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Mouse Mast Cell Protease-4 Deteriorates Renal Function by Contributing to Inflammation and Fibrosis in Immune Complex-Mediated Glomerulonephritis

Lisa Scandizzi,*†§ Walid Beghdadi,*† Eric Daugas,*†‡ Magnus Åbrink,§ Neeraj Tiwari,*† Cristiana Brochetta,*† Julien Claver,*† Nassim Arouche,†,*/details§ Xingxing Zang,|| Marina Pretolani,†,* Renato C. Monteiro,*† Gunnar Pejler,§ and Ulrich Blank*†

Mast cells exert protective effects in experimental antiglomerular basement membrane-induced glomerulonephritis (GN), yet the responsible mediators have not been identified. In this study, we investigated the role of mouse mast cell protease (mMCP)-4, the functional homolog of human chymase, using mMCP-4–deficient mice. Compared with wild type animals, mMCP-4–deficient mice exhibited lower proteinuria, blood creatinine, and blood urea nitrogen levels, indicating an aggravating role of mMCP-4. Kidney histology confirmed less severe renal damage in mMCP-4–deficient mice with reduced deposits, glomerular and interstitial cellularity, and fibrosis scores. High amounts of mMCP-4 were detected in renal capsules, but not in the whole kidney, from wild type mice. Its expression in renal capsules was markedly decreased after GN induction, suggesting that locally released enzyme by degranulated mast cells could contribute to the functional and physiopathological hallmarks of GN. Supporting a proinflammatory role, glomerular and interstitial macrophage and T cell infiltration, levels of proinflammatory TNF and MCP-1 mRNA, and the expression of the profibrotic peptide angiotensin II together with type I collagen were markedly downregulated in kidneys of mMCP-4–deficient mice. We conclude that mMCP-4 chymase, contrary to the global anti-inflammatory action of mast cells, aggravates GN by promoting kidney inflammation. These results highlight the complexity of mast cell-mediated inflammatory actions and suggest that chymase inhibition may represent a novel therapeutic target in GN. The Journal of Immunology, 2010, 185: 624–633.

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*Institut National de la Santé et de la Recherche Médicale U699; †Université Paris 7, Faculté de Médecine Paris Diderot-Site Xavier Bichat; ‡Service de Néphrologie, Groupe Hospitalier Universitaire Bichat-Claude Bernard, Assistance Publique-Hôpitaux de Paris; §Institut National de la Santé et de la Recherche Médicale U700, Paris, France; ‡Department of Medical Biochemistry and Microbiology, Uppsala University; §Department of Anatomy, Physiology and Biochemistry, Swedish University of Agricultural Sciences, Uppsala, Sweden; and †Department of Microbiology and Immunology, Albert Einstein College of Medicine, New York, NY 10461

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Address correspondence and reprint requests to Dr. Ulrich Blank, Institut National de la Santé et de la Recherche Médicale U699, Université Paris 7, Faculté de Médecine Denis Diderot, Site Xavier Bichat, 16 Rue Henri Huchard, 75780 Paris Cedex 18, France. E-mail address: ulrich.blank@inserm.fr

Abbreviations used in this paper: ACE, angiotensin-converting enzyme; Ang, angiotensin; anti-GBM, antiglomerular basement membrane; BMDM, bone marrow-derived macrophage; BMMC, bone marrow-derived mast cell; BUN, blood urea nitrogen; DTH, delayed-type hypersensitivity; GN, glomerulonephritis; mMCP, mouse mast cell protease; NRS, normal rabbit serum; PAS, periodic acid-Schiff; RT, room temperature; SA, streptavidin; WT, wild type.
mast cells promoted glomerular expression of adhesion molecules and enhanced Th1-dependent effector mechanisms. Overall, these observations indicate that mast cells can exert beneficial or detrimental effects, depending on a given pathological context. Therefore, further studies are needed to clarify the mechanisms by which mast cells affect renal disease parameters.

The murine counterpart of human mast cell chymase, mMCP-4, was shown to attract granulocytes and macrophages (17, 18), to regulate homeostatic intestinal epithelial migration and barrier function (19), and to convert Ang I into Ang II, a vasoactive peptide with potent inflammatory and fibrogenic properties (20, 21). These observations suggest that mMCP-4 may play an important role in tissue inflammation and remodeling in the kidney. To explore this issue, we examined the role of mMCP-4 in the accelerated model of anti-GBM-induced GN using mMCP-4–deficient mice. We show that these mice develop less severe disease compared with their wild type (WT) counterparts. This effect involved the ability of mMCP-4 to mediate inflammatory and fibrotic processes in the kidney.

Materials and Methods

Induction of anti-GBM–induced GN

Anti-GBM serum was produced in rabbits following immunization with purified mouse glomeruli obtained according to established procedures (22). An accelerated model of anti-GBM Ab-induced GN was used (23, 24). Briefly, mice were preimmunized i.p. with normal rabbit IgG (0.5 mg/20 g body weight) (Southern Biotechnology Associates, Birmingham, AL) and CFA (Sigma-Aldrich, St. Louis, MO) 5 d before an i.v. administration of anti-GBM serum through the retro-orbital vein at a dose of 0.2 ml/20 g. For control purposes, NRS was also used. Mice were sacrificed at day 3 to evaluate early changes of the heterologous phase following deposition of rabbit anti-GBM or at day 14 to evaluate late changes provoked by the developing immune response to the injected Abs. No sign of GN was observed when NRS was administered into WT and mMCP-4–deficient mice. Perfused kidneys were fixed in 10% formalin and embedded in paraffin. Some portions of the kidneys were also frozen in Tissue-Tek OCT (Polysciences, Warrington, PA) compound for immunohistochemical and immunofluorescence analysis. Other portions were lysed in RA1 buffer (Nucleospin, Macherey-Nagel, Hoerdt, France) for quantitative RT-PCR experiments or in buffer for protein analysis, as described below. Experimental groups included male mMCP-4–deficient mice and their WT littermates between weeks 7 and 10 wk of age. All experiments were approved by a local ethics committee.

Mouse strains and reconstitution experiments

Previously generated mMCP-4 knockout mice (25) were backcrossed into the C57BL/6J background, shipped from Uppsala, and a founder colony was independently established and housed under strictly controlled pathogen-free conditions at the mouse facilities of IFR02 at the Bichat Medical School. Homozygous mMCP-4–deficient and WT mice were screened by PCR using the following primers: forward primer 5′-GGGCCAGCTCA-3′, reverse primer 5′-GGTGATCTATCTCCAGATGGGCCATGTAAGGGCG-3′, and second reverse primer 5′-GGTGATCTATCTCCAGATGGGCCATGTAAGGGCG-3′. They yielded a 380-bp and a 967-bp fragment for knockout and WT genes, respectively. Amplification was performed overnight with shuttle PCR using TaKaRa enzyme (Takara Bio, Shiga, Japan), with annealing and melting temperatures of 68˚C and 98˚C. C57BL/6-Tg(OT-II)-RAG1 tm1Mom mice were bred in the C57BL/6J background, shipped from Uppsala, and a founder colony was approved by a local ethics committee.

Evaluation of systolic blood pressure

Systolic blood pressure of WT and mMCP-4–deficient mice (n = 6) before and after induction of anti-GBM GN was measured by the tail-cuff method, adapted to the mouse using Chart 4.1.1 software (ADInstruments, Chalgrove, U.K.) (26). To avoid variations in blood pressure that occur during the day cycle, all measurements were carried out between 10 AM and 12 PM. Ten measurements from each mouse were taken on three consecutive days.

Immunoblotting

Tissue lysates were prepared in 50 mM Tris-HCl (pH 8) containing 1% SDS and 5% glycerol. Lysates were migrated on a 12% SDS-PAGE, transferred onto nitrocellulose membrane (Schleicher & Schuell Microscience, Dassel, Germany), as described (27). Membranes were blocked with 4% BSA for 1 h, followed by incubation for 1 h at room temperature (RT) with the following primary Abs: a rabbit anti-serum to mMCP-4 (a kind gift from Lars Hellman, Uppsala University) (28), mouse mAb JRK directed to the β-chain of the high-affinity IgE R, and a mouse mAb anti-β-actin (Sigma-Aldrich). After several washes, blots were incubated with donkey anti-rabbit IgG HRP (1/30,000) or goat anti-mouse IgG HRP (1/20,000) (Jackson ImmunoResearch Laboratories, Newmarket, U.K.) for 45 min and were developed by ECL (GE, Paris, France).

Cell cultures

Bone marrow-derived macrophages (BMDMs) were obtained by isolating bone marrow cells from femurs and tibias of C57BL/6J mice, as described (30). Cells were resuspended at a concentration of 1 × 10⁶ cells/ml in BMDM complete medium (DMEM/Glutamax; BioWhittaker, Walkersville, MD), supplemented with 10% FCS, 10% L cell-conditioned medium (as a source of M-CSF-1), 100 U/ml penicillin G, and 100 μg/ml streptomycin (Life Technologies, Rockville, MD), supplemented with 15% FCS, 10% L cell-conditioned medium (as a source of M-CSF-1), 100 U/ml penicillin G, and 100 μg/ml streptomycin (Life Technologies, Rockville, MD) and cultured at 37˚C in a humidified 5% CO₂ incubator in 75-ml flasks. After 24 h, the nonadherent cells were removed, counted, plated on bacteriologic (nontissue cultured) plastic plates at a concentration of 1 × 10⁶ cells/ml, and cultured in BMDM complete medium that was replaced every 3 d. BMDMs were used between days 7 and 8.

BMMCs were obtained by isolating bone marrow precursors from femurs and tibias of WT and mMCP-4–deficient mice, as described (31). Cells were grown at 37˚C in a humidified 5% CO₂ incubator in complete BMMC medium (IMDM, 15% FCS, 25 mM HEPES [pH 7.4], 1 mM sodium pyruvate, 1% nonessential amino acid, 54 μM 2-ME with 100 U/ml penicillin, and 100 μg/ml streptomycin) (Invitrogen, Cergy Pontoise, France) containing recombinant murine IL-3 and stem cell factor (10 ng/ml for each cytokine) (PeproTech, Paris, France) for quantitative RT-PCR experiments or in buffer for protein analysis, as described below. Experimental groups included male mMCP-4–deficient mice and their WT littermates between weeks 7 and 10 wk of age. All experiments were approved by a local ethics committee.
Assessment of humoral and delayed-type hypersensitivity immune responses

Sera from mice were collected at indicated days before and after the induction of anti-GBM GN. Sera from mice injected with NRS were used as a control. To detect normal rabbit IgG in serum, 96-well plates were coated overnight with Fab(½)1 goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1 μg/ml in PBS (100 μl/well). As a standard, wells were incubated with defined concentrations of rabbit IgG (Southern Biotechnology Associates). After washing in 0.05% Tween-20 in PBS (pH 7.2), serum samples (100 μl/well) diluted in PBS/1% BSA were added for 1 h at RT. After washing, biotinylated donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories) (1/100 in PBS/1% BSA) was added for 2 h at RT before the final addition of streptavidin-SA-HRP diluted 1/200 (R&D Systems, Minneapolis, MN) for 20 min at RT. ELISA was developed by the addition of the substrate, and the reaction was stopped by adding 1 N HCl. The OD of each well was determined using a microplate reader set at 450 nm. To detect normal rabbit IgG in serum, 96-well plates were incubated overnight with 100 μl/well rabbit IgG (Southern Biotechnology Associates) at a final concentration of 1 μg/ml in PBS. After washing, serum samples (100 μl/well) diluted in PBS/1% BSA were added for 1 h at RT. After washing, biotinylated donkey anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories) (1/200) was added for 20 min at RT. ELISAs were developed as described above.

To evaluate dermal delayed-type hypersensitivity (DTH) to rabbit Ig, mice were challenged, 13 d after induction of GN, by injecting 250 μg normal rabbit control mouse IgG into the right and left hind footpad, respectively. The swelling response was determined at 24 h using a micrometer. Hypersensitivity reaction was determined as the difference in swelling response between the right and left footpads.

Evaluation of proteinuria, blood urea nitrogen, and creatinine levels

Spot samples of urine were collected at indicated days. Urinary protein concentrations (g/l) were normalized to urinary creatinine concentrations (mmol/l) by calculating the ratio of urinary protein per urinary creatinine. Proteinuria, blood urea nitrogen (BUN), and blood or urinary creatinine levels were determined using commercially available kits (Olympus Diagnostics, Hamburg, Germany) by means of an autoanalyzer (AU400; Olympus Diagnostics), as described (32).

Histological and immunohistochemical analysis

To assess histological changes by light microscopy, tissue sections were stained with periodic acid-Schiff (PAS) reagent and Masson’s trichrome. They were counted using a C-mount. Deposits appear as pink- and green-stained areas for PAS and Masson’s trichrome staining, respectively, which contrast with adjacent glomerular structures. Glomerular deposits were scored in a minimum of 30 glomeruli per mouse as follows: 0 = no deposition of Masson’s trichrome; 1 = up to one third of the cross-sectional area of the glomerulus is Masson’s trichrome; 2 = one third to two thirds of the cross-sectional area of the glomerulus is Masson’s trichrome; and 3 = more than two thirds of the cross-sectional area of the glomerulus is Masson’s trichrome. Glomerular proliferation, interstitial infiltration, interstitial fibrosis, and tubular necrosis were quantified using a semiquantitative score (0–3): 0 = absence of lesions, 1 < 25%, 2 = 25–50%, and 3 > 50% of lesions.

Mast cells in indicated tissues were evaluated after fixation in 10% formalin and staining with toluidine blue. They were counted using 3–6 objective) per experimental condition. Frozen kidney sections were also stained with Texas red goat anti-rabbit IgG or FITC goat anti-mouse IgG (Southern Biotechnology Associates) to evaluate heterologous rabbit or autologous mouse anti-rabbit Abs deposited in the glomeruli, respectively, using immunofluorescence light microscopy. Fibrin deposition was determined using direct staining with FITC-labeled antifibrin Ab (Nordic Immunological Laboratories, Tilburg, The Netherlands). Type I collagen and Ang II accumulation were evaluated, respectively, using a goat anti-type I collagen plus FITC-labeled donkey anti-goat Ab (Southern Biotechnology Associates) and a guinea pig anti-Ang II Ab (Peninsula Laboratories, Weil am Rhein, Germany), followed by staining with tetramethylrhodamine isothiocyanate-labeled donkey anti-guinea pig Ab (Jackson ImmunoResearch Laboratories) for 30 min. Quantitative analysis of images was performed on a minimum of 30 high-power fields using NIH ImageJ software (National Institutes of Health, Bethesda, MD).

Transwell cell-migration assay

Chemotaxis of BMDMs and CD4+ T cells was measured by assessing migration through a polycarbonate filter with 8 μm and 5 μm pore size, respectively, in a 24-well microchemotaxis chamber (3422; Corning Costar, Cambridge, MA). One hundred microliters BMDM cell suspension (1 × 106 cells/ml resuspended in IMDM medium, 5% FCS, 15% L cell-conditioned medium, 100 U/ml penicillin, and 100 μg/ml streptomycin) or

FIGURE 1. Expression of mMCP-4 in tissues of WT and mMCP-4-deficient (−/−) mice. Lysates (20 μg/lane) of indicated tissues were migrated on SDS-PAGE and analyzed by immunoblotting using anti-mMCP-4 Ab. For loading controls, blots were probed with anti-β-actin Ab.

FIGURE 2. Mice deficient in mMCP-4 have less decline in renal function. WT mice (n = 9), mMCP-4−−/− mice (n = 13) (A), or in a separate experiment, mast cell-deficient KitW−/KitW− mice reconstituted with WT BMMCs (n = 6) or mMCP4−−/− BMMCs (n = 6) (D) were subjected to an accelerated model of anti-GBM–induced GN. The ratio of urinary protein to urinary creatinine was determined. WT mice (n = 3 at day 3 and n = 9 at day 14), mMCP-4−−/− mice (n = 3 at day 3 and n = 13 at day 14) (B, C), or in a separate experiment, mast-cell-deficient mice reconstituted with WT BMMCs (n = 6) or mMCP-4-deficient BMMCs (n = 6) (E, F) were subjected to an accelerated model of anti-GBM–induced GN. Blood creatinine and BUN were measured after induction of anti-GBM–induced GN on the days indicated. Data are mean ± SEM of urine or blood samples in each group. *p < 0.05.
Spleen cells freshly isolated (1 × 10⁶ cells/ml) in IMDM medium, 5% FCS, 25 mM HEPES [pH 7.4], 1 mM sodium pyruvate, 1% nonessential amino acid, 54 μM 2-ME with 100 U/ml penicillin, and 100 μg/ml streptomycin) from C57BL/6-Tg(OT-II)-RAG1tm1Mom mice were added to the upper chamber. Anti-IgE-sensitized BMMCs (1 × 10⁶ cell/ml) derived from WT and mMCP-4–deficient mice were stimulated for 30 min with DNP-HSA (10 ng/ml) and 600 μl supernatant was added to the lower chamber (in duplicate). One hundred nanograms per milliliter of murine MCP-1 (R&D Systems) in 5% serum complete BMMC medium and anti-DNP IgE-sensitized BMMCs (1 × 10⁶ cell/ml), stimulated for 2 h with DNP-HSA (10 ng/ml), were used as positive control in separate lower chambers for the macrophage and for the T cell chemotaxis experiments, respectively. Chambers were incubated at 37°C and 5% CO₂ for indicated times. At the end of the incubation period, the total cell number in the lower chamber were determined by staining with specific Abs and subsequent FACs analysis. The chemotaxis index for BMDMs or CD4⁺ T cells migrated toward the CD4⁺ T cells migrated toward the lower chamber were determined by staining with specific Abs and subsequent FACs analysis. Flow cytometry for BMMCs or CD4⁺ T cells was determined as the ratio of migrated cells per well/cells migrated to wells containing medium from unstimulated BMMCs.

Flow cytometry

To evaluate the purity of BMMCs, cells were washed in FACS buffer (ice-cold 0.5% BSA in PBS) and stained with rat anti-mouse c-Kit–biotin and hamster anti-mouse FcRII-biotin Abs (both from eBioscience, San Diego, CA), followed by SA-PE (Southern Biotechnology Associates). For T cell chemotaxis experiments, migrated cells in the lower chamber were washed in FACS buffer and stained with anti-CD4 (L3T4) conjugated to allophycocyanin (eBioscience). To prevent unspecific binding, all samples were preincubated with Fc-Block or unlabeled isotype-matched unspecific Abs (eBioscience). Data were acquired using a FACS Calibur flow cytometer (BD Biosciences) according to standard protocols.

RT-PCR and real-time PCR

RNA was isolated from the cortex region of kidney of anti-GBM–treated mMCP-4–deficient and WT mice using Nucleospin purification system (Macherey-Nagel). Kidneys from mice injected with NRS were used as a control. The first-strand cDNA was synthesized from 2 μg total RNA using reverse transcriptase and 1 μmol/d oligonucleotide-dT primer. Each cDNA sample was amplified by using specific primers to detect MCP-1 (sense 5'-GGCTCACGGCATGCATTAAA-3' and antisense 5'-CTTACTCATTTGGATCATCTTGCT-3'), TNF (sense 5'-CTGTCCTACGTTAAGCAGCTT-3' and antisense 5'-GGTCTGGCCCATATAAGTGG-3'), IL-1β (sense 5'-CTGTTCTTCCCTGGAGGC-3' and antisense 5'-CAAGCTATATGTCCTGCA-3'), and ribosomal protein L13a (RPL13a) (sense 5'-GTTGGCCCTGTGACT-3' and antisense 5'-CGATAGTGACTTGGCCCTTTT-3'). Real-time PCR was performed in SYBR Green I dye (Sigma-Aldrich). Measurements of the threshold cycles were made at the end of each extension step by the second-derivative method, using LightCycler software, version 4.0 (Stratagene, La Jolla, CA). Melting-curve analysis was performed to validate the identity of peaks of interest in all samples. Absolute quantification of the number of copies was calculated by determining half-maximal fluorescence values and comparing them to an internal standard of amplification with a known number of copies.

Data analysis

Data are expressed as mean ± SEM of the indicated number of experiments. Statistical analysis was performed using an unpaired Student t test; values of p < 0.05 were considered significant.

Results

mMCP-4 deficiency reduces renal disease in anti-GBM–induced GN

The expression of mMCP-4 was assessed by immunoblotting in kidney, ear, and renal capsule lysates from WT and mMCP-4–deficient mice (Fig. 1). Although mMCP-4 was prominent in ear and renal capsule, no mMCP-4 was detected in total kidney tissue, likely reflecting the relative paucity of mast cells in the kidney (12). As expected, mMCP-4 was absent in tissues from mMCP-4–deficient animals.

The role of mMCP-4 in anti-GBM–induced GN was examined in WT and mMCP-4–deficient mice using an accelerated model

FIGURE 3. Mice deficient in mMCP-4 exhibit less deterioration in kidney morphology. A, Representative histological analysis of Masson’s trichrome- (1,3,4) and PAS- (2) stained sections of anti-GBM kidneys in the late autologous phase (day 14) of WT and mMCP-4–/– mice, as well as WT control mice that had received NRS (original magnifications; panel 1, 10× objective; panel 2, 100× objective; panels 3, 4, 40× objective). Less extensive histological lesions are seen in all of the kidney compartments from mMCP-4–/– mice compared with WT mice. Progression of glomerular injury is characterized by subendothelial deposits (arrows in panel 2 and left arrow in panel 3), intraglomerular hypercellularity (lower right arrow in panel 2), tubular necrosis (arrows in panel 4) and mononuclear cell infiltrate in the interstitium (left pictures, right arrow on panel 3 and panel 4). B, Scoring of PAS+ and Masson’s trichrome+ material at day 14 in WT (n = 9) or mMCP-4–/– mice (n = 13) after injection of anti-GBM Ab. ∗∗p < 0.05; ∗∗∗p < 0.02. C, Scoring of Masson’s trichrome+ material at day 14 after injection of anti-GBM Ab of mast cell-deficient Kit⁺/Kit⁻ mice reconstituted with WT BMMCs (n = 6) or mMCP-4–deficient BMMCs (n = 6) subjected to an accelerated model of anti-GBM–induced GN in separate experiments. Data are mean ± SEM of urine or blood samples in each group.
(23, 24). Both mouse strains developed marked proteinuria and showed high levels of blood creatinine and BUN following the injection of anti-GBM serum (Fig. 2A–C). However, these parameters were significantly reduced in mMCP-4–deficient mice, in particular at the late (autologous) phase of the disease (days 10–14), whereas no differences in proteinuria (Fig. 2A) were noted at the time of the early (heterologous) phase (day 3). Both mouse strains injected with NRS failed to develop functional features of GN (data not shown). Together, these results demonstrated that mMCP-4 contributed to GN progression. Although mMCP-4 is a mast cell-specific enzyme, its absence may lead indirectly to developmental defects or affect other cell types, which could contribute to GN progression (33). To examine this possibility, mast cell-deficient Kit<sup>W-sh</sup>/Kit<sup>W-sh</sup> mice were reconstituted with BMMCs obtained from WT or mMCP-4–deficient mice for 12 wk before inducing anti-GBM GN. In agreement with the above studies, mice reconstituted with mMCP-4–deficient mast cells showed a tendency to lower proteinuria and lower blood creatinine levels and had significantly decreased BUN (Fig. 3B). Results in age-matched WT and mMCP-4–deficient mice were comparable (data not shown). This supports the conclusion that mast cell-secreted mMCP-4 is directly responsible for the observed effects.

**mMCP-4 aggravates histological features of anti-GBM–induced GN**

To delineate the involvement of mMCP-4 in kidney damage, we performed PAS and Masson’s trichrome staining in kidney tissue sections from WT and mMCP-4–deficient mice sacrificed at day 14 after anti-GBM. Both groups of mice showed significant renal damage compared with NRS-treated mice (Fig. 3A). However, semiquantitative evaluation showed lower deposits and cellularity in the glomerulus of mMCP-4–deficient mice compared with their WT counterparts (Fig. 3B). This was accompanied by a decrease in cellular infiltration, interstitial fibrosis, and tubular necrosis. Similar results were observed when mast cell-deficient Kit<sup>W-sh</sup>/Kit<sup>W-sh</sup> mice reconstituted with WT or mMCP-4–deficient BMMCs were examined (Fig. 3C). These findings indicate that mMCP-4 directly modulates renal damage in this model.

**Evaluation of immune responses in WT and mMCP-4–deficient mice**

We next examined whether mMCP-4–deficient mice had altered humoral immune responses to injected rabbit IgG Abs compared with WT mice. Evaluation of frozen kidney sections at day 14 showed no significant differences in rabbit IgG or mouse anti-rabbit IgG glomerular deposits between WT and mMCP-4–deficient mice (Fig. 4A). Similarly, serum levels of rabbit IgG were comparable at day 3 and they had decreased to a similar extent by day 14 (Fig. 4B, left panel). Finally, the amounts of mouse anti-rabbit IgG at day 14 were indistinguishable between the two mouse strains (Fig. 4B, right panel). No significant levels of mouse anti-rabbit Abs were detected prior to the injection of anti-GBM (data not shown). These results demonstrated that deficiency in mMCP-4 fails to alter the deposition of heterologous Abs and the onset of the humoral immune response to injected Abs. We also evaluated T cell responses by measuring dermal DTH activity to rabbit Ig. As shown in Fig. 4C, no differences in dermal DTH responses became apparent between WT and mMCP-4–deficient mice.

**mMCP-4 is released locally from mast cells in renal capsules after induction of GN**

Large amounts of mMCP-4 are expressed in renal capsule tissue, whereas we were unable to detect it in renal tissue before (Fig. 1) or after induction of GN (not shown). To determine whether mast cells present in kidney capsules became activated to release mMCP-4 after the induction of anti-GBM GN, mMCP-4 expression was assessed by immunoblotting of tissue lysates collected from WT mice before and after the induction of GN. Fig. 5A shows that a marked decrease (≥90%) in mMCP-4 levels in the renal capsules was observed at day 14 after the injection of anti-GBM Abs. Under these conditions, mast cells were still present, as demonstrated by the expression of FcεRI β-chain, which recognizes degranulated and nondegranulated mast cells. In agreement, histological analysis showed a decrease in toluidine blue-stained mast cells in renal capsule tissue sections after anti-GBM treatment, likely reflecting extensive mast cell degranulation (Fig. 5B). By contrast, mast cell numbers did not significantly change in other tissues, including ear, skin, and tongue (Fig. 5C). Likewise, mMCP-4 levels in ear tissue were not significantly altered after GN induction (Fig. 5D). Mast cell numbers were comparable between WT and mMCP-4–deficient mice (Fig. 5E). Taken together, these results suggest that mast cells released mMCP-4 locally in renal capsules upon anti-GBM–induced GN.

**Inflammatory cytokines are decreased in kidneys of mMCP-4–deficient mice**

To establish whether mMCP-4 contributed to anti-GBM–induced kidney inflammation by sustaining the local production of inflammatory cytokines and chemokines, we assessed mRNA steady-state levels of MCP-1, TGF-β, TNF, and IL-1β in kidney
tissue extracts (34). All of the transcripts analyzed were elevated at day 14 in GN-stimulated WT and mMCP-4–deficient mice compared with NRS-injected mice (Fig. 6). mMCP-4–deficient mice had significantly lower levels of MCP-1 and TNF mRNA than WT mice, a trend toward a decrease in the levels of IL-1β, but no significant changes in the expression of TGF-β. These results further support the view that mMCP-4 promotes a proinflammatory response that favors disease progression.

Role of mMCP-4 in mediating inflammatory cell infiltration

We next examined the glomerular and tubulointerstitial inflammatory infiltrate in kidneys from anti-GBM–stimulated mice, in particular macrophages and T cells, which are the main inflammatory cell types that play a role in this disease model (35, 36). We found that mMCP-4–deficient mice had slightly, but significantly, lower numbers of macrophages within the glomeruli, but not in the interstitial space, compared with their WT counterparts (Fig. 7A). Similarly, intraglomerular and interstitial CD4+ T cells were markedly decreased in mMCP-4–deficient mice compared with WT mice (Fig. 7B). To examine whether mMCP-4 had chemotactic activity toward these cells, we determined the chemotactic potential of supernatants collected from short-term IgE-activated WT or mMCP-4–deficient BMMCs toward macrophages or CD4+ T cells using an in vitro transwell-migration assay. As shown in Fig. 7C, supernatants from mMCP-4–deficient BMMCs had a significantly lower macrophage chemotactic activity than those collected from WT BMMCs. Supernatants from short-term IgE-activated BMMCs also induced significant migration of CD4+ T cells; however, no differences were seen between WT and mMCP-4–deficient cells. Together, these results suggest that mMCP-4 contributes to kidney inflammation by promoting the infiltration and accumulation of macrophages and CD4+ T cells. This may be secondary to the increased inflammatory processes initiated, but it may also involve a direct recruitment of macrophages by mMCP-4 released locally from capsule tissue.

Renal fibrosis is attenuated in mMCP-4–deficient mice

Next, a series of immunohistochemical studies was performed to analyze the possible consequences of mMCP-4 deficiency on known mMCP-4 target proteins, such as type I collagen, fibrin, and Ang II, which are associated with the renal-remodeling process (5, 9). Examination of kidney tissue sections from anti-GBM–treated mice showed a prominent increase of glomerular fibrin and interstitial type I collagen deposits at day 14 compared with control Ab-treated kidneys (Fig. 8A). The amounts of type I collagen in the interstitium were nearly absent in mMCP-4–deficient mice, whereas those of fibrin were unaffected. Finally, Ang II in the interstitium was nearly absent in mMCP-4–deficient mice; its levels were similar to those observed in NRS-injected mice (Fig. 8B). By contrast, WT animals attained significant levels of Ang II, which appeared as a punctuate staining in localized areas through-

**FIGURE 5.** mMCP-4 expression in renal capsules before and after the induction of anti-GBM GN and mast cell quantification. A, Lysates (20 μg/lane) of renal capsule tissue from NRS- and anti-GBM-treated WT mice or an equivalent of 1 × 107 BMMC control lysates were migrated in parallel on 12% SDS-PAGE and analyzed by immunoblotting using anti-mMCP-4 or anti-FcγRI β-chain Abs. Numbers indicate relative expression levels compared with the first lane, which was set arbitrarily to 1. B, Several granulated mast cells stained with toluidine blue (arrows) can be observed in renal capsule tissue from control (NRS)-treated WT mice, whereas they are absent in anti-GBM–treated mice. Objective magnifications are indicated. Insets show high magnification (100× objective) of intact granulated and degranulated mast cells (taken in a different field), the latter being observed often after anti-GBM treatment. Respective quantification of toluidine blue-stained mast cells in healthy NRS-treated mice (n = 6), anti-GBM–treated WT mice (n = 6), and mMCP-4−/− mice (n = 6) is reported in the far right panel.

***p < 0.001. C, Quantification of toluidine blue-stained mast cells in indicated tissues. All data are mean ± SEM. D, Lysates (20 μg/lane) of ear tissue from NRS- and anti-GBM–treated WT mice were migrated in parallel on SDS-PAGE and analyzed by immunoblotting using anti-mMCP-4 or anti-β-actin. Numbers indicate relative expression levels compared with the first lane, which was set arbitrarily to 1.

**FIGURE 6.** The expression of proinflammatory cytokine and chemokine mRNA in kidneys is lower in mMCP-4–deficient mice. The cDNAs from the cortex region of kidney of mMCP-4−/− and WT mice, as well as from control (NRS)-treated WT mice, were used to evaluate the expression of MCP-1, TGF-β, TNF, and IL-1β mRNA by real-time PCR. The level of all genes is expressed as the number of copies. Data are mean ± SEM of a minimum of six mice in each group. *p < 0.05. Similar results were obtained when the number of copies was compared with the housekeeping gene RPL13a.
out the interstitial space. Because chymase-mediated Ang II generation has been implicated in blood pressure regulation with a potential impact on GN development (37), we also analyzed systolic blood pressure in a separate experiment. However, no significant differences between the two types of mice were seen before or after the induction of GN (Fig. 9). Together, these findings indicated that mMCP-4 mediates type I collagen deposition in kidneys and local Ang II generation, both of which are associated with the fibrotic process (38).

Discussion

Mast cells produce a large variety of mediators with beneficial and deleterious roles in inflammatory diseases, depending on the pathophysiological context (1–3, 39). Recently, we and other investigators showed that mast cells protect against anti-GBM–induced GN; however, relevant mast cell mediators involved have remained unclear (14, 15). Protection was seen in the absence of any detectable mast cell infiltration into kidneys, indicating that these cells could act systemically (14, 15). In the

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** Role of mMCP-4 in kidney macrophage and CD4+ T cells infiltration in anti-GBM GN. Day 14 cryostat kidney sections from anti-GBM–treated WT mice (n = 9) and mMCP-4−/− mice (n = 13), as well as NRS-treated WT control mice (n = 6), were stained using anti-CD11b (A) or anti-CD4 (B) Abs (left panels). Cells infiltrating glomeruli and interstitium were quantified (middle and right panels). Data are mean ± SEM in each group. Objective magnifications for glomerulus and interstitium are indicated in the right panels. p, 0.05; ppp, 0.001.

![Figure 8](http://www.jimmunol.org/)

**FIGURE 8.** mMCP-4–deficient mice have decreased glomerular and interstitial deposits of type I collagen and Ang II. At day 14, cryostat kidney sections from anti-GBM–stimulated mMCP-4−/− mice (n = 13) and WT mice (n = 9), as well as from NRS-treated WT control mice (n = 10) were stained with anti-fibrin Ab (A), anti-type I collagen Ab (B), or anti-Ang II Ab (C) and were analyzed by immunofluorescence light microscopy (left panels). Fluorescence intensities were quantified (right panels). Objective magnifications for glomerulus and interstitium are indicated in the right panels. Data are mean ± SEM per group. *p < 0.05.
was measured by the tail-cuff method. All measurements (10/mouse) were found in renal capsules, with levels decreasing markedly after detection of mMCP-4 and mast cells in renal interstitium, even after GN.

Analysis of renal parameters following disease induction clearly showed that the absence of mMCP-4 resulted in less severe kidney disease, with lower proteinuria and decreased serum parameters, such as BUN and creatinine. This difference became apparent at the late stage (autologous phase) of GN development, whereas the extent of the disease was similar in the two mouse strains during the early stage (heterologous phase). Attenuation of renal disease was confirmed by histological analysis showing less cellularity and fewer deposits in the glomeruli and lower infiltration and tubular necrosis scores in the interstitium of mMCP-4–deficient mice. Although these findings contrast with the reported protective effect of mMCP-4–deficient mast cells on the extent of the disease was similar in the two mouse strains during the early stage (heterologous phase). Attenuation of renal disease was confirmed by histological analysis showing less cellularity and fewer deposits in the glomeruli and lower infiltration and tubular necrosis scores in the interstitium of mMCP-4–deficient mice. Although these findings contrast with the reported protective effect of mMCP-4–deficient mast cells on the development of glomerular disease, indicating that mMCP-4 has a detrimental role in GN.

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Although we did not see any baseline effects on kidney development, kidney histology and renal function, in NRS-treated mMCP-4–deficient mice, mMCP-4 could have unknown developmental effects or affect the activity of other cells types. To analyze this question directly, we reconstituted mast cell-deficient mice with WT or mMCP-4–deficient mast cells before the induction of anti-GBM–induced GN. Our data confirmed the less deteriorated renal phenotype in mice reconstituted with mMCP-4–deficient mast cells, further supporting a direct effect of mast cell-derived mMCP-4 in renal disease progression. Although the observed effects of mMCP-4 are compatible with a role of mMCP-4 in kidney disease progression, we were unable to detect mMCP-4 and mast cells in renal interstitium, even after GN induction. Interestingly, however, large amounts of mMCP-4 were found in renal capsules, with levels decreasing markedly after the induction of GN in conjunction with extensive mast cell degranulation. The agents responsible for this degranulation remain unknown, but could be multiple, because of the induced inflammatory response in anti-GBM kidneys. Once released, mMCP-4 may function in the renal microenvironment to induce its biological effects. This is supported by the fact that no such mMCP-4 release and degranulation were noticed in distant ear tissues. Similarly, although the numbers of countable mast cells decreased in the capsular, likely as a result of their degranulated phenotype, these numbers were stable in other tissues (ear, skin, and tongue) examined in WT and mMCP-4–deficient mice. Thus, our data indicate that mMCP-4 contributes locally to the aggravation of GN by mediating a variety of proinflammatory effects. In agreement, lower levels of TNF and MCP-1, and to a lesser extent, of IL-1β mRNA, were observed in mMCP-4–deficient mice compared with WT mice. All of these cytokines/chemokines were shown to have a pathogenic role in anti-GBM–induced GN (35, 41, 42). Although we cannot explain the mechanism behind the TNF and MCP-1 induction, possible scenarios include direct cell activation of kidney resident cells through G protein-coupled protease-activated receptors (40, 43) or indirect effects through the general activation of infiltrating cells.

Moreover, mice lacking mMCP-4 revealed an impaired infiltration of inflammatory macrophages and CD4+ T cell upon anti-GBM treatment compared with their WT counterparts. In vitro experiments showed that supernatants of short-term IgE-activated WT BMMCs, which contained mediators stored in secretory granules, exhibited heightened macrophage chemotactic activity compared with mMCP-4–deficient BMMCs. This is in agreement with previous studies showing potent chemotactic properties of human chymase and mMCP-4 for several types of leukocytes (17, 18). These supernatants also promoted the migration of CD4+ T cells, although no specific mMCP-4–mediated enhancement was noticed. Altogether, these results suggested that the increased T cell infiltration in WT mice is likely the consequence of the generally aggravated inflammatory response, whereas for macrophage infiltration, the possibility exists that mMCP-4 diffused from capsules participates in the recruitment.

To establish whether mMCP-4 interferes with the remodeling component of anti-GBM–induced GN, we examined the expression of Ang II, a peptide with multiple proinflammatory roles that promotes proliferation and fibrogenesis in the kidney (21, 44). The increase in local expression of interstitial Ang II observed in WT mice upon anti-GBM stimulation was not apparent in mMCP-4–deficient animals. Because human chymase and mMCP-4 can directly generate Ang II from Ang I as an alternative to the angiotensin-converting enzyme (ACE) pathway (10, 45), these data suggest that mMCP-4 is directly responsible for Ang II generation in vivo. This notion is also in agreement with data showing that ACE-knockout mice do not display any major reduction in tissue Ang II, in contrast to its levels in the circulation, indicating that ACE-independent Ang II-generation mechanisms, such as through chymase, are active (45). Based on the reported effects of chymase-mediated Ang II generation on blood pressure regulation that might contribute to the aggravation of GN (37, 46), we measured systolic blood pressure in WT and mMCP-4–deficient animals before and after the induction of GN. However, no measurable differences were found within the time frame examined, suggesting that blood pressure regulation does not contribute to the observed aggravation. Our findings extend previous observations suggesting that human chymase and mMCP-4 act as an alternate pathway to ACE for Ang II production in vivo, as shown in experimental and human nephropathies (46–49). Histological analysis of kidney tissue sections also revealed a partial inhibition of type I collagen deposition in mMCP-4–deficient mice, an intriguing finding that differed from the known ability of mMCP-4 to trigger MMP-9 activity, which degrades collagen (9). However, during the inflammatory phase, mMCP-4–induced Ang II may represent the mechanism involved in this inhibition, because Ang II was shown.
to strongly contribute to the progression of renal disease and fibrosis development (21, 24).

Overall, our current results demonstrating that inflammatory and fibrotic responses in the kidney were markedly attenuated in the absence of mMCP-4 contrast with previous findings showing that mast cells exert global protective properties against renal diseases (14, 15). One explanation for this discrepancy is that mast cells, in addition to mMCP-4, can release a set of mediators, including tryptase, serglycin proteoglycans, and biogenic amines, with multiple potential functions in relation to GN, as well as a plethora of cytokines and growth factors that may counteract the pathological effects of this chymase. In addition, mast cells may be beneficial or detrimental, depending on the stage of disease. Indeed, following the administration of anti-GMB Abs, WT mice showed a high capacity to initiate repair functions and to activate the fibrinolytic system (14), suggesting that mast cells may decrease inflammation at early time-points. Such early functions do not involve mMCP-4, because no protective effects were seen in mMCP-4−/− deficient mice at the initial stages of GN. Finally, mast cells may display immunoregulatory functions leading to downregulation of inflammatory responses, as observed in other disease models (1–3, 39, 50, 51). Thus, the boundary between beneficial and detrimental functions can be tight, as also indicated by previous data showing that mast cells can be protective or detrimental in GN, depending on the experimental model (16). Similar contrasting examples were also reported in other pathophysiological conditions, such as venom protein-induced fatal anaphylaxis, in which the response is mediated by mast cells, but their proteases serve to detoxify these proteins (52). In addition, mast cell proteases may have protective effects during sepsis (53) or in allergic airway inflammation (54), but they can also aggravate disease by promoting exacerbating inflammation (28, 33, 55), as also shown in this study.

In conclusion, we demonstrated that mice lacking mMCP-4 had fewer functional and histological signs of GN, particularly kidney dysfunction (21, 24). This study therefore supports our previous findings showing that mast cells lacking mMCP-4, because no protective effects were seen in mMCP-4−/− deficient mice at the initial stages of GN. Finally, mast cells may display immunoregulatory functions leading to downregulation of inflammatory responses, as observed in other disease models (1–3, 39, 50, 51). Thus, the boundary between beneficial and detrimental functions can be tight, as also indicated by previous data showing that mast cells can be protective or detrimental in GN, depending on the experimental model (16). Similar contrasting examples were also reported in other pathophysiological conditions, such as venom protein-induced fatal anaphylaxis, in which the response is mediated by mast cells, but their proteases serve to detoxify these proteins (52). In addition, mast cell proteases may have protective effects during sepsis (53) or in allergic airway inflammation (54), but they can also aggravate disease by promoting exacerbating inflammation (28, 33, 55), as also shown in this study.

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