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Mast cells limit extracellular levels of IL-13 via a serglycin proteoglycan-serine protease axis

Abstract: Mast cell (MC) granules contain large amounts of proteases of the chymase, tryptase and carboxypeptidase A (MC-CPA) type that are stored in complex with serglycin, a proteoglycan with heparin side chains. Hence, serglycin-protease complexes are released upon MC degranulation and may influence local inflammation. Here we explored the possibility that a serglycin-protease axis may regulate levels of IL-13, a cytokine involved in allergic asthma. Indeed, we found that wild-type MCs efficiently degraded exogenous or endogenously produced IL-13 upon degranulation, whereas serglycin$^{-/-}$ MCs completely lacked this ability. Moreover, MC-mediated IL-13 degradation was blocked both by a serine protease inhibitor and by a heparin antagonist, which suggests that IL-13 degradation is catalyzed by serglycin-dependent serine proteases and that optimal IL-13 degradation is dependent on both the serglycin and the protease component of the serglycin-protease complex. Moreover, IL-13 degradation was abrogated in MC-CPA$^{-/-}$ MC cultures, but was normal in cultures of MCs with an inactivating mutation of MC-CPA, which suggests that the IL-13-degrading serine proteases rely on MC-CPA protein. Together, our data implicate a serglycin-serine protease axis in the regulation of extracellular levels of IL-13. Reduction of IL-13 levels through this mechanism possibly can provide a protective function in the context of allergic inflammation.

Keywords: allergy; cytokine; mast cell; proteoglycan; serine protease.

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Introduction

Mast cells (MCs) are multifunctional cells with the capacity to regulate immune responses, in addition to their role as effector cells during inflammatory conditions and infections (Galli et al., 2008). MCs operate through a wide variety of bioactive mediators that they release in response to activation by, for example, FcεRI crosslinking (Lundequist and Pejler, 2011). Proteases are stored in exceedingly high amounts in the secretory granules of MCs, and they are released as fully active enzymes upon degranulation (Pejler et al., 2007). MC proteases could therefore have a profound influence on local inflammatory reactions. However, the exact contribution of individual proteases in disease progression under various inflammatory settings is only beginning to be uncovered (Pejler et al., 2010; Caughey, 2011). Based on their cleavage specificity, MC proteases can be divided into three main groups: chymases, tryptases and MC carboxypeptidase A (MC-CPA; CPA3) [reviewed in (Pejler et al., 2007, 2009b)].

We recently demonstrated that a murine chymase, mouse MC protease (mMCP)-4, protects against bronchial hyperreactivity and airway inflammation in a model of allergic airway inflammation (Waern et al., 2009). This is intriguing considering that MCs, as such, are detrimental in the context of allergic disease (Bradding et al., 2006; Galli et al., 2008; Feyerabend et al., 2011), and suggests that released MC proteases may have a down-regulatory

impact on the otherwise pro-inflammatory potential of MCs. The exact mechanism by which MC proteases confer protection in this allergic model is not known. However, a plausible scenario is that chymase and/or other MC proteases may regulate the course of an inflammatory reaction through proteolytic action on bioactive proteins, such as cytokines.

IL-13 is a key cytokine known to play a vital role in the development of allergic airway responses, e.g., by its ability to induce IgE production, bronchial hyperreactivity, eosinophil infiltration and mucus production (Grunig et al., 1998; Wills-Karp et al., 1998; Zhu et al., 1999). Moreover, elevated levels of IL-13 have been found in human asthmatics, and polymorphisms in the IL-13 gene are correlated to asthma susceptibility (Humbert et al., 1997; Vercelli, 2008). IL-13 is predominantly expressed by type-2 T helper cells but can also be expressed by other cell types, including MCs (Toru et al., 1998; Gessner et al., 2005).

The storage and activities of MC proteases are, to a large extent, influenced by serglycin (Pejler et al., 2009a; Kolset and Pejler, 2011), a proteoglycan species composed of a small protein core to which strongly negatively charged side chains of heparin (or chondroitin sulfate) type are attached. Primarily, serglycin proteoglycans are known to serve as storage matrices for several of the MC proteases, including mMCP-4, mMCP-5, mMCP-6 and MC-CPA, as shown by the absence of these proteases in MCs from serglycin-deficient mice (Åbrink et al., 2004). In addition, several reports show that serglycin (or purified heparin) can enhance the actual activity of the MC proteases, either by promoting protease assembly (Hallgren et al., 2004) or by facilitating MC protease-catalyzed cleavage of certain substrates (Pejler and Sadler, 1999; Tchougounova and Pejler, 2001). However, the release of MC proteases may occur independently of serglycin proteoglycan, at least during an *in vivo* response to parasite infection (Sawesi et al., 2010).

To explore the ability of a putative serglycin-protease axis to regulate IL-13 levels, we used peritoneal cell-derived MCs (PCMCs) from a number of mouse strains that were deficient in either serglycin or in various serglycin-dependent MC proteases. We demonstrated that FcεRI-activated PCMCs can efficiently degrade both exogenously added and endogenously produced IL-13. Moreover, the data suggest that IL-13 degradation is mediated by serglycin-dependent serine proteases and that optimal proteolytic cleavage requires that the proteases are complex-bound to heparin, with heparin representing the functional entity of serglycin.

Results

Phenotype of cultured peritoneal MCs

To obtain mature MCs expressing high levels of proteases, we used peritoneal cell-derived MCs (PCMCs) cultured for 4 weeks in medium containing stem cell factor (SCF) as described by Malbec et al. (2007). Wild-type (WT) MCs displayed typical MC phenotypes as shown by intense metachromatic staining (Figure 1A), surface expression of c-kit and FcεRI (Figure 1B) and the presence of MC-specific proteases, i.e., mMCP-4, -5, -6 and MC-CPA (Figure 1C). Earlier studies have shown that mMCP-4, -5, -6 and MC-CPA are strictly dependent on serglycin for their storage in secretory granules (Henningsson et al., 2002; Åbrink et al., 2004; Henningsson et al., 2006), and here we demonstrate the absence of these proteases in serglycin⁻/⁻ PCMCs (Figure 1C). Genetic deficiency of either mMCP-4 or mMCP-6 did not substantially affect levels of other MC proteases in PCMCs, whereas MC-CPA-deficiency was accompanied by a total lack of mMCP-5, as expected due to the known interdependency of these proteases for proper storage (Feyerabend et al., 2005; Younan et al., 2010). Normal morphology and staining properties were seen in PCMCs derived from mMCP-4⁻/⁻, mMCP-6⁻/⁻, MC-CPA⁻/⁻ and MC-CPAmut (mice with an inactivating mutation in the active site of MC-CPA) (Figure 1A). However, as serglycin⁻/⁻ PCMCs lack the negatively charged glycosaminoglycans (heparin) binding to cationic dyes, these cells displayed no metachromatic staining of secretory granules (Figure 1A).

MC-mediated reduction of IL-13 is serglycin-dependent

Next, we used PCMC cultures to explore the possibility that MC proteases regulate extracellular IL-13 levels. The processing of exogenously added IL-13 was studied in WT and serglycin⁻/⁻ PCMC cultures in which MC degranulation was induced by either IgE receptor crosslinking or with a calcium ionophore (A23187). At 4 h after degranulation, there was already a substantial (77%) decrease in IL-13 levels in WT MC cultures, whereas levels of IL-13 were essentially unaltered in control wells containing non-stimulated MCs or medium alone (Figure 2). In contrast to WT MCs, degranulated serglycin⁻/⁻ MCs were unable to reduce the amount of IL-13, and high levels of IL-13 remained in the supernatant, even after 24 h (Figure 2). These findings demonstrate that MCs can efficiently reduce external IL-13 upon degranulation and that this process is totally dependent on the presence of serglycin proteoglycan.
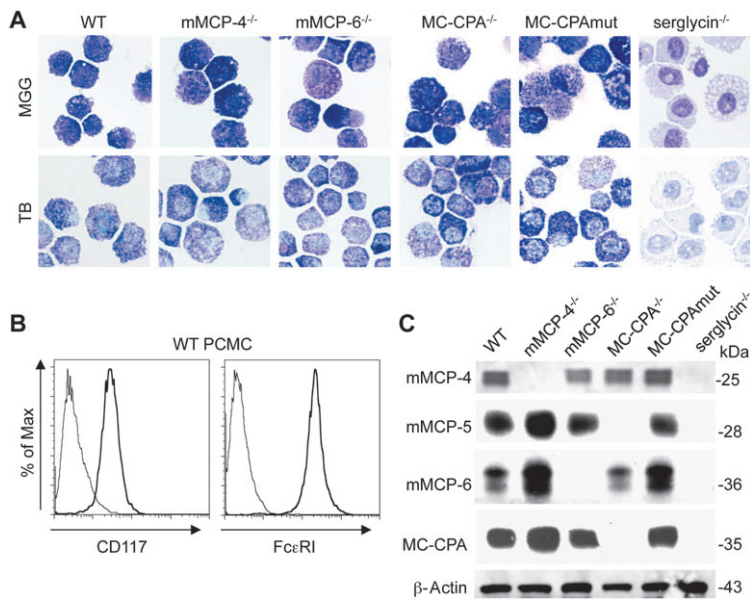
Figure 1 Phenotypic characterization of peritoneal cell-derived MCs (PCMCs).
(A) PCMCs stained with May-Grünwald/Giemsa (MGG, *upper panel*) or toluidine blue (TB, *lower panel*) display altered morphology in serglycin$^{-/-}$ mice and normal morphology in mMCP-4$^{-/-}$, mMCP-6$^{-/-}$, MC-CPA$^{-/-}$ and MC-CPAmut mice. (B) Confirmation of MC phenotype of WT PCMCs by flow cytometry. Expression of c-kit (CD117) and FcεRI (*thick lines*) are shown together with corresponding isotype controls (*thin lines*). (C) Western blot analyses of protease content of PCMCs from WT, mMCP-4$^{-/-}$, mMCP-6$^{-/-}$, MC-CPA$^{-/-}$, MC-CPAmut and serglycin$^{-/-}$ mice. Samples added to each well correspond to 2.7×10^3 cells, and β-actin was used as a loading control.
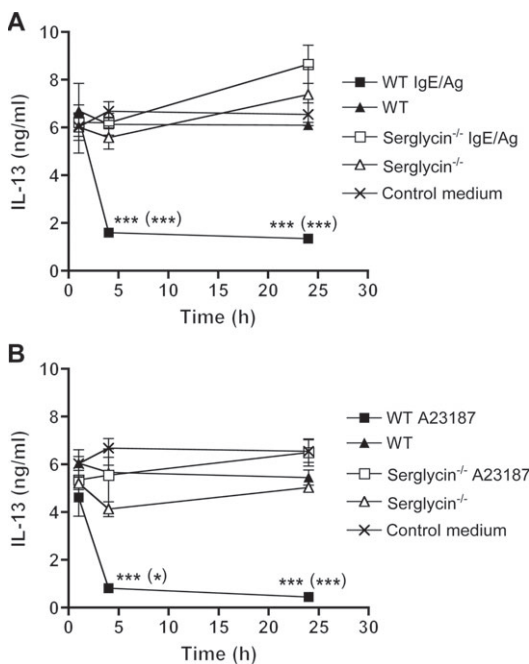

Figure 2 IL-13 degradation by PCMCs is serglycin-dependent. Degranulation of WT or serglycin$^{-/-}$ PCMCs was induced by IgE-anti-TNP and OVA-TNP (IgE/Ag; A) or by calcium ionophore A23187 (B) in the presence of IL-13. Levels of IL-13 in the supernatant were measured with ELISA at 1 h, 4 h and 24 h after stimulation. Data are expressed as means±SEM (n=3). ***, $p<0.001$ for stimulated vs. non-stimulated WT cultures, and (*), $p<0.05$; (***), $p<0.001$ for stimulated WT vs. stimulated serglycin$^{-/-}$ cultures.

Proteolytic cleavage of IL-13 by MC serine proteases

To investigate whether the MC-mediated reduction of IL-13 is due to proteolytic cleavage and, if so, to identify the type of enzyme involved, we used a panel of enzyme inhibitors, including Pefabloc SC (serine proteases), EDTA (metalloproteases), E-64 (cysteine proteases) and Pepstatin A (aspartic acid proteases). Incubation with the serine protease inhibitor effectively blocked IL-13 degradation after MC activation with either IgE receptor crosslinking or calcium ionophore (Figure 3). In contrast, inhibitors of metalloproteases, cysteine proteases and aspartic acid proteases were unable to prevent IL-13 reduction (Figure 3). These results suggest that the reduction of IL-13 levels is due to proteolytic activities mediated by serglycin-dependent serine proteases released upon MC degranulation.

Degradation of external IL-13 is abrogated in MC-CPA$^{-/-}$ MC cultures

The inability of serglycin$^{-/-}$ MCs to degrade IL-13 (Figure 2) implicated the serglycin-dependent proteases, i.e., mMCP-4, -5, -6 or MC-CPA (Pejler et al., 2010). To analyze this further, we measured IL-13 degradation in cultures of
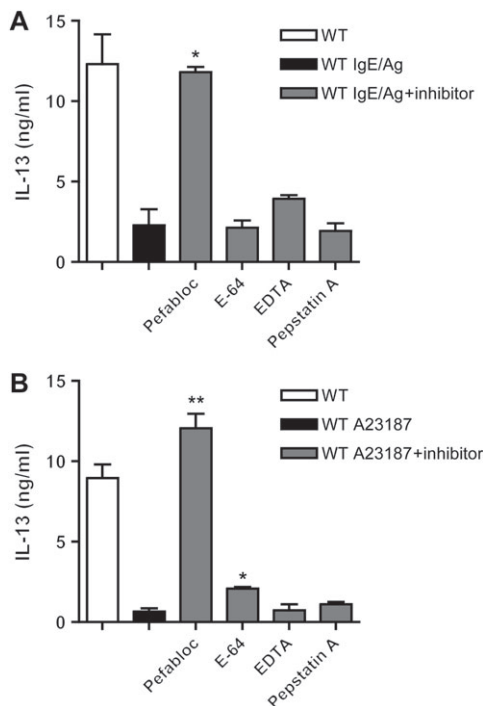
Figure 3 IL-13 degradation by PCMCs is serine protease-dependent. Degranulation of PCMCs was induced by IgE-receptor crosslinking (IgE/Ag; A) or by calcium ionophore A23187 (B) in the presence of IL-13 and inhibitors of serine proteases (Pefabloc SC), metalloproteases (EDTA), cysteine proteases (E-64) or aspartic acid proteases (Pepstatin A). Levels of IL-13 in supernatants 24 h after degranulations were measured by ELISA. Data are expressed as means±SEM. *, $p<0.05$; **, $p<0.01$ for cultures with inhibitor vs. no inhibitor.

PCMCs from mice lacking individual proteases (see Figure 1C). PCMCs were generated from mMCP-4$^{-/-}$, mMCP-6$^{-/-}$, MC-CPA$^{-/-}$ and MC-CPAmut mice, and the levels of exogenously added IL-13 were measured at 1 h, 4 h and 24 h post-stimulation. Given that Pefabloc SC could inhibit IL-13 degradation (Figure 3), we considered two of the major serine proteases, i.e., mMCP-4 and mMCP-6, to be likely candidates exerting the proteolytic cleavage of IL-13. Upon IgE crosslinking, both mMCP-4$^{-/-}$ and mMCP-6$^{-/-}$ PCMCs degraded IL-13, at least to the same extent as did WT cells (Figure 4A and B), which indicates that efficient processing of IL-13 can occur, even in the absence of either of these two enzymes. Notably, non-stimulated WT cells exhibit a weak IL-13-degrading activity that is significantly reduced in the absence of mMCP-4 (Figure 4A).

Next, we assessed the role of MC-CPA in the degradation of IL-13. As shown in Figure 4C and D, MC-CPA$^{-/-}$ MCs had a severely diminished capacity to degrade IL-13, whereas MC-CPAmut MCs degraded IL-13 almost as efficiently as WT MCs. Moreover, Western blot analyses demonstrated a dramatic decrease in the amount of visible IL-13 upon treatment of WT MCs with IgE and antigen,

whereas no reduction in IL-13 levels was seen for activated MC-CPA$^{-/-}$ MCs (Figure 4E). Altogether, these findings indicate that the degradation of IL-13 is dependent on the presence of MC-CPA protein. However, the preserved IL-13 degradation seen in MCs from MC-CPAmut mice indicates that proteolytic activity of MC-CPA is not required. The latter notion was also supported by the inability of a metalloprotease inhibitor to prevent IL-13 degradation (Figure 3).

Endogenously produced IL-13 is reduced in MC-CPA$^{-/-}$ and mMCP-4$^{-/-}$ MCs

MCs exhibit the capacity to endogenously express and secrete IL-13 (Toru et al., 1998; Gessner et al., 2005), and we hypothesized that proteolytic cleavage of endogenous IL-13 by MC proteases could represent a means for intrinsic regulation. To explore this possibility, we analyzed IL-13 levels in cultures of FcεRI-activated MCs (without adding exogenous IL-13). We found that the amounts of endogenously produced IL-13 were significantly higher in cultures of FcεRI-activated MC-CPA$^{-/-}$ MCs compared to the IL-13 levels in cultures of WT or MC-CPAmut MCs (Figure 5A and B), which suggests a role for MC-CPA protein in the degradation of endogenous IL-13. Moreover, there was a significantly higher amount of endogenous IL-13 in mMCP-4$^{-/-}$ MC cultures than in WT cultures 4 h after degranulation (Figure 5B). By contrast, the levels of IL-13 mRNA, as measured by qPCR, were not higher in activated MC-CPA$^{-/-}$ or mMCP-4$^{-/-}$ MCs, than in corresponding WT MCs (data not shown). Thus, our data suggests that both MC-CPA and mMCP-4 are involved in the regulation of endogenously produced IL-13 and that the regulation of IL-13 levels occurs at the protein- rather than at the transcriptional level.

Recombinant mMCP-5 does not reconstitute IL-13 degradation by MC-CPA$^{-/-}$ MCs

Previous studies have shown that the absence of MC-CPA protein (MC-CPA$^{-/-}$) leads to a secondary loss of mMCP-5 at the protein level (mMCP-4 and mMCP-6 storage is unaffected), whereas mMCP-5 storage is preserved in MC-CPAmut MCs (Feyerabend et al., 2005; Schneider et al., 2007). This, together with the known dependence of mMCP-5 on serglycin for storage (Younan et al., 2010), strongly implicated mMCP-5 (or other MC-CPA-dependent proteases) in the degradation of IL-13 by activated MCs. We therefore assessed the capacity of recombinant mMCP-5 to degrade
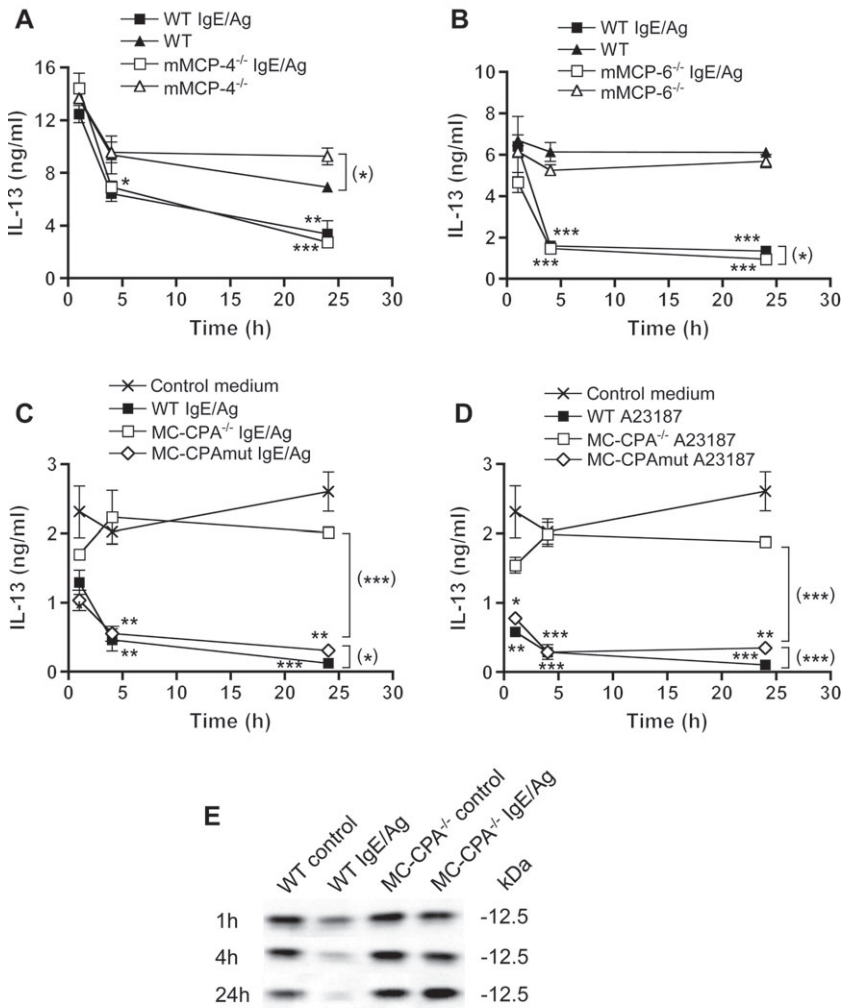
Figure 4 Degradation of exogenous IL-13 is abrogated in MC-CPA$^{-/-}$ PCMC cultures. PCMCs from mMCP-4$^{-/-}$ (A), mMCP-6$^{-/-}$ (B), MC-CPA$^{-/-}$ (C–E) or MC-CPAmut mice (C, D) and from their WT controls were degranulated by IgE-crosslinking (IgE/Ag) or by calcium ionophore (A23187) in the presence of IL-13. Levels of IL-13 in the supernatant at 1 h, 4 h and 24 h after stimulation were measured with ELISA (A–D) or Western blot analysis (E). Data are from representative experiments and are expressed as means±SEM (n=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ for stimulated vs. non-stimulated cultures or control medium. (*), $p < 0.05$; (***), $p < 0.001$ for differences between stimulated cultures from various mouse strains as indicated.

IL-13. Enzymatic activity of purified recombinant mMCP-5 was first confirmed by the cleavage of four different recombinant protein substrates (Andersson et al., 2009). Three of them were elastase substrates containing either valine, isoleucine or alanine in the cleavable position (VLL**V**SEVL, VLL**I**SEVL, VLL**A**SEVL). The fourth substrate was a chymase substrate containing a phenylalanine in the cleavable position (VLL**F**SEVL). The N or C terminal of the cleavable amino acids was based on an extensive phage display analysis of the rat counterpart of mMCP-5 (rMCP-5) (Karlson et al., 2003). mMCP-5 cleaved both the valine and isoleucine substrates very well, the alanine substrate between 5 and 10 times less efficiently and no cleavage was seen with the chymase substrate. This confirms the activity of the recombinant mMCP-5 enzyme and the elastase specificity (Kunori et al., 2002). However, recombinant mMCP-5 at the same concentration failed to degrade recombinant IL-13 (Figure 6A). MC proteases normally operate in close contact with the heparin chains of serglycin proteoglycan. To mimic this situation, we added heparin to facilitate proteolytic cleavage by mMCP-5. However, even in the presence of heparin, there was no detectable IL-13 degradation by mMCP-5 (not shown). Moreover, purified mMCP-5 added to PCMCs was unable to restore the loss of IL-13 degradation in MC-CPA$^{-/-}$ cultures (Figure 6B).

In addition to mMCP-4, -5, and -6, murine MCs may store and release significant amounts of other serine proteases,
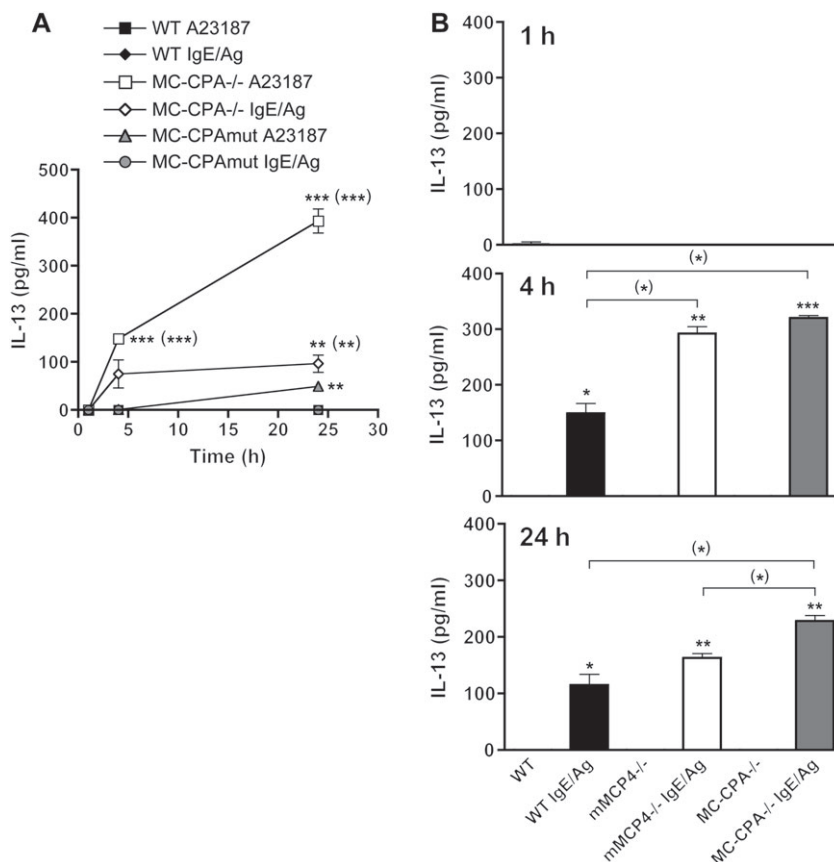
Figure 5 Degradation of endogenous IL-13 is deficient in MC-CPA$^{-/-}$ and mMCP-4$^{-/-}$ PCMC cultures.
PCMCs from MC-CPA$^{-/-}$, MC-CPAmut or mMCP-4$^{-/-}$ mice and from their respective WT controls were degranulated by IgE crosslinking (IgE/Ag) or by calcium ionophore A23187, and levels of endogenously produced IL-13 were analyzed by ELISA up to 24 h after stimulation. Data are from representative experiments and are expressed as means±SEM (n=3). (A) **, $p<0.01$; ***, $p<0.001$ vs. WT cultures and (**), $p<0.01$; (***), $p<0.001$ for MC-CPA$^{-/-}$ vs. MC-CPAmut. (B) *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ vs. non-stimulated controls and (*), $p<0.05$ for differences between stimulated cultures from various mouse strains as indicated.

e.g., granzyme B and cathepsin G, of which granzyme B has been shown to be stored in a serglycin-dependent manner (Grujic et al., 2005). To explore whether any of these proteases have a role in the serglycin/MC-CPA-dependent degradation of IL-13, we investigated whether recombinant granzyme B or human cathepsin G could degrade IL-13. Whereas granzyme B did not cause any detectable IL-13 degradation, human cathepsin G caused an almost complete loss of added IL-13 (Figure 7A), the latter result being in agreement with previous findings (Zhao et al., 2005). These results suggested that cathepsin G expressed by the PCMCs may be a candidate protease mediating the serglycin/MC-CPA-dependent degradation of IL-13. To evaluate this possibility, we first examined whether cathepsin G is expressed by PCMCs and if its storage is serglycin- and/or MC-CPA-dependent. On Western blot analysis, a band corresponding to cathepsin G was clearly detectable in PCMCs from WT, mMCP-4$^{-/-}$ and MC-CPAmut mice, but was virtually absent in MC-CPA$^{-/-}$ PCMCs (Figure 7B). In

serglycin$^{-/-}$ PCMCs, only a faint band corresponding to full-size cathepsin G was observed along with the presence of a lower molecular weight band, the latter possibly corresponding to partially degraded cathepsin G (Figure 7B). These data suggest that cathepsin G is expressed by PCMCs and that it is stored in a serglycin- and MC-CPA-dependent fashion, i.e., an expression pattern mimicking that of the putative serglycin- and MC-CPA-dependent IL-13-degrading proteases. To further examine this, we assessed the effect of a cathepsin G inhibitor on PCMC-mediated IL-13 degradation. However, cathepsin G inhibition using an inhibitor of the human enzyme failed to block PCMC-mediated IL-13 degradation. Notably though, human and murine cathepsin G differ substantially within their respective active sites, human cathepsin G having dual trypsin- and chymotrypsin-like activity, whereas murine cathepsin G has sole chymotrypsin-like activity (Raymond et al., 2010). Thus, we cannot exclude that the available inhibitor of human cathepsin G has poor efficacy towards the murine counterpart,
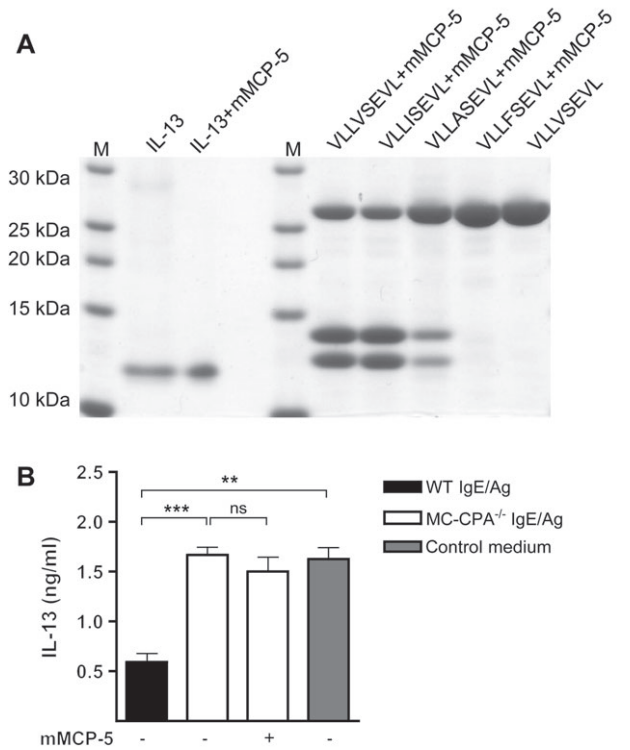
Figure 6 Recombinant mMCP-5 does not degrade IL-13.
(A; left part) Recombinant murine IL-13 (1 μg) was incubated for 3 h with recombinant mMCP-5 (0.4 μg), followed by SDS-PAGE and staining of the gel with Coomassie brilliant blue. (A; right part) To ascertain that mMCP-5 was enzymatically active, recombinant mMCP-5 (0.08 μg) was incubated with four different recombinant substrates for 3 h followed by SDS-PAGE and Coomassie brilliant blue staining. Three of the four were elastase substrates containing either valine, isoleucine or alanine in the cleavable position (VLL-VSEVL, VLLISEVL, VLLASEVL). The fourth substrate was a chymase substrate containing a phenylalanine in the cleavable position (VLLFSEVL). The valine- and isoleucine-containing substrates were partly cleaved, confirming the elastase activity of mMCP-5. The alanine-containing substrate was cleaved 5–10 times less efficiently, whereas the phenyalanine-containing substrate, indicating chymase activity, was not cleaved. (B) MC-CPA$^{-/-}$ or WT PCMCs were degranulated by IgE crosslinking (IgE/Ag) in the presence of IL-13, and in the presence or absence of recombinant mMCP-5 (4 μg/ml). Levels of IL-13 in the supernatant 24 h after stimulation were analyzed by ELISA. Data are from representative experiments and are expressed as mean±SEM (n=3).

and thus we cannot completely rule out that IL-13 is, in fact, degraded by PCMC-expressed (murine) cathepsin G.

IL-13-degrading activity is partly cell-bound and requires functional heparin

Upon MC degranulation, the released proteases may either remain attached to the cell surface or diffuse into the supernatant. To identify whether the proteolytic activity was cell surface-bound or not, we isolated cell-free supernatants from cultures of degranulated MCs, and the ability of these supernatants to degrade IL-13 was compared with that of intact cultures (containing both cells and supernatant). Although IL-13 was degraded to some extent in cell-free supernatants, the degradation was most pronounced in whole cultures, which indicates that the proteases responsible for cleavage of IL-13 are localized partly to the cell surface (Figure 8A).

Optimal MC-dependent IL-13 degradation requires heparin

Several reports suggest that MC proteases are dependent on interaction with heparin (or other strongly negatively charged glycosaminoglycans) for optimal proteolysis of certain substrates (Pejler and Sadler, 1999; Tchougounova et al., 2001; Tchougounova and Pejler, 2001). To investigate the role of heparin (as part of the serglycin proteoglycan) in MC-mediated degradation of IL-13, we added the heparin antagonist protamine to MC cultures. Addition of protamine to MC cultures degranulated with IgE + antigen inhibited IL-13 degradation in a dose-dependent manner (Figure 8B). Protamine also blocked IL-13 degradation in cultures stimulated with calcium ionophore, but had no effect on IL-13 levels when added to non-stimulated cells or to cell-free medium. These results suggest that proteolysis of IL-13 by MC serine proteases is dependent on the heparin side chains attached to the serglycin protein core.

Discussion

MC proteases are released as mature enzymes in high amounts upon FcεRI- crosslinking, and they may exert both beneficial and detrimental activities depending on the specific context. Here, we show that a serglycin proteoglycan-serine protease axis, unleashed by FcεRI-activation of peritoneal cell-derived MCs, mediates efficient degradation of both endogenously produced and exogenously added IL-13. Importantly, the data indicate that serglycin (heparin) contributes to the degradation mechanism not only by promoting the actual storage of the IL-13-degrading proteases, but also by presenting IL-13 to the executioner protease.

Murine MCs can be cultured *in vitro* using various protocols, of which bone-marrow-derived MCs have been most widely used. However, in the present investigation, we
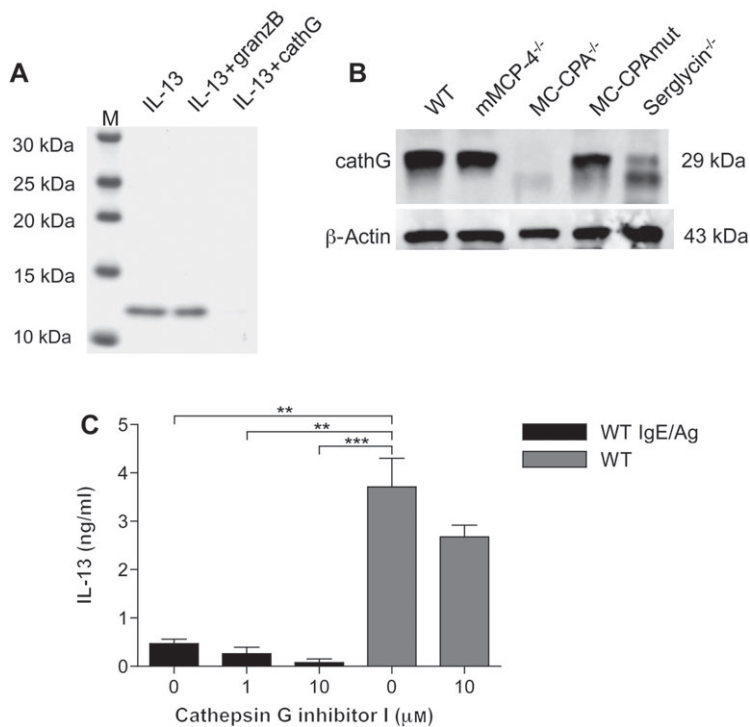
Figure 7 Cathepsin G and granzyme B in IL-13 degradation.
(A) Recombinant murine IL-13 (1 μg) was incubated for 3 h with either recombinant murine granzyme B (0.2 μg) or human cathepsin G (0.2 μg) as indicated, followed by SDS-PAGE and Coomassie brilliant blue staining. (B) PCMCs from WT, mMCP-4$^{-/-}$, MC-CPA$^{-/-}$, MC-CPAmut and serglycin$^{-/-}$ mice were analyzed for levels of cathepsin G protein using Western blot analysis. β-Actin was used as a loading control. (C) WT PCMCs, either control (gray bars) or activated with IgE + antigen (IgE/Ag; black bars), were incubated with recombinant murine IL-13 (10 ng) ±1 or 10 μM of an inhibitor of human cathepsin G as indicated. Residual levels of IL-13 after 24 h were measured using ELISA. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

used PCMCs as these cells display a more mature phenotype and express higher levels of granule-stored proteases (Malbec et al., 2007). We confirmed the mature features of PCMCs as described earlier (Malbec et al., 2007), i.e., surface expression of IgE and c-kit as well as strong metachromatic staining. Moreover, we analyzed the presence of individual MC proteases at the protein level and found significant amounts of mMCP-4, -5, -6 and MC-CPA in WT PCMCs. As PCMCs contain all types of MC proteases, they resemble those MCs found in tissues involved in allergic inflammation, e.g., in the skin, in murine lungs and in the smooth muscle layer around airways of asthmatic patients (Bradding et al., 2006; Waern et al., 2009).

In this study, we found that MC-mediated degradation of IL-13 was completely dependent on the presence of serglycin proteoglycan. This was demonstrated by the inability of serglycin$^{-/-}$ MCs to reduce IL-13 levels upon degranulation induced by FcεRI crosslinking or calcium ionophore. A plausible explanation for this finding was that the lack of a number of preformed MC proteases in serglycin$^{-/-}$ MCs caused their inability to degrade IL-13. Another possibility is that the reduction in IL-13 levels was

merely due to binding of added IL-13 to serglycin proteoglycan on the cell surface, resulting in reduced levels in supernatants. We therefore assessed the involvement of proteolytic cleavage by using a panel of protease inhibitors, and showed that a serine protease inhibitor could prevent IL-13 reduction, whereas inhibitors of metalloproteases, cysteine proteases and aspartic acid proteases had no effect. Taken together, these findings strongly suggested that the IL-13 reduction seen in FcεRI-activated murine MCs is due to proteolytic events, mediated by one or several serglycin-dependent serine proteases expressed by murine MCs, i.e., by mMCP-4, -5, -6 or by other as yet non-identified proteases that rely on serglycin for storage.

Considering the protective role of mMCP-4 in the allergic airway inflammation that we reported earlier (Waern et al., 2009), it was of particular interest to evaluate the contribution of mMCP-4 to IL-13 degradation. We noticed that mMCP-4$^{-/-}$ MCs did not degrade endogenously produced IL-13 as efficiently as did WT MCs, whereas there was only a minor difference between mMCP-4$^{-/-}$ and WT MCs in the ability to degrade exogenously added IL-13. mMCP-4 can thus promote IL-13 degradation, a finding that is in
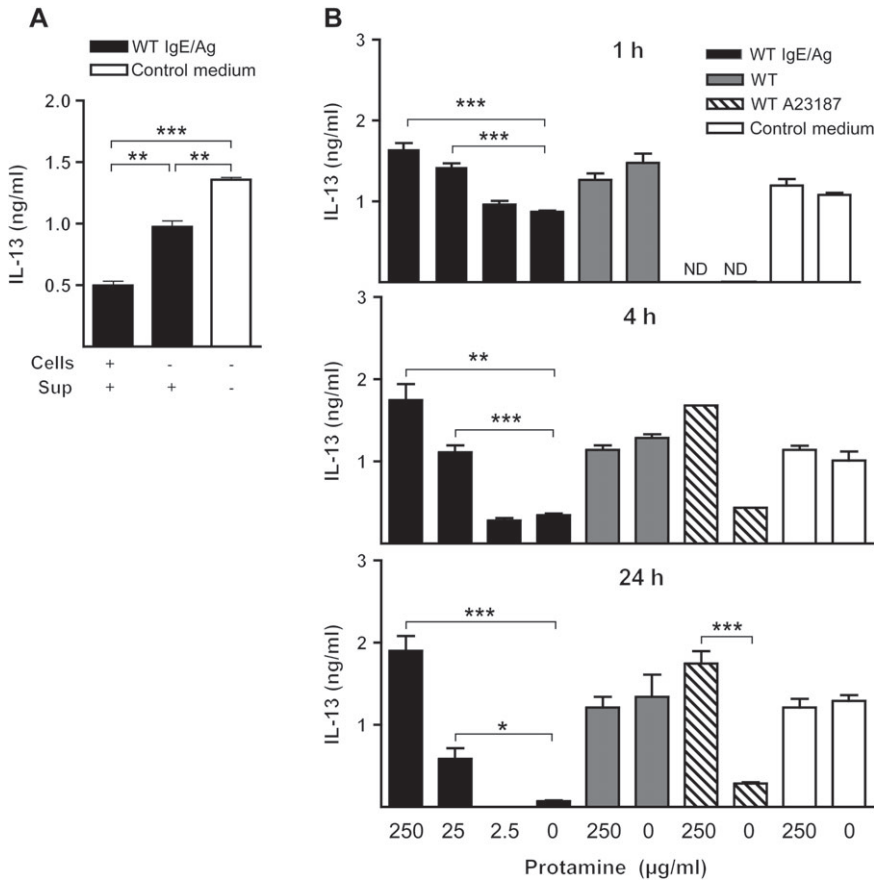
Figure 8 IL-13 degradation by MCs requires functional heparin and is partly cell bound.
(A) WT PCMCs were degranulated by IgE crosslinking (IgE/Ag). IL-13 was either added to medium alone, to cell-free supernatants from degranulated cells (sup) or to whole cultures containing both cells and supernatants and incubated for 24 h. (B), WT PCMCs were degranulated by IgE crosslinking (IgE/Ag) or by calcium ionophore (A23187) in the presence of IL-13. Protamine, a heparin antagonist, was added as indicated. IL-13 levels were analyzed by ELISA and are shown as means±SEM (n=3).

agreement with previous studies showing that human chymase has the ability to degrade IL-13 (Zhao et al., 2005). However, it is important to emphasize that the absence of mMCP-4 resulted in only a partial blockade of IL-13 degradation, which suggests the contribution of other (serglycin-dependent) MC serine proteases to the total impact of MCs on IL-13 levels. We may therefore envisage a scenario in which multiple serglycin-dependent proteases may act on IL-13 and, most likely, on other pro-inflammatory cytokines as well, thereby serving a regulatory function by preventing accumulation of excessive amounts of potentially harmful cytokines. Accordingly, the absence of individual serglycin-dependent proteases (e.g., mMCP-4, -6) may result in only limited effects (or no effects) on total cytokine-degrading capacity due to redundancy and overlapping functions among individual serglycin-dependent proteases, whereas a global absence of the serglycin-protease axis will lead to a profound impairment in the ability of MCs to regulate cytokines such as IL-13.

As shown here, MCs have the capacity both to generate and to degrade IL-13, which raises the intriguing question of whether MCs are net producers or degraders of IL-13. Although we cannot with certainty clarify this issue, it is conceivable that IL-13 generation and degradation, respectively, may occur during different timeframes during an inflammatory reaction. Conceivably, IL-13 production and release into the exterior may occur in the early phase of an inflammatory response, whereas IL-13 degradation is a subsequent event serving to prevent excessive effects of secreted IL-13.

An intriguing finding of this study was that IL-13 degradation was completely dependent on the presence of MC-CPA protein. However, a role for MC-CPA catalytic activity in the degradation process was excluded as MCs expressing inactive MC-CPA or MCs treated with a metalloprotease inhibitor exhibited essentially normal IL-13 degradation. These findings, together with the total blockade of IL-13 degradation by a serine protease inhibitor, suggest

that the putative IL-13-degrading proteases are strictly dependent on the presence of MC-CPA at the protein level. However, the exact nature of the IL-13-degrading protease activity is still intriguing.

Several reports suggest that MC proteases, in particular chymases, are dependent on interaction with heparin (serglycin) for optimal proteolysis of certain substrates. The mechanism for this effect involves simultaneous binding of the MC protease and its substrate to the same heparin (serglycin) molecule. Thereby, the heparin chain will bring the enzyme and substrate in close contact and in this way facilitate proteolytic cleavage. In line with this notion, heparin has been shown to preferentially facilitate the cleavage of heparin (serglycin)-binding substrates, e.g., fibronectin and thrombin (Tchougounova et al., 2001; Tchougounova and Pejler, 2001). To evaluate whether such a mechanism is operative in the MC-mediated degradation of IL-13, we used a polycationic heparin antagonist (protamine) that blocks the anionic sites of the serglycin moiety of the putative serglycin-MC protease complex without affecting the active site of the putative IL-13-degrading protease(s). Indeed, protamine was shown to inhibit the degradation of IL-13, which suggests that IL-13 degradation is facilitated by the simultaneous binding of IL-13 and IL-13-degrading MC proteases to serglycin. This scenario would thereby imply that IL-13 has the ability to interact with heparin, a notion that may be supported by the previously reported ability of heparin to modulate IL-13-mediated activities (Kanabar et al., 2008).

The interaction between MC proteases and the heparin side chains of serglycin proteoglycan may also influence the location of proteases in the extracellular environment when released upon degranulation, e.g., proteases with strong binding to heparin would be localized close to the MC or even bound to the cell surface, whereas those with weaker binding would be allowed to diffuse away from the MC surface. In support of such a scenario, our data suggest that the IL-13-degrading proteases are partly attached to the cell surface upon MC degranulation. This notion is also supported by previous studies showing that mMCP-6 (Ghildyal et al., 1996) and mMCP-4 (Tchougounova and Pejler, 2001), both of which have high affinity for serglycin (Pejler and Maccarana, 1994; Hallgren et al., 2004), remain largely associated with the cell surface after MC degranulation.

In summary, the present findings implicate serglycin and its complex-bound proteases in down-regulating extracellular levels of pathogenic cytokines. Conceivably, the serglycin-protease axis may have a major impact on the outcome of inflammatory processes in which MCs

participate, by limiting excessive effects of cytokines expressed either intrinsically by MCs or by other inflammatory cells.

Materials and methods

Mice

Serglycin$^{-/-}$ (Åbrink et al., 2004), mMCP-4$^{-/-}$ (Tchougounova et al., 2003), mMCP-6$^{-/-}$ (Shin et al., 2008) and MC-CPA$^{-/-}$ (Feyerabend et al., 2005) mice, all on a C57BL/6 genetic background, were previously described. Mc-cpa^{Y356L,E378A} (MC-CPAmut) are knock-in mice with catalytically inactive MC-CPA (Schneider et al., 2007). WT C57BL/6 mice were used as controls. Mice were bred and maintained in Uppsala Biomedical Centre. Animal experiments were approved by the local ethics committee.

Generation of PCMCs

Peritoneal cell-derived MCs (PCMCs) were established according to a protocol described by Malbec et al. (2007)). In short, PCMCs were established by culture of peritoneal cells in DMEM plus GlutaMAX (Gibco, Invitrogen, Paisley, UK) supplemented with 10% supernatant of stem cell factor-transfected Chinese hamster ovary cells (a gift from Dr. M. Daeron, Pasteur Institute, France), 10% FBS, 60 µg/ml streptomycin, 50 µg/ml penicillin, 100 µM MEM non-essential amino acids and 50 µM 2-mercaptoethanol (Gibco). The medium was changed every 4 to 5 days. Cultures of 4–5 weeks were used for all experiments.

Cytospin and staining

Cells were collected on cytospin slides (600 rpm for 5 min on a Cytospin 2, Shandon Southern Products Ltd, Runcorn, UK). Slides were stained for 5 min in concentrated May-Grünwald (Merck, Darmstadt, Germany) and for 1 min in a 2-fold dilution of May-Grünwald followed by 15 min in 2.5% Giemsa (Merck). For metachromatic staining, slides were placed in a 2% toluidine blue solution (Sigma Aldrich, St Louis, MO, USA) containing 1% sodium chloride (pH 2.4). After staining, the slides were washed in H$_2$O and air-dried.

Flow cytometry

Flow cytometry was used to analyze cell surface expression of CD117 and FcεRI on PCMCs. WT PCMCs were cultured overnight in the presence of 5 µg/ml murine IgE anti-TNP (BD Biosciences Pharmingen, San Jose, CA, USA) to saturate unbound FcεRI. Samples of 0.5×10^6 cells in PBS containing 0.5% FBS were stained for 30 min on ice with 0.5 µg FITC-labeled rat anti-mouse IgE, PE-labeled rat anti-CD117 or with rat isotype controls (ImmunoTools, Friesoythe, Germany). Cells were washed twice and then analyzed using a FACScan flow cytometer and the CELLQuest 3.3 software (Becton Dickinson, San Jose, CA, USA).

Western blot analyses

Western blot was performed as previously described (Waern et al., 2010). Briefly, cell extracts from PCMCs were separated under reducing conditions on 12% SDS-PAGE gels and blotted onto polyvinylidene difluoride membranes. After blocking, membranes were stained over night at 4°C with rabbit antisera towards mMCP-4, mMCP-5, mMCP-6, MC-CPA or cathepsin G (dilution 1:400 in LI-COR blocking buffer, LI-COR Biosciences, Cambridge, UK). As a loading control, β-actin was detected using a goat polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA, dilution 1:200). Bound primary antibodies were detected with IR Dye 800-conjugated donkey anti-rabbit Ig or IR Dye 800-conjugated donkey anti-goat Ig (LI-COR Biosciences, dilution 1:1000). Visualization was performed using Odyssey IR imaging system (LI-COR Biosciences).

IL-13 degradation

PCMCs were degranulated via crosslinking of FcεRI. IgE anti-TNP (BD Biosciences Pharmingen) was added to 1×10^6 PCMCs at a concentration of 1 µg/ml followed by incubation overnight at 37°C. Cells were washed three times and resuspended with DMEM supplemented as above. Ovalbumin (OVA)-TNP was added to a final concentration of 0.4 µg/ml to 1×10^6 cells/ml. For calcium ionophore-mediated degranulation, A23187 (Sigma Aldrich) was added (2 µm final concentration) to 1×10^6 cells/ml. Mouse recombinant interleukin-13 (mrIL-13) (PeproTech, London, UK) was added at 2–10 ng/ml to the PCMCs immediately after stimulation with either IgE anti-TNP, A23187 or to non-stimulated control cells. As an additional control, IL-13 was added to wells containing medium without cells. No external IL-13 was added when degradation of endogenous IL-13 was analyzed. Experiments were performed in triplicate and supernatants were collected at 1, 4 and 24 h after stimulation. To assess the localisation of IL-13 degradation, supernatant from IgE anti-TNP-stimulated MCs was separated from cells by centrifugation and incubated with 2 ng/ml mrIL-13 for 24 h.

Detection of IL-13

Levels of IL-13 were analyzed by ELISA according to the manufacturer's instructions (PeproTech). In a separate experiment, IL-13 was detected by Western blot analyses using rabbit anti-murine IL-13 (PeproTech).

Protease inhibitors and heparin antagonist

PCMCs were degranulated with IgE crosslinking or A23187 in the presence of exogenous IL-13 with the following inhibitors added to separate wells: 2 mm Pefabloc SC (Pentapharm LTD, Basel, Switzerland), 10 mm EDTA (Merck), 20 µm E-64 (Boehringer Mannheim Biochemicals, Mannheim, Germany), 10 µg/ml Pepstatin A (USB, Cleveland, OH, USA) or 10 µm Cathepsin G Inhibitor I (Merck). To block heparin, protamine (Sigma) was added at three different concentrations (2.5, 25 and 250 µg/ml). Supernatants were collected at 1, 4 and 24 h after stimulation and analyzed by IL-13 ELISA.

Generation and analyses of recombinant mMCP-5

A region encoding a six-histidine tag and an enterokinase site was added to the 5′ end of the coding region of mMCP-5. The sequence was ordered from Genscript Corporation (Piscataway, NJ, USA) as a fragment inserted in the vector pUC 57, with EcoRI and XhoI sites in the 5′ and 3′ ends, respectively. This fragment was excised from the vector by cleavage with EcoRI and XhoI and inserted in the mammalian expression vector pCEP-Pu2 (Vernersson et al., 2002). Following transfection of this construct into HEK-293 EBNA cells and selection with puromycin (1.5 µg/ml final concentration), a producer cell line was obtained. This cell line secreted inactive histidine-tagged mMCP-5 into the conditioned medium and the recombinant protein could be purified on IMAC Ni chelating columns (Qiagen, Hilden, Germany). A more detailed description of transfection and purification procedures can be found in Andersson et al. (2009, 2010). To obtain an enzymatically active mMCP-5, the protease was cleaved with enterokinase for 3 h at 37°C. (Roche Diagnostics, Mannheim, Germany). Following enterokinase cleavage, the activity of the enzyme was tested against a new type of recombinant substrate containing a cleavable sequence inserted in a linker region between two thioredoxin proteins. The enzyme cleaves in the linker region and generates two fragments of approximately 12 kDa in size (Figure 6A). The extent of cleavage can be monitored by SDS-PAGE analysis. Based on our previous analysis by phage display of the rat counterpart of mMCP-5 (rMCP-5) (Karlson et al., 2003), we constructed four different recombinant substrates with the following cleavable linker sequences: VLL**V**SEVL, VLL**I**SEVL, VLL**A**SEVL and VLL**F**SEVL. The first three of these four are elastase substrates with valine, isoleucine or alanine in the cleavable position, whereas the fourth substrate is a chymase substrate with a phenylalanine in the cleavable position. All four recombinant proteins were produced in *E.coli Rosetta gami* cells, and the protein was purified on Ni chelating columns according to previously published procedures (Andersson et al., 2009, 2010).

Statistical analysis

Statistical significance was calculated with Student's *t*-test, using the GraphPad Prism 4.0 (GraphPad Software, Inc, San Diego, CA, USA). All *p*-values <0.05 were considered to be significant.

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