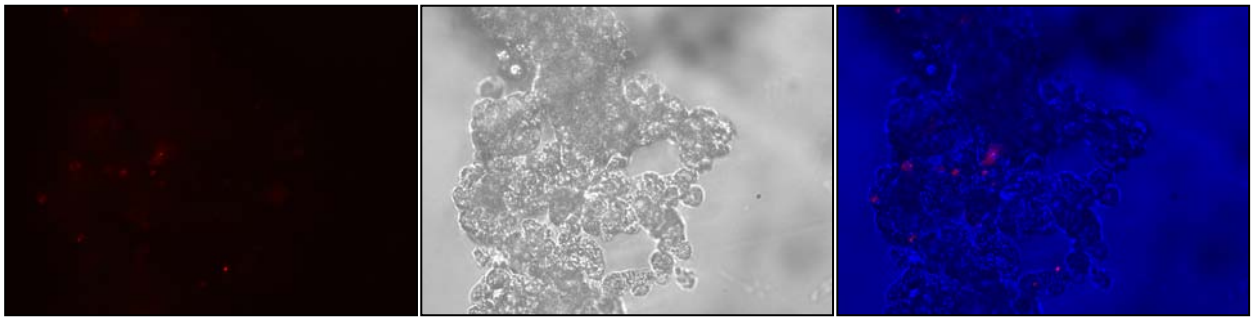
Blood + *S. aureus*



Blood + Protease inhibitors + *S. aureus*



Blood + EDTA + *S. aureus*



Fluorescence views Bright field views Overlapped views

Supplementary Fig. 5