1 *Leishmania major* **degrades murine CXCL1 – an immune evasion strategy**

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25 **Running title:** *Leishmania spp.* degrades CXCL1

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27 **Summary sentence:** *L. major* metalloproteases can specifically degrade murine

28 CXCL1 to subvert the host immune response and establish infection.

29

30   **Abstract**

31   Leishmaniasis is a global health problem with an estimated report of 2 million new cases

32   every year and more than 1 billion people at risk of contracting this disease in endemic

33   areas. The innate immune system plays a central role in controlling *L. major* infection by

34   initiating a signaling cascade that results in production of pro-inflammatory cytokines and

35   recruitment of both innate and adaptive immune cells. Upon infection with *L. major*,

36   CXCL1 is produced locally and plays an important role in the recruitment of neutrophils

37   to the site of infection. Herein, we report that *L. major* specifically targets murine CXCL1

38   for degradation. The degradation of CXCL1 is not dependent on host factors as *L. major*

39   can directly degrade recombinant CXCL1 in a cell-free system. Using mass

40   spectrometry, we discovered that the *L. major* protease cleaves at the C-terminal end of

41   murine CXCL1. Finally, our data suggest that *L. major* metalloproteases are involved in

42   the direct cleavage and degradation of CXCL1, and a synthetic peptide spanning the

43   CXCL1 cleavage site can be used to inhibit *L. major* metalloprotease activity. In

44   conclusion, our study has identified an immune evasion strategy employed by *L. major*

45   to evade innate immune responses in mice, likely reservoirs in the endemic areas, and

46   further highlights that targeting these *L. major* metalloproteases may be important in

47   controlling infection within the reservoir population and transmittance of the disease.

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Authors' summary

Our study discovered a highly specific role for *L. major* metalloprotease in cleaving and degrading murine CXCL1. Indeed, *L. major* metalloprotease did not cleave murine CXCL2 or human CXCL1, CXCL2 and CXCL8. CXCL1 is a critical chemokine required for neutrophil recruitment to the site of infection; thus, we propose that this metalloprotease may have evolved to evade immune responses specifically in the murine host. We have further identified that the C-terminal end on CXCL1 is targeted for cleavage by the *L. major* metalloprotease. Finally, this cleavage site information was used to design peptides that are able to inhibit CXCL1 degradation by *L. major*. Our study highlights an immune evasion strategy utilized by *L. major* to establish infection within a murine host.

Introduction

Leishmania spp. are unicellular eukaryotic protozoan parasites that are transmitted to mammalian hosts by sandfly (*Phlebotomine and Lutzomyia spp.*) bites [1]. Upon transmission of *L. major* promastigotes (the infectious stage for mammalian hosts with a long slender body and an anterior flagellin), the promastigotes are quickly taken up by neutrophils, macrophages and keratinocytes [2-6]. Within the macrophages, *Leishmania spp.* promastigotes hijack the phagocytic vacuole and transform into amastigotes (round body lacking an anterior flagellin) [7]. The *Leishmania spp.* amastigotes then proliferate within the vacuole and establish infection within the host [8, 9]. While a mammalian host-vector system is the major mode of *Leishmania spp.* transmission, several studies have reported a vertical transmission of these parasites in mammalian hosts, from a pregnant female to its offspring [10-13]. Specifically, *Leishmania spp.* infection has been found to be endemic in foxhound dog populations in the United States, where the vectors are not present [14-17]. Given that *Leishmania spp.* can infect several hosts, including rodents and dogs (in addition to humans) [18], these studies demonstrate how these parasites can remain endemic even with strategies to eradicate sandflies.

Our immune system is extremely efficient in killing pathogens. Professional phagocytes such as macrophages and neutrophils phagocytose and kill the invading pathogen in the intracellular phagosome-lysosome compartment. In addition, these phagocytes also respond to the foreign pathogens by secreting pro-inflammatory cytokines and chemokines to recruit neutrophils to the site of infection [19-21]. *Leishmania spp.* have evolved to evade the host immune response by using its armada of virulence factors to

84   avoid host killing [22]. The two major virulence factors of *Leishmania spp.* include

85   leishmanolysin metalloprotease glycoprotein 63 (GP63) and lipophosphoglycan (LPG),

86   and both have been extensively studied for their roles in immune evasion [23, 24].

87   GP63 and LPG inhibit the formation of the membrane attack complex to evade

88   complement-mediated lysis [25, 26], inhibit acidification of leishmania containing

89   vacuoles [27, 28], and dampen host immune signaling pathways [29, 30] for

90   establishing infection within the mammalian host. In addition, *Leishmania spp.* can

91   hijack the host immune responses to establish infections as shown by *Leishmania*

92   *chagasi (L. chagasi)*-mediated activation of TGF-β and *Leishmania major (L. major)*-

93   induced activation of NLRP3 inflammasome, events that promote *L. major* survival and

94   pathology [31-33]. Here, we have identified one such immune evasion mechanism

95   employed by *L. major*.

96

97   *L. major* is the most common *Leishmania spp.* and a major cause of cutaneous

98   leishmaniasis that affects 600,000-1,000,000 people globally [34]. Once deposited at

99   the site of a sandfly bite, *L. major* promastigotes are taken up by the resident

100   macrophages and keratinocytes, which then secrete essential cytokines and

101   chemokines to elicit an innate immune response [35]. One of the earliest chemokines

102   that are produced in the skin in response to *L. major* include CXCL1 [36]. CXCL1 is a

103   functional homolog of human IL-8 in mice and a potent neutrophil chemoattractant [37-

104   41]. Keratinocytes [3], neutrophils [42] and macrophages [43, 44] have all been

105   suggested to produce CXCL1 (or IL-8 in humans) in response to *Leishmania spp.*

106   infection. Several studies have shown that neutrophils infiltrate the site of *Leishmania*

107   *spp.* infection as early as 1-hour post-infection and are important for optimal resolution

108   of the infection [21, 45]. Given the importance of CXCL1 in modulating the early innate

109   immune response, we reasoned that *L. major* targets CXCL1 to evade early host innate

110   immune responses.

111

112   In this current study, we have identified murine CXCL1 as a highly specific substrate for

113   *L. major* metalloprotease and a possible immune evasion strategy employed by this

114   parasite to establish a successful infection within the murine host. We further report that

115   *L. major* promotes proteolytic cleavage of murine CXCL1 at the C-terminal end to

116   initiate its degradation. Finally, we have designed a synthetic peptide spanning the

117   CXCL1 cleavage site that inhibits *L. major* protease activity. In conclusion, our study

118   has uncovered a specific mechanism employed by *L. major* to degrade murine CXCL1

119   which may help the parasite to establish infection within the murine host.

120

121

122   **Results**

123   ***L. major* infection abrogates LPS-induced CXCL1 production by bone marrow-**

124   **derived macrophages.**

125   To investigate how *L. major* impacts innate immune responses elicited by

126   macrophages, we stimulated bone marrow-derived macrophages (BMDM) with

127   lipopolysaccharide (LPS) in the presence or absence of *L. major* (WHOM/IR/-173)

128   infection following established protocol [32]. As detailed in the experimental outline (**Fig.**

129   **1A**), BMDM were stimulated with 20 ng/ml LPS in the presence or absence of 20 MOI *L.*

130   *major* promastigotes for 48 hours. LPS stimulation of BMDM induced robust production

131   of IL-6, TNF and CXCL1. Simultaneous LPS stimulation and *L. major* infection did not

132   impact the production of IL-6 and TNF by BMDM whereas CXCL1 levels were

133   significantly blunted (**Fig. 1A**). These data suggest that *L. major* specifically targets

134   LPS-induced CXCL1 production by BMDM.

135

136   Previous studies have shown that *L. major* targets signaling pathways to evade immune

137   responses and establish infection [[29]]. To examine whether *L. major* specifically

138   targeted CXCL1 expression and production, we designed an experiment whereby

139   BMDM were infected with 20 MOI *L. major* for 6 hours, extracellular *L. major* were

140   washed, then stimulated with 20 ng/ml LPS for the next 42 hours (**Fig. 1B**).

141   Interestingly, *L. major*-infected BMDM produced equal levels of CXCL1 when compared

142   to controls (**Fig. 1B**). These results show that inhibition of CXCL1 by *L. major* may not

143   occur through modulation of intracellular signaling pathway that promotes CXCL1

144   expression and/or production by BMDM.

145

CXCL1 detection is significantly reduced by *L. major* in a cell free system

147 Given that *L. major* pre-infection of BMDM prior to LPS stimulation did not affect CXCL1

148 production, we posited that *L. major* regulates secreted CXCL1 in the extracellular

149 milieu. To this end, we used supernatants derived from LPS-stimulated BMDM as a

150 source of CXCL1 and cultured 500 μl of these cell-free supernatants with 20×10^6 *L.*

151 *major* promastigotes for 24 hours (**Fig. 2A**). While levels of IL-6 and TNF detected in the

152 supernatants remained unchanged, levels of CXCL1 were significantly reduced after

153 addition of *L. major* to the supernatants. The reduced levels of CXCL1 were not due to

154 its short half-life in the culture, as the levels of CXCL1 in the cell free supernatants were

155 stable up to 48 hours (**Supplemental Fig. 1**). Thus, *L. major* directly dampens CXCL1

156 detection.

157

158 We next examined whether the observed effect of *L. major* on CXCL1 detection was

159 dependent on live parasites. To this end, we cultured supernatants from LPS-stimulated

160 BMDM with *L. major* lysates (*Lm* lysate: generated by 3× freeze-thaw cycles of *L. major*)

161 or supernatants from *L. major* culture (*Lm* sup: supernatant collected from stationary

162 phase of *L. major* growth; **Fig. 2B**). Similar to the addition of live *L. major*, addition of

163 *Lm* lysate or *Lm* sup also reduced CXCL1 in the supernatants while IL-6 and TNF

164 remained unaffected (**Fig. 2B**). These results demonstrate that: first, *L. major* need not

165 be alive to dampen CXCL1 detection; second, *L. major* lysate can dampen CXCL1

166 detection; and finally, *L. major* secreted factors dampen CXCL1 detection. Boiling *Lm*

167 lysate or *Lm* sup for 20 minutes at 100°C rescued CXCL1 detected in the supernatants,

168   suggesting that the responsible *L. major* components are susceptible to heat treatment

169   (**Supplemental Fig. 2**). Given the sensitivity to heat treatment, we propose that the

170   CXCL1 regulating *L. major* components are proteinaceous.

171

172   ***L. major* reduces recombinant murine CXCL1 (rm-CXCL1) levels in a cell free**

173   **system**

174   Macrophages secrete several hundreds of different proteins that may indirectly alter our

175   observed effect of *L. major* components on CXCL1 [46]. To this end, we obtained

176   recombinant murine CXCL1 (rm-CXCL1, Tonbo Biosciences, San Diego, CA) which

177   was stable in culture up to 48 hours (**Supplemental Fig. 3**). Importantly, *Lm* sup

178   addition to rm-CXCL1 dampened its detection by ELISA in a time-dependent manner,

179   and boiling *Lm* sup rescued rm-CXCL1 detection (**Supplemental Fig. 3**).

180

181   To further investigate the specificity of *L. major* in reducing rm-CXCL1 levels, we

182   examined rm-CXCL2, rh-CXCL1, rh-CXCL2 and rh-CXCL8 sequence homology by

183   CLUSTAL-W alignment and found significant homology between these recombinant

184   proteins. Despite the significant homology, *L. major* failed to reduce levels of rm-

185   CXCL2, rh-CXCL1, rh-CXCL2 or rh-CXCL8, demonstrating specific regulation of rm-

186   CXCL1 (**Fig. 3, B-F**). As expected, *L. major* did not inhibit rm-TNF levels (**Fig. 3G**).

187   Given the specific nature of *L. major* (WHOM/IR/-170) in dampening rm-CXCL1 levels,

188   we next determined whether this activity was specific to the WHOM/IR/-170 isolate.

189   However, supernatant from *L. major* (IA0, isolated from a patient in Iowa who acquired

190   *L. major* in Iraq [47]) was also able to inhibit rm-CXCL1 detection when compared to the

191   WHOM/IR/-170 strain (**Fig. 3H**). Furthermore, exosomes from the *L. major* supernatants

192   from both isolates were able to reduce rm-CXCL1 levels, suggesting that the active

193   components are also present in the *L. major*-derived vesicles (**Fig. 3H**).

194

195   ***L. major* inactivates biological activity of CXCL1**

196   Our data show that *L. major* reduced CXCL1 levels as demonstrated by ELISA (**Figs. 1-**

197   **3**). Possible reasons why CXCL1 levels were reduced when incubated with *L. major*

198   include: 1) *L. major* proteins bind to CXCL1, limiting the ability of anti-CXCL1 antibody in

199   ELISA to interact with CXCL1 or 2) *L. major* proteases cleaves and degrades CXCL1.

200   Considering both outcomes, it is possible that CXCL1, either masked or cleaved by *L.*

201   *major* components, could still be biologically active. To this end, we performed a

202   functional assay where rm-CXCL1 or rm-CXCL1+*Lm* lysate was used to stimulate

203   BMDM (**Fig. 4A**). As demonstrated previously, when rm-CXCL1 is incubated with *L.*

204   *major*, rm-CXCL1 levels are significantly reduced (**Fig. 4B**). *Lm* lysate alone has very

205   little stimulatory activity as demonstrated by mRNA induction of *Cxcl1*, *Tnf* and *Il6* (**Fig.**

206   **4, C-E**). As expected, rm-CXCL1 induced modest increase of *Cxcl1*, *Tnf* and *Il6* but rm-

207   CXCL1+*Lm* lysate failed to induce *Cxcl1*, *Tnf* and *Il6* mRNA (**Fig. 4, C-E**). These results

208   altogether demonstrate that *L. major* inactivates biological activity of rm-CXCL1.

209

210   ***L. major* cleaves CXCL1 at the C-terminal end to promote its degradation**

211   To determine whether CXCL1 is degraded by *L. major*, we incubated rm-CXCL1 with

212   *Lm* lysate for various time periods and examined rm-CXCL1 by silver staining (**Fig. 5A**).

213   Rm-CXCL1 was detected at approximately 10KDa by silver stain. Interestingly, when

214   incubated with *Lm* lysate rm-CXCL1 showed two bands as early as 30 minutes after

215   incubation, suggesting *Lm* lysate-mediated cleavage of rm-CXCL1 (**Fig. 5A and**

216   **Supplemental Fig. 4**). By 2 hours, only the cleaved rm-CXCL1 was observed and this

217   cleaved band intensity decreased over time, suggesting further degradation (**Fig. 5A**).

218   *L. major*-mediated cleavage of rm-CXCL1 was specific because similar experiments

219   done with rm-CXCL2, rh-CXCL1 and rh-CXCL2 did not result in cleavage or

220   degradation of these recombinant proteins (**Fig. 5B**).

221

222   Based on the cleavage pattern of rm-CXCL1 (less than 1KDa shift), cleavage either at

223   the N-terminal or C-terminal end would result in a large fragment (detected by silver

224   stain as the lower band, **Fig. 5A**) and a smaller fragment (which could not be detected

225   by silver stain; **Fig. 5C**). To identify the cleavage product and site, we processed the

226   full-length rm-CXCL1 and *Lm* lysate-cleaved rm-CXCL1 bands by trypsin digestion and

227   performed mass spectrometry to determine the sequence of the bands (**Supplemental**

228   **Fig. 5**). The tryptic peptide sequence readouts of full-length rm-CXCL1 covered the

229   whole rm-CXCL1 peptide sequence (**Supplemental Fig. 5B**), while the peptide

230   coverage of cleaved rm-CXCL1 covered all of the rm-CXCL1 peptide sequence except

231   the last 7 amino acids (MLKGVPK) at the C-terminal end (**Supplemental Fig. 5C**).

232   Thus, *L. major* cleaves rm-CXCL1 after lysine 65 (K65) residue that results in a large N-

233   terminal rm-CXCL1 fragment lacking 7 amino acids at the C-terminal end (**Fig. 5D**).

234

235   ***L. major* metalloprotease cleaves CXCL1**

236   *L. major* has several proteases that enable it to survive within a cell and establish

237   infection [48]. Metalloproteases and cysteine, serine and aspartic proteases are the

238   major proteases described in *L. major* [48]. To examine whether cleavage activity was

239   dependent on proteases, we first treated our rm-CXCL1 + *Lm* sup culture with pan

240   protease inhibitor (Roche and Sigma) (**Fig.6, A-C**). Roche cOmplete protease inhibitor

241   is a broad inhibitor of proteases including serine, cysteine and metalloproteases, and

242   Sigma P8340 inhibitor is reported to inhibit serine, cysteine, acid proteases and

243   aminopeptidases. However, the presence of protease inhibitors from Roche (cOmplete)

244   or Sigma (P8340) did not inhibit *Lm* sup-mediated degradation of rm-CXCL1 as

245   demonstrated by ELISA (**Fig. 6, B and C, and Supplemental Fig. 6**). Marimastat, a

246   broad inhibitor of matrix metalloproteases [49], did not rescue the degradation of rm-

247   CXCL1 by *Lm* sup (**Supplemental Fig. 6B**). EDTA treatment, which chelates metal ions

248   such as Ca^{2+} and Fe^{3+} (and thus can inhibit certain metalloprotease), did not rescue rm-

249   CXCL1 cleavage by *Lm* sup (**Fig. 6, D and E**). Interestingly, 1,10-Phenanthroline, a

250   Zn^{2+} metalloprotease inhibitor [50], rescued rm-CXCL1 degradation by *Lm* sup (**Fig. 6, F**

251   **and G**). While the addition of 1,10-Phenanthroline resulted in the upward shift of rm-

252   CXCL1 bands in SDS-PAGE gels; importantly, no cleavage or degradation of rm-

253   CXCL1 was observed (**Fig. 6G**).

254

255   Several studies have shown that 1,10-Phenanthroline inhibits GP63, a Zn^{2+}

256   metalloprotease present on all *Leishmania spp* and is often used in biochemical assays

257   to inhibit non-specific proteolytic activity of GP63 [51]. To determine whether GP63 was

258   involved in specific cleavage of rm-CXCL1, we immunoprecipitated (IP) GP63 from *Lm*

259   lysate using anti-GP63 antibody (**Fig. 6H**). Immunoblotting the GP63+ve and GP63-ve

260   IP fractions for GP63 showed that GP63 was exclusively present in the IP+ve fraction

261   and not present in the IP-ve fraction demonstrating successful immunoprecipitation of

262   GP63 (**Fig. 6H**). More importantly, when these fractions were cultured with rm-CXCL1,

263   the IP-ve (*Lm* lysate lacking GP63) but not the IP+ve (i.e. GP63) fraction degraded rm-

264   CXCL1 suggesting a role for GP63-independent metalloprotease in the specific

265   degradation of rm-CXCL1 (**Fig. 6I**).

266

267   **Synthetic peptide spanning the CXCL1 cleavage site inhibits *L. major* proteolytic**

268   **activity against rm-CXCL1**

269   Our data suggest that *L. major* Zn^{2+} metalloprotease specifically cleaves rm-CXCL1

270   after the K65 residue leaving a 7-mer amino acid sequence (MLKGVPK). To further

271   examine the specificity of this *L. major* metalloprotease, we designed a blocking peptide

272   that covered this cleavage site (i.e. the last 15 amino acid sequences of rm-CXCL1; **Fig.**

273   **7A**). The addition of blocking peptide was able to rescue *Lm* lysate-mediated

274   degradation of rm-CXCL1 in a dose-dependent manner (**Fig. 7B**). The peptide

275   sequence from the signal peptide region of rm-CXCL1 (peptide #1 and peptide #2, Fig.

276   7A) did not inhibit *Lm* lysate-mediated degradation of rm-CXCL1, demonstrating the

277   specificity of the blocking peptide (**Fig. 7B**). These data demonstrate that a synthetic

278   peptide spanning the murine CXCL1 cleavage site can competitively inhibit *L. major*-

279   mediated rm-CXCL1 degradation.

280

281 **Discussion**

282 CXCL1 is a potent neutrophil chemoattractant and is rapidly upregulated following

283 *Leishmania spp.* infection in the skin [36]; however, the role of CXCL1 during

284 *Leishmania spp.* infection of mice in vivo has been understudied. Charmoy et al showed

285 that mice deficient in CXCL1 have a slight increase in lesion size and reduced numbers

286 of neutrophil infiltrates in chronic lesions, but the overall pathology remained similar

287 between WT and *Cxcl1*$^{-/-}$ mice [33]. However, it should be noted that the study used the

288 *L. major* Seidman strain (*Lm*Sd) that causes a non-healing infection in C57BL/6 mice

289 [33]. Thus, more thorough analyses are needed to determine the precise role of CXCL1

290 during *L. major* infection in mice and humans.

291

292 Importantly, even depletion of neutrophils using anti-Gr1 neutralizing antibody does not

293 always lead to increased parasitic burden, lesion or pathology [21]. While some studies

294 with acute depletion of neutrophils reported worsened pathology during *L. major*

295 infections, other studies have shown no role or even amelioration of disease pathology

296 [5, 52-60]. Thus, mouse strain, *L. major* strain, route of infection and timing of CXCL1

297 release may all contribute to the fate of the infection and overall pathology. In line with

298 this thought, two independent *L. major* strains were tested in our study, and both strains

299 degraded CXCL1 in our experimental systems. Whether this degradation of murine

300 CXCL1 is a specific feature of *L. major* or a more general feature of all *Leishmania spp.*

301 will need further investigation.

302

303   Our initial observation demonstrated that *L. major* in culture with macrophages

304   effectively inhibited LPS-induced CXCL1 production. Further examination showed that

305   intracellular *L. major* within the macrophages did not inhibit LPS-induced CXCL1

306   production. These experiments suggest that *L. major* does not interfere with signaling

307   pathways that promote CXCL1 production but directly regulates secreted CXCL1 in the

308   extracellular milieu. *L. major* are present as metacyclic promastigotes in sandflies [61].

309   When infected into the mammalian host, *L. major* infect macrophages and resides

310   within the vacuole where they transform into amastigotes, multiply and establish

311   infection [62]. Because *L. major*-infected macrophages which contain amastigotes did

312   not inhibit CXCL1 production, it could be posited that only the promastigote form of *L.*

313   *major* degrades CXCL1. However, genome microarray analysis shows that the majority

314   of genes are constitutively expressed in both *L. major* promastigotes and amastigotes

315   (>90%) and therefore the major genes and virulent factors expressed by these different

316   stages of *L. major* may not be different [63]. Thus, both *L. major* promastigotes and

317   amastigotes may similarly cleave and degrade murine CXCL1 in an in vitro assay. In

318   addition, infected macrophages can secrete exosomes containing *L. major* proteins [64,

319   65], which could degrade murine CXCL1. We have consistently shown that *L. major*

320   secreted microvesicles (i.e. exosomes) contain these metalloproteases that can

321   degrade murine CXCL1. Physiologically, *L. major* promastigotes released during a

322   sandfly bite may act locally to limit acute CXCL1 produced in response to the infection.

323   Once *L. major* are phagocytosed and transformed into amastigotes, they are physically

324   separated from the CXCL1 due to their location (amastigotes are in the vacuole while

16

325   CXCL1 are secreted by the macrophages and are extracellular) and may only impact

326   CXCL1 production through the *L. major* component laden exosomes.

327

328   Our studies suggest that a yet-unknown metalloprotease from *L. major* cleaves murine

329   CXCL1. Given that 1,10-Phenanthroline (Zn^{2+} chelator) but not EDTA (Ca^{2+} and Fe^{3+}

330   metal ion chelator) or Marimastat (Matrix metalloprotease inhibitor) rescued *L. major*-

331   mediated CXCL1 degradation, it is possible that the unknown metalloprotease is a Zn^{2+}

332   metalloprotease. Our studies also demonstrate that the *L. major* metalloprotease is

333   highly specific for murine CXCL1 in that its closest murine homolog, CXCL2, or human

334   homologs, CXCL1, CXCL2 or CXCL8, were not degraded. We hypothesize that this

335   particular *L. major* metalloprotease may have evolved to specifically evade host immune

336   response in rodents. Given that human CXCL1 homologs are not susceptible to this

337   metalloprotease, one can argue that our results may not have any importance from a

338   public health standpoint, but we reason otherwise. Our results are highly relevant to

339   public health because rodents are ubiquitous, serve as a reservoir for *Leishmania spp*.

340   and we highlight a rodent-specific *L. major* evolution in targeting murine CXCL1 [66].

341

342   Our studies demonstrate that the murine CXCL1 is first cleaved at the C-terminal end

343   after K65 releasing a 7aa residue. CXCL1 cleavage occurs as early as 30 minutes of

344   incubation with *L. major* and by 4 hours the cleavage is complete in that only the

345   cleaved bands are observed. Interestingly, we did not observe any accumulation of the

346   cleaved CXCL1 band, suggesting continuous degradation of the cleaved form. While

347   our data clearly demonstrate that the unknown *L. major* metalloprotease mediates

348   cleavage of murine CXCL1, how the cleaved CXCL1 is further degraded will be the

349   subject of future investigation. The cleaved murine CXCL1 (lacking the C-terminal 7 aa)

350   may be highly unstable and undergo spontaneous degradation overtime. Alternatively,

351   the cleaved murine CXCL1 may be susceptible to additional *L. major* proteases which

352   promote its subsequent degradation.

353

354   In conclusion, we have identified an immune evasion strategy utilized by *L. major* that is

355   highly specific to murine CXCL1 (**Fig. 7C**). Specifically, *L. major*-associated

356   metalloprotease cleaved murine CXCL1 at K65 residue and released a C-terminal 7

357   amino acid fragment to promote its degradation. Finally, we have designed a peptide

358   spanning the cleavage site of CXCL1 that inhibited murine CXCL1 cleavage by *L.*

359   *major*. Our study altogether uncovered an immune evasion strategy employed by *L.*

360   *major* that may have evolved in rodents and highlights how parasites may utilize diverse

361   immune evasion strategy to establish infection within its diverse mammalian hosts.

362

363 **Methods**

364 **Ethics statement**

365 Experimental procedures that utilized mice were all approved by the University of Iowa

366 Animal Care and Use Committee (Approved Animal Protocol # 7042004 – PI Dr.

367 Prajwal Gurung) and performed in accordance to the Office of Laboratory Animal

368 Welfare guidelines and the PHS Policy on Humane Care and Use of Laboratory

369 Animals.

370

371 **BMDM culture**

372 BMDMs were prepared as described previously (32). Briefly, bone marrow cells were

373 harvested from the hind limbs of BALB/c mice (Jackson Laboratory, Stock No. 000651)

374 and cultured in L cell-conditioned IMDM medium supplemented with 10% FBS, 1%

375 nonessential amino acid, and 1% penicillin-streptomycin for 5-7 d to differentiate into

376 macrophages. BMDMs were counted and seeded at 1×10^6 cells in 12-well cell culture

377 plates in IMDM media containing 10% FBS, 1% nonessential amino acids, and 1%

378 penicillin-streptomycin. BMDMs were primed with LPS (20 ng/ml) and infected with 20

379 MOI *L. major* promastigotes for 24 and 48 hours (Supplemental Figure 1). For some

380 experiments, BMDMs were LPS primed to generate supernatant containing cytokines

381 for in vitro biochemical analysis with *L. major* supernatant (*Lm* sup) and *L. major* lysate

382 (*Lm* lysate). BMDMs were also treated with rm-CXCL1 (20 ng/ml) or rm-CXCL1+*Lm* sup

383 preparations in some experiments.

384

385 **L. major culture**

386   *L. major* strains WHOM/IR/-173 [67] and IA0 [47] were grown in T-25 flasks with M199

387   media supplemented with 10% FBS, 5% HEPES and 1% penicillin-streptomycin at room

388   temperature. BMDMs were infected with 20 MOI of *L. major* promastigotes for 48 hours.

389   Conditioned *L. major* supernatant (*Lm* sup) was prepared after *L. major* reached the

390   stable growth phase at approximately 20×10^6 / ml. Following centrifugation (3000×g) to

391   pellet *L. major*, supernatants were collected and filtered using a 0.2μm vacuum filter to

392   harvest *Lm* sup. *Lm* lysate was prepared by collecting the *L. major* pellet, washing 3

393   times with PBS followed by 3 freeze-thaw cycles of 7×10^9 *L. major* /ml in PBS. For the

394   preparation of *L. major* exosomes, promastigotes were grown to stable phase and

395   changed to serum-free media and incubated at room temperature overnight.

396   Supernatant was obtained as above and then separated using a 100kDa molecular

397   weight Amicon ultra-15 centrifugal filter (MilliporeSigma, Burlington, MA). The

398   concentrated vesicles were washed twice with PBS by ultracentrifugation at 110,000xg

399   at 4°C for 1 hour (Beckman Optma MAX-XP Ultracentrifuge, rotor TLA 120.2; Beckman

400   Coulter Inc., USA). The final pellet (exosomes) was resuspended in PBS and used in in

401   vitro biochemistry assays.

402

403   **Electrophoresis and in vitro biochemistry**

404   For in vitro reactions, 125ng of total recombinant protein was separated on 4-20% or

405   10-20% Novex WedgeWell Tris-Glycine precast gels (Thermofisher, Waltham, MA).

406   For silver stain, the Pierce Mass Spec compatible silver stain kit was used per

407   manufacturer's instructions (Thermofisher). Synthetic peptides were generated and

408   purchased from Pierce Biotechnology (Thermofisher) and are composed of the

409   following sequences: peptide#1: CAALLLLATSRLA; peptide#2: RLATGAPIAN;

410   blocking peptide: LVQKIVQKMLKGVPK. For Western blot analysis a semi-dry transfer

411   was completed using trans-blot turbo system (Bio-Rad, Hercules, CA) with Immobilon

412   PVDF membrane. The membranes were blocked in 5% non-fat milk for 1 hour at room

413   temperature. Membranes were probed with sheep polyclonal antibody Sp180 raised

414   against *L. chagasi* promastigote GP63 at a concentration 1:1500 incubated overnight at

415   4°C. The membranes were then probed with HRP-tagged secondary antibodies at room

416   temperature for 1 hour and developed using Immobilon Forte Western HRP Substrate

417   (MilliporeSigma) and imaged with an Odyssey Fc Infrared Imaging System (LI-COR

418   Bioscience, Lincoln, NE). Immunoprecipitation was performed using Protein G

419   Dynabeads and Leishmania GP63 monoclonal antibody clone 96-126 (Thermofisher).

420   As instructed by the manufacturer, 50μL beads were washed in PBS with 0.02% Tween

421   20 and bound to 5μg antibody for at least 10 minutes. Following a wash, the antibody

422   bound beads were incubated with *Lm* lysate for another 10 minutes and finally either

423   antigen-antibody binding was negated by acidic (pH 2.8) 50mM Glycine treatment or

424   bead bound antibody-antigen complexes were used directly for biochemistry.

425

426   **Mass spectrometry**

427   *In-gel trypsin digestion:* The gel was stained using a Pierce mass spec compatible silver

428   stain kit (Thermofisher) per manufacturer directions. A procedure slightly modified than

429   that described by Yu et al [68] was used for in-gel digestion. Briefly, the targeted protein

430   bands from SDS-PAGE gel were manually excised, cut into 1mm^3 pieces, and washed

431   in 100mM ammonium bicarbonate:acetonitrile (1:1, v/v) and 25mM ammonium

432 bicarbonate /acetonitrile (1:1, v/v), respectively, to achieve complete de-staining. The

433 gel pieces were further treated with acetonitrile (ACN), to effectively "dry" the gel

434 segments and then reduced in 50μl of 10mM DTT at 56 °C for 60 min. Gel-trapped

435 proteins were alkylated with 55mM chloroacetamide (CAM) for 30 min at room

436 temperature. The gel pieces were washed with 25mM ammonium bicarbonate:

437 acetonitrile (1:1, v/v) twice to remove excess DTT and CAM. 50μl of cold trypsin solution

438 at 10ng/μl in 25mM ammonium bicarbonate was then added to the gel pieces and they

439 were allowed to swell on ice for 60 min. Digestion was conducted at 37°C for 16 h.

440 Peptide extraction was performed three times, adding 100µL of 50% acetonitrile/0.1%

441 formic acid for 0.5 h, combining the supernatants. The combined extracts were

442 concentrated in a lyophilizer and rehydrated with 15 μl of mobile phase A.

443

444 *LC-MS/MS*: Mass spectrometry data were collected using an Orbitrap Fusion Lumos

445 mass spectrometer (Thermofisher) coupled to an Easy-nLC-1200™ System (Proxeon

446 P/N LC1400). The autosampler is set to aspirate 3μl (estimated 0.3ug) of reconstituted

447 digest and load the solution on a 2.5cm C18 trap (New Objective, P/N IT100-25H002)

448 coupled to waste, HV or analytical column through a microcross assembly (IDEX, P/N

449 UH-752). Peptides are desalted on the trap using 16μl mobile phase A for 4 min. The

450 waste valve is then blocked and a gradient begins flowing at 0.4μl/min through a self-

451 packed analytical column, 10cm in length × 75μm id. The fused silica column was

452 tapered from 100μm ID (Polymicro) to ~8μm at the tip using a Sutter P-2000 laser puller

453 then packed with 2.7micron Halo C18 particles using a He-pressurized SS cylinder.

454 Peptides were separated in-line with the mass spectrometer using a 70 min gradient

455   composed of linear and static segments wherein buffer A is 0.1% formic acid and B is

456   95%ACN, 0.1% formic acid. The gradient begins first hold at 4% for 3 min then makes

457   the following transitions (%B, min): (2, 0), (35, 46), (60, 56), (98, 62), (98, 70).

458

459   *Tandem mass spectrometry on the Thermo Q-Exactive hf*: Data acquisitions begin with

460   a survey scan (m/z 380 -1800) acquired on a Q-Exactive Orbitrap mass spectrometer

461   (Thermofisher) at a resolution of 120,000 in the off-axis Orbitrap segment (MS1) with

462   automatic gain control (AGC) set to 3E06 and a maximum injection time of 50 ms. MS1

463   scans were acquired every 3 sec during the 70-min gradient described above. The most

464   abundant precursors were selected among 2-6 charge state ions at a 1E05 AGC and 70

465   ms maximum injection time. Ions were isolated with a 1.6 Th window using the multi-

466   segment quadrupole and subjected to dynamic exclusion for 30 sec if they were

467   targeted twice in the prior 30 sec. Selected ions were then subjected to high energy

468   collision-induced dissociation (HCD) in the ion routing multipole (IRM). Targeted

469   precursors were fragmented by (HCD) at 30% collision energy in the IRM. HCD

470   fragment ions were analyzed using the Orbitrap (AGC 1.2E05, maximum injection time

471   110 ms, and resolution set to 30,000 at 400 Th). Both MS2 data were recorded as

472   centroid and the MS1 survey scans were recorded in profile mode.

473

474   *Proteomic searches*: Initial spectral searches were performed with both Mascot version

475   2.6.2 (MatrixScience) and Byonic search engines (Protein Metrics ver. 2.8.2). Search

476   databases were composed of the Uniprot KB for species 10090 (mouse) downloaded

477   February 6, 2016 containing 58436 sequences. In either search, an equal number of

478   decoy entries were created and searched simultaneously by reversing the original

479   entries in the target database. Precursor mass tolerance was set to 5 ppm and

480   fragments were searched at 10 ppm. A fixed 57 Da modification was assumed for

481   cysteine residues while variable oxidation was allowed at methionine. A variable GG

482   modification at lysine was set to monitor ubiquitylation and potential phosphorylation

483   was accessed at Ser and Thr residues. The false discovery rate was maintained at 1%

484   by tracking matches to the decoy database. Both Mascot and Byonic search results

485   were combined and validated using Scaffold ver. 4.8.5 (Proteome Software). Protein

486   assignments required a minimum of two peptides established at 70% probability (local

487   FDR algorithm) and an overall 95% protein probability (assigned by Protein Prophet).

488   Approximately 300 protein families (including common contaminants) were assigned at

489   a total FDR to 1.2%. Proteins were annotated with GO terms from goa_uniprot_all.gaf

490   downloaded on May 3, 2017.

491

492   **Immunoassays and recombinant proteins**

493   Cytokine ELISAs and multiplex immunoassays were performed according to

494   manufacturer instructions. Mouse and human CXCL1 and CXCL2 as well as human IL-

495   8 (CXCL8) were obtained from R&D Systems (Minneapolis, MN) and multiplex CXCL1,

496   IL-6, and TNF were completed using ProcartaPlex assays (Thermofisher) which were

497   run on the BioRad Bio-Plex (Luminex, Austin, TX). We obtained our recombinant

498   proteins from Tonbo biosciences which included mouse CXCL1, CXCL2, and TNF as

499   well as human CXCL1, CXCL2, and CXCL8.

500

501 **PCR methods**

502 RNA was extracted from BMDM cultures in TRIzol reagent (Thermofisher) followed by

503 chloroform extraction and isopropanol precipitation. The extracted RNA was reverse-

504 transcribed into cDNA by using qScript Supermix (Quanta Biosciences, Beverly, MA).

505 Real-time quantitative PCR was performed on Eppendorf realplex EPgradient S

506 Mastercycler (Eppendorf, Germany) using PerfeCTa SYBR Green SuperMix ROX

507 (Quanta Biosciences) and the appropriate primers. The sequences of the quantitative

508 RT-PCR primers are as follows: *mCXCL1:* 5'-CAATGAGCTGCGCTGTCAGTG-3', 5'-

509 CTTGGGGACACCTTTTAGCATC-3'; *mTnf*: 5'-CATCTTCTCAAAATTCGAGTGACAA-

510 3', 5'-TGGGAGTAGACAAGGTACAACCC-3'; *mIL-6:* 5'-

511 CAAGAAAGACAAAGCCAGAGTC-3', 5'-GAAATTGGGGTAGGAAGGAC-3'.

512 Threshold cycle Ct values were normalized to GAPDH, and gene fold change was

513 determined by the relative comparison method, relative to the 0 h time point.

514

515 **Statistical analysis and alignment**

516 GraphPad Prism 8.0 software was used for data analysis and figure presentation. Data

517 are shown as mean ± SEM. Statistical significance was determined by *t* tests (two-tailed

518 and Mann-Whitney) for two groups, and one-way ANOVA (with Dunnett's or Tukey's

519 multiple comparisons tests) for three or more groups. For the purpose of alignment

520 presentation, sequences were obtained from the protein manufacturer or the NCBI

521 protein database and figures were prepared using Jalview 2 [69].

522

523 **Authorships**

524 M.S.Y, B.P., L.M. performed the experiments. M.S.Y, M.E.W., and P.G. analyzed the

525 data. R.M.P. performed the mass spectrometry and analysis of CXCL1 cleavage bands.

526 M.S.Y. and P.G. wrote the manuscript with input from all authors. P.G. designed and

527 conceptualized the study.

528

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538

539 **Conflict of Interest**

540 The authors have no conflict of interest to declare.

541

542

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820
821
822

Figure legends

Figure 1: *L. major* diminishes LPS-induced CXCL1 release by BMDMs. (A) BMDMs were treated with LPS (20 ng/ml) in the presence or absence of *L. major* (20 MOI) for 48h and cell culture supernatants were analyzed for the indicated cytokines. (B) BMDMs were treated with PBS or *L. major* (20 MOI) for 6h. Then, the cells were washed to remove extracellular *L. major* and stimulated with LPS for next 42h and cell culture supernatants were subjected to cytokines analysis as in (A). Data are representative of at least three independent experiments. Results are represented as mean $\pm$ SEM. ***P<0.001.

Figure 2: *L. major* inhibits CXCL1 detection in a cell free system. (A) Conditioned supernatants from 24h LPS (20 ng/ml)-stimulated BMDMs were treated with or without *L. major* for 24h and cytokines were analyzed by ELISA. (B) 24h LPS treated conditioned media from BMDMs were treated with or without *L. major*, *L. major* lysate (*Lm* lysate), or *L. major* culture supernatant (*Lm* sup) for 24h and cytokines were measured as in (A). Data are representative of at least three independent experiments. Results are represented as mean $\pm$ SEM. ****P<0.0001.

Figure 3: Selective inhibitory activity of *L. major* on recombinant murine CXCL1 (A) Sequence alignment of recombinant murine (rm) and recombinant human (rh) CXCL1, CXCL2, and CXCL8. (B) Rm-CXCL1, (C) rh-CXCL1, (D) rm-CXCL2, (E) rh-CXCL2, (F) rh-CXCL8, and (G) rm-TNF were incubated with or without *Lm* sup for indicated time-points and ELISA was performed to quantify the mentioned cytokine/chemokines. (H)

847   Rm-CXCL1 was incubated with or without *Lm* sup or *Lm* exosomes derived from indicated

848   strains of *L. major* for 24h, and CXCL1 quantification was performed as in (G). Data are

849   representative of at least three independent experiments. Results are represented as

850   mean $\pm$ SEM. ****P<0.0001.

851

852   **Figure 4: Biological activity of *L. major*-treated CXCL1 is diminished** (A) Schematic

853   representation of experimental design. (B) Recombinant murine CXCL1 was incubated

854   with *Lm* lysate for 24h and CXCL1 levels was determined by ELISA. (C) BMDMs were

855   treated *Lm* lysate, rm-CXCL1, or *Lm* lysate + rm-CXCL1 from (B) for indicated time-points

856   and mRNA expression of pro-inflammatory genes were evaluated by qPCR. Data are

857   representative of at least three independent experiments.

858

859   **Figure 5: *L. major* cleaves CXCL1 at the C-terminal end.** (A) A silver-stained SDS-

860   PAGE demonstrate time-dependent cleavage of rm-CXCL1. Rm-CXCL1 were left alone

861   or treated with *Lm* lysate for the indicated time points. Rm-CXCL1 bands were enlarged

862   to show the cleavage products on right. (B) Indicated chemokines were treated with *Lm*

863   lysate for indicated time-points and subjected to SDS-PAGE followed by silver-staining.

864   (C) Hypothesized *L. major* cleavage site on rm-CXCL1 based on the observed cleaved

865   rm-CXCL1 bands in (A and B). Arrow denotes possible cleavage site on CXCL1 (D)

866   Identification of CXCL1 cleavage site by Mass Spectrometry analysis (raw data presented

867   in Supplemental Fig. 4). Arrow represents cleavage site on rm-CXCL1 targeted by *L.*

868   *major*. Data in A and B are representative of at least three independent experiments.

869

870   **Figure 6: *L. major* metalloprotease cleaves CXCL1.** (A) Recombinant CXCL1 was

871   incubated with or without *Lm* sup containing (B) Roche cOmplete protease inhibitor, or

872   (C) Sigma P8340 protease inhibitor for indicated time-points and subjected to CXCL1

873   quantification using ELISA. (D) Recombinant CXCL1 was treated with *Lm* sup in th

874   presence of (E) EDTA or (F) 1,10-Phenanthroline for indicated time-points and CXCL1

875   degradation determined by ELISA. (G) Recombinant CXCL1 was treated with *Lm* sup in

876   the presence of 1,10-Phenanthroline for indicated time-points and CXCL1 degradation

877   determined by silver staining. (H) *Lm* lysate was immunoprecipitated with GP63 antibody

878   and Western blot was carried out to evaluate the levels of GP63 protein. (I) The fractions

879   from (H) were incubated with rm-CXCL1 for indicated time-points and ELISA was

880   performed to quantify the levels of CXCL1. Data are representative of at least three

881   independent experiments. Results are represented as mean ± SEM. ***P<0.001,

882   ****P<0.0001.

883

884

885   **Figure 7: Synthetic peptide spanning rm-CXCL1 cleavage site inhibits *L. major*-**

886   **mediated CXCL1 degradation.** (A) Schematic of synthetic peptide generation.

887   Alignment of full length murine CXCL1 with mature murine CXCL1 demonstrating the

888   schematic for generation of peptide#1, peptide#2 and blocking peptide. Peptide#1 and

889   peptide#2 were generated from the N-terminal region of full length murine CXCL1.

890   Blocking peptide spanning the *L. major* cleavage site on murine CXCL1 were generated.

891   *Lm* lysate-mediated degradation of rm-CXCL1 in the presence of (B) Activity of synthetic

892   peptides from (A) in inhibiting L. major-mediated degradation of rm-CXCL1. (C) Model of

893   *L. major* metalloprotease function on CXCL1 during infection. Data are representative of

894   at least three independent experiments. Results are represented as mean $\pm$ SEM.

895   ****P<0.0001.

896   **Supplemental Figure 1: CXCL1, IL-6, and TNF secreted by LPS-activated BMDMs**

897   **are stable in cell culture conditions.** Conditioned supernatants from 24h LPS (20

898   ng/ml) stimulated BMDMs were collected and further cultured for 0, 24, or 48h and stability

899   of CXCL1, IL-6 and TNF in the culture were determined by ELISA. Data are representative

900   of at least three independent experiments. Results are represented as mean $\pm$ SEM.

901

902   **Supplemental Figure 2: Heat inactivation of *Lm* lysate or *Lm* sup rescues CXCL1**

903   **detection.** (A) Experimental Design. (B) Conditioned supernatants from 24h LPS (20

904   ng/ml) stimulated BMDMs were collected and subjected to 24h treatment with control or

905   boiled (20 min at 100ºC) *Lm* lysate or *Lm* sup. Levels of CXCL1, IL-6 and TNF were

906   determined by ELISA. Results are represented as mean $\pm$ SEM. ****P<0.0001.

907

908   **Supplemental Figure 3: *L. major* reduces rm-CXCL1 levels in culture.** Rm-CXCL1

909   were left alone or treated with *Lm* sup (control or boiled) for up to 48 hours in culture. The

910   quantity of rm-CXCL1 in the culture at 0, 24 and 48 h were determined by ELISA. Data

911   are representative of at least three independent experiments. Results are represented as

912   mean $\pm$ SEM.

913

914   **Supplemental Figure 4:** Acute time course of *L. major*-induced CXCL1 cleavage. Rm-

915   CXCL1 were left alone or treated with *Lm* lysate for acute time points as indicated to

916   determine the precise time course of rm-CXCL1 cleavage. Silver staining was used to

917   visualize rm-CXCL1. Data are representative of at least three independent experiments.

918

919   **Supplemental Figure 5: Identification of CXCL1 cleavage site and cleaved products.**

920   (A) A silver-stained SDS-PAGE showing cleavage of full length (BAND 1) and cleaved

921   rm-CXCL1 (BAND 2). (B) Mass spectrometry analysis of BAND 1 (full length CXCL1) and

922   tryptic peptide coverage analysis confirm a full length rm-CXCL1. (C) Mass spectrometry

923   analysis of BAND 2 (cleaved CXCL1) and tryptic peptide coverage analysis confirm a C-

924   terminal end cleavage after K65 residue.

925

926   **Supplemental Figure 6: Proteolytic activity of *L. major* on CXCL1 is not prevented**

927   **by Roche cOmplete Inhibitor or Marimastat** (A) Conditioned supernatants from 24h

928   LPS (20 ng/ml)-stimulated BMDMs were collected and treated with *Lm* sup in the

929   presence or absence of Roche cOmplete protease inhibitor. Levels of CXCL1, IL-6 and

930   TNF were determined by ELISA. (B) Rm-CXCL1 was treated with *Lm* sup in the presence

931   or absence of Marimastat (10μM) for indicated time-points and ELISA was performed to

932   evaluate the levels of CXCL1. Data are representative of at least three independent

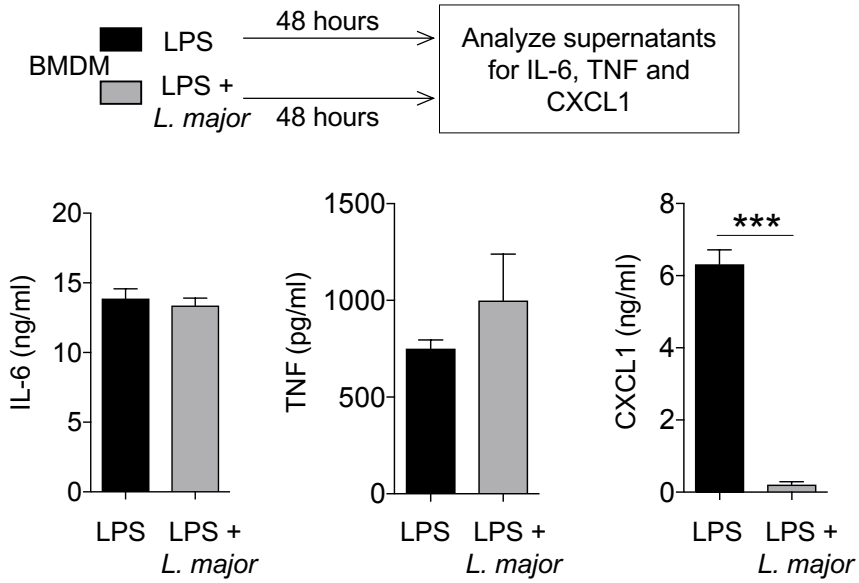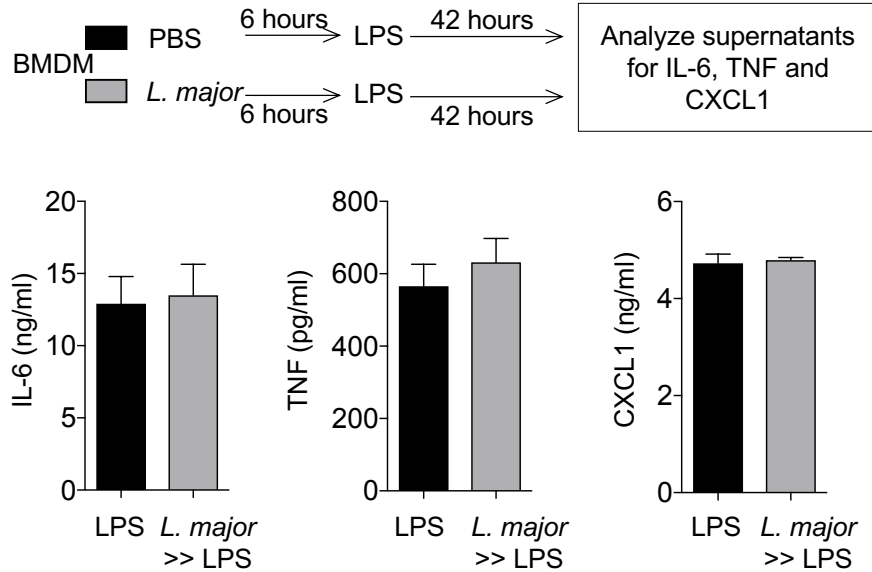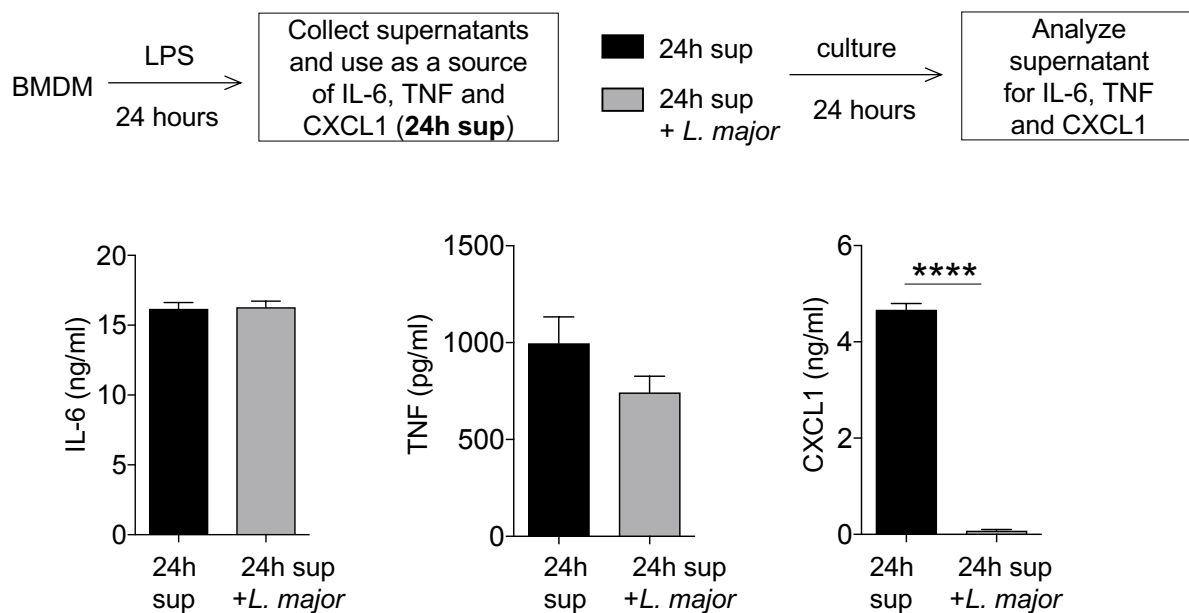933   experiments. Results are represented as mean $\pm$ SEM. ***P<0.001, ****P<0.0001.

934

A

BMDM
- LPS → 48 hours →
- LPS + *L. major* → 48 hours →

Analyze supernatants for IL-6, TNF and CXCL1

IL-6 (ng/ml) — LPS, LPS + *L. major*

TNF (pg/ml) — LPS, LPS + *L. major*

CXCL1 (ng/ml) — LPS, LPS + *L. major* — ***

B

BMDM
- PBS → 6 hours → LPS → 42 hours →
- *L. major* → 6 hours → LPS → 42 hours →

Analyze supernatants for IL-6, TNF and CXCL1

IL-6 (ng/ml) — LPS, *L. major* >> LPS

TNF (pg/ml) — LPS, *L. major* >> LPS

CXCL1 (ng/ml) — LPS, *L. major* >> LPS

Figure 1

A

BMDM →(LPS / 24 hours)→ Collect supernatants and use as a source of IL-6, TNF and CXCL1 (**24h sup**)

■ 24h sup
□ 24h sup + *L. major*

→(culture / 24 hours)→ Analyze supernatant for IL-6, TNF and CXCL1

IL-6 (ng/ml) — 24h sup, 24h sup +*L. major*

TNF (pg/ml) — 24h sup, 24h sup +*L. major*

CXCL1 (ng/ml) — 24h sup, 24h sup +*L. major* ****

B

BMDM →(LPS / 24 hours)→ Collect supernatants and use as a source of IL-6, TNF and CXCL1 (**24h sup**)

■ 24h sup
■ 24h sup + *Lm*
□ 24h sup + *Lm Lysate*
□ 24h sup + *Lm* sup

→(culture / 24 hours)→ Analyze supernatant for IL-6, TNF and CXCL1

IL-6 (ng/ml) — Media, *Lm*, *Lm Lysate*, *Lm* sup

TNF (pg/ml) — Media, *Lm*, *Lm Lysate*, *Lm* sup

CXCL1 (ng/ml) — Media, *Lm*, *Lm Lysate*, *Lm* sup ****/****/****

Figure 2

Figure 3

A

■ *Lm Lysate*

■ rm-CXCL1

■ rm-CXCL1 + *Lm Lysate*

culture
⟶
24 hours

Analyze supernatant for CXCL1 degradation (**shown in B**) and use these as a source for BMDM stimulation in **C - E**.

B

rm-CXCL1 (pg/ml)

****

Media + rm-CXCL1

Lm Lysate + rm-CXCL1

C

Cxcl1 (fold induction)

Time (min)

D

Tnf (fold induction)

Time (min)

E

Il6 (fold induction)

Time (min)

■ BMDM + *Lm Lysate*
● BMDM + rm-CXCL1
▲ BMDM + rm-CXCL1 + *Lm Lysate*

Figure 4

A

MW (KDa) — Marker, rm-CXCL1, rm-CXCL1 + *Lm Lysate* (1, 2, 4, 12h), *Lm lysate*

250
150
100
75
50
37
25
20
15
10

rm-CXCL1 bands

rm-CXCL1 · rmCXCL1 + *Lm Lysate* (1h, 2h, 4h, 12h)

Full length rm-CXCL1 (Upper band)

Cleaved rm-CXCL1 (lower band)

B

MW (KDa) — Marker

rm-CXCL1 + *Lm Lysate* — 0, 1, 2h
rm-CXCL2 + *Lm Lysate* — 0, 1, 2h
rh-CXCL1 + *Lm Lysate* — 0, 1, 2h
rh-CXCL2 + *Lm Lysate* — 0, 1, 2h

250
150
100
75
50
37
25
20
15
10

rm-CXCL1 · rm-CXCL2 · rh-CXCL1 · rh-CXCL2

C

rm-CXCL1

NH₂ ——————— COOH

N-terminal cleavage
NH₂ ——————— COOH

C-terminal cleavage
NH₂ ——————— COOH

LM protease possible cleavage site

D

Full length rm-CXCL1

APIANELRCQCLQTMAGIHLKNIQSLKVLPSGPHCTQTEVI
ATLKNGREACLDPEAPLVQKIVQKMLKGVPK

Cleaved rm-CXCL1

APIANELRCQCLQTMAGIHLKNIQSLKVLPSGPHCTQTEVI
ATLKNGREACLDPEAPLVQKIVQK

Figure 5

Figure 6

A

Full length m-CXCL1 MIPATRSLLCAALLLLATSRLATGAPIANELRCQCLQTMAGIHLKNIQSLKVLPSGPHCTQTEVIATLKNGREACLDPEAPLVQKIVQKMLKGVPK
rm-CXCL1   - APIANELRCQCLQTMAGIHLKNIQSLKVLPSGPHCTQTEVIATLKNGREACLDPEAPLVQKIVQKMLKGVPK
Peptide 1   - - - - - - - - -CAALLLLATSRLA- -
Peptide 2   - - - - - - - - - - - - - - - - - - -RLATGAPIAN- -
Blocking Peptide -LVQKIVQKMLKGVPK

Full length m-CXCL1 LCAALLLLATSRLATGAPIANELR   KNGREACLDPEAPLVQKIVQKMLKGVPK
Rm-CXCL1   - - - - - - - - - - - - -APIANELR   KNGREACLDPEAPLVQKIVQKMLKGVPK
Peptide 1   -CAALLLLATSRLA- - - - - - - - - -   -
Peptide 2   - - - - - - - - - -RLATGAPIAN- - -   -
Blocking Peptide -   - - - - - - - - - -LVQKIVQKMLKGVPK

B

C

Figure 7

A

BMDM $\xrightarrow[\text{24 hours}]{\text{LPS}}$ Collect supernatants and use as a source of IL-6, TNF and CXCL1 (**24h sup**)
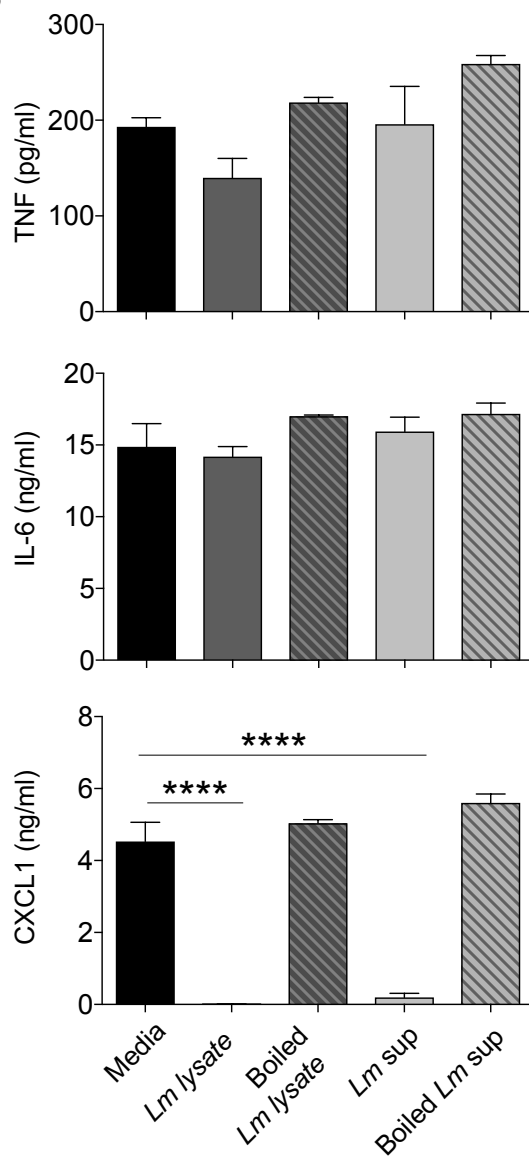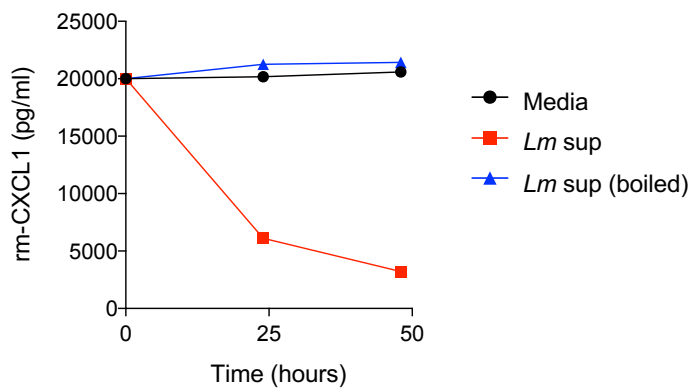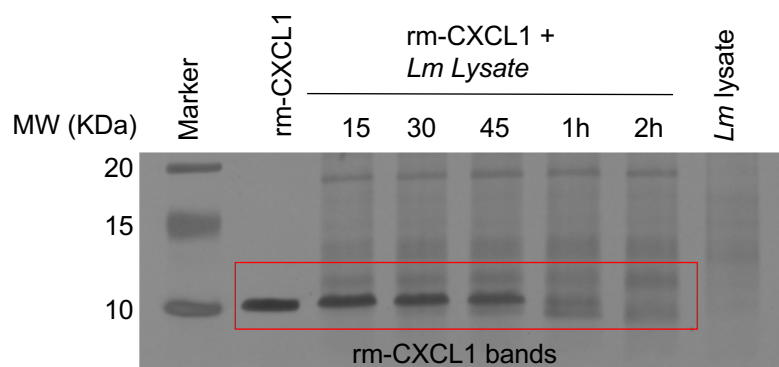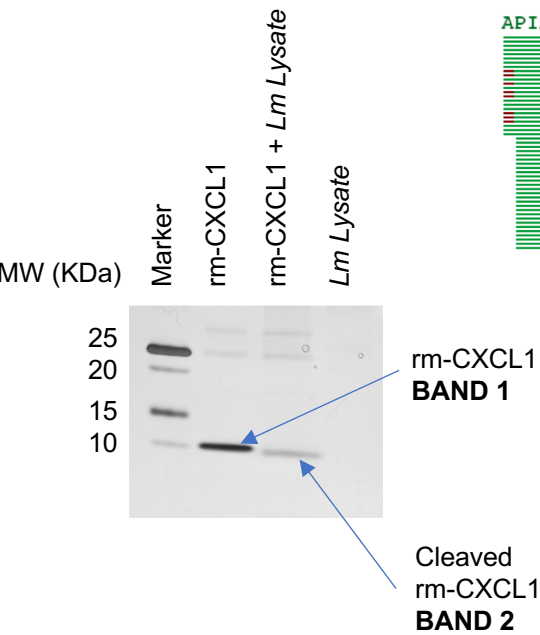
■ 24h sup

■ 24h sup + *Lm Lysate*

▨ 24h sup + Boiled *Lm Lysate*

▨ 24h sup + *Lm* sup

▨ 24h sup + Boiled *Lm* sup

B

Supplemental Figure 2

A

MW (KDa)

Marker
rm-CXCL1
rm-CXCL1 + *Lm Lysate*
Lm Lysate

25
20
15
10

rm-CXCL1
BAND 1

Cleaved
rm-CXCL1
BAND 2

B

BAND 1 – peptide reads for rm-CXCL1

>tr|A2RTH0-2|A2RTH0-2_MOUSE C-X-C motif chemokine minus first 24 AA OS=Mu

APIANELRCQCLQTMAGIHLKNIQSLKVLPSGPHCTQTEVIATLKNGREACLDPEAPLVQKIVQKMLKGVPK

C

BAND 2 – peptide reads for rm-CXCL1

>tr|A2RTH0-2|A2RTH0-2_MOUSE C-X-C motif chemokine minus first 24 AA OS=Mu

APIANELRCQCLQTMAGIHLKNIQSLKVLPSGPHCTQTEVIATLKNGREACLDPEAPLVQKIVQKMLKGVPK

Supplemental Figure 5

A

BMDM → LPS / 24 hours → Collect supernatants and use as a source of IL-6, TNF and CXCL1 (**24h sup**)

■ 24h sup
▨ 24h sup + *Lm* sup
▨ 24h sup + *Lm* sup + Roche cOmplete Protease Inhibitor (PI)

B