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Metalloelastase is required for macrophage-mediated proteolysis and matrix invasion in mice

(targeted gene disruption/elastin/basement membrane)

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ABSTRACT **Macrophages secrete a variety of proteinases that are thought to participate in remodeling of the extracellular matrix associated with inflammatory processes. We have eliminated expression of the macrophage metalloelastase (MME) gene by targeted disruption to assess the role of this protein in macrophage-mediated proteolysis. We found that the macrophages of MME-deficient (MME −/−) mice have a markedly diminished capacity to degrade extracellular matrix components. In addition, MME −/− macrophages are essentially unable to penetrate reconstituted basement membranes *in vitro* and *in vivo*. MME is therefore required for macrophage-mediated extracellular matrix proteolysis and tissue invasion.**

Macrophages produce a variety of cysteine, serine, and metalloproteinases that are involved with the physiologic tissue remodeling associated with inflammation and wound repair. However, proteolytic activity released by macrophages can also lead to pathologic tissue destruction. Metalloproteinases constitute a family of structurally related matrix-degrading proteinases including the collagenases, stromelysins, gelatinases, matrilysin, membrane-type metalloproteinases, and macrophage metalloelastase. These enzymes require zinc for catalytic activity and are inhibited by the tissue inhibitors of metalloproteinases (1, 2). Macrophage metalloelastase (MME) is characterized by macrophage-specific expression and the capacity to hydrolyze a broad spectrum of substrates (3, 4). To determine the contribution of metalloelastase to macrophage-mediated proteolysis, we generated mice deficient in metalloelastase (MME −/−) by targeted mutagenesis.

MATERIALS AND METHODS

Generation of the Targeting Construct. A genomic clone encoding MME was isolated from a mouse 129/SvJ library (Stratagene) by using the cDNA as a probe. The targeting vector, constructed in pBluescript KS II, was composed of a 1.7-kb *Eco*RI fragment encompassing the proximal 5′ flanking region, exon 1, and part of intron 1. The PGK-neo cassette was subcloned into an adjacent *Xho* I site with the transcriptional orientation of PGK-neo opposite to that of MME. The 3′ portion of the construct was a 3.2-kb *Kpn* I fragment extending from the 3′ end of exon 2 to a site within intron 4. This construct was linearized with *Not* I and used to electroporate RW4 embryonic stem cells (129/SvJ derived by R.L.W. and T.J.L.) as described (5). One in 40 clones resistant to G418 selection underwent appropriate homologous recombination. Two targeted clones from separate transfections were used to generate two lines of mutant mice. Primers used for PCR were located in exon 1 (5′-CTGCCTGTGGGGCTGCTCCCAT-3′) and exon 3 (5′-ATCCTCACGCTTCATGTCCG-3′).

Isolation of Peritoneal Macrophages and Peritoneal Fluid. Macrophages were recruited into the peritoneum by an intraperitoneal (i.p.) injection of Brewer's thioglycollate medium (Difco) as described (6). Macrophages and ascites fluid were collected by peritoneal lavage 5 days later. Macrophages were used directly for experiments or maintained in culture as described below.

Protein Analysis. Approximately 5×10^6 peritoneal macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal calf serum for 24 h, washed, and cultured in serum-free medium for an additional 48 h. Serum-free macrophage conditioned medium and peritoneal lavage fluid (equal amounts concentrated 50-fold) from +/+, −/+, and −/− mice were subjected to substrate gel electrophoresis using casein as described (7) and Western blot analysis using enhanced chemiluminescence. A monospecific polyclonal antibody directed against the catalytic domain of recombinant MME expressed in *Escherichia coli* was generated in rabbits. This antibody (1:2000 dilution) was used for Western blot analysis.

RNA Analysis by Northern Blot Hybridization. Total cellular RNA was isolated from 2×10^7 thioglycollate-stimulated peritoneal macrophages by the guanidinium/acid phenol extraction method. Northern blot analysis of the RNA was performed using full-length MME cDNA as the probe.

Degradation of Insoluble Elastin by Macrophages. Bovine ligament elastin (Elastin Products, Owensville, MO) was radiolabeled with sodium boro [^{3}H]hydride as described (8). Peritoneal macrophages were harvested by lavage 5 days after thioglycollate injection. Cells were plated on elastin-coated dishes at 10^6 cells per well in DMEM containing 10% fetal bovine serum and cultured for 72 h. Elastin degradation was quantified by measuring solubilization of insoluble [^{3}H]elastin. Data are expressed as micrograms of elastin degraded (±SD) of triplicate samples. These calculations are based on measurements of [^{3}H]elastin (cpm), corrected for medium-only blanks. The radiolabeled elastin used for these studies had a specific activity of ≈1900 cpm/μg.

Invasion of Basement Membrane Matrigel by Peritoneal Macrophages *in Vitro*. Peritoneal macrophages (10^5 cells per well) were plated on filters coated with an 8-μm-thick layer of Matrigel (Becton Dickinson) in DMEM containing 10% fetal bovine serum. The Matrigel matrix has been isolated from Engelbreth–Holm–Swarm mouse tumor cell medium (9, 10). The lower compartment of the Boyden chamber contained the

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Abbreviations: MMP, matrix metalloproteinase; MME, mouse macrophage metalloelastase.

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same medium with the murine monocyte chemotactic protein JE/MCP-1 (R&D Systems) at 10 ng/ml. After 5 days in culture, filters were washed according to the manufacturer's instructions and cells having traversed to the undersurface of the filter were visualized by light microscopy (×400) with a modified Wright's stain and counted.

Invasion of Basement Membrane Matrigel by Peritoneal Macrophages *in Vivo*. Matrigel (100 μl) was evenly injected into 125-mm^3 sponges. Mice were anesthetized with ketamine/Avertin and sponges were introduced i.p. or s.c. (at both the back and abdominal areas). After 10 days the sponges were removed and fixed in 10% formalin. Midsponge slices were placed on glass slides and processed for hematoxylin/eosin staining. Cells were identified morphologically and by murine mac-3 immunostaining (11). Cell counts and differentials of five random high power fields in the center of each slide were performed by the same blinded observer.

RESULTS AND DISCUSSION

Metalloelastase-deficient (MME −/−) mice were generated by targeted disruption of the MME gene in embryonic stem cells (Fig. 1). A mutation was generated in the MME genomic locus by replacing most of exon 2 with a PGK-neo cassette. Embryonic stem cell clones carrying this mutation obtained from two transfections were injected into C57BL/6J blastocysts and chimeric mice were created. This mutation was transmitted into the germ line, and two lines of mice homozygous for this mutation (MME −/−) were generated. Activated macrophages isolated from these mice by peritoneal lavage completely lacked MME mRNA (Fig. 2*A*). Moreover, this mutation entirely eliminated detectable expression of MME protein in macrophages and inflammatory peritoneal fluid (Fig. 2 *B–D*). MME −/− mice undergo normal embryonic and postnatal development in the absence of inflammatory stress. Resting hematopoiesis and myelomonocytic development are also normal. However, MME −/− mothers have litter sizes only 60% that of wild-type mice [MME −/−, 5.7 pups per litter (12 litters); wild type, 9.8 pups per litter (32 litters)]. This abnormality is most likely due to placental abnormalities induced by MME deficiency; this enzyme is also normally expressed at high levels in the human placenta (14).

Thioglycollate injection into the peritoneum results in an inflammatory response with accumulation and activation of neutrophils, lymphocytes, and macrophages (6). To assess the spectrum of proteinases secreted by thioglycollate-stimulated macrophages, these cells were isolated and cultured, and the conditioned medium was subjected to substrate gel zymography (7) by using casein as the substrate. All major zones of lysis observed in MME +/+ medium were decreased in MME −/+ medium and absent in MME −/− medium (Fig. 2*C*). Thus, nearly all secreted caseinolytic activity is MME-dependent; the different bands on the gel most likely represent different forms of MME (activated within the gel during incubation). This is supported by immunoblots that identified MME comigrating with caseinolytic bands. These bands can also be converted by autocatalytic processing to 22 kDa, the molecular mass of fully processed metalloelastase (ref. 3 and data not shown).

Casein zymography was also performed directly on the peritoneal fluid to assess the spectrum of proteolytic enzymes released by all cell types in response to this inflammatory stimulus (Fig. 2*D*). Prominent MME activity was detected in fluid from wild-type (MME +/+) but not MME −/− mice. MME migrated at 22 kDa, suggesting that it was fully processed *in vivo*. There were very few other caseinolytic bands. One faint band at ≈60 kDa was inhibited by EDTA and subsequently identified as matrix metalloproteinase (MMP) 13

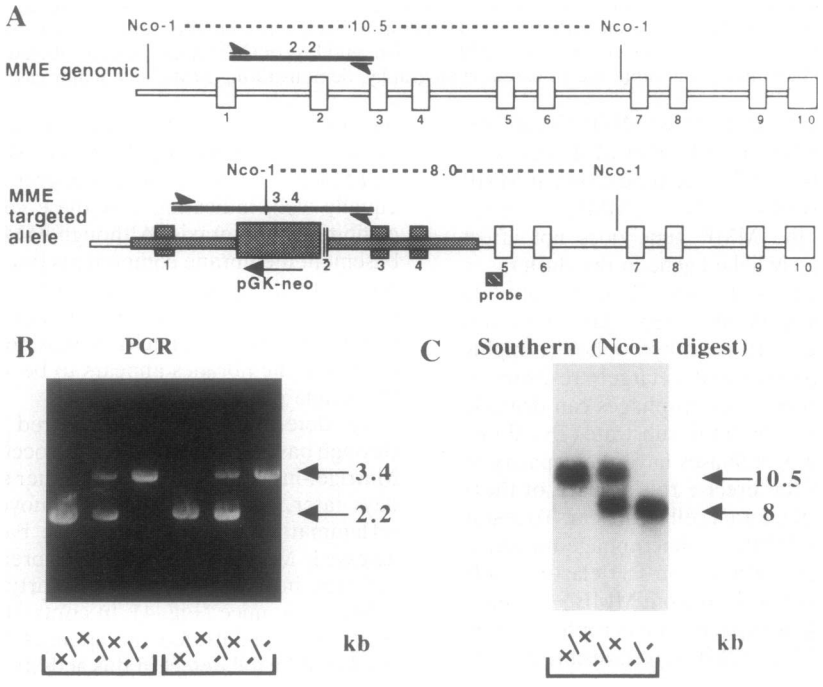

FIG. 1. Targeted disruption of the MME gene. (*A Upper*) The genomic organization of the MME gene. (*Lower*) The targeting construct (shaded region) was constructed as described below replacing most of exon 2 (and part of intron 1) with the neomycin phosphotransferase cDNA driven by the phosphoglycerate kinase promoter (PGK-neo) as a selectable marker (12, 13). RW-4 embryonic stem cell clones carrying this mutation were injected into C57BL/6J blastocysts, and chimeric mice were created. This mutation was transmitted into the germ line, and MME-deficient (MME −/−) mice were generated. The location of *Nco* I sites, the unique external Southern blot hybridization probe, and PCR fragments used for screening are also shown. (*B*) Tail genomic DNA derived from progeny of heterozygous mice was amplified by PCR using primers located in exons 1 and 3, which span the PGK-neo cassette. PCR fragments representing the normal and mutant alleles are 2.2 and 3.4 kb, respectively. (*C*) Southern blots of tail DNA digested with *Nco* I and hybridized with the external probe (*A*) demonstrate 10.5- and 8-kb *Nco* I bands representing wild-type and mutant alleles, respectively.

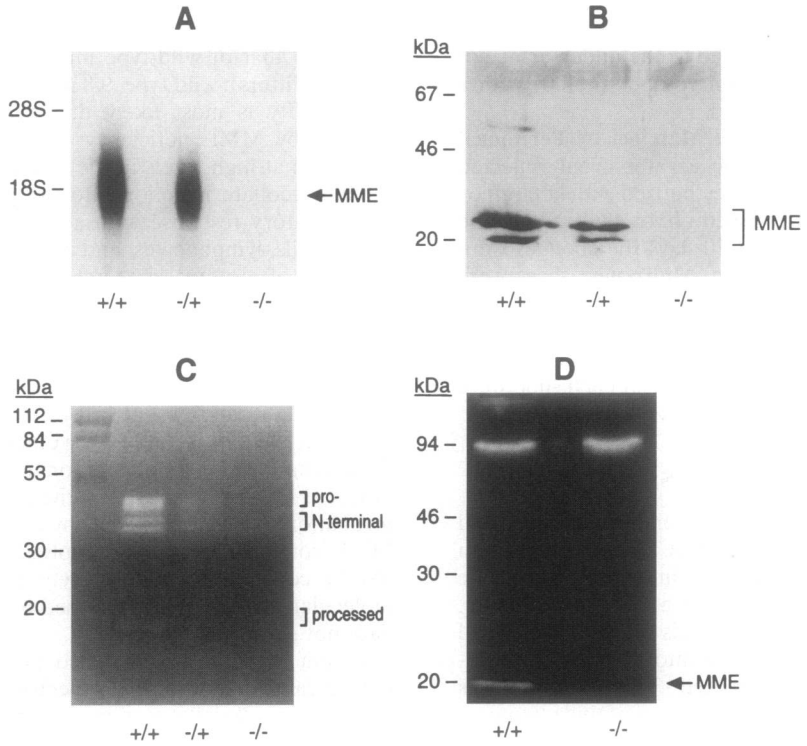

FIG. 2. Expression of MME in peritoneal macrophages and peritoneal fluid. (*A*) Identification of MME RNA. A 5-μg aliquot of total cellular RNA from peritoneal macrophages of wild-type (+/+), heterozygous (−/+), and deficient (−/−) mice was subjected to Northern blot hybridization by using the MME cDNA as the probe. Note the absence of MME mRNA in −/− macrophages. (*B*) Identification of MME protein by Western blot analysis. Equal amounts of thioglycollate-stimulated peritoneal fluid of wild-type (+/+), heterozygous (−/+), and MME-deficient (−/−) mice was analyzed by Western blot analysis using an antibody directed against MME. Note the doublet at 20–22 kDa characteristic of metalloelastase (3, 4) and its absence in −/− mice. (*C*) The spectrum of proteinases secreted by cultured macrophages was determined by casein zymography. Areas of casein degradation are visualized as clear zones of lysis after Coomassie blue staining. Nearly all of the casein-degrading activity secreted by macrophages was MME-dependent; different sized bands most likely represent different activated and processed forms of MME (54-kDa proenzyme labeled pro, loss of proenzyme domain labeled N-terminal, and loss of C-terminal domain labeled processed). (*D*) Peritoneal lavage fluid (used for Western blots in *B*) was subjected to casein zymography, demonstrating MME as a major caseinolytic product *in vivo*.

(collagenase 3) by immunoblots. In humans, MMP-13 and metalloelastase genes are closely linked with other MMP genes on chromosome 11q22.2/22.3 (14, 15). If mice have a similar MMP cluster, then the presence of MMP-13 activity in MME −/− mice suggests that disruption of the MME gene does not affect expression of at least one closely linked gene in the cluster.

Elastin is the major component of elastic fibers that provide resilience to tissues (including blood vessels, skin, and lung) that undergo dynamic stress. It is a hydrophobic, highly crosslinked, and insoluble protein that is largely resistant to proteolytic cleavage. Nevertheless, macrophages can degrade elastin when cultured in contact with this substrate (16). While several purified macrophage proteinases have the capacity to solubilize elastin (3, 17–20), the precise role of each of these proteinases in the context of the intact cell is unclear. To assess the relative contribution of MME to macrophage-mediated elastolysis, macrophages were cultured on [^{3}H]elastin at pH 7.4. Remarkably, macrophages derived from MME −/− mice exhibited <5% of the elastolytic capacity of macrophages from MME +/+ mice (Fig. 3*A*). Thus, MME is responsible directly and perhaps in part indirectly (via activation of other elastases or degradation of their inhibitors) for nearly all murine macrophage-derived elastolytic capacity.

Thioglycollate-stimulated peritoneal macrophages were also tested for their capacity to penetrate a reconstituted basement membrane composed of an 8-μm-thick Matrigel-coated micropore filter (9, 10) in response to monocyte chemoattractant protein 1 (MCP-1) (21) (Fig. 3*B*). Macrophages lacking MME, in contrast to MME +/+ macrophages, were almost entirely unable to penetrate this basement membrane. In the absence of the Matrigel coating, however, MME −/− macrophages and MME +/+ macrophages migrated in response to MCP-1 equally well, indicating that these cells are fully capable of exhibiting chemotaxis. Although MME is very active against basement membrane components [including fibronectin, laminin, entactin, proteoglycans, and type IV collagen, (T. J. Gronski and S.D.S., unpublished observations)], it is perhaps surprising that the capacity of murine macrophages to invade basement membranes appears to be so heavily dependent on this single proteinase.

To determine whether impaired macrophage migration through basement membrane also occurred *in vivo*, we inserted Matrigel-impregnated sponges either s.c. or i.p. into mice. Ten days later, the sponges were removed and the capacity of inflammatory cells to invade the basement membrane was assessed. Macrophages were the predominant inflammatory cell-type invading the sponges (particularly i.p.) in wild-type MME +/+ mice (Fig. 4). In contrast, very few mononuclear phagocytes penetrated the sponges in MME −/− mice, as predicted by the defect in this activity of these macrophages in culture. However, other inflammatory cells invaded the matrix equally in both MME −/− and MME +/+ mice.

In summary, macrophages from mice lacking metalloelastase have markedly impaired capacity to degrade elastin and other substrates. Furthermore, these MME −/− macrophages cannot penetrate reconstituted basement membranes *in vitro* or *in vivo*. However, this observation does not extend to peripheral blood monocytes. Monocytes do not express MME, yet they presumably extravasate to tissues normally in

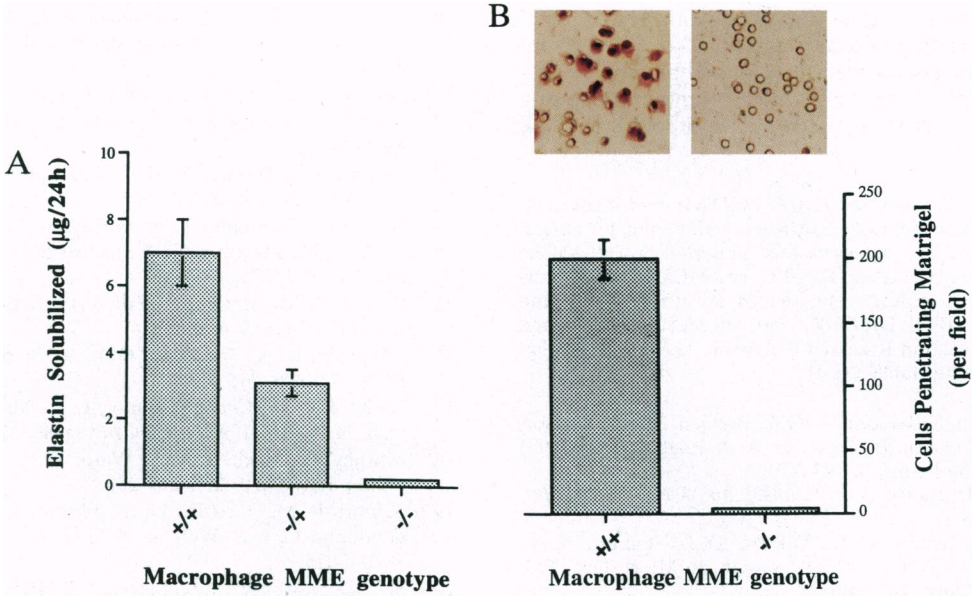

FIG. 3. MME-dependent degradation of elastin and penetration of basement membranes by peritoneal macrophages. (*A*) Degradation of elastin by peritoneal macrophages. Peritoneal macrophages from +/+, −/+, and −/− mice were incubated on [^{3}H]elastin-coated plates. Aliquots of conditioned medium were centrifuged, and the ^{3}H activity in supernatants was quantified to assess the amount of elastin degraded as a function of time. Greater than 95% of the elastin degraded was MME-dependent, with −/+ (heterozygous) macrophages degrading ≈50% the elastin of +/+ (wild-type) macrophages. (*B*) Invasion of basement membrane Matrigel by peritoneal macrophages. Peritoneal macrophages were cultured on Matrigel-coated micropore filters to assess their ability to traverse this reconstituted basement membrane. The figures on the right show the corresponding undersurfaces of filters from typical experiments. In separate experiments, MME −/− macrophages displayed the same migration as +/+ macrophages in chemotaxis assays to monocyte chemotactic protein 1 (data not shown).

MME −/− mice. As monocytes differentiate into macrophages, they alter their proteinase profile, acquiring the capacity to synthesize several metalloproteinases and losing their serine proteinase content (22).

Macrophage-mediated proteolysis is believed to be important in physiologic conditions such as extracellular matrix remodeling in wound repair. Abnormal expression of macrophage proteinases leads to tissue damage that contributes to

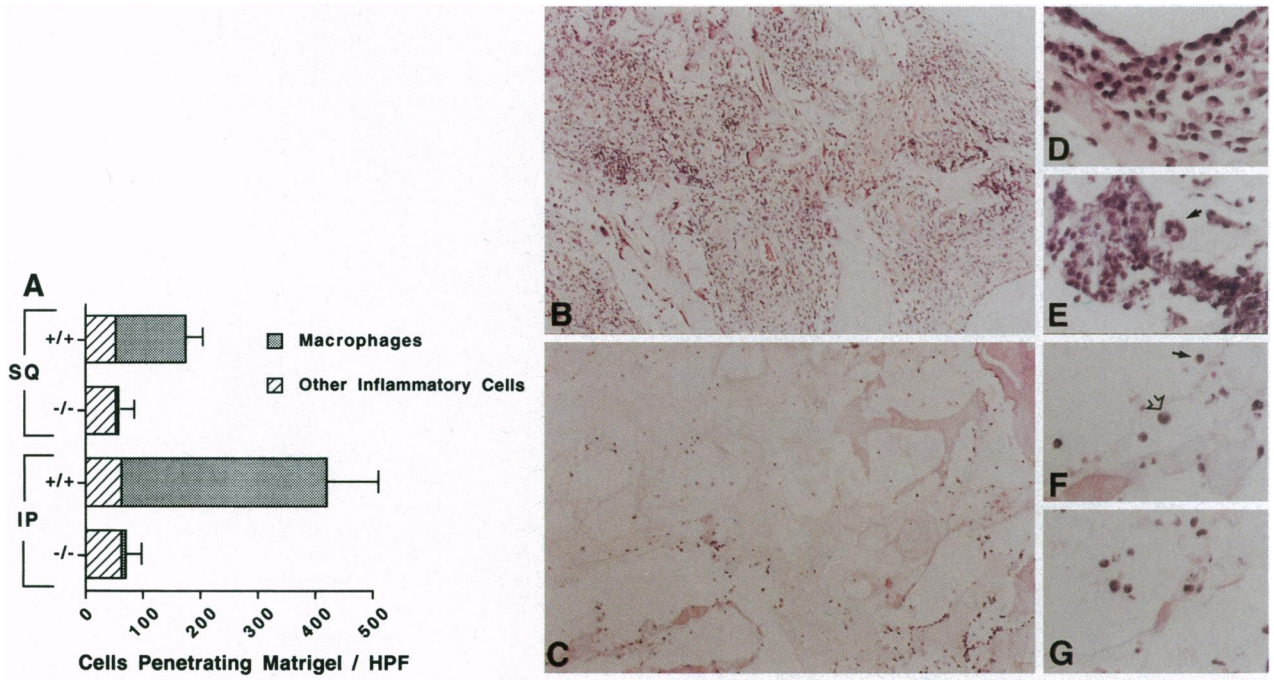

FIG. 4. Capacity of wild-type and MME-deficient macrophages to penetrate a Matrigel basement membrane *in vivo*. (*A*) Equal amounts of Matrigel were incorporated into sponges (125 mm^3) that were inserted into +/+ and −/− mice either subcutaneously (SQ) or intraperitoneally (IP) for 10 days. Inflammatory cells penetrating toward the center of the sponge were counted and identified. Numbers of penetrating macrophages were markedly diminished in MME −/− mice. Data represent the cell counts and differentials of five mice for each group with standard deviations of total cell counts. (*B–G*) Hematoxylin/eosin staining of the centers of Matrigel-impregnated sponges inserted i.p. for 10 days. *B* (×150), *D* (×300), and *E* (×300) demonstrate findings in wild-type (+/+) mice. The majority of the cells are macrophages (confirmed with mac-3 immunostaining, data not shown) with occasional formation of epithelioid giant cells (arrow in *E*). *C* (×150), *F* (×300), and *G* (×300) demonstrate findings in MME −/− mice. There are significantly fewer cells penetrating the matrix, and the majority of the cells are neutrophils (open arrow in *F*) and lymphocytes (solid arrow in *F*) with few macrophages present.

the pathogenesis of the leading causes of death in developed countries (23), including coronary artery (24) and cerebral vascular diseases, metastatic cancer (25), and pulmonary emphysema (26). MME −/− mice should be helpful for defining the importance of macrophage-mediated proteolysis in these disease states.

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