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The Chymase Mouse Mast Cell Protease 4 Degrades TNF, Limits Inflammation, and Promotes Survival in a Model of Sepsis

Adrian M. Piliponsky,*† Ching-Cheng Chen,*‡ Eon J. Rios,* Piper M. Treuting,§ Asha Lahiri,† Magnus Abrink,¶ Gunnar Pejler,‖ Mindy Tsai,* and Stephen J. Galli*

From the Department of Pathology, Stanford University School of Medicine, Stanford, California; the Center for Immunity and Immunotherapies,† Seattle Children's Research Institute, Seattle, Washington; the Division of Hematopoietic Stem Cell and Leukemia Research,‡ Beckman Research Institute, City of Hope National Medical Center, Duarte, California; the Department of Comparative Medicine & Histology and Imaging Core,§ School of Medicine, University of Washington, Seattle, Washington; and the Departments of Biomedical Sciences and Veterinary Public Health¶ and Anatomy, Physiology and Biochemistry,‖ Swedish University of Agricultural Sciences, Uppsala, Sweden*

Mouse mast cell protease 4 (mMCP-4), the mouse counterpart of human mast cell chymase, is thought to have proinflammatory effects in innate or adaptive immune responses associated with mast cell activation. However, human chymase can degrade the proinflammatory cytokine TNF, a mediator that can be produced by mast cells and many other cell types. We found that mMCP-4 can reduce levels of mouse mast cell-derived TNF *in vitro* through degradation of transmembrane and soluble TNF. We assessed the effects of interactions between mMCP-4 and TNF *in vivo* by analyzing the features of a classic model of polymicrobial sepsis, cecal ligation and puncture (CLP), in C57BL/6J-mMCP-4-deficient mice versus C57BL/6J wild-type mice, and in C57BL/6J-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice containing adoptively transferred mast cells that were either wild type or lacked mMCP-4, TNF, or both mediators. The mMCP-4-deficient mice exhibited increased levels of intraperitoneal TNF, higher numbers of peritoneal neutrophils, and increased acute kidney injury after CLP, and also had significantly higher mortality after this procedure. Our findings support the conclusion that mMCP-4 can enhance survival after CLP at least in part by limiting detrimental effects of TNF, and suggest that mast cell chymase may represent an important negative regulator of TNF *in vivo*. (Am J Pathol 2012, 181: 875–886; http://dx.doi.org/10.1016/j.ajpath.2012.05.013)

Sepsis is a complex and often fatal disorder that is considered to represent a dysregulated host response to infection.[1,2] There are approximately 750,000 cases of sepsis per year in the United States, with mortality ranging from 20% to 50%,[3] but the factors responsible for the pathology and fatal outcome of sepsis are not yet fully understood. Accordingly, there is much interest in identifying additional molecules that promote or restrain the excessive inflammatory response and other pathology observed in this disorder.[4]

Mast cells are derived from hematopoietic progenitor cells and are widely distributed in tissues. Mast cells are best known for their roles in anaphylaxis, atopic asthma, and other IgE-associated allergic disorders.[5,6] In addition to such detrimental roles, mast cells have been shown to have a variety of beneficial functions in the host, including promoting host resistance in certain models of bacterial or parasite infection[5,6] and enhancing resistance to certain animal venoms.[7–10] However, mast cell functions in such settings may depend, in part, on the strain background of the mice studied,[11] and strain background can also influence the mast cell content of particular mediators, including cytoplasmic granule-associated proteases.[12,13]

Mouse strain background also can importantly influence the outcome of cecal ligation and puncture (CLP),[14,15] a well-established and extensively used

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Address reprint requests to Adrian M. Piliponsky, Ph.D., Center for Immunity and Immunotherapies, Seattle Children's Research Institute, Department of Pediatrics, University of Washington, 1900 9th Ave., Seattle, WA 98101, or to Stephen J. Galli, M.D., Department of Pathology, L-235, Stanford University School of Medicine, 300 Pasteur Dr., Stanford, CA 94305-5324. E-mail: adrian.piliponsky@seattlechildrens.org or sgalli@stanford.edu.

model of sepsis.[16] For example, we found that C57BL/6 mice subjected to a severe model of CLP exhibited increased intraperitoneal TNF levels and neutrophil numbers, and significantly reduced survival rates, compared with (WB × C57BL/6)F_1 mice (hereafter referred to as WBB6F_1 mice).[15] These observations are consistent with the hypothesis that the intense inflammatory response that develops after CLP in C57BL/6 mice can contribute to the increased mortality in this model of sepsis.

Individual mast cell-associated proteases have been shown to contribute to effective host responses to bacterial infections,[17,18] either through the recruitment of inflammatory cells[19] or via the degradation of toxic mediators generated by the host in response to the infection.[7,20] However, the potential role of the mouse counterpart of human chymase, mouse mast cell protease-4 (mMCP-4), during sepsis has not previously been investigated. Here, we report evidence for a previously unsuspected role for mMCP-4 as an anti-inflammatory protease that can contribute to reduced levels of TNF, diminished inflammation, and improved survival after CLP.

Materials and Methods

Mice

C57BL/6-*Kit*$^{W\text{-}sh/+}$ mice (*Kit*$^{W\text{-}sh/+}$ mice)[21] were a generous gift from Peter Besmer (Molecular Biology Program, Memorial Sloan-Kettering Cancer Center and Cornell University Graduate School of Medical Sciences). We backcrossed *Kit*$^{W\text{-}sh/+}$ mice with C57BL/6J (B6J) mice for 11 generations to produce B6J-*Kit*$^{W\text{-}sh/+}$ mice and intercrossed B6J-*Kit*$^{W\text{-}sh/+}$ mice to produce mast cell-deficient B6J-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. mMCP-4-deficient (*Mcpt4*$^{-/-}$),[22] TNF-deficient (*Tnf*$^{-/-}$),[23] and mMCP-4-deficient and TNF-deficient (*Mcpt4*$^{-/-}$*Tnf*$^{-/-}$) mice on the C57BL/6J background were bred and maintained at the Stanford University Research Animal Facility. C57BL/6J mice were purchased from the Jackson Laboratory (Sacramento, CA). Unless specified otherwise, all experiments were performed using male mice that were 12 weeks old at the beginning of the experiment. All animal care and experimentation was conducted in accord with current National Institutes of Health guidelines and with the approval of the Stanford University and the Seattle Children's Research Institute Institutional Animal Care and Use Committee.

Mast Cell Engraftment of Mast Cell-Deficient Mice

Some B6J-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (4 to 6 weeks old) were repaired of their mast cell deficiency selectively and locally by the intraperitoneal injection of growth factor-dependent, congenic bone marrow derived-cultured mast cells (BMCMCs). Briefly, femoral bone marrow cells from B6J wild-type (WT), *Mcpt4*$^{-/-}$, *Tnf*$^{-/-}$ or *Mcpt4*$^{-/-}$*Tnf*$^{-/-}$ mice were maintained *in vitro* for approximately 4 weeks in IL-3-containing medium until mast cells represented >95% of the total cells as indicated by May-Grünwald-Giemsa staining. BMCMCs (1.5×10^6 in 200 μL of PBS) were injected intraperitoneally (with a 27-gauge needle), and the mice were used for experiments, together with strain-, sex-, and age-matched mast cell-deficient mice, 4 to 6 weeks after adoptive transfer of BMCMCs. The numbers of peritoneal mast cells (PMCs) were similar in B6J-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice that had been engrafted with B6J WT (2.4 ± 1.2% of total cells in the peritoneal lavage fluid), mMCP-4-deficient (1.9 ± 0.8%), TNF-deficient (2.1 ± 0.8%), or both mMCP-4-deficient and TNF-deficient mast cells (2.4 ± 0.1%); the distribution and numbers of mast cells in the mesentery (ie, mesenteric windows) of these four groups of mice were also similar (2.3 ± 0.9, 2.3 ± 0.8, 2.5 ± 0.4, and 2.0 ± 0.4 mast cells/mm^2, respectively).

Cecal Ligation and Puncture

CLP was performed as described previously,[15] to induce a moderately severe model of CLP in which 20% to 50% of the WT mice die within 4 days after CLP. Briefly, mice were deeply anesthetized by an intramuscular injection of 100 mg/kg ketamine and 20 mg/kg xylazine, and the cecum was exposed by a 1- to 2-cm midline incision on the anterior abdomen and subjected to ligation of the distal half of the cecum and single puncture (with a 22-gauge needle) of the ligated segment, The cecum was then replaced into the abdomen, 1 mL of sterile saline (pyrogen-free 0.9% NaCl) was administrated into the peritoneal cavity, and the incision was closed using 9-mm steel wound clips. Mice were observed for mortality at least four times daily. Mice that were clearly moribund were euthanized by CO_2 inhalation.

Assessment of Acute Kidney Injury

Acute kidney injury (AKI), defined as tubules lined by vacuolated degenerating to necrotic epithelium with variable intraluminal sloughed cells, was scored as described by Wang et al,[24] with modifications. Briefly, H&E-stained coronal sections of each kidney from the mice were scanned at ×200 magnification for AKI within the cortex and outer stripe of the outer zone of the medulla. It is well established clinically that severe kidney dysfunction may be associated with only subtle and perhaps patchy morphological changes.[25] Because of the multifocal distribution of the lesions in mice subjected to CLP, one section from each kidney was scanned in its entirety, and the region in each kidney section (1× sections per kidney) that was the most severely affected with AKI was scored (some regions scored from sections of kidneys from *Mcpt4*$^{+/+}$ mice exhibited no evidence of AKI).[24] Similar scores for AKI were obtained when a single observer repeatedly scored the same slides, and when the slides were scored independently by a second observer (data not shown). The top score, obtained in the most severely affected individual section, was 4 (*Mcpt4*$^{-/-}$ after CLP), with 46% to 75% of tubules affected within the field (×200 magnification); the lowest score obtained was 0 (*Mcpt4*$^{+/+}$ at baseline). PAS-stained sections were examined to confirm changes noted on H&E and to assess the integrity of the brush border. Images were captured with Nikon Eclipse 80i microscope equipped with CFI

Plan Apo objectives and a Nikon Digital Sight DS-Fi1 12-megapixel camera and Nikon Basic Elements software version 3.0 (Melville, NY). Raw images were edited for brightness in Adobe Photoshop Elements version 8.0 (San Jose, CA).

Blood Urea Nitrogen Measurements

Blood urea nitrogen (BUN) levels were assessed using a commercially available kit (Teco Diagnostics, Anaheim, CA).

Evaluation of Mast Cells in the Peritoneal Cavity and Mesenteric Windows

To harvest peritoneal cells, 2 mL of Hank's buffered saline solution buffer containing 10 U/mL heparin and 1% fetal calf serum were injected into the peritoneal cavity, and the abdomen was massaged gently for 30 seconds. Fluid containing peritoneal cells was aspirated, and the cells were cytocentrifuged onto glass slides and stained with May-Grünwald-Giemsa to identify and quantify the percentages of various cell populations. To evaluate mast cells in the mesenteric windows, approximately four to five mesenteric windows from approximately the same locations in each mouse were arranged on slides and fixed for 1 hour in Carnoy's solution (3:2:1 v/v/v of ethanol, chloroform, and acetic acid). Tissues were stained with Csaba stain, which contains both safranin (red, thought to identify mature mast cells) and alcian blue (thought to identify less mature mast cells), which bind to mast cell granules. Cytocentrifuge preparations were evaluated for mast cell percentage, and slides of mesenteric windows were examined by an observer not aware of the identity of the individual mice, to assess presence, numbers, and distribution of mast cells in the mesentery.

PMC Purification

Peritoneal cells were layered onto 1.5 mL of 23% Histodenz (Sigma-Aldrich, St. Louis, MO) in Hank's buffered saline solution containing 10 U/mL heparin and 1% fetal calf serum and were centrifuged at 1350 rpm (469 × *g*) for 15 minutes at room temperature. After a washing, cells were stained with Toluidine Blue. PMCs represented 85% to 90% of the cells recovered.

Generation of Peritoneal Cell-Derived Mast Cells

To generate peritoneal cell-derived mast cells (PCMCs), peritoneal cells from *Mcpt4*$^{+/+}$ and *Mcpt4*$^{-/-}$ mice were maintained *in vitro* for 2 weeks in medium containing IL-3 (10 ng/mL) and stem cell factor (SCF; 50 ng/mL) until mast cells represented >95% of the total nonadherent cells, as indicated by May-Grünwald-Giemsa staining.[26]

Quantification of Leukocytes

Neutrophils (Gr-1^{high}/F4/80^{-} cells) and macrophages (Gr-1^{+}/F4/80^{+} cells) in the peritoneal fluid were analyzed by flow cytometry. Briefly, red blood cells were lysed with ACK lysis buffer for 5 minutes, and total cell numbers were counted using a hemocytometer. Cells were blocked with unconjugated anti-CD16/CD32 on ice for 5 minutes and then were stained with a combination of fluorescein isothiocyanate-labeled anti-Gr-1 (RB6-8C5, 2.5 μg/mL) and APC-labeled anti-F4/80 (BM8, 4 μg/mL) antibodies (eBioscience, San Diego, CA) on ice for 15 minutes for the detection of neutrophils and macrophages. The expression of cell surface markers was analyzed on a FACSCalibur system (BD Biosciences, San Jose, CA) using FlowJo software version 8.8.6 (Tree Star, Ashland, OR). Gates for subpopulations of cells were based on unstained cells, as well as cells stained with a single color to determine compensation and nonspecific fluorescence. Propidium iodide was used to detect dead cells. Only cells negative for propidium iodide were used for analysis.

Quantification of Bacterial CFUs

Dilutions of peritoneal fluids or blood were performed and samples were plated on LB agar for peritoneal fluids or tryptose blood agar (BD Biosciences) for blood. Colonies were counted after overnight incubation at 37°C.

β-Hexosaminidase Release Assay

BMCMCs were sensitized with 2 μg/mL of anti-dinitrophenyl (anti-DNP) IgE monoclonal antibody (mAb) (H1-ε-26)[27] by overnight incubation at 37°C. The cells were then washed with Tyrode's buffer [10 mmol/L HEPES pH 7.4, 130 mmol/L NaCl, 5 mmol/L KCl, 1.4 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 0.1% glucose, and 0.1% bovine serum albumin (fraction V; Sigma-Aldrich)] and resuspended at 8×10^6 cells/mL. Next, 25 μL of a 2× concentration of stimulus [to achieve final concentrations of: 0, 10, and 100 ng/mL dinitrophenylated human serum albumin (DNP-HSA; Sigma-Aldrich)] was added to the wells of a 96-well V-bottom plate (Costar; Corning Life Sciences, Lowell, MA), and then 25 μL of 8×10^6 cells/mL IgE-sensitized BMCMCs was added and incubated at 37°C for 1 hour. After centrifugation, supernatants were collected. The supernatants from nonstimulated IgE-sensitized BMCMCs treated with 50 μL of 0.5% (v/v) Triton X-100 (Sigma-Aldrich) were used to measure the maximal (100%) cellular β-hexosaminidase content, to which the experimental samples were normalized. β-Hexosaminidase release was quantified by enzyme immunoassay with *p*-nitrophenyl-*N*-acetyl-β-D-glucosamine (Sigma-Aldrich) substrate, as follows: 10 μL of culture supernatant was added to the wells of a 96-well flat-bottom plate; 50 μL of 1.3 mg/mL *p*-nitrophenyl-*N*-acetyl-β-D-glucosamine solution in 100 mmol/L sodium citrate, pH 4.5, was added, and the plate was incubated at room temperature for 15 to 30 minutes. Next, 140 μL of 200 mmol/L glycine, pH 7, was added to stop the reaction, and the optical density (OD_{405}) was determined.

Cytokine Release Assays

BMCMCs (1×10^5/100 μL) were sensitized with IgE mAb to DNP (H1-ε-26)[27] (2 μg/mL) overnight at 37°C and then

were challenged with DNP-HSA (10 ng/mL) for 18 hours at 37°C. Cell supernatants were then collected for cytokine measurements.

Cytokine Measurements

TNF, IL-6, and IFN-γ levels were measured by enzyme-linked immunosorbent assay (BD OptEIA ELISA; BD Biosciences). The assay detection limits were 15 pg/mL.

Angiotensin II Measurements

Plasma angiotensin II levels were measured by ELISA (Cayman Chemical, Ann Arbor, MI). The detection limit for this assay was 1.5 pg/mL. Angiotensin II-forming activity associated with the mouse aorta was measured as described previously.[28] Briefly, aortic tissue was homogenized in 2 mL of 50 mmol/L NaH_2PO_4 buffer, pH 7.4, using a homogenizer (PRO Scientific, Oxford, CT) and centrifuged at 30,000 $\times$ g for 20 minutes. Pellets were resuspended in 0.5 mL of buffer, and 3.84 μL of 130 ng/mL angiotensin I was added. Next, 5 μL of this solution was added to 35 μL of assay buffer (20 mmol/L Tris-HCl, pH 8.0, containing 0.5 mol/L KCl and 0.01% Triton X-100) and incubated at 37°C for 40 minutes. The reaction was terminated with the addition of 300 μL of ethanol. Precipitated proteins were removed by centrifugation, and the supernatants were dried and then resuspended in ELISA buffer for quantification of angiotensin II.

Intracellular Staining of TNF

BMCMCs (1×10^5/100 μL) were sensitized with IgE mAb to DNP (H1-ε026)[27] (2 μg/mL) overnight at 37°C and then were washed and challenged with DNP-HSA (10 ng/mL) in the presence of 2 μmol/L monensin for 6, 12, or 18 hours, respectively. Cells were blocked with unconjugated anti-CD16/CD32 on ice for 5 minutes, then incubated on ice for 30 minutes with a combination of fluorescein isothiocyanate-labeled anti-FcεRIα (MAR-1, 2.5 μg/mL; eBioscience) and phycoerythrin-labeled anti-c-Kit (2B8; 1 μg/mL) (BD Biosciences) antibodies for mast cell detection. The cells were washed, fixed in PBS containing 4% paraformaldehyde for 20 minutes at room temperature, washed with a permeabilization buffer [0.1% saponin (Sigma-Aldrich) in the staining buffer], and incubated with Alexa Fluor 647-labeled-anti-mouse TNF (MP6-XT22; 2 μg/mL) (eBioscience). The expression of cell surface markers and TNF was analyzed by fluorescence-activated cell sorting. The expression of cell surface markers was analyzed on a FACSCalibur system (BD Biosciences) using FlowJo software version 8.8.6 (Tree Star).

Analysis of Chymotryptic Activity

Peritoneal lavage cells containing 5×10^4 PMCs were solubilized in 200 μL lysis buffer (PBS/2 mol/L NaCl, 0.5% Triton X-100), and 10 μL of the peritoneal lavage cell lysates was mixed with 90 μL H_2O, followed by the addition of 20 μL of 1.8 mmol/L solution (in H_2O) of chromogenic substrate for chymotrypsin-like proteases (Chromogenix S-2586; DiaPharma, West Chester, OH). The absorbance at 405 nm was monitored with a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA).

Analysis of Cathepsin G Activity

Freshly harvested mouse femoral bone marrow cells (1×10^7 cells) and PCMCs (1×10^6 cells) were solubilized in 100 μL lysis buffer (1% Nonidet P-40 in PBS). Cathepsin G activity was measured using 100 μL of cell lysates with 1 μL of 1.5 mmol/L of fluorogenic substrate for cathepsin G (ABZ-TPFSGQ-EDDnp) in a total volume of 200 μL. ABZ-TPFSGQ-EDDnp hydrolysis was monitored by measuring fluorescence at λ_{ex} = 320 nm and λ_{em} = 420 nm using a microplate fluorescence reader (SpectraMax Gemini; Molecular Devices).

Degradation of sTNF and proTNF

We incubated 1 μg of mouse recombinant soluble TNF (sTNF; PeproTech, Rocky Hill, NJ) or 500 ng human recombinant proTNF (R&D Systems, Minneapolis, MN) in the presence or absence of TAPI-1 (10 μg/mL), with PMC lysates obtained from *Mcpt4*$^{+/+}$ or *Mcpt4*$^{-/-}$ mice [2×10^5 cells/100 μL of lysis buffer (PBS/2 mol/L NaCl, 0.5% Triton X-100)] for 1.5 hours at 37°C. Cleaved fragments were visualized by SDS-PAGE.

RT-PCR

RNA (50 ng) was isolated from cells with an RNeasy mini kit (Qiagen, Valencia, CA) and converted to first-strand cDNA with oligo(dT) primers (Ambion; Life Technologies-Applied Biosystems, Foster City, CA) and Sensiscript Reverse Transcriptase (Qiagen), Ambion RETROscript (Life Technologies-Applied Biosystems), or iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Taq DNA polymerase (Life Technologies-Invitrogen, Carlsbad, CA) and the following primer pairs were used for amplification of mMCP-4: 5′-GACAGAATCCACACAGCAGAAG-3′ (forward) and 5′-CCTCCAGAGTCTCCCTTGTATG-3′ (reverse). The resulting PCR products were resolved on 1.5% agarose gels.

Quantitative PCR

BMCMCs (1×10^5/100 μL) were sensitized with IgE mAb to DNP (H1-ε-26)[27] (2 μg/mL) overnight at 37°C and then were washed and challenged with DNP-HSA (10 ng/mL) for 0.5, 2, or 6 hours. cDNA was analyzed for quantitative expression levels of TNF with Fast SYBR Green master mix on a 7500 Fast real-time PCR system (Life Technologies-Applied Biosystems) with the following primers: 5′-AAATGGCCTCCCTCTCATCAG-3′ (forward) and 5′-GCTTGTCACTCGAATTTTGAGAAG-3′ (reverse). Results were analyzed using the ΔC_T method normalized to *GAPDH*.

Western Blot Analysis

The cell sTNF or proTNF degradation products were denatured by boiling for 1 minute with sample buffer (2.5% SDS 10% glycerol and 5% mercaptoethanol). Lysates were separated by SDS/PAGE [10% bis-tris gel with 2-(*N*-morpholino)ethanesulfonic acid running buffer; Life Technologies-Invitrogen], electroblotted onto Invitrolon polyvinylidene difluoride membranes (Life Technologies-Invitrogen), and then probed with antibodies against TNF (Cell Signaling Technology, Danvers, MA), cathepsin G (Abnova, Taipei, Taiwan; Walnut, CA), and mMCP-4 and GAPDH (Research Diagnostics, Concord, MA).

Statistical Analysis

We assessed differences in the survival rates after CLP using the Mantel-Haenszel log-rank test. All other data were analyzed for statistical significance using the Mann-Whitney *U*-test. $P < 0.05$ was considered statistically significant. Unless otherwise specified, all data are presented as means ± SEM.

Results

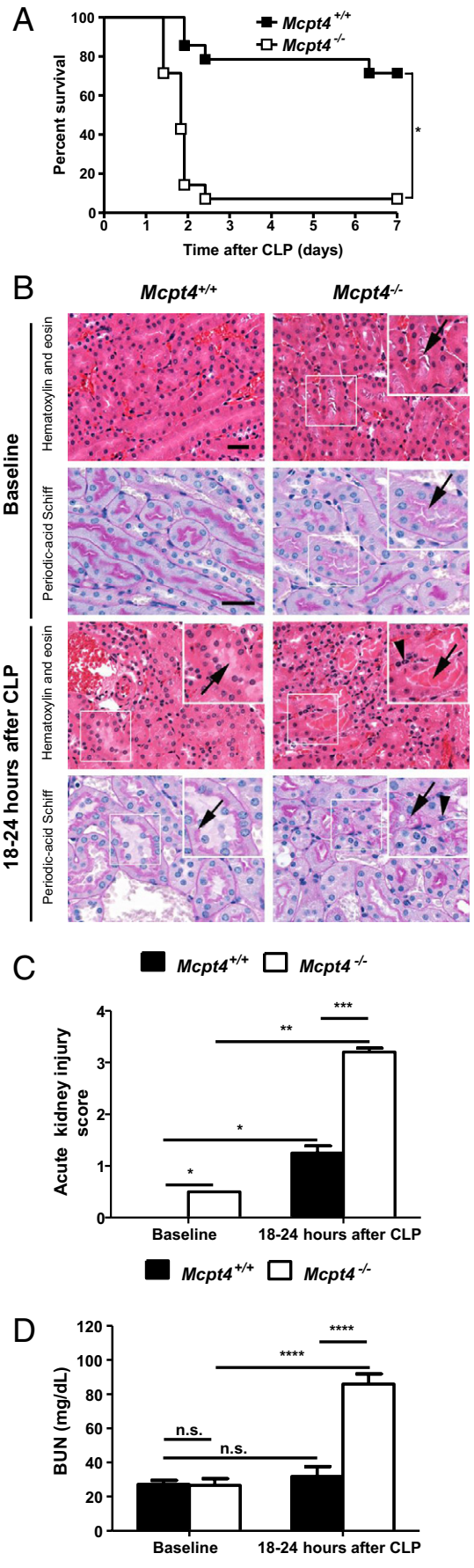
Higher Expression and Activity of mMCP-4 in WBB6F$_1$ than in C57BL/6J Mast Cells

Mast cells of WBB6F$_1$ mice have been reported to express higher levels of mRNA for mMCP-4, compared with mast cells of B6J mice.[12,13] We confirmed that WBB6F$_1$ BMCMCs express higher levels of mMCP-4 mRNA than B6J BMCMCs; we also found that levels of mMCP-4 protein were higher in WBB6F$_1$ than in B6J BMCMCs (see Supplemental Figure S1, A and B, at *http://ajp.amjpathol.org*). Moreover, peritoneal lavage cells of WBB6F$_1$ mice exhibited higher levels of chymotryptic activity than did B6J peritoneal lavage cells (see Supplemental Figure S1C at *http://ajp.amjpathol.org*). These data suggested to us that there may be an association between levels of mMCP-4 expression and the outcome of CLP. B6J mice exhibit low survival rates after severe CLP,[15] and their mast cells express low levels of mMCP-4 (see Supplemental Figure S1 at *http://ajp.amjpathol.org*), whereas WBB6F$_1$ mice exhibited high survival rates after severe CLP,[15] and their mast cells express high levels of mMCP-4 (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). We therefore decided to assess the role of mMCP-4 in CLP.

mMCP-4-Deficient Mice Exhibit Reduced Survival after CLP

To assess whether mMCP-4 can influence the outcome of sepsis, we compared responses to CLP in WT (*Mcpt4*$^{+/+}$) and in mMCP-4-deficient (*Mcpt4*$^{-/-}$) mice on the B6J background. Deletion of mMCP-4 in *Mcpt4*$^{-/-}$ mice does not influence the activities of other mast cell proteases analyzed, such as tryptase or mast cell carboxypeptidase A (MC-CPA).[22] We also searched for cathepsin G protein and enzymatic activity in PCMCs and, as a control, in freshly isolated femoral bone marrow cells, which contain neutrophils that express cathepsin G[29]. We readily detected cathepsin G protein and enzymatic activity in the bone marrow cells of both *Mcpt4*$^{+/+}$ and *Mcpt4*$^{-/-}$ mice (see Supplemental Figure S2 at *http://ajp.amjpathol.org*). By contrast, when we tested similar amounts of protein derived from PCMCs, we detected neither cathepsin G protein nor enzymatic activity in mast cells derived from either *Mcpt4*$^{+/+}$ or *Mcpt4*$^{-/-}$ mice (see Supplemental Figure S2 at *http://ajp.amjpathol.org*). *Mcpt4*$^{-/-}$ mice resemble WT mice in general appearance, gross anatomy, body weight, reproduction, and overt behavior.[22] Moreover, we observed that baseline blood levels of alanine aminotransferase (60.3 ± 13.9 versus 77.3 ± 13.0 U/L), BUN (30.8 ± 2.1 versus 30.9 ± 5.1 mg/dL), and CO_2 (21.8 ± 1.1 versus 18.5 ± 0.3 mmol/L) were similar in *Mcpt4*$^{+/+}$ and *Mcpt4*$^{-/-}$ mice, which provides evidence that liver, kidney, and lung functions are not grossly affected by the lack of chymase under normal conditions. We also verified that the numbers of PMCs were similar in *Mcpt4*$^{+/+}$ and *Mcpt4*$^{-/-}$ mice (3.8 ± 0.5% and 3.9 ± 0.3% of total peritoneal cells, respectively; $P = 0.96$, $n = 9$ or 10 mice per group), as was the distribution of mast cells in the mesentery (ie, mesenteric windows) of these mice (4.2 ± 0.8 and 5.5 ± 1.2 mast cells/mm^2, respectively; $P = 0.56$, $n = 7$ or 8 mice per group).

Nonetheless, *Mcpt4*$^{-/-}$ mice exhibited markedly reduced survival in a protocol of moderate CLP, compared with *Mcpt4*$^{+/+}$ mice (7% versus 71% survival at 1 week after CLP; $P < 0.0001$) (Figure 1A). Sepsis and septic shock are the most important causes of acute renal failure (ARF) in critically ill patients, and account for more than 50% of cases of ARF in the ICU.[30] We found that *Mcpt4*$^{-/-}$ mice were more susceptible to ARF after CLP, compared with *Mcpt4*$^{+/+}$ mice (Figure 1, B–D). Lesions of acute kidney injury (AKI) in mice subjected to CLP were increased in kidney sections from *Mcpt4*$^{-/-}$, compared with *Mctp4*$^{+/+}$ mice or compared with kidney sections from mice not subjected to CLP. Characteristic lesions seen in the CLP group included mild proximal renal tubular swelling and apical blebbing with loss of PAS$^+$ brush border, some vacuolation, and PAS$^+$ intracytoplasmic hyaline droplets (Figure 1B), consistent with degeneration. In some tubules, epithelial damage progressed to necrosis and/or apoptosis, with intraluminal sloughing of hyalinized necrotic cells, and a mild increase in neutrophils within renal vasculature (Figure 1B). The average severity of kidney injury in mice subjected to CLP (scored as 0 to 4, with 4 = most severe) was 3.2 in the *Mcpt4*$^{-/-}$ mice, compared with 1.2 in *Mcpt4*$^{+/+}$ mice (Figure 1C). The histological findings are consistent with BUN levels, which were markedly increased in *Mcpt4*$^{-/-}$ mice, relative to those of *Mcpt4*$^{+/+}$ mice, at 18 to 24 hours after CLP (Figure 1D). In contrast, at baseline *Mcpt4*$^{-/-}$ mice had some histological lesions within the proximal renal tubular epithelium (scored minimally at 0.5); however, there was no detectable increase in BUN levels, suggesting that this histological lesion does not affect renal function and may represent transient and reversible metabolic changes to the individual affected tubules that were



not widespread enough to change overall renal function. Together, the histological and BUN levels support the conclusion that *Mcpt4*$^{-/-}$ mice develop more severe renal dysfunction after CLP than do *Mcpt4*$^{+/+}$mice.

These observations indicate that chymase has direct or indirect effects that can significantly increase survival in mice after CLP of moderate severity and can have a protective role in the kidneys of mice subjected to this procedure.

mMCP-4-Deficient Mice Have Increased Neutrophil Accumulation after CLP

In sepsis, both the efficiency of bacterial clearance and the magnitude of the inflammatory response contribute to the host's outcome. Although neutrophils are essential for bacterial defense and clearance, excessive amounts of activated neutrophils can exacerbate tissue damage after infection.[31] We therefore investigated whether chymase might influence numbers of neutrophils at the site of infection.

We found significantly increased numbers of neutrophils in the peritoneal cavity of *Mcpt4*$^{-/-}$ mice at 6, 18 to 24, and 48 hours after CLP, compared with the values for *Mcpt4*$^{+/+}$ mice at the same time points (Figure 2A). By contrast, the numbers of macrophages in the peritoneum did not differ significantly between *Mcpt4*$^{+/+}$ and *Mcpt4*$^{-/-}$ mice at the same time points (Figure 2B).

It has recently been reported that another mast cell chymase, mMCP-2, has antibacterial activity.[32] In the present study, however, there was a trend toward lower levels of bacterial CFUs in the peritoneal cavities of *Mcpt4*$^{-/-}$ mice than in those of *Mcpt4*$^{+/+}$ mice at 18 to 24 hours after CLP (34,363 ± 17,789 CFU versus 108,309 ± 56,064 CFU, respectively) and at 48 hours after CLP (133,177 ± 131,766 versus 411,200 ± 247,481 CFU, respectively). With the caveat that not all of the potentially pathogenic cecal bacteria present would be expected to grow under the condi-

Figure 1. mMCP-4 can enhance mouse survival after CLP. **A:** Survival after CLP (50% ligation; single puncture with a 22-gauge needle) in *Mcpt4*$^{+/+}$ mice (n = 14) and *Mcpt4*$^{-/-}$ mice (n = 15). *$P < 0.0001$ versus *Mcpt4*$^{-/-}$ mice. **B:** Representative images of kidneys at baseline and at 18 to 24 hours after CLP in *Mcpt4*$^{+/+}$ and *Mcpt4*$^{-/-}$ mice. At baseline, the *Mcpt4*$^{-/-}$ kidneys have minimal tubular lesions with intratubular apical laminated hyaline material as indicated by H&E staining (**arrow**) and mildly irregular PAS staining of the brush border (**arrow**), whereas corresponding sections from *Mcpt4*$^{+/+}$ kidneys appear normal. At 18 to 24 hours after CLP, the sections of *Mcpt4*$^{+/+}$ kidney exhibit mild proximal renal tubular swelling and apical blebbing under H&E staining (**arrow**) with some vacuolation (not shown), as well as mild irregular PAS staining and ectasia of affected tubules (**arrow**). In *Mcpt-4*$^{-/-}$ kidney, CLP resulted in moderate multifocal acute renal tubular necrosis with intraluminal sloughing of hyalinized necrotic debris as indicated by H&E staining (**arrow**), occasional apoptotic cells (not shown), and a mild increase in neutrophils within renal vasculature as indicated by both H&E and PAS staining (**arrowheads**). Degenerate epithelial cells contain numerous PAS^{+} cytoplasmic droplets (**arrow**). **Insets** are digitally magnified portions of the same section, corresponding to the boxed region. **C:** Scoring of acute kidney injury in H&E-stained sections at baseline and at 18 to 24 hours after CLP in *Mcpt4*$^{+/+}$ mice (n = 3 or 4 per group) and *Mcpt4*$^{-/-}$ mice (n = 3 to 10 per group). **D:** Blood urea nitrogen (BUN) levels at baseline and at 18 to 24 hours in *Mcpt4*$^{+/+}$ mice (n = 9 or 10 per group) and *Mcpt4*$^{-/-}$ mice (n = 9 or 10 per group). Data were pooled from three independent experiments, each of which gave similar results. In **C** and **D**, *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, ****$P < 0.0001$. n.s., not significant ($P > 0.05$). Scale bar = 50 μm. Original magnification: ×400 (H&E); ×600 (PAS).

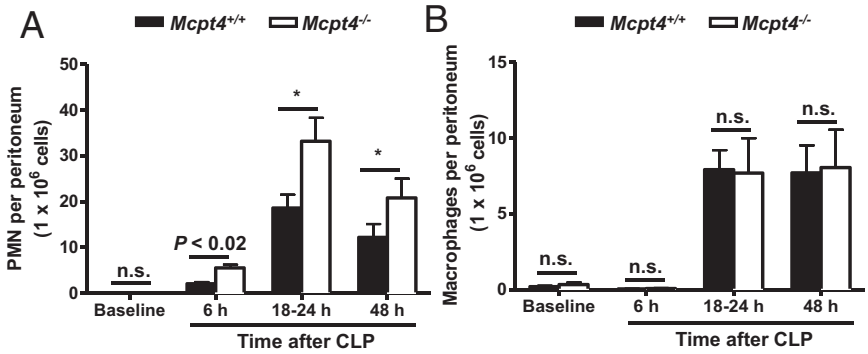


Figure 2. mMCP-4-deficient mice have increased numbers of intraperitoneal neutrophils after CLP. Numbers of neutrophils [polymorphonuclear leukocytes (PMN)] (**A**) and macrophages (**B**) in the live cell population were analyzed by flow cytometry ($Gr\text{-}1^{high}/F4/80^{-}$ cells for PMN and $Gr\text{-}1^{high}/F4/80^{+}$ for macrophages) in the peritoneal lavage fluid at baseline, and at 6, 18 to 24, and 48 hours after CLP, in $Mcpt4^{+/+}$ mice (n = 6 to 15 per group) and $Mcpt4^{-/-}$ mice (n = 5 to 20 per group). Data were pooled from three independent experiments, each of which gave similar results. $^{*}P < 0.05$; n.s. = not significant ($P > 0.05$).

tions we used for quantifying bacterial CFUs,[33] the present findings do not support the hypothesis that mMCP-4 is required for effective bacterial clearance after CLP. Taken together, our observations suggest that an excessive inflammatory response, but not defective bacterial clearance, may contribute to the decreased survival of $Mcpt4^{-/-}$ versus $Mcpt4^{+/+}$ mice after CLP. A similar dissociation between effects on mouse survival and bacterial CFU levels after CLP was observed in the dipeptidyl peptidase I (DPP-I)-deficient mouse, in which mMCP-4 and certain other enzymes remain in their inactive form.[34] In that study, however, the DPP-I-deficient mice had both improved survival and higher levels of bacterial CFUs after CLP, compared with the corresponding WT mice.[34]

mMCP-4-Deficient Mice Have Elevated Levels of TNF after CLP

High levels of TNF are associated with a poor outcome in both mouse and human sepsis.[35,36] We found that the accumulation of neutrophils in $Mcpt4^{-/-}$ mice subjected to CLP was associated with increased intraperitoneal levels of TNF at 6 hours after CLP (Figure 3A). Mediators that are generated at later stages of the septic process, such as IL-6 (which can enhance survival in the CLP model[33]) and IFN-γ, were slightly but not significantly increased in $Mcpt4^{-/-}$ mice at 6, 18 to 24, or 48 hours after CLP (Figure 3, B and C). These observations indicate that mMCP-4 can have direct or indirect effects that result in lower levels of TNF at the initial stages of the bacterial infection caused by CLP.

mMCP-4 Can Down-Regulate TNF Generation by Mast Cells through Degradation of Soluble and Membrane TNF

In vitro studies using mouse recombinant proteases[34] or analyzing the effects of nonspecific protease inhibitors on human mast cells[37,38] indicate that mast cell proteases may function to reduce the levels of certain cytokines and chemokines produced by mast cells. Using BMCMCs from $Mcpt4^{+/+}$ and $Mcpt4^{-/-}$ mice, we assessed the potential influence of mouse chymase on the generation of TNF. After immunological stimulation with IgE and specific antigen, supernatants from $Mcpt4^{-/-}$ BMCMCs had higher amounts of TNF than those from $Mctp4^{+/+}$ BMCMCs (Figure 4A). By contrast, such supernatants exhibited no significant differences in the amounts of IL-6 (see Supplemental Figure S3A at *http://ajp.amjpathol.org*) or in the percentage release of the preformed mediator, β-hexosaminidase (see Supplemental Figure S3B at *http://ajp.amjpathol.org*). These findings indicate either that the absence of mMCP-4 permits mast cells to release larger amounts of TNF and/or that the TNF released by mMCP-4-deficient mast cells persists longer once outside the cells.

Analysis of IgE/DNP-HSA-activated BMCMCs obtained from $Mcpt4^{+/+}$ or $Mcpt4^{-/-}$ mice indicates that chymase does not substantially influence TNF production at the levels of transcription (see Supplemental Figure S4A at *http://ajp.amjpathol.org*) or translation (see Supplemental Figure S4B at *http://ajp.amjpathol.org*). However, as noted above, prior studies using

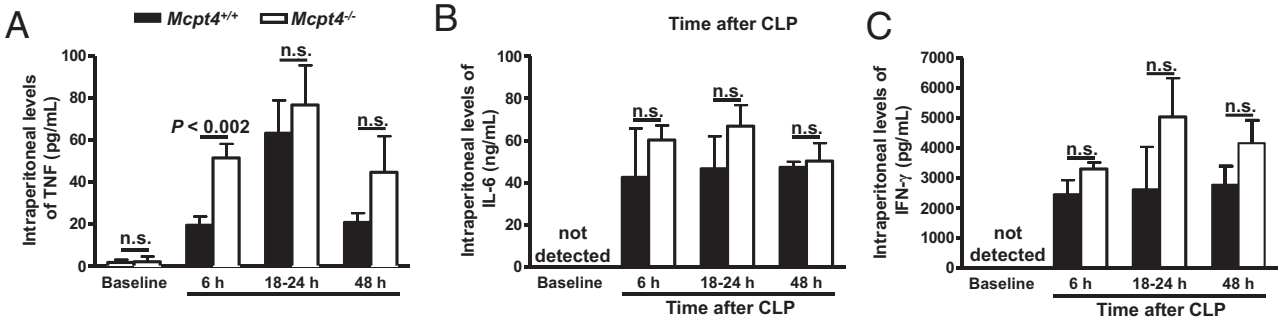


Figure 3. mMCP-4-deficient mice have increased amounts of intraperitoneal TNF after CLP. Amounts of TNF (**A**), IL-6 (**B**), and IFN-γ (**C**) in the peritoneal lavage fluid at baseline, and at 6, 18 to 24, and 48 hours after CLP were quantified in $Mcpt4^{+/+}$ mice (n = 3 to 15 per group) and $Mcpt4^{-/-}$ mice (n = 3 to 20 per group). Data were pooled from three independent experiments, each of which gave similar results. n.s. = not significant ($P > 0.05$).

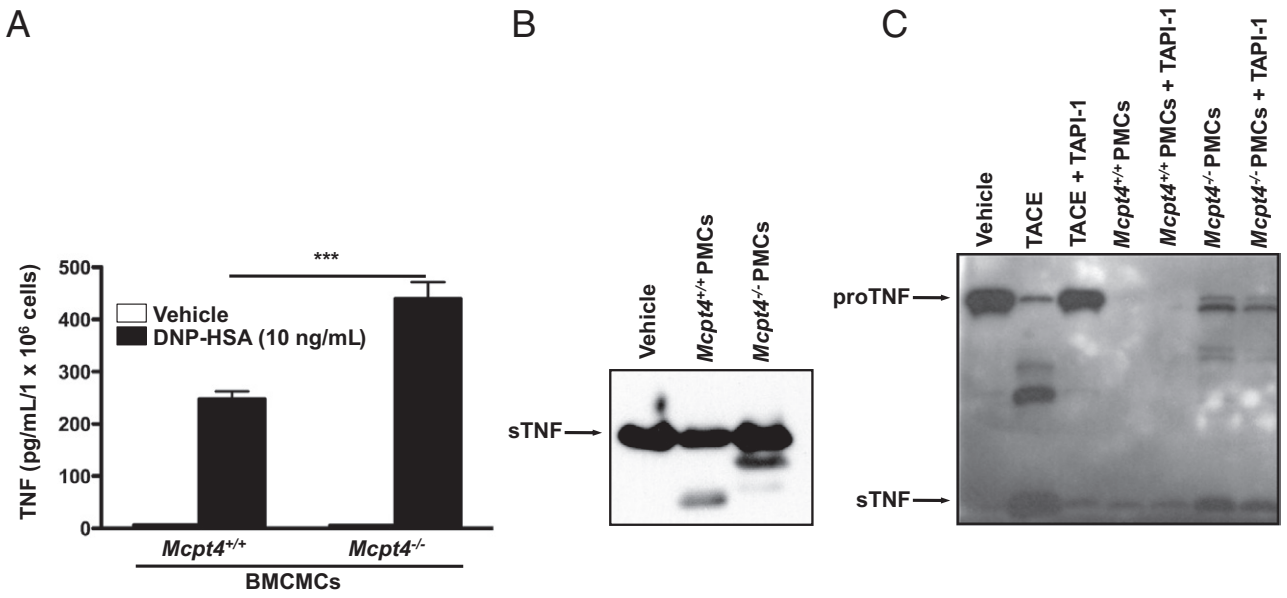


Figure 4. mMCP-4 can limit the amounts of TNF generated by mast cells through degradation of proTNF and sTNF. **A:** TNF production by *Mcpt4*$^{+/+}$ and *Mcpt4*$^{-/-}$ BMCMCs. Cells (1×10^5/100 μL) were sensitized with IgE mAb to DNP (2 μg/mL) overnight at 37°C and then were challenged with DNP-HSA (10 ng/mL) for 18 hours at 37°C. ***$P < 0.001$. **B** and **C:** Lysates of peritoneal mast cells (PMCs) isolated from WT (*Mcpt4*$^{+/+}$) or mMCP-4-deficient (*Mcpt4*$^{-/-}$) mice were incubated with mouse recombinant sTNF (**B**) or proTNF in the presence or absence of an inhibitor of TACE (TAPI-1) (**C**) for 1.5 hours at 37°C and subjected to Western blot analysis. proTNF (**C**) was incubated with mouse recombinant TACE in the presence or absence of TAPI-1 to show that this inhibitor can significantly impair TACE ability to generate sTNF from proTNF under the conditions examined. Data were pooled from three independent experiments, each of which gave similar results (**A**) or are representative of two independent experiments, each of which gave similar results (**B** and **C**). $n = 3$ per group (**A**).

mouse recombinant mast cell proteases and nonspecific protease inhibitors suggested to us that mast cell proteases might be able to degrade certain mast cell-derived cytokines and chemokines.[34,37,38] Based on the reported substrate specificity of chymase, the amino acid sequence of TNF includes potential cleavage sites for mMCP-4.[39] We therefore hypothesized that mMCP-4 can degrade TNF secreted by mast cells.

By Western blot analysis, we found that lysates of PMCs from *Mcpt4*$^{+/+}$ mice caused more extensive degradation of sTNF than did lysates of *Mcpt4*$^{-/-}$ PMCs, indicating that mMCP-4 can degrade sTNF (Figure 4B). TNF is synthesized as a transmembrane protein (proTNF; 26 kDa) and then is cleaved to its soluble form (sTNF; 17 kDa). The TNF-α-converting enzyme (TACE; also known as ADAM17),[40] a member of the ADAM (disintegrin and metalloprotease domain-containing) family of metalloprotease-disintegrins,[41] can catalyze the conversion of proTNF to sTNF. This function was markedly reduced in the presence of the TACE inhibitor TAPI-1 (Figure 4C). Lysates of PMCs from *Mcpt4*$^{-/-}$ mice were impaired in their ability to degrade proTNF, and similar results were obtained in the presence or absence of TAPI-1 (Figure 4C). These findings indicate that *Mcpt4*$^{+/+}$ and *Mcpt4*$^{-/-}$ PMC lysates can convert proTNF to sTNF in the absence of TACE activity, but that *Mcpt4*$^{+/+}$ PMC lysates can do this, and can degrade sTNF, more efficiently than lysates of *Mcpt4*$^{-/-}$ PMCs (Figure 4C). These findings indicate that mMCP-4 can down-regulate the levels of TNF detected in mouse mast cell supernatants by degradation of both proTNF and sTNF.

The Increased Mortality after CLP Associated with a Lack of mMCP-4 Is Reduced in Mice Whose Mast Cells Lack TNF

Mast cell granules contain preformed, bioactive TNF that is available for rapid release in response to a triggering event,[42] which makes mast cells a potentially important early source of TNF in sepsis. Galli and colleagues,[13] using a mast cell knock-in approach,[43] found that a beneficial role of mast cells in CLP of moderate severity can occur independently of mast cell-derived TNF; moreover, their data suggested that production of large amounts of mast cell-derived TNF can impair survival after severe CLP by promoting a dysregulated inflammatory response associated with increased numbers of intraperitoneal neutrophils.

To investigate possible interactions between mast cell-derived TNF and mast cell mMCP-4 in CLP, we engrafted various mast cell populations into genetically mast cell-deficient C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ (*Kit*$^{W\text{-}sh/W\text{-}sh}$) mice. Specifically, we prepared mast cell-engrafted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice whose PMCs were either WT (*Mcpt4*$^{+/+}$) or lacked the ability to produce mMCP-4 (*Mcpt4*$^{-/-}$), or TNF (*Tnf*$^{-/-}$), or both mMCP-4 and TNF (*Mcpt4*$^{-/-}$*Tnf*$^{-/-}$). Survival after CLP was significantly improved in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice that had been selectively engrafted intraperitoneally with *Mcpt4*$^{-/-}$*Tnf*$^{-/-}$ BMCMCs than in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice that had been engrafted intraperitoneally with *Mcpt4*$^{-/-}$ BMCMCs (33% versus 8% survival at day 7 after CLP, respectively; $P < 0.005$) (Figure 5A). Moreover, survival after CLP was also improved in *Mcpt4*$^{+/+}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, compared with *Mcpt4*$^{-/-}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (45%

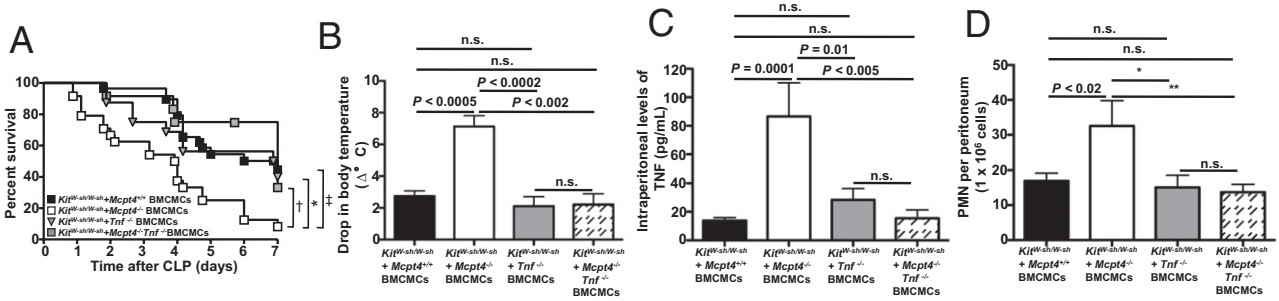


Figure 5. In the absence of mMCP-4, mast cell-derived TNF can contribute to increased amounts of intraperitoneal TNF and numbers of peritoneal neutrophils, and decreased survival, after CLP. **A:** Survival after CLP (50% ligation; single puncture with a 22-gauge needle) in *Mcpt4*$^{+/+}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ ($n = 19$), *Mcpt4*$^{-/-}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ ($n = 24$), *Tnf*$^{-/-}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ ($n = 12$), and *Mcpt4*$^{-/-}$ *Tnf*$^{-/-}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ ($n = 12$). Survival for *Mcpt4*$^{-/-}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice was significantly reduced, compared with each of the other three types. $^{*}P < 0.02$; $^{\dagger}P < 0.005$; $^{\ddagger}P < 0.0005$. **B–D:** Change in rectal temperature (**B**), amounts of TNF (**C**), and numbers of neutrophils [polymorphonuclear leukocytes (PMN) in the live cell population analyzed by flow cytometry; Gr-1^{high}/F4/80^{-} cells] (**D**) in the peritoneal lavage fluid at 18 to 24 hours after CLP in *Mcpt4*$^{+/+}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ ($n = 10$ or 11), *Mcpt4*$^{-/-}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ ($n = 10$ or 11), *Tnf*$^{-/-}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ ($n = 8$ to 10), and *Mcpt4*$^{-/-}$ *Tnf*$^{-/-}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ ($n = 5$ to 7). Data were pooled from three independent experiments, each of which gave similar results. $^{*}P < 0.05$, $^{**}P < 0.01$; n.s., not significant ($P > 0.05$).

versus 8% survival at day 7 after CLP) ($P < 0.0005$). However, survival in *Mcpt4*$^{+/+}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (45% at day 7 after CLP) did not differ significantly different from that in *Mcpt4*$^{-/-}$*Tnf*$^{-/-}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (33% at day 7 after CLP) ($P = 0.97$) or in *Tnf*$^{-/-}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (40% at day 7 after CLP) ($P = 0.38$).

We also assessed features of inflammation in these groups of mice at 18 to 24 hours after CLP. We chose this time point because *Mcpt4*$^{-/-}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice exhibited a significant drop in body temperature 18 to 24 hours after CLP, compared with the other groups of mice (Figure 5B), which correlated with the increased mortality of *Mcpt4*$^{-/-}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (Figure 5A). At 18 to 24 hours after CLP, the three groups with the best survival after CLP (ie, *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice engrafted with *Mcpt4*$^{+/+}$, *Tnf*$^{-/-}$, or *Mcpt4*$^{-/-}$*Tnf*$^{-/-}$ BMCMCs) (Figure 5A) exhibited significantly lower amounts of TNF (Figure 5C) and numbers of neutrophils (Figure 5D) in the peritoneal cavity, compared with *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice engrafted with *Mcpt4*$^{-/-}$ BMCMCs. Moreover, when the engrafted mast cells contained mMCP-4, the presence or absence of mast cell-derived TNF made no significant difference in intraperitoneal levels of TNF or in neutrophil numbers in mast cell-engrafted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (Figure 5, C and D). This finding suggests that mast cell-derived TNF does not represent a major contributor to the TNF levels measured in this model of CLP at 18 to 24 hours after the procedure in mice with normal levels of mast cell mMCP-4.

Discussion

Of the various chymases expressed by mouse mast cells, mMCP-4, which has been recognized as the functional homolog of human chymase, is the major source of chymotrypsin-like activity in the mouse peritoneal cavity,[22] the site where bacterial infection is initiated during CLP. Our studies of mMCP-4-deficient mice indicate that mMCP-4 can help to reduce or prevent the development of ARF, as well as markedly improve survival, in mice subjected to CLP (Figure 1). Notably, mMCP-4 is not detected in total kidney tissue, likely reflecting a general paucity of mast cells in the kidney.[44] Indeed, Toluidine Blue staining of kidney sections from *Mcpt4*$^{+/+}$ and *Mcpt4*$^{-/-}$ mice subjected to CLP revealed no mast cells. These observations indicate that the chymase-dependent preservation of kidney function after CLP does not require that mMCP-4 be generated within the kidney, but instead appears to reflect effects of mast cell activation and release of mMCP-4 outside of the kidney.

Notably, although the CLP model used in the present study was associated with ARF, it induced only patchy morphological evidence of kidney injury. It is well known in clinical medicine that assays of renal function (ie, serum levels of electrolytes, BUN, creatinine) may detect profound renal dysfunction that cannot be appreciated under light microscopy.[45] In this respect, CLP-associated ARF may represent a form of AKI in mice that is more subtle (at least from the standpoint of kidney histopathology), and therefore more similar to clinical AKI in humans, than that associated with other models.[46,47]

We do not know the mechanism by which a lack of mMCP-4 contributes to kidney dysfunction in CLP. However, it is known that the development of ARF in sepsis is associated with increased amounts of plasma angiotensin II,[48] and we found substantially increased levels of plasma angiotensin II after CLP in *Mcpt4*$^{-/-}$ mice, compared with *Mcpt4*$^{+/+}$ mice (see Supplemental Figure S5 at *http://ajp.amjpathol.org*). *In vitro* studies indicate that mouse chymase can enhance both the formation and degradation of angiotensin II.[28,49] We found that, compared with the corresponding WT mice, naïve *Mcpt4*$^{-/-}$ mice (like naïve genetically mast cell-deficient WBB6F$_1$-*Kit*$^{W/W\text{-}v}$ mice[28]) exhibited reduced formation of angiotensin II in aortic tissues: 9.4 ± 3.5 pg/mL of angiotensin II/mg of protein contained in aortic tissues from *Mcpt4*$^{+/+}$, compared with <1.5 pg/mL for *Mcpt4*$^{-/-}$ mice ($P = 0.06$, $n =$ 3 per group). It is possible, therefore, that the increased plasma levels of angiotensin II in septic *Mcpt4*$^{-/-}$ mice reflect either reduced degradation of angiotensin II in the absence of mMCP-4 and/or the robust activation of the renin-angiotensin-aldosterone system as part of an intrinsic response to sepsis-associated circulatory failure in these animals.[50]

Overall, our evidence supports the hypothesis that mMCP-4 exerts a novel protective effect in mice subjected to CLP by restraining the development of an excessive inflammatory response that contributes to kidney dysfunction and mortality in the septic mice. In contrast to *Mcpt4*$^{-/-}$ mice, DPP-I knockout mice (*Ctsc*$^{-/-}$) that express an inactive form of chymase (and certain other proteases), exhibited better survival rates after CLP than did WT mice.[34] The discrepancy between our findings with *Mcpt4*$^{-/-}$ mice and those reported for DPP-I knockout mice may be related to two potentially important differences between the two studies. First, in addition to being required for the activation of chymase, DPP-I is also required for the activation of several other serine proteases,[34] some of which might have negative effects on survival in sepsis that counteract the positive effects of chymase. Second, the DPP-I knockout mice were subjected to a more severe model of CLP than we used for analyses of the *Mcpt4*$^{-/-}$ versus *Mcpt4*$^{+/+}$ mice, in that nearly 80% of the WT mice died by 48 hours after CLP in the study of DPP-I knockout versus WT mice, as opposed to only 29% of the WT mice in the present study. CLP models of different severities could result in the generation of different profiles and/or amounts of substrates available for proteases, contributing to different outcomes during sepsis.

We found that mMCP-4 can limit the magnitude of the neutrophil response triggered by CLP (Figure 2A). The reduction in neutrophil numbers in WT mice, compared with mMCP-4-deficient mice, was associated with lower levels of TNF in the peritoneal cavity during CLP in the WT mice (Figures 3A and 5C). Neutrophils can produce TNF and can contribute to mortality in the lipopolysaccharide-induced model of sepsis.[51] It is possible, therefore, that mMCP-4 can down-regulate TNF levels indirectly, through effects that result in limiting the number of TNF-producing neutrophils recruited to the peritoneal cavity during sepsis. However, our *in vitro* data indicate that mMCP-4 can also degrade both proTNF and sTNF (Figure 4, B and C), and these findings are consistent with the possibility that degradation of proTNF and sTNF by mMCP-4 may limit the levels and biological effects of TNF *in vivo*. In fact, our *in vivo* data comparing the features of CLP in mice whose adoptively transferred mast cells have TNF but do or do not contain mMCP-4 indicate that mast cell-derived TNF can impair survival and can promote inflammation in this model of CLP if the mast cells lack chymase (Figure 5). Thus, it is possible that mMCP-4 can limit levels of TNF during CLP by two distinct mechanisms: degrading proTNF and sTNF from whatever sources, and having direct or indirect effects that limit neutrophil numbers in this setting and that therefore limit levels of neutrophil-derived TNF.

In the present study, we focused on interactions between mMCP-4 and TNF. However, based on the substrate specificity of chymase, many other mediators generated during sepsis may represent additional potential substrates of mMCP-4. For example, potential chymase cleavage sites are present in mouse IL-6,[39] but *in vitro* studies indicate that mMCP-4 is unable to degrade this cytokine.[34] Moreover, we did not detect significant differences in the amounts of IL-6 and IFN-γ generated in the presence or absence of chymase after CLP (Figure 3B). Unlike IL-6 and IFN-γ, TNF is one of the earliest mediators to be generated during the host response to CLP,[52] suggesting that TNF may be more available than IL-6 as a substrate for chymase when the protease is rapidly released after mast cell activation early in the reaction to CLP.[53]

It is important to point out that the newly identified beneficial role for mMCP-4 as a negative regulator of inflammation in the CLP model differs substantially from the roles for this enzyme that have been discovered in models of abdominal aortic aneurysm,[54] glomerulonephritis,[44] or arthritis,[55] in which mMCP-4 seems to increase inflammation. It was recently shown, however, that mMCP-4 can reduce airway inflammation and airway hyperreactivity in a mouse model of allergic airway inflammation, findings consistent with an anti-inflammatory role for mMCP-4 in this setting.[56] Such divergent roles for mMCP-4 may reflect differences in the types and/or availability of endogenous chymase substrates in these conditions, about which little is known as yet. Indeed, for mMCP-4, as well as for other proteases, characterizing in detail the profile of endogenous protease substrates present in different physiological or pathological settings will be essential to extend our understanding of the functions of these proteases in such conditions.[57]

Acknowledgments

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