METHODS

Reagents. Pro- and active forms of human MMP2, 3, 7, 8, 9 and 13, and MT1-MMP (also known as MMP14) were obtained from Chemicon. Synthetic peptides (University of Pittsburgh protein core) with >95% purity were generated for: mouse MMP12 CTD I, 344-SRNQLFLFKDEKYWLINNLV-363 (known as SR-20); mouse MMP12 CTD II, 370-RSIYSLGFSASVKKVDAAVF-389; human SR-20, ARNQVFLFKDDKYWLISNLR; MMP13 CTD, 343-SRDLMFIFRGR KFWALNGYD-362; SR-20 mutant peptide, 344-SRNXLFLF*SGRQ*YW LINNLV-363, with italic residues replacing KDEK. Recombinant mouse MMP12 CTD was generated in pET 20b vector with a 6-histidine tag. Three fragments of mouse MMP12 CTD were generated: I, 280-SSPPSTFCHQSLSF DAVTTVGEKIFFFKDWFFWWKLPGSPATNITSISSIWPSIPSGIQAAYEI-342; II, 343-ESRNQLFLFKDEKYWLINNLVPEPHYPRSIYSLGFSASVKKVDAAVFD PLRQKVYFFVDKHY-404; and III, 405-WRYDVRQELMDPAYPKLISTHFPG IKPKIDAVLYFKRHYYIFQGAYQLEYDPLFRRVTKTLKSTSWFGC-473. Vectors were transformed into *E. coli* BL2(DE3) LysE (Novagen). Protein was resuspended in 6 M urea, 300 mM NaCl, 50 mM Na_2HPO_4/NaH_2PO_4 at pH 8.0, and purified using Talon binding resin (Clontech). After dialysis against 0.75 M urea, 300 mM NaCl, 50 mM Na_2HPO_4/NaH_2PO_4 at pH 7.4, recombinant protein was verified by western blotting using 6-histidine residue antibody (Invitrogen).

Mice. *Mmp12*$^{-/-}$ mice previously generated in our laboratory have been described elsewhere[10]. All mice were maintained on a 129/SvJ background and housed in a sterile barrier facility. All experiments were approved by the Harvard Standing Committee for Animal Research and used age- and sex-matched controls.

Bacteria. *Staphylococcus aureus, K. pneumoniae* and *S. enteriditis* (clinical isolates) and *E. coli* (K1 strain) were grown in TSB for 18 h at 37 °C. Bacteria in mid-log phase growth were centrifuged at 1,800*g* for 10 min, washed, and resuspended in PBS. Bacterial concentration was determined by counting c.f.u. from limiting dilutions after an 18-h incubation at 37 °C on antibiotic-free LB plates.

***In vivo* models of infection.** Peritonitis: i.p. injection of either 1×10^8 c.f.u. *S. aureus* ($n = 40$, each group from six experiments) or 1×10^8 c.f.u. *E. coli* (*Mmp12*$^{-/-}$, $n = 15$; wild type, $n = 16$ from three experiments). Peritoneal fluid, liver, blood and lungs were collected from a subset of mice ($n = 4$ each group) and c.f.u. were determined from homogenates. In a separate experiment, peritoneal fluid macrophage and neutrophil counts were measured from peritoneal lavage using a haemocytometer and Wright-stained cytospins ($n = 6$ each group).

Haematogenous infection: TVI of 1×10^8 c.f.u. *S. aureus* into *Mmp12*$^{-/-}$ ($n = 16$) and wild-type control ($n = 13$) mice (from three experiments). Lungs, spleen and kidneys were removed to determine c.f.u.

Pneumonia: 1×10^8 c.f.u. *S. aureus* i.t. into *Mmp12*$^{-/-}$ ($n = 36$) and wild-type control ($n = 30$) mice (from six experiments). The mortality was recorded over a 14-day time course, as described earlier. *Mmp12*$^{-/-}$ and wild-type mice ($n = 8$, each group) were also subjected to a sub-lethal dose i.t. (1×10^6 c.f.u.) of *S. aureus*. Lung homogenates were generated at 2 and 24 h after instillation. Organ homogenates were made in 1 ml of 50 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$ at pH 7.4 containing a proteinase inhibitor cocktail (Sigma). Haematoxylin and eosin, Brown and Breen-modified Gram stain, and macrophage-specific mac-3 immunostaining (Invitrogen) were performed on 5-mm thick sections.

***In vitro* bacterial killing assays.** Bacteria were incubated with MMP3, MMP7, MMP8, MMP9, MMP12, MMP12 CTD, MMP13, MMP13 CTD, and MT1-MMP in either 5% TSB or RPMI plus 10% FCS, in separate experiments. $CaCl_2$ (100 mM) was added to reactions with peptides, which were solubilized in 10% dimethylsulphoxide (DMSO). Direct bactericidal activity of MMP12 CTD was assessed using fluorescent probes Syto 59 and S-7020 (Molecular Probes) at concentrations of 5 μM and 20 μM, respectively. Quantification of dead versus total cells was performed using Metamorph image analysis software from three experiments.

***In vitro* bacterial killing by murine peritoneal macrophages.** Thioglycollate-stimulated peritoneal macrophages were resuspended in DMEM with 5% FCS (without antibiotics), and plated at a concentration of 2.5×10^5 cells per well in a 24-well plate. Cytospin analysis confirmed >95% pure macrophages, with the remainder being comprised of neutrophils and fibroblasts. *Staphylococcus aureus* (1×10^6 c.f.u.) was added for a 60-min incubation. At this time ($t = 0$), the cells were washed with PBS to clear non-adherent bacteria. The cells were lysed 30 and 90 min later with 0.2% Triton X-100 and c.f.u. were measured. Experiment was performed three times. Teflon-coated wells (Costar) were used for both scanning electron microscopy and transmission electron microscopy. Immuno-electron-microscopy used an MMP12 antibody generated in our laboratory (1:100) with a gold-labelled (2-nm particles) secondary to rabbit.

Liposomal release assay. *Staphylococcus aureus* was grown to mid-log phase, centrifuged, pelleted and freeze-fractured with dry ice. Chloroform/methanol (2:1) was added to a final volume of 5 ml. The mixture was agitated for 20 min in an orbital shaker at 25 °C, and centrifuged at 700*g* for 10 min before removing the lipid phase. The chloroform was evaporated under vacuum. Bacterial membrane lipids were hydrated in 1 mM $CaCl_2$, 10 mM MOPS, 100 mM KCl at pH 7.2. Membranes were then freeze-fractured and incubated in the presence of fluorescent Calcium Green-1 Dextran conjugates 3000 MW (Molecular Probes). Bacterial membrane vesicles were incubated in the presence and absence of MMP12 C-terminal protein (20 μg ml^{-1}) for 60 min. Results are from three experiments.

MMP12 processing. Full-length pro-MMP12 was incubated with or without *S. aureus* in RPMI for 10, 20 or 30 min. The reactions were centrifuged at 8,000*g* and the supernatant was subjected to western blotting using an antibody directed against the MMP12 CTD (Santa Cruz).

Structural studies. In the absence of an experimental structure of mouse MMP12 CTD, a homology model was constructed on the basis of the crystal structures of related full-length MMPs. This strategy was followed instead of considering only CTD structures to get further information on the relative orientations of the pro-, catalytic domain and the CTD. An initial search with PSI-Blast (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) of the mouse pro-MMP12 sequence (UniProt database entry P34960) against the Protein Data Bank (PDB) using standard values identified human pro-MMP1 (PDB accession 1su3) as the closest homologue with an E-value of 9E-110 and 48% sequence identity. On the basis of this experimental structure, models for mouse pro-MMP12 covering residues 55–462 were generated by using programs CPHmodels 2.0 (http://www.cbs.dtu.dk/services/CPHmodels) and Swiss Model (http://swissmodel.expasy.org). In addition, human pro-MMP1, the catalytic domain of human MMP12 (PDB accession 1JK3), together with other reported experimental MMP structures encompassing the catalytic and/or the haemopexin-like domain, that is, human pro-MMP2 (PDB accession 1CK7), human pro-MMP2 in complex with TIMP2 (PDB accession 1GXD), porcine MMP1 (PDB accession 1FBL), and human MMP13 (PDB accession 1PEX) were further superimposed on the homology model for structure assessment.

Statistics. Kaplan–Meier life survival curve analysis was performed using the Prism Data Analysis software, which uses the log-rank test for curve comparison analysis. For all other experiments, the data are expressed as the mean value and s.d. Statistical significance was determined using the Student's *t*-test (two-tailed distribution with a two sample equal variance). $P < 0.05$ was considered significant.