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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. Furthermore, sGPs were highly resistant to degradation by human tryptase while human chymase degraded a 65kDa sGP and, wild-type mouse ear tissue extracts degraded several protein bands in the 10 to 75kDa range. In striking contrast, sGPs and ESPs were found to increase the enzymatic activity of human and mouse tryptase and to reduce the activity of human and mouse chymase. Conclusion: Our finding suggests that *Giardia* ssp. via enhancement or reduction of mast cell protease activity may modulate mast cell-driven intestinal immune responses. ESP-mediated modulation of the mast cell specific proteases may also increase degradation of tight junctions, which may be beneficial for *Giardia* ssp. during infection.

Keywords	mast cell; tryptase; chymase; soluble <i>Giardia</i> proteins (sGPs); <i>Giardia</i> excretory-secretory proteins (ESPs); intestinal parasite
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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 ***Giardia* excretory-secretory proteins modulate the enzymatic activities of mast cell**
2 **chymase and tryptase**

3

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13 **Abstract**

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32 protease activity may modulate mast cell-driven intestinal immune responses. ESP-mediated
33 modulation of the mast cell specific proteases may also increase degradation of tight junctions,
34 which may be beneficial for *Giardia ssp.* during infection.

35

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

38 **Introduction**

39 Mast cell activation and degranulation to microbial and parasitic infections may occur by
40 several mechanisms, *i.e.* via PAMPs and alarmins (DAMPs) acting over pattern recognition
41 receptors, or via complement- and antibody-dependent mechanisms [1]. When mast cells
42 degranulate large quantities of preformed mediators are released, *e.g.* the mast cell specific
43 proteases. Of the mast cell specific proteases tryptases and chymases may constitute up to as
44 much as 35-50% of the mast cell protein content [2]. In the mouse mast cells predominantly
45 express four different chymases: in mucosal tissue mast cells express the mouse mast cell
46 protease (mMCP)-1 (*Mcpt1*) with chymotrypsin-like activity and mMCP-2 (*Mcpt2*) with as
47 yet no identified enzymatic activity and; in connective tissue mast cells express the mMCP-4
48 (*Mcpt4*) with chymotrypsin-like activity and mMCP-5 (*Mcpt5*) with elastase-like activity. In
49 contrast, human mast cells express only one chymase gene and the closest functional ortholog
50 in mice is the chymase mMCP-4. Mouse and human mast cells express three tryptase genes,
51 *i.e.* the mouse mast cell proteases (mMCP)-6 *Mcpt6* which is closely related to human
52 TPSAB1/A1 and, mMCP-7 (*Tpsab1*) closely related to human TPSD1 as well as mTMT
53 (*Tpsg1*) closely related to the human TPSG1 gene [3]. The C57BL/6 mouse strain carries a
54 disrupted mMCP-7 gene due to a splicing defect and a gene knockout of mMCP-6 has been
55 introduced on the C57BL/6 background. The lack of both mMCP-6 and mMCP-7 caused no
56 major problems for unchallenged mice suggesting that the mast cell specific tryptases are not
57 essential for survival. However, challenged mMCP-6-deficient mice displayed significant
58 inability to recruit eosinophils in chronically *Trichinella spiralis* infected skeletal muscle
59 tissue [4]. In another recent study, it was suggested that the termination of the helminth
60 *Strongyloides ratti* infection required the presence of mucosal mast cells, because infection
61 lasted longer in CPA3^{Cre} mice which lack mucosal and connective tissue mast cells than in
62 *Mcpt5*^{Cre} R-DTA mice which only lack connective tissue mast cells [5].

63
64 *Giardia intestinalis* is a relatively prevalent non-invasive intestinal protozoan parasite with
65 zoonotic potential found worldwide that can cause diarrhea and growth stunting in humans
66 and animals [6]. *G. intestinalis* parasite antigens, either on the surface or excreted-secreted
67 products, have been shown to be immunogenic [7]. *Giardia*-infection and *Giardia* excretory-
68 secretory proteins (ESPs) induced IL-4, IL-5 and IL-10 cytokine responses as well as IgG and
69 IgE antibody responses in BALB/c mice [8]. ESPs induced IL-8 production in a human
70 gastrointestinal cell line (HT-29) via activation of p38, ERK1/2, nuclear factor kappaB and
71 activator protein 1 [9]. Several other chemokines (*e.g.* CXCL 1-3, CCL2 and 20) are up-
72 regulated by ESPs in differentiated Caco-2 cells [10]. In addition, antibody responses to
73 several *Giardia* glycoproteins have been identified in the serum of immunized BALB/c mice
74 [11]. Furthermore, glycoproteins with immunomodulatory capacity and proteolytic activity
75 have been identified in the excretory-secretory proteins from *Giardia* trophozoites [10, 12-18].
76 During infections with *Giardia ssp.* mast cells are recruited to the intestine [19] and compared
77 to wild-type mice c-kit-dependent mast cell-deficient mice (c-kit^{w/wv}) and anti-c-kit mast cell
78 depleted mice showed increased parasite burden and failure to produce parasite-specific IgA
79 antibodies [20, 21]. In addition, mast cell specific proteases were among the most obviously
80 induced transcripts in the small intestinal tissue at 13 days post infection [22]. Mast cells are
81 also recruited to the small intestinal mucosa during *Giardia* infections in humans [23], gerbils
82 [24, 25] and rats [26]. Stimulation of the rat mast cell line HRMC with soluble *Giardia*
83 protein extracts (sGPs) which overlap extensively in protein content with *Giardia* ESPs [10,
84 18], triggered mast cell activation and up-regulation of tryptase [21, 27]. Collectively, these
85 results suggest that mast cells and the mast cell specific proteases may play a significant role
86 in the host immune responses against *G. intestinalis*. However, if *Giardia* via secretion of
87 ESPs and other soluble proteins directly interacts with the mast cell specific proteases remain

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

92 **Material and methods**

93 *Ethics and Mice*

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

103

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

105 To obtain bone marrow derived mucosal-like mast cells (BM-MMCs), bone marrow cells was
106 collected from femur and tibia. The cells were washed two times in PBS and cultured in
107 complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 1%
108 PEST, 2 mM L-glutamine, 5ng/ml mouse interleukin (IL) -9 (ImmunoTools), 1ng/ml
109 recombinant human transforming growth factor beta (TGF-beta, ImmunoTools), 1ng/ml
110 mouse IL-3 (ImmunoTools) and 50ng/ml mouse stem cell factor (SCF, ImmunoTools). After
111 two weeks >99% of the cells showed BM-MMCs characteristics as verified by May-
112 Grünwald/Giemsa staining. The BM-MMCs were washed three times in PBS and seeded in
113 duplicates (first experiment) or triplicates (second experiment) at 2x10⁶ BM-MMCs/ml in
114 HBSS and challenged with different concentrations (25 ng/ml, 100 ng/ml and 1µg/ml) of
115 soluble protein extracts from three different *Giardia* isolates (GS, WB and H3). After 6h or
116 24h incubation (at 37°C, 5% CO₂), supernatants were collected and frozen at -20°C until used.

117

118 ***Preparations of soluble Giardia proteins and Giardia excretory-secretory proteins***

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

129

130 *Giardia* excretory-secretory proteins (ESPs) were obtained from *G. intestinalis* as described
131 [10]. In brief, WB and GS trophozoites cultured for 48 h at 37°C in TYDK medium were
132 rinsed three times with warm and serum-free RPMI-1640 medium (Sigma, St. Louis, MO,
133 USA) to eliminate non-attached or dead trophozoites. Thereafter, adherent trophozoites were
134 incubated with RPMI-1640 medium supplemented with 11.4 mM L-cysteine hydrochloride
135 monohydrate, 55.5 mM glucose, 11.4 mM ascorbic acid, 1 mM sodium pyruvate (Gibco),
136 22.8 mM L-arginine, 2mM Glutamax (Gibco) and 1x MEM essential amino acids. The final
137 pH of the supplemented media was set at 6.8 and the trophozoites were incubated for 6h at
138 37°C. Trophozoite viability was assessed at 90% and culture supernatants were harvested by
139 centrifugation at 930xg for 10 min at 4°C, filtered through Amicon® Ultra 15 mL centrifugal
140 filters with 3kDa cut-off (Merck-Millipore, Darmstadt, Germany), concentrated down to 200-

141 300µl with a final concentration of approximately 1µg/µl of ESPs, and stored at -80°C until
142 used.

143

144 ***ELISA assay for IL-6 detection***

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

148

149 ***β-hexosaminidase measurement***

150 For the β-hexosaminidase assay, 20µl of cell culture supernatants were incubated with 80µl of
151 1mM substrate (p-nitrophenyl N-acetyl-beta-D-glucosamine, #487052, Merck KGaA,
152 Germany) dissolved in citrate buffer (0.05M citric acid and trisodium citrate, pH 4.5) for 1
153 hour at 37°C, followed by addition of 200µl 0.05M sodium carbonate reaction buffer (Na₂CO₃
154 and NaHCO₃, pH 10.0). Absorbance was measured at 405nm.

155

156 ***Cell viability***

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

159

160 ***Purification of mouse proteolytic ear tissue protein extracts***

161 The purification method was as described before [29]. Briefly, ear tissues from mMCP-6^{+/+}
162 (n=10 per preparation) and mMCP-6^{-/-} mice (n=10 per preparation) were frozen in liquid
163 nitrogen and crushed into a tissue powder with a mortar and pestle and then transferred to a
164 15 ml tube. To enrich for mast cell protease activities, the ear tissue powder was first
165 extracted with a low salt lysis buffer (PBS/1% Triton X-100) to remove the fraction of ear

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

173

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

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

182

183 ***SDS-PAGE electrophoresis and colloidal Coomassie blue staining***

184 The 3h and overnight samples containing the 20 µg sGPs incubated with or without
185 proteolytic activity was loaded on SDS-PAGE gels and the gels were run according to
186 standard procedures. The gels were stained in Coomassie solution (0.1% Coomassie brilliant
187 blue R-250/50% methanol/ 10% Acetic acid) for at least 4 hours, and de-stained in 10% acetic
188 acid/40% EtOH/50% dH₂O for 30 min with several changes of the de-staining solution.
189 Photos of the de-stained gels were taken with the Odyssey CLx imaging system (Germany).

190

191 ***Soluble Giardia proteins and mast cell protease activity***

192 Early work showed that the activity of the chymase and tryptase could be affected by salt
193 concentrations (NaCl) as well as pH [30-32]. Therefore, in all our experiments the NaCl
194 concentrations were kept in the physiological range of 0.15 to 0.20 M NaCl and the pH was
195 kept at ≈ 7.4 .

196

197 To address if *Giardia* via sGPs or ESPs could block or modulate the tryptase activity, 5 μ g
198 ESPs from the GS and WB isolates or approximately 20, 10, 5 and 1 μ g of WB, GS and H3
199 sGPs (5 μ g/ μ l) were mixed with 0.1 μ g of recombinant human tryptase (rHT) or approximately
200 15 μ g of crude high salt ear tissue extracts from mMCP-6^{+/+} and mMCP-6^{-/-} mice (tryptase-
201 deficient mice) and 20 μ l of the tryptase substrate S-2288 (H-D-Ile-Pro-Arg-pNA,
202 Chromogenic, Sweden) at a final concentration of 1mM, and PBS to give the total 120 μ l
203 reaction volume. Enzymatic activity of tryptase was measured as hydrolysis of S-2288 and
204 monitored spectrophotometrically at 405 nm in a microplate reader. The change in optical
205 density over time (delta OD) and the substrate conversion rate (delta OD per minute) was
206 calculated.

207

208 To determine if *Giardia* via sGPs or ESPs affected the chymase activity, 5 μ g ESPs of GS and
209 WB or approximately 10 μ g of WB, GS and H3 sGPs were mixed with 0.05 μ g of
210 recombinant human chymase (rCh) or approximately 5 μ g of crude high salt ear tissue extracts
211 from mMCP-6^{+/+} mice and 20 μ l of the chymase substrate L-1595 (Suc-Ala-His-Pro-Phe-pNA,
212 Bachem, Switzerland) at a final concentration of 1mM, and ddH₂O to give the total 120 μ l
213 reaction volume. Enzymatic activity of chymase was measured as hydrolysis of L-1595 and
214 monitored spectrophotometrically at 405 nm in a microplate reader. The change in optical
215 density (OD) over time (deltaOD) was calculated.

216

217 *Statistical analysis*

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

220 **Results**

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

222 Mast cells and mast cell proteases are important for control of infection with *Giardia spp.* [19,
223 20], and mast cells may degranulate in response to soluble proteins from protozoan parasites
224 [33]. To address a direct interaction between mouse mast cells and *G. intestinalis*, bone
225 marrow derived mucosal-like mast cells (BM-MMCs) were challenged with increasing
226 concentrations of soluble proteins (sGPs) from *G. intestinalis* GS-, WB-, and H3- isolates. As
227 a read out of mast cell activation and mast cell degranulation the levels of IL-6, and tryptase
228 and beta-hexosaminidase activity were determined in the BM-MMC supernatants. Challenge
229 with the three *Giardia*-isolates induced a significant increase in IL-6 release at 6h and at 24h
230 (Fig 1a, b), and a small but significantly increased tryptase activity with 1 microgram of sGPs
231 (Fig. 1c, d). The challenge with sGPs did not induce degranulation of the BM-MMC (Fig 1e, f
232 and data not shown) and the cell viability remained equally high in control cells and sGP-
233 challenged cells (Fig 1g, h). Together our data suggests that sGPs can induce mast cell
234 activation and that the challenge does not induce mast cell degranulation but may induce
235 piecemeal secretion of tryptase.

236

237 ***Soluble Giardia proteins (sGPs) are not extensively degraded by human or mouse mast cell*** 238 ***proteases.***

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

245 to degrade most of the sGPs after 24h (Fig. 2c, lanes 2, 4, 6), suggesting that sGPs are poor
246 target substrates for the mast cell specific tryptase and chymase. In contrast, the high salt ear
247 tissue proteolytic extracts showed a diffuse degradation activity of the sGPs in the 10 to
248 75kDa range, *e.g.* a \approx 45kDa protein was significantly reduced (Fig. 2 a, b, lanes 5, 8, 11),
249 suggesting that the mixture of proteolytic enzymes in the ear tissue extracts can degrade sGPs.
250 Note also that the sGPs did not carry any major intrinsic degradation activity.

251

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

258

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

261 Next we investigated if sGPs and *Giardia* excretory-secretory proteins (ESPs) could have a
262 modulatory effect on the mast cell protease activities. Surprisingly, we observed an enhancing
263 effect on the tryptase activity (Fig. 3) and the enhancing effect on rHT activity was dose
264 dependent and required the addition of $> 5\mu\text{g}$ of sGPs (Fig. 3a). Addition of $20\mu\text{g}$ GS, WB
265 and H3 sGPs resulted in a significant increase of rHT activity over 60 minutes, where the
266 sGPs-effect on the S-2288 substrate conversion was evident after 15 minutes, and the
267 substrate conversion rate significantly was increased up to at least 50 minutes after addition of
268 sGPs (Fig. 3b, c). Note that the GS, WB and H3 sGPs showed no intrinsic activity for the S-
269 2288 substrate. To evaluate if the observed increased activity of rHT is due to proteins found

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

277

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

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

293

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

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

304 **Discussion**

305 Previous studies have suggested that mast cells play an important role during infection with
306 *Giardia*. Mast cell-deficient and mast cell-depleted mice showed clearance failure of the GS
307 isolate (assemblage B) and recruited mast cell numbers increased in the intestinal villi and
308 crypt of mice infected with the GS isolate [19, 20, 34]. Furthermore, the mast cell specific
309 proteases CPA3 as well as *Mcpt1*, *Mcpt2* and CMA2 were reported to be up-regulated during
310 infection with *Giardia*, suggesting that both connective and mucosal tissue type mast cells
311 have increased activity in the intestinal tissue in response to *Giardia* [22].

312

313 However, it still remains unknown if the *Giardia* WB (assemblage A) or H3 (assemblage B)
314 isolates cause mast cell activation *in vivo* and, the potential direct interactions of *Giardia* with
315 the mast cell specific proteases have previously not been explored. First, to study potential
316 interactions between *Giardia* and mast cells we cultured mouse bone marrow derived
317 mucosal-like mast cells (BM-MMCs) expressing both connective and mucosal mast cell
318 specific proteases [35] to investigate the potential activation of mast cells after challenge with
319 *G. intestinalis* sGPs. Challenge with *Giardia* sGPs caused IL-6 secretion after 6h suggesting
320 that our cultured BM-MMCs do respond to *Giardia* sGPs. In addition, the low levels of
321 tryptase activity in BM-MMCs supernatants suggested that *Giardia* does not cause strong
322 mast cell degranulation, but instead may cause increased piecemeal secretion of tryptase as
323 augmented tryptase activity was only evident after challenge with sGPs. The observation that
324 *Giardia* sGPs did not cause degranulation of the mast cells, *i.e.* as indicated with very low
325 tryptase activity, was further supported by a similarly low beta-hexosaminidase activity in the
326 supernatants of un-challenged and challenged BM-MMCs. Previous studies using a rat
327 hybridoma mast cell line (HRMC) with a mucosal phenotype showed that IL-6 was secreted
328 24h after challenge [21, 27]. In contrast to our results, these studies suggested that the level of

329 tryptase only increased beneath the mast cell cell-membrane in response to *Giardia* challenge
330 [21, 27].

331

332 Several studies have demonstrated that *Giardia* soluble proteins (sGPs) contain cytosolic,
333 cytoskeletal, surface and excretory-secretory proteins (ESPs), and that the secretion of ESPs
334 will be induced upon contact with epithelial cells in the small intestine [10, 18, 36, 37], and
335 that some of the ESPs may penetrate into the host tissue [10, 17]. Previous studies suggested
336 an extensive over-lap in protein content between the two *in vitro* prepared fractions. One
337 difference, ESPs contained several surface proteins that were not found in the sGPs fraction
338 [10, 18, 38]. In line with this extensive over-lap we see similar effects of ESP and sGPs on
339 tryptase activity (Figure 3 and 4). For chymase the inhibitory effect of sGPs was not evident
340 with the ESPs (Figure 5). The observed difference could depend on several things, for
341 example that ESPs may lack the chymase interfering protein(s). Alternatively, *Giardia*
342 expresses several cysteine proteases/cathepsin-like proteases (CPs) in the ESPs that
343 potentially could degrade chymase. However, these CPs are subjected to rapid auto-
344 degradation [17]. So, lack of chymase-inhibition with ESPs could depend on the rapid auto-
345 inactivation of the CPs.

346

347 *Giardia* has been found to secrete several different proteins (ESPs) at low levels in axenic
348 culture and, in the interaction with human intestinal epithelial cells (IECs) the ESPs have the
349 capacity to modulate the host innate immune response, *e.g.* it was found that ESPs could
350 stimulate a preferential Th2 response [39], degrade chemokines and induce the anti-
351 inflammatory protein tristetraprolin (TTP) [10]. The ESP fraction from the WB isolate
352 contains around 200 different proteins [10] and most of these proteins are also found in the
353 soluble *Giardia* protein fractions [18]. It is difficult to generate high levels of ESPs from

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

356

357 We therefore investigated a potential interaction of the mast cell specific proteases trypsin
358 and chymase with *Giardia* proteins. Collectively our findings suggest that *Giardia* via release
359 of ESPs may directly affect the mast cell trypsin activity. Therefore, it is possible that due to
360 the disturbed intestinal epithelial barrier caused by the *Giardia* infection [17], *Giardia* ESPs
361 can reach into the intestinal tissue and thereby increase the level and activity of the mast cell
362 trypsin. An increase in trypsin activity can in turn even more increase the epithelial leakage
363 [40], thereby resulting in a leaky gut that can induce diarrhea and post-infectious symptoms
364 [41-43]. Several post-infectious symptoms can be induced after *Giardia* infections; irritable
365 bowel syndrome (IBS), chronic fatigue syndrome, skin and food allergies and reactive
366 arthritis [44-49]. Mast cells could be very important in the allergies induced after a *Giardia*
367 infection and humans and mice infected by *Giardia* induce *Giardia*-specific IgE responses
368 and ESPs have been suggested to be involved in IgE induction [8, 36, 50]. It will be
369 interesting to follow up the role of mast cells in the induction of post-infectious symptoms in
370 larger studies.

371

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

379 were highly resistant to degradation with tryptase, however the resistance of most of the
380 soluble *Giardia* proteins to degradation by mast cell chymase was an unexpected finding.
381
382 The mast cell specific chymase is monomeric and the extended target site for chymase is eight
383 amino acids long (P4, P3, P2, P1, P1', P2', P3', P4'). Chymase prefers to cut after aromatic
384 amino acids phenylalanine (F), tryptophan (W) or tyrosine (Y) located in the P1 position and
385 usually require aspartic acid (D) or glutamic acid (E) in the P2' position [51-53]. However,
386 physiologic targets for degradation by chymase remain elusive but some virulence factors and
387 alarmins seems to be major targets [29, 54]. In addition, activation of matrix metalloproteinase
388 (MMP)-9 and angiotensin seems to require chymase [55]. Chymase binds strongly to heparin
389 (or chondroitin sulphate E) and it has been suggested that chymase and heparin forms a
390 functional complex that can leave the mast cell after degranulation and move into
391 inflammatory or other body sites. For example, in bladder infection with uropathogenic
392 bacteria chymase is released by mast cell degranulation, and then the chymase migrates and
393 enter into the infected umbrella epithelial cells lining the bladder wall to induce apoptosis,
394 causing the epithelial cells to shed as a protective measure [56]. Furthermore, a detrimental
395 action of the chymase is in the course of glomerulonephritis where mast cells are distantly
396 found in the kidney capsule [57]. In bronchial asthma, mast cell chymase impairs bronchial
397 epithelium integrity through degradation of cell junction molecules, *i.e.* occluding, claudin-4,
398 ZO-1 and E-cadherin in epithelial cells [58]. In the intestine the expression and secretion of
399 chymase will have effect on the epithelial barrier function via protease-activated receptor
400 (PAR)-2 activation and matrix metalloproteinase (MMP)-2 expression and activation [59].
401
402 Tryptase is the predominant serine proteinase of mast cells and heparin stabilizes the activity
403 of the functional monomeric tetramer [60], where the four substrate pockets facing inwards to

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

410

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

417

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

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

430

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

440

441 **Author contributions**

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

445

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450 **Figure legends**

451 **Figure 1. Soluble *Giardia* proteins induce secretion of IL-6 and tryptase in bone marrow**
452 **derived mucosal type mast cells (BM-MMCs).** To determine if *Giardia* activates mast cells
453 1×10^6 BM-MMCs seeded in 0.5 ml HBSS were challenged with three concentrations, 25ng/ml,
454 100ng/ml and 1000ng/ml, of soluble *Giardia* proteins (sGPs) from the GS-, WB- and H3-
455 isolates. The levels of IL-6 (**a, b** with N=5), tryptase activity (**c, d** with N=5), and beta-
456 hexosaminidase activity (**e, f** with N=3) were determined in supernatants collected at 6h (a, c,
457 e) and 24h (b, d, f). Tryptase activity was evaluated by the conversion of the substrate S-2288
458 and recorded as changes in optical density (OD) at 405 nm. BM-MMCs viability was scored
459 by trypan blue exclusion at 6h (**g**) and 24h (**h**). Data is pooled from two independent
460 experiments with BM-MMCs derived from a total of five individual mice (N=5), in duplicate
461 cultures (from two mice in first experiment, n=4) or triplicate cultures (from three mice in
462 second experiment, n=9). Data are shown as mean \pm SEM and statistical analysis conducted
463 by the non-parametric Mann-Whitney U test with significant difference indicated as * P<0.05,
464 ** P<0.01, *** P< 0.005, **** P< 0.001 versus un-challenged control.

465

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

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

478

479 **Figure 3. Soluble *Giardia* proteins and excretory-secretory proteins mediate**

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

494

495 **Figure 4. Soluble *Giardia* proteins enhance mouse mast cell tryptase activity.** sGPs were

496 incubated with or without 0,02µg of rHT or with 15µg of ear tissue extracts from WT mice
497 (bulk ear tissue extracts, n=10) and mMCP6^{-/-} mice (bulk ear tissue extracts, n=10) at room
498 temperature, respectively. (**a**) GS (upper panels), (**b**) WB (middle panels) and (**c**) H3 (lower
499 panels). The **left panels** in a, b, c show the tryptase activity of the high salt mouse ear tissue

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

511

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

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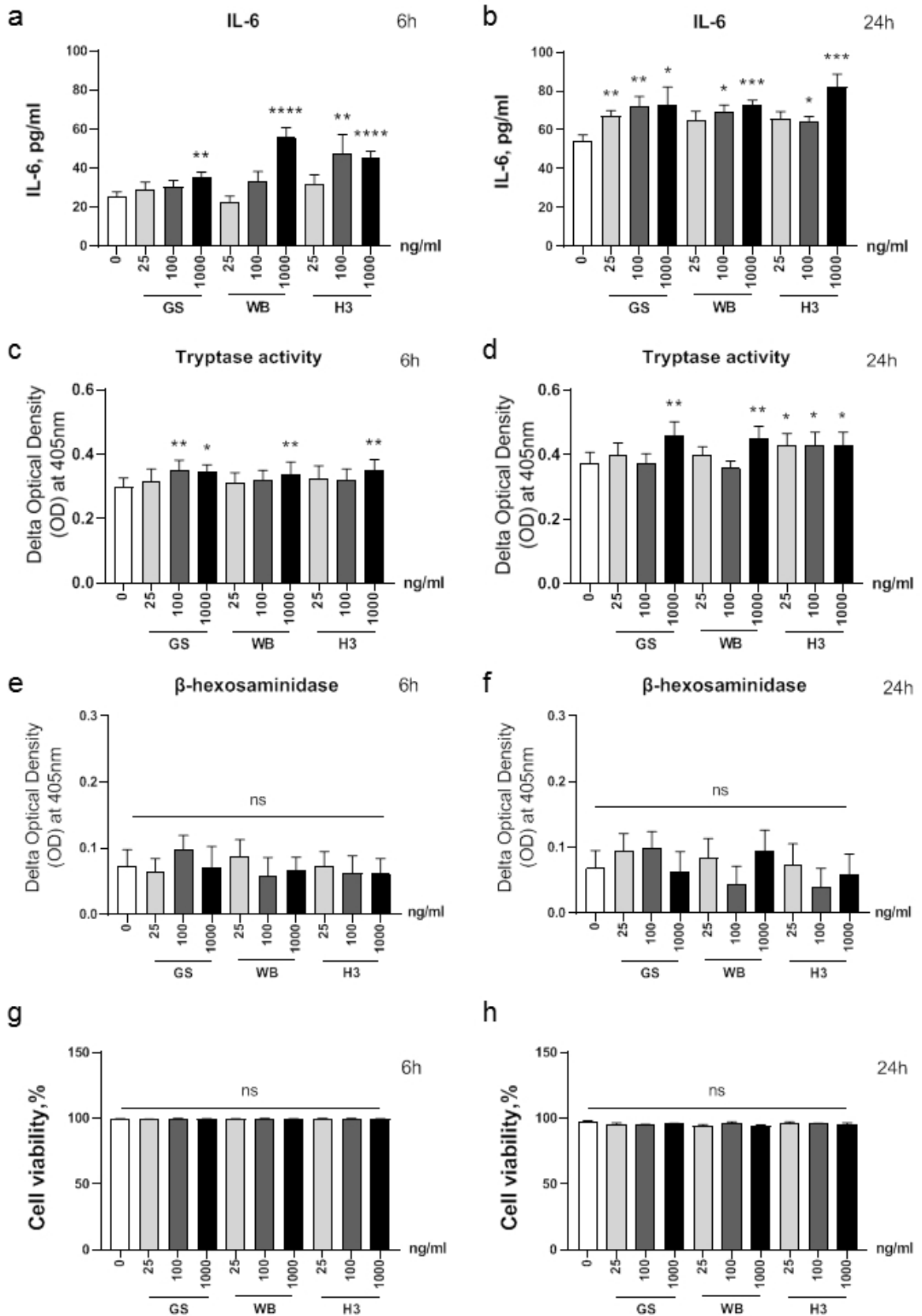
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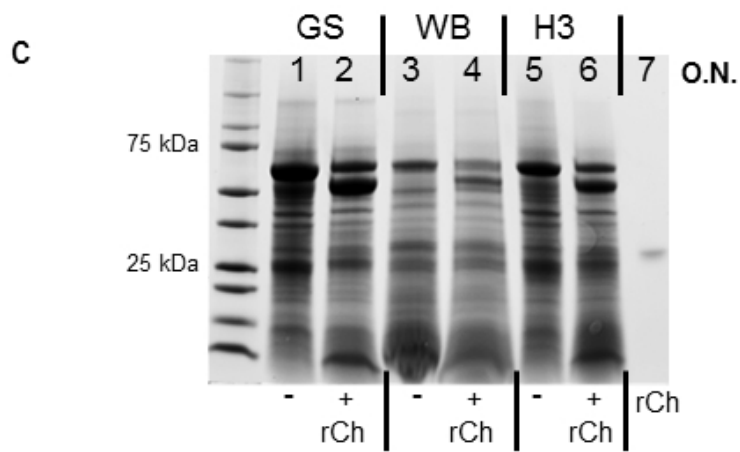
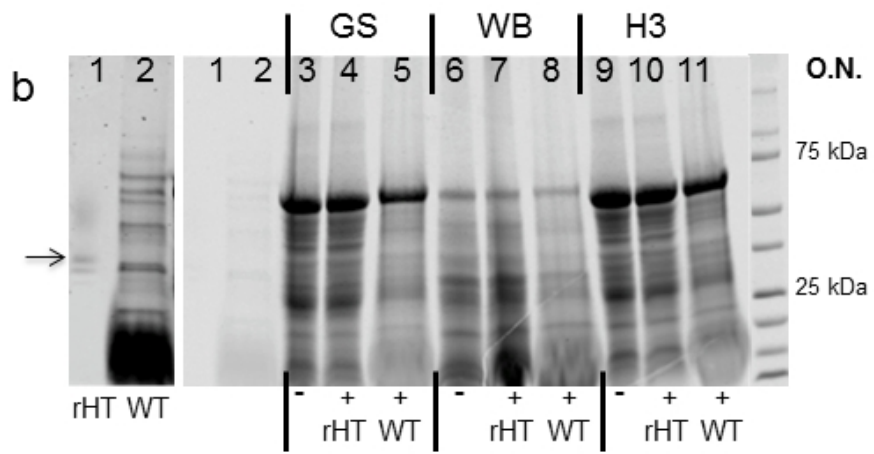
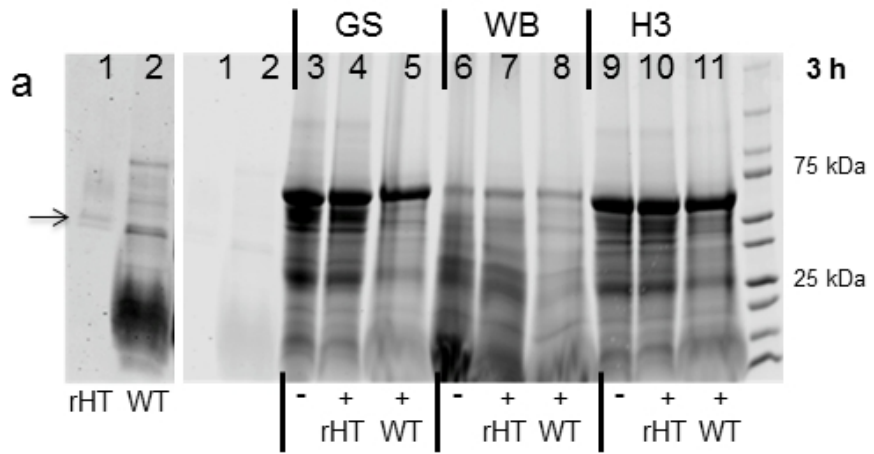
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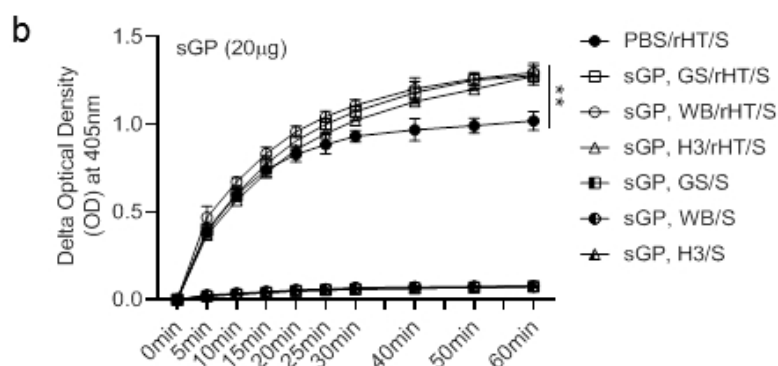
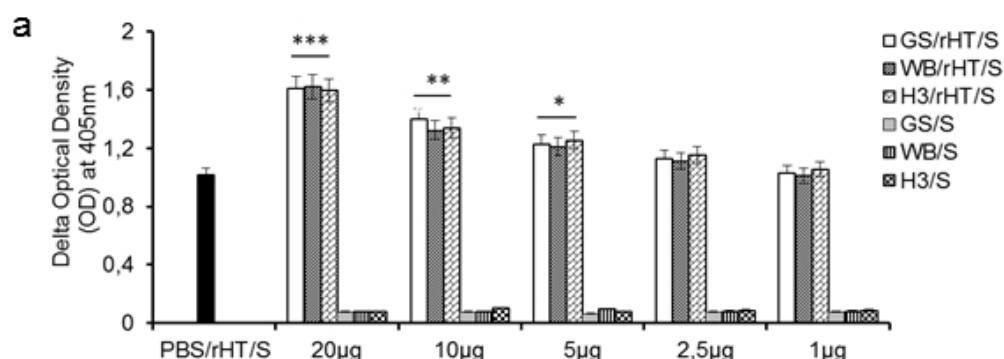
New Figure 1.



New Figure 2.

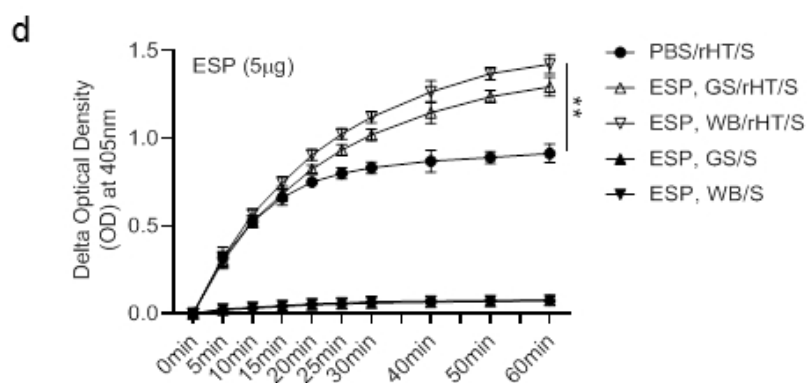


New Figure 3.



c

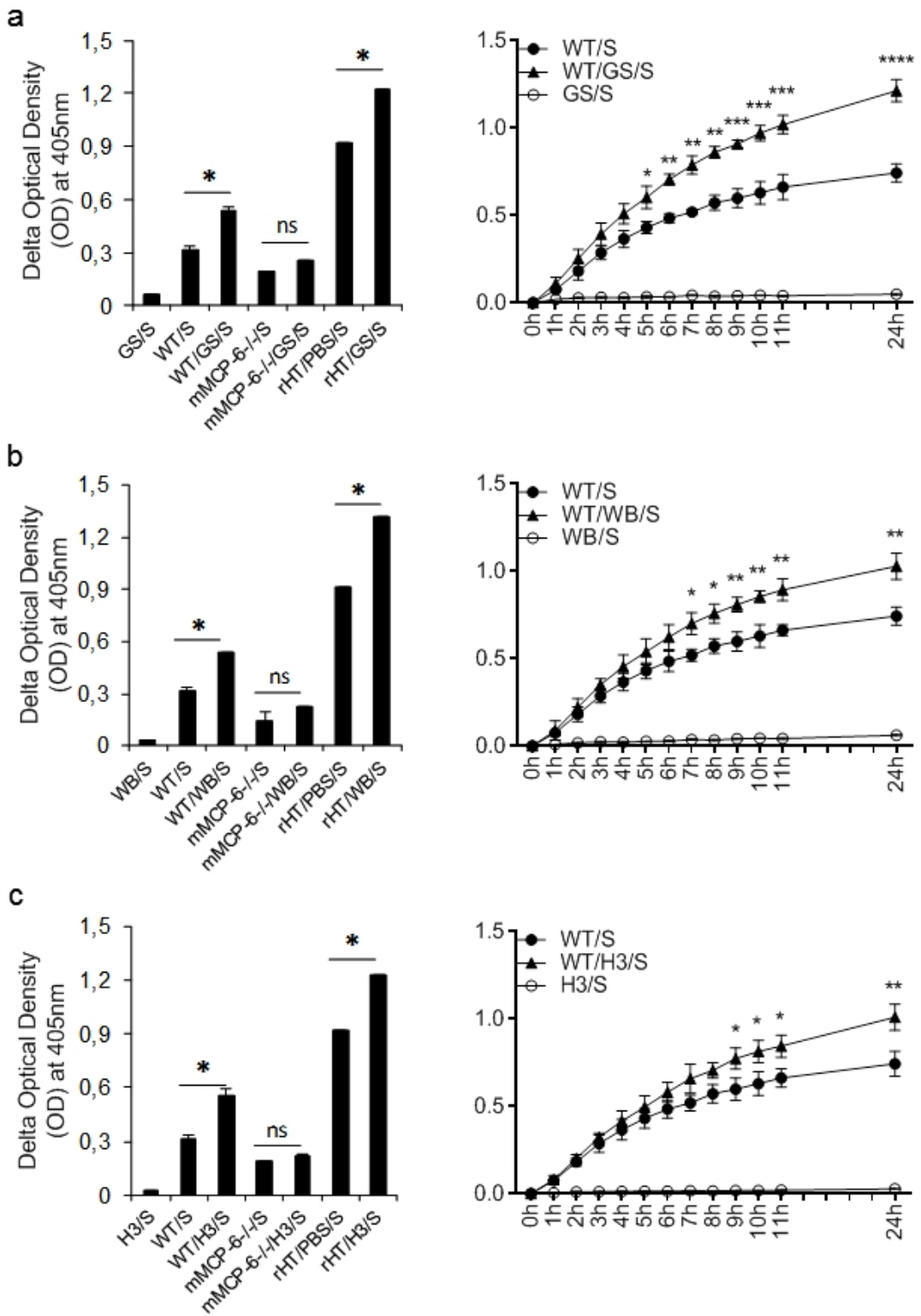
minute (m)	5m-0m	10m-5m	15m-10m	20m-15m	25m-20m	30m-25m	40m-30m	50m-40m	60m-50m
PBS/rHT/S	77.6±2.8	42.1±1.8	28.6±1.4	17.2±2.8	11.4±1.4	9.4±1.4	7.0±1.8	4.7±1.1	5.5±1.1
rHT/GS/S	79.6±3.5	41.3±1.8	34.1±1.1*	26.1±6.0**	18.8±0***	14.6±2.8***	21.6±2.0***	14.3±2.6***	5.1±2.5
rHT/WB/S	83.9±7.4	40.4±7.1	32.2±0.7*	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***



e

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

New Figure 4.



New Figure 5.

