Mast Cell Tryptase Potentiates Neutrophil Extracellular Trap Formation

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Abstract

Previous research has indicated an intimate functional communication between mast cells (MCs) and neutrophils during inflammatory conditions, but the nature of such communication is not fully understood. Activated neutrophils are known to release DNA-containing extracellular traps (neutrophil extracellular traps [NETs]) and, based on the known ability of tryptase to interact with negatively charged polymers, we here hypothesized that tryptase might interact with NET-contained DNA and thereby regulate NET formation. In support of this, we showed that tryptase markedly enhances NET formation in phorbol myristate acetate-activated human neutrophils. Moreover, tryptase was found to bind vividly to the NETs, to cause proteolysis of core histones and to cause a reduction in the levels of citrullinated histone-3. Secretome analysis revealed that tryptase caused increased release of numerous neutrophil granule compounds, including gelatinase, lactoferrin, and myeloperoxidase. We also show that DNA can induce the tetrameric, active organization of tryptase, suggesting that NET-contained DNA can maintain tryptase activity in the extracellular milieu. In line with such a scenario, DNA-stabilized tryptase was shown to efficiently degrade numerous pro-inflammatory compounds. Finally, we showed that tryptase is associated with NET formation in vivo in a melanoma setting and that NET formation in vivo is attenuated in mice lacking tryptase expression. Altogether, these findings reveal that NET formation can be regulated by MC tryptase, thus introducing a novel mechanism of communication between MCs and neutrophils.

Introduction

Mast cells (MCs) are hematopoietic immune cells with an impact on numerous physiological and pathological settings. For example, MCs are major players in allergic conditions and can also contribute to the pathology of numerous other pathological settings, including rheumatoid arthritis, cardiovascular complications, bone disease, fibrosis, and various inflammatory conditions of the skin [1–6]. On the other hand, MCs are also known to have...
beneficial functions, including a major role in degrading various toxins and also in the host defense against parasitic and bacterial insults [7, 8].

There is considerable evidence that MCs can have an impact on neutrophils. This was originally proposed from models of sepsis, where that lack of MCs was associated with reduced recruitment of neutrophils into the peritoneal cavity of mice, and it was suggested that the ability of MCs to recruit neutrophils is dependent on their release of TNF [9, 10]. This notion has recently gained further support by showing that MCs can directly release TNF into the circulation, thereby promoting neutrophil recruitment [11]. Further, it has been suggested that the ability of MCs to recruit neutrophils is dependent on their release of neutrophil-attracting CXCL1/CXCL2 chemokines [12], whereas another study linked the neutrophil-attracting capacity of MCs to their expression of IL-33 [13]. It has also been shown that MCs can enhance neutrophil recruitment in the context of allograft rejection [14] and in several other settings [15–20]. However, adding complexity to this issue, it has been shown that MCs can have a dampening impact on neutrophil recruitment in a setting of acute ischemic kidney injury [21], and MC products have been shown to be cytotoxic for neutrophils [22] and to limit neutrophil recruitment by degrading TNF [23].

When neutrophils have been attracted to the site of bacterial challenge, they will participate in the clearance of the invading bacteria. This can occur by phagocytosis and by the release of antibacterial compounds. Additionally, it is now established that neutrophils can neutralize bacteria by releasing extracellular traps (neutrophil extracellular traps [NETs]), a meshwork containing DNA, other nuclear constituents and compounds derived from the neutrophil secretory granules [24–26].

Although the collective evidence from previous research has firmly linked MCs to processes in which neutrophils are recruited, there is limited knowledge of whether MCs can have a more direct impact on neutrophil function. Under inflammatory conditions, MCs are prone to undergo degranulation, in response to various secretagogues that might be released during such conditions [27]. When MCs degranulate, they release large quantities of preformed mediators that are stored within the MC secretory granules. These include histamine and other biogenic amines, various cytokines (such as TNF), growth factors, proteoglycans as well as remarkably large amounts of neutral proteases, the latter including tryptase, chymase, and carboxypeptidase A3 (CPA3) [28–30]. Hence, neutrophils invading the inflammatory tissues will be exposed to these granule mediators. A plausible scenario could thus be that the function of neutrophils might be influenced through their interaction with the preformed mediators that are released by degranulating MCs in the inflammatory milieu.

MC tryptase is a tetrameric serine protease, in which all of the active sites are facing a narrow central pore. Due to this organization, tryptase is resistant to all known macromolecular protease inhibitors and has a relatively narrow substrate cleavage profile [31, 32]. It is established that the tryptase tetramer is stabilized by interaction with heparin proteoglycan, carrying a high negative charge density due to extensive sulfation and carboxylation [33]. However, in a recent study, we showed that tryptase can additionally be stabilized by DNA [34]. Considering that NET formation is a prominent event during inflammatory conditions and that such conditions also are associated with a massive release of tryptase from MCs, we here hypothesized that MC tryptase may interact with the DNA component of NETs and that tryptase potentially might affect neutrophils through such an interaction. Indeed, the findings presented here indicate that tryptase can have a major impact on NETs, introducing a novel mechanism by which MCs can influence the function of neutrophils under inflammatory conditions.

Materials and Methods

Reagents

Recombinant human (rh) cytokines and chemokines were purchased from ImmunoTools (Friesoythe, Germany). rh β-tryptase was prepared as described [35]. Porcine intestinal muco- cosa heparin, phorbol myristate acetate (PMA), calcium ionophore A23187, and lipopolysaccharide from *Escherichia coli* O111:B4 (lipopolysaccharide [LPS]) were from Sigma-Aldrich (Steinheim, Germany). N-formyl-Met-Leu-Phe (fMLP) was from Tocris (Abingdon, UK). NucBlue™ live ReadyProbes™ reagent (Hoechst-33342), NucGreen™ dead 488 ReadyProbes™ reagent (Sytox-green), ProLong™ diamond antifade mountant, goat anti-rat Alexa-647 antibody, goat anti-rabbit Alexa-555 antibody and BS³ (bis[sulfosuccinimidyl]suberate) were from Thermo Fisher Scientific (Waltham, MA, USA). Rat anti-mouse Ly-6G and Ly-6C antibody were from BD-Pharmingen (San Jose, CA, USA), rabbit anti-human histone histone 2A (H2A), H2B, H3, and H4 antibodies were from Abcam (Cambridge, UK) and rabbit anti-human histone H3 citrulline Arg2, -8 and -17 antibody was from Novus (Centennial, CO, USA).

Cleavage of Cytokines by Recombinant Human β-Tryptase

rh tryptase (1 ng/μL) was prepared in PBS (pH 7.2) alone, or either with DNA (∼10 ng/μL) or heparin (∼10 ng/μL) and incubated for 1 h on a shaker at room temperature. Cytokines and chemokines were dissolved in sterile water or PBS, following the instructions from the supplier (ImmunoTools); the final concentra-
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Dilutions were 0.1–0.25 μg/μL. Subsequently, 5 μL (~1.25 μg) of the cytokine in a 1.5 mL tube was mixed with either rh β-tryptase (~50 ng) alone, DNA-stabilized β-tryptase or heparin-stabilized β-tryptase, followed by incubation at 37°C for 2.5 h. Controls were prepared by replacing β-tryptase with sterile water or PBS. After incubation for 2.5 h, the reactions were terminated by the addition of 3 μL of 4x SDS-PAGE sample buffer (including 0.5 μL of β-mercaptoethanol) to each sample, followed by heating for 7 min at 85°C. The reaction mixtures were then analyzed on ~20% mini-protein tggx stain-free gels (Bio-Rad, Hercules, CA, USA). To visualize the proteins, the gels were stained overnight in colloidal Coomassie staining solution and washed with H2O for 2–4 h.

Tryptase Activity

rh β-tryptase (1 ng/μL), in PBS (pH 7.2; 100 μL total volume) was incubated either alone or in the presence of heparin or DNA (double-stranded). Incubations were performed in 96-well plates. The plates were incubated for 1 h at 37°C, followed by measurement of absorbance after adding 20 μL from a stock solution (10 μM) of the chromogenic substrate S-2288 (Chromogenix, Milan, Italy). Tryptase activity was monitored by reading the absorbance at 405 nm over 60 min using a microplate reader (M200-TECAN Infinite). Assays were performed in triplicates.

DNA Isolation

DNA was isolated from HMC-1 cells using the QIAamp DNA mini kit, following the manufacturer’s instructions.

Isolation of Human Neutrophils

Freshly isolated human neutrophils (0.2 × 10⁶ cells/mL in RPMI) were distributed in triplicates into 24-well plates. PMA was used at a final concentration of 50 nM and β-tryptase at 100 nM. The slides were incubated at 37°C (5% CO₂) for 2 h. Supernatants were removed, samples were fixed with 4% paraformaldehyde, and stained with NucBlue™ live ReadyProbes™. Samples were washed 3× with PBS and mounted using ProLong™ diamond antifade mountant. Images were acquired using 63× NA 1.40 oil objective on Zeiss LSM700 confocal microscope. Images were analyzed using Imaris software (Bitplane, Zurich, Switzerland).

Immunohistochemistry

Freshly isolated human neutrophils (0.2 × 10⁶ cells/mL in RPMI) were distributed in duplicates into 8-chamber polystyrene vessel tissue culture-treated glass slides. PMA was used at a final concentration of 50 nM and β-tryptase at 100 nM. The slides were incubated at 37°C (5% CO₂) for 2 h. Supernatants were removed, samples were fixed and permeabilized with methanol for 10 min, washed 3× with PBS and blocked for 15 min at room temperature with 10% goat serum in PBS. Primary antibody was incubated overnight at 4°C; slides were washed 3× with PBS and incubated with the secondary antibody for 1 h at room temperature. After 3× washing with PBS, slides were mounted using ProLong™ Diamond Antifade Mountant. Images were acquired using Zeiss LSM700 confocal microscope and a Zeiss LSM710 SIM-Elyra super-resolution microscope with automatic segmentation (Zeiss, Oberkochen, Germany), or a Nikon 90i, fluorescent microscope with ×20 objective (Nikon, Melville, NY). Images were analyzed using ImageJ open source image processing software and Imaris software (Bitplane).

B16F10 Tumor Sections

B16F10 tumors from wild-type and Mcpt6 knockout mice were obtained as described [36]. Tissue sections from paraffin-embedded tissue (5 μm) were deparaffinized and first stained with NucGreen™ Dead 488 ReadyProbes, followed by NucBlue™ Live ReadyProbes. Samples were then fixed and permeabilized with methanol for 10 min, washed 3× with PBS and blocked for 15 min at room temperature with 10% goat serum in PBS. Tissue sections were then incubated overnight at 4°C with rat anti-mouse Ly-6G and Ly-6C antibody diluted 1:500 in PBS. Sections were washed 3 times in PBS and incubated for 1 h at room temperature with goat anti-rat Alexa-647 antibody (1:1,000 in PBS), washed 3× with PBS and incubated at room temperature for 2 h with rabbit anti-Mcpt6 immune serum (1:500 in PBS). Sections were washed 3× with PBS and incubated for 1 h at room temperature with goat anti-rabbit Alexa-555 antibody (1:1,000 in PBS). Samples were washed 3× with PBS and mounted using ProLong™ Diamond Antifade.
Mountant. Images were acquired using a Zeiss LSM700 confocal microscope and were analyzed using Imaris software. The animal experiments were approved by the relevant Ethical Committee (Uppsala djurförsöksåsetiska nämnd Dnr 5.8.18-04096/2019).

**Tryptase Tetramerization**

1.5-mL tubes containing rh β-tryptase (1 μg) alone or with increasing concentrations of heparin or DNA in PBS were incubated for 15 min at 37°C in an orbital shaker. Samples were adjusted to 30 μL final volume. After incubation, 10 μL of BS³ cross-linker (10 mg/mL) was added, followed by incubation for 5 min at room temperature. Ten microliters of 5× concentrated Laemmli buffer was added, and samples were analyzed by PAGE.

**Mass Spectrometry**

Supernatants from untreated neutrophils or neutrophils treated with 50 nM PMA and/or 100 nM β-tryptase were collected. The samples were reduced, alkylated, and digested with trypsin according to a standard operating procedure. Next, the collected peptides were vacuum centrifuged to dryness using a SpeedVac system. Thereafter, the samples were resolved in 30 μL 0.1% formic acid and further diluted 4×. The resulting peptides were separated in reversed-phase on a C18-column and electrospayed on line to a QExactive Plus Orbitrap mass spectrometer (Thermo Finnigan) with 35 min gradient. Tandem mass spectrometry was performed applying HCD. Database searches were performed using the Se-quest algorithm, embedded in Proteome Discoverer 1.4 (Thermo Fisher Scientific) against the database consisted of *Homo sapiens* proteome extracted from Uniprot, Release 2019. The search parameters were set to enzyme: Trypsin. Fixed modification was carbamidomethyl, and variable modifications were oxidation (M) and deamidated (NQ). The search criteria for protein identification were set to at least 2 matching peptides of 95% confidence level per protein.

**Statistics**

All analyses were performed in GraphPad Prism 7.0 software (GraphPad Software Inc., San Diego, CA, USA) using unpaired t test. Results shown are presented as mean values ± SEM. A p value ≤0.05 was considered statistically significant.
**Results**

**PMA Induces NET Formation in Human Peripheral Blood-Derived Neutrophils**

Neutrophils were purified from human peripheral blood by density gradient centrifugation. To induce NET formation, the neutrophils were treated with PMA, an established method to induce NET formation [24]. As seen in Figures 1a and 3, addition of PMA to the neutrophils caused the cells to attach to the substratum, indicating cellular activation. Moreover, PMA induced a time-dependent release of DNA-containing structures with a morphological appearance strongly reminiscent of NETs (Fig. 1a, b, 3). Previous studies have revealed that citrullination of histone-3 (H3) at Arg2, -8, and -17 is a hallmark event during NET formation [37]. To further substantiate that the morphological effects observed after PMA treatment of the neutrophils represents NET formation, we stained the activated cells with an antibody-recognizing citrullinated H3 (H3cit). Indeed, PMA-treatment of the neutrophils caused a marked increase in H3 citrullination (Fig. 1c).

**Tryptase Causes Exaggerated NET Formation in Human Neutrophils**

To assess whether tryptase can affect NET formation, we conducted experiments in which NETs were induced in the absence or presence of rh β-tryptase. Tryptase alone did not induce any major morphological effects on the neutrophils, and no apparent NET formation could be observed (Fig. 2a–c). However, when tryptase was added together with PMA, we noted that NET formation was
**Fig. 3.** Tryptase binds to NETs. Human neutrophils were treated with 50 nM PMA for different time periods as indicated, followed by 15 min incubation with 100 nM β-tryptase labeled with Alexa-488, and Hoechst-33342 staining. Three dimensional-images were generated from z-stack confocal sections. Scale bars, 10 μm.
markedly more pronounced than neutrophils that had been treated with PMA only (Fig. 2a–c; online suppl. Fig. 1; see www.karger.com/doi/10.1159/000520972 for all online suppl. material). Moreover, whereas neutrophils treated with PMA only retained considerable nuclear integrity, a striking observation was that neutrophils treated with PMA + tryptase displayed a completely disintegrated nuclear architecture (Fig. 2a–c). Taken together, these experiments suggest that tryptase has a strong potentiating effect on NET formation induced by PMA-treatment of human neutrophils.

NET formation was also seen in response to a calcium ionophore (A23187) and in response to N-fMLP, the latter representing a bacteria-derived neutrophil-activating peptide, and tryptase was shown to augment NET formation in response to both of these agents (online suppl. Fig. 2). In contrast, NET formation was not seen in response to bacterial LPS, either in the absence or presence of tryptase (online suppl. Fig. 2).

Tryptase binds strongly to heparin within the granules of MCs and is important for activation and tetramer formation of tryptase [38]. We thus considered the possibility that heparin might affect the ability of tryptase to promote NET formation. However, the ability of tryptase to promote NET formation was not affected by the presence of heparin (online suppl. Fig. 2).

**Tryptase Binds to NETs**

The findings above suggest that MC tryptase has a strong potentiating effect on NET formation. To approach the mechanism behind this, we first investigated whether tryptase has the ability to interact with the NETs. To this end, we labeled recombinant β-tryptase with Alexa-488, and then added the labeled tryptase to PMA-
activated human neutrophils followed by confocal microscopy analysis. As seen in Figure 3, tryptase consistently associated with PMA-induced NETs. In fact, the labeled tryptase largely overlapped with NETs throughout the neutrophil population, suggesting that tryptase binds efficiently to the NETs. However, we noted that tryptase positivity was not uniformly distributed within the individual NETs, being concentrated in distinct areas of the NET structures whereas adjacent regions within the NETs were tryptase-negative (Fig. 3).

Tryptase Causes Core Histone Truncation, Reduction of Citrullinated H3, and Release of Neutrophil Granule Constituents in PMA-Activated Human Neutrophils

To further elucidate the mechanism behind the impact of MC tryptase on NETs, we considered the possibility that tryptase achieves this effect by executing proteolytic effects. Based on our previous observation that tryptase can execute core histone truncation in the nucleus of MCs [39], we assessed whether tryptase could cause proteolysis of core histones in the PMA-treated human neutrophils. As seen in Figure 4a, all of the core histones (H2A, H2B, H3, and H4) showed minimal signs of degradation in the absence of PMA. Similarly, the core histones were largely intact also after PMA activation of the neutrophils. In contrast, marked core histone truncation was seen after treatment of the PMA-treated neutrophils with tryptase. Distinct proteolytic fragments were observed for H2B, H3, and H4 after treatment with tryptase, along with a reduced intensity of the bands corresponding to the intact histones. For H2A, a marked reduction in the levels of intact protein was seen after treatment with tryptase, whereas distinct proteolytic fragments could not be seen (Fig. 4a). Most likely, the latter observation is explained by degradation into smaller fragments that were not detected by the antibody.

Having shown that tryptase can regulate NET formation, we next asked whether this could be associated with effects on the levels of H3cit. As shown in Figure 4b, whereas extracellular traps from PMA-activated neutrophils were positive for H3cit, the levels of H3cit were markedly reduced after treatment with tryptase. This suggests that tryptase has the ability to cleave off H3 N-terminal tails carrying these modifications.

To provide further insight into the mechanism by which tryptase affects the NETs, we assessed whether tryptase affected the release of proteins from PMA-treated neutrophils. To this end, we analyzed the conditioned media from untreated, PMA-treated, tryptase treated and PMA + tryptase-treated neutrophils for alterations in the proteome. This analysis revealed little release of neutrophil granule compounds in untreated neutrophils, and also only minimal release after treatment with PMA only (Table 1). Under these conditions,
Fig. 5. DNA maintains the active tetrameric state of tryptase and promotes tryptase-mediated proteolysis of inflammatory cytokines. 

(a) rh β-tryptase was incubated alone or in the presence of increasing concentrations of heparin or DNA (15 min; 37°C), followed by cross-linking with BS3 and SDS-PAGE analysis. Tryptase monomers and tetramers are indicated by arrows.

(b) rh β-tryptase (1 ng/μL) was incubated alone or in the presence of either heparin (10 ng/μL) or DNA (10 ng/μL; room temperature; 1 h). Residual tryptase enzymatic activity was then measured using the chromogenic substrate S-2288.

(c) rh cytokines/chemokines were incubated either alone (control) or in the presence of tryptase, DNA-stabilized tryptase or heparin-stabilized tryptase as indicated, followed by SDS-PAGE analysis.
defensin-1 appeared to be the major neutrophil product released. In contrast, we noted that the treatment with tryp­tase (only) resulted in a more substantial release of neutrophil granule proteins, including lactoferrin, gelatinase, and lysozyme. An even more pronounced release of the corresponding granule proteins was seen after treatment with tryp­tase combined with PMA (Table 1). When cells were treated with PMA + tryp­tase in the presence of a tryp­tase inhibitor (Nafamostat), the pattern of released neutrophil proteins was similar as after treatment with PMA only (Table 1), indicating that the ability of tryp­tase to cause the release of neutrophil granule compounds is dependent on its proteolytic activity. Taken together, these findings suggest that tryp­tase has the capacity to modify the secretome of PMA-activated neutrophils.

DNA Maintains the Active Tetrameric State of Tryptase and Promotes Tryptase-Mediated Proteolysis of Inflammatory Cytokines

Our findings suggest that tryp­tase has the capacity to bind to NETs, most likely as a result of electrostatic inter­actions with the DNA moiety of these structures. Next, we evaluated whether DNA can affect the function of tryp­tase. To this end, we first assessed whether DNA can maintain the tetrameric and active organiza­tion of tryp­tase. Indeed, as shown by native gel elec­tro­phoresis, the tryp­tase tetramer was preserved in the presence of DNA, and as expected also in the presence of heparin (Fig. 5a). In contrast, tetrameric tryp­tase was undetectable in the absence of DNA or heparin (Fig. 5a). Stabilization of tryp­tase by the respective compounds was also verified by measurements of enzymatic activ­ity (Fig. 5b).

To assess whether the association of tryp­tase with DNA affects its ability to act proteolytically on macro­molecular targets, we assessed whether DNA-stabilized tryp­tase can degrade a number of cytokines and other pro-infl ammatory compounds. Out of 73 tested candi­dates (online suppl. Table 1), we noted proteolytic effects of tryp­tase on only 8 different compounds (IFNγ, TSLP, TRAIL, IL-20, Gal7-His, KGF-2, IL-21, and OSM) (Fig. 5c). Notably, DNA and heparin differed somewhat in their ability to promote tryp­tase-catalyzed degrada­tion of the respective compounds. Thus, whereas DNA-stabilized tryp­tase readily degraded TRAIL and IL-21, heparin-stabilized tryp­tase was less efficient in this respect. Conversely, heparin-stabilized tryp­tase appeared more effective in the degradation of TSLP versus tryp­tase stabilized by DNA (Fig. 5c).

Tryptase Promotes NET Formation in vivo

In the next set of experiments, we asked whether tryp­tase is associated with NETs formed in vivo, and whether our observed effect of tryp­tase on NET features can be translated into in vivo conditions. Melanomas are known to be associated with extensive inflammatory infiltrates, consisting of various immune cell types in­cluding, among others, MCs and neutrophils [36, 40]. Moreover, we showed recently that MCs populating melanoma tumors show extensive signs of activation and can release tryp­tase into the tumor milieu [36]. We thus reasoned that tryp­tase, released from tumor-assoc­iated MCs, may engage in contacts with NETs that are possibly formed in the inflammatory context of the tu­mor. As shown in Figure 6a and online supplementary Figure 3, we indeed identified an abundance of neutro­phils in the tumor stroma, as judged by Gr1 (Ly-6G/6C) staining. Moreover, we observed that the tumor-assoc­iated neutrophils frequently showed signs of NET for­mation, as judged by the presence of extracellular DNA arranged as NET-like fibers (assessed with a cell non­permeable DNA probe; SYTOX-green) (Fig. 6a, c). We also noted that tryp­tase was frequently associated with the NETs (Fig. 6a, c). To evaluate the possible function­al impact of tryp­tase on NET formation in vivo, we an­alyzed corresponding tumors from tryp­tase-deficient (Mcpt6+/−) mice. This analysis revealed that NET for­mation was dramatically reduced in tumor tissue from tryp­tase-deficient versus wild-type animals (Fig. 6b; on­line suppl. Fig. 3). In fact, NET formation could not be detected in any of 4 investigated sections taken from tumors of Mcpt6+/− mice. Hence, these data suggest that tryp­tase interacts with NETs in vivo and that tryp­tase has a major impact on NET formation in a melanoma setting in vivo.

Discussion

Previous research has suggested that there is an inti­mate relationship between MCs and neutrophils, as man­i­fested by the known ability of MCs to secrete cytokines and chemokines having the capacity to recruit neutro­phils [9–20]. With this work, we introduce an additional level of communication between these 2 cell types, by demon­strating that MC tryp­tase, a major component of the MC secretory granules, has the ability to promote the formation of NETs. Importantly, tryp­tase alone did not induce extracellular trap formation in neutrophils. How­ever, when tryp­tase was added in the presence of neutro-
phil-activating stimulus, NET formation was markedly more pronounced than if neutrophil-activating stimulus was added alone.

The mechanism underlying the ability of tryptase to potentiate NET generation is intriguing. NET formation is a complex process, with a hallmark event being chromatin decondensation [25]. Such chromatin decondensation is thought to depend on the citrullination of H3 at Arg2, -8, and -17 [37]. This causes neutralization of the positive charge at these positions, leading to weakened histone-DNA interactions, which will promote NET formation. Here, we showed that tryptase has the capacity to truncate the NET-contained core histones, which is in line with our previous observations where

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**Fig. 6.** Tryptase is associated with NETs in vivo and NET formation in vivo is attenuated in mice lacking tryptase expression. Sections from subcutaneous B16F10 melanoma tumors were stained for neutrophils (Ly-6G/6C), Mcpt6, extracellular DNA (SYTOX-green; cell-impermeable) and with Hoechst 33342 (depicts nuclear DNA; cell-permeable). The images represent 3D images generated from z-stacks. Sections from wild-type (WT) (a) and tryptase-deficient (Mcpt6 <sup>−/−</sup>) (b) mice were analyzed. a–c Sections were first stained with SYTOX-green, followed by Hoechst-33342. Cells were then permeabilized and stained for Ly-6G/6C (Gr-1) and Mcpt6. a Tumor section from WT mice showing the presence of activated neutrophils and extensive NET formation (green). Note the presence of Mcpt6 in close contact with NETs. b Tumor section from Mcpt6 <sup>−/−</sup> mice showing neutrophils with attenuated NET formation. Note the absence of Mcpt6 staining. c Higher magnification of tumors from WT mice showing neutrophils and NET formation in association with Mcpt6. Scale bars, 10 μm. Mcpt6, MC tryptase; WT, wild-type.
Tryptase was shown to execute cell-intrinsic core histone truncation in the nucleus of MCs [41]. In our previous studies, we showed that tryptase cleaves the corresponding core histones in the N-terminal regions, and it is notable that the proteolytic histone fragments observed in the previous study closely resemble those seen in the present work (see [41] and Fig. 4a). Based on these observations, we can therefore propose that the core histone truncation observed in the present study represents cleavages in the N-terminal portions of the core histones. Most likely, this will result in the proteolytical removal of Arg residues that are sites for citrullination, which may serve to reduce the positive charge of H3, in turn promoting NET formation. In support of this scenario, we show that tryptase causes a reduction in the levels of N-terminal histone citrullination (at Arg2, -8, and -17). Altogether, we may thus propose that tryptase has the capacity to cleave off N-terminal ends of core histones, which can lead to decreased interaction of histones with DNA, in turn contributing to DNA decondensation and thereby promoting NET formation. Notably, this scenario resembles the mechanism by which neutrophil elastase has been shown to promote NET formation [42].

Intriguingly, it has been shown that MCs can form extracellular traps with antimicrobial activity [43]. Based on the present study, we may thus propose that tryptase might have the capacity to regulate the generation of such MCs-derived extracellular traps. However, this remains to be investigated.

Notably, tryptase did not localize to the nuclei of the neutrophils. This suggests that tryptase acts on core histones after their exit from the nucleus, rather than by entering the neutrophil nuclei and carrying out histone proteolysis within this compartment. It is also notable that tryptase was associated with distinct subregions of the NETs, rather than being uniformly distributed over the entire NET structures. It is known from previous studies that tryptase binds strongly to polyanions of glycosaminoglycan type [38], and we recently showed that tryptase also can bind to and be stabilized by DNA [34]. Hence, a likely scenario could be that tryptase binds to the NETs through electrostatic interactions with the DNA moiety of the NETs. Through this interaction, tryptase may become stabilized and may gain access to proteolytic targets such as the core histones.

The impact of tryptase on NETs could have multiple pathophysiological consequences. As shown here, DNA-stabilized tryptase has a strong capacity to carry out proteolysis of various inflammatory cytokines; in fact, DNA-associated tryptase was even more efficient at cleaving TRAIL and IL-21 than were heparin-stabilized tryptase. Hence, a likely scenario could be that NET-associated tryptase can regulate the cytokine profile in pathological contexts where both MCs and neutrophils are present and are activated. We also noted that tryptase has the ability to cause increased release of neutrophil granule compounds into the surrounding milieu, with possible consequences related to the impact of such compounds on the inflammatory outcome. Another likely scenario is that promotion of NET formation by tryptase can result in a more efficient clearance of bacteria through NET-dependent mechanisms.

On a different angle, we here show that tryptase is indispensable for NET formation in the inflammatory milieu of melanoma tumors in vivo. Of note, we have recently shown that tryptase has a protective impact in a melanoma model [36], and there are also clinical studies pointing to a positive association between high tryptase levels and favorable clinical outcome in melanoma [44]. Although the exact mechanisms behind these findings are not clear, we may, based on the present investigation, speculate that the impact of tryptase on melanoma outcome could be related to its ability to regulate NET formation and functionality.

Statement of Ethics

The experiments on human neutrophils were approved by the relevant Ethical Committee (Etikprövningsmyndigheten Dnr 2020-05080). The animal experiments were approved by the relevant Ethical Committee (Uppsala djurförsöksnämnden Dnr 5.8.18-04096/2019).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

G.P. designed and planned the study, interpreted data and wrote the manuscript; S.A. performed experiments and wrote parts of the article; M.G. performed experiments; J.A. performed
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Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding authors.
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