Serglycin-dependent mast cell-specific proteases and their potential interactions with *Giardia intestinalis*

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Abstract

Mast cells are located in mucosal and connective tissues throughout the body where they play important roles in immune reactions towards infections. Mast cell deficiency results in failure of clearance of the non-invasive intestinal protozoan parasite *Giardia intestinalis* in mice, but the mast cell-driven mechanisms remain poorly understood. It is well known that pathogen-activated mast cells can release large number of inflammatory mediators, including the mast cell specific proteases and the serglycin (SG) proteoglycan. In this thesis the functional roles of the SG-dependent mast cell proteases tryptase and chymase during *G. intestinalis* were addressed, both *in vitro* and *in vivo.*

In vitro, soluble *Giardia* proteins (sGPs) were demonstrated to stimulate mucosal-like mast cells to release IL-6 and tryptase, and both sGPs and excretory-secretory proteins (ESPs) were found to enhance human and mouse tryptase activity, but in contrast sGPs inhibited chymase activity. These finding suggest that proteins secreted by *Giardia* can modulate the activity of both chymase and tryptase.

In vivo, oral gavage with *Giardia* trophozoites was performed to challenge chymase mMCP-4-deficient as well as SG-deficient mice. Clinical scoring and weight data were recorded in a blinded fashion. Fecal samples collected from individual mice every second day were evaluated with nested PCR to confirm successful *Giardia* infection. The small intestines, serum and tail tissue were sampled from each mice and evaluated for: cytokine and chemokine expression, morphological changes and genotype. Infection of mature adult mice with *G. intestinalis* caused a more rapid significant weight loss in the mMCP-4-deficient than in the mMCP-$4^{+/+}$ mice. In young SG-deficient female mice the infection with *G. intestinalis* caused a significantly reduced weight gain, suggesting a sex- and SG-dependent response to the infection. Serum levels of IL-6 matched the weight changes in both the mMCP-4 and the SG mouse strains. A regulatory role of mMCP-4 and SG, respectively on the intestinal inflammatory cytokine responses was indicated by real-time PCR. Furthermore, increased numbers of intestinal goblet cells and granulocytes, unaltered myeloperoxidase activity and a decreased neutrophil elastase activity were found in the *G. intestinalis*-infected mice, suggesting that secreted *Giardia* proteins can also modulate neutrophil protease activities.

Key words: mast cell proteases, chymase/mMCP-4, tryptase/mMCP-6, serglycin proteoglycan, *Giardia intestinalis,* excretory-secretory proteins (ESPs), soluble *Giardia* proteins (sGPs)

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Serglycin-beroende mastcellsproteaser och *Giardia intestinalis* interaktioner

Mastceller sitter strategiskt placerade kroppen, framförallt i slemhinnor och bindväv nära "utsidan", och kan snabbt svara på angrepp av patogener genom att starta det medfödda immunsvaret. Mastceller har visats vara viktiga för att bekämpa infektion med den icke invasiva intestinala protozo-parasiten *Giardia intestinalis*, men de mastcells-drivna mekanismerna är fortsatt oklara. Vid infektion aktiveras mastceller och frisätter en mängd inflammatoriska mediatorer, till exempel mastcell-specifika proteaser och serglycin (SG) proteoglykaner. Här undersökte jag vilken roll de SG-beroende mastcellsproteaserna kymas och tryptas har vid infektion med *G. intestinalis*.

Mukosa-liknande mastceller stimulerade med lösliga *Giardia* proteiner (sGPs) *in vitro* frisatte IL-6 och små mängder tryptas. Både sGPs och "excretory-secretory proteins" (ESPs) förstärkte den enzymatiska aktiviteten hos tryptas, medan kymas-aktiviteten inhiberades vid interaktion med sGPs, vilket indikerar att utsöndrade proteiner från *Giardia* kan modulera aktiviteten av kymas och tryptas.

In vivo studerades de SG-beroende mastcellsproteaserna via gavage-challenge med *G. intestinalis* trophozoiter i kymas-defekta (mMCP-4$^{-/-}$) och serglycin-defekta (SG$^{-/-}$) möss. I helt blindade försök noterades viktdata och djurens allmänna hälsotillstånd. Feces insamlades för att påvisa *Giardia* DNA med "nested PCR" vilket konfirmerade att de experimentella infektionerna fungerade. Vid experimentets avslut insamlades tunntarm, serum och svansvävnad för bestämning av uttrycket av cytokiner och kemokiner, morfologiska förändringar samt bestämning av mössens genotyp. Infektion med *G. intestinalis* gav en snabbare viktminskning i vuxna mMCP-4$^{-/-}$ än i mMCP-4$^{+/+}$ möss. Infektion med *G. intestinalis* i unga möss gav ett signifikant avbrott i viktökning framförallt hos de SG$^{-/-}$ honmössen, vilket indikerar ett köns- och SG- beroende svar mot infektionen. IL-6 nivåerna i serum matchade viktförändringarna i mMCP-4 och SG musstammarna. Det inflammatoriska svaret i tarmen och den potentiella reglerande rollen av kymas och SG utvärderades med qPCR. Vid infektion med *G. intestinalis* ökade antalet bägarceller och granulocyter i tarmen. Myeloperoxidas-aktiviteten var oförändrad medan neutrofilelastas-aktiviteten minskade vilket indikerar att utsöndrade *Giardia* proteiner också kan modulera aktiviteten hos neutrofila proteaser.

Key words: mast cell proteases, chymase/mMCP-4, tryptase/mMCP-6, serglycin proteoglycan, *Giardia intestinalis,* excretory-secretory proteins (ESPs), soluble *Giardia* proteins (sGPs)

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Dedication

To my loved parents for always supporting me.

To those whom gave me hands many times on the road of life.

And to myself for finally being ready.

Be a practical man, do with sincere.

My respectable teachers:

Juxiong Liu and Lichen Wang

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Zhiqiang Li, Dimitra Peirasmaki, Staffan Svärd, and Magnus Åbrink* (2019). *Giardia* excretory-secretory proteins modulate the enzymatic activities of mast cell chymase and tryptase. *Molecular Immunology*, 114, 535-544.

II Zhiqiang Li[#], Dimitra Peirasmaki[#], Staffan Svärd* and Magnus Åbrink* (2019). The chymase mouse mast cell protease 4 regulates intestinal cytokines in mature adult mice infected with *Giardia intestinalis*. (manuscript)

III Zhiqiang Li, Dimitra Peirasmaki, Staffan Svärd and Magnus Åbrink* (2019). The mouse serglycin proteoglycan regulates immune responses in a sex-specific direction during infection with *Giardia intestinalis*. (manuscript)

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Abbreviations

CCL	Chemokine (C-C motif) ligand
CPA3	Carboxypeptidase A3
CPs	Cysteine proteases
CTMC	Connective tissue mast cell
CXCL	Chemokine (C-X-C motif) ligand
DCs	Dendritic cells
ESPs	Giardia excretory-secretory proteins
GAG	Glycosaminoglycan
GRPs	*Giardia*-released Proteins
IFN-γ	Interferon gamma
IL	Interleukin
MMC	Mucosal mast cells
MMPs	Matrix metallopeptidases
mMCP	Mouse mast cell protease
NE	Neutrophil elastase
NOS	Nitric oxide synthase
PBS	Phosphate buffered saline
PG	Proteoglycan
rCh	Recombinant human chymase
rHT	Recombinant human tryptase
SG	Serglycin
SPs	*Giardia* soluble proteins
TGF-β	Transforming frowth factor beta
TNF-α	Tumor necrosis factor alpha

Serglycin-dependent mast cell-specific proteases and their potential interactions with *Giardia intestinalis*

1 Introduction:

Mast cells are traditionally known for their role in allergic reactions but have lately also been recognized for their importance in the immune response against several infectious diseases. Because of their location in mucosal and connective tissues mast cells can affect the inflammatory response and immunity during intestinal parasitic infections such as *Giardia intestinalis* that causes the diarrheal disease giardiasis. *Giardia intestinalis* is a non-invasive intestinal protozoan parasite, *i.e.* it stays attached to the luminal side of the epithelium of the small intestine. In the intestine *G. intestinalis* secretes antigens and modulatory proteins that influence both the innate and the adaptive immune reactions of the host. In line with this the clearance of *G. intestinalis* in mice requires mast cells, but the exact mechanisms by which mast cells combat *G. intestinalis* remain poorly understood. Activation of mast cells can cause release of a large number of preformed secretory granule-stored inflammatory mediators. These mediators include the mast cell-specific proteases (*i.e.* tryptase and chymase) and the proteoglycan serglycin (SG) that is essential for storage of the proteases. In the present thesis the functional role of mast cell specific proteases in giardiasis is addressed. For that purpose, a combination of *in vivo* and *in vitro* experimental models has been applied including mouse strains deficient in SG or in the chymase murine mast-cell protease-4 (mMCP-4).

1.1 Mast cells in the immune system

Mast cells execute important roles during the immune response, particularly during the innate immune responses. Immature progenitor mast cells, derived from hematopoietic stem cells in the bone marrow, circulate in the blood before extravasation and final differentiation and maturation in the tissue. Mast cells are widely distributed throughout the body, especially in connective and vascular tissues of the skin, airway and digestive tract, as well as in mucosal tissues. Mast cells usually reside

close to blood vessels, nerves, smooth muscles, secretory glands and hair follicles. With this location mast cell-degranulation and release of potent inflammatory mediators will have immediate effects on the surrounding tissue. Classical activation of mast cells is via crosslinking of the IgE/FcεRI-receptor complex, but mast cells can be activated by many other stimulus, *e.g.* by toxins (like snake venom), complement components, alarmins (like IL-33), as well as by physical damage caused by high temperature, ionizing radiation and trauma. The released mast cell-mediators will participate in many different aspects of the immune responses, *e.g.* regulating the recruitment and activation of neutrophils, eosinophils, macrophages, and affecting some activities of T-cells and B-cells (Cardamone *et al.* 2016).

1.2 Mast cell mediators

Mature tissue resident mast cells produce and store large amounts of highly reactive preformed mediators such as histamine, SG proteoglycans (PGs), and the mast cell-specific proteases in their inducible secretory granules. After mast cell-activation leading to degranulation these mediators are released causing instant changes in the tissue. In addition, within minutes after activation mast cells start to synthesize eicosanoids and within hours post activation mast cells secrete newly synthesized cytokines and chemokines. Here follows a short description of some of these mediators.

1.2.1 Mast cell proteases

Tryptases and chymases, and to some extent carboxypeptidase A3 (CPA), are considered as mast cell-specific proteases, but their expression patterns are dependent on the species. In humans, mast cells can be divided into two subclasses: "MC_T" expressing tryptase only and "MC_{TC}" expressing tryptase, chymase and CPA3. Similarly, rat and mouse connective tissue mast cells (CTMCs) express tryptases, chymases and CPA3, while rodent mucosal mast cells (MMCs) expresses chymases only (Pejler *et al.* 2007).

1.2.2 Mast cell tryptases

Mast cells express at least four different tryptase genes, *i.e* the α-, β-, γ-, δ-tryptase genes. Of these the α- and β-tryptases are probably the most abundant proteases expressed by mast cells. The α-tryptase can be further

classified into αI- and αII-tryptase and the β-tryptases into βI-, βII- and βIII-tryptase (Miller *et al.* 1990; Pallaoro *et al.* 1999). α-tryptases and β-tryptases are usually forming tetrameric serine proteinases, with their monomer active catalytic sites facing inwards, forming a rather narrow proteasome-like central pore that only allows entry of linear peptides. However, β-tryptases, which can exist both as monomers and tetramers, appear to be the main form of tryptase stored in mast cell-granules. Tetramerization of tryptase monomers occurs only when pH values are below 6.5 (Hallgren and Pejler 2006; Hallgren *et al.* 2000), suggesting that once released into the tissue with pH ≈7 it will dissociate into monomers.

Tryptase constitutes approximately twenty percent of the protein content in human mast cells (Abraham 2002; McEuen *et al.* 1996). It has been suggested as a valid marker for mast cell-activation and to play crucial roles in inflammatory diseases or during infections. For instance, mast cell tryptase secretion and tryptase-positive mast cells are increased in patients with ulcerative colitis or irritable bowel syndrome (Raithel *et al.* 2001; Sohn *et al.* 2014). In mice, secretion of the orthologous β-tryptase mMCP-6 comprises an integral link in the chronic immune response, contributing to eosinophil recruitment in striated muscle tissue during infection with the parasitic nematode *Trichinella spiralis* (Shin *et al.* 2008). In addition, mMCP-6 is needed for full-blown airway hyper-responsiveness (Cui *et al.* 2014) and is highly expressed in airway mast cells after exposure to cigarette smoke (Mortaz *et al.* 2012). Another isoform of mouse β-tryptase, mMCP-7, showed up-regulated expression after induction of activin A and transforming growth factor-beta 1(TGF-beta 1) (Funaba *et al.* 2003; Funaba *et al.* 2006). Recombinant mMCP-7 can initiate angiogenesis *in vitro* in matrix gel embedded endothelial cells by degrading the integrin subunits αv and β1 (de Souza Junior *et al.* 2019).

1.2.3 Mast cell chymases

The monomeric human chymase, with the substrate-binding pocket located on the "surface", is exclusively expressed by CTMCs and was isolated already in 1959 (Benditt and Arase 1959). Chymases are classified into α- and β-chymases and while humans only express one α-chymase (Caughey *et al.* 1991) mouse MMCs express two β-chymases, *i.e.* mMCP-1 and mMCP-2 whereas mouse CTMCs mainly express one β-chymase mMCP-4 and one α-chymase mMCP-5 (Huang *et al.* 1991; Reynolds *et al.* 1990).

An evolutionary phylogenetic comparison based on nucleotide and protein sequences of human chymase *vs* the mouse chymases suggested that the mouse chymase mMCP-5 is orthologous to the human chymase. However, functional studies of mMCP-4 and mMCP-5 revealed that the mMCP-4 chymase is the closest functional orthologue to human chymase, in terms of extended substrate specificity and tissue location (reviewed by (Pejler *et al.* 2007). In contrast, the mouse chymase mMCP-5 chymase was shown to have elastolytic activity (Karlson *et al.* 2003; Thorpe *et al.* 2018).

The storage of very high levels of active chymase in human mast cells suggests that this mast cell-specific protease exerts a major role in mast cell-mediated diseases (Krishnaswamy *et al.* 2001). The chymases are mainly released after mast cell degranulation, and the human chymase enables microvascular leakage in the skin and promotes tissue accumulation of inflammatory cells (He and Walls 1998a, 1998b). Human chymase also cleave the cytokines IL-1, -15, -18 and -33 (Fu *et al.* 2017), and degrade cell to cell junction molecules, *e.g.* occludin, claudin-4, ZO-1 and E-cadherin in epithelial cells (Zhou *et al.* 2018). In experimental infections in mouse models, expression of the mucosal chymase mMCP-1 is up-regulated in the jejunum after infection with *Nippostrongylus brasiliensis* (Scudamore *et al.* 1997) and clearance of the nematode *T. spiralis* is delayed in mice lacking mMCP-1 (Knight *et al.* 2000). The other mucosal chymase mMCP-2 is also expressed and released by MMCs, but to date very little is known about the biological function of mMCP-2 (Pemberton *et al.* 2003). For the murine CTMC-proteases the chymase mMCP-4 has three functions; degrade the alarmins heat shock protein 70, biglycan, HMGB1, IL-33 (Roy *et al.* 2014) and TNF-α (Piliponsky *et al.* 2012), activate pro-matrix metalloproteinase (MMP)-2 and pro-MMP-9 (Tchougounova *et al.* 2005), and inactivate thrombin (Pejler and Karlstrom 1993; Tchougounova 2003). These studies all suggest an important role of the chymase mMCP-4 and, by using the mouse knockout model, it has been shown that chymase/mMCP-4 contributes to various disease conditions (see Table 1 for some examples). The elastase mMCP-5 is much less well studied in disease settings but seem to contribute to ischemia-reperfusion injury of mouse skeletal muscle (Abonia *et al.* 2005), to wound healing after skin burn injury (Younan *et al.* 2010; Bankova *et al.* 2014), and to the inflammatory levels in experimental rheumatoid arthritis (Stevens *et al.* 2017).

Table 1. *Mast cell chymase mMCP-4 associated diseases*

Diseases	**Comments**	**References**
Myocardial infarction	mMCP-4 not only protects heart function and survival but also alleviates cardiac dysfunction. mMCP-4 could degrade insulin-like growth factor-1 (IGF-1) to promote cell death.	Houde *et al.* 2018; Wang *et al.* 2019; Tejada *et al.* 2016
Traumatic spinal cord	mMCP-4 plays protective role in traumatic spinal cord damage by cleaving MCP-1, IL-6, IL-13 and by suppressing scar formation	Vangansewinkel *et al.* 2019; Nelissen *et al.* 2014
Melanoma colonization	In a mouse model with a triple knockout of CPA, mMCP-4 and mMCP-6 melanoma colonization of the lungs was increased	Grujic *et al.* 2017
Atherosclerosis	Inhibition of mMCP-4 reduces lesion spreading and helps stabilization of the advanced plaque	Houde *et al.* 2016
Wound healing	mMCP-4, together with mMCP-5 and mMCP-6, plays a critical role in microdeformational wound therapy in the proliferative phase of healing	Succar *et al.* 2014
Lung inflammation	Inflammation and fibrosis levels in bleomycin-induced lung inflammation are reduced in mice with a genetic deletion of mMCP-4	Reber *et al.* 2014
Epidermal injury	mMCP-4 disrupts the tight junction claudin-4 to mediate epidermal injury	Bankova *et al.* 2014
Sepsis	mMCP-4 partly enhances survival of sepsis by degrading Tκα and limiting inflammation.	Piliponsky *et al.* 2012
Skin tumour	During tumour progression, the expression of CPA and other mast cell proteases increased.	de Souza *et al.* 2012
Bullous pemphigoid	mMCP-4 cause activation of MMP-9 and degradation of the transmembrane protein BP180 leading to blisters by detaching the basement membrane from the cornified skin	Lin *et al.* 2011
Autoimmune arthritis	mMCP-4 null mice immunized with collagen II shows less cartilage destruction, pannus formation and mononuclear cell infiltration	Magnusson *et al.* 2009
Venom	The release of mast cells CPA3 and other proteases can enhance innate defense by degradation of snake and honeybee toxins, e.g. endothelin, sarafotoxin.	Akahoshi *et al.* 2011; Rivera 2006; Metz *et al.* 2006; Schneider *et al.* 2007
Scleroderma	The chymase inhibitor SUN-C8257 reduces the upregulated mMCP-4 expression during the development of skin fibrosis	Kakizoe *et al.* 2001; Shiota *et al.* 2005

1.2.4 Mast cell carboxypeptidase A3

Carboxypeptidase A3 (CPA3) is also stored in an active form in mast cell granules. Murine CTMCs express CPA3 (Lutzelschwab *et al.* 1997; MacDonald *et al.* 1998; Serafin *et al.* 1991; Serafin *et al.* 1987), and also in humans the expression of CPA3 seems to be restricted to the CTMCs (Irani *et al.* 1991; Weidner 1993). The development of a CPA3 knockout mouse strain has supported in depth studies of CPA3 function. However, a classical direct knockout of CPA3 resulted in a reduction of the mMCP-5 protein level as well, but no reduction of the other mast cell expressed chymases and tryptases was noted (Feyerabend *et al.* 2005). Conversely, the deletion of mMCP-5 caused an inability of CPA3 storage (Stevens *et al.* 1996). These results suggest that CPA3 and mMCP-5 may form a complex and that their retention in the mast cell granules depends on each other. Based on this, the CPA3-deficient mouse strain is not a perfect tool for studying the complex aspects of CPA3 function *in vivo*. To counteract this, another CPA3 knockout mouse strain, in which the enzymatic activity CPA3 is inactivated, was developed. This mouse strain makes an enzymatically dead CPA3 and retains a normal level of active mMCP-5 (Schneider *et al.* 2007).

1.2.5 Other proteases expressed in mast cells

Mast cells can also express other proteases, *e.g.* several of the aspartic proteases cathepsins, as well as granzymes and matrix metalloproteinases (MMPs). In bone marrow derived mast cells cathepsin C and S regulate the levels of CPA3 protein and activity (Henningsson *et al.* 2003) and mast cell-expressed cathepsin G has been associated with heart diseases (Jahanyar *et al.* 2007; Helske *et al.* 2006; Lewicki *et al.* 2018). Mast cells express a few granzymes, *i.e.* granzyme B, D and H, where granzyme B can decrease efficacy of anti-angiogenic therapy (Wroblewski *et al.* 2017), cause cell death, increase vascular permeability and leukocyte extravasation (Pardo *et al.* 2007). The expression of granzyme D in murine mast cells and granzyme H in human mast cells were recently described (Ronnberg *et al.* 2013; Ronnberg *et al.* 2014). The MMPs, which are not only expressed by mast cells, are implicated in many physiological as well as pathological situations. The major functional roles of MMPs are to degrade extracellular matrix components. In disease, MMPs are involved in inflammation (Nissinen and Kahari 2014) and can directly affect the

tumour microenvironment and the tumour cell invasiveness (Komi and Redegeld 2019). If all these other proteases are retained in mast cell granules remains to be explored.

1.2.6 Proteoglycans

Proteoglycans (PGs) consist of a core protein attaching several highly negatively charged GAG chains, which pending on the cell type could be heparin, heparan sulphate or/and different chondroitin sulphate chains. The negative charge of the GAG chains will attract binding of cationic mediators, *e.g.* chemokines, cytokines, histamine and several different proteases. This feature also provides the strong staining properties seen in CTMCs when stained with cationic dyes such as toluidine blue. Mast cells can express several different PGs, such as syndecan, perlecan (Jung *et al.* 2013; Higashi *et al.* 2018) and serglycin (SG). Ablation of the SG gene resulted in the loss of staining by cationic dyes in CTMCs, and severe defects in the storage of the highly positively charged mast cell proteases (Abrink *et al.* 2004). Furthermore, SG is important for the enzymatic activities of the mast cell proteases, where heparin attached to the SG protein core is essential for the mast cell chymase and tryptase activity (Pejler and Sadler 1999). In addition, tryptase requires heparin to stabilize its tetramer structure and maintain its active state (Hallgren *et al.* 2000; Hallgren *et al.* 2005). Thus, tryptase and chymase activity rates will be decreased if the GAGs on the SG core protein are degraded. In contrast, there is no direct evidence that the CPA3 activity is dependent on GAGs. However, the processing of pro-CPA3 into active CPA3 is catalysed by cathepsin E, which depends on heparin for correct storage (Henningsson *et al.* 2005), thus demonstrating that CPA3 enzymatic activity indirectly depends on the SG PG. In addition, the SG PG also influences the dissemination of the proteases from the secretory granule matrix when released at mast cell degranulation. However, mainly the tryptase mMCP-6 and the chymase mMCP-4, but not the mMCP-7 tryptase or the mMCP-1 chymase, have a high affinity to heparin which retain them in the close vicinity of the degranulated mast cell (Ghildyal *et al.* 1996; Schwartz *et al.* 1981; Tchougounova and Pejler 2001).

1.2.7 Other preformed mediators

Mast cells also produce biogenic amines such as histamine, serotonin and dopamine. Histamine is often used as an indicator of mast cell activation in the clinic. Histamine exerts its biological roles in physiological and pathological situations via binding to four G protein-coupled receptors, *i.e.* H1, H2, H3 and H4 (Panula *et al.* 2015; Seifert *et al.* 2013). Serotonin, known as 5-hydroxytryptophan, exerts its effect via binding and activation of seven receptors 5-HT_1 through 5-HT_7 (Lanfumey and Hamon 2004). Dopamine, derived from many cell types, is involved in the control of neuroendocrine secretion, cognition and locomotion mediated by binding to its receptors D1, D2, D3 and D4 (Jaber *et al.* 1996). Interestingly, the storage of histamine, serotonin and dopamine in mast cells relies on the expression of the SG PG (Ringvall *et al.* 2008; Ronnberg *et al.* 2012). However, the biogenic amines are not the focus of this thesis and have recently been reviewed extensively elsewhere (Hu and Chen 2017).

1.2.8 Eicosanoids

Activated mast cells (via IgE/FcεRI-receptor engagement) also release lipid-derived mediators, *e.g.* prostaglandins and leukotrienes. Prostaglandins are hormone-like substances involved in the control of blood pressure and smooth muscle contraction and regulation of gastric juice secretion. The prostaglandin D2 secreted by mast cells can activate eosinophils (Raible *et al.* 1992) and exacerbates food antigen-induced mast cell hyperplasia (Guo *et al.* 2010), but may attenuate anaphylactic reactions (Nakamura *et al.* 2017). Leukotriene B4 and leukotriene C4 can be produced by mast cells and are involved in allergic and asthmatic reactions. Leukotriene B4 is a chemoattractant for neutrophils and mast cell progenitors and recruits eosinophils during infection with *Toxocara canis* (Weller *et al.* 2005; Carlos *et al.* 2011). Leukotriene C4 secreted by mast cells can recruit neutrophils involved in bacterial clearance (Malaviya and Abraham 2000).

1.2.9 Cytokines and chemokines.

In a few hours after activation, intrinsically or extrinsically stimulated mast cells can release a vast array of different cytokines and chemokines, *e.g.* IFN-γ, TNF-α, IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-33, CCL4, CCL5, CXCL8, CXCL10, GM-CSF, and TGF-β. Bone marrow-derived mast cells

release IL-1β, IL-6, IL-10, IL-13, IFN-γ, TNF-α, GM-CSF, VEGF and CCL8 when cultured *in vitro* in the presence of LPS (Palaska *et al.* 2016; Dong *et al.* 2019; Supajatura *et al.* 2002; Okumura *et al.* 2003). After stimulation with the alarmin IL-33, *in vitro* derived mast cells produce IL-2, IL-5, IL-6, IL-10, IL-13, GM-CSF, TGF-β1, VEGF, CCL1, CCL2, CCL3, CCL17, CCL22 and CXCL8 (Morita *et al.* 2015; Hsu *et al.* 2010; Allakhverdi *et al.* 2007; Theoharides *et al.* 2010; Ndaw *et al.* 2017). Together, these results suggest important roles of the mast cells in inflammation, via secretion of cytokines and chemokines as thoroughly and recently discussed elsewhere (Mukai *et al.* 2018).

2 Giardia

Intestinal parasites have had millions of years to evolve regulatory mechanisms that circumvent the immune response, allowing for the longevity of the infection characteristically observed during intestinal parasitic infestations (King and Li 2018). Epidemiological studies have clearly shown that immunomodulatory products from parasites can interfere with and even prevent allergic inflammation, and parasitic infections can also affect the efficiency of vaccination (Maizels *et al.* 2018). Thus, studies aimed at exploring the mechanisms and pathways in immune reactions affected by the parasite immunomodulatory molecules will be increasingly important, for example when developing new therapeutic strategies.

2.1 The protozoan parasite *Giardia intestinalis*

Giardia intestinalis (also named *G. lamblia* or *G. duodenalis*) was first reported in 1681 by Antonie van Leeuwenhoek, known as the `the father of microbiology` (Dobell 1920). The parasite was named *Giardia lamblia* in 1915 to commemorate the meaningful work on *Giardia* by professor Alfred M. Giard in Paris and the physician Vilem D. Lambl in Prague (Ford 2005). It is estimated that *G. intestinalis* (hereafter referred to as *Giardia*) contributes to symptomatic infections (“giardiasis”) in 200 million people per year, mainly in Asia, Africa and Latin America per year (Yason and Rivera 2007). Giardiasis is characterized by gastrointestinal

symptoms; abdominal cramps, watery diarrhea, epigastric pain, nausea, vomiting and constitutional symptoms; malaise, fatigue, anorexia, weight loss. The symptoms usually start from the first or second week after infection and last for two to six weeks (Pennardt *et al.* 2006). *Giardia* can be transmitted between human and mammals, and so far symptomatic infection cases have been reported in many kinds of livestock, sports and companion animals and even in wild life. To date, eight well-defined assemblages (A to H) of *Giardia* have been identified. Assemblage A and B are zoonotic and can infect various species, but A and B are the only assemblages that infect humans (Caccio and Ryan 2008; Lasek-Nesselquist *et al.* 2010). In contrast, other assemblages are fairly species specific, where C and D are specific for dogs and other canids, E for hoofed animals, F for cats, G for rats and H might be specific for marine mammals (Souza *et al.* 2007; Lasek-Nesselquist *et al.* 2010).

2.2 The life cycle of *Giardia*

A key for *Giardia* in long-term survival is its strong ability to adapt to harsh surroundings via an environmentally-induced shifting into two main life forms, the cysts and the trophozoites. Both forms can produce infection in the host but usually the much more long lived and persistent cysts start the infection after the host has ingested contaminated food, water or drinks. The mature cysts survive the strong acidic environment of the stomach and migrate to the small intestine, where they attach to the epithelium and asexually multiply by binary fission. Four binuclear trophozoites released by each quadrinuclear cyst continue replication in the crypts of the duodenum and upper part of jejunum, and when the numbers of trophozoites increases it causes giardiasis. Meanwhile, some *Giardia* trophozoites reform into cysts in the ileum, most likely due to starvation for nutrient. This explains why both cysts and trophozoites forms can be found in faeces from infected individuals. *Giardia* trophozoites cannot survive long in the environment while *Giardia* cysts can persist for many months in moist surroundings, waiting for a new host to restart the infectious lifecycle. The general outline for *Giardia* lifecycle is shown in Figure 1.

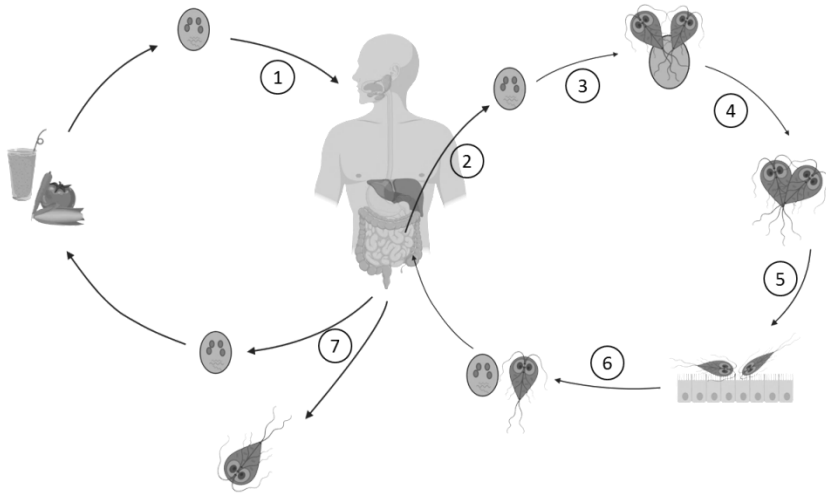

Figure 1. General overview of the *Giardia* life cycle.1) *Giardia* cysts are ingested by the host. 2) migration to intestine. 3) one generate into four trophozoites. 4) replication of trophozoites. 5) attachment to epithelia cells. 6) encystation or not. 7) excreted with feces, trophozoites will die, cysts will wait for the new host. (Created with Biorender.com)

2.3 *Giardia* proteins

The detailed mechanisms explaining how the non-invasive *Giardia* parasite influences and interacts with the host are largely unknown. Proteins secreted by *Giardia* likely exert key roles during host interaction procedures. Several different *in vitro* protocols on obtaining *Giardia* protein extracts have been established.

2.3.1 Giardia Excretory-Secretory Proteins (ESPs)

It has been shown that *Giardia* secret proteins that interact with epithelial cells. In 1991, ESPs were obtained for the first time (Guy *et al.* 1991). Then in the following years, ESPs collected with the same procedure have shown several main functions, *e.g.* to degrade several chemokines, including CXCL1, CXCL2, CXCL3, IL-8, CCL2 and CCL20 (Liu *et al.* 2018); to induce local immunity via enhancement of T helper cell activity (Kaur *et al.* 1999); and to stimulate Th2 immune response leading to intestinal morphological changes (Jimenez *et al.* 2004). Several functional

antigens in ESPs have been identified. Indeed, it was found that some glycoproteins and cysteine proteases in the ESPs could induce antibody production in sera (Kaur *et al.* 2001; Jimenez *et al.* 2007; Jimenez *et al.* 2009). Several other potentially important candidates with immunomodulatory capacity in the ESP fractions were also reported (Shant *et al.* 2002; Shant *et al.* 2004, 2005).

2.3.2 Soluble Giardia Proteins (sGPs)

In addition to ESPs, sGPs are used in the studies of *Giardia*-proteins inducing or modulating immune responses. sGPs can be obtained after simple sonication or lysis of *Giardia* trophozoites, and such sGPs can hydrolyse a variety of protein substrates, including human immunoglobulins (Parenti 1989). Purified sGPs fractions have selective antigen activities and can cause disaccharide deficiencies, as well as release of cytokines and activation of mast cells (Chaudhuri *et al.* 1997; Mohammed and Faubert 1995; Munoz-Cruz *et al.* 2018). sGPs can also induce production of specific antibodies and cytokines (Jimenez *et al.* 2014; Munoz-Cruz *et al.* 2018).

2.3.3 Giardia-Released Proteins (GRPs) in vitro

Unlike ESPs and sGPs that were purified from *Giardia* trophozoites, GRPs are proteins released after interaction of *Giardia* trophozoites with host intestinal/colon epithelial cell lines such as HT-29, HCT-8 and Caco-2. The interaction with intestinal cell lines showed that GRPs from the GS isolate (assemblage B) could up-regulate a wide range of genes in Caco-2 cells, genes that are implicated in regulation of the cell cycle, apoptosis, inflammatory signalling and the maintenance of the mucin layer and the microvilli structure of the small intestine. In addition, the GRPs induced secretion of IL-8, CXCL1, and CCL20 in the Caco-2 cell line, and were found to degrade cytokines and chemokines *e.g.* IL-8, IL-1α, TNF-α, CXCL1 and CXCL3 (Ma'ayeh *et al.* 2018). In experiments using the HT-29 cell line for identification of virulence factors in *Giardia*, proteomic analysis showed that around 25 secreted or membrane-associated GRPs were up-regulated (Emery *et al.* 2016). Furthermore, GRPs can induce caspase-dependent apoptosis as well as tight junction disruption in the HCT-8 cell line (Panaro *et al.* 2007; Koh *et al.* 2013).

Giardia proteins may play pivotal roles during the infection. A large number of *Giardia* proteins have been identified and associated with infection, for example Variant-Specific Surface Proteins (VSPs), surface antigens (SA), cyst wall proteins (CWPs), the family of cysteine proteases (CPs), as well as proteins in encystation specific vesicles (ESV) and in the endoplasmic reticulums (ERs) (Dubourg *et al.* 2018; Ma'ayeh *et al.* 2017; Liu *et al.* 2018; Rafferty and Dayer 2015; Emery *et al.* 2016; Hehl and Marti 2004). Some of these proteins have been studied in more detail: *e.g.* the *Giardia* enolase, a glycolytic enzyme which is involved in regulation of *Giardia* excystation (Castillo-Romero *et al.* 2012);

3 Host responses in the small intestine during infection with *Giardia intestinalis*

The small intestine can be divided into four main structural components; 1) the intestinal lumen, 2) the outer and inner mucus layer, 3) the epithelial cell barrier and 4) the lamina propria. In general, in defence against exogenous antigens the intestinal lumen is thought to be a biological barrier where the luminal microbiota to some extent can provide protection, *e.g.* via reduction of the numbers or exclusion of microbial invaders. In the outer and inner mucus layers mucins, secreted by goblet cells, are responsible for the prevention of further invasion of pathogens. The intestinal epithelial cells clear antigens not only by brushing them out but also by releasing antimicrobial factors and inflammatory mediators, *e.g.* chemokines and alarmins, that also induce the recruitment of immune cells. In the lamina propria sentinel cells, *i.e.* macrophages, mast cells and dendritic cells (DCs) reside, and these cells will enhance and control the inflammatory process.

3.1 The intestinal epithelial cell barrier

The intestinal epithelial cell barrier, a single layer of cells along the gastrointestinal tract textured into villi and crypts, consists of goblet cells, epithelial cells, microfold (M) cells, stem cells and Paneth cells as well as Tuft cells forming an innate defence barrier against exogenous toxins, pathogens and antigens (Bischoff *et al.* 2014; Groschwitz and Hogan 2009).

The intestinal epithelial cell barrier is primarily also implicated in selective absorption of nutrients and water. The tight junctions, a complex protein network that connects the intestinal epithelial cells and excludes foreign matters from accessing the body (Forster 2008; Schneeberger and Lynch 1992), can be degraded by proteases secreted by invaders, *e.g.* by the cathepsins secreted by *Giardia* (Liu *et al.* 2018).

Defects in the mucosal barrier and/or permeability of the epithelial layers are implicated in gut-associated diseases, such as irritable bowel syndrome and inflammatory bowel disease (Scaldaferri *et al.* 2012; Ohman *et al.* 2015). The main function of goblet cells is to secret mucins to the outer and inner mucus layers, and the secretion will be increased upon infection-induced changes. It has been indicated that secretion of mucins, in particular MUC2, plays a key role in the regulation of the ensuing immune responses in the intestine (Birchenough *et al.* 2015). Paneth cells also contribute to the barrier function through secretion of antimicrobial proteins (AMPs), *e.g.* lysozyme, cathelicidins, defensins and secretory phospholipase A2. AMPs can selectively disrupt the bacterial cell walls (Peterson and Artis 2014; Bevins and Salzman 2011; Gallo and Hooper 2012). Studies of the intestinal epithelial cell layers revealed expression and secretion of IL-25 and the alarmin IL-33, two cytokines that induced a series of inflammatory immune responses in the early stages of microbial infection (Li *et al.* 2019; Bouchery 2019; Tahaghoghi-Hajghorbani *et al.* 2019; Xiao *et al.* 2019).

3.2 *Giardia* and the mucus layers

When *Giardia* that experienced excystation migrates to the intestine from the stomach, mucins secreted by goblet cells will adhere to *Giardia*. The main function of mucins is to protect the mucosal epithelial cells and to remove invaders or harmful substances preventing them from touching the epithelial cell layer. Little is known about the importance of mucins during a *Giardia* infection, except that *Giardia* could damage the intestinal mucus layer through degradation of Mucin-2, leading to an increased translocation of bacteria (Chen *et al.* 2013). Interestingly, infected mucin-2-deficient mice showed increased numbers of jejunal *Giardia* trophozoites and an impairment of weight gain as compared with wild type mice (Amat *et al.* 2017). Furthermore, Mucin-2 was reported to be

involved in protection against intestinal infection since the lack of the Mucin-2 gene contributed to exacerbated inflammatory intestinal responses (Hasnain *et al.* 2010).

3.3 *Giardia* and the intestinal epithelial cell barrier

Giardia is not an invasive protozoan parasite, so in order to take advantage of the bio-available nutrient they need to attach to the surface of the epithelium cell layer. The attachment is achieved with a suction-based mechanism using an adhesive disc containing around 100 proteins, *e.g.* tubulin and giardins, among other proteins (Ankarklev *et al.* 2010; Faubert 2000; Woessner and Dawson 2012). Attached tropozoites synthesize and secrete ESPs, *e.g.* cysteine proteases, to disrupt intestinal epithelial cell tight junctions or to induce apoptosis in the epithelial cells (Ringqvist *et al.* 2008; Ma'ayeh *et al.* 2017; Scott *et al.* 2002). Thus, it is speculated that infection with *Giardia* can increase the intestinal epithelial cell permeability, leading to a "leaky" gut. In response, epithelial cells may secret anti-parasitic factors such as lysozyme, which may contribute to the clearance of *Giardia* (Eckmann 2003; Tako *et al.* 2013).

3.4 *Giardia* and interactions with immune cells in lamina propria

The lamina propria located between the epithelial cell barrier and the connective tissue is also important for preventing the entry and spreading of pathogens (Wershil and Furuta 2008; Tlaskalova-Hogenova *et al.* 2004). In response to *Giardia* antigens immune sentinel cells in the lamina propria can secret several cytokines and chemokines, which will induce the recruitment or activation of different innate and adaptive immune cells, *e.g.* macrophages, dendritic cells, eosinophils, neutrophils, mast cells and several different innate and adaptive lymphoid cells.

3.4.1 Giardia and macrophage interactions

The macrophage, one important member of the sentinel innate immune cells, is associated with the killing of *Giardia* trophozoites. Earlier studies found that macrophages were effector cells and by mediating enterocyte damage they contributed to the removal of the *Giardia* infection (Kanwar

et al. 1987; Goyal *et al.* 1993). *In vitro* experiments showed that macrophages could ingest *Giardia* trophozoites and release TNF-α and IFN-γ in response to the *Giardia* challenge (Hill and Pearson 1987; Hill and Pohl 1990; Kaplan *et al.* 1985; Belosevic and Daniels 1992). Furthermore, the killing rate of macrophages could be inhibited by a nitric oxide synthase inhibitor (Fernandes and Assreuy 1997).

3.4.2 Giardia and dendritic cell interactions

Dendritic cells (DCs) are also considered to be sentinel cells. In the lamina propria DCs with elongated dendrites to increase efficient surveillance will recognize and endocytose *Giardia* antigens, before migrating to draining lymph nodes where they activate naïve T-cells (Lopez-Romero *et al.* 2015). DCs challenged with *Giardia* trophozoites or ESPs increased the proliferation of T cells *in vitro* (Grit *et al.* 2014). A recent study showed a correlation between malnutrition and the ensuing DC cytokine response. The authors showed that *Giardia* could attenuate bacterial endotoxin induced IL-23 expression in bone marrow derived DCs (Burgess *et al.* 2019).

3.4.3 Giardia and mast cell interactions

Mast cells are the third type of sentinel cells. *Giardia* infection induces mast cell activation and recruitment to the small intestine in mice (Halliez *et al.* 2016; Li *et al.* 2016). Mast cell-deficient mice showed a significantly delayed elimination of the infection, suggesting that mast cells are important for the control of the infection (Li *et al.* 2004). In line with this, mice deficient in mannose-binding lectin or in complement receptor factor 3a displayed a delayed parasite clearance, reduced mast cells sub-mucosal recruitment and reduced ex-vivo T cell responses at resolution of an infection with *Giardia* (Li *et al.* 2016). *In vivo*, C3a is rapidly degraded to C3a-desArg protein which will not cause mast cell activation (Lohman *et al.* 2017). Thus in experimental infection models *Giardia*-induced C3a could stimulate mast cells to degranulate and to release cytokines. In vitro, *Giardia*-activated mast cells were found to release histamine, IL-6 and TNF-α, and increase the expression of tryptase, but degranulation of mast cells was not found (Munoz-Cruz *et al.* 2010). In addition, *Giardia* can also directly activate mast cells by the release of ADI and its metabolic product, citrulline (Munoz-Cruz *et al.* 2018), although the direct

interaction of *Giardia*-derived molecules does not seem to cause mast cell degranulation (Munoz-Cruz *et al.* 2018; Munoz-Cruz *et al.* 2010).

3.4.4 Giardia and neutrophil interactions

Recruited neutrophils also seem to play key roles during the *Giardia* infection. However, the specific roles of neutrophils remain poorly understood. Two neutrophil defensins HNP-1 and NP-2 were found to reduce the viability of *Giardia* trophozoites (Aley *et al.* 1994), and the adherence of *Giardia* to the epithelial cell layer could be inhibited by two neutrophil derived enzymes, lysozyme and β-glucuronidase (Crouch *et al.* 1991). Another interesting finding is that *Giardia* trophozoites adhered poorly to neutrophils when heat inactivated complement components or monoclonal antibodies directed towards low affinity Fc receptors were used (Arbo *et al.* 2006). Furthermore, although neutrophil infiltration was reported to be increased in human patients and in BABL/C mice infected with *Giardia* (Abbas *et al.* 1994; Chen *et al.* 2013), the number of MPO positive neutrophils in infected C57BL/6 mice was found to be similar to the uninfected control mice (Cotton. *et al.* 2014). This suggests that *Giardia* potentially could block neutrophil activity *in vivo*. In line with this it was found that *Giardia* ESPs such as cathepsin B efficiently degraded the chemokine IL-8/CXCL8 and thus could attenuate neutrophil recruitment (Cotton. *et al.* 2014; Cotton *et al.* 2014).

3.4.5 Giardia and interactions with T and B cells

T cells are reported to play pivotal roles in the immune response to *Giardia* infections. T-cell-deficient mice failed clearance of *Giardia* (Singer and Nash 2000). *Ex vivo* experiments showed that challenge with *Giardia* induced the proliferation and/or activation of lymphocytes or blood CD4-positive (CD4+) and/or CD8+ T cells (Ebert 1999; Gottstein *et al.* 1991; Hanevik *et al.* 2011; Hanevik *et al.* 2012). The activation of T cells through complement factor 3a is important for *Giardia* control (Li *et al.* 2016) and helper T cells but not cytotoxic T cells were reported to be required for elimination of *Giardia* (Heyworth *et al.* 1987; Ermak *et al.* 1988; Khanna *et al.* 1990; Vinayak *et al.* 1989). Furthermore, *Giardia*-stimulated bovine monocyte-derived DCs induced the proliferation of CD3-negative γδ-T-cells, and CD4+ and CD8+ TCRαβ+ T-cells (Grit *et al.* 2014). Interestingly, *Giardia* antigens in the molecular weight range of 90-

110, 65-77 and 40-64 kDa were shown to activate T cells (Astiazaran-Garcia *et al.* 2009).

In addition, B cells may also play a significant role in the host defence against infection with *Giardia*. The number of B cells was increased in mice with orally administrated *Giardia* (Lee *et al.* 2009). However, as compared with T-cell-deficient mice, which completely failed to clear *Giardia*, B-cell-deficient mice showed a capacity to control *Giardia* (Singer and Nash 2000; Langford *et al.* 2002).

3.4.6 Giardia and regulation of cytokines / chemokines

The important roles of cytokines and chemokines during infection with *Giardia* cannot be ignored (reviewed in detail (Lopez-Romero *et al.* 2015)). *Giardia* causes a mixture of Th1/Th2/Th17 immune responses and the importance of several cytokines in giardiasis was addressed using IL-17A Receptor-deficient mice as well as IL-6, IL-9 or TNF-α -deficient mice, which all were found to display a delayed clearance of the *Giardia* infection (Li *et al.* 2004; Zhou *et al.* 2007; Dann *et al.* 2015). Cytokines and chemokines that display altered expression levels during giardiasis in different models are summarized in Table 2.

Table 2. Cytokines and chemokines involved in Giardia infection.

Cytokines and Chemokines	**Induction by *Giardia***	**Model materials**	**References**
TNF, IFN-γ, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-17	Up regulated	Gerbils	Serradell *et al.* 2018
IL-8, IL-1α, TNF-α, CXCL1, CXCL2, CXCL3, CCL20	Degraded	Caco-2 intestinal epithelial cells	Liu *et al.* 2018
TNF-α, IL-12, IL-6, IL-2, IFN-γ	Increased	$TLR2^{-/-}$, $TLR4^{-/-}$ mice, DCs	Lee *et al.* 2014
IL-4, IL-5, IL-10	Increased	Spleen cells and intestinal fluids	Jimenez *et al.* 2014
IL-8	Increased	Caco-2 intestinal epithelial cells	Fisher *et al.* 2013
IL-4	Up regulated	IL-6-deficient mice	Bienz *et al.* 2003
CCL2, CCL20, CXCL1, CXCL2, CXCL3	Up regulated	Caco-2 intestinal epithelial cells	Roxstrom-Lindquist *et al.* 2005

4 Hypothesis and aims

The hypothesis of this thesis was that the mast cell-specific proteases chymase and tryptase expressed by connective tissue mast cells could affect the intestinal immune responses towards infection with the non-invasive protozoan parasite *Giardia intestinalis*.

The aims were to investigate and characterize potential interactions between the connective tissue mast cell-specific proteases and secreted *Giardia* proteins, and to characterize the intestinal immune responses to infections with *G. intestinalis* in knockout mouse models, lacking the chymase mouse mast cell protease-4 or the serglycin proteoglycan.

The specific objectives were to:

- Establish *in vitro* mast cell culture models for studies of the interactions with *G. intestinalis*.
- Establish an *in vivo* gavage-infection model in the mMCP-4 and the SG proteoglycan knockout mouse models
- Analyze the immune responses to the *G. intestinalis* infection in the knockout mouse models

5 Present investigations

In this thesis I have used both *in vitro* and *in vivo* experimental models to address the impact of *G. intestinalis* on intestinal mast cells. To elucidate the functions of the mast cell-specific chymase as well as the serglycin during infection with *G. intestinalis* I have used genetically targeted mouse strains deficient for mMCP-4 or SG. The advantages with mouse knockout models, compared with cell cultures, are that the concerted actions of the immune response can be addressed. During infection with *G. intestinalis*, which is not invasive, a direct interaction with the chymase may be hard to envisage. However, the trophozoites attached to the epithelial cells in the small intestine will secrete immunomodulatory proteins into the villi, and after activation of the crypt-dwelling CTMCs the chymase can diffuse into the villi and potentially makes an interaction with the infiltrating *Giardia*proteins.

5.1 In vitro experiments: interactions of mast cell proteases and *Giardia* proteins

Here I investigated if potential interactions between the mast cells as well as the mast cell specific proteases and *Giardia* proteins exist. To address the outcome of such interactions I cultured mouse bone marrow cells according to two slightly different cell culture protocols. CTMCs were derived in IL-3 and stem cell factor (SCF) conditioned media and MMCs in IL-3, SCF, TGF-β and IL-9 conditioned media. I then challenged the CTMCs and MMCs with soluble *Giardia* proteins (sGPs) to evaluate the activation status of the mast cells. I also investigated if mast cell tryptase or chymase could degrade any of the sGPs. The protein bands degraded by chymase were sent for proteomics to identify the *Giardia* proteins (Table 3). Furthermore, I studied if sGPs or ESPs could affect the enzymatic activities of the human and the mouse mast cell specific proteases. The potential fractions of sGPs that influenced on the tryptase activity were subjected to sorting by anion-exchange and size-exclusion chromatography

(Figure 2). Proteomics were performed to identify the proteins in the fractions that affected the tryptase activity (Table 4).

Table 3. Top 10 Giardia proteins probably degraded by rCh

Accession	Description	MW[kDa]	calc. pI
V6U7H9	Phosphoglucomutase /Phosphomannomutase	73,5	5,50
A0A132NU12	Putative Alanine aminotransferase	58,4	7,68
V6U395	Arginine deiminase	64,3	6,54
Q24967	Immunoglobulin heavy chain binding protein	72,6	5,10
C6LZK0	Malic enzyme	61,3	7,20
V6TS77	Tenascin family protein	61,2	4,67
C6LR45	Tenascin-like protein	64,7	4,59
V6U3S0	High cysteine membrane EGF-like protein	62,0	5,10
V6TT28	Variant-specific surface protein	73,1	5,41
V6TZJ9	EGF-like cyst protein	63,2	4,37

Table 4. Top 5 proteins in each band possibly enhancing mast cell tryptase activity

Protein bands	Accession	Description	MW [kDa]	calc. pI
Upper	C6LRY8	UPL-1	34,4	7,30
	V6U387	Thioredoxin reductase	33,9	6,61
	V6TU22	Serine/threonine-protein phosphatase	35,5	5,45
	C6LPK2	Ornithine carbamoyl transferase	36,5	7,03
	V6U471	Cathepsin B-like cysteine proteinase	35,2	5,52
Middle	V6TSI5	Alpha1-giardin	33,8	6,73
	V6U387	Thioredoxin reductase	33,9	6,61
	Q2QBT8	14-3-3 protein	28,6	5,17
	V6TX12	Cathepsin B-like cysteine proteinase	32,8	5,12
	A0A132NVX4	Uncharacterized protein	30,0	4,53
Lower	Q2QBT8	14-3-3 protein	28,6	5,17
	A0A1L7B7I7	Beta-giardin	23,2	5,16
	Q01832	Protein C4	22,3	8,54
	C6LQF2	Eukaryotic translation initiation factor 6	26,7	5,55
	V6U362	Proteasome endopeptidase complex	27,5	5,29

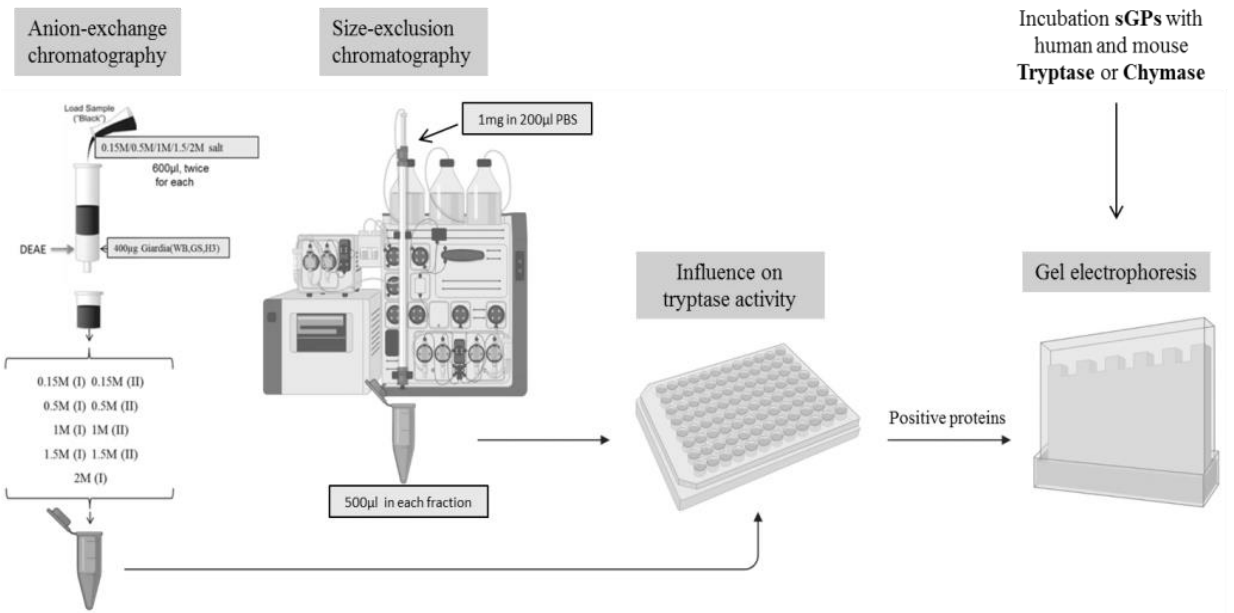

Figure 2. The outline for the in vitro *Giardia* mast cell protease interaction experiments. After establishing the impact of *Giardia* sGPs on the tryptase activity, the sGPs was further purified and fractionated by anion-exchange and size-exclusion chromatography. The effect of eluted fractions on recombinant human tryptase (rHT) was determined then the proteins having a positive influence on the rHT activity were visualized on gel. (Created with biorender.com)

5.2 In vivo experiments: mast cell protease-deficient mice and infection with *Giardia*

5.2.1 Kit dependent mast cell deficient mice

To date, naturally occurring mutations of the mice that selectively delete only mast cells have not been found. Kit tyrosine kinase, encoded by the c-kit gene, is the receptor for stem cell factor (SCF) that regulates the maturation, survival, proliferation and functional responses of mast cells (Galli *et al.* 2015). C-kit is highly expressed not only in mast cells but also in other cell types such as hematopoietic stem cells and immune cells. Thus, mice carrying mutations in the c-kit gene usually show mast cell deficiency as well as other cell type abnormalities (Galli *et al.* 2015). The two mouse strains $Kit^{W/W-v}$ and $Kit^{W-sh/W-sh}$ have commonly been used for the analysis of potential functions of mast cells *in vivo*. Compared with the $Kit^{W/W-v}$ mouse strain, the $Kit^{W-sh/W-sh}$ mouse strain has fewer abnormalities with relatively increased numbers of neutrophils and basophils, and is neither sterile nor anemic (Galli *et al.* 2015). The $Kit^{W/W-v}$ mouse strain has been used to claim that mast cells are important for protection against and elimination of the *Giardia*-infection (Li *et al.* 2004). In addition, it was shown that c-kit depleted mice, *i.e.* mast cell deficient, showed similarly increased numbers of parasites as the $Kit^{W/W-v}$ mice (Li *et al.* 2004). However, to date there are no reports on that $Kit^{W-sh/W-sh}$ mice was used for the study of mast cells in *Giardia* infection *in vivo*. I therefore used the $Kit^{W-sh/W-sh}$ mice to establish the *Giardia* infection mouse model and to evaluate if this mouse strain had the same response profile as the $Kit^{W/W-v}$ mouse strain. In the vast majority of the *Giardia*-infection studies, to date, the authors have used mice in the age range of 3 to 10 weeks. These young mice are in their growing phase and will usually gain weight even when they are infected with *G. intestinalis*. The outcome of the infection in older full-grown (mature adult) mice is much less studied. Therefore, I used aged/mature adults (22-36 weeks old) $Kit^{W-sh/W-sh}$ mice to address the response to *G. intestinalis* in adult mice lacking mast cells.

5.2.2 The mouse Mast Cell Protease-4-deficient and the Serglycin-deficient mouse strains

The mMCP-4 and the SG-deficient mouse strains were conceived, constructed and produced in Uppsala. The targeted deletion of both mMCP-4 and SG involved the replacement of exon 1 with a Neomycin-cassette, which however was not floxed. Thus, the resulting targeted mouse strains carry the Neomycin-cassette and if the Neomcyin-promotor produces any off-target effects the best control of the homozygote deficient mice may in fact be the heterozygote mice.

Initial characterization of the mMCP-4-deficient mouse strain showed similar levels of mMCP-4 protein in the mMCP-$4^{+/+}$ and mMCP-$4^{+/-}$ mice, and that the deletion of the mMCP-4 protein did not affect the numbers of mast cells or the expression of the other connective tissue mast cell-specific proteases in the mMCP-$4^{-/-}$ mice (Tchougounova *et al.* 2003).

The deletion of SG resulted in multiple changes in the storage of cationic mediators, *e.g.* storage of histamine and serotonin, and the proteases mMCP-4, mMCP-5, mMCP-6 and CPA in mast cells (Abrink *et al.* 2004). However, when assessing the expression of neighboring genes in the $SG^{+/-}$ and $SG^{-/-}$ mice no major differences was found (Roy *et al.* 2016), suggesting that the $SG^{+/-}$ mice is as valid as the $SG^{+/+}$ mice as a control for the SG-deficiency.

The mouse strains referred to above, *i.e.* $Kit^{W\text{-}sh/W\text{-}sh}$, mMCP-4, or SG on the C57BL/6J Taconic genetic background (backcrossed for more than 20 generations) were used to establish the *Giardia intestinalis* trophozoite gavage-infection mouse models. Clinical scoring in the mMCP-4-deficient and the SG-deficient mice was performed in a blinded fashion, *i.e.* the genotypes were not known to the assessor. Since infection with *G. intestinalis* potentially could cause weight loss or reduced weight gain, weight data were recorded before infection (day 0) and then every second or third day. From infected and control mice feces, tail tissue, serum, and intestines (jejunum and duodenum) were collected (Figure 3).

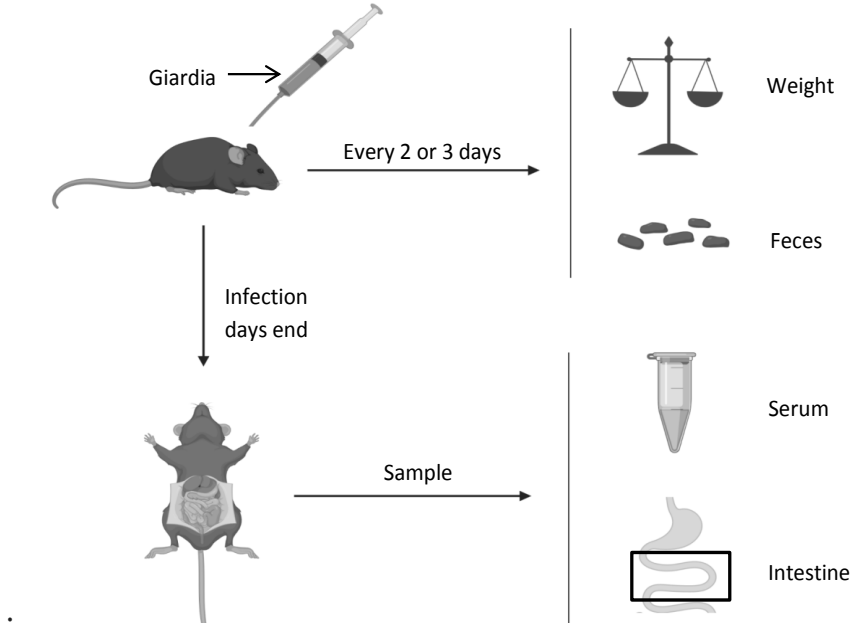

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Figure 3. The procedures of in vivo *Giardia* infections and the sampling of tissues. 10^6 *Giardia* trophozoites were administered to the different knockout mouse strains by oral gavage. Weight data and fecal samples were collected every two or three days post infection. At the experimental endpoint, serum and intestines were sampled for each mouse. (Created with biorender.com)

6 Results and discussion

Supplementary results are shown here to support **Paper I-III**

6.1 *Giardia* excretory-secretory proteins modulate the enzymatic activities of mast cell chymase and tryptase (Paper I)

6.1.1 The interaction of soluble Giardia proteins (sGPs) with mast cells

Previous studies have shown that sGPs can activate rat mucosal-like mast cell lines (Munoz-Cruz *et al.* 2010; Munoz-Cruz *et al.* 2018). In Paper I mouse bone marrow-derived connective tissue mast cells (CTMCs) and mucosal-like mast cells (MMCs) were challenged with sGPs to study the activation of MMCs. Interestingly, no clear activation of the CTMCs (Figure 4) occurred whereas sGPs from the three different isolates (GS, WB, H3) caused very similar activation of the MMC, with increased IL-6 production and tryptase activity. However, the activation induced by sGPs was very minor and, probably sGPs did not cause degranulation of the MMCs since no change of the activity of the granule-stored mast cell-activation marker β-hexosaminidase was found in the collected supernatants.

6.1.2 The interaction of soluble Giardia proteins (sGPs) with mast cell proteases

To study if any of the sGPs were degraded by mast cell proteases, recombinant human tryptase (rHT), recombinant human chymase (rCh) or by wild-type mouse ear tissue proteolytic extracts were used. Strikingly, sGPs were not extensively degraded by the recombinant human proteases or by the ear tissue mouse mast cell proteases. This was unexpected since such degradation has previously been observed in protein extracts derived from the protozoan *Toxoplasma gondii*, and the parasitic worms *Heligosomoides polygyrus* and *Trichinella spiralis* (Roy *et al.* 2016).

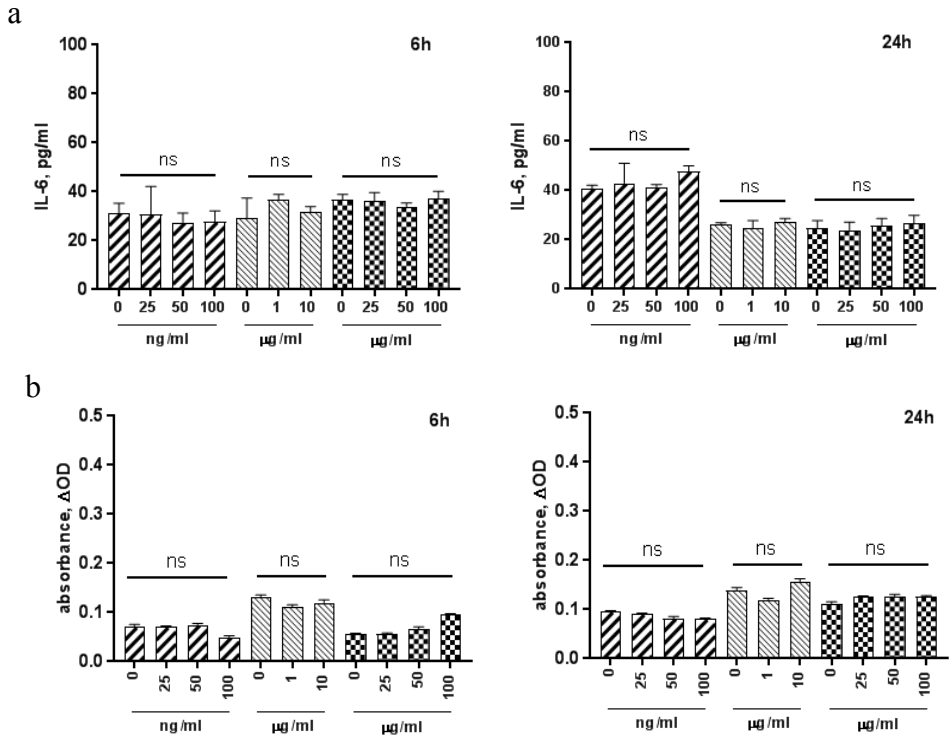


Figure 4. IL-6 level and tryptase activity in connective tissue mast cell supernatants after challenge with soluble *Giardia* proteins. $1x10^6$/ml BMMCs were incubated without or with sGPs (ranging from 25ng/ml to a 100µg/ml in concentration), then supernatant was collected at time point 6h and 24h after cells were stimulated to be used for measurement of (**a**) IL-6 and (**b**) tryptase activity(difference of optical density (OD) between 3h and 0h was calculated).

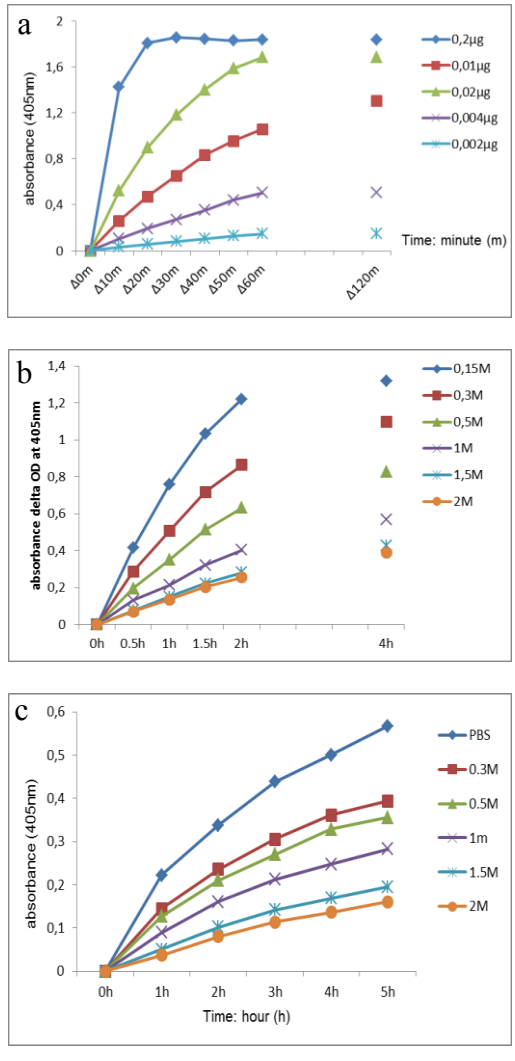


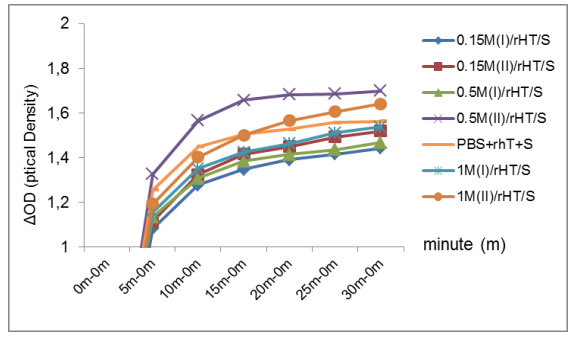
Figure 5. Optimization of working concentration of rHT and the effect of NaCl-concentration on rHT and rCh. a) Different amounts of rHT in 10 µl PBS reacted with 20µl of the substrate S-2288 + 90µl PBS. **b**) The optimal activity of 0.01µg rHT for the substrate S-2288 in different PBS+ NaCl-concentrations. **c**) The optimal activity of 0.05µg rCh for the substrate L-1595 in different PBS+ NaCl-concentrations. In (a, b, c) changes in the absorbance was measured at 405nm every 10 minutes, 30 minutes or 1 hour, respectively, for 2, 4 and 5 hours.

To further study potentially functional interactions between the mast cell specific proteases and *Giardia* proteins, the concentrations of rHT, as well as the salt concentration (NaCl) previously shown to affect the proteolytic activities of tryptase and chymase were optimized (Figure 5). In the absence of heparin high NaCl-concentrations maintain the stability of the tryptase tetramer, however, the tryptase tetramer will decay into monomers if stored long term in high NaCl concentrations also slow down the activity rate of tryptase (Pereira *et al.* 1998; Hallgren 2001). Optimal conditions for the enzyme reactions were determined to be: PBS containing 0.15M NaCl; 0.01µg of the recombinant human tryptase and 0.05µg of the recombinant human chymase. These conditions were used in the further interaction studies using Giardia-derived soluble proteins (sGPs) or excretory-secretory proteins (ESPs). Surprisingly, an enhancing effect of both sGPs and ESPs on the activity of rHT and mouse ear tissue extracts was observed for the chromogenic substrate S-2288. In contrast, sGPs were found to significantly inhibit the rCh activity and the WT mouse ear tissue extract activity for the substrate L-1595. These results suggest that sGPs and ESPs potentially can modulate mast cell protease activities *in vivo* and thus may affect mast cell driven immune responses.

6.1.3 Sorting out the interacting proteins

Two chromatography methods were used in the following attempts to sort out and identify the sGPs that affected the tryptase activity. Using anion-exchange chromatography fractions of sGPs were eluted by increasing concentrations of NaCl, step vise from 0.15 M to 2.0 M NaCl. The second fraction eluted with 0.5 M NaCl significantly enhanced the rHT activity and the SDS-PAGE gel electrophoresis showed proteins in this fraction with molecular weights ranging from 10 to 35 kDa (Figure 6). A second independent experiment repeating the anion-exchange fractional purification of the GS, H3 and WB *Giardia*-isolate proteins gave very similar results as those presented in Figure 6. The *Giardia*-proteins were also fractionated with size-exclusion chromatography. Interestingly, the size-exclusion fractions numbered 26, 29, 32, 33, 34, 35, 36, 37, and 38 showed significant enhancement of the rHT activity (Figure 7).

a



b

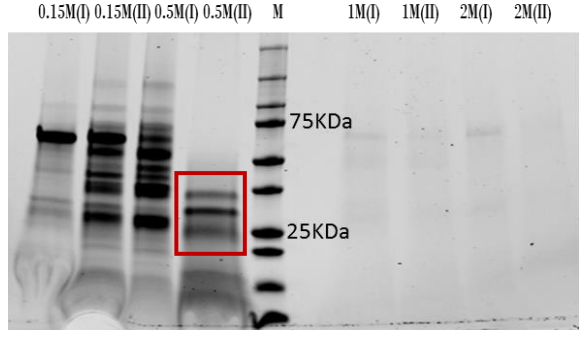


Figure 6. Anion-exchange fractions eluted at 0.5M NaCl enhances the activity of rHT. 400µg of sGPs in PBS from the GS, WB and H3 *Giardia* isolates were loaded onto a 0,5 ml DEAE Sephacel^{TM} column (GE Healthcare) and the bound sGPs were stepwise eluted twice by 0.15M, 0.5M, 1M and 2M NaCl in PBS. **a)** The eluted fractions were then incubated with 0.2µg rHT (old batch) + the chromogenic substrate S-2288, and optical density (OD) was measured every 5 minutes up to 30 minutes, and all OD values were normalized to the OD value at 0 minute. **b)** The different fractions of sGPs were checked by SDS-PAGE gel electrophoresis. The protein bands in the red frame were cut out from the gel and identified by proteomics. In panel a & b representative results of the GS *Giardia* isolate fractions is presented. Very similar (identical) results were obtained with the H3 and WB *Giardia* isolates (data not shown).

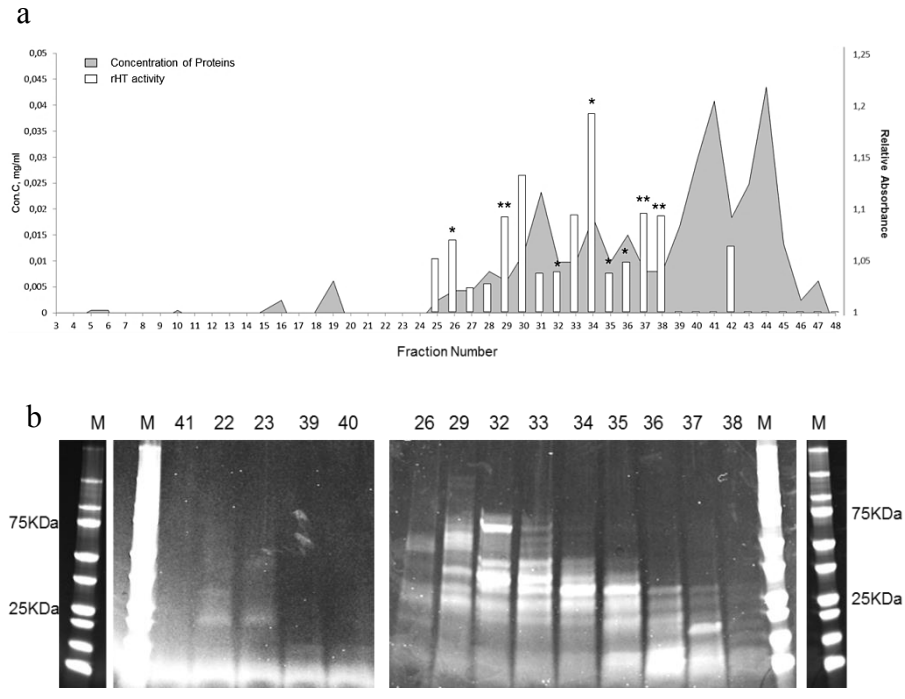


Figure 7. Fractions enhancing activity of rHT. 1mg of GS sGPs were eluted in a size-exclusion chromatography HPLC-system. **a)** Each fraction with detectable protein levels was then tested for enhancement of the rHT activity. Protein fractions were then incubated with 0.2µg rHT (old batch) + the chromogenic substrate S-2288, and optical density (OD) was measured every 5 minutes up to 30 minutes, and all OD values were normalized to the OD value at 0 minute. **b)** The fractions containing proteins and with an enhancement effect were checked by SDS-PAGE gel electrophoresis.

The visualization of proteins in these fractions indicated that the molecular weight of interesting proteins was in the range of 15 to 35 kDa. Attempts were therefore made to identify the 15-35 kDa *Giardia*-proteins (red frame, with three major protein bands) in Figure 6 using a proteomics approach. The top 5 proteins of each band, with a molecular weight of 20-35 kDa, are shown in Table 4. In addition, the 65 kDa protein band in the sGPs that potentially could be degraded by rCh was sent for proteomic identification. The top 10 candidate proteins in the 65kDa band are shown in Table 3.

Some of these candidate proteins shown in Tables 3 and 4 have been reported to be important in *Giardia*. It was found that thioredoxin reductase of *Giardia* could catalyse the reduction of drug (metronidazole and furazolidone) (Leitsch *et al.* 2016). The serine/threonine-protein phosphatase was shown to be involved in *Giardia* differentiation, where it was upregulated in the early stage of excystation (Lauwaet *et al.* 2007). Ornithine carbamoyl transferase catalyze citrulline to produce ATP (Galkin *et al.* 2009). *Giardia* cathepsin B which is important for *Giardia´*s encystation and excystation (DuBois *et al.* 2008) can degrade epithelial cell tight junction proteins and chemokines (Cotton *et al.* 2014; Liu *et al.* 2018). Alpha1-giardin, a structural protein of *Giardia* trophozoite, was shown to be a potential vaccine antigen candidate in a murine model (Jenikova *et al.* 2011). The 14-3-3 protein involved in *Giardia* differentiation opens up for anti-*giardia* drug design (Fiorillo *et al.* 2014). Beta-giardin gene detection by nested PCR is constantly used for identification of *Giardia* in most infected animal species and humans worldwide, but the functional role of beta-giardin is still unclear. Phosphoglucomutase can be utilized by *Giardia* to isomerase glucose-6-phosphate to glucose-1-phosphate to make glycophosphophoinositol anchors (Mitra *et al.* 2010). The arginine deiminase (ADI), a metabolic enzyme secreted by *Giardia* can consume arginine causing the reduction of epithelial cell proliferation (Stadelmann *et al.* 2012), and ADI induces MMCs to release significant levels of IL-6 and TNF-α (Munoz-Cruz *et al.* 2018). Tenascin was suggested to be implicated in the regulation of *Giardia* trophozoite attachment (Emery *et al.* 2016). Epidermal growth factor-like protein may be also involved in *Giardia* encystation (Chiu *et al.* 2010). Variant-specific surface proteins could induce strong antibody responses (Serradell *et al.* 2018). In addition, there are additional secreted

Giardia proteins shown to have immunogenic capacity, as reviewed in (Lopez-Romero *et al.* 2015).

Considering the functions of these proteins, it would be interesting to know which of the *Giardia* protein(s) that influences on the tryptase and chymase enzyme activities. This would further increase the knowledge on how mast cell proteases interact with *Giardia*.

6.2 Experimental *Giardia* infection in c-kit dependent mast cell deficient mice causes increased weight loss

To increase the infection rate it has been common to use antibiotic pre-treatment of the mice during experimental infections with *Giardia* trophozoites from human isolates. However, pre-treatment with antibiotics will reduce the microbial load of the intestine and produces a very artificial milieu for the studies of *Giardia* and the ensuing host immune reactions. Thus, to avoid this we established a gavage model without antibiotic pre-treatment of the mice to be used for our studies of the mast cell specific proteases. Furthermore, most studies used young mice (≈6-8 weeks old) or very young mice (≈3-6 weeks old) to investigate potential weight changes and immune regulatory mechanisms. Therefore, mature adult mice (*i.e.* >18 weeks old mice) which have reached a plateau in their weight gain were included to study potential age-related weight losses and immune regulatory mechanisms.

The gavage model was initially evaluated with mature adult congenic $Kit^{+/+}$ and $Kit^{-/-}$ littermate mice infected with the *Giardia* GS isolate (Figure 8a and b), where we scored weight changes every second day up to day 18. From day 8 and onwards the weight change in relation to the day of infection differed significantly between the two groups, indicating that the c-kit dependent mast cell-deficient mice were more vulnerable to the infection. Thus, an experimental *Giardia*-infection model, without antibiotic treatment of the mice and suitable for mast cell studies was established and applied in the subsequent studies.

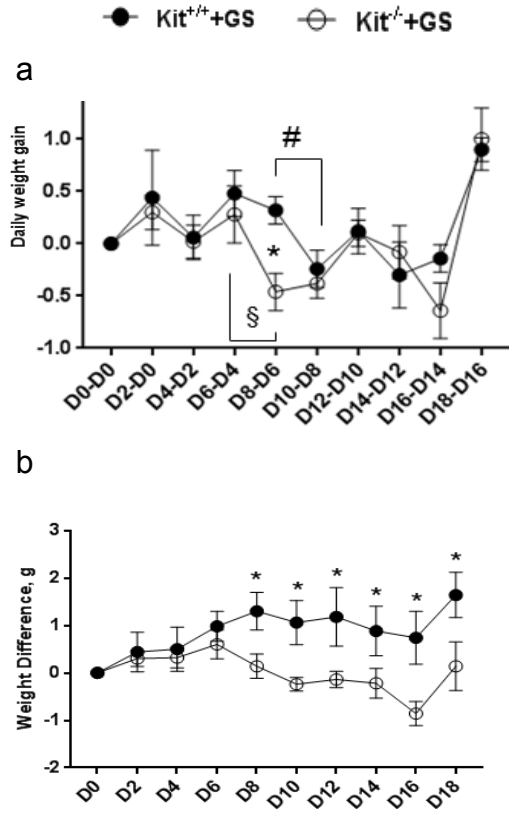


Figure 8. *Giardia* intestinalis infections in c-kit dependent mast cell deficient mice without antibiotic treatment. Successful establishment of the infection model in age grouped wild-type congenic $Kit^{+/+}$ and $Kit^{-/-}$ ($Kit^{W\text{-}sh/W\text{-}sh}$) littermate mice. Mice were infected by gavage with ≈10^6 *Giardia* intestinalis trophozoites. Weight data (in gram) and feces were collected every second day before the mice were euthanized day 18. **a)** Weight changes in-between days in 22-36 weeks old $Kit^{+/+}$ (n=5) and $Kit^{-/-}$ (n=5) mice during infection with the *Giardia*. A statistical analysis weight changes between D4-D6, D6-D8, as well as D8-D10 is shown. * indicate a significant p value between $Kit^{+/+}$ and $Kit^{-/-}$ mice. # indicate significant changes in infected $Kit^{+/+}$ mice between day 8 and day 10, § indicate changes in infected $Kit^{-/-}$ mice between day 6 and day 8, (#, §, *) p value <0.05. (b) Weight changes relative to initial weight at day 0 was determined. * indicate a significant p value <0.05 between $Kit^{+/+}$ and $Kit^{-/-}$ mice.

6.3 The chymase mouse mast cell protease-4 regulates intestinal cytokine responses in mature adult mice infected with *Giardia intestinalis* (Paper II)

The role of mMCP-4 at infection with *Giardia intestinalis* was studied using the chymase mMCP-4-knockout mouse strain. Littermate mMCP-$4^{-/-}$ and mMCP-$4^{+/+}$ mice were infected with 10^6 *Giardia* trophozoites by oral gavage and uninfected littermates were used as un-treated controls. The infection was followed for 8 or 13 days by recording clinical signs, weight data and collection of feces. Genotypes of the infected mice were determined after the experimental endpoint. At endpoint, serum samples and samples from the small intestine were collected. The intestinal samples were further divided into smaller pieces, for RNA extraction and subsequent PCR-analysis, for morphological studies, for DNA extraction and for extraction of proteins and subsequent biochemical analysis including enzymatic assays.

6.3.1 Weight loss in Giardia-infected mature adult mice

Feces and intestinal DNA samples were subjected to a nested PCR to detect *Giardia* DNA. Successful infection was shown in all gavage-infected mice. Importantly, uninfected control mice did not catch the infection during the experimental period. Since IL-6 can affect the outcome of giardiasis, the serum levels of IL-6 were measured and found to be increased in infected mMCP-$4^{-/-}$ and mMCP-$4^{+/+}$ mice as compared to control mice, further supporting a successful infection in all mice as confirmed by the nested PCR. Recent reports show that *Giardia* is a significant factor in the induction of reduced weight gain and stunting of young children in low-resource settings (Donowitz et al. 2016; Rogawski et al. 2018). In paper II I found that infected mice showed significant weight loss compared to uninfected mice. Interestingly, between day 2 and day 4 post infection an increased weight loss in mMCP-$4^{-/-}$ mice was observed, as compared with mMCP-$4^{+/+}$ mice, a difference between genotypes that was lost at endpoint, day 8 or day 13. According to the weight data, it would be interesting to study the intestinal infection status or immune responses caused by *Giardia* at day 2 or 4 in infected mice.

However, the current experimental design focused on day 8 as this has been reported as the peak infection day for *Giardia* in mice.

6.3.2 Significantly decreased neutrophil elastase (NE) activity in Giardia- infected mice

The morphological assessment of the small intestines at day 8 showed no or very minor differences between mMCP-$4^{-/-}$ and mMCP-$4^{+/+}$ mice. Compared with uninfected mice the *Giardia*-infection caused a similarly increased goblet cell counts and a minor increase in granulocyte counts in both genotypes. However, the increase in granulocyte counts were not reflected by increased myeloperoxidase or neutrophil elastase (NE) activity. In contrast, the *Giardia* infection caused a significantly decreased intestinal NE activity, an observation that was confirmed *in vitro*, using soluble *Giardia* proteins and recombinant human NE. Thus, *Giardia* seems capable to inhibit the two proteases NE and chymase (see paper I) expressed by two different innate immunity cell types, *i.e.* neutrophils and mast cells.

6.3.3 The mMCP-4 regulates intestinal cytokines and chemokines in mature adult mice infected with Giardia intestinalis

To address what innate and adaptive immune responses *Giardia* can cause in the absence of mMCP-4 in mice the transcriptional expression profile of intestinal cytokines and chemokines was determined by qPCR. On the whole, the intestinal immune response to *Giardia* was more pronounced on day 8 than day 13 post infection. An extensive screening for expression of cytokines and chemokines in the small intestines of the control and infected mice showed an overall increase of the expression levels in infected mice. Strikingly, lack of the chymase mMCP-4 caused a significantly reduced upregulation of the expression of IL-6, TNF-α, CCL2, CXCL2, IL-2, IL-5, IL-17a, IL-17c and IL-10. In addition, IL-4 was significantly downregulated in the chymase-deficient mice. The onset and the chymase-dependent regulation of IL-25 and IL-33 in infected mice is a new finding, and suggests that chymase is involved in the early onset and recruitment of innate lymphoid cells. Furthermore, the reduced cytokine expression in mice lacking mMCP-4 suggests that chymase is involved in the regulation of inflammatory cytokine response profiles. If this regulation is direct or indirect remain to be determined.

In previous studies research groups most often used 5 to 10 weeks old mice for their experimental infections with *G.* intestinalis. The mature adult mice (>18 weeks old) that I have used is one of the first studies, to my knowledge, that experimentally address the effect of age of mice during a *Giardia*-infection. The level of cytokines and chemokines can be affected by age or sex during parasite infections (Lechner *et al.* 2013; Roberts *et al.* 2001). For example, an *ex vivo* experiment showed that the expression levels of chemokines and cytokines in peripheral blood mononuclear cells and umbilical cord blood cells sampled from neonates, children, adults, and the elderly were pronouncedly different (Lechner *et al.* 2013). In addition, it was found that the immune cells that expressed specific sex hormone receptors, for example for progesterone or estrogen, when challenged with LPS responded by reduced production of cytokines (Roberts *et al.* 2001). To reduce the influence of age variation the mice selected for qPCR analysis were around 20 weeks old. In addition, since the trends of weight loss of the mature adult females and males were found to be similar post *Giardia*-infection, the data obtained from females and males, *e.g.* in the qPCR, were pooled for the respective genotypes. Instead I focused on the potential differences in cytokine expression at day 8, *i.e.* peak day of infection, versus day 13 when elimination of the infection should have been initiated.

6.4 The mouse serglycin proteoglycan regulates immune responses in a sex-specific direction during infection with *Giardia intestinalis* (paper III)

Many of the preformed mast cell specific mediators, *e.g.* mMCP-4, mMCP-6 and CPA3, rely on the serglycin (SG) proteoglycan for correct storage and function. The SG-knockout mouse strain was therefore used to address the combinatorial effects of multiple protease deficiency upon combating a *Giardia*-infection.

Infection procedures and sampling of the mice were performed as described in Paper II. In brief, 7 to12 weeks old serglycin-deficient ($SG^{-/-}$) and serglycin-competent ($SG^{+/+}$) littermate mice were infected by oral gavage with 10^6 *Giardia* trophozoites. PBS-challenged littermate mice of similar ages were used as controls. Infection was followed for 12 days,

where clinical and weight data scoring, and collection of feces from infected mice was performed. Genotypes of the infected mice were determined after the experimental endpoint. At endpoint serum and the small intestine of all mice were sampled. The small intestines were collected and divided separately for RNA and protein extraction, and for studies of morphological changes.

6.4.1 Reduced weight gain in Giardia-infected mice

Successful infection was shown in all gavage-infected mice by a nested PCR detection for *Giardia* in fecal samples and increased IL-6 levels in serum. Compared with PBS-gavage control mice, infection with *Giardia* caused a reduced weight gain before 7 days post infection (dpi). In addition, the infected $SG^{-/-}$ mice showed a delayed and reduced weight gain starting from 5 dpi as compared to the infected $SG^{+/+}$ mice. Interestingly, when the weight data were sorted by sex, infected females showed more obvious reduction of weight gain. According to the weight data, it would be attractive to also determine the intestinal infection status and the resulting immune responses at early days, especially day 5, in the infected mice. However, in the current experimental design I focused on day 12 as this has been commonly used for the study of *Giardia* in mice.

6.4.2 Increased goblet cells as well as granulocytes counts and decreased neutrophil elastase activity

The clinical scoring of mice showed only very mild symptoms, with mice producing "softer" feces around three dpi. Morphological assessment of the small intestines at 12 dpi showed higher numbers of goblet cells and granulocytes in mice infected with *Giardia* in comparison to the control mice, with only minor differences between $SG^{-/-}$ and $SG^{+/+}$ infected mice. The neutrophil elastase (NE) activity in the intestinal tissue was significantly inhibited in infected mice (a similar finding to what I show in Paper II), with no difference between the $SG^{+/+}$ and $SG^{-/-}$ mice. Collectively, these results suggest that the NE activity is not SG-dependent during infection and that *Giardia* may cause the reduction of the NE activity observed.

6.4.3 The intestinal cytokines and chemokines regulated by SG in mice infected with Giardia intestinalis is sex dependent

To address what impact SG could have on the immune responses during experimental infection with *G. intestinalis* I evaluated the intestinal cytokine and chemokine expression levels by qPCR. According to the weight data, the reduced weight loss of infected young mice was mainly contributed to by the female mice. Therefore, I sorted the mice into sex groups. Interestingly, only minor differences of detected gene expressions between infected $SG^{+/+}$ and $SG^{-/-}$ mice was found. The lack of SG in female mice resulted in a significant reduction of CXCL2 and IL-6, and a trend towards downregulation of IL-25, IL-33, TNF-α and NOS2, as well as a trend towards upregulation of CXCL1 and IFN-γ. In male mice lacking SG a significant upregulation of NOS1, IL-2, IL-4, IL-5, and a trend towards upregulation of IL-25, IL-33, IL-6, IL-10, and NOS2, as well as a significant downregulation of IL-9 was observed. A striking difference in the expression of IL-25, IL-33, IL-6, NOS1, NOS2, IL-2, IFN-γ, IL-4, IL-5 and IL-9 between the infected $SG^{-/-}$ male and female mice were found. This suggests that serglycin (SG) may regulate intestinal immune responses in a sex-dependent fashion.

7 Conclusions and future perspectives

The studies included in this thesis cover the interactions between *Giardia intestinalis* and the mast cell specific proteases, *i.e.* tryptase and chymase. We present *in vitro* results showing that the sGPs and ESPs proteins could modulate the activity of the human and mouse mast cell proteases (Paper I) and that sGPs could inhibit the neutrophil elastase activity (Paper II). Collectively, these findings suggest that *Giardia* via excretory-secretory proteins could affect the immune system. Furthermore, Giardia-infected mature adult mice lost weight whereas young mice did not show reduced weight gain. In addition, the transcriptional gene expression levels of intestinal chemokines and cytokines were significantly reduced in adult mice lacking mMCP-4. Compared to the young mice, the mature adult mice also showed a slightly different cytokine profile which could depend on the age of mice (data not shown and Paper II). In paper III the Giardia infection caused a reduced weight gain mainly in females, with a more pronounced effect on the SG-deficient females. The SG-deficiency as well as the sex of the mice affected the expression levels of intestinal chemokines and cytokines in the infected females and males (Paper III). The increase of IL-6 in serum was associated with the weight change. In mMCP-$4^{+/+}$ and mMCP-$4^{-/-}$ mice the Giardia infection caused similar weight loss and had similar IL-6 levels at 8 dpi and 13 dpi. In the $SG^{+/+}$ and $SG^{-/-}$ mice the serum IL-6 levels were dependent on SG and the SG-deficient females showed no weight gain at 12 dpi (Paper II and Paper III). Our studies lay the foundation for further addressing the mechanisms on how mast cells provide protection during host defense against *Giardia*.

7.1 Roles of mast cell proteases in *Giardia* infection

In this thesis we have addressed the importance of chymase towards *Giardia* through in *vivo* and *in vitro* experiments. The functional interaction of mast cell tryptase with *Giardia* should be further studied *in vivo* based on the *in vitro* finding where *Giardia* proteins could enhance

the enzyme activity of tryptase. Furthermore, mast cells express several other proteases besides tryptase and chymase; *e.g.* CPA, cathepsins, granzyme B and MMPs. So it would also be interesting to determine the potential interactions between other mast cell expressed proteases and *Giardia,* to obtain more knowledge on the role of the mast cell in connection to the *Giardia* infection.

7.2 Actions of immune cells at the core of mast cells during *Giardia* infection

Not only mast cells but also other innate and adaptive immune cells such as macrophage, neutrophil, dendritic cells, T and B cells have been shown to be involved in host defensive mechanisms during infection with *Giardia*. Since all these different immune cells will act in a complex network of direct cell to cell-interactions and via cytokine signaling it would be interesting to study which of the receptors in the cell to cell-interactions and which of the cytokines that are essential to mount a successful immune response towards the *Giardia* infection.

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Popular science summary

Giardiasis is caused by the non-invasive intestinal protozoan parasite *Giardia intestinalis*. The parasite *G. intestinalis* can be found worldwide, but occur more frequently in developing countries. Main symptoms of an infection include abnormal stomach pain, vomiting, and diarrhea. The parasite can be transmitted among domestic animals, wild life and humans, usually via contaminated food or drinks. Mast cells were reported to be recruited during infection with *Giardia*, but what the mast cells do during the infection is still poorly understood. It is well known that mast cells secret or release proteases which protects against pathogenic bacteria, viruses and parasites.

In this Thesis I addressed the potential roles of the mast cell-specific proteases in response to *Giardia*, using both *in vitro* and *in vivo* experimental models. I found that the enzymatic activities of the human and mouse mast cell-specific proteases were affected by soluble *Giardia* proteins (sGPs). For example, the tryptase activity was enhanced while the chymase activity was inhibited when sGPs were added. This suggests that *Giardia* during infection can release proteins with immunomodulatory capacity to change the activity of the mast cells residing in the small intestine. Previous studies have demonstrated that tryptase can activate the protease activated receptor 2 and, that chymase as well as some proteins secreted by *Giardia* can degrade tight junction proteins in the intestine. These actions will influence the permeability of the small intestine, effects that probably account for the mechanism on how *Giardia* causes long-term diarrhea.

When using experimental mouse models, I found that infection with *Giardia* caused increased weight loss in mature adult knockout mice lacking the mouse mast cell protease (mMCP)-4 gene. The mouse mMCP-4 is the functional homologue of the human chymase, and the loss of mMCP-4 resulted in significantly changed regulation of the intestinal expression of cytokines and chemokines. These results indicate that the mast cell chymase definitely is involved in intestinal immunity during infection with *Giardia*.

In addition, I found that young knockout female mice lacking the serglycin proteoglycan gene were more affected by the infection, with significantly reduced weight gain. The lack of the serglycin proteoglycan resulted in sex-specific changes of the intestinal cytokine and chemokines expression. Female mice lacking the serglycin proteoglycan showed lower expression of T helper cell cytokines. These results indicate that both sex-specific and serglycin-dependent intestinal immune responses are mounted during an infection with *Giardia*.

In summary, soluble proteins released by *Giardia* can induce intestinal immune responses, and these immune responses are clearly modulated by the mast cell chymase mMCP-4 as well as by the serglycin proteoglycan. As next steps it would be interesting to identify the soluble *Giardia* proteins that interacts with the mast cell proteases, and to further study the mechanisms on how the chymase mMCP-4 and the serglycin proteoglycan exert their functions in vivo. Furthermore, I would like to explore if other mast cell proteases are affected when facing *Giardia*.

Populärvetenskaplig sammanfattning

Giardia intestinalis är en icke invasiv encellig tarmparasit som orsakar sjukdomen Giardiasis. Parasiten *G. intestinalis* har global spridning men är vanligare och orsakar fler sjukdomsfall i utvecklingsländer. Parasiten kan också orsaka sjukdom i våra husdjur och vilda djur, där vi eller djuren vanligen får i sig parasiten via kontaminerad mat eller dryck. De huvudsakliga symtomen är magkramper, kräkningar och diarré.

Ökad mastcellsaktivitet och ett mastcellberoende skydd mot infektionen har påvisats vid Giardiasis, men vad exakt mastceller gör under infektionen är fortfarande tämligen oklart. Mastceller utsöndrar flera olika specifika proteaser (såsom tryptas och kymas) vilka ger skydd mot sjukdomar orsakade av bakterier, virus, och parasiter. I avhandlingen har jag undersökt de mastcellspecifika proteasernas möjliga roll i immunsvaret mot *G. intestinalis.*

I den första studien upptäckte jag att aktiviteten av proteaserna tryptas och kymas påverkades av utsöndrade *Giardia* proteiner. Aktiviteten av tryptas ökade och aktiviteten av kymas minskade i kontakt med de utsöndrade *Giardia* proteinerna. Detta fynd visar att *G. intestinalis* vid infektion troligen kan modulera det mastcellsdrivna inflammationsvaret i tarmen.

I studie II använde jag knockoutmöss för att undersöka hur avsaknad av kymas påverkade immunsvaret i tarmen vid infektion med *G. intestinalis*. Jag undersökte framförallt immunsvaret mot *G. intestinalis* i gamla möss då detta inte undersökts förut. I möss som saknade kymaset ”mouse mast cell protease” (mMCP)-4 fann jag en signifikant förändrad cytokinprofil i tarmen, där uttrycket av många undersökta cytokiner var reducerat i jämförelse med de möss som hade kymaset kvar i tarmen. Detta visar att kymas i gamla möss bidrar till immunsvaret i tarmen vid Giardiasis.

I studie III använde jag knockoutmöss för att undersöka hur avsaknad av serglycin påverkade immunsvaret i tarmen vid infektion med *G. intestinalis*. Proteoglykanen serglycin är viktig i flera immuncelltyper för inlagring i granula av flera olika positivt laddade proteaser. Avsaknad av

serglycin ger därför upphov till starkt reducerade mängder av många av dessa proteaser i immuncellerna. I unga honmöss som saknade serglycin fann jag en starkt reducerad viktminskning och en i huvudsak nedreglerad cytokinprofil i tarmen, jämfört med de honmöss som hade serglycin kvar i tarmen. Vidare visade hanmöss som saknar serglycin ett ökat uttryck av de flesta av de undersökta cytokinerna. Denna studie visar att både serglycin och kön kan påverka immunsvaret i tarmen under Giardiasis.

Sammanfattningsvis så verkar utsöndrade *Giardia* proteiner inducera och reglera immunsvaret i tarmen. Dessutom påverkar kymas och serglycin immunsvaret. I mina nästa steg skulle jag vilja identifiera de utsöndrade *Giardia* proteinerna som påverkar mastcellsproteaserna, och mer i detalj undersöka hur kymas och serglycin utför sina funktioner *in vivo.* Det skulle också vara intressant att undersöka de andra mastcells-specifika proteaserna i svaret mot *Giardia.*

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

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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 65 kDa sGP and, wild-type mouse ear tissue extracts degraded several protein bands in the 10 to 75 kDa 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.

1. 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 (Redegeld et al., 2018). 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 (Pejler et al., 2010). 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 (Pejler et al., 2007). 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

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chronically *Trichinella spiralis* infected skeletal muscle tissue (Shin et al., 2008). 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 (Reitz et al., 2017).

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 (Luján and Svärd, 2011). *G. intestinalis* parasite antigens, either on the surface or excreted-secreted products, have been shown to be immunogenic (Kaur et al., 1999). *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 (Jimenez et al., 2014). 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 (Lee et al., 2012). Several other chemokines (*e.g.* CXCL 1–3, CCL2 and 20) are up-regulated by ESPs in differentiated Caco-2 cells (Ma'ayeh et al., 2017). In addition, antibody responses to several *Giardia* glycoproteins have been identified in the serum of immunized BALB/c mice (Jimenez et al., 2007). Furthermore, glycoproteins with immunomodulatory capacity and proteolytic activity have been identified in the excretory-secretory proteins from *Giardia* trophozoites (Ma'ayeh et al., 2017; Ward et al., 1988; Ortega-Barria et al., 1990; Hiltpold et al., 2000; de Carvalho et al., 2008; Cabrera-Licona et al., 2017; Liu et al., 2018; Dubourg et al., 2018). During infections with *Giardia* ssp. mast cells are recruited to the intestine (Li et al., 2016) 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 (Li et al., 2004; Munoz-Cruz et al., 2010). In addition, mast cell specific proteases were among the most obviously induced transcripts in the small intestinal tissue at 13 days post infection (Tako et al., 2013). Mast cells are also recruited to the small intestinal mucosa during *Giardia* infections in humans (Nicolov et al., 1983), gerbils (Leitch et al., 1993; Hardin et al., 1997) and rats (Halliez et al., 2016). Stimulation of the rat mast cell line HRMC with soluble *Giardia* protein extracts (sGPs) which overlap extensively in protein content with *Giardia* ESPs (Ma'ayeh et al., 2017; Dubourg et al., 2018), triggered mast cell activation and up-regulation of tryptase (Munoz-Cruz et al., 2010; Munoz-Cruz et al., 2018). 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.

2. Material and methods

2.1. 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 (Shin et al., 2008) 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*.

2.2. 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, 5 ng/ml mouse interleukin (IL) -9 (ImmunoTools), 1 ng/ml recombinant human transforming growth factor beta (TGF-beta, ImmunoTools), 1 ng/ml mouse IL-3 (ImmunoTools) and 50 ng/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 2×10^6 BM-MMCs/ml in HBSS and challenged with different concentrations (25 ng/ml, 100 ng/ml and 1 μg/ml) of soluble protein extracts from three different *Giardia* isolates (GS, WB and H3). After 6 h or 24 h incubation (at 37 °C, 5% CO_2), supernatants were collected and frozen at −20 °C until used.

2.3. 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 (Keister, 1983). Trophozoites were collected after three washing steps with cold, sterile phosphate-buffered saline (PBS) by pelleting with centrifugation at 931 × *g* at 4 °C for 10 min. The pellet was re-suspended in PBS, followed by sonication (3 times for 30 s at 50 W) and centrifuged at 14462 × *g* at 4 °C for 15 min to remove cell debris. The supernatants containing approximately 5 μg/μl of sGPs were kept at −80 °C until used.

Giardia excretory-secretory proteins (ESPs) were obtained from *G. intestinalis* as described (Ma'ayeh et al., 2017). In brief, WB and GS trophozoites cultured for 48 h at 37 °C 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, 2 mM 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 6 h at 37 °C. Trophozoite viability was assessed at 90% and culture supernatants were harvested by centrifugation at 930 × *g* for 10 min at 4 °C, filtered through Amicon® Ultra 15 ml centrifugal filters with 3 kDa cut-off (Merck-Millipore, Darmstadt, Germany), concentrated down to 200–300 μl with a final concentration of approximately 1 μg/μl of ESPs, and stored at −80 °C until used.

2.4. 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.

2.5. β-hexosaminidase measurement

For the β-hexosaminidase assay, 20 μl of cell culture supernatants were incubated with 80 μl of 1 mM substrate (p-nitrophenyl N-acetyl-beta-D-glucosamine, #487052, Merck KGaA, Germany) dissolved in

citrate buffer (0.05 M citric acid and trisodium citrate, pH 4.5) for 1 h at 37 °C, followed by addition of 200 µl 0.05 M sodium carbonate reaction buffer (Na_2CO_3 and $NaHCO_3$, pH 10.0). Absorbance was measured at 405 nm.

2.6. Cell viability

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

2.7. Purification of mouse proteolytic ear tissue protein extracts

The purification method was as described before (Roy et al., 2014). 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 3000 rpm for 10 min. The fragmented pelleted ear tissues were then extracted with a high salt lysis buffer (PBS/2 M NaCl/1% Triton X-100). After shaking for 30 min at room temperature and centrifugation at 3000 rpm for 10 min, high salt supernatants containing enriched mast cell protease activities were collected and kept at −20 °C until used.

2.8. Degradation assay of GS, WB, and H3 soluble Giardia proteins

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

2.9. SDS-PAGE electrophoresis and colloidal Coomassie blue staining

The 3 h 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 h, 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).

2.10. 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 (McEuen et al., 1995; Ren et al., 1998; Addington and Johnson, 1996). 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, 5 µg ESPs from the GS and WB isolates or approximately 20, 10, 5 and 1 µg of WB, GS and H3 sGPs (5 µg/µl) were mixed with 0.1 µg of recombinant human tryptase (rHT) or approximately 15 µg of crude high salt ear tissue extracts from mMCP-$6^{+/+}$ and mMCP-$6^{-/-}$ mice (tryptase-deficient mice) and 20 µl of the tryptase substrate S-2288 (H-D-Ile-Pro-Arg-pNA, Chromogenic, Sweden) at a final concentration of 1 mM, and PBS to give the total 120 µl 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, 5 µg ESPs of GS and WB or approximately 10 µg of WB, GS and H3 sGPs were mixed with 0.05 µg of recombinant human chymase (rCh) or approximately 5 µg of crude high salt ear tissue extracts from mMCP-$6^{+/+}$ mice and 20 µl of the chymase substrate L-1595 (Suc-Ala-His-Pro-Phe-pNA, Bachem, Switzerland) at a final concentration of 1 mM, and ddH_2O to give the total 120 µl 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.

2.11. 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.

3. Results

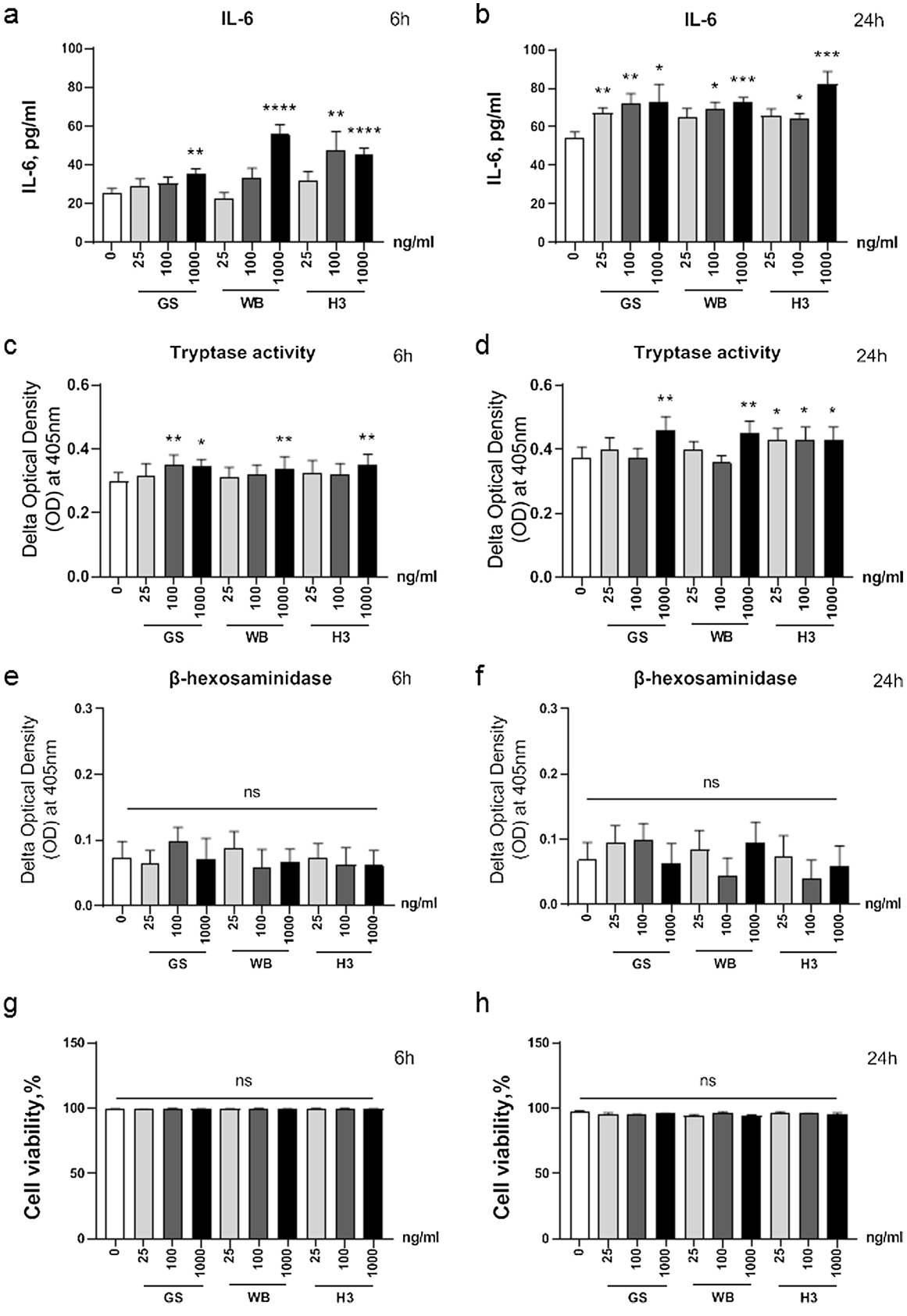
3.1. Soluble Giardia proteins (sGPs) only cause minor mast cell activation

Mast cells and mast cell proteases are important for control of infection with *Giardia* spp. (Li et al., 2016; Li et al., 2004), and mast cells may degranulate in response to soluble proteins from protozoan parasites (Bidri et al., 1997). 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 6 h and at 24 h (Fig. 1a, b), and a small but significantly increased tryptase activity with 1 µg 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.

3.2. 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 h (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 24 h (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 10–75 kDa range, *e.g.* a ≈45kDa protein was significantly reduced (Fig. 2a, 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



(caption on next page)

Fig. 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 1×10^6 BM-MMCs seeded in 0.5 ml HBSS were challenged with three concentrations, 25 ng/ml, 100 ng/ml and 1000 ng/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 6 h (a, c, e) and 24 h (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 6 h (g) and 24 h (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 < 0.001, ****, P < 0.0001 versus un-challenged control.

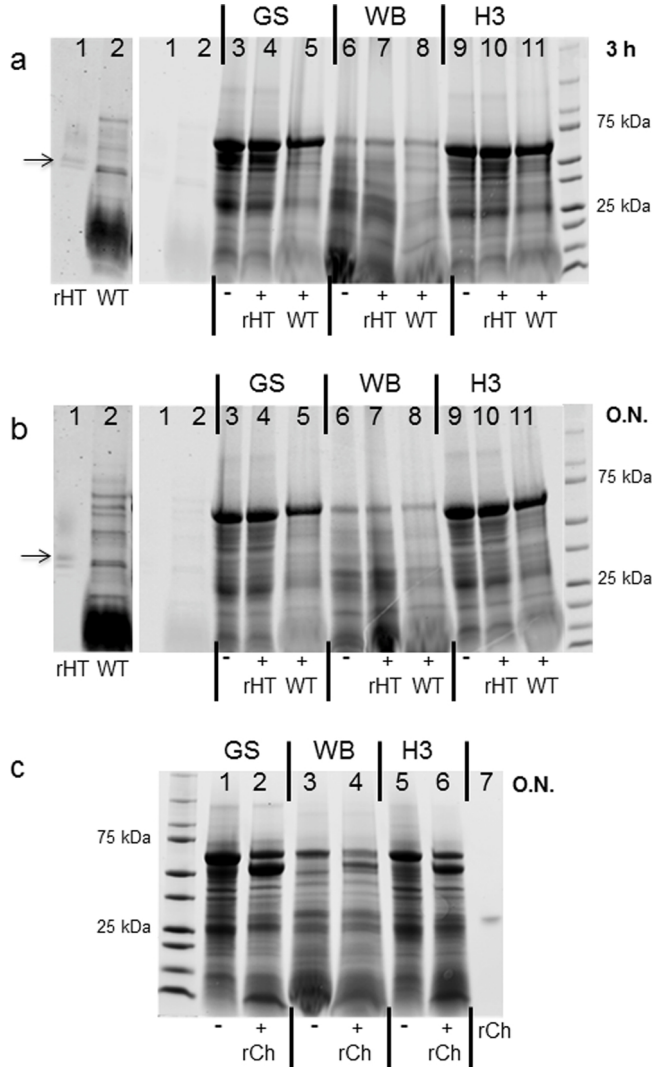


Fig. 2. Soluble *Giardia* proteins are not extensively degraded by human tryptase or chymase, or by wild type mouse ear tissue proteolytic extracts. To determine if mast cell proteases can degrade *Giardia* proteins, 20 µg of soluble *Giardia* proteins (sGPs) were incubated with 0.2 µg recombinant human tryptase (rHT, arrow) or with 5 µg of proteolytic mouse ear tissue extracts (WT, pooled ear tissue extracts, n = 10) for (a) 3 h 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) 20 µg of soluble *Giardia* proteins of the GS, WB and H3 isolates were incubated without or with 0.4 µg 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).

for these mast cell proteases to cut. However, the top 10 secreted peptides from WB and GS trophozoites (Ma'ayeh et al., 2017) 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.

3.3. 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 > 5 µg of sGPs (Fig. 3a). Addition of 20 µg GS, WB and H3 sGPs resulted in a significant increase of rHT activity over 60 min, where the sGPs-effect on the S-2288 substrate conversion was evident after 15 min, and the substrate conversion rate significantly was increased up to at least 50 min 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 (Ma'ayeh et al., 2017) 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 min and the activity rate were still significantly higher after 60 min (Fig. 3e).

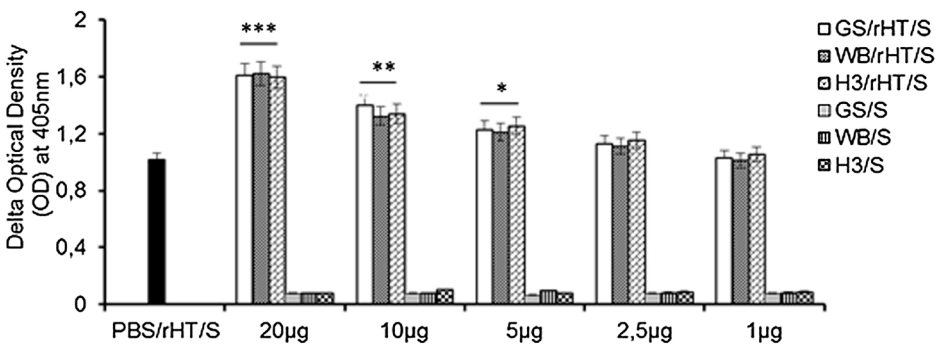
3.4. Soluble Giardia proteins (sGPs) enhance mouse mast cell tryptase activity

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).

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

Finally we investigated if sGPs and ESPs could modulate the mast cell chymase activity. Interestingly, and in contrast to the enhancing

a



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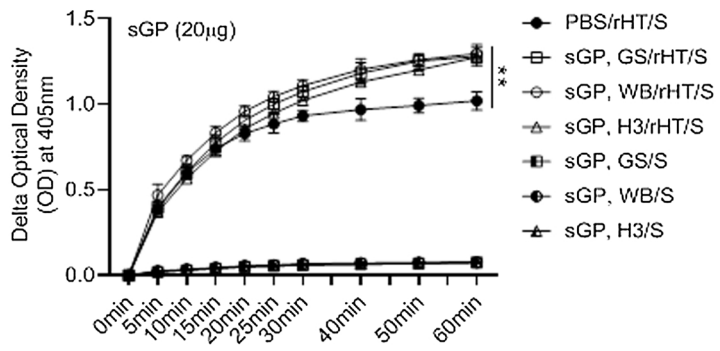
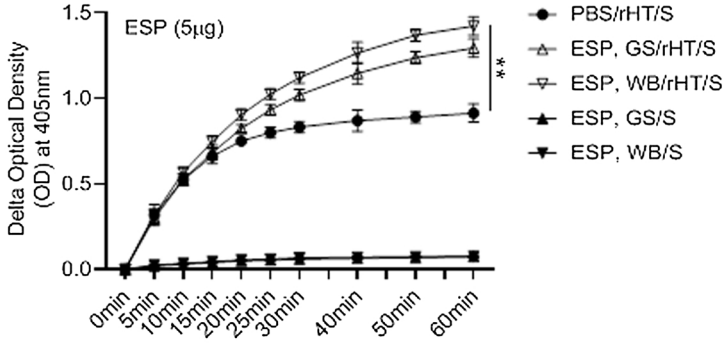


c

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*

Fig. 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 5 µg of GS and WB excretory-secretory protein (ESP) (c, d) were incubated with or without 0.02 µg of rHT. The change in optical density (OD) was measured at 405 nm 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 min and maintained a significantly increased rate also at 60 min. 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: *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

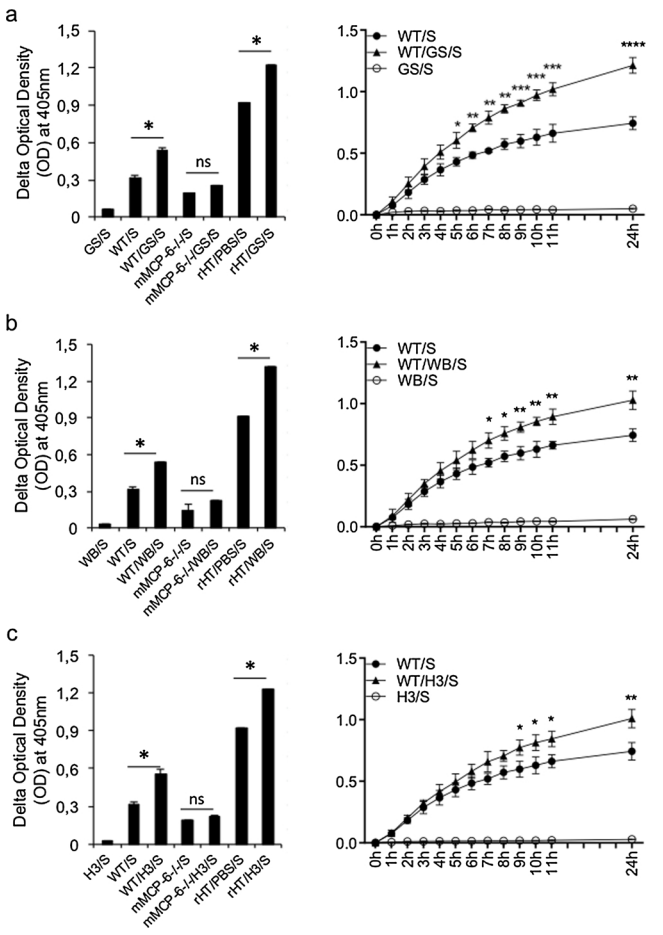


Fig. 4. Soluble *Giardia* proteins enhance mouse mast cell tryptase activity. sGPs were incubated with or without 002 µg of rHT or with 15 µg of ear tissue extracts from WT mice (bulk ear tissue extracts, n = 10) and mMCP6$^{-/-}$ mice (bulk ear tissue extracts, n = 10) at room temperature, respectively. (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 10 µg sGPs from the GS, WB and H3 isolates. rHT was included as a positive control. Change in optical density (OD) at 405 nm was measured at time point 0 h and 4 h 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 5 µg of sGPs from the GS, WB and H3 isolates. Change in optical density (OD) at 405 nm was measured every hour up to 11 h and after 24 h. 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 *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$.

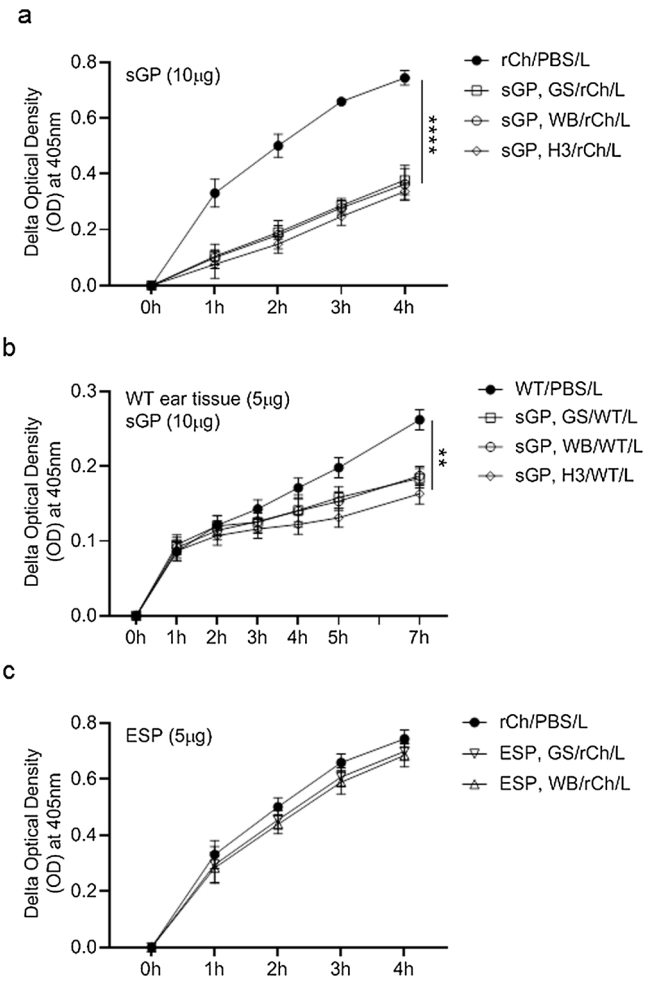


Fig. 5. Soluble *Giardia* proteins inhibit human and mouse chymase activity. (a) The activity of 0.05 µg of recombinant human chymase (rCh) incubated with or without 10 µg of GS, WB and H3 sGPs. (b) The chymase activity in 5µg wild-type mouse ear tissue (WT, pooled ear tissue extracts, n = 10) with or without 10 µg of GS, WB and H3 sGPs. (c) The activity of 0.05 µg rCh incubated with or without 5µg of GS and WB ESPs. The change in optical density (OD) at 405 nm 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. **, $P < 0.01$, ****, $P < 0.0001$.

effect on tryptase, addition of 10 µg of GS, WB and H3 sGPs significantly inhibited the rCh activity (Fig. 5a) and resulted in inhibition of chymase activity also in the WT mouse ear tissue extracts (Fig. 5b). Note that sGPs have no intrinsic activity for the L-1595 chymase substrate (not shown). To evaluate if the observed reduced activity of rCh is due to *Giardia* proteins found in the ESP fraction we finally used ESPs from the *Giardia* isolates GS and WB. However, the addition of ESPs did not significantly affect the rCh activity (Fig. 5c).

4. 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 (Li et al., 2016; Li et al., 2004; von Allmen et al., 2006). 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* (Tako et al., 2013).

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 (Braga et al., 2007) to investigate the potential activation of mast cells after challenge with *G. intestinalis* sGPs. Challenge with *Giardia* sGPs caused IL-6 secretion after 6 h 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 24 h after challenge (Munoz-Cruz et al., 2010; Munoz-Cruz et al., 2018). In contrast to our results, these studies suggested that the level of tryptase only increased beneath the mast cell cell-membrane in response to *Giardia* challenge (Munoz-Cruz et al., 2010; Munoz-Cruz et al., 2018).

Several studies have demonstrated that *Giardia* soluble proteins (sGPs) contain cytosolic, cytoskeletal, surface and excretory-secretory proteins (ESPs), and that the secretion of ESPs will be induced upon contact with epithelial cells in the small intestine (Ma'ayeh et al., 2017; Dubourg et al., 2018; Jimenez et al., 2009; Ringqvist et al., 2008), and that some of the ESPs may penetrate into the host tissue (Ma'ayeh et al., 2017; Liu et al., 2018). Previous studies suggested an extensive over-lap in protein content between the two *in vitro* prepared fractions. One difference, ESPs contained several surface proteins that were not found in the sGPs fraction (Ma'ayeh et al., 2017; Dubourg et al., 2018; Emery et al., 2016). In line with this extensive over-lap we see similar effects of ESP and sGPs on tryptase activity (Figs. 3 and 4). For chymase the inhibitory effect of sGPs was not evident with the ESPs (Fig. 5). The observed difference could depend on several things, for example that ESPs may lack the chymase interfering protein(s). Alternatively, *Giardia* expresses several cysteine proteases/cathepsin-like proteases (CPs) in the ESPs that potentially could degrade chymase. However, these CPs are subjected to rapid auto-degradation (Liu et al., 2018). So, lack of chymase-inhibition with ESPs could depend on the rapid auto-inactivation of the CPs.

Giardia has been found to secrete several different proteins (ESPs) at low levels in axenic culture and, in the interaction with human intestinal epithelial cells (IECs) the ESPs have the capacity to modulate the host innate immune response, *e.g.* it was found that ESPs could stimulate a preferential Th2 response (Jimenez et al., 2004), degrade chemokines and induce the anti-inflammatory protein tristetraprolin (TTP) (Ma'ayeh et al., 2017). The ESP fraction from the WB isolate contains around 200 different proteins (Ma'ayeh et al., 2017) and most of these proteins are also found in the soluble *Giardia* protein fractions (Dubourg et al., 2018). It is difficult to generate high levels of ESPs from *Giardia* due to the low level of secretion (Ma'ayeh et al., 2017), 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 (Liu et al., 2018), *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 (Fernandez-Blanco et al., 2015), thereby resulting in a leaky gut that can induce diarrhea and post-infectious symptoms (Fink and Singer, 2017; Allain et al., 2019; Halliez and Buret, 2013). Several post-infectious symptoms can be induced after *Giardia* infections; irritable bowel syndrome (IBS), chronic fatigue syndrome, skin and food allergies and reactive arthritis (Litleskare et al., 2019; Litleskare et al., 2018; Painter et al., 2017; Di Prisco et al., 1998; Chirila et al., 1981; Mahmoud et al., 2004). 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 (Jimenez et al., 2014; Jimenez et al., 2009; Matowicka-Karna et al., 2009). 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 (Roy et al., 2014). 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 (Roy et al., 2014). 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 (Andersson et al., 2008; Andersson et al., 2009; Andersson et al., 2010). However, physiologic targets for degradation by chymase remain elusive but some virulence factors and alarmins seems to be major targets (Roy et al., 2014; Fu et al., 2017). In addition, activation of matrix metalloprotease (MMP)-9 and angiotensin seems to require chymase (Tchougounova et al., 2005). 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 (Choi et al., 2016). Furthermore, a detrimental action of the chymase is in the course of glomerulonephritis where mast cells are distantly found in the kidney capsule (Scandiuzzi et al., 2010). In bronchial asthma, mast cell chymase impairs bronchial epithelium integrity through degradation of cell junction molecules, *i.e.* occludin, claudin-4, ZO-1 and E-cadherin in epithelial cells (Zhou et al., 2018). 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 (Groschwitz et al., 2013).

Tryptase is the predominant serine proteinase of mast cells and heparin stabilizes the activity of the functional monomeric tetramer (Pereira et al., 1998), 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 (Molino et al., 1997). 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 (Ma'ayeh et al., 2017) 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 (Fu et al., 2017). 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.

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