Manuscript Details

Manuscript number	MIMM_2019_213_R1
Title	Giardia excretory-secretory proteins modulate the enzymatic activities of mast cell chymase and tryptase
Article type	Full Length Article

Abstract

Background: Mast cells are involved in the host immune response controlling infection with the non-invasive intestinal protozoan parasite Giardia intestinalis. Experimental infections in rodents with G. intestinalis showed increased intestinal expression of mucosal and connective mast cell specific proteases suggesting that both mucosal and connective tissue mast cells are recruited and activated during infection. During infection Giardia excretory-secretory proteins (ESPs) with immunomodulatory capacity are released. However, studies investigating potential interactions between Giardia ESPs and the connective tissue mast cell specific serine proteases, i.e. human chymase and mouse mast cell protease (mMCP)-4 and, human and mouse tryptase (mMCP-6) remain scarce. Results: We first investigated if soluble Giardia proteins (sGPs), which over-lap extensively in protein content with ESP fractions, from the isolates GS, WB and H3, could induce mast cell activation. sGPs induced a minor activation of bone marrow derived mucosal-like mast cells, as indicated by increased IL-6 secretion. Furthermore, sGPs were highly resistant to degradation by human tryptase while human chymase degraded a 65kDa sGP and, wild-type mouse ear tissue extracts degraded several protein bands in the 10 to 75kDa range. In striking contrast, sGPs and ESPs were found to increase the enzymatic activity of human and mouse tryptase and to reduce the activity of human and mouse chymase. Conclusion: Our finding suggests that Giardia ssp. via enhancement or reduction of mast cell protease activity may modulate mast cell-driven intestinal immune responses. ESP-mediated modulation of the mast cell specific proteases may also increase degradation of tight junctions, which may be beneficial for Giardia ssp. during infection.

Keywords	mast cell; tryptase; chymase; soluble Giardia proteins (sGPs); Giardia excretory-secretory proteins (ESPs); intestinal parasite
Taxonomy	Health Sciences, Natural Sciences

Corresponding Author	Magnus Åbrink
Corresponding Author's Institution	Section for Immunology
Order of Authors	Zhiqiang Li, Dimitra Peirasmaki, Staffan Svard, Magnus Åbrink

Suggested reviewers	Andre Buret, Lars Eckmann, Andrew F Walls

Submission Files Included in this PDF

File Name [File Type]

Letter to editor Molecular Immunology.doc [Cover Letter]

Response to reviewers.docx [Response to Reviewers]

R1 Highlights Molecular Immunology.docx [Highlights]

R1 Li et al. Abstract.docx [Abstract]

R1 Li et al. 2019-07-22x.docx [Manuscript File]

Figure 1 Li et al..tiff [Figure]

Figure 2 Li et al..tiff [Figure]

Figure 3 Li et al..tiff [Figure]

Figure 4 Li et al..tiff [Figure]

Figure 5 Li et al..tiff [Figure]

Abstract

Background: Mast cells are involved in the host immune response controlling infection with the non-invasive intestinal protozoan parasite *Giardia intestinalis*. Experimental infections in rodents with *G. intestinalis* showed increased intestinal expression of mucosal and connective mast cell specific proteases suggesting that both mucosal and connective tissue mast cells are recruited and activated during infection. During infection *Giardia* excretory-secretory proteins (ESPs) with immunomodulatory capacity are released. However, studies investigating potential interactions between *Giardia* ESPs and the connective tissue mast cell specific serine proteases, *i.e.* human chymase and mouse mast cell protease (mMCP)-4 and, human and mouse tryptase (mMCP-6) remain scarce.

Results: We first investigated if soluble *Giardia* proteins (sGPs), which over-lap extensively in protein content with ESP fractions, from the isolates GS, WB and H3, could induce mast cell activation. sGPs induced a minor activation of bone marrow derived mucosal-like mast cells, as indicated by increased IL-6 secretion and no degranulation. Furthermore, sGPs were highly resistant to degradation by human tryptase while human chymase degraded a 65kDa sGP and, wild-type mouse ear tissue extracts degraded several protein bands in the 10 to 75kDa range. In striking contrast, sGPs and ESPs were found to increase the enzymatic activity of human and mouse tryptase and to reduce the activity of human and mouse chymase.

Conclusion: Our finding suggests that *Giardia ssp.* via enhancement or reduction of mast cell protease activity may modulate mast cell-driven intestinal immune responses. ESP-mediated modulation of the mast cell specific proteases may also increase degradation of tight junctions, which may be beneficial for *Giardia ssp.* during infection.

Giardia excretory-secretory proteins modulate the enzymatic activities of mast cell chymase and tryptase

Zhiqiang Li,[1] Dimitra Peirasmaki,[2] Staffan Svärd,[2] and Magnus Åbrink[1]*

1. Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, SE-75007 Uppsala, Sweden

2. Department of Cell and Molecular Biology, Uppsala University, SE-75124 Uppsala, Sweden

* Corresponding author: Magnus Åbrink, Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, VHC, Box 7028, SE-75007 Uppsala, Sweden, e-mail address: magnus.abrink@slu.se

Abstract

Background: Mast cells are involved in the host immune response controlling infection with the non-invasive intestinal protozoan parasite *Giardia intestinalis*. Experimental infections in rodents with *G. intestinalis* showed increased intestinal expression of mucosal and connective mast cell specific proteases suggesting that both mucosal and connective tissue mast cells are recruited and activated during infection. During infection *Giardia* excretory-secretory proteins (ESPs) with immunomodulatory capacity are released. However, studies investigating potential interactions between *Giardia* ESPs and the connective tissue mast cell specific serine proteases, *i.e.* human chymase and mouse mast cell protease (mMCP)-4 and, human and mouse tryptase (mMCP-6) remain scarce.

Results: We first investigated if soluble *Giardia* proteins (sGPs), which over-lap extensively in protein content with ESP fractions, from the isolates GS, WB and H3, could induce mast cell activation. sGPs induced a minor activation of bone marrow derived mucosal-like mast cells, as indicated by increased IL-6 secretion and no degranulation. Furthermore, sGPs were highly resistant to degradation by human tryptase while human chymase degraded a 65kDa sGP and, wild-type mouse ear tissue extracts degraded several protein bands in the 10 to 75kDa range. In striking contrast, sGPs and ESPs were found to increase the enzymatic activity of human and mouse tryptase and to reduce the activity of human and mouse chymase.

Conclusion: Our finding suggests that *Giardia ssp.* via enhancement or reduction of mast cell protease activity may modulate mast cell-driven intestinal immune responses. ESP-mediated modulation of the mast cell specific proteases may also increase degradation of tight junctions, which may be beneficial for *Giardia ssp.* during infection.

Keywords: Mast cell, Tryptase, Chymase, Infection, *Giardia ssp.,* Parasite, Intestine, *Giardia* excretory-secretory proteins (ESPs)

Introduction

Mast cell activation and degranulation to microbial and parasitic infections may occur by several mechanisms, *i.e.* via PAMPs and alarmins (DAMPs) acting over pattern recognition receptors, or via complement- and antibody-dependent mechanisms [1]. When mast cells degranulate large quantities of preformed mediators are released, *e.g.* the mast cell specific proteases. Of the mast cell specific proteases tryptases and chymases may constitute up to as much as 35-50% of the mast cell protein content [2]. In the mouse mast cells predominantly express four different chymases: in mucosal tissue mast cells express the mouse mast cell protease (mMCP)-1 (*Mcpt*1) with chymotrypsin-like activity and mMCP-2 (*Mcpt*2) with as yet no identified enzymatic activity and; in connective tissue mast cells express the mMCP-4 (*Mcpt*4) with chymotrypsin-like activity and mMCP-5 (*Mcpt*5) with elastase-like activity. In contrast, human mast cells express only one chymase gene and the closest functional ortholog in mice is the chymase mMCP-4. Mouse and human mast cells express three tryptase genes, *i.e.* the mouse mast cell proteases (mMCP)-6 *Mcpt*6 which is closely related to human TPSAB1/A1 and, mMCP-7 (*Tpsab*1) closely related to human TPSD1 as well as mTMT (*Tpsg*1) closely related to the human TPSG1 gene [3]. The C57BL/6 mouse strain carries a disrupted mMCP-7 gene due to a splicing defect and a gene knockout of mMCP-6 has been introduced on the C57BL/6 background. The lack of both mMCP-6 and mMCP-7 caused no major problems for unchallenged mice suggesting that the mast cell specific tryptases are not essential for survival. However, challenged mMCP-6-deficient mice displayed significant inability to recruit eosinophils in chronically *Trichinella spiralis* infected skeletal muscle tissue [4]. In another recent study, it was suggested that the termination of the helminth *Strongyloides ratti* infection required the presence of mucosal mast cells, because infection lasted longer in CPA3^{Cre} mice which lack mucosal and connective tissue mast cells than in *Mcpt*5^{Cre} R-DTA mice which only lack connective tissue mast cells [5].

Giardia intestinalis is a relatively prevalent non-invasive intestinal protozoan parasite with zoonotic potential found worldwide that can cause diarrhea and growth stunting in humans and animals [6]. *G. intestinalis* parasite antigens, either on the surface or excreted-secreted products, have been shown to be immunogenic [7]. *Giardia*-infection and *Giardia* excretory-secretory proteins (ESPs) induced IL-4, IL-5 and IL-10 cytokine responses as well as IgG and IgE antibody responses in BALB/c mice [8]. ESPs induced IL-8 production in a human gastrointestinal cell line (HT-29) via activation of p38, ERK1/2, nuclear factor kappaB and activator protein 1 [9]. Several other chemokines (*e.g.* CXCL 1-3, CCL2 and 20) are up-regulated by ESPs in differentiated Caco-2 cells [10]. In addition, antibody responses to several *Giardia* glycoproteins have been identified in the serum of immunized BALB/c mice [11]. Furthermore, glycoproteins with immunomodulatory capacity and proteolytic activity have been identified in the excretory-secretory proteins from *Giardia* trophozoites [10, 12-18]. During infections with *Giardia ssp.* mast cells are recruited to the intestine [19] and compared to wild-type mice c-kit-dependent mast cell-deficient mice (c-kit$^{w/wv}$) and anti-c-kit mast cell depleted mice showed increased parasite burden and failure to produce parasite-specific IgA antibodies [20, 21]. In addition, mast cell specific proteases were among the most obviously induced transcripts in the small intestinal tissue at 13 days post infection [22]. Mast cells are also recruited to the small intestinal mucosa during *Giardia* infections in humans [23], gerbils [24, 25] and rats [26]. Stimulation of the rat mast cell line HRMC with soluble *Giardia* protein extracts (sGPs) which overlap extensively in protein content with *Giardia* ESPs [10, 18], triggered mast cell activation and up-regulation of tryptase [21, 27]. Collectively, these results suggest that mast cells and the mast cell specific proteases may play a significant role in the host immune responses against *G. intestinalis*. However, if *Giardia* via secretion of ESPs and other soluble proteins directly interacts with the mast cell specific proteases remain

unknown. Therefore, in this study we investigated the activities of human and mouse mast cell tryptase and chymase towards soluble *Giardia* protein extracts (sGPs) from the isolates GS and H3 (both assemblage B) and WB (assemblage A) and, ESPs from the GS and WB isolates.

Material and methods

Ethics and Mice

Animals were kept in agreement with the Swedish Animal Welfare Act under the permission C140/15 granted by Uppsala District Court. Heterozygote mice of the mouse mast cell protease 6-deficient (mMCP-$6^{-/-}$) mouse strain on the C57BL/6J Taconic background were crossed to produce littermate mMCP-$6^{+/+}$ and mMCP-$6^{-/-}$ mice from which ear tissue materials to be used in the enzymatic protease activity assays were collected. Bone marrow for generation of bone marrow-derived mucosal-like mast cells (BM-MMC) was obtained from five of the in house bred mMCP-$6^{+/+}$ C57BL/6J Taconic mice. All mice were housed at the Faculty of Veterinary Medicine and Animal Science, SLU, Uppsala, Sweden under specific pathogen free conditions in an enriched environment and provided food and water ad libitum.

Bone marrow derived mast cells: preparation, culture and in vitro stimulation

To obtain bone marrow derived mucosal-like mast cells (BM-MMCs), bone marrow cells was collected from femur and tibia. The cells were washed two times in PBS and cultured in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 1% PEST, 2 mM L-glutamine, 5ng/ml mouse interleukin (IL) -9 (ImmunoTools), 1ng/ml recombinant human transforming growth factor beta (TGF-beta, ImmunoTools), 1ng/ml mouse IL-3 (ImmunoTools) and 50ng/ml mouse stem cell factor (SCF, ImmunoTools). After two weeks >99% of the cells showed BM-MMCs characteristics as verified by May-Grünwald/Giemsa staining. The BM-MMCs were washed three times in PBS and seeded in duplicates (first experiment) or triplicates (second experiment) at $2x10^6$ BM-MMCs/ml in HBSS and challenged with different concentrations (25 ng/ml, 100 ng/ml and 1µg/ml) of soluble protein extracts from three different *Giardia* isolates (GS, WB and H3). After 6h or 24h incubation (at 37^0C, 5% CO_2), supernatants were collected and frozen at -20^0C until used.

Preparations of soluble Giardia proteins and Giardia excretory-secretory proteins

To get soluble *Giardia* proteins (sGPs), *Giardia* assemblage A (WB-C6, ATTC 50803) and B (GS/M, ATTC 50581 and H3) trophozoites were grown separately at 37 °C in Diamond- and Keister media (TYDK media) supplemented with 10% sterile bile, 10% heat inactivated bovine serum (FBS, Gibco, Thermo FisherScientific, MA, USA) and 1% Ferric ammonium citrate solution with the final pH adjusted to 6.8 [28]. Trophozoites were collected after three washing steps with cold, sterile phosphate-buffered saline (PBS) by pelleting with centrifugation at 931xg at 4^0C for 10 minutes. The pellet was re-suspended in PBS, followed by sonication (3 times for 30 seconds at 50 Watts) and centrifuged at 14462xg at 4 ^{0}C for 15 minutes to remove cell debris. The supernatants containing approximately 5µg/µl of sGPs were kept at -80^0C until used.

Giardia excretory-secretory proteins (ESPs) were obtained from *G. intestinalis* as described [10]. In brief, WB and GS trophozoites cultured for 48 h at 37^0C in TYDK medium were rinsed three times with warm and serum-free RPMI-1640 medium (Sigma, St. Louis, MO, USA) to eliminate non-attached or dead trophozoites. Thereafter, adherent trophozoites were incubated with RPMI-1640 medium supplemented with 11.4 mM L-cysteine hydrochloride monohydrate, 55.5 mM glucose, 11.4 mM ascorbic acid, 1 mM sodium pyruvate (Gibco), 22.8 mM L-arginine, 2mM Glutamax (Gibco) and 1x MEM essential amino acids. The final pH of the supplemented media was set at 6.8 and the trophozoites were incubated for 6h at 37^0C. Trophozoite viability was assessed at 90% and culture supernatants were harvested by centrifugation at 930xg for 10 min at 4^0C, filtered through Amicon® Ultra 15 mL centrifugal filters with 3kDa cut-off (Merck-Millipore, Darmstadt, Germany), concentrated down to 200-

300µl with a final concentration of approximately 1µg/µl of ESPs, and stored at -80^{0}C until used.

ELISA assay for IL-6 detection

The concentration of IL-6 was determined in supernatants from *Giardia*-challenged and un-challenged BM-MMCs using a mouse IL-6 ELISA developmental kit (#900-T50, PeproTech), according to supplier´s protocol.

β-hexosaminidase measurement

For the β-hexosaminidase assay, 20µl of cell culture supernatants were incubated with 80µl of 1mM substrate (p-nitrophenyl N-acetyl-beta-D-glucosamine, #487052, Merck KGaA, Germany) dissolved in citrate buffer (0.05M citric acid and trisodium citrate, pH 4.5) for 1 hour at 37^{0}C, followed by addition of 200µl 0.05M sodium carbonate reaction buffer (Na_2CO_3 and $NaHCO_3$, pH 10.0). Absorbance was measured at 405nm.

Cell viability

BM-MMCs were stained for five minutes with 0.02% tryptan blue, dead blue cells were counted and cell viability for BM-MMCs was calculated.

Purification of mouse proteolytic ear tissue protein extracts

The purification method was as described before [29]. Briefly, ear tissues from mMCP-6$^{+/+}$ (n=10 per preparation) and mMCP-6$^{-/-}$ mice (n=10 per preparation) were frozen in liquid nitrogen and crushed into a tissue powder with a mortar and pestle and then transferred to a 15 ml tube. To enrich for mast cell protease activities, the ear tissue powder was first extracted with a low salt lysis buffer (PBS/1% Triton X-100) to remove the fraction of ear

tissue proteins not binding to negatively charged glycosaminoglycan chains found on proteoglycans. After 30 min on a rocking table at ambient temperature, lysed tissues were centrifuged at 3000rpm for 10 minutes. The fragmented pelleted ear tissues were then extracted with a high salt lysis buffer (PBS/2M NaCl/1% Triton X-100). After shaking for 30 min at room temperature and centrifugation at 3000 rpm for 10 minutes, high salt supernatants containing enriched mast cell protease activities were collected and kept at -20^{0}C until used.

Degradation assay of GS, WB, and H3 soluble Giardia Proteins

Enzymatic degradation of sGPs was analyzed by mixing 20μg of sGPs with 0.2μg of recombinant human tryptase (rHT, Promega) or with 0.05μg or 0.4μg recombinant human chymase (rCh, a kind gift from Lars Hellman, ICM, Uppsala University), or with 5μg of crude wild-type ear tissue extracts and incubated at 37^{0}C for 3 hours or overnight. As a control of intrinsic degradation activity in sGPs 20 μg of sGPs were incubated at 37^{0}C for 3 hours or overnight. Enzymatic and intrinsic degradation of the sGPs was visualized on colloidal Coomassie blue stained SDS-PAGE gels.

SDS-PAGE electrophoresis and colloidal Coomassie blue staining

The 3h and overnight samples containing the 20 μg sGPs incubated with or without proteolytic activity was loaded on SDS-PAGE gels and the gels were run according to standard procedures. The gels were stained in Coomassie solution (0.1% Coomassie brilliant blue R-250/50% methanol/ 10% Acetic acid) for at least 4 hours, and de-stained in 10% acetic acid/40% EtOH/50% dH_2O for 30 min with several changes of the de-staining solution. Photos of the de-stained gels were taken with the Odyssey CLx imaging system (Germany).

Soluble Giardia proteins and mast cell protease activity

Early work showed that the activity of the chymase and tryptase could be affected by salt concentrations (NaCl) as well as pH [30-32]. Therefore, in all our experiments the NaCl concentrations were kept in the physiological range of 0.15 to 0.20 M NaCl and the pH was kept at ≈7,4.

To address if *Giardia* via sGPs or ESPs could block or modulate the tryptase activity, 5µg ESPs from the GS and WB isolates or approximately 20, 10, 5 and 1µg of WB, GS and H3 sGPs (5µg/µl) were mixed with 0.1µg of recombinant human tryptase (rHT) or approximately 15µg of crude high salt ear tissue extracts from mMCP-$6^{+/+}$ and mMCP-$6^{-/-}$ mice (tryptase-deficient mice) and 20µl of the tryptase substrate S-2288 (H-D-Ile-Pro-Arg-pNA, Chromogenic, Sweden) at a final concentration of 1mM, and PBS to give the total 120µl reaction volume. Enzymatic activity of tryptase was measured as hydrolysis of S-2288 and monitored spectrophotometrically at 405 nm in a microplate reader. The change in optical density over time (delta OD) and the substrate conversion rate (delta OD per minute) was calculated.

To determine if *Giardia* via sGPs or ESPs affected the chymase activity, 5µg ESPs of GS and WB or approximately 10 µg of WB, GS and H3 sGPs were mixed with 0.05µg of recombinant human chymase (rCh) or approximately 5µg of crude high salt ear tissue extracts from mMCP-$6^{+/+}$ mice and 20µl of the chymase substrate L-1595 (Suc-Ala-His-Pro-Phe-pNA, Bachem, Switzerland) at a final concentration of 1mM, and ddH_2O to give the total 120µl reaction volume. Enzymatic activity of chymase was measured as hydrolysis of L-1595 and monitored spectrophotometrically at 405 nm in a microplate reader. The change in optical density (OD) over time (deltaOD) was calculated.

Statistical analysis

Statistical analysis of data was performed with GraphPad Prism Software using the non-parametric Mann-Whitney U test. P values ≤ 0.05 were considered significant.

Results

Soluble Giardia proteins (sGPs) only cause minor mast cell activation

Mast cells and mast cell proteases are important for control of infection with *Giardia spp*. [19, 20], and mast cells may degranulate in response to soluble proteins from protozoan parasites [33]. To address a direct interaction between mouse mast cells and *G. intestinalis*, bone marrow derived mucosal-like mast cells (BM-MMCs) were challenged with increasing concentrations of soluble proteins (sGPs) from *G. intestinalis* GS-, WB-, and H3- isolates. As a read out of mast cell activation and mast cell degranulation the levels of IL-6, and tryptase and beta-hexosaminidase activity were determined in the BM-MMC supernatants. Challenge with the three *Giardia*-isolates induced a significant increase in IL-6 release at 6h and at 24h (Fig 1a, b), and a small but significantly increased tryptase activity with 1 microgram of sGPs (Fig. 1c, d). The challenge with sGPs did not induce degranulation of the BM-MMC (Fig 1e, f and data not shown) and the cell viability remained equally high in control cells and sGP-challenged cells (Fig 1g, h). Together our data suggests that sGPs can induce mast cell activation and that the challenge does not induce mast cell degranulation but may induce piecemeal secretion of tryptase.

Soluble Giardia proteins (sGPs) are not extensively degraded by human or mouse mast cell proteases.

To investigate the potential interactions between the mast cell specific proteases and *Giardia* proteins we next studied if any of the sGPs were degraded by recombinant human tryptase (rHT) or wild-type proteolytic mouse ear tissue extracts. rHT did not induce any major degradation of the GS-, WB-, H3- sGPs after 3 hours (Fig. 2a, lanes 4, 7, 10) or after extended overnight incubation (Fig. 2b, lanes 4, 7, 10). Furthermore, except for a 65 kDa sized protein a high concentration of recombinant human mast cell chymase (rCh) also failed

to degrade most of the sGPs after 24h (Fig. 2c, lanes 2, 4, 6), suggesting that sGPs are poor target substrates for the mast cell specific tryptase and chymase. In contrast, the high salt ear tissue proteolytic extracts showed a diffuse degradation activity of the sGPs in the 10 to 75kDa range, *e.g.* a ≈45kDa protein was significantly reduced (Fig. 2 a, b, lanes 5, 8, 11), suggesting that the mixture of proteolytic enzymes in the ear tissue extracts can degrade sGPs. Note also that the sGPs did not carry any major intrinsic degradation activity.

The lack of degradation by chymase and tryptase could indicate that *Giardia* proteins are devoid of the defined extended target sites required for these mast cell proteases to cut. However, the top 10 secreted peptides from WB and GS trophozoites [10] all contained several of the potential chymase and tryptase target sites (not shown), but it is possible that these sites are hidden in the three dimensional protein structure. Alternatively, sGPs may block the proteolytic activities of the mast cell specific tryptase and chymase.

Soluble Giardia proteins (sGPs) and excretory-secretory proteins (ESPs) enhance recombinant human tryptase activity

Next we investigated if sGPs and *Giardia* excretory-secretory proteins (ESPs) could have a modulatory effect on the mast cell protease activities. Surprisingly, we observed an enhancing effect on the tryptase activity (Fig. 3) and the enhancing effect on rHT activity was dose dependent and required the addition of > 5µg of sGPs (Fig. 3a). Addition of 20µg GS, WB and H3 sGPs resulted in a significant increase of rHT activity over 60 minutes, where the sGPs-effect on the S-2288 substrate conversion was evident after 15 minutes, and the substrate conversion rate significantly was increased up to at least 50 minutes after addition of sGPs (Fig. 3b, c). Note that the GS, WB and H3 sGPs showed no intrinsic activity for the S-2288 substrate. To evaluate if the observed increased activity of rHT is due to proteins found

in the *Giardia* excretory-secretory proteins (ESPs) we next used purified ESPs [10] from the *Giardia* isolates GS and WB. Addition of ESPs also gave a significantly increased S-2288 substrate conversion rate for rHT (Fig. 3d), suggesting that the protein(s) responsible for the enhancement activity is to be found in the ESPs. Note that the GS and WB ESPs showed no intrinsic activity for the S-2288 substrate. Compared with the control (PBS/rHT/S), addition of both GS and WB ESPs significantly increased enzymatic activity of rHT already after 15 minutes and the activity rate were still significantly higher after 60 minutes (Fig. 3e).

Soluble Giardia proteins (sGPs) enhance mouse mast cell tryptase activity

Seeing the enhancement effect on the human tryptase activity, we next assessed if secreted *Giardia* proteins could also enhance the mouse mast cell tryptase activity. High salt ear tissue protein extracts from wild-type mice were incubated with sGPs derived from the GS (Fig. 4a), WB (Fig. 4b) or H3 (Fig. 4c) isolates. High salt ear tissue protein extracts from the mMCP-6$^{-/-}$ mice as well as rHT were included as negative and positive controls, respectively. Addition of the three sGP-isolates induced a significantly increased mouse tryptase activity (Fig. 5a-c, left panels). As expected ear tissue protein extracts from mMCP-6$^{-/-}$ mice showed little tryptase activity (S-2288 substrate conversion rate) and no significantly increased substrate conversion after addition of sGPs, suggesting that the substrate S-2288 is relevant for measurement of tryptase activity even in complex protein mixtures (Fig. 4a-c, left panels). The enhancement of mouse mast cell tryptase activity over time after addition of sGPs was also studied. We found that the OD values for (sGPs/WT/S-2288) were significantly increased compared to control (WT/S-2288) suggesting that all three sGP-isolates over time can enhance tryptase activity or, alternatively, increase the stability of the tryptase tetramer (Fig. 4a-c, right panels).

Soluble Giardia proteins (sGPs) reduce human and mouse mast cell chymase activity

295 Finally we investigated if sGPs and ESPs could modulate the mast cell chymase activity.
296 Interestingly, and in contrast to the enhancing effect on tryptase, addition of 10µg of GS, WB
297 and H3 sGPs significantly inhibited the rCh activity (Fig. 5a) and resulted in inhibition of
298 chymase activity also in the WT mouse ear tissue extracts (Fig. 5b). Note that sGPs have no
299 intrinsic activity for the L-1595 chymase substrate (not shown). To evaluate if the observed
300 reduced activity of rCh is due to *Giardia* proteins found in the ESP fraction we finally used
301 ESPs from the *Giardia* isolates GS and WB. However, the addition of ESPs did not
302 significantly affect the rCh activity (Fig. 5c).
303

Discussion

Previous studies have suggested that mast cells play an important role during infection with *Giardia*. Mast cell-deficient and mast cell-depleted mice showed clearance failure of the GS isolate (assemblage B) and recruited mast cell numbers increased in the intestinal villi and crypt of mice infected with the GS isolate [19, 20, 34]. Furthermore, the mast cell specific proteases CPA3 as well as *Mcpt*1, *Mcpt*2 and CMA2 were reported to be up-regulated during infection with *Giardia*, suggesting that both connective and mucosal tissue type mast cells have increased activity in the intestinal tissue in response to *Giardia* [22].

However, it still remains unknown if the *Giardia* WB (assemblage A) or H3 (assemblage B) isolates cause mast cell activation *in vivo* and, the potential direct interactions of *Giardia* with the mast cell specific proteases have previously not been explored. First, to study potential interactions between *Giardia* and mast cells we cultured mouse bone marrow derived mucosal-like mast cells (BM-MMCs) expressing both connective and mucosal mast cell specific proteases [35] to investigate the potential activation of mast cells after challenge with *G. intestinalis* sGPs. Challenge with *Giardia* sGPs caused IL-6 secretion after 6h suggesting that our cultured BM-MMCs do respond to *Giardia* sGPs. In addition, the low levels of tryptase activity in BM-MMCs supernatants suggested that *Giardia* does not cause strong mast cell degranulation, but instead may cause increased piecemeal secretion of tryptase as augmented tryptase activity was only evident after challenge with sGPs. The observation that *Giardia* sGPs did not cause degranulation of the mast cells, *i.e.* as indicated with very low tryptase activity, was further supported by a similarly low beta-hexosaminidase activity in the supernatants of un-challenged and challenged BM-MMCs. Previous studies using a rat hybridoma mast cell line (HRMC) with a mucosal phenotype showed that IL-6 was secreted 24h after challenge [21, 27]. In contrast to our results, these studies suggested that the level of

329 tryptase only increased beneath the mast cell cell-membrane in response to *Giardia* challenge
330 [21, 27].
331
332 Several studies have demonstrated that *Giardia* soluble proteins (sGPs) contain cytosolic,
333 cytoskeletal, surface and excretory-secretory proteins (ESPs), and that the secretion of ESPs
334 will be induced upon contact with epithelial cells in the small intestine [10, 18, 36, 37], and
335 that some of the ESPs may penetrate into the host tissue [10, 17]. Previous studies suggested
336 an extensive over-lap in protein content between the two *in vitro* prepared fractions. One
337 difference, ESPs contained several surface proteins that were not found in the sGPs fraction
338 [10, 18, 38]. In line with this extensive over-lap we see similar effects of ESP and sGPs on
339 tryptase activity (Figure 3 and 4). For chymase the inhibitory effect of sGPs was not evident
340 with the ESPs (Figure 5). The observed difference could depend on several things, for
341 example that ESPs may lack the chymase interfering protein(s). Alternatively, *Giardia*
342 expresses several cysteine proteases/cathepsin-like proteases (CPs) in the ESPs that
343 potentially could degrade chymase. However, these CPs are subjected to rapid auto-
344 degradation [17]. So, lack of chymase-inhibition with ESPs could depend on the rapid auto-
345 inactivation of the CPs.
346
347 *Giardia* has been found to secrete several different proteins (ESPs) at low levels in axenic
348 culture and, in the interaction with human intestinal epithelial cells (IECs) the ESPs have the
349 capacity to modulate the host innate immune response, *e.g.* it was found that ESPs could
350 stimulate a preferential Th2 response [39], degrade chemokines and induce the anti-
351 inflammatory protein tristetraprolin (TTP) [10]. The ESP fraction from the WB isolate
352 contains around 200 different proteins [10] and most of these proteins are also found in the
353 soluble *Giardia* protein fractions [18]. It is difficult to generate high levels of ESPs from

Giardia due to the low level of secretion [10], this has led to the use of soluble proteins as a substitute for ESPs in most experiments looking at *Giardia*-immune cell interactions.

We therefore investigated a potential interaction of the mast cell specific proteases tryptase and chymase with *Giardia* proteins. Collectively our findings suggest that *Giardia* via release of ESPs may directly affect the mast cell tryptase activity. Therefore, it is possible that due to the disturbed intestinal epithelial barrier caused by the *Giardia* infection [17], *Giardia* ESPs can reach into the intestinal tissue and thereby increase the level and activity of the mast cell tryptase. An increase in tryptase activity can in turn even more increase the epithelial leakage [40], thereby resulting in a leaky gut that can induce diarrhea and post-infectious symptoms [41-43]. Several post-infectious symptoms can be induced after *Giardia* infections; irritable bowel syndrome (IBS), chronic fatigue syndrome, skin and food allergies and reactive arthritis [44-49]. Mast cells could be very important in the allergies induced after a *Giardia* infection and humans and mice infected by *Giardia* induce *Giardia*-specific IgE responses and ESPs have been suggested to be involved in IgE induction [8, 36, 50]. It will be interesting to follow up the role of mast cells in the induction of post-infectious symptoms in larger studies.

It is well known that activation of mast cells during infection release extensive amounts of preformed mediators. The mast cell proteases can play regulatory roles through degradation of parasite antigens, *e.g.* the *T. spiralis* heat shock protein 70, as well as through activation or inactivation of endogenous cytokines and alarmins, *i.e.* biglycan, HMGB1 and IL-33 all seem to be rapidly degraded by the mast cell specific chymase [29]. However, while chymase rapidly degraded most *T. spiralis* proteins as well as the alarmins and the HSP70, tryptase was not very effective in the degradation of these proteins [29]. In line with this, *Giardia* sGPs

were highly resistant to degradation with tryptase, however the resistance of most of the soluble *Giardia* proteins to degradation by mast cell chymase was an unexpected finding.

The mast cell specific chymase is monomeric and the extended target site for chymase is eight amino acids long (P4, P3, P2, P1, P1', P2', P3', P4'). Chymase prefers to cut after aromatic amino acids phenylalanine (F), tryptophan (W) or tyrosine (Y) located in the P1 position and usually require aspartic acid (D) or glutamic acid (E) in the P2' position [51-53]. However, physiologic targets for degradation by chymase remain elusive but some virulence factors and alarmins seems to be major targets [29, 54]. In addition, activation of matrix metalloprotease (MMP)-9 and angiotensin seems to require chymase [55]. Chymase binds strongly to heparin (or chondrotin sulphate E) and it has been suggested that chymase and heparin forms a functional complex that can leave the mast cell after degranulation and move into inflammatory or other body sites. For example, in bladder infection with uropathogenic bacteria chymase is released by mast cell degranulation, and then the chymase migrates and enter into the infected umbrella epithelial cells lining the bladder wall to induce apoptosis, causing the epitehlial cells to shed as a protective measure [56]. Furthermore, a detrimental action of the chymase is in the course of glomerulonephritis where mast cells are distantly found in the kidney capsule [57]. In bronchial asthma, mast cell chymase impairs bronchial epithelium integrity through degradation of cell junction molecules, *i.e.* occluding, claudin-4, ZO-1 and E-cadherin in epithelial cells [58]. In the intestine the expression and secretion of chymase will have effect on the epithelial barrier function via protease-activated receptor (PAR)-2 activation and matrix metalloproteinase (MMP)-2 expression and activation [59].

Tryptase is the predominant serine proteinase of mast cells and heparin stabilizes the activity of the functional monomeric tetramer [60], where the four substrate pockets facing inwards to

form a narrow proteasome-like structure. Tryptase preferentially targets ”linear” proteins displaying a stretch of positively charged amino acids and will cut after arginine (R) or lysine (K) in its three amino acid target site (K/R + X + K/R). Physiologic targets for tryptase include, *e.g.* PAR-2 [61]. The narrow pore in tetrameric tryptase would not allow entry of bulky proteins and thus this could explain that no or very low degradation of soluble *Giardia* proteins was observed.

A screen for potential chymase and tryptase target sites of the 15 most abundantly secreted proteins from *Giardia* WB and GS trophozoites [10] identified several potential target sites in each of the 15 secreted *Giardia* proteins. This suggests that most soluble *Giardia* proteins lack accessible surface exposed extended target sites for the mast cell specific chymase [54]. Alternatively, resistance to degradation can partly be explained by that sGPs directly reduce the activity of chymase.

In summary, we here showed that sGPs do not cause significant mast cell degranulation and that sGPs were not significantly degraded by tryptase or chymase. The lack of degradation suggested that ESPs and sGPs could inhibit the activity of the mast cell proteases. In line with this observation sGPs induced a significant inhibition of chymase activity. In contrast, we found that ESPs and sGPs specifically increased the mast cell tryptase activity, suggesting that as yet unidentified protein(s) in ESPs and sGPs may stabilize tryptase tetramers, thereby increasing the tryptase activity. We now aim to identify and characterize the *Giardia*-proteins providing the increased effects of the tryptase activity. In addition, future studies using heat inactivation of identified candidate proteins and EPSs as well as cathepsin inhibitors, *e.g.* E64, would be interesting and could address if intact and properly folded ESPs or enzymatic

activities in the ESPs are required for the observed enhancement of tryptase and inhibition of chymase.

Speculative, our result suggests that *Giardia* may affect the intestinal tissue via increased stability of tetrameric tryptase or direct enhancement of tetrameric tryptase activity as well as via inhibition of chymase, and this potential immunomodulation could be beneficial for *Giardia* since mast cell tryptase has been shown to control intestinal paracellular permeability and the enhancement of tryptase activity may lead to a leaky intestinal tissue. Finally, the inhibition of chymase may serve to retain intestinal barrier functions and reduce chymase driven inflammatory symptoms. However, the exact roles of the different mast cell specific proteases during giardiasis remains an open question and will require extensive *in vivo* experimentation to be resolved.

Author contributions

MÅ conceived the study and supervised the experiments; ZL performed experiments; DP and SS provided the Giardia proteins and advise; ZL, DP, SS, MÅ analyzed the data; MÅ and ZL wrote the manuscript and all authors edited, revised and approved the final version.

Acknowledgement

We thank Caroline Fossum for critical reading of the manuscript and helpful discussions. ZL received a scholarship from the Chinese Scholarship Council (CSC). This research was funded by VR-M to SS.

Figure legends

Figure 1. Soluble *Giardia* proteins induce secretion of IL-6 and tryptase in bone marrow derived mucosal type mast cells (BM-MMCs). To determine if *Giardia* activates mast cells $1x10^6$ BM-MMCs seeded in 0.5 ml HBSS were challenged with three concentrations, 25ng/ml, 100ng/ml and 1000ng/ml, of soluble *Giardia* proteins (sGPs) from the GS-, WB- and H3-isolates. The levels of IL-6 **(a, b** with N=5**),** tryptase activity **(c, d** with N=5**),** and beta-hexosaminidase activity **(e, f** with N=3**)** were determined in supernatants collected at 6h (a, c, e) and 24h (b, d, f). Tryptase activity was evaluated by the conversion of the substrate S-2288 and recorded as changes in optical density (OD) at 405 nm. BM-MMCs viability was scored by trypan blue exclusion at 6h **(g)** and 24h **(h)**. Data is pooled from two independent experiments with BM-MMCs derived from a total of five individual mice (N=5), in duplicate cultures (from two mice in first experiment, n=4) or triplicate cultures (from three mice in second experiment, n=9). Data are shown as mean ±SEM and statistical analysis conducted by the non-parametric Mann-Whitney U test with significant difference indicated as * $P<0.05$, ** $P<0.01$, *** $P< 0.005$, **** $P< 0.001$versus un-challenged control.

Figure 2. Soluble *Giardia* proteins are not extensively degraded by human tryptase or chymase, or by wild type mouse ear tissue proteolytic extracts. To determine if mast cell proteases can degrade *Giardia* proteins, 20µg of soluble *Giardia* proteins (sGPs) were incubated with 0.2µg recombinant human tryptase (rHT, arrow) or with 5µg of proteolytic mouse ear tissue extracts (WT, pooled ear tissue extracts, n=10) for **(a)** 3 hours and **(b)** overnight (O.N.). Left panels in **(a)** and **(b)** are longer exposures of the gels to visualize the loading of rHT (arrows) and WT ear tissue extracts. In panels **a** and **b** lanes are numbered and loaded as follows (**1**- rHT; **2**- WT; **3**- GS; **4**- GS+rHT; **5**- GS+WT; **6**- WB; **7**- WB+rHT; **8**- WB+WT; **9**- H3; **10**- H3+rHT; **11**- H3+WT). In **(c)** 20µg of soluble *Giardia* proteins of the

GS, WB and H3 isolates were incubated without or with 0.4µg recombinant human chymase (rCh) overnight. In panel **c** lanes are numbered and loaded as follows (**1**- GS; **2**- GS+rCh; **3**- WB; **4**- WB+rCh; **5**- H3; **6**- H3+rCh; **7**- rCh).

Figure 3. Soluble *Giardia* proteins and excretory-secretory proteins mediate enhancement of human tryptase activity. Different concentrations of soluble *Giardia* proteins (sGPs) from the GS, WB and H3 isolates **(a, b)** or 5µg of GS and WB excretory-secretory protein (ESP) **(c, d)** were incubated with or without 0.02µg of rHT. The change in optical density (OD) was measured at 405nm after adding the substrate (S-2288, S) and the difference over time (deltaOD) calculated. Note that sGPs and ESPs have no intrinsic activity for the S-2288 substrate. In (a) a representative experiment out of >5 independent experiments is shown and in (b and c) a representative experiment out of 3 independent experiments is shown. In (d) the enzyme activity rate in figure c was determined as milli-delta OD per minute. Note that addition of ESPs significantly increased the tryptase activity rate already after 15 minutes and maintained a significantly increased rate also at 60 minutes. Representative data from two independent experiments with triplicates for each condition are shown as mean ±SEM, and statistical analysis was conducted by the non-parametric Mann-Whitney U test. Statistical significances compared to PBS/rHT/S: *, $P<0.05$, **, $P<0.01$, ***, $P<0.005$.

Figure 4. Soluble *Giardia* proteins enhance mouse mast cell tryptase activity. sGPs were incubated with or without 0,02µg of rHT or with 15µg of ear tissue extracts from WT mice (bulk ear tissue extracts, n=10) and mMCP6$^{-/-}$ mice (bulk ear tissue extracts, n=10) at room temperature, respectively. **(a**) GS (upper panels), **(b)** WB (middle panels) and **(c)** H3 (lower panels). The **left panels** in a, b, c show the tryptase activity of the high salt mouse ear tissue

extracts mixed with or without 10µg sGPs from the GS, WB and H3 isolates. rHT was included as a positive control. Change in optical density (OD) at 405nm was measured at time point 0h and 4h after the S-2288 substrate was added. Data are shown as mean ±SEM and statistically significant enhancement compared to WT ear tissue extracts indicated with *, P<0.05. The **right panels** in a, b, c show the activity of WT ear tissue extracts (WT) mixed with or without 5µg of sGPs from the GS, WB and H3 isolates. Change in optical density (OD) at 405nm was measured every hour up to 11h and after 24h. Note that GS, WB and H3 sGPs have no intrinsic activity for the S-2288 substrate. Pooled data from two independent experiments with triplicates for each condition are shown as mean ±SEM and, statistically significant enhancement compared to WT ear tissue extracts without sGPs indicated with *, P<0.05, **, P<0.01, ***, P<0.001.

Figure 5. Soluble *Giardia* proteins inhibit human and mouse chymase activity. (**a**) The activity of 0.05µg of recombinant human chymase (rCh) incubated with or without 10µg of GS, WB and H3 sGPs. (**b**) The chymase activity in 5µg wild-type mouse ear tissue (WT, pooled ear tissue extracts, n=10) with or without 10µg of GS, WB and H3 sGPs. **(c)** The activity of 0.05µg rCh incubated with or without 5µg of GS and WB ESPs. The change in optical density (OD) at 405nm was measured after adding the chymase substrate (L-1595, L) and the difference over time (delta OD) calculated. Pooled data from two independent experiments with triplicates for each condition are shown as mean ±SEM.

References

1. Redegeld, F.A., et al., *Non-IgE mediated mast cell activation.* Immunol Rev, 2018. **282**(1): p. 87-113.
2. Pejler, G., et al., *Mast cell proteases: multifaceted regulators of inflammatory disease.* Blood, 2010. **115**(24): p. 4981-90.
3. Pejler, G., et al., *Mast cell proteases.* Adv Immunol, 2007. **95**: p. 167-255.
4. Shin, K., et al., *Mouse mast cell tryptase mMCP-6 is a critical link between adaptive and innate immunity in the chronic phase of Trichinella spiralis infection.* J Immunol, 2008. **180**(7): p. 4885-91.
5. Reitz, M., et al., *Mucosal mast cells are indispensable for the timely termination of Strongyloides ratti infection.* Mucosal Immunol, 2017. **10**(2): p. 481-492.
6. Luján, H.D. and S. Svärd, *Giardia: A model organism*. 2011: Springer Science & Business Media.
7. Kaur, H., et al., *Immune effector responses to an excretory-secretory product of Giardia lamblia.* FEMS Immunol Med Microbiol, 1999. **23**(2): p. 93-105.
8. Jimenez, J.C., et al., *Antibody and cytokine responses to Giardia excretory/secretory proteins in Giardia intestinalis-infected BALB/c mice.* Parasitol Res, 2014. **113**(7): p. 2709-18.
9. Lee, H.Y., et al., *Excretory-secretory products of Giardia lamblia induce interleukin-8 production in human colonic cells via activation of p38, ERK1/2, NF-kappaB and AP-1.* Parasite Immunol, 2012. **34**(4): p. 183-98.
10. Ma'ayeh, S.Y., et al., *Characterization of the Giardia intestinalis secretome during interaction with human intestinal epithelial cells: The impact on host cells.* PLoS Negl Trop Dis, 2017. **11**(12): p. e0006120.

11. Jimenez, J.C., et al., *Excreted/secreted glycoproteins of G. intestinalis play an essential role in the antibody response.* Parasitol Res, 2007. **100**(4): p. 715-20.
12. Ward, H.D., et al., *Biology of Giardia lamblia. Detection of N-acetyl-D-glucosamine as the only surface saccharide moiety and identification of two distinct subsets of trophozoites by lectin binding.* J Exp Med, 1988. **167**(1): p. 73-88.
13. Ortega-Barria, E., et al., *N-acetyl-D-glucosamine is present in cysts and trophozoites of Giardia lamblia and serves as receptor for wheatgerm agglutinin.* Mol Biochem Parasitol, 1990. **43**(2): p. 151-65.
14. Hiltpold, A., et al., *Glycosylation and palmitoylation are common modifications of giardia variant surface proteins.* Mol Biochem Parasitol, 2000. **109**(1): p. 61-5.
15. de Carvalho, T.B., et al., *Protease activity in extracellular products secreted in vitro by trophozoites of Giardia duodenalis.* Parasitol Res, 2008. **104**(1): p. 185-90.
16. Cabrera-Licona, A., et al., *Expression and secretion of the Giardia duodenalis variant surface protein 9B10A by transfected trophozoites causes damage to epithelial cell monolayers mediated by protease activity.* Exp Parasitol, 2017. **179**: p. 49-64.
17. Liu, J., et al., *Secreted Giardia intestinalis cysteine proteases disrupt intestinal epithelial cell junctional complexes and degrade chemokines.* Virulence, 2018. **9**(1): p. 879-894.
18. Dubourg, A., et al., *Giardia secretome highlights secreted tenascins as a key component of pathogenesis.* Gigascience, 2018. **7**(3): p. 1-13.
19. Li, E., E.A. Tako, and S.M. Singer, *Complement activation by Giardia duodenalis parasites through the lectin pathway contributes to mast cell responses and parasite control.* Infection and immunity, 2016. **84**(4): p. 1092-1099.
20. Li, E., et al., *Mast cell-dependent control of Giardia lamblia infections in mice.* Infect Immun, 2004. **72**(11): p. 6642-9.

21. Munoz-Cruz, S., et al., *Giardia lamblia: interleukin 6 and tumor necrosis factor-alpha release from mast cells induced through an Ig-independent pathway.* Exp Parasitol, 2010. **126**(3): p. 298-303.
22. Tako, E.A., et al., *Transcriptomic analysis of the host response to Giardia duodenalis infection reveals redundant mechanisms for parasite control.* MBio, 2013. **4**(6): p. e00660-13.
23. Nicolov, N.P., et al., *Nodular lymphoid hyperplasia of the jejunal mucosa associated with hypogammaglobulinemia. Increased number of mast cells in the jejunal mucosa.* Dtsch Z Verdau Stoffwechselkr, 1983. **43**(4): p. 157-63.
24. Leitch, G.J., et al., *Effects of protein malnutrition on experimental giardiasis in the Mongolian gerbil.* Scand J Gastroenterol, 1993. **28**(10): p. 885-93.
25. Hardin, J.A., et al., *Mast cell hyperplasia and increased macromolecular uptake in an animal model of giardiasis.* J Parasitol, 1997. **83**(5): p. 908-12.
26. Halliez, M.C., et al., *Giardia duodenalis induces paracellular bacterial translocation and causes postinfectious visceral hypersensitivity.* Am J Physiol Gastrointest Liver Physiol, 2016. **310**(8): p. G574-85.
27. Munoz-Cruz, S., et al., *Giardia lamblia: identification of molecules that contribute to direct mast cell activation.* Parasitol Res, 2018. **117**(8): p. 2555-2567.
28. Keister, D.B., *Axenic culture of Giardia lamblia in TYI-S-33 medium supplemented with bile.* Trans R Soc Trop Med Hyg, 1983. **77**(4): p. 487-8.
29. Roy, A., et al., *Mast cell chymase degrades the alarmins heat shock protein 70, biglycan, HMGB1, and interleukin-33 (IL-33) and limits danger-induced inflammation.* J Biol Chem, 2014. **289**(1): p. 237-50.
30. McEuen, A.R., B. Sharma, and A.F. Walls, *Regulation of the activity of human chymase during storage and release from mast cells: the contributions of inorganic*

cations, pH, heparin and histamine. Biochim Biophys Acta, 1995. **1267**(2-3): p. 115-21.

31. Ren, S., K. Sakai, and L.B. Schwartz, *Regulation of human mast cell beta-tryptase: conversion of inactive monomer to active tetramer at acid pH.* J Immunol, 1998. **160**(9): p. 4561-9.

32. Addington, A.K. and D.A. Johnson, *Inactivation of human lung tryptase: evidence for a re-activatable tetrameric intermediate and active monomers.* Biochemistry, 1996. **35**(42): p. 13511-13518.

33. Bidri, M., et al., *Evidence for direct interaction between mast cells and Leishmania parasites.* Parasite Immunol, 1997. **19**(10): p. 475-83.

34. von Allmen, N., et al., *Acute trichinellosis increases susceptibility to Giardia lamblia infection in the mouse model.* Parasitology, 2006. **133**(Pt 2): p. 139-49.

35. Braga, T., et al., *Serglycin proteoglycan is required for secretory granule integrity in mucosal mast cells.* Biochem J, 2007. **403**(1): p. 49-57.

36. Jimenez, J.C., et al., *Antibody and cytokine responses in BALB/c mice immunized with the excreted/secreted proteins of Giardia intestinalis: the role of cysteine proteases.* Ann Trop Med Parasitol, 2009. **103**(8): p. 693-703.

37. Ringqvist, E., et al., *Release of metabolic enzymes by Giardia in response to interaction with intestinal epithelial cells.* Mol Biochem Parasitol, 2008. **159**(2): p. 85-91.

38. Emery, S.J., et al., *Induction of virulence factors in Giardia duodenalis independent of host attachment.* Sci Rep, 2016. **6**: p. 20765.

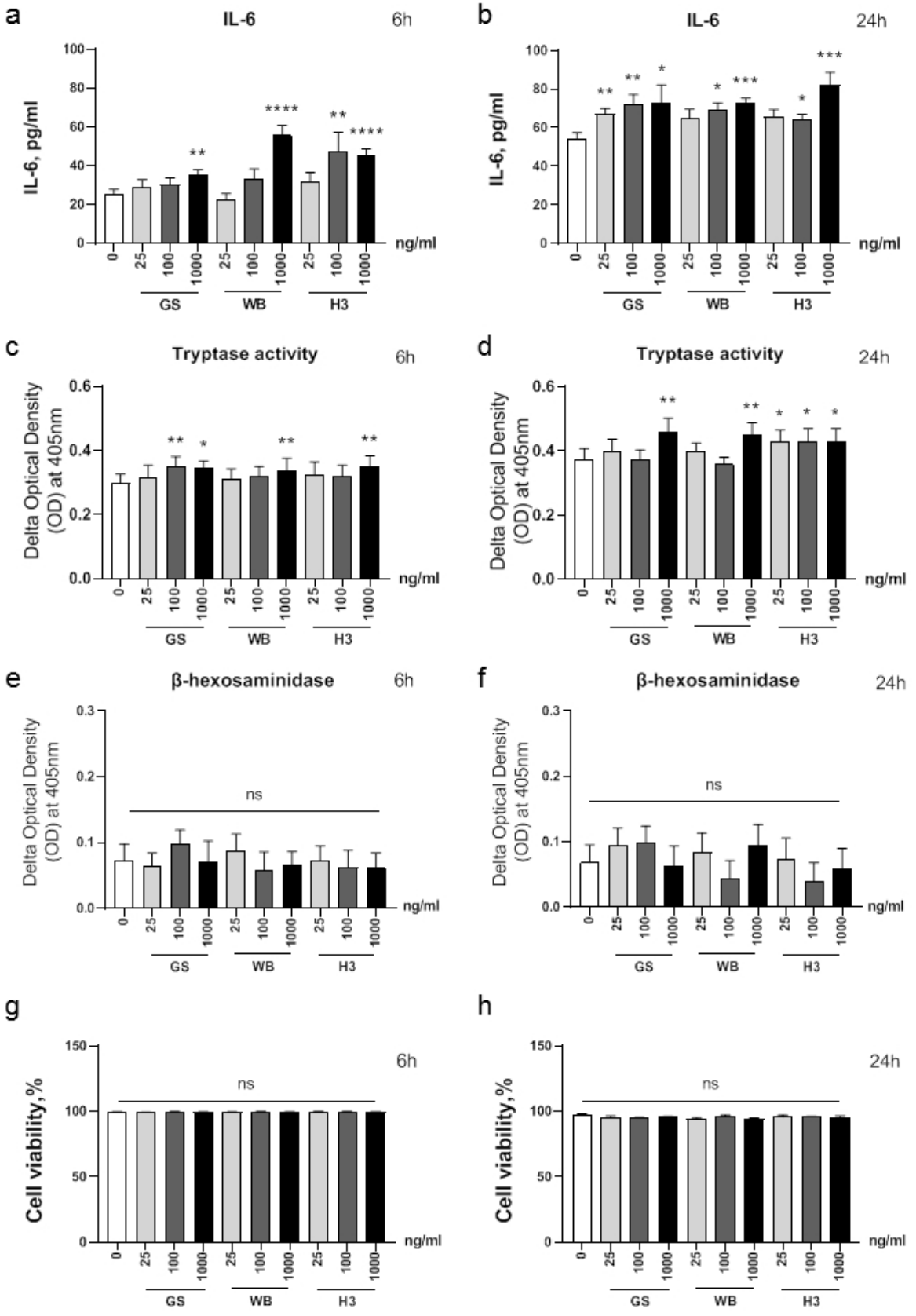
39. Jimenez, J.C., et al., *Systemic and mucosal responses to oral administration of excretory and secretory antigens from Giardia intestinalis.* Clin Diagn Lab Immunol, 2004. **11**(1): p. 152-60.

40. Fernandez-Blanco, J.A., et al., *Changes in Epithelial Barrier Function in Response to Parasitic Infection: Implications for IBD Pathogenesis.* J Crohns Colitis, 2015. **9**(6): p. 463-76.
41. Fink, M.Y. and S.M. Singer, *The Intersection of Immune Responses, Microbiota, and Pathogenesis in Giardiasis.* Trends Parasitol, 2017. **33**(11): p. 901-913.
42. Allain, T., E. Fekete, and A.G. Buret, *Giardia Cysteine Proteases: The Teeth behind the Smile.* Trends Parasitol, 2019.
43. Halliez, M.C. and A.G. Buret, *Extra-intestinal and long term consequences of Giardia duodenalis infections.* World J Gastroenterol, 2013. **19**(47): p. 8974-85.
44. Litleskare, S., et al., *Quality of life and its association with irritable bowel syndrome and fatigue ten years after giardiasis.* Neurogastroenterol Motil, 2019. **31**(5): p. e13559.
45. Litleskare, S., et al., *Prevalence of Irritable Bowel Syndrome and Chronic Fatigue 10 Years After Giardia Infection.* Clin Gastroenterol Hepatol, 2018. **16**(7): p. 1064-1072 e4.
46. Painter, J.E., S.A. Collier, and J.W. Gargano, *Association between Giardia and arthritis or joint pain in a large health insurance cohort: could it be reactive arthritis?* Epidemiol Infect, 2017. **145**(3): p. 471-477.
47. Di Prisco, M.C., et al., *Association between giardiasis and allergy.* Ann Allergy Asthma Immunol, 1998. **81**(3): p. 261-5.
48. Chirila, M., D. Panaitescu, and T. Capraru, *Frequency of Giardia lamblia in certain allergic syndromes.* Med Interne, 1981. **19**(4): p. 367-72.
49. Mahmoud, M.S., A.A. Salem, and M.M. Rifaat, *Human giardiasis as an etiology of skin allergy: the role of adhesion molecules and interleukin-6.* J Egypt Soc Parasitol, 2004. **34**(3): p. 723-37.

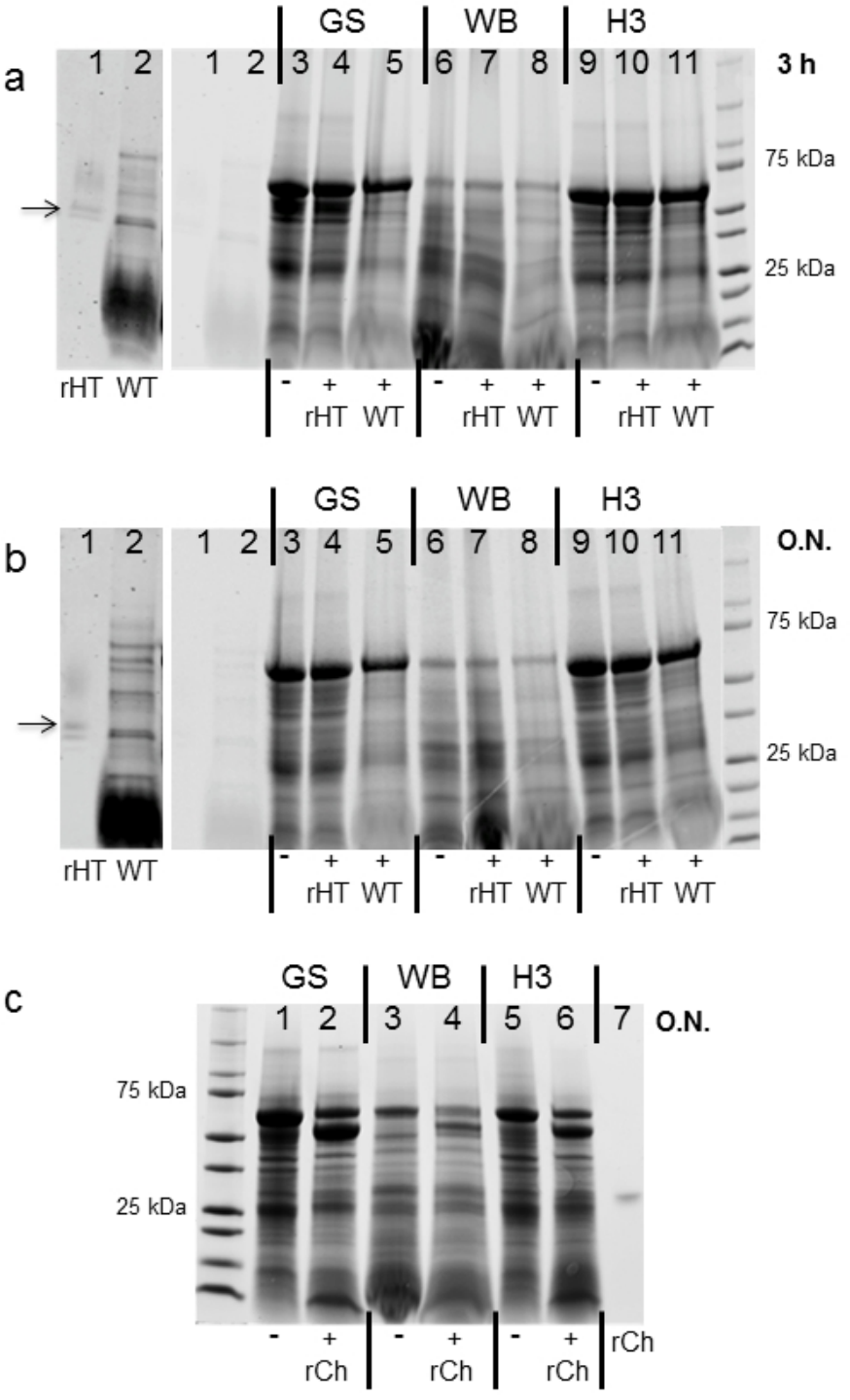
50. Matowicka-Karna, J., V. Dymicka-Piekarska, and H. Kemona, *IFN-gamma, IL-5, IL-6 and IgE in patients infected with Giardia intestinalis.* Folia Histochem Cytobiol, 2009. **47**(1): p. 93-7.
51. Andersson, M.K., et al., *Extended cleavage specificity of mMCP-1, the major mucosal mast cell protease in mouse-high specificity indicates high substrate selectivity.* Mol Immunol, 2008. **45**(9): p. 2548-58.
52. Andersson, M.K., et al., *The extended substrate specificity of the human mast cell chymase reveals a serine protease with well-defined substrate recognition profile.* Int Immunol, 2009. **21**(1): p. 95-104.
53. Andersson, M.K., M. Thorpe, and L. Hellman, *Arg143 and Lys192 of the human mast cell chymase mediate the preference for acidic amino acids in position P2' of substrates.* FEBS J, 2010. **277**(10): p. 2255-67.
54. Fu, Z., et al., *Highly Selective Cleavage of Cytokines and Chemokines by the Human Mast Cell Chymase and Neutrophil Cathepsin G.* J Immunol, 2017. **198**(4): p. 1474-1483.
55. Tchougounova, E., et al., *A key role for mast cell chymase in the activation of pro-matrix metalloprotease-9 and pro-matrix metalloprotease-2.* J Biol Chem, 2005. **280**(10): p. 9291-6.
56. Choi, H.W., et al., *Loss of Bladder Epithelium Induced by Cytolytic Mast Cell Granules.* Immunity, 2016. **45**(6): p. 1258-1269.
57. Scandiuzzi, L., et al., *Mouse mast cell protease-4 deteriorates renal function by contributing to inflammation and fibrosis in immune complex-mediated glomerulonephritis.* J Immunol, 2010. **185**(1): p. 624-33.
58. Zhou, X., et al., *Mast cell chymase impairs bronchial epithelium integrity by degrading cell junction molecules of epithelial cells.* Allergy, 2018.

59. Groschwitz, K.R., et al., *Chymase-mediated intestinal epithelial permeability is regulated by a protease-activating receptor/matrix metalloproteinase-2-dependent mechanism.* Am J Physiol Gastrointest Liver Physiol, 2013. **304**(5): p. G479-89.
60. Pereira, P.J., et al., *Human beta-tryptase is a ring-like tetramer with active sites facing a central pore.* Nature, 1998. **392**(6673): p. 306-11.
61. Molino, M., et al., *Interactions of mast cell tryptase with thrombin receptors and PAR-2.* J Biol Chem, 1997. **272**(7): p. 4043-9.

New Figure 1.

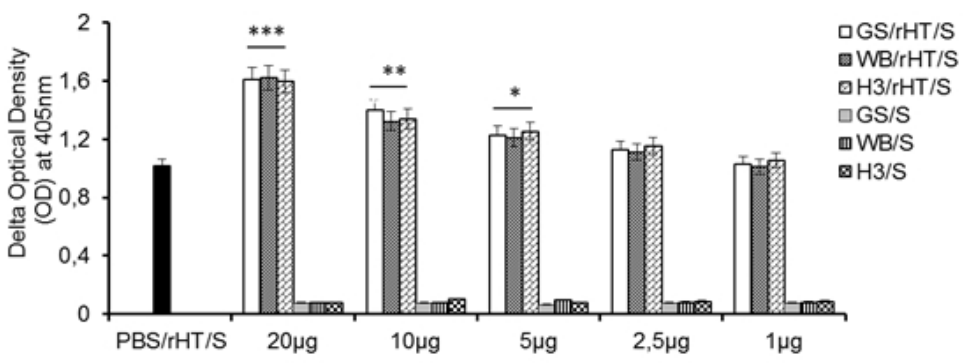

a
IL-6
6h
IL-6, pg/ml
ng/ml
GS
WB
H3
b
IL-6
24h
IL-6, pg/ml
ng/ml
GS
WB
H3
c
Tryptase activity
6h
Delta Optical Density (OD) at 405nm
ng/ml
GS
WB
H3
d
Tryptase activity
24h
Delta Optical Density (OD) at 405nm
ng/ml
GS
WB
H3
e
β-hexosaminidase
6h
Delta Optical Density (OD) at 405nm
ns
ng/ml
GS
WB
H3
f
β-hexosaminidase
24h
Delta Optical Density (OD) at 405nm
ns
ng/ml
GS
WB
H3
g
6h
Cell viability,%
ns
ng/ml
GS
WB
H3
h
24h
Cell viability,%
ns
ng/ml
GS
WB
H3

New Figure 2.

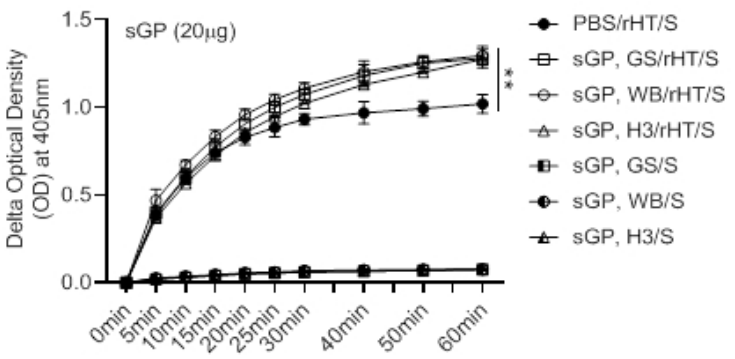

a
GS
WB
H3
1 2
1 2 3 4 5 6 7 8 9 10 11
3 h
75 kDa
25 kDa
rHT WT
- + + - + + - + +
rHT WT rHT WT rHT WT
b
GS
WB
H3
1 2
1 2 3 4 5 6 7 8 9 10 11
O.N.
75 kDa
25 kDa
rHT WT
- + + - + + - + +
rHT WT rHT WT rHT WT
c
GS
WB
H3
1 2 3 4 5 6 7
O.N.
75 kDa
25 kDa
- + - + - + rCh
rCh rCh rCh

New Figure 3.

a



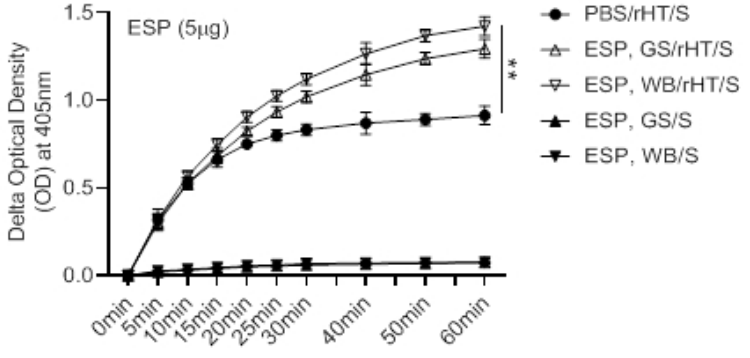
b



c

minute (m)	5m-0m	10m-5m	15m-10m	20m-15m	25m-20m	30m-25m	40m-30m	50m-40m	60m-50m
PBS/rHT/S	77.6±2.8	42.1±1.8	28.6±1.4	17.2±2.8	11.4±1.4	9.4±1.4	7.0±1.8	4.7±1.1	5.5±1.1
rHT/GS/S	79.6±3.5	41.3±1.8	34.1±1.1*	26.1±6.0**	18.8±0***	14.6±2.8***	21.6±2.0***	14.3±2.6***	5.1±2.5
rHT/WB/S	83.9±7.4	40.4±7.1	32.2±0.7*	25.0±3.5**	17.2±1.4***	12.9±2.1**	18.9±1.2***	11.4±2.8***	6.9±2.1*
rHT/H3/S	73.8±7.1	39.4±3.5	32.0±1.4*	25.8±4.9**	18.4±0***	14.9±2.0***	18.9±2.4***	13.9±1.8***	15.0±2.4***

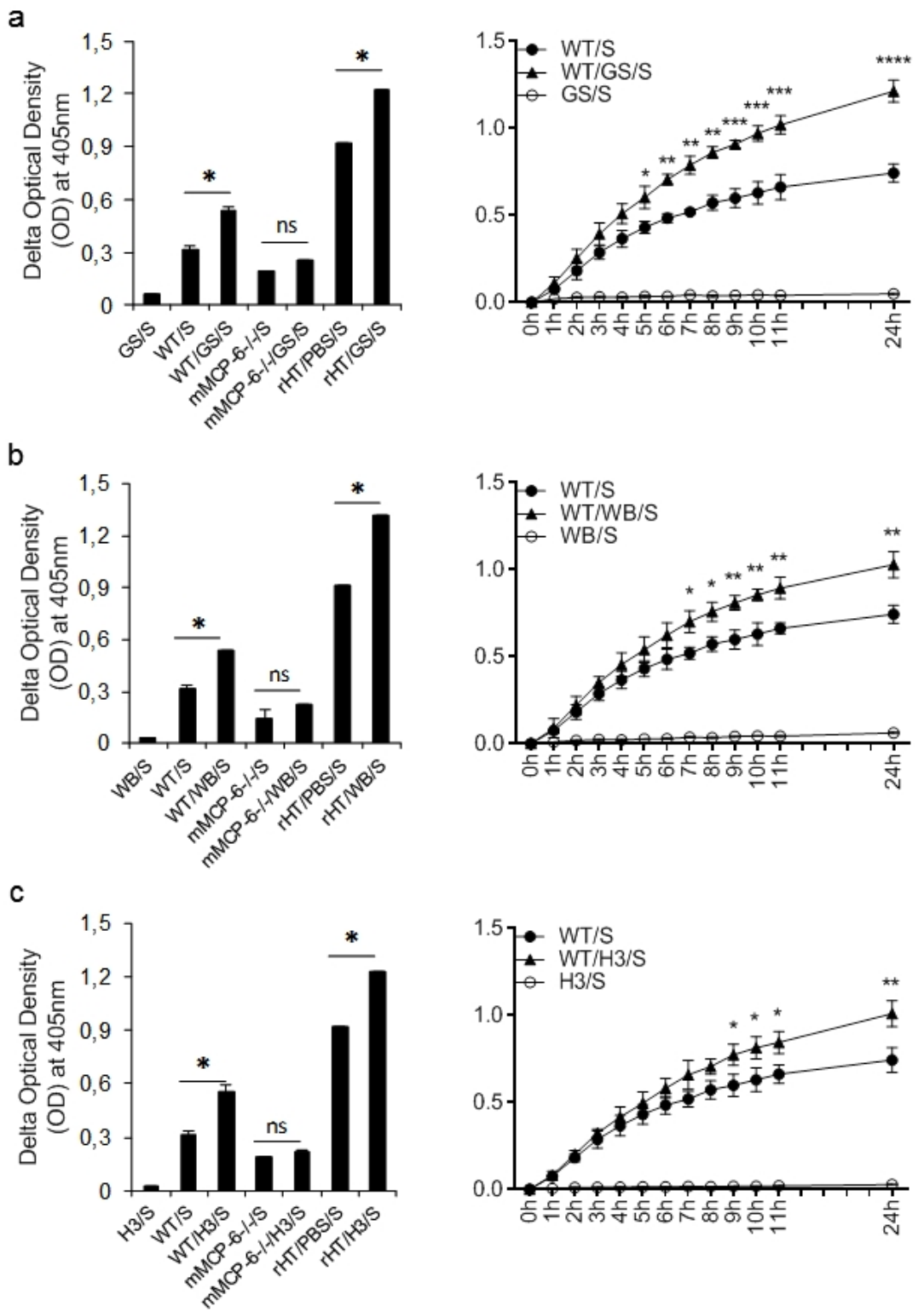
d



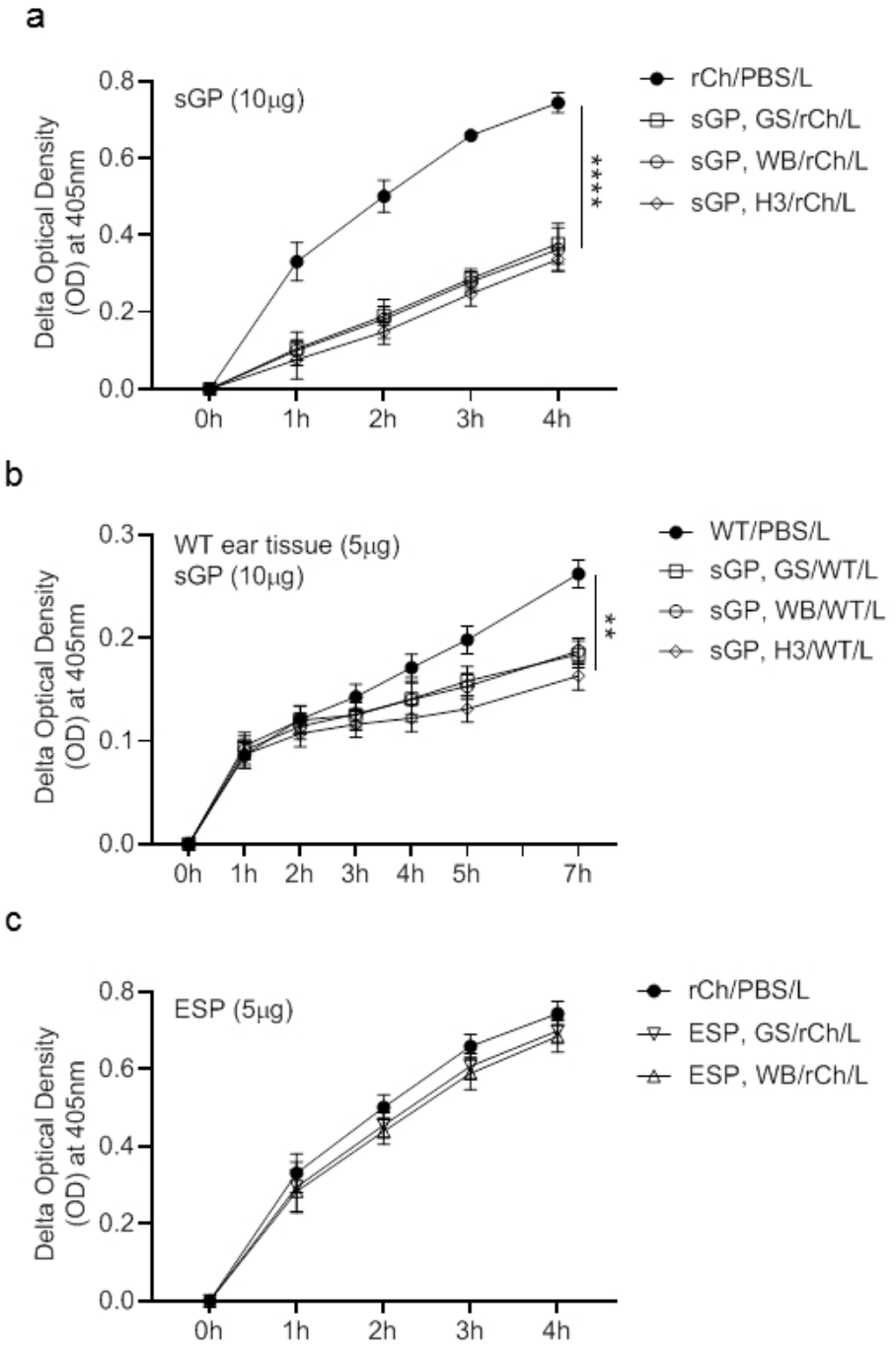
e

minute (m)	5m-0m	10m-5m	15m-10m	20m-15m	25m-20m	30m-25m	40m-30m	50m-40m	60m-50m
PBS/rHT/S	63.6±2.9	41.6±0	27.1±0.9	17.8±0.6	9.8±1.7	6.4±0	3.8±0	2.1±1.4	2.4±0.9
GS/rHT/S	59.8±5.8	45.2±14.4	31.7±3.2*	28.0±3.6**	21.9±2.0***	17.3±4.9**	12.7±0.3***	9.1±1.6***	5.7±2.0*
WB/rHT/S	63.8±0.6	49.9±3.8	35.6±9.2*	31.5±0.9**	24.1±3.8***	19.2±5.8**	14.5±13.6***	10.3±8.1***	5.4±2.3*

New Figure 4.


a
Delta Optical Density (OD) at 405nm
1,5
1,2
0,9
0,6
0,3
0
*
*
ns
*
GS/S
WT/S
WT/GS/S
mMCP-6-/-/S
mMCP-6-/-/GS/S
rHT/PBS/S
rHT/GS/S
WT/S
WT/GS/S
GS/S
1.5
1.0
0.5
0.0
0h
1h
2h
3h
4h
5h
6h
7h
8h
9h
10h
11h
24h
b
Delta Optical Density (OD) at 405nm
WB/S
WT/S
WT/WB/S
mMCP-6-/-/S
mMCP-6-/-/WB/S
rHT/PBS/S
rHT/WB/S
WT/S
WT/WB/S
WB/S
c
Delta Optical Density (OD) at 405nm
H3/S
WT/S
WT/H3/S
mMCP-6-/-/S
mMCP-6-/-/H3/S
rHT/PBS/S
rHT/H3/S
WT/S
WT/H3/S
H3/S

New Figure 5.


a
sGP (10μg)
Delta Optical Density (OD) at 405nm
0.8
0.6
0.4
0.2
0.0
0h
1h
2h
3h
4h
****
rCh/PBS/L
sGP, GS/rCh/L
sGP, WB/rCh/L
sGP, H3/rCh/L
b
WT ear tissue (5μg)
sGP (10μg)
Delta Optical Density (OD) at 405nm
0.3
0.2
0.1
0.0
0h
1h
2h
3h
4h
5h
7h
**
WT/PBS/L
sGP, GS/WT/L
sGP, WB/WT/L
sGP, H3/WT/L
c
ESP (5μg)
Delta Optical Density (OD) at 405nm
0.8
0.6
0.4
0.2
0.0
0h
1h
2h
3h
4h
rCh/PBS/L
ESP, GS/rCh/L
ESP, WB/rCh/L