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PMX-53 as a dual CD88 antagonist and an agonist for Mas-related gene 2 (MrgX2) in Human mast cells

Hariharan Subramanian, Sakeen W. Kashem, Sarah J. Collington, Hongchang Qu, John D. Lambris and Hydar Ali

Department of Pathology, School of Dental Medicine (H.S, S.W.K, S.J.C, H.A), Department of Pathology and Laboratory Medicine, School of Medicine (H.Q, J.D.L), University of Pennsylvania, Philadelphia, Pennsylvania 19104

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Running Title: Human mast cell degranulation via MrgX GPCR

Address for correspondence:
Hydar Ali, Ph.D.,
Department of Pathology,
University of Pennsylvania School of Dental Medicine,
240 South 40th Street,
Philadelphia, PA, 19104-6030.
Phone: 215-573-1993
Fax: 215-573-2050
E-mail: alih@upenn.edu.

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Abbreviations used in this paper: GPCR, G protein coupled receptor; CST, cortistatin-14; Mrg, Mas-related gene; BAM-22P, Bovine Adrenal Medulla Docosapeptide; GPA-2, G protein antagonist 2; PTx, pertussis toxin; SP, substance P and HA, hemagglutinin

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Abstract

Human mast cells express the G protein coupled receptor (GPCR) for C5a (CD88). Previous studies indicated that C5a could cause mast cell degranulation, at least in part, via a mechanism similar to that proposed for basic neuropeptides such as substance P, possibly involving Mas-related gene 2 (MrgX2). We therefore sought to more clearly define the receptor specificity for C5a-induced mast cell degranulation. We found that a human mast cell line, LAD2 and $CD34^+$ cell-derived primary mast cells express functional MrgX1 and MrgX2 but an immature human mast cell line HMC-1 does not. A potent CD88 antagonist, PMX-53 (10 nM) inhibited C5a-induced Ca^{2+} mobilization in HMC-1 cells but at higher concentrations (≥ 30 nM) it caused degranulation in LAD2 mast cells, $CD34^+$ cell-derived mast cells and RBL-2H3 cells stably expressing MrgX2. PMX-53 did not, however, activate RBL-2H3 cells expressing MrgX1. Although C5a induced degranulation in LAD2 and $CD34^+$ cell-derived mast cells, it did not activate RBL-2H3 cells expressing MrgX1 or MrgX2. Replacement of Trp with Ala and Arg with dArg abolished PMX-53's ability to inhibit C5a-induced Ca^{2+} mobilization in HMC-1 cells and to cause degranulation in RBL-2H3 cells expressing MrgX2. These findings demonstrate that C5a does not utilize MrgX1 or MrgX2 for mast cell degranulation. Moreover, it reveals the novel finding that PMX-53 functions as a potent CD88 antagonist and a low affinity ligand for MrgX2. Furthermore, Trp and Arg residues are required for PMX53's ability to act both as a CD88 antagonist and MrgX2 agonist.

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Introduction

The anaphylatoxin C5a is generated as a byproduct of complement activation, which interacts with its cognate cell surface G protein coupled receptor (GPCR; CD88) to activate neutrophils and macrophages (Guo and Ward, 2005; Tomhave et al., 1994). C5a induces chemotaxis of a human mast cell line, HMC-1 via a pertussis toxin-sensitive G protein (Hartmann et al., 1997; Nilsson et al., 1996). In purified human skin mast cells and a subpopulation of human lung mast cells, C5a induces mast cell degranulation (Oskeritzian et al., 2005). C5a also causes degranulation and chemokine expression in a newly developed human mast cell line, LAD2 cells (Venkatesha et al., 2005). Although CD88 are expressed in human mast cells, previous studies, suggested that effects of C5a on mast cell degranulation may involve pathways independent of cell surface receptors (el-Lati et al., 1994; Oskeritzian et al., 2005).

Human C5a is a 74-residue glycopolypeptide that consists of two distinct structural domains, the N-terminal core (residues 1-63) that promotes CD88 recognition and the C-terminal region (residues 65-74) which constitutes the receptor activation domain. A large number of peptide CD88 agonists and antagonists have recently been synthesized and tested both *in vitro* and *in vivo*. A cyclic hexapeptide, Ac-Phe-[Orn-Pro-dCha-Trp-Arg], which is based on the terminal amino acid sequence of C5a is a potent CD88 antagonist. It inhibits C5a-induced responses in human neutrophil and monocytes/macrophages *in vitro* (Haynes et al., 2000; Woodruff et al., 2004; Woodruff et al., 2001) and protects rodents from a number of experimental inflammatory diseases such as ischemia reperfusion injury, neurodegeneration, arthritis and immune-complex-mediated inflammation (Kohl, 2006; Qu et al., 2009; Woodruff et al., 2004; Woodruff et al., 2006). Surprisingly, the effects of these peptides on human mast cells have not been determined.

Polybasic molecules such as compound 48/80, substance P (SP) and mastoparan induce substantial degranulation in mast cells. Previous studies indicated that the mechanism of action of basic

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secretagogues involves their insertion into plasma membrane and direct activation of G proteins (Ferry et al., 2002; Mousli et al., 1994). Studies with human skin mast cells indicated that C5a-induced mast cell degranulation involve direct activation of G proteins similar to that proposed for polybasic compounds (el-Lati et al., 1994).

Recently, a large family of GPCRs called Mas-related genes (Mrgs, also known as sensory neuron-specific receptors, SNSR) has been identified in rodents (Dong et al., 2001; Lembo et al., 2002). These receptors are selectively expressed in small-diameter sensory neurons of dorsal root ganglia and are thought to be involved in the sensation and modulation of pain. Based on homology analysis the ~50 mouse Mrg receptors have been subdivided into MrgD and three subfamilies termed MrgA, MrgB, and MrgC (Dong et al., 2001; Lembo et al., 2002). However, there is no information available as to which of these Mrg receptors are expressed in murine mast cells. A subgroup of these receptors (MrgX1 - MrgX4), are expressed in human neurons (Burstein et al., 2006; Dong et al., 2001). Interestingly, there is very little sequence homology between the human and mouse receptors. Tatemoto et al. (Tatemoto et al., 2006) recently showed that MrgX1 and MrgX2 are expressed in human cord blood-derived mast cells (CBMC) and that compound 48/80 as well as substance P activate MrgX2 but not MrgX1. These findings raise the interesting possibility that C5a could activate human mast cells, at least in part, via MrgX2.

The purpose of this study was to determine the receptor specificity of C5a-induced mast cell degranulation. To achieve this objective, we utilized a number of human mast cell lines, primary $CD34^+$ cell-derived mast cells, murine mast cells, transfected RBL-2H3 cells as well as C5a and a potent antagonist of CD88, PMX-53. Using these systems, we demonstrate that C5a does not utilize MrgX1 or MrgX2 for mast cell degranulation but PMX-53 acts as a dual CD88 antagonist and an MrgX2 agonist. Studies with PMX-53 allowed us to identify specific amino acid residues within PMX-53 that are required for CD88 agonist and MrgX2 antagonist activities.

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Materials

All cell culture reagents and pertussis toxin were purchased from Invitrogen (Gaithersburg, MD). Human IgE was purchased from EMD Biosciences (San Diego, CA). Monoclonal anti-DNP specific IgE and anti-human IgE were purchased from Sigma Life Sciences, Inc (St. Louis, MO). Amaxa cell transfection kits and reagents were purchased from Lonza (Gaithersburg, MD). Plasmids encoding hemagglutinin (HA)-tagged human MrgX1, and MrgX2 in pReceiver–M06 vector were obtained from Genecopeia (Rockville, MD). All recombinant human cytokines were purchased from Peprotech (Rocky Hill, NJ). G Protein Antagonist 2 (GPA2) was obtained from Enzo Life Sciences, Inc (Farmingdale, NY). Cortistatin-14 (CST) and Bovine Adrenal Medulla Docosapeptide (BAM-22P) were obtained from American Peptide (Vista, California). Native complement C5a was from Complement Technology (Tyler, Texas).

Synthesis and purification of CD88 peptides.

Linear peptides were synthesized on an Applied Biosystem 433A Peptide Synthesizer using Fmoc chemistry. If necessary, lactam bridge was formed in solution as described previously (Finch et al., 1999). All peptides were purified using reverse-phase HPLC. Their mass was confirmed using MALDI-TOF.

Differentiation of human mast cells from CD34$^+$ progenitors and culture of mast cell lines

Human CD34$^+$ progenitors were cultured in StemPro-34 medium supplemented with L-glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 µg/mL), rhSCF (100 ng/mL), rhIL-6 (100 ng/mL) and rhIL-3 (30 ng/mL) (first week only). Hemidepletions were performed weekly with media containing rhSCF (100 ng/mL) and rhIL-6 (100 ng/mL). Cells were used for experiments after 7-10 weeks in culture (Radinger et al.; Venkatesha et al., 2005). LAD2 cells were maintained in StemPro-34 medium containing nutrient supplements (Invitrogen) supplemented with L-glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 µg/mL) and 100 ng/mL rhSCF (Kirshenbaum et al., 2003; Radinger et al.). Cell culture medium was hemi-depleted weekly with fresh culture medium (Kirshenbaum et al., 2003). Human mast cell line,

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HMC-1 cells were cultured in Iscove's modified Eagle's medium supplemented with 10% FCS, L-glutamine (2 mM), penicillin (100 IU/mL) and streptomycin (100 µg/mL). RBL-2H3 cells were maintained as monolayer cultures in Dulbeccos modified Eagle's medium (DMEM) supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 IU/mL) and streptomycin (100 µg/mL) (Ali et al., 1994).

Stable transfection of RBL-2H3 cells

RBL-2H3 cells were detached with versene, washed twice with DMEM and 1 x 10^6 cells were transfected with plasmids encoding HA-tagged MrgX1 or MrgX2, using the Amaxa nucleofector device and Amaxa kit V according to the manufacturer's protocol. Following nucleofection, cells were cultured in the presence of G418 (1mg/mL) and cells expressing equivalent receptors were sorted using an anti-HA specific antibody 12CA5/FITC-conjugated anti-mouse-IgG and used for studies on degranulation and Ca^{2+} mobilization.

Culture of murine bone marrow-derived mast cells and isolation of peritoneal mast cells

Murine BMMC were obtained by flushing bone marrow cells from the femurs of C57BL6 mice, and were cultured for 4–6 weeks in IMDM supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (100 units/mL), streptomycin (100 mg/mL) and rmIL-3 (10 ng/mL). The homogeneity of the mast cells was confirmed by acid toluidine blue staining. More than 95% pure BMMC population was used for these studies. For peritoneal mast cells, mixed population of peritoneal cells were obtained by lavage, incubated with IgE for 16 h and used for degranulation studies.

RT-PCR

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Total RNA from mast cells was extracted using TRIzol (Invitrogen), treated with DNAse I, and reverse transcribed to cDNA using first strand cDNA synthesis kit (GE). The cDNAs were amplified with primers specific for MrgX1 and MrgX2 (Origene, Rockville, MD). Human β-actin primers were used as an internal control for the PCR reaction.

Calcium mobilization

Ca^{2+} mobilization was determined as described previously (Ali et al., 2000; Ali et al., 1993). Briefly, cells (human mast cells; 0.2 x 10^6) (RBL-2H3 and murine bone marrow-derived mast cells; 1.0 x 10^6) were loaded with 1 μM indo-1 AM in the presence of 1 μM pluronic F-127 for 30 min at room temperature. Cells were washed and resuspended in 1.5 mL of HEPES-buffered saline. Ca^{2+} mobilization was measured in a Hitachi F-2500 spectrophotometer with an excitation wavelength of 355 nm and an emission wavelength of 410 nm (Ali et al., 2000).

Degranulation assay

$CD34^+$-derived mast cells, LAD2 cells (5×10^3) and RBL-2H3 cells, murine mast cells (5×10^4) were seeded into 96-well plates overnight in the presence of human IgE (1μg/mL) or DNP-specific mouse IgE (1μg/mL), respectively. The following day, cells were washed and incubated in a total volume of 50 μL of buffer containing 0.1% BSA and exposed to anti-human IgE (human mast cells), DNP-BSA (RBL-2H3, murine mast cells) or different concentrations of peptides. For total β-hexosaminidase release, control cells were lysed in 50 μL of 0.1% Triton X-100. Aliquots (20 μL) of supernatants or cell lysates were incubated with 20 μL of 1 mM p-nitrophenyl-N-acetyl-β-D-glucosamine for 1.5 h at 37°C. Reaction was stopped by adding 250 μL of a 0.1 M Na_2CO_3/0.1 M $NaHCO_3$ buffer and absorbance was measured at 405 nm (Ali et al., 1994).

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RESULTS

C5a and CD88 antagonist PMX-53 induce degranulation in human LAD2 mast cells

C5a induces degranulation in human skin mast cells, a subpopulation of lung mast cells and LAD2 cells (Oskeritzian et al., 2005; Venkatesha et al., 2005). For our initial studies, we tested the effects of purified C5a and a synthetic CD88 agonist peptide C5aP (Table 1) on degranulation in LAD2 mast cells. As shown in Fig. 1, both C5a and C5aP induced mast cell degranulation in a dose-dependent manner. Surprisingly, we found that CD88 antagonist PMX-53 caused degranulation in LAD2 mast cells and this response was dose-dependent reaching a maximum of ~60% at a concentration of 1 μM. A scrambled linear PMX-53 peptide (PMX-53S) also induced degranulation in LAD2 mast cells but the magnitude of the response was lower (Fig. 1). Interestingly, a scrambled control peptide in which the Trp-Arg was replaced with Ala-dArg (PMX-53C) did not induce degranulation even at a concentration of 1 μM (Fig. 1). Consistent with degranulation, PMX-53 (Fig 2A) and PMX-53S (Fig 2B) induced Ca^{2+} mobilization at concentrations of 100 nM and 1 μM. Not surprisingly, PMX-53C at a concentration of 100 nM was inactive and at 1 μM induced only a small and variable response (Fig 2C). These findings suggest PMX-53 (Fig 1-2) can activate human mast cells and requires the presence of hydrophobic residue for activity (Table 1).

PMX-53 is a potent CD88 antagonist and inhibits C5a-induced neutrophil myeloperoxidase release and chemotaxis with IC_{50} values of 22 nM and 75 nM, respectively (Haynes et al., 2000; March et al., 2004). We therefore sought to determine if the PMX-53 used in the present study displayed CD88 inhibitory activity. Given that PMX-53 induced signaling and degranulation in the mature mast cell line LAD2 cells, we tested its effects on C5a-induced Ca^{2+} mobilization in HMC-1 cells. In untreated cells, C5a (10 nM) and C3a (10 nM) induced transient Ca^{2+} mobilization responses of similar magnitude (Fig. 3A). Preincubation with PMX-53 (10 nM) almost completely inhibited Ca^{2+} response to C5a but had no effect on C3a-induced response (Fig. 3B). By contrast, PMX-53C (Fig 3C) and PMX-53S (Fig 3D) had no

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effect on either C5a or C3a-induced responses. Consistent with these results PMX-53 (10 nM) also inhibited C5a-induced degranulation response in RBL-2H3 cells expressing CD88 (Fig 3E). These findings demonstrate that the ability of PMX-53 to induce degranulation in LAD2 mast cells (Fig. 1) is independent of CD88.

PMX-53 and C5a activate human mast cells via cell surface GPCR

We utilized two approaches to determine whether PMX-53-induced mast cell degranulation occurs via a GPCR or by the direct activation of G proteins. A substance P-related peptide (known as G protein antagonist 2; GPA-2; pGlu-Gln-dTrp-Phe-dTrp-dTrp-Met-NH_2) inhibits M_2 muscarinic cholinergic receptor-mediated G protein Gαo/Gαi activation in vesicles containing purified proteins (Mukai et al., 1992). This peptide does not directly inhibit G protein activity but blocks the ability of the receptor to activate G proteins (Mukai et al., 1992). We found that GPA-2, at a concentration of 1 μM, caused a substantial inhibition of C5a and PMX-53-induced degranulation in LAD2 cells but had little or no effect on IgE-mediated response (Fig. 4A). Unlike GPA-2, pertussis toxin (PTx), covalently modifies Gαo/Gαi family of G proteins to prevent their activation by GPCRs. We found that PTx also inhibited C5a and PMX-53 but not IgE-mediated degranulation (Fig. 4B). These findings in total suggest that C5a and PMX-53 activate PTx-sensitive GPCRs to induce degranulation in human LAD2 mast cells.

PMX-53 activates human mast cells via MrgX2 but C5a does not

GPCRs constitute the largest family of receptors with ~1000 members and ~50% have no known ligands and are classified as orphan receptors. Ligands for the previously orphan GPCRs, MrgX1 and MrgX2 have recently been identified as neuropeptides BAM-22P and the cyclic neuropeptide cortistatin-14 (CST, Table 1) respectively. Emerging evidence suggests that MrgX2 is activated by short peptides containing amino acids Pro, Phe, Trp and Arg/Lys but the presence of negatively charged residues such as Asp and Glu results in loss of activity (Nothacker et al., 2005; Robas et al., 2003). Based on the sequence

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comparison of PMX-53 with BAM-22P and CST (Table 1), we postulated that PMX-53 could activate human mast cells via MrgX2. Human cord blood-derived mast cells (CBMC) express MrgX1 and MrgX2 (Tatemoto et al., 2006). Because LAD2 cells display a mature phenotype similar to CBMC (Kirshenbaum et al., 2003), we suspected that this cell could also express MrgX1 and MrgX2. Indeed, we found that mRNA for MrgX1 and MrgX2 are expressed in LAD2 mast cells (Fig. 5A). By contrast, the immature human mast cell line HMC-1, expressed MrgX1 and MrgX2 at low levels (Fig. 5A). This difference reflected in the abilities of known ligands for MrgX1 and MrgX2 to activate LAD2 and HMC-1 cells. Thus, while BAM-22P and CST (ligands for MrgX1 and MrgX2, respectively) caused sustained Ca^{2+} mobilization and degranulation in LAD2 cells, they were essentially without effect in HMC-1 cells (Fig. 5B, C, & D).

To determine the relevance of studies using human LAD2 cells, we performed selected confirmatory experiments in primary human CD 34^{+}-derived mast cells. Transcripts for MrgX1 and MrgX2 are expressed in $CD34^{+}$-derived mast cells (Fig. 6A inset). Furthermore, PMX-53 and PMX-53S caused substantial degranulation in $CD34^{+}$ mast cells (Fig. 6A). As in LAD2 mast cells, while C5a caused a transient Ca^{2+} mobilization, PMX-53 (Fig. 6B) and PMX-53S (Fig. 6C) promoted sustained Ca^{2+} responses. Again, as in LAD2 cells, PMX-53C did not induce Ca^{2+} mobilization and degranulation in $CD34^{+}$ mast cells (Fig. 6A and D). These findings clearly demonstrate that the data obtained in LAD2 mast cell line is biologically relevant and does not reflect an artifact due to immortalization.

A rodent mast cell line, RBL-2H3 cells did not respond to BAM-22P and CST (data not shown), indicating that MrgX1 and MrgX2 are not expressed in these cells. To precisely determine the role of MrgX receptors on C5a and PMX-53-induced mast cell degranulation, we generated stable transfectants in RBL-2H3 cells expressing HA-tagged MrgX2 and for control MrgX1 (Fig. 7A & B). We used a HA-specific antibody to determine cell surface receptor expression. Cells expressing equivalent MrgX1 and

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MrgX2 were used for degranulation and signaling studies. Cells expressing MrgX2 responded to CST, PMX-53, PMX-53S and substance P for degranulation but PMX-53C had no effect (Fig. 7B). Interestingly, PMX-53 caused a sustained Ca^{2+} mobilization in RBL-2H3 cells expressing MrgX2, similar in magnitude and duration to that observed in LAD2 cells and $CD34^{+}$-derived mast cells (Fig. 7C). The effects of PMX-53, PMX-53S and substance P were specific for MrgX2. Thus, RBL-2H3 cells expressing MrgX1, responded to its known ligand BAM-22P for Ca^{2+} mobilization and degranulation but PMX-53, PMX-53S or substance P had no effect (Fig. 7A & C). Interestingly, C5a at a concentration of 1 μM, which induced ~60% degranulation in LAD2 mast cells, did not cause any noticeable degranulation in RBL-2H3 cells expressing MrgX1 or MrgX2 (Fig. 7 A & B).

Murine peritoneal and bone marrow-derived mast cells do not respond to PMX-53

In contrast to human, ~50 Mrg sequences have been identified in the mouse (Dong et al., 2001; Lembo et al., 2002). Interestingly, there is very little sequence homology between the human and mouse receptors. Furthermore, there is no information available as to which of these Mrg receptors are expressed in murine mast cells. We therefore sought to determine if agonists for human MrgX2 could activate murine mast cells. For these experiments, we used murine bone marrow-derived mast cells (BMMC) and murine peritoneal mast cells (PMC). As shown in Fig.8A, while antigen/IgE caused degranulation in BMMC and PMC, PMX-53 and CST (1 μM) were without effect. Similarly, BMMCs did not respond to PMX-53 or CST (1 μM) for Ca^{2+} mobilization but antigen/IgE caused a robust response (Fig 8B).

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DISCUSSION

Previous studies indicated that C5a could induce mast cell degranulation, at least in part, via a CD88 independent pathway, possibly involving MrgX2 (el-Lati et al., 1994; Tatemoto et al., 2006). In the present study, we utilized a potent CD88 antagonist, $CD34^+$-derived primary mast cells, two human mast cell lines, primary murine mast cells as well as RBL-2H3 cells stably expressing human CD88, MrgX1 and MrgX2 to determine the receptor specificity of C5a-induced mast cell degranulation. We found that although C5a does not utilize MrgX1/Mrgx2 for mast cell degranulation, PMX-53 functions both as a potent CD88 antagonist and a low affinity ligand for MrgX2. The effect of PMX-53 on mast cell activation appears to be specific for human mast cells as murine mast cells, which do not express MrgX2 are unresponsive to activation by PMX-53. Our studies with PMX-53 analogs demonstrate that Trp and Arg residues are required for its activity both as a CD88 antagonist and MrgX2 agonist.

PMX-53 is a potent CD88 antagonist in human neutrophils and macrophages *in vitro* and protects rodents from a number of experimental inflammatory diseases (Arumugam et al., 2002; Arumugam et al., 2004; Strachan et al., 2001; Woodruff et al., 2006; Woodruff et al., 2002). Currently, it is under clinical trial for osteoarthritis. Human mast cells endogenously express CD88 and respond to C5a for signaling and mediator release (Oskeritzian et al., 2005) . Using HMC-1 cells and RBL-2H3 cells expressing CD88, we have shown that PMX-53 (10 nM) selectively inhibits C5a, but not C3a-induced responses. However, utilization of LAD2 cells, $CD34^+$ mast cells and MrgX transfected RBL-2H3 cells clearly demonstrated that PMX-53, at concentration higher than that required to inhibit CD88, causes mast cell degranulation via MrgX2. MrgX2 is a low affinity receptor and responds to CST and substance P for mast cell degranulation and reporter gene activity in transfected Pch12 cells with EC_{50} values of ≥30 nM (Robas et al., 2003; Tatemoto et al., 2006). Our finding that PMX-53 promotes degranulation in human mast cells at concentrations of ≥30 nM is consistent with MrgX2 activation. This was confirmed in studies with RBL-2H3 cells which showed that PMX-53 activated MrgX2 and not MrgX1. These findings suggest that

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PMX-53 is a dual action molecule; it is a high affinity antagonist of CD88 but a low affinity agonist of MrgX2. It is noteworthy that there is very little sequence homology between human MrgX2 and any of the known mouse Mrg receptors (Dong et al., 2001; Lembo et al., 2002). Accordingly, we found that CST, a known ligand for MrgX2 and PMX-53 failed to induce degranulation in murine BMMC and peritoneal mast cells. Thus, the effect of PMX53 appears to be specific for human MrgX2.

An important shared property of the peptides that activate mast cells via MrgX2 is that they possess amino acids with hydrophobic and positively charged side chains (Table 1). Emerging evidence suggests that MrgX2 is activated by short peptides containing four amino acids Pro,Phe,Trp and Arg/Lys (Nothacker et al., 2005; Robas et al., 2003). The demonstration that a scrambled linear PMX-53, which has no antagonist activity against CD88, caused mast cell degranulation, albeit at higher concentration, suggests that cyclic nature of the peptide is not essential for its agonist activity towards MrgX2. In addition, we found that replacement of Trp-Arg with Ala-dArg in a cyclic PMX-53 peptide resulted in loss of both the ability to inhibit C5a-induced Ca^{2+} mobilization in HMC-1 cells and to induce degranulation in LAD2 mast cells. This study thus identified the hexapeptide PMX-53 as an agonist for MrgX2, which requires Trp and Arg residues for activity. It is unclear why PMX-53 did not induce degranulation in RBL-2H3 cells expressing MrgX1. It is noteworthy that BAM-22P, a ligand that activates MrgX1, possesses negative changed amino acid residues in close proximity to the hydrophobic Trp residues (Glu-Trp-Trp-Met-Asp; Table 1). Our finding that BAM-22P caused degranulation in RBL-2H3 cells expressing MrgX1 but PMX-53 did not suggest that there are important differences in binding properties of these receptors.

An interesting and consistent finding of the present study was that while C5a caused transient Ca^{2+} mobilization, agonists for MrgX1 and MrgX2 induced more sustained responses in human mast cells and transfected RBL-2H3 cells. The reason for this difference is unclear but could reflect differences in

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the phosphorylation and desensitization of these receptors. CD88 undergoes phosphorylation in response to agonist stimulation resulting in their desensitization (Braun et al., 2003; Christophe et al., 2000). Furthermore, a phosphorylation-deficient mutants of CD88 are resistant to desensitization (Pollok-Kopp et al., 2006). A recent study showed that MrgX1 is one of the few GPCRs that does not associate with β-arrestin and is resistant to agonist-induced receptor internalization (Solinski et al.). Thus, the possibility that MrgX2 receptor is also resistant to agonist-induced phosphorylation, β-arrestin recruitment and desensitization remains to be determined.

Our attempt to identify the mechanisms by which C5a causes mast cell degranulation led us to the unexpected finding that CD88 antagonist PMX-53 induces degranulation in human mast cells via MrgX2. Outside the dorsal root ganglia, MrgX2 is expressed only in human mast cells (Tatemoto et al., 2006). Furthermore, we have shown a striking selectivity of PMX-53 for promoting degranulation in human but not rodent mast cells. It is noteworthy that while PMX-53 has been shown to be effective in modulating a number of experimental inflammatory diseases in animal models, one study reported that orally administered PMX-53, despite reaching serum levels high enough for C5aR-blocking activity, did not reduce synovial inflammation in humans with rheumatoid arthritis (Vergunst et al., 2007). It is therefore possible that lack of its effectiveness in humans could reflect opposing effects on blocking CD88 and activating MrgX2 in human mast cells. Given that mast cells play important roles in a number inflammatory diseases, caution should be exercised in using peptides containing amphiphilic residues for therapeutic purposes.

MrgX2 is a low affinity and low specificity GPCR which can be activated by short amphiphilic peptides. It is currently unknown if this receptor is activated by endogenous peptides produced in the context of innate immunity and inflammation. However, human defensins, which are amphiphilic peptides, induce mast cell degranulation at concentrations similar to those observed for PMX-53 (Chen et

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al., 2007; Niyonsaba et al.). This raises the interesting possibility that defensins produced by epithelial cells during microbial infection may activate human mast cells via MrgX2 to promote innate immunity. It is therefore possible that the dual effect of PMX-53 could be pharmacologically relevant in promoting both anti-inflammatory activity and innate immunity. Thus, at low concentrations, PMX-53 could block inflammation by acting as a CD88 antagonist but at higher concentration it can promote innate immunity by mimicking the actions of defensins on mast cell activation. These exciting possibilities will be subjects of our future investigations.

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Author contributions:

Participated in research design: Subramanian, Kashem, Ali

Conducted experiments: Subramanian, Kashem, Collington

Contributed new reagents or analytical tools: Qu, Lambris

Performed data analysis: Subramanian, Kashem, Collington

Wrote or contributed to the writing of the manuscript: Subramanian, Collington, Ali

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Footnotes

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Figure Legends

Fig. 1: C5a, C5aP and CD88 antagonist PMX-53 induce degranulation in human mast cells. LAD2 mast cells were stimulated with different concentrations of native C5a, C5aP, PMX-53, scrambled PMX-53 (PMX-53S) or a control PMX-53 (PMX-53C) and percent degranulation (β-hexosaminidase release) was determined. Data are mean ± SEM of n=3. Statistical significance was determined by two-way ANOVA with Bonferroni's post test. * indicates $p<0.05$.

Fig. 2: CD88 antagonist PMX-53 induces Ca^{2+} mobilization in LAD2 mast cells. Cells were incubated with Indo-1AM and stimulated with increasing concentrations of PMX-53 (A), PMX-53S (B), PMX-53C (C) and intracellular Ca^{2+} mobilization was determined. Data shown are representative of 3 similar experiments.

Fig. 3: PMX-53 inhibits C5a-induced responses in HMC-1 and RBL-2H3 cells expressing CD88. (A) HMC-1 cells were incubated with Indo-1AM and stimulated sequentially with C5a (10 nM) and C3a (10 nM) and intracellular Ca^{2+} mobilization was determined. Cells were exposed to PMX-53 (10 nM) (B), PMX-53C (C) or PMX-53S (D) 100 seconds prior to stimulation with C5a and C3a and Ca^{2+} mobilization determined. (E) RBL-2H3 cells stably expressing CD88 were pretreated with vehicle or PMX-53 (10 nM) and exposed to C5a (1nM) and percent degranulation (β-hexosaminidase release) was determined. Data shown are representative of 3 similar experiments. Data are mean ± SEM of n=3. Statistical significance was determined by two-way ANOVA with Bonferroni's post test. * indicates $p<0.05$.

Fig. 4: PMX-53 causes degranulation in human mast cells via GPCR. LAD2 mast cells were pretreated with vehicle, G protein antagonist (GPA-2; 1 μM, 30 min, A) or Pertussis toxin (PTx; 100

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ng/ml, 16h, B). Cells were stimulated with C5aP (10 nM) and PMX-53 (100 nM) and degranulation was determined. In all experiments LAD2 cells were also stimulated by IgE/anti-IgE as a non-GPCR control. Data are mean ± SEM of n=3. Statistical significance was determined by one-way ANOVA with Dunnett's post test to compare differences between vehicle and GPA-2 or PTx treatment. * indicates $p<0.05$.

Fig 5: LAD2 cells express functional MrgX1 and MrgX2 receptors but HMC-1 cells do not. (A) MrgX1 and MrgX2 receptor expression was determined in LAD2 and HMC-1 cells by RT-PCR. Ligands for MrgX1 and MrgX2 BAM-22P (1 μM) and cortistatin (CST) (1 μM) respectively, induced sustained Ca^{2+} mobilization (B) and degranulation (C) in LAD2 mast cells. Statistical significance was determined by two-way ANOVA with Bonferroni's post test. * indicates $p<0.05$. (D) BAM-22P did not induce Ca^{2+} mobilization and CST induced modest Ca^{2+} mobilization in HMC-1 cells. C3a, 10 nM was used as a positive control. Data shown are representative of three similar experiments.

Fig 6: Human $CD34^+$-derived primary mast cells express MrgX1 and MrgX2 and respond to PMX-53 for degranulation and Ca^{2+} mobilization. Human $CD34^+$-derived primary mast cells were stimulated with C5aP, PMX-53, scrambled PMX-53 (PMX-53S) and control PMX-53 (PMX-53C) (1 μM) and percent degranulation (β-hexosaminidase release) was determined (A). MrgX1 and MrgX2 receptor expression was determined in $CD34^+$-derived primary mast cells by RT-PCR (A, inset). C5a (B), PMX-53 (C) and PMX-53S but not PMX-53C (D) induced Ca^{2+} mobilization in $CD34^+$-derived mast cells. Data shown are representative of three similar experiments. Statistical significance was determined by one-way ANOVA with Dunnett's post test. * indicates $p<0.05$.

Fig. 7: PMX-53 utilizes MrgX2 to induce mast cell degranulation. RBL-2H3 cells stably expressing (A) MrgX1 or (B) MrgX2 were incubated with anti-DNP specific IgE (1 μg/mL, 16 hours) and stimulated

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with the indicated peptides (1 μM), substance P (SP, 1 μM) or antigen (DNP-BSA, 30 ng/mL) for 30 min and β-hexosaminidase release was measured. (C and D) MrgX1 and MrgX2-expressing cells were also incubated with Indo-1AM and stimulated with selected peptides (1 μM) and intracellular calcium mobilization was determined. Data shown are representative of three similar experiments. Statistical significance was determined by one-way ANOVA with Dunnett's post test. * indicates $p<0.05$.

Fig. 8: PMX-53 and CST do not activate murine peritoneal and bone marrow-derived mast cells. (A) Murine bone marrow-derived and peritoneal mast cells were incubated with DNP specific mouse IgE (1 μg/mL, 16 hours). Cells were exposed to buffer (control), PMX-53 (1 μM), CST (1 μM) or DNP-BSA (30 ng/mL) for 30 minutes and β-hexosaminidase release was measured. Data are mean ± SEM of n=3. (B) Murine bone marrow-derived mast cells were incubated with DNP specific mouse IgE (1 μg/mL, 16 hours). Cells were incubated with Indo-1AM and were exposed to PMX-53 (1 μM), CST (1 μM) and DNP-BSA (100 ng/ml) and intracellular Ca^{2+} mobilization was determined. Data shown are representative of three similar experiments. Statistical significance was determined by one-way ANOVA with Dunnett's post test. * indicates $p<0.05$.

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Table I: Amino acid sequences of the peptides used

Peptides	Amino acid sequence
C5a peptide (C5aP)	Tyr-Ser-Phe-Lys-Pro-Met-Pro-Leu-dAla-Arg
PMX-53 A	c-Phe-[Orn-Pro-dCha-Trp-Arg]
PMX-53 control peptide (PMX-53C)	Ac-Phe-[Orn-Pro-dCha-Ala-dArg]
PMX-53 scrambled peptide (PMX-53S)	Ac-dCha-Pro-Trp-Phe-Arg-Orn-NH_2
BAM-22P	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys-Arg-Tyr-Gly
Cortistatin-14 (CST)	Pro-[Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Ser-Ser-Cys]-Lys
Substance-P A	rg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2

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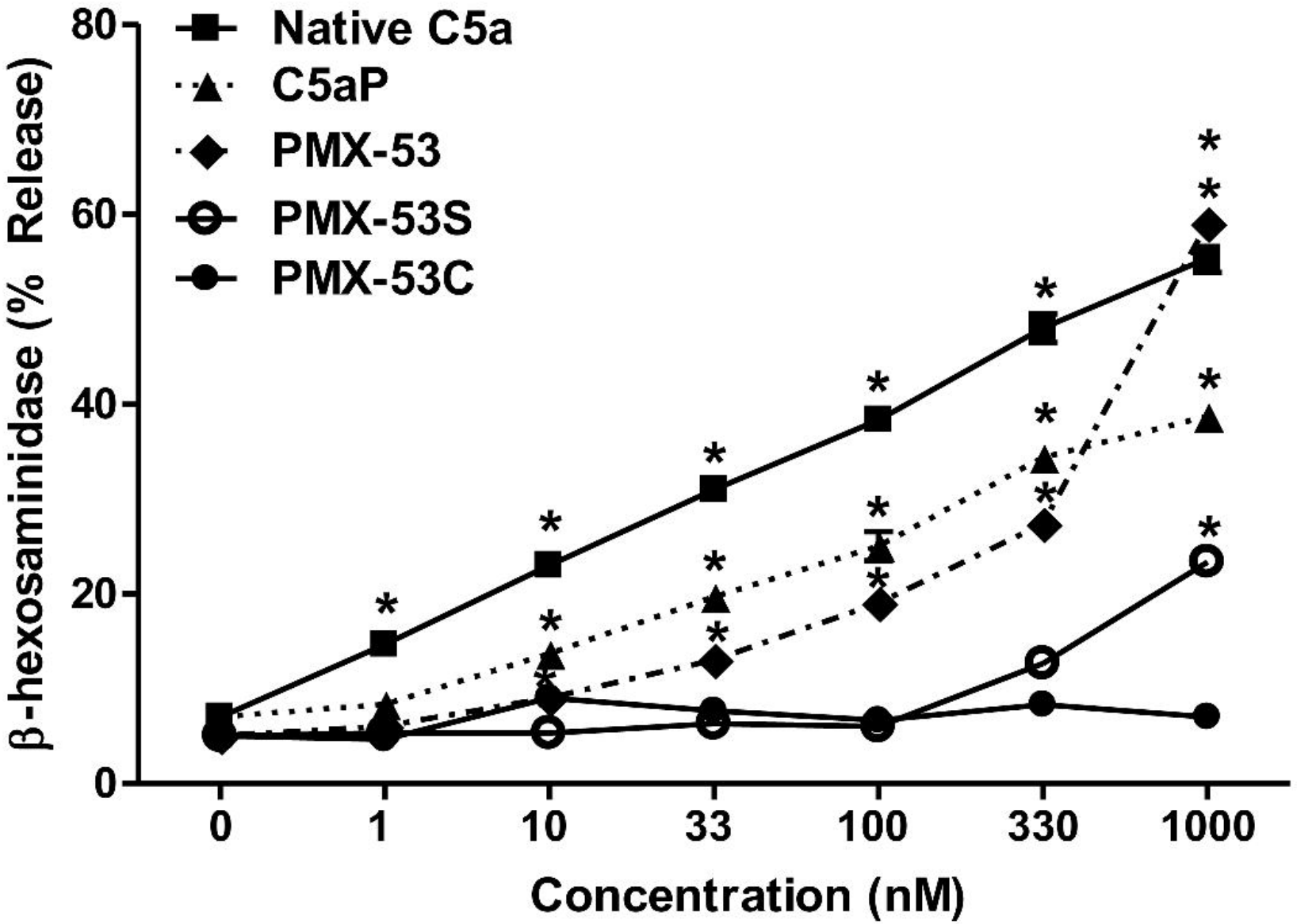
Native C5a
C5aP
PMX-53
PMX-53S
PMX-53C
β-hexosaminidase (% Release)
0
20
40
60
80
0
1
10
33
100
330
1000
Concentration (nM)

Figure 1

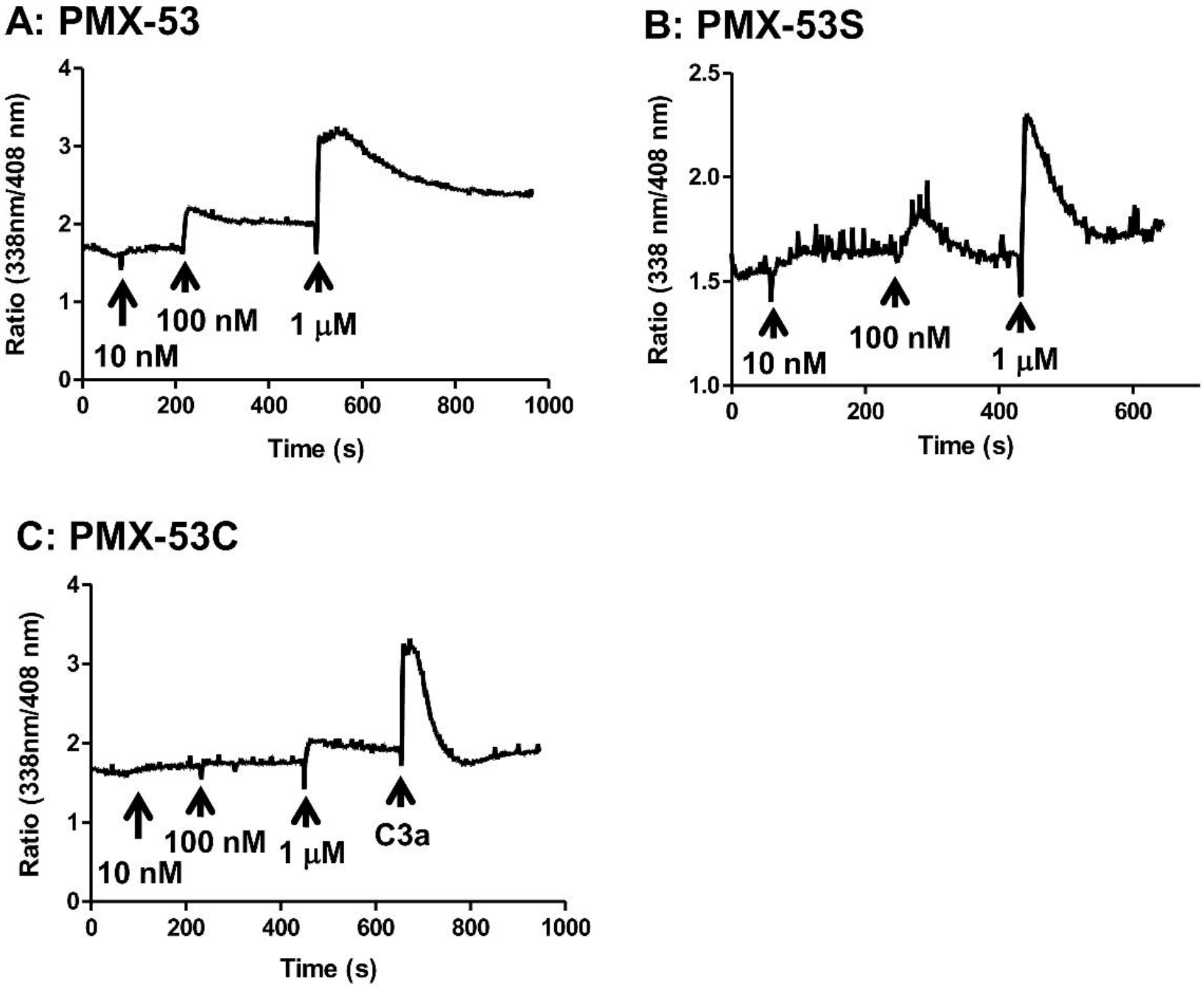
A: PMX-53
Ratio (338nm/408 nm)
Time (s)
10 nM
100 nM
1 μM
B: PMX-53S
Ratio (338 nm/408 nm)
Time (s)
10 nM
100 nM
1 μM
C: PMX-53C
Ratio (338nm/408 nm)
Time (s)
10 nM
100 nM
1 μM
C3a

Figure 2

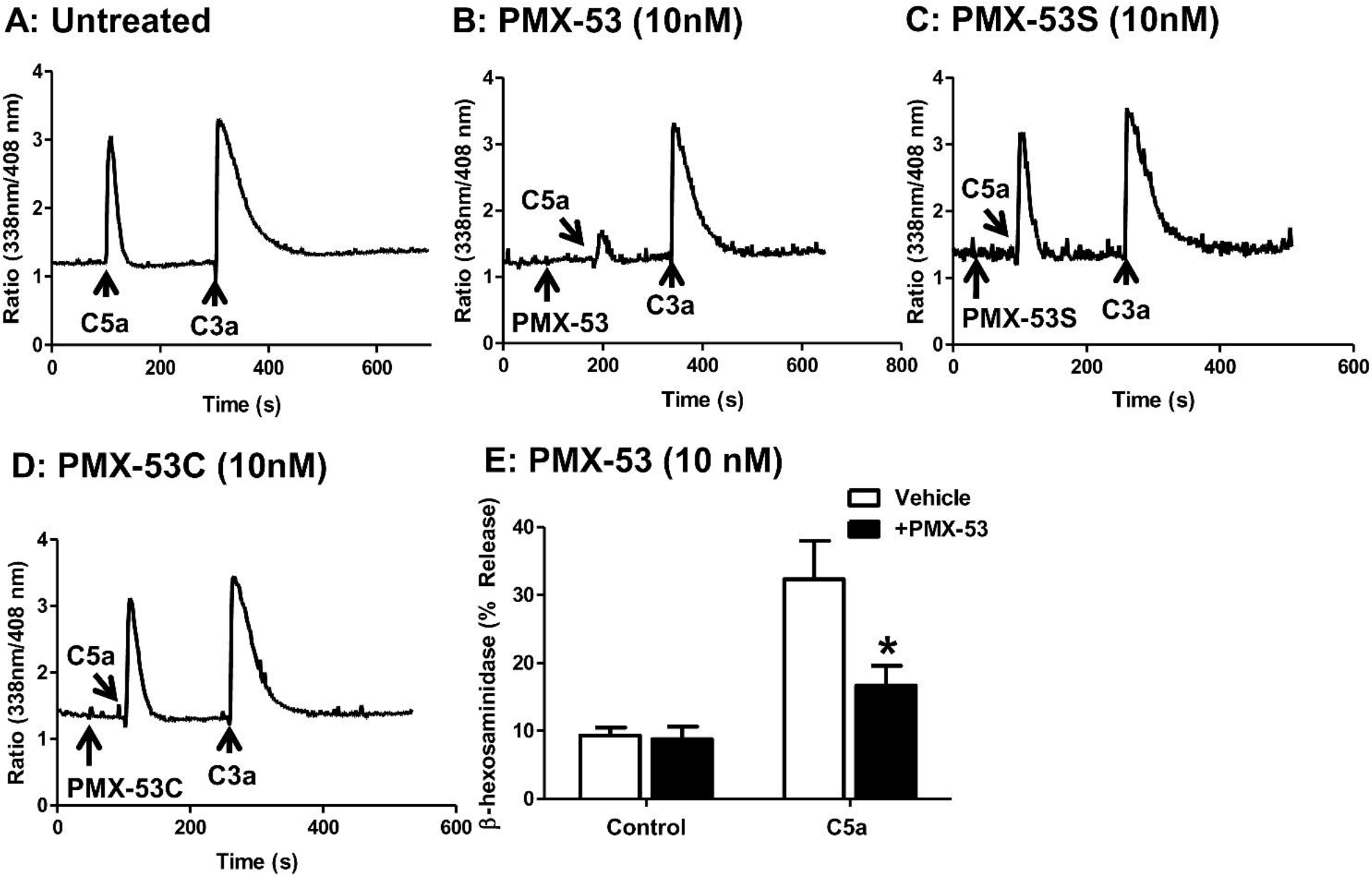
A: Untreated
B: PMX-53 (10nM)
C: PMX-53S (10nM)
Ratio (338nm/408 nm)
Time (s)
C5a
C3a
PMX-53
PMX-53S
D: PMX-53C (10nM)
PMX-53C
E: PMX-53 (10 nM)
Vehicle
+PMX-53
β-hexosaminidase (% Release)
Control
C5a
*

Figure 3

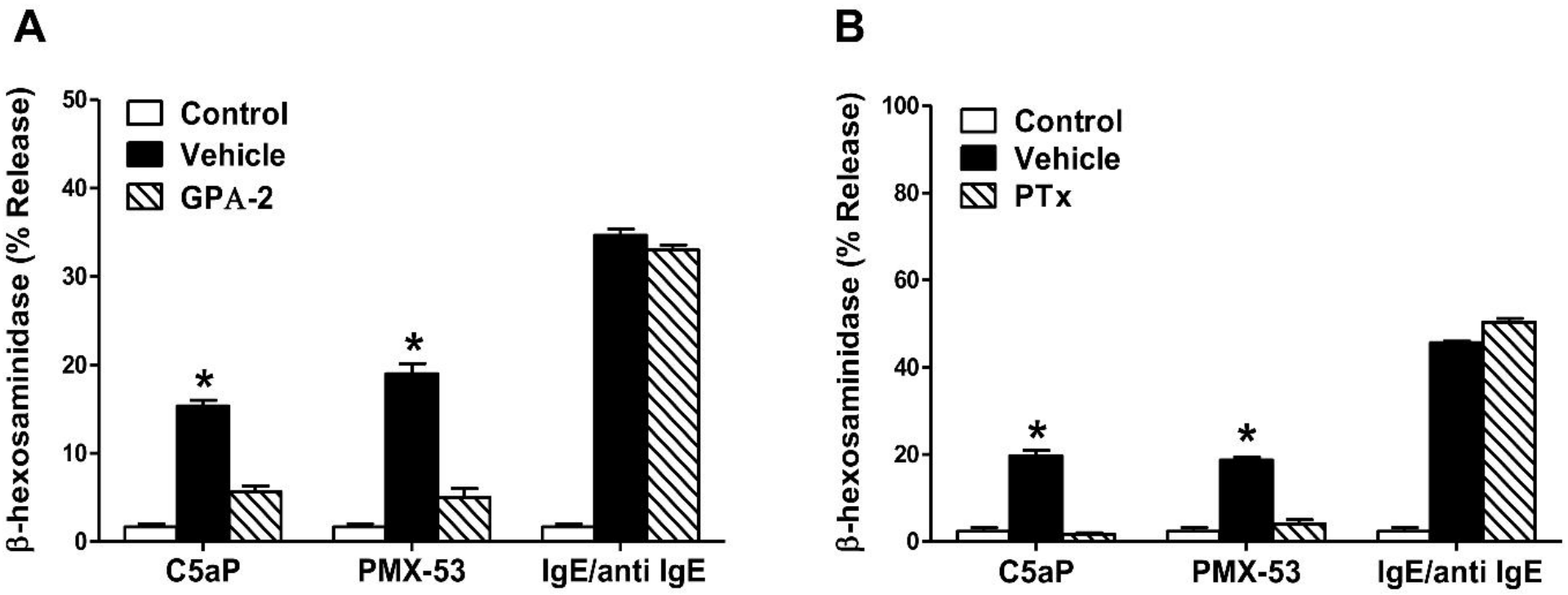
A
β-hexosaminidase (% Release)
50
40
30
20
10
0
Control
Vehicle
GPA-2
*
*
C5aP
PMX-53
IgE/anti IgE
B
β-hexosaminidase (% Release)
100
80
60
40
20
0
Control
Vehicle
PTx
*
*
C5aP
PMX-53
IgE/anti IgE

Figure 4

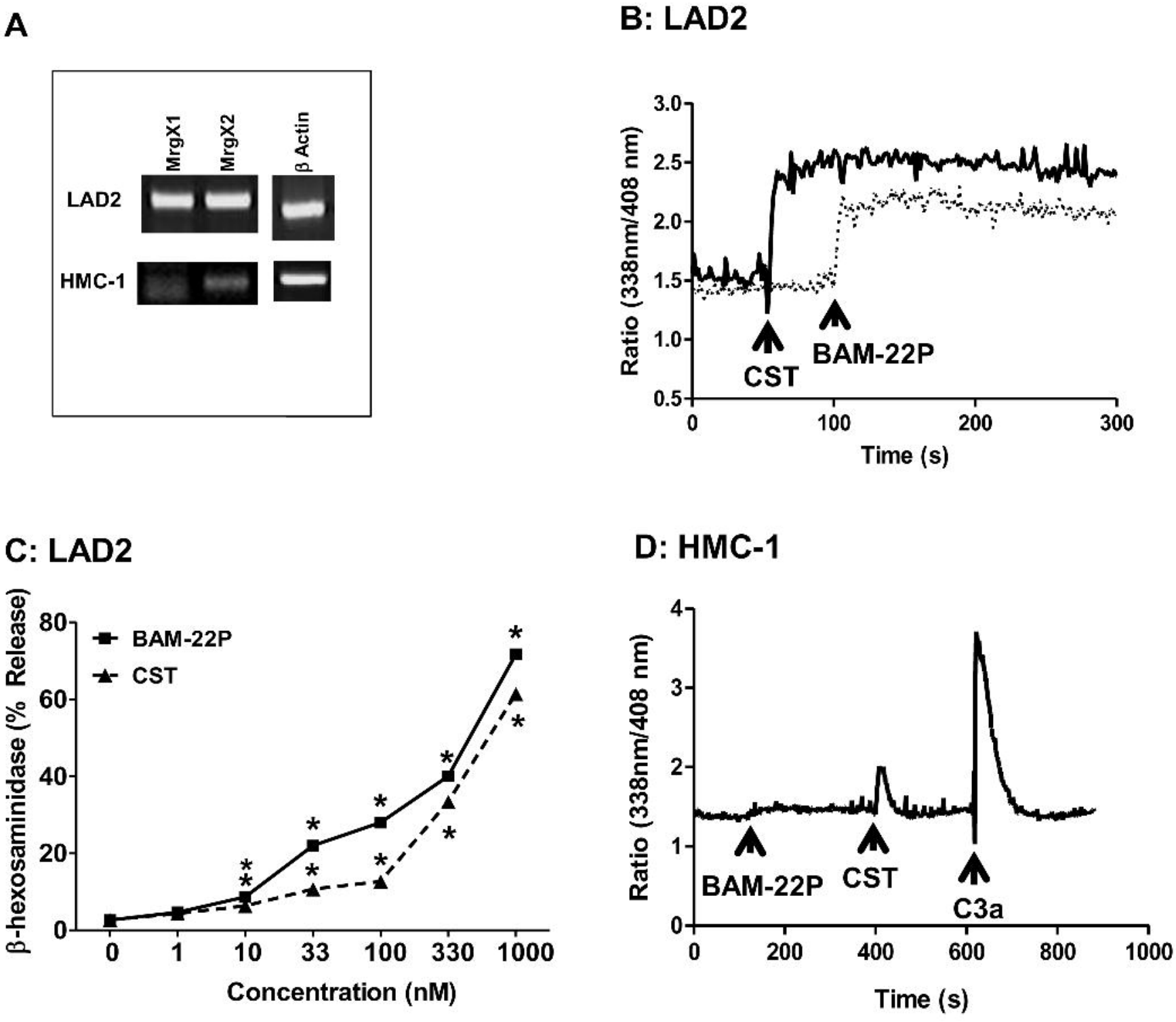
A
MrgX1
MrgX2
β Actin
LAD2
HMC-1
B: LAD2
Ratio (338nm/408 nm)
3.0
2.5
2.0
1.5
1.0
0.5
CST
BAM-22P
0
100
200
300
Time (s)
C: LAD2
β-hexosaminidase (% Release)
80
60
40
20
0
BAM-22P
CST
0
1
10
33
100
330
1000
Concentration (nM)
D: HMC-1
Ratio (338nm/408 nm)
4
3
2
1
0
BAM-22P
CST
C3a
0
200
400
600
800
1000
Time (s)

Figure 5

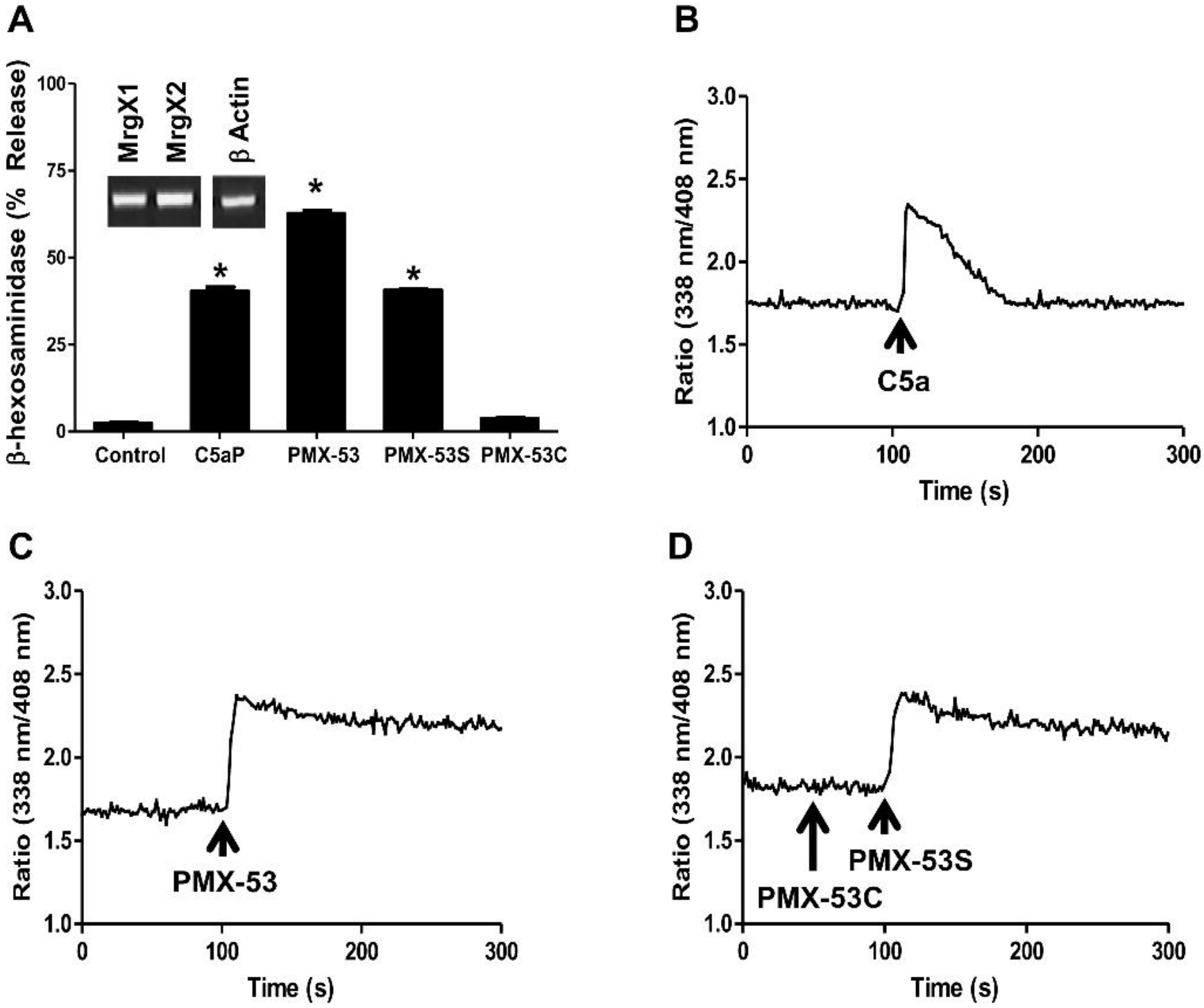
A
β-hexosaminidase (% Release)
100
75
50
25
0
MrgX1
MrgX2
β Actin
*
*
*
Control
C5aP
PMX-53
PMX-53S
PMX-53C
B
Ratio (338 nm/408 nm)
3.0
2.5
2.0
1.5
1.0
C5a
0
100
200
300
Time (s)
C
Ratio (338 nm/408 nm)
PMX-53
Time (s)
D
Ratio (338 nm/408 nm)
PMX-53S
PMX-53C
Time (s)

Figure 6

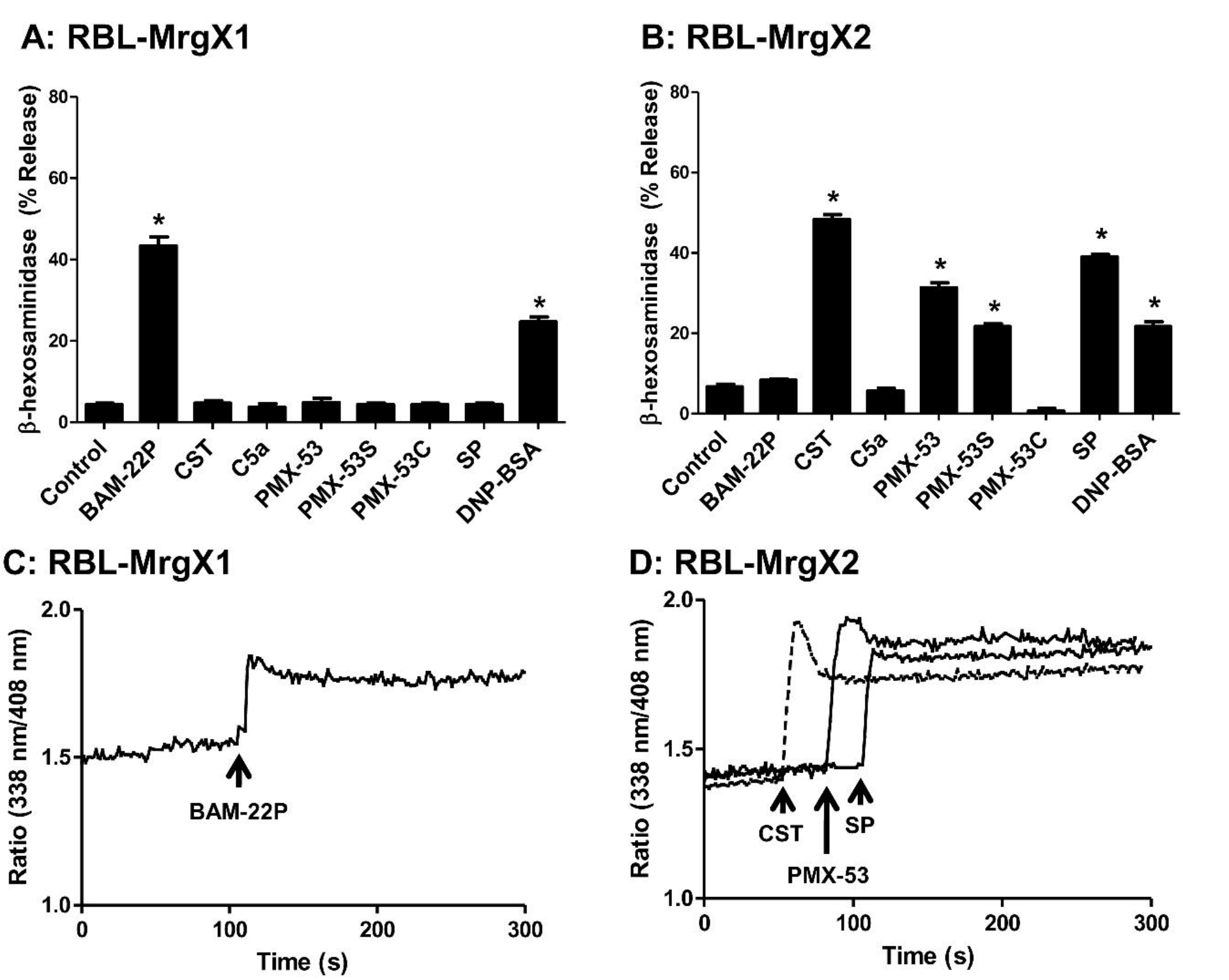
A: RBL-MrgX1
β-hexosaminidase (% Release)
0
20
40
60
80
*
*
Control
BAM-22P
CST
C5a
PMX-53
PMX-53S
PMX-53C
SP
DNP-BSA
B: RBL-MrgX2
β-hexosaminidase (% Release)
0
20
40
60
80
*
*
*
*
*
Control
BAM-22P
CST
C5a
PMX-53
PMX-53S
PMX-53C
SP
DNP-BSA
C: RBL-MrgX1
Ratio (338 nm/408 nm)
1.0
1.5
2.0
BAM-22P
0
100
200
300
Time (s)
D: RBL-MrgX2
Ratio (338 nm/408 nm)
1.0
1.5
2.0
CST
SP
PMX-53
0
100
200
300
Time (s)

Figure 7

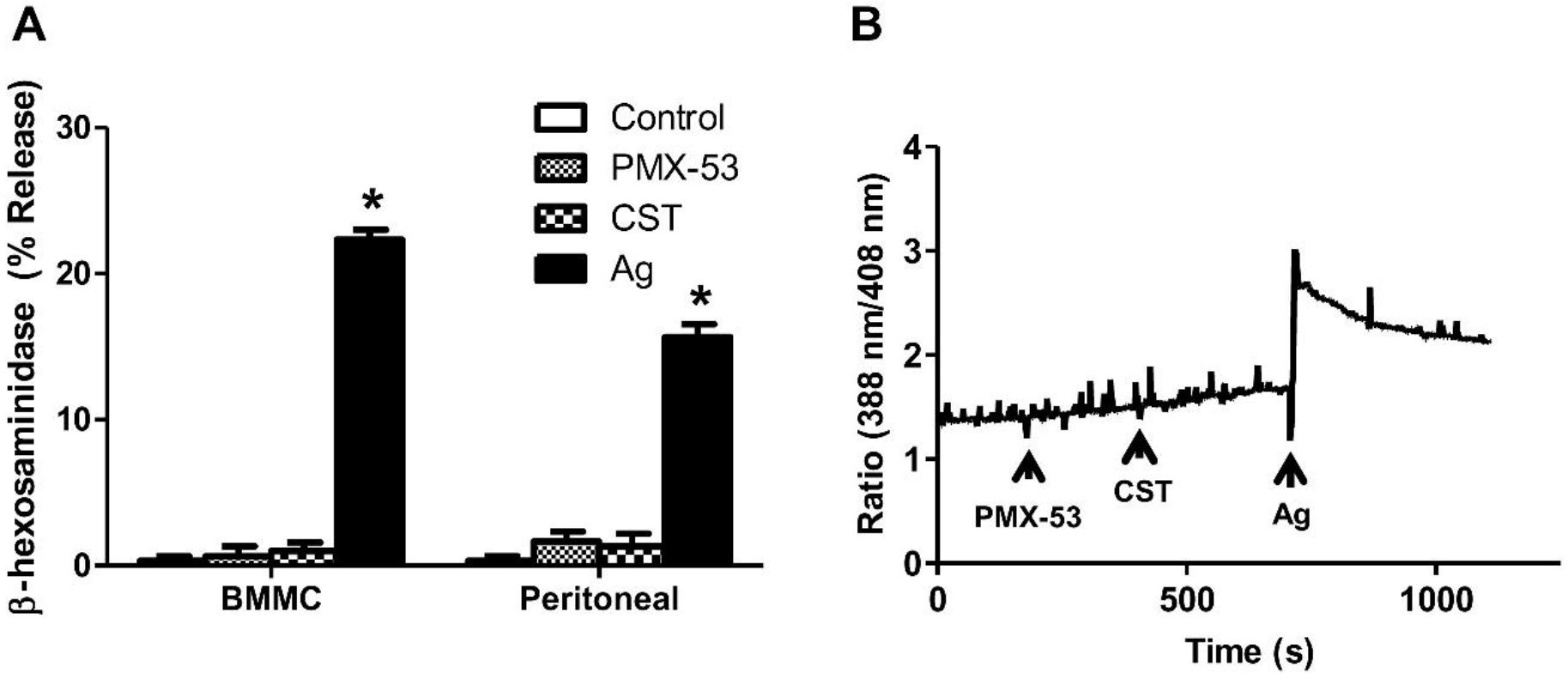
A
β-hexosaminidase (% Release)
30
20
10
0
Control
PMX-53
CST
Ag
*
*
BMMC
Peritoneal
B
Ratio (388 nm/408 nm)
4
3
2
1
0
PMX-53
CST
Ag
0
500
1000
Time (s)

Figure 8