Cathepsin G Recruits Osteoclast Precursors via Proteolytic Activation of Protease-Activated Receptor-1

Thomas J. Wilson, Kalyan C. Nannuru, and Rakesh K. Singh

Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska

Abstract

Metastatic breast cancer shows extreme tropism for the bone microenvironment, leading to the establishment of osteolytic metastases. Perpetuation of tumor-induced osteolysis requires a continuous supply of osteoclast precursors migrating into the bone microenvironment that can subsequently differentiate into mature osteoclasts and resorb bone. Thus, identification and subsequent targeting of chemoketactants of osteoclast precursors that are up-regulated at the tumor-bone interface represents a potential avenue to interrupt osteolysis. We report that cathepsin G, a serine protease, plays a vital role in the bone microenvironment by modulating tumor-stromal interaction in a manner that favors tumor establishment and regulates chemotaxis of monocytes, a subset of which has the potential to differentiate into osteoclasts. Our data show that cathepsin G–induced chemotaxis of monocytes is mediated by proteolytic activation of protease-activated receptor-1 (PAR-1). Attenuation of PAR-1 activation abrogates cathepsin G–mediated induction of monocyte chemotaxis. We also show that in vivo inhibition of cathepsin G reduces the number of CD11b+ osteoclast precursors and mature osteoclasts at the tumor-bone interface. Together, these data suggest that therapeutic targeting of both PAR-1 signaling in osteoclast precursors as well as cathepsin G at the tumor-bone interface has the potential to reduce osteolysis by inhibiting the recruitment, differentiation, and activation of osteoclast precursors. [Cancer Res 2009;69(7):3188–95]

Introduction

Nearly 200,000 women in the United States are diagnosed with breast cancer annually and over 40,000 women every year ultimately succumb to breast cancer (1). Of those that succumb to their disease, nearly all are found to have bone metastases that are predominantly osteolytic in nature (2). These osteolytic lesions carry significant consequences, including hypercalcemia, increased risk of pathologic fracture, and intractable bone pain (2), which both dramatically decrease the quality of life and increase mortality for breast cancer patients. Thus, understanding the complex dialogue that occurs between tumor cells and cells of the bone microenvironment that distort normal bone physiology in a way that favors osteolysis becomes important for designing new therapeutic strategies to combat this serious health problem.

As malignant tumor cells enter the bone microenvironment, they release signaling molecules that initiate a vicious cycle that promotes both tumor cell growth and osteolysis (2, 3). The rate-limiting step of this cycle is the signaling that occurs between osteoblasts and osteoclasts because this requires cell to cell contact to bring receptor activator of nuclear factor-κB ligand (RANKL) on the surface of osteoblasts and RANK on the surface of osteoclast precursors into contact. Our recent data show that this requirement can be bypassed via cleavage of RANKL from the cell surface of osteoblasts by cathepsin G, generating soluble RANKL (sRANKL) that relieves the necessity of cell-cell contact and allows widespread osteoclast activation and enhanced osteolysis (4). Cathepsin G expression is up-regulated at the tumor-bone interface of mammary tumor–induced osteolytic lesions (4). However, even with the increased generation of sRANKL, there must be continuous recruitment of osteoclast precursors that can become activated for osteolysis to continue to occur. Identification of a chemoketactant that is up-regulated at the tumor-bone interface that is responsible for recruiting osteoclast precursors would represent a point in the cycle that could potentially be disrupted with therapeutic targeting. Cathepsin G has previously been shown to be a chemoketactant for monocytes, and a subset of monocytes serves as osteoclast precursors (5). Thus, up-regulation of cathepsin G at the tumor-bone interface is potentially important in perpetuating osteolysis via recruitment of monocytes. Consequently, in this report, we sought to confirm that cathepsin G is a chemoketactant for osteoclast precursors and then sought to delineate the signaling pathway used by cathepsin G in the chemoketaction of osteoclast precursors.

One receptor family that has been previously implicated in protease-induced chemoketaction is the protease-activated receptors (PAR). These G protein–coupled receptors (GPCR) are activated when the NH2 terminus is cleaved by proteases generating a tethered ligand that can bind intramolecularly and initiate signaling (6). The mammalian genome encodes four members of the PAR family (6). This family of receptors is involved both directly and indirectly in the chemoketaxis of a variety of cell types. Direct activation of the PARs has been shown to affect the migration of eosinophils (7), monocytes (8), and breast cancer cells (9–12). The PAR family of receptors has also been shown to indirectly influence the migration of prostate cancer cells and monocytes through increased production of monocye chemokettractant protein-1 (MCP-1; refs. 13, 14). Here, we report that cathepsin G–mediated recruitment of osteoclast precursors requires proteolytic activation of PAR-1, which potentially increases osteolysis by indirectly increasing the number of activated osteoclasts.

Materials and Methods

Cathepsin G chemotaxis assay. Human peripheral blood mononuclear cells (PBMC) were isolated from blood from healthy donors using...
Fico/Lite-Monocytes (Atlanta Biologicals) per standard procedures. The blood samples were obtained using protocols approved by the Institutional Review Board of the University of Nebraska Medical Center. A chemotaxis assay was set up using a three-tiered chemotaxis chamber (Neuro Probe) and the following chemoattractants: HBSS alone (negative control); 10 and 100 ng/mL of MCP-1 (positive control); and 100 ng/mL, 1 μg/mL, 5 μg/mL, and 10 μg/mL of cathepsin G. Each chemoattractant was plated in triplicate. For each well, ten 250-μm fields were counted.

N-tosyl-L-phenylalanine chloromethyl ketone inhibition chemotaxis assay. To test whether the proteolytic activity of cathepsin G is necessary for its ability to serve as a chemoattractant, the chemotaxis assay was repeated using HBSS, 1 μg/mL cathepsin G, and 1 μg/mL cathepsin G preincubated with 1 mmol/L N-tosyl-L-phenylalanine chloromethyl ketone (TPCK; Sigma-Aldrich) for 30 min at 37°C. TPCK is an inhibitor of the chymotrypsin-like group of proteases and is a potent inhibitor of cathepsin G (4, 15, 16). TPCK has also previously been shown to be nontoxic to osteoclast precursors/osteoclasts (4).

PAR expression in monocytes. Human PBMCs were isolated as described. Total RNA was isolated using Trizol reagent (Invitrogen). Total RNA (5 μg) was used for reverse transcription. First-strand cDNA was generated using oligo(dT)$_{18}$ (Fermentas) and SuperScript II RT (Invitrogen). The resulting cDNA (2 μL; 1:10 dilution) was used in the reactions with gene-specific primers. The following gene-specific primers were used: PAR-1, 5′-GTGATTGGCAGTTTGGGTCT-3′ and 5′-GCCAGACAGTGAAGGAAGC-3′; PAR-2, 5′-TGCTAGCAGCCTCTCTTCC-3′ and 5′-CCAGTGAGGACAGATGCAAG-3′; PAR-3, 5′-GGCACAGGAGGCAAATTC-3′ and 5′-CTGCAAAGATGAGGGCTTTC-3′; PAR-4, 5′-CAGACTTCTGGAGAGATGCAGA-3′ and 5′-GGTGTGTTGTGATCCTGTTGG-3′; and glyceraldehyde-3-phosphate dehydrogenase, 5′-AGCCTCGTCCCTAGACAAAA-3′ and 5′-GATGAACGTCTCCACATCTCG-3′. PCRs were carried out using a PTC-100 Programmable Thermal Controller (MJ Research) under optimal conditions. PCR fragments were separated on 2% ethidium bromide containing agarose gel and analyzed using a MultiImage Light Cabinet (Alpha Innotech Corp.).

PAR activation chemotaxis assay. To determine if PAR-1 activation leads to chemotaxis of monocytes, the chemotaxis assay was repeated using HBSS alone (negative control), 10 and 100 ng/mL of MCP-1 (positive control), 1 and 5 μg/mL of cathepsin G, and 1 and 5 μg/mL of PAR-1 agonist (AnaSpec). Because PBMCs do not express PAR-2, PAR-2 agonist (AnaSpec) was also used as a negative control at concentrations of 1 and 5 μg/mL.

**Figure 1.** Cathepsin G is a chemoattractant for monocytes via a proteolytic mechanism. A, cathepsin G showed a concentration-dependent induction of monocyte chemotaxis. At the highest concentrations, it induced chemotaxis similar to MCP-1, a known monocyte chemoattractant. Bars, SD. ***, significantly different from cells incubated with medium alone by post hoc analysis using Bonferroni’s correction. B, representative fields from HBSS alone, MCP-1 (10 ng/mL), and cathepsin G (1 μg/mL), respectively. C, TPCK, an inhibitor of the proteolytic activity of cathepsin G, effectively eliminated cathepsin G–induced chemotaxis of monocytes. Bars, SD. ***, significantly different by post hoc analysis using Bonferroni’s correction.
PAR-1 inhibition chemotaxis assay. To evaluate the role of PAR-1 in cathepsin-G-mediated chemotaxis, the chemotaxis assay was repeated using HBSS (negative control), 5 μg/mL cathepsin G, 5 μg/mL cathepsin G plus blocking anti–PAR-1 antibody, and 5 μg/mL cathepsin G plus nonblocking anti–PAR-1 antibody. The blocking antibody (Santa Cruz Biotechnology) and the nonblocking antibody (Santa Cruz Biotechnology) were both used at a final dilution of 1:200 (17).

Pertussis toxin/cholera toxin inhibition chemotaxis assay. To determine what subunits of the GPCR are activated during cathepsin-G–mediated chemotaxis, the chemotaxis assay was repeated using HBSS, 5 μg/mL cathepsin G, and 5 μg/mL PAR-1 agonist using monocytes that had been preincubated with 100 ng/mL pertussis toxin (PTX; Sigma-Aldrich) for 2 h at 37°C, monocytes that had been preincubated with 10 μg/mL cholera toxin (CTX) for 2 h at 37°C, or monocytes not pretreated.

Conditioned medium treatment of RAW 264.7 and Cl66 cells. RAW 264.7, a murine monocyte/macrophage cell line, and Cl66 mammary tumor cells were seeded onto 100-mm dishes at a density of 1 × 10^6 per dish. Cells were grown in DMEM plus 10% fetal bovine serum (FBS), 1% vitamins, 1% l-glutamine, and 0.08% gentamicin (DMEM complete medium). The cells were incubated overnight. The following day, the concentration of FBS was reduced to 1%. The cells were then allowed to grow for 48 h, at which time conditioned medium was collected for each of the cell lines.

RAW 264.7 cells were then seeded onto six-well plates at a density of 5,000/cm². The cells were then grown in DMEM complete medium plus 10%, 25%, or 50% Cl66-conditioned medium. Simultaneously, Cl66 cells were seeded onto six-well plates at a density of 5,000/cm². The cells were then grown in DMEM complete medium plus 10%, 25%, or 50% conditioned medium from RAW 264.7 cells. At the end of 4 d, total RNA was isolated as previously described and quantitative reverse transcription–PCR was done for cathepsin G using gene-specific primers.

In vivo inhibition of cathepsin G using TPCK. Cathepsin G function was inhibited using TPCK in a murine bone invasion model as previously described (4). Cl66 tumor cells (1 × 10^6) mixed with growth factor–reduced Matrigel were implanted on the dorsal skin flap over the calvaria of female BALB/c mice. Tumor growth was monitored twice a week. Beginning 7 d after tumor implantation, mice were injected s.c. with TPCK at 50 mg/kg/d (n = 4) or 50 µL DMSO (n = 4) for 21 d. Mice were sacrificed at day 31 after implantation and necropsied for examination of osteolytic lesions. All studies were done in accordance with the Institutional Animal Use and Care Committee of the University of Nebraska Medical Center.

Immunohistochemistry for CD11b. CD11b is a marker for osteoclast precursors (18–25). Sections from TPCK-treated animals or control (DMSO)–treated animals were rehydrated and endogenous peroxidase activity was quenched. Antigen retrieval was then performed by boiling sections in 10 mMOL/L sodium citrate buffer (pH 6.0) for 11 min. Sections were blocked using goat serum. Sections were then incubated overnight at 4°C with antibody directed against CD11b (Caltag Laboratories) diluted 1:20 in blocking solution. After washing, sections were incubated for 1 h at room temperature with biotinylated anti-rat IgG diluted 1:200. After washing again, sections were incubated with avidin–biotin complex (Vectastain ABC, Vector Laboratories) for 45 min at room temperature. Sections were then counterstained with hematoxylin. These sections were then mounted with 50% glycerol. Sections were then counterstained with hematoxylin. Sections were then photographed using 50% glycerol. Sections were then photographed using 50% glycerol. Sections were then photographed using 50% glycerol.
a series of xylenes and ethanols. Then, the slides were incubated in tartrate-resistant acid phosphatase (TRAP) staining solution (Sigma-Aldrich) at 37°C for 2 h. After washing with deionized water for 10 min, counter-staining was done with Gill Hematoxylin 3 solution for 2 min. Slides were washed with running tap water for 10 min and then mounted with aqueous mount. The total number of TRAP-positive, multinucleated osteoclasts over the entire length of the tumor-bone interface was then counted for each section using a light microscope. The total number of osteoclasts was then divided by the length of the tumor-bone interface to get the number of osteoclasts per millimeter of tumor-bone interface.

Statistical analysis. ANOVA was performed for statistical comparison of multiple groups. P < 0.05 was considered significant. If significance was found in the ANOVA, then post hoc analysis was performed to compare specific groups using Bonferroni’s correction, where the significance level was determined by dividing 0.05 by the number of comparisons to be made. Where only two groups were tested, the Student’s t test was used. P < 0.05 was considered significant.

Results
Cathepsin G is a chemoattractant for monocytes. MCP-1 is known to induce monocyte chemoattraction. It was used in a monocyte chemotaxis assay as a positive control, whereas HBSS alone was used as a negative control. Cathepsin G showed a concentration-dependent induction of monocyte chemotaxis from 100 ng/mL to 10 μg/mL (Fig. 1A and B). Even in response to as little as 100 ng/mL cathepsin G, induction of chemotaxis was statistically significant compared with control. The highest concentration used, 10 μg/mL, induced chemotaxis at a level similar to 100 ng/mL MCP-1 (Fig. 1A and B).

Cathepsin G-induced chemotaxis of monocytes occurs via a proteolytic mechanism. Next, we used TPCK, an inhibitor of the proteolytic activity of cathepsin G, to examine whether proteolytic activity is necessary for induction of chemotaxis. We observed a

Figure 4. Cathepsin G–mediated activation of PAR-1 signals through the Gαi/o subunits. A, PTX, an inhibitor of the Gαi/o subunits of the GPCR, eliminated the ability of cathepsin G to induce monocyte chemotaxis, whereas CTX, an inhibitor of the Gαs subunit, had no effect. ***, significantly different by post hoc analysis using Bonferroni’s correction. B, PTX reduced PAR-1 agonist–induced monocyte chemotaxis, whereas CTX had no effect. Bars, SD. *** significantly different by post hoc analysis using Bonferroni’s correction.

Figure 5. Source of cathepsin G. A, cathepsin G expression was tested by PCR in human PBMCs (lane 1), RAW 264.7 (a murine monocyte/macrophage cell line; lane 2), 4T1 (lane 3), Cl66 (lane 4), and Cl66M2 (murine breast adenocarcinoma cell lines; lane 5). All cell lines expressed cathepsin G at the RNA level. B, Cl66 cells were cultured with conditioned medium from RAW 264.7 cells and the expression of cathepsin G was compared with Cl66 baseline expression. RAW 264.7–conditioned medium (CM) increased expression of cathepsin G in Cl66. Bars, SD. *, P < 0.01. C, RAW 264.7 cells were cultured with conditioned medium from Cl66 cells and the expression of cathepsin G was compared with RAW 264.7 baseline expression. Cl66-conditioned medium markedly increased expression of cathepsin G in RAW 264.7. Bars, SD. *, P < 0.05; **, P < 0.01. D, for each of the treatment groups and at baseline, expression of cathepsin G was higher in RAW 264.7 cells (dotted columns) than in Cl66 cells (striped columns).
significant inhibition of cathepsin G–induced chemotaxis of monocytes following TPCK treatment (Fig. 1C). These data show that induction of chemotaxis of monocytes by cathepsin G occurs via a proteolytic mechanism.

**Monocytes express PAR-1 and PAR-3.** PCR was used to examine the expression of the PARs by monocytes. Monocytes express both PAR-1 and PAR-3 but do not express PAR-2 or PAR-4 at the mRNA level (Fig. 2A). PAR-1 was expressed at a higher level than PAR-3.

**PAR-1 activation induces chemotaxis of monocytes.** To determine if PAR-1 activation is potentially involved in cathepsin G–induced chemotaxis of monocytes, a PAR-1 peptide agonist was used to evaluate chemotaxis. A PAR-2 peptide agonist was also used as a negative control because monocytes do not express PAR-2. As expected, the PAR-2 agonist did not induce chemotaxis of monocytes and did not differ from HBSS alone (Fig. 2B). The PAR-1 agonist did induce chemotaxis of monocytes at a level that was statistically significant compared with control but did not induce the same level of chemotaxis as cathepsin G at similar concentrations (Fig. 2B).

**Cathepsin G induces chemotaxis of monocytes via proteolytic cleavage of PAR-1.** To attempt to show that the mechanism by which cathepsin G induces chemotaxis of monocytes is via proteolytic cleavage of PAR-1, blocking antibody to PAR-1 was used. Blocking antibody to PAR-1 significantly reduced cathepsin G–mediated chemotaxis, although it did not eliminate it (Fig. 3). A nonblocking antibody to PAR-1 had no effect on the ability of cathepsin G to induce chemotaxis of monocytes (Fig. 3), confirming...
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that proteolytic cleavage of PAR-1 mediates cathepsin G–induced chemotaxis of monocytes.

**Cathepsin G–mediated activation of PAR-1 results in signaling via G\textsubscript{i/o}.** The PARs are GPCRs that are known to signal through a variety of pathways (26, 27). PTX is a known inhibitor of the G\textsubscript{i/o} subunits of GPCRs, whereas CTX is a known inhibitor of the G\textsubscript{s} subunit of GPCRs (28). PTX eliminated the ability of the PAR-1 agonist to induce chemotaxis, whereas CTX had no effect, suggesting that PAR-1 is inducing chemotaxis via the G\textsubscript{i/o} subunits (Fig. 4). Similarly, PTX reduced cathepsin G–induced chemotaxis, whereas CTX had no effect, suggesting that cathepsin G–induced chemotaxis occurs, at least partially, through a G\textsubscript{i/o} subunit–mediated mechanism (Fig. 4).

**Interaction of mammary tumor cells with osteoclast precursors regulates cathepsin G expression.** To determine which cells up-regulate the expression of cathepsin G during the migration of breast tumor cells into the bone microenvironment, we examined whether conditioned medium from Cl66 cells modulates cathepsin G expression by osteoclast precursors and vice versa. First, cathepsin G baseline mRNA expression was examined in human PBMCs and RAW 264.7 cells, a murine monocyte/macrophage cell line, as well as in the mammary tumor cell lines 4T1, Cl66, and Cl66M2. All cell lines expressed cathepsin G at the mRNA level (Fig. 5A). Cl66 cells increased expression of cathepsin G when treated with RAW 264.7–conditioned medium (Fig. 5B). Similarly, RAW 264.7 cells increased cathepsin G expression when treated with Cl66-conditioned medium (Fig. 5C). Although Cl66 cells showed more marked up-regulation of cathepsin G expression, the baseline expression was higher in RAW 264.7 cells (Fig. 5D). Thus, RAW 264.7 cells treated with Cl66-conditioned medium showed higher overall expression of cathepsin G than Cl66 cells treated with RAW 264.7–conditioned medium.

**Inhibition of cathepsin G in vivo reduces osteoclast precursors at the tumor-bone interface.** We used CD11b as a marker for osteoclast precursors (18–25). We stained sections from the tumor-bone interface of mice bearing Cl66 tumors treated with either control treatment (DMSO) or TPCK, a cathepsin G inhibitor. There were significantly less CD11b+ osteoclast precursors at the tumor-bone interface of mice bearing Cl66 tumors treated with the G\textsubscript{i/o} subunit, preventing further activation (28). Monocytes pretreated with CTX did not show reduced chemotaxis in response to cathepsin G. PTX significantly reduced cathepsin G–mediated induction of chemotaxis, which shows the involvement of the G\textsubscript{i/o} subunits.

We have previously reported that cathepsin G is up-regulated at the tumor-bone interface of mammary tumor–induced osteolytic lesions (4). To evaluate the source of cathepsin G in the bone microenvironment, we looked at expression in 4T1, Cl66, and Cl66M2 (murine breast cancer cell lines), RAW 264.7 cells (murine monocyte/macrophage cell line), and human PBMCs. All cell lines tested expressed cathepsin G at the mRNA level. We observed up-regulation of cathepsin G in both RAW 264.7 cells treated with Cl66-conditioned medium and Cl66 cells treated with RAW 264.7–conditioned medium, suggesting that both osteoclasts and tumor cells likely contribute to the observed up-regulation of cathepsin G at the tumor-bone interface. Although the most significant up-regulation was observed in the Cl66 cells treated with RAW 264.7–conditioned medium, the baseline expression of cathepsin G was higher in RAW 264.7 cells. Consequently, RAW 264.7 cells treated with Cl66-conditioned medium showed higher expression of cathepsin G than did Cl66 cells treated
with RAW 264.7–conditioned medium. Hence, although both Cl66 cells and osteoclasts up-regulate cathepsin G expression as tumor cells interact with the bone microenvironment, osteoclasts likely make the most significant contribution. This agrees with our previously reported immunohistochemistry data that showed moderate positivity for cathepsin G in Cl66 cells at the tumor-bone interface and strong positivity for cathepsin G in osteoclasts (4).

Serine proteases such as cathepsin G are stored in azurophilic granules of neutrophils, and the major physiologic function of these proteinases is commonly thought to be the intralysosomal degradation of engulfed debris or microorganisms. However, it has become evident that cathepsin G plays a crucial role in extracellular proteolytic processes at sites of inflammation (4, 29–36). Our immunohistochemical analysis shows diffused cytoplasmic cathepsin G expression in osteoclasts and malignant cells at the tumor-bone interface (4). In our *in vitro* coculture experiments, we observed cathepsin G activity in cell-free supernatant (data not shown). A previous report has shown that cathepsin G is found in gingival tissue during periodontal disease (37). Furthermore, activation of neutrophils with inflammatory cytokines induced release of cathepsin G into the extracellular space and increased the membrane-bound form of catalytically active cathepsin G (5, 32, 34–37).

These data, taken as a whole, suggested that inhibition of cathepsin G *in vivo* would potentially reduce the number of osteoclast precursors at the tumor-bone interface, which would have the potential to reduce osteolysis. We have previously reported that inhibition of cathepsin G *in vivo* does, in fact, significantly reduce the bone destruction index (4). Here, we show that one potential mechanism for the observed reduction in the bone destruction index, in addition to the reduction in sRANKL that we previously reported (4), is a reduction in the number of osteoclast precursors at the tumor-bone interface. We used CD11b as a marker for osteoclast precursors (18–25) and observed significantly fewer CD11b+ osteoclast precursors in TPCK-treated animals, suggesting that cathepsin G is responsible for the recruitment of osteoclast precursors to the site of mammary tumor–induced osteolysis.

The question remaining to be answered was whether reduction of CD11b+ cells at the tumor-bone interface would translate into a reduction in the number of mature osteoclasts. We did observe significantly fewer mature osteoclasts at the tumor-bone interface of TPCK-treated animals. This suggests that inhibition of cathepsin G does, in fact, reduce the number of osteoclast precursor cells migrating to the tumor-bone interface, which in turn depletes the pool of osteoclast precursors and reduces the number of osteoclasts.

Little is known about cathepsin G in the physiologic bone microenvironment. In fact, cathepsin G knockout mice have no morphologic defects in bone development (38), suggesting that cathepsin G may be an ideal target in mammary tumor–induced bone lesions as side effects would likely be minimal. Furthermore, despite apparently normal bone development in cathepsin G knockout mice and no known physiologic roles for cathepsin G in the bone microenvironment, other pathophysiologic roles in the bone microenvironment have been established, including periodontitis (39), rheumatoid arthritis (40), and tumor-induced osteolysis (4).

In conclusion, we report that cathepsin G serves as a chemottractant for osteoclast precursors via proteolytic activation of PAR-1. The up-regulation of cathepsin G at the tumor-bone interface of mammary tumor–induced osteolytic lesions thus serves to increase osteoclast differentiation via the generation of sRANKL (4) as well as by ensuring there is a continuous supply of osteoclast precursors that can be differentiated by the newly generated sRANKL. Other factors, including interleukin-8, are also likely involved. However, for the first time, we report the involvement of cathepsin G and have delineated portions of its downstream signaling pathway.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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