



Published in final edited form as:

J Allergy Clin Immunol. 2017 January ; 139(1): 323–334. doi:10.1016/j.jaci.2016.03.051.

Proteome analysis of mast cell releasates reveals a role for chymase in the regulation of coagulation factor XIIIa levels via proteolytic degradation

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Abstract

Background—Mast cells are significantly involved in IgE-mediated allergic reactions; however, their roles in health and disease are incompletely understood.

Objective—We aimed to define the proteome contained in mast cell releasates upon activation to better understand the factors secreted by mast cells that are relevant to the contribution of mast cells in diseases.

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Disclosure of Conflict of Interest: The authors declare no competing financial interests.

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Methods—Bone marrow derived-cultured mast cells (BMCMCs) and peritoneal cell-derived mast cells (PCMCs) were used as “surrogates” for mucosal and connective tissue mast cells, respectively, and their releasate proteomes were analyzed by mass spectrometry.

Results—Our studies showed that BMCMCs and PCMCs produced substantially different releasates following IgE-mediated activation. Moreover, we observed that the transglutaminase, coagulation factor XIIIa, was one of the most abundant proteins contained in the BMCMC releasates. Mast cell-deficient mice exhibited increased FXIIIa plasma and activity levels as well as reduced bleeding times, indicating that mast cells are more efficient in their ability to down-regulate FXIIIa than in contributing to its amounts and functions in homeostatic conditions. We found that human chymase and mouse mast cell protease (mMCP)-4 (the mouse homologue of human chymase) had the ability to reduce FXIIIa levels and function via proteolytic degradation. Moreover, we found that chymase deficiency led to increased FXIIIa amounts and activity, as well as reduced bleeding times in homeostatic conditions and during sepsis.

Conclusions—Our study indicates that the mast cell protease content can shape its releasate proteome. Moreover, we found that chymase plays an important role in the regulation of FXIIIa via proteolytic degradation.

Keywords

Mast cells; proteases; chymase; proteomics

INTRODUCTION

Mast cells are hematopoietic progenitor-derived, granule-containing immune cells that are widely distributed in tissues that interact with the external environment, such as the skin and mucosal tissues. Although it is well known that mast cells are significantly involved in IgE-mediated allergic reactions, recent studies have shown that mast cells and their granule proteases have pleiotropic regulatory roles in other immunological responses and diseases, such as in bacterial and parasite infections, sepsis, auto-immune disease, and cancer.¹ Many of these studies, however, were biased to investigate mouse models in which mast cells were hypothesized to play key roles based on *in vitro* studies or conditions in which mast cell numbers or their mediators were increased, suggesting that our understanding of the contribution of mast cells to health and disease is limited. In this study, we adopted an unbiased approach with the potential to provide a more comprehensive assessment of how mast cells may influence biological processes by characterizing mast cell releasate proteomes via mass spectrometry analysis.

Mast cells express protease profiles that vary amongst species and different mast cell subsets. In humans, mast cells either express tryptase (α and β) only (known as the MC_T subclass) or tryptase, chymase, and mast cell carboxypeptidase A (CPA) (known as the MC_{TC} subclass). In mice, mast cells are divided into connective tissue (CTMC) and mucosal mast cell (MMC) subtypes. CTMCs predominantly express the β -chymase, mouse mast cell protease 4 [mMCP-4] and the α -chymase, mMCP-5, whereas MMCs predominantly express two different β -chymases, mMCP-1 and mMCP-2. Additionally, in the C57BL/6 mouse background, CTMCs also express a tetrameric tryptase (mMCP-6) as well as CPA.² We and

others have shown that mast cell proteases can cleave certain mediators released by mast cells into inactive or active fragments. For example, we reported that mMCP-4, the mouse homolog of human chymase in substrate specificity,³ prevents hyper-inflammation in severe sepsis via proteolytic degradation of tumor necrosis factor (TNF).⁴ Consequently, it is important to understand how mast cell phenotypes and their protease content may shape their mediator release profile upon activation. Herein, we characterized the releasates generated by bone marrow derived-cultured mast cells (BMCMCs) and peritoneal cell-derived mast cells (PCMCs) following IgE-mediated activation as “surrogates” for MMCs and CTMCs, respectively.^{5,6}

Our proteomics studies indicate that BMCMCs and PCMCs produced substantially different releasates. As a specific example, we observed that the transglutaminase, coagulation factor XIIIa (FXIIIa), was one of the most abundant proteins in the IgE-mediated BMCMC releasate. In contrast, the PCMC releasate did not contain FXIIIa. Therefore, we investigated the involvement of CTMC-specific proteases in the down-regulation of FXIIIa, and we observed that mast cell chymase could proteolytically degrade FXIIIa and diminish its function during homeostatic and septic states.

METHODS

For detailed methods, including the mice used, experimental protocols and procedures, and statistical analysis, please see the Methods section in this article’s Online Repository www.jacionline.org.

RESULTS

Proteome profiling of mast cell-releasates

To define the BMCMC and PCMC releasates following IgE-mediated activation, we utilized a mass spectrometry shotgun proteomics approach. Specifically, IgE-DNP sensitized BMCMCs and PCMCs were stimulated with DNP-HSA antigen for 6h. Then, the supernatants were collected, concentrated, and subjected to LC-MS/MS to identify the IgE-mediated differentially produced proteins from these MCs (Fig. 1A). We identified 91 proteins that were unique to PCMCs (Table 1 and Suppl. Table 1), 390 proteins that were unique to BMCMCs (Table 2 and Suppl. Table 2), and 674 proteins that were extracellularly produced by both BMCMCs and PCMCs (Table 3 and Suppl. Table 3) (Fig. 1B). We associated the proteins identified in the releasates with functional annotations. By gene ontology (GO) analysis, the commonly enriched biological process categories in the BMCMC and PCMC releasates were linked to metabolic processes (e.g., carbohydrate/protein metabolism and glycolysis) as well as intracellular protein trafficking, protein folding, cell structure and motility, and endocytosis (Table 3 + Suppl. Table 4). Synthesis of newly formed mediators and mediator secretion are two main mast cell functions upon activation that may require the metabolic and actin cytoskeleton remodeling processes observed in our GO analysis. Processes associated with protein alteration, such as amino acid, protein metabolism and proteolysis were the main processes found amongst the unique proteins contained in the PCMC releasates (Suppl. Table 5). The enriched categories from the unique BMCMC releasates (not found in the PCMC releasates) were associated with

blood clotting, immune response regulation (e.g., cytokine and chemokine mediated signaling pathways), and immunity and defense (Suppl. Table 6). These data suggest not only that BMCMCs produce factors related to immune response regulation but also that PCMCs either do not produce these factors or they are more efficient in their down-regulation via proteolysis.

Mast cells produce and release FXIIIa upon activation

Our proteomics study revealed that the transglutaminase FXIIIa was one of the most abundant proteins released by BMCMCs upon activation. Transglutaminases are multifunctional proteins (e.g., enzymatic and scaffolding functions) that are known to regulate cellular processes in homeostatic and disease states.⁷ FXIIIa is one of 9 transglutaminases in humans, all of which (with the exception of band 4.2, which is catalytically inactive) share the same catalytic site sequence. FXIIIa becomes catalytically active once it dissociates from FXIIIb and a 37 AA peptide is cleaved from its N-terminus (the active form of FXIIIa is called FXIIIa). FXIIIa is best known for its involvement in fibrin cross-linking for clot stabilization, and it is the only transglutaminase known to be involved in the coagulation cascade.⁸ FXIIIa-deficiency can result in moderate to severe hemorrhagic diathesis.⁹ Furthermore, FXIIIa can also contribute to processes in which mast cells play a key role, such as wound healing, cell migration and innate immunity.¹⁰ FXIIIa is present in the cytoplasm of platelets and monocytes/macrophages;¹¹ however, FXIIIa expression has never been reported in mast cells.

By using western blot analysis, we estimated that BMCMCs contained 800 fg FXIIIa per cell (Suppl. Fig. 1), which is around 10-fold greater than what is reported for platelets.¹² There is evidence for FXIIIa entering the alternative secretory pathway; however, its secretion by an immune cell has not been demonstrated.¹³ Our mass spectrometry data (Table 2) and western blot analysis (Fig. 2A) indicate that IgE/antigen-activated BMCMCs secrete FXIIIa upon activation.

In contrast to monocytes,¹⁴ BMCMCs did not display increased FXIIIa mRNA expression after activation (Fig. 2B) suggesting that FXIIIa protein is pre-formed in mast cells. Indeed, we observed that intracellular FXIIIa exhibited a typical granular pattern in BMCMCs (Fig. 2C), confirming that FXIIIa is stored in mast cell granules.

Mast cell deficiency leads to increased FXIIIa levels and reduced bleeding time

To investigate the relative contribution of mast cells to FXIIIa plasma levels in homeostatic conditions, we examined the *Cpa3*-Cre; *ROSA^{DTA/DTA}* mast cell- and basophil-deficient mice (a c-kit independent mast cell- and basophil-deficient strain).¹⁵ We found that *Cpa3*-Cre; *ROSA^{DTA/DTA}* mice exhibited increased FXIIIa plasma (Figs. 3A and 3B) and FXIIIa activity levels (Fig. 3C) indicating that mast cells and/or basophils significantly contribute to down-regulation of FXIIIa levels rather than to contribute to production of FXIIIa circulating pool in naïve mice. To determine whether basophils contribute to FXIIIa down-regulation we used *Mcpt8^{DTR}* mice, which express DTR only in basophils and in which basophils can be selectively depleted after a single i.p. injection of DT.¹⁶ Plasma from basophil-depleted mice (*Mcpt8^{DTR/+}*) exhibited similar plasma FXIIIa amounts as basophil

competent mice (*Mcpt8^{+/+}*) (Suppl. Fig. 2) indicating that mast cells, and not basophils, down-regulate FXIIIa under homeostatic conditions.

To determine the physiological impact of the increased FXIIIa and FXIIIa activity levels due to mast cell deficiency, we assessed bleeding times in *Cpa3-Cre; ROSA^{DTA/DTA}* mice because bleeding time is a measurement that is directly linked to active FXIIIa levels.¹⁷ We observed that *Cpa3-Cre; ROSA^{DTA/DTA}* mice exhibited reduced bleeding times than their littermate controls (Fig. 3D) suggesting that mast cell-mediated down-regulation of FXIIIa amounts can influence clotting.

Overall, our findings indicate that despite the ability of mast cells to produce FXIIIa, mast cells mainly contribute to the down-regulation of FXIIIa levels and consequently reduce clotting during homeostatic conditions.

Mouse chymase down-regulates FXIIIa levels and function via proteolysis

PCMC lysates contain lower FXIIIa amounts than BMCMC lysates (240 fg FXIIIa per cell). Moreover, our mass spectrometry data revealed that PCMCs do not generate detectable amounts of FXIIIa in their supernatants upon activation in contrast to BMCMCs (Table 1, Fig. 4). This observation led us to hypothesize that PCMCs are more efficient in FXIIIa down-regulation than in its production. Therefore, we decided to investigate mechanisms by which PCMCs down-regulate FXIIIa levels. Prior studies using mouse recombinant mast cell proteases and non-specific protease inhibitors suggested that mast cell proteases are able to down-regulate certain cytokines and chemokines via proteolytic degradation.^{18–21} Our proteomics data showed that IgE/antigen-activated PCMCs generate releasates that are richer in their protease content than releasates generated by BMCMCs. In fact, mMCP-4, the mouse homologue of human chymase,²² was one of the most abundant proteins found in the PCMC releasate and one of the least abundant amongst the BMCMC releasate proteins (Tables 1 and 2, Suppl. Tables 1 and 2, and Suppl. Fig. 3). This finding is in agreement with previous reports by us and other groups that BMCMCs generated from mice in the C57BL/6 background express low mMCP-4 levels.^{4, 23, 24} As the FXIIIa amino acid sequence contains potential cleavage sites for chymase,³ we investigated whether mMCP-4 can down-regulate FXIIIa via proteolysis. To test this hypothesis, we used mMCP-4-deficient mice (*Mcpt4^{-/-}* mice) which do not exhibit any marked defects in the concentrations of other mast cell proteases, including mMCP-5, -6 or carboxypeptidase A, in their peritoneal mast cells.²² Our mass spectrometry data indicated that *Mcpt4^{+/+}* PCMC releasates contained lower FXIIIa spectral count levels than the releasates generated by *Mcpt4^{-/-}* PCMCs (Fig. 4A). Moreover, we found that the *Mcpt4^{+/+}* PCMC lysates caused more extensive FXIIIa degradation than the *Mcpt4^{-/-}* PCMC lysates (Fig. 4B), indicating that chymase contributes to FXIIIa fragmentation. Analysis of IgE/DNP-HSA-activated PCMCs that were obtained from *Mcpt4^{+/+}* or *Mcpt4^{-/-}* mice indicated that chymase does not substantially influence FXIIIa mRNA production (Suppl. Fig. 4), indicating that FXIIIa down-regulation by chymase mainly occurs at the post-transcriptional level.

Similarly to the mast cell- and basophil-deficient mouse, *Mcpt4^{-/-}* mice exhibited increased FXIIIa plasma (Figs. 4C and 4D) and FXIIIa activity levels (Fig. 4E) indicating that chymase significantly contribute to down-regulate FXIIIa levels and function. Moreover,

Mcpt4^{-/-} mice exhibited reduced bleeding times than *Mcpt4*^{+/+} mice (Fig. 4F) suggesting that chymase-mediated down-regulation of FXIIIa amounts also influences clotting.

Therefore, our data suggest that FXIIIa may be a potential target for mMCP-4 proteolytic activity, and hence mast cells contribute to FXIIIa down-regulation.

Human chymase degrades FXIIIa and diminishes its activity

mMCP-4 and human chymase share similar substrate specificity.²⁵ Therefore, we hypothesized that human chymase may also have the ability to down-regulate FXIIIa via proteolysis. FXIIIa that was incubated with human skin chymase led to extensive fragmentation of FXIIIa, but not FXIIIb (Fig. 5A and mass spectrometry data not shown). We performed *in vitro* cleavage reactions containing FXIIIa and increasing chymase concentrations to identify chymase cleavage sites. By focusing on the peptides identified in the FXIIIa samples that were incubated with the lowest utilized chymase concentration (0.001U), we identified peptides containing non-tryptic termini that represent 6 potential chymase cleavage sites (Fig. 5B). Using label-free quantitative mass spectrometry, we found that these putative chymase cleavage products increased in abundance with increasing chymase concentrations, as depicted for the cleavage site at F574 (Fig. 5C). Additionally, for 5 out of 6 of these high affinity cleavage sites, we found that chymase cleavage followed aromatic residues (P1) (Fig. 5D). Furthermore, the amino acids that were two residues upstream and downstream of the cleavage site (P3 and P2') were enriched for aliphatic or negatively charged residues (Fig. 5D).

These data are consistent with the reported substrate specificity for human chymase²⁵ and suggest that human chymase is sufficient to specifically cleave FXIIIa.

Interestingly, our mass spectrometric analysis of FXIIIa degradation showed that human chymase did not cleave FXIIIa at all of the potential cleavage sites. One of the possible explanations is that chymase accessibility to certain cleavage sites may be reduced by the presence of charged or bulky amino acids in P4-P2 or P3'-P4'. In fact, cleavage at F574 may not be favored by chymase over other sites according to substrate specificity; however, 3D structure analysis revealed that F574 is a very accessible site for chymase cleavage (data not shown). To confirm that the FXIIIa fragmentation data obtained with our mass spectrometry analysis were derived from chymase activity, we designed a peptide (GVP) spanning the chymase site, F574, which could compete with FXIIIa for chymase-mediated cleavage (Suppl. Fig. 5A). Our *in vitro* data indicated that GVP could prevent FXIIIa degradation by human chymase and *Mcpt4*^{+/+} PCMC lysates (Suppl. Figs. 5B and 5C) indicating that FXIIIa fragmentation was caused by chymase proteolytic activity.

To assess whether chymase-mediated FXIIIa degradation leads to the generation of peptides with reduced biological activity, we performed a ROTEM clot lysis assay with the FXIIIa digests. Chymase alone had no effect on fibrin clot formation by thromboelastography, indicating that chymase could not cleave and inactivate thrombin-activated FXIIIa when it was added simultaneously with FXIIIa and thrombin to this assay (data not shown). FXIIIa incubation with chymase prior to ROTEM impaired the ability for FXIIIa to strengthen the fibrin clot (Fig. 5E); however, it did not affect clot stability (Fig. 5F) demonstrating that

FXIIIa cleavage by chymase leads to the generation of FXIIIa fragments with reduced biological activity.

Chymase-deficient mice exhibit increased plasma FXIIIa levels and activity during experimental sepsis

Taking into account our *in vitro* data, we investigated whether chymase-FXIIIa interactions influence disease outcome. For this purpose, we investigated chymase-FXIIIa interactions in sepsis because fibrinolysis and its down-regulation is a key component of the septic coagulation response, and increased FXIIIa activity levels correlate with disease severity and fatality in sepsis.²⁶ *Mcpt4^{+/+}* mice exhibited a significant reduction in FXIIIa plasma and activity levels when compared with naïve *Mcpt4^{+/+}* mice (Figs. 4C, 6A and 6B) at 24 h after experimental sepsis induced by cecal ligation and puncture (CLP). This observation seemed to indicate that FXIIIa is consumed during sepsis as observed for other coagulation factors in this disorder.²⁷ *Mcpt4^{-/-}* mice exhibited reduced downregulation of FXIIIa plasma and activity levels when compared with naïve *Mcpt4^{-/-}* mice or *Mcpt4^{+/+}* mice at 24 h after CLP (Figs. 4A, 6A and 6B) indicating that FXIIIa consumption during sepsis is partially mediated by chymase. On the contrary, *Mcpt4^{+/+}* and *Mcpt4^{-/-}* mice exhibited similar plasma fibrinogen levels after CLP (Suppl. Fig. 6), suggesting that the chymase-mediated effects on FXIIIa activity levels were not an indirect result of changes in upstream mediators of the coagulation cascade.

We also observed that the *Mcpt4^{-/-}* mice did not have increased bleeding times, as were observed with the *Mcpt4^{+/+}* mice (Fig. 6D), suggesting that mMCP-4 mediated downregulation of FXIIIa plasma and activity levels at 24 h after CLP impaired their capacity to stop bleeding. Therefore, these data demonstrate that mMCP-4 not only degrades FXIIIa *in vitro* but it also reduces its function, which likely causes alterations in the clotting system during sepsis.

DISCUSSION

Accumulating *in vitro* and *in vivo* evidence indicates that mast cell functions may be much broader than their well-defined contribution to IgE-associated immune-responses. In this study, we characterized mast cell releasates in order to further understand the contribution of mast cells to health and disease. For this purpose, we used proteomics as an unbiased approach to characterize mast cell secretomes. Previous studies used mass spectrometry analysis to dissect signaling pathways that are involved in IgE-mediated mast cell activation;^{28,29,30} however, a characterization of the mediators that are released by mast cells upon activation has not yet been reported. Therefore, we characterized the releasates generated by BMCMCs and PCMCs upon IgE/antigen-mediated activation. BMCMCs and PCMCs were utilized because these cells can be generated in the large numbers that are required for mass spectrometry analysis (25 million cells/treatment), and they are the closest *in vitro* “surrogates” to the two main murine mast cell subtypes, MMCs and CTMCs. Moreover, our BMCMC and PCMC culture conditions have been standardized and validated by us and other groups. However, we cannot dismiss the fact that the local microenvironment and the mechanisms of mast cell activation (IgE-dependent and IgE-

independent) can influence the spectrum of mediators produced and released by mast cells from different locations during normal and pathological conditions. For example, we found that BMCMCs released reduced amounts of mast cell proteases upon IgE/antigen activation; however, MMCs can up-regulate certain proteases, such as the mast cell chymase, mMCP-1, during parasitic infections or following exposure to IL-9 or IL-10.³¹ Therefore, further studies using alternative mass spectrometry methods that allow for low cell number usage will be important to replicate our work using freshly isolated cells from naïve and challenged mice. In the meanwhile, the validation of unexpected or unknown mast cell mediators that are found studying cultured mast cells may be possible by independent verification of their expression using databases such as the one provided by the Immunological Genome Project (ImmGen, <http://www.immgen.org/databrowser/>) when complemented with targeted proteomics. By using Immgen, we retrieved information that freshly isolated mast cells from the trachea, tongue, esophagus, skin, and peritoneum express FXIII A mRNA, as we found for BMCMCs and PCMCs.

The mechanisms that regulate and/or shape the extracellular proteomes that are generated by different mast cell populations are unclear. We found that releasates from IgE/antigen-activated PCMCs were enriched in mediators that are associated with proteolysis as a main biological function (Table 1). In contrast, releasates from IgE/antigen-activated BMCMC contained a large number of mediators with immune regulatory capabilities and reduced proteolytic activity. From this observation, we hypothesized that proteolytic cleavage is a mechanism that can shape the profile of mediators produced by mast cells. For example, a mast cell type with a reduced number and/or diversity of proteases, such as BMCMCs, may be prone to regulate immune responses through the production of mediators, such as cytokines or chemokines, after IgE/antigen-mediated activation. Our proteomics findings were especially notable when we investigated how mast cells that express different protease profiles could differentially regulate the levels of the same mediator, FXIII A, and affect its function during healthy and septic conditions. We focused our attention on FXIII A due to its high abundance in the BMCMC releasates and undetectable levels in the PCMC releasates. Based on our proteomics findings pertaining to the protease profiles, we hypothesized that while BMCMCs contribute to FXIII A production, PCMCs mainly contribute to FXIII A degradation via proteolysis upon IgE-mediated activation.

The capability of mast cells to secrete FXIII A suggests that these cells may contribute to plasma FXIII A levels in addition to the FXIII A that is potentially produced by platelets and other myeloid cells.³² However, we found that mast cell-deficient mice exhibited increased total and active FXIII A plasma levels (Figs. 3A, 3B and 3C). Moreover, mast cell deficient mice also exhibited reduced bleeding times (Fig. 3D).¹⁷ Overall, these observations suggest that mast cells may significantly influence clotting processes through FXIII A down-regulation. Our findings with mMCP-4 deficient mice (Fig. 4) suggest that chymase is involved in the down-regulation of FXIII A amounts and function under homeostatic conditions.

It has been shown that murine³³ and rat chymase³⁴ can be constitutively secreted by naïve mast cells. Therefore, we think that constant or intermittent release of mast cell-derived chymase downregulates FXIII A amounts and function. The mechanism by which mast cells

release chymase in unchallenged rodents is unknown but it may involve piecemeal degranulation as observed for mast cells from the human intestine.³⁵

What is the relevance of FXIIIa-chymase interactions during disease? To answer this question, we utilized the CLP model of sepsis because diminished fibrinolysis is a key component of the septic coagulation response³⁶ and because we observed that chymase plays a key role in protecting from sepsis by proteolytically cleaving inflammatory mediators that exacerbate the disease.⁴ We found that chymase deficiency in mice subjected to CLP partially restored their plasma FXIIIa levels to those observed in naïve mice (Figs. 3B and 6B), suggesting that chymase released from activated mast cells may degrade FXIIIa during CLP. Notably, neutrophil elastase can also inactivate FXIIIa,³⁷ which makes our findings even more noteworthy considering that *Mcpt4*^{-/-} mice exhibit an augmented hyperinflammatory response, which is mainly neutrophilic, during CLP.⁴ Septic *Mcpt4*^{-/-} mice exhibited reduced bleeding times, likely as a consequence of their increased FXIIIa activity levels (Figs. 6C and 6D). However, we did not detect fibrin deposition in the kidneys of *Mcpt4*^{-/-} mice, which is indicative of septic disseminated intravascular coagulation (DIC), a potential cause of acute kidney injury that *Mcpt4*^{-/-} mice develop after CLP.⁴ This evidence suggests that the increased FXIIIa activity levels we observed in the chymase-deficient mice did not cause significant coagulopathy alterations. These observations are consistent with the fact that severe CLP-induced sepsis, as is the septic phenotype that *Mcpt4*^{-/-} mice develop after CLP surgery, is a weaker inducer of DIC.³⁸ FXIII-deficiency impairs myeloid cell migration^{39,40} and inflammation after myocardial infarction.⁴¹ Therefore, FXIIIa may be able to contribute to the hyper-inflammatory response that *Mcpt4*^{-/-} mice develop after CLP,⁴ by inducing myeloid cell mobilization.

We can only speculate for now on when mast cell-mediated production of FXIIIa may influence disease outcomes or may overcome its degradation by chymase. For example, mast cell-derived FXIIIa may prevent pathogen dissemination that contributes to pathogen entrapment into clots,⁴² or contribute to the coagulopathies that are observed with systemic mastocytosis.⁴³ However, we should point out that this assumption comes with the caveats that mast cells produce other mediators with the ability to influence coagulation, such as heparin and tryptase⁴⁴ and there are additional cellular sources of FXIIIa.⁸ Moreover, mast cell activation in non-allergic disorders may involve certain IgE-independent stimuli to which CTMCs may be more responsive than MMCs, leading to a more pronounced reduction in FXIIIa levels and activity than that observed after IgE-dependent mast cell activation. Therefore, we believe that further studies will be necessary to assess the contribution of mast cell-derived FXIIIa to health and disease, ideally using mice with FXIIIa-deficiency in mast cell-specific subsets.

The relevance of our murine findings regarding the contribution of FXIIIa-mast cell interactions in human homeostasis and disease still requires investigation. We performed a pilot study in which we investigated FXIIIa expression in nasal polyp mast cells from chronic rhinosinusitis patients. We found that a subset of mast cells located in the epithelium submucosa and around the glands of nasal polyps expressed FXIIIa (Suppl. Fig. 7). Interestingly, FXIIIa expressing cells within glands are rare. These observations correlate with the chymase expression levels that were recently reported for mast cells in nasal polyps,

in which chymase expression levels were low in mast cells that were located in the epithelium and high for mast cells located in the nasal polyp glands.⁴⁵ These findings raise questions regarding the contribution of mast cells in nasal polyp development. In fact, our findings suggest that gland mast cells may have a beneficial effect by reducing excessive fibrin deposition in nasal polyps via chymase-mediated proteolytic degradation of FXIIIa that is produced by either mast cells themselves or by M2 macrophages.⁴⁶

Conclusions

Overall, our studies in mice and humans suggest that mast cells have the ability to produce and degrade FXIIIa depending on their chymase expression profile: mast cells expressing chymase degrade FXIIIa, while mast cells that do not express chymase mainly produce FXIIIa. We believe that our findings exemplify a new level of complexity for mast cells in health and disease: the mast cell protease content can shape its extracellular mediator profile.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding: Research for this work was supported by grants for A.M.P. from the National Institute of Health (NIH) (HL113351-01) and American Heart Association (12GRNT9680021), for R.G.J. from the NIH (5R00HL103768-04), and a fellowship for N.J.S. from the American Association of Immunologists (2015 AAI Careers in Immunology Fellowship). N.J.W. is supported, in part, by the National Center for Advancing Translational Sciences (NCATS) (grant KL2 TR000421), a component of the NIH. S.R.R. was supported by the Parker B. Francis Fellowship. M.A. was supported by the Swedish Research Council. This work is supported in part by the University of Washington's Proteomics Resource (UWPR95794).

We thank Dr. Piper Treuting for the critical advice regarding histological studies with chymase-deficient mice and Dr. Hajime Karasuyama for the *Mcpt-8-DTR* mice.

Abbreviations used

BMCMCs	bone marrow derived-cultured mast cells
PCMCs	peritoneal cell-derived mast cells
FXIIIa	coagulation factor XIIIa
mMCP	mouse mast cell protease
CPA	carboxypeptidase A
MMC	mucosal mast cells
MCTC	connective tissue mast cells
CLP	cecal ligation and puncture
DNP-HSA	2, 4-Dinitrophenyl-Human Serum Albumin
DIC	disseminated intravascular coagulation

LC-MS/MS liquid chromatography-mass spectrometry-mass spectrometry

ROTEM rotational thromboelastometry

TNF tumor necrosis factor

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Clinical implications

Unbiased characterization of mast cell releasates by mass spectrometry analysis has the potential to provide a better understanding of the contribution of mast cells to health and disease.

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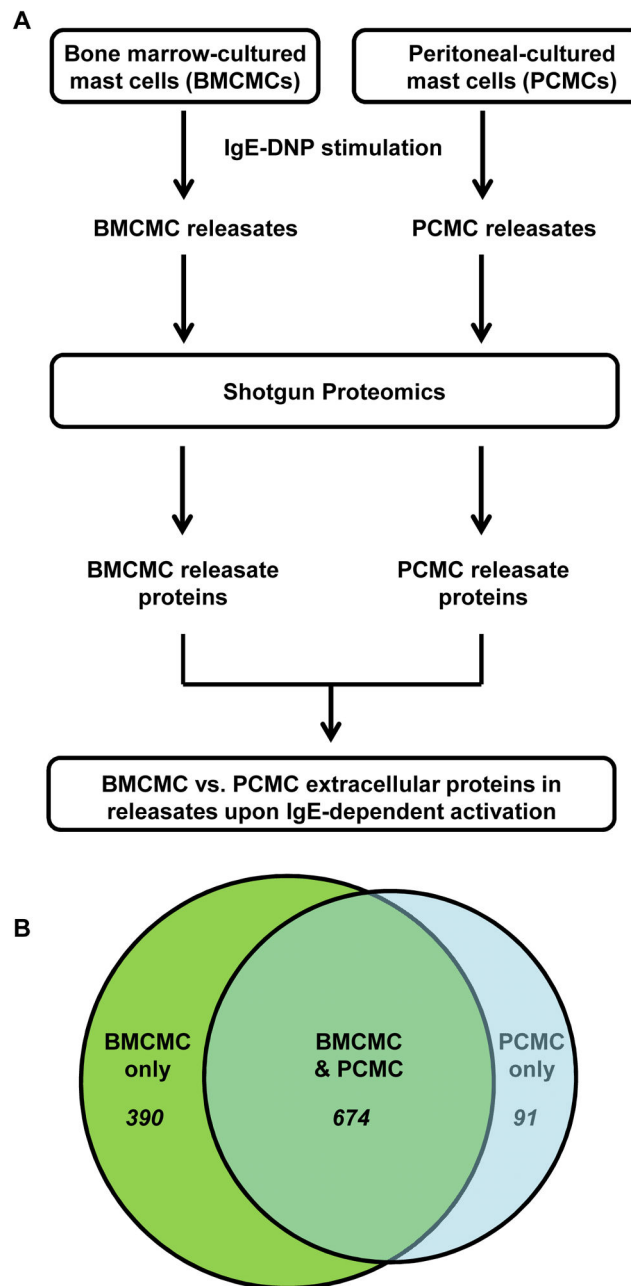


Figure 1. Differentially produced extracellular proteins from bone marrow compared with peritoneal derived mast cells

(A) A schematic overview of the approach used to define the releasates generated by BMCMCs and PCMCs following IgE-mediated activation. (B) A Venn diagram showing the number of shared and exclusive proteins amongst the BMCMC and PCMC releasates.

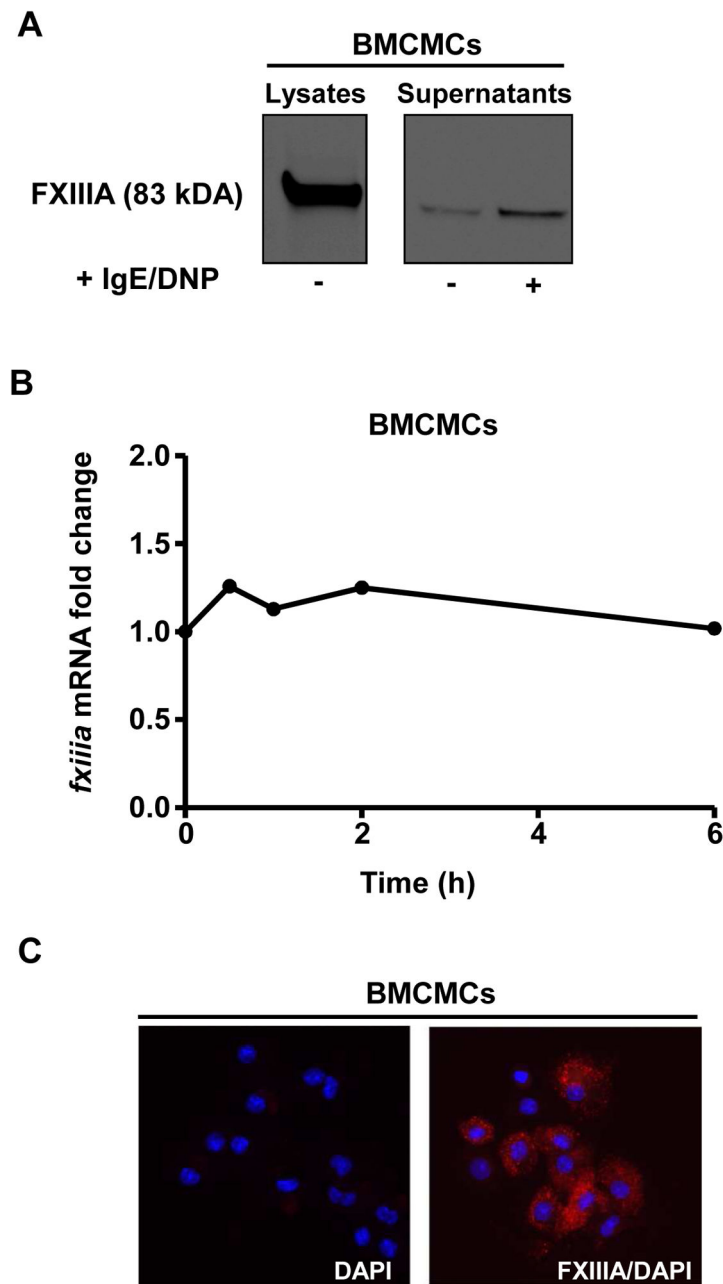


Figure 2. Mast cells produce and release FXIII A

BMCMCs were sensitized with IgE mAb to DNP (2 $\mu\text{g}/\text{ml}$) overnight and then were challenged with DNP-HSA (100 ng/ml) for 6 h (A) or for the indicated time points (B). (A) FXIII A (83 kDa) was observed by Western blot analysis in BMCMC protein lysate and their extracellular supernatant. The data are representative of 2 independent experiments. (B) FXIII A expression was analyzed by qPCR. The FXIII A versus GAPDH mRNA expression level for the BMCMCs that were incubated in media alone was assigned a value of 1.0 for calculation of the corresponding IgE/antigen stimulated BMCMC results. The data were pooled from the 3 independent experiments. (C) Immunofluorescence of fixed and

permeabilized BMCs labeled first with anti-FXIIIa antibody and then with Alexa Fluor® 594-conjugated anti-mouse donkey polyclonal antibody (red). The cells were mounted with anti-fade reagent containing DAPI for nuclear staining (blue). The images are representative of 2 experiments.

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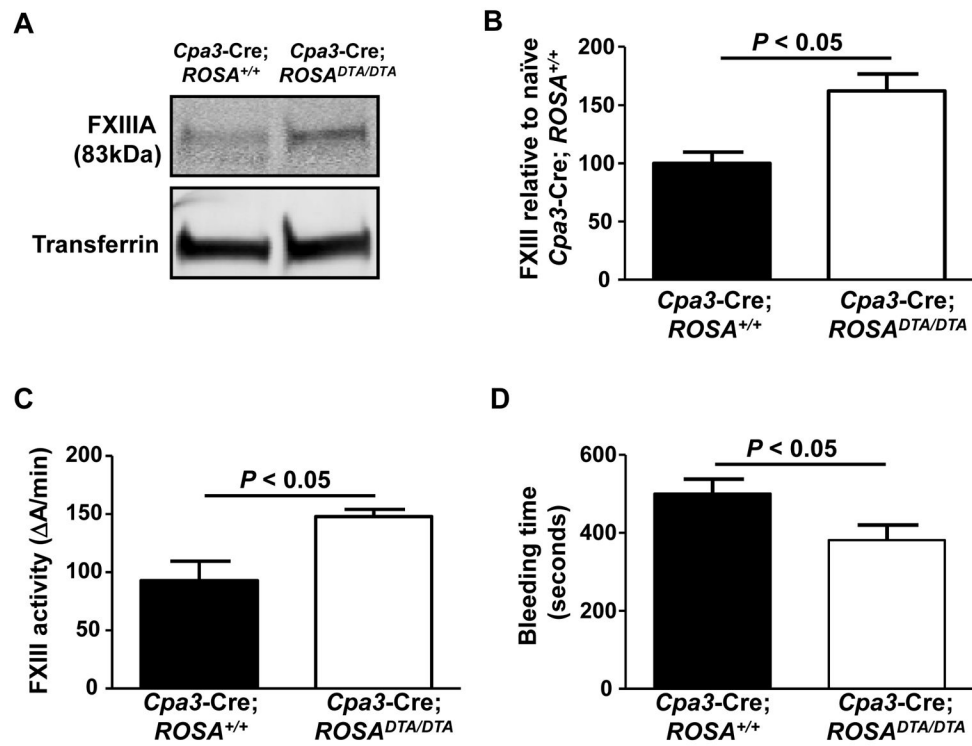


Figure 3. *Cpa3-Cre; ROSA^{DTA/DTA}* mast cell- and basophil-deficient mice exhibit increased plasma FXIII amounts and activity, as well as reduced bleeding time (A) Western blot and (B) densitometry analyses for plasma FXIIIa levels in *Cpa3-Cre; ROSA^{+/+}* ($n = 4$) and *Cpa3-Cre; ROSA^{DTA/DTA}* mice ($n = 7$). (C–D) FXIIIa activity levels (C) and tail tip bleeding times (D) in *Cpa3-Cre; ROSA^{+/+}* ($n = 11–13$) and *Cpa3-Cre; ROSA^{DTA/DTA}* mice ($n = 7–20$).

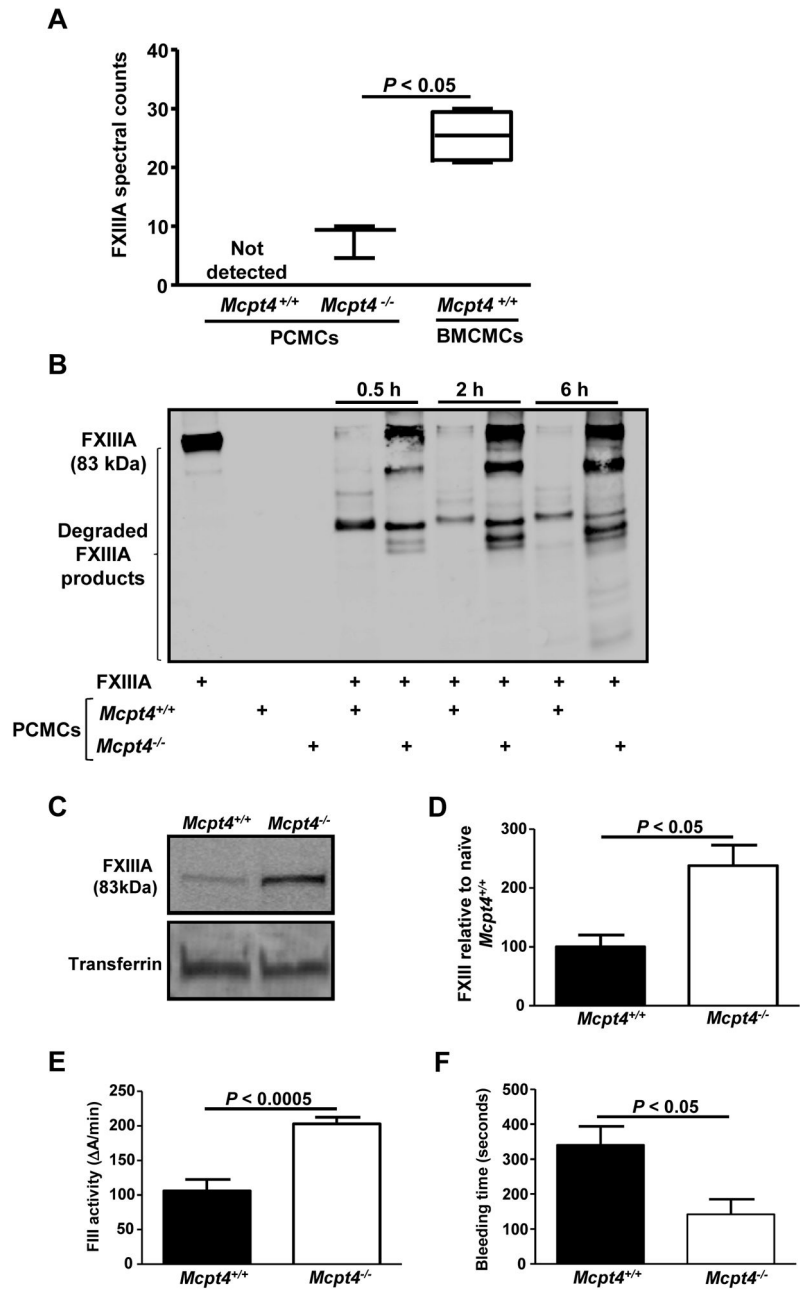


Figure 4. Mouse chymase (mMCP-4) down-regulates FXIIIa plasma levels and activity via proteolytic degradation and contributes to prolonged bleeding times under homeostatic conditions

(A) Wild type (*Mcpt4*^{+/+}) (*n* = 5) and mMCP-4-deficient (*Mcpt4*^{-/-}) (*n* = 3) mouse PCMCs and *Mcpt4*^{+/+} BMCMCs (*n* = 6) were sensitized with IgE mAb to DNP (2 μg/ml) overnight and then were challenged with DNP-HSA (100 ng/ml) for 6h. The cell supernatants were collected, concentrated, and subjected to shotgun proteomics. The FXIIIa abundance is expressed in spectral counts. (B) PCMC lysates were incubated with human FXIIIa for the indicated time points at 37° C. The FXIIIa levels and their fragments were assessed with Western blot analysis. The data are representative of those obtained in 2 independent

experiments. **(C)** Western blot analysis and **(D)** densitometry values for plasma FXIIIa levels in *Mcpt4*^{+/+} ($n = 5$) and *Mcpt4*^{-/-} mice ($n = 5$). **(E-F)** FXIIIa activity levels **(E)** and tail tip bleeding times **(F)** in *Mcpt4*^{+/+} ($n = 8-9$) and *Mcpt4*^{-/-} mice ($n = 5-8$).

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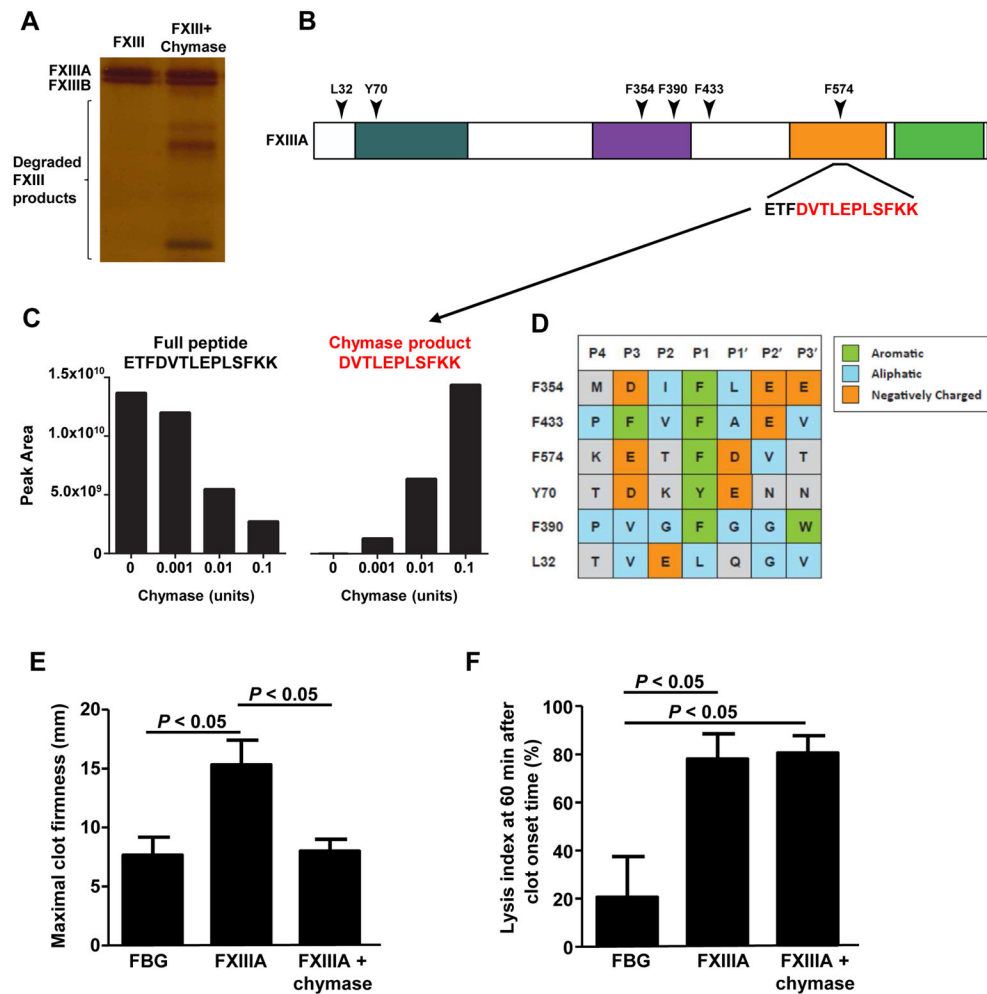


Figure 5. Human chymase degrades FXIIIa and diminishes its activity

(A) Human FXIIIa was incubated with human skin chymase for 6 h at 37 °C. FXIIIa fragmentation was assessed by silver stain analysis. The data are representative of those obtained from 3 independent experiments. (B) A human FXIIIa domain map, including the conserved transglutaminase regions and the location of the detected chymase cleavage sites are shown. (C) FXIIIa was incubated with increasing concentrations of human skin chymase for 6 hours at 37 °C and then subjected to LC-MS/MS. Six chymase cleavage sites in FXIIIa produced a marked dose-response to the increasing chymase levels, as exemplified for cleavage site F574. (D) An approximation of the consensus peptide sequence alignment patterns containing the detected chymase cleavage sites. Chymase cleavage occurs between P1 and P1'. The peptide sequences shown were sorted in order of their negatively charged and aliphatic residues. (E–F) ROTEM results are reported as maximal clot firmness (MCF) (E), which represents fibrin polymerization, FXIIIa activity, and the lysis index 60 (LI60) (F), which represents the percentage of the remaining clot in relation to the MCF value at 60 min after clot onset time. The data were pooled from 3 independent experiments.

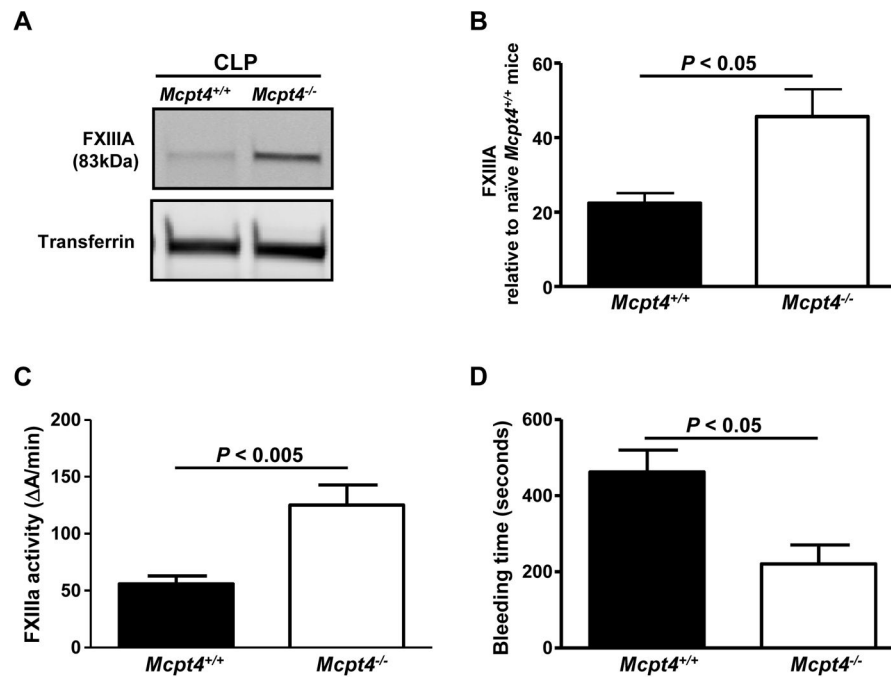


Figure 6. mMCP-4 diminishes FXIIIa amounts and FXIII activity levels in mice during experimental sepsis
(A) Western blot analysis and **(B)** densitometry values for FXIIIa plasma levels, **(C)** FXIIIa plasma activity levels, and **(D)** bleeding times at 24 h after CLP in *Mcpt4*^{+/+} ($n = 5-9$) and *Mcpt4*^{-/-} mice ($n = 3-8$).

Table 1
Top 20 unique PCMC releasate proteins

Ex-vivo IgE-activated mouse peritoneal-derived mast cell extracellular proteins that were not observed in the IgE-activated bone marrow-derived mast cell supernatants (top 20 proteins identified, according to spectral counts).

Gene name	UniProt ID	Uniprot description	Normalized PCMC spectral counts (avg.)	Normalized BMCMC spectral counts (avg.)
Mcpt4	MCPT4_MOUSE	Mast cell protease 4 (mMCP-4)	62.34	0.81
Ahnak	E9Q616_MOUSE	Protein Ahnak	20.38	0.81
Flna	FLNA_MOUSE	Filamin-A	19.54	0.00
Mcpt1	Q496V0_MOUSE	Mast cell protease 1	15.14	0.00
Aoah	AOAH_MOUSE	Acyloxyacyl hydrolase	12.82	0.40
Cpq	CBPQ_MOUSE	Carboxypeptidase Q	12.66	0.00
Galc	GALC_MOUSE	Galactocerebrosidase	12.27	0.28
Ecm1	ECM1_MOUSE	Extracellular matrix protein 1	12.17	0.00
Ddah2	DDAH2_MOUSE	N(G),N(G)-dimethylarginine dimethylaminohydrolase 2	8.65	0.81
Papss2	PAPS2_MOUSE	Bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthase 2	8.64	0.00
Lap3	AMPL_MOUSE	Cytosol aminopeptidase	8.56	0.00
Syng1	SNG1_MOUSE	Synaptogyrin-1	7.53	0.81
Ckb	KCRB_MOUSE	Creatine kinase B-type	7.06	0.00
Aars	SYA_MOUSE	Alanine--tRNA ligase, cytoplasmic	6.45	0.13
Rbp1	Q58EU7_MOUSE	Rbp1 protein	6.38	0.00
Ide	IDE_MOUSE	Insulin-degrading enzyme	6.19	0.27
Kv2a7	KV2A7_MOUSE	Ig kappa chain V-II region 26-10	5.89	0.67
Ptprg	PTPRG_MOUSE	Receptor-type tyrosine-protein phosphatase gamma	5.60	0.00
Prkar2b	KAP3_MOUSE	cAMP-dependent protein kinase type II-beta regulatory subunit	5.50	0.14
Pacsin2	Q3TDA7_MOUSE	Protein kinase C and casein kinase substrate in neurons 2,	5.04	0.68

Table 2
Top 20 unique BMCMC releasate proteins

Ex-vivo IgE-activated mouse bone marrow-derived mast cell extracellular proteins that were not observed in the IgE-activated peritoneal-derived mast cell supernatants (top 20 proteins identified, according to spectral counts).

Gene name	UniProt ID	Uniprot description	Normalized PCMC spectral counts (avg.)	Normalized BMCMC spectral counts (avg.)
Scin	ADSV_MOUSE	Adeverin	0.26	66.88
Mcpt8	Q3UWB6_MOUSE	Mast cell protease 8	0.00	41.26
Akr1c18	Q3U538_MOUSE	Aldo-keto reductase family 1, member C18	0.00	33.53
Napsa	Q3U7H1_MOUSE	Putative uncharacterized protein	0.00	29.36
Jak1	JAK1_MOUSE	Tyrosine-protein kinase JAK1	0.82	22.41
F13a1	F13A_MOUSE	Coagulation factor XIII A chain	0.00	21.94
Hist1h2af	H2A1F_MOUSE	Histone H2A type 1-F	0.26	21.19
Il4ra	Q3U905_MOUSE	Interleukin 4 receptor, alpha	0.26	19.09
Hist1h2bb	H2B1B_MOUSE	Histone H2B type 1-B	0.00	19.00
Ifitm1	IFM1_MOUSE	Interferon-induced transmembrane protein 1	0.00	18.63
Anxa2	Q542G9_MOUSE	Annexin	0.27	18.36
Stat5a	Q9JIA0_MOUSE	Signal transducer and activator of transcription 5A	0.00	17.14
Ccl6	SY06_MOUSE	C-C motif chemokine 6	0.00	16.35
Upp1	UPP1_MOUSE	Uridine phosphorylase 1	0.00	14.67
Ctsg	Q059V7_MOUSE	Cathepsin G	0.58	14.66
Myo1g	MYO1G_MOUSE	Unconventional myosin-Ig	0.00	12.29
Tgfb1	Q4FJL1_MOUSE	Tgfb1 protein	0.86	11.64
Atp6v0a1	VPP1_MOUSE	V-type proton ATPase 116 kDa subunit a isoform 1	0.58	11.15
Tmem59	TMM59_MOUSE	Transmembrane protein 59	0.56	10.64
Aldh9a1	AL9A1_MOUSE	4-trimethylaminobutyraldehyde dehydrogenase	0.58	10.11

Table 3

Top 20 shared PCMC and BMCMC releasate proteins

Extracellular proteins that were observed in both ex-vivo IgE-activated mouse bone marrow-derived mast cell and peritoneal-derived mast cell (PCMC) supernatants (top 20 protein PCMC proteins identified, according to mean shared protein spectral counts).

Gene name	UniProt ID	Uniprot description	Normalized PCMC spectral counts (avg.)	Normalized BMCMC spectral counts (avg.)	Mean spectral counts of shared proteins
ALB	ALBU_BOVIN	Serum albumin	190.95	209.58	200.27
Anpep	AMPN_MOUSE	Aminopeptidase N	213.66	170.67	192.17
Man2b1	MA2B1_MOUSE	Lysosomal alpha-mannosidase	162.04	195.21	178.63
Grn	Q544Y8_MOUSE	Granulin	137.44	103.40	120.42
Cpa3	Q542E3_MOUSE	Carboxypeptidase A3, mast cell	166.55	46.79	106.67
Man2b2	MA2B2_MOUSE	Epididymis-specific alpha-mannosidase	142.38	48.62	95.50
Hexb	Q3TXR9_MOUSE	Putative uncharacterized protein	138.51	42.62	90.56
Glb1	Q3TAW7_MOUSE	Beta-galactosidase	106.38	55.50	80.94
Naglu	A2BFA6_MOUSE	Alpha-N-acetylglucosaminidase	105.93	38.96	72.44
Cma1	CMA1_MOUSE	Mast cell protease 5	113.96	30.30	72.13
Pkm	KPYM_MOUSE	Pyruvate kinase isozymes M1/M2	45.40	87.59	66.50
Tln1	TLN1_MOUSE	Talin-1	80.15	51.73	65.94
Tpsb2	B5A5B2_MOUSE	Tryptase beta 2	106.77	24.32	65.54
Pdcd6ip	PDC61_MOUSE	Programmed cell death 6-interacting protein	89.03	39.59	64.31
Lcp1	PLSL_MOUSE	Plastin-2	61.34	64.68	63.01
Iqgap1	IQGAI_MOUSE	Ras GTPase-activating-like protein IQGAP1	79.60	44.75	62.18
Myh9	MYH9_MOUSE	Myosin-9	41.59	81.99	61.79
Aldoa	Q5FWB7_MOUSE	Fructose-bisphosphate aldolase	68.73	53.66	61.19
Lgals3bp	LG3BP_MOUSE	Galectin-3-binding protein	64.19	51.36	57.78
Vwa5a	VMA5A_MOUSE	von Willebrand factor A domain-containing protein 5A	87.43	26.63	57.03