1 **Guanylate-binding Protein 1 (GBP1) Contributes to the Immunity of Human**

2 **Mesenchymal Stromal Cells against *Toxoplasma gondii***

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35   version.

36

38

Abstract

Mesenchymal stromal cells (MSCs) have recently been shown to play important roles in mammalian host defenses against intracellular pathogens, but the molecular mechanism still needs to be clarified. We confirmed that human MSCs (hMSCs) pre-stimulated with IFN-γ showed a significant and dose-dependent ability to inhibit the growth of two types of *Toxoplasma gondii* (type I strain RH/GFP or type II strain PLK/RED). However, in contrast to previous reports, the anti-*T. gondii* activity of hMSCs was not mediated by indoleamine 2,3-dioxygenase (IDO). Genome-wide RNA-seq analysis revealed that IFN-γ increased the expression of the p65 family of guanylate-binding proteins (hGBPs) in hMSCs, especially hGBP1. To analyze the functional role of hGBPs, stable knockdowns of hGBP1, -2, -5 in hMSCs were established using a lentiviral transfection system. hGBP1 knockdown in hMSCs resulted in a significant loss of the anti-*T. gondii* host defense property, compared with hMSCs infected with non-targetted control sequences. hGBP2 and -5 knockdowns had no effect. Moreover, the hGBP1 accumulation on the parasitophorous vacuole (PV) membranes of IFN-γ-stimulated hMSCs might protect against *T. gondii* infection. Taken together, our results suggest that hGBP1 plays a pivotal role in anti-*T. gondii* protection of hMSCs and may shed new light on clarifying the mechanism of host defense properties of hMSCs.

3

57   **Significance**

58   Mesenchymal stem cells (MSCs) are thought to be derived from pericytes and exhibit a

59   cellular, autonomous antimicrobial effector function which provides therapeutic potential

60   against infectious diseases. However, the molecular mechanism remains unknown. Here, we

61   demonstrate that human guanylate-binding protein 1 (hGBP1) is a key protective factor

62   against *T. gondii* infection in human MSCs (hMSCs). The recruitment of hGBP1 to the

63   parasitophorous vacuole membrane in IFN-γ-stimulated hMSCs significantly inhibited *T.*

64   *gondii* replication. Thus, our current study reveals an important function of hGBP1 in the

65   defense against *T. gondii* and may shed new light on clarifying the mechanism of host

66   defense properties of hMSCs.

67

68 **/body**

69 Mesenchymal stromal cells (MSCs) comprise a heterogeneous cell population endowed with

70 multi-lineage differentiation potential and extensive immunomodulatory properties. MSCs

71 have been successfully used to prevent and treat immune disorders, such as graft-versus-host

72 disease, and emerging preclinical studies suggest that they might also protect against

73 infectious challenges (1, 2). Recent studies showed that MSCs are located in the perivascular

74 niche and constitute a subset of pericytes that are involved in both pathogen recognition and

75 early inflammatory events (3). MSCs appear to impede pathogen growth and reduce the

76 microbial burden by inhibiting growth through soluble factors or by enhancing the

77 antimicrobial function of immune cells, as shown both *in vitro* and *in vivo* (2-5). For example,

78 Nemeth et al. reported that mouse MSCs (mMSCs) prolonged the survival of septic mice and

79 improved their organ (kidney, liver, and pancreas) functions (5). They did this by enhancing

80 IL-10 production from murine alveolar macrophages via MSC-secreted cyclooxygenase-2

81 (COX2) and prostaglandin E2 (PGE2) (5). Data from murine colitis models have shown that

82 human adipose-derived MSCs protect against dextran-induced colitis by decreasing the

83 secretion of pro-inflammatory cytokines and chemokines (6). However, the antimicrobial

84 effector molecules in vertebrate MSCs are not universally the same (4-11). The antimicrobial

85 effect of unstimulated hMSCs is mediated by the cathelicidin, LL-37 (4), as shown both *in*

86 *vitro* and *in vivo*. In IFN-γ-stimulated hMSCs, by contrast, the antibacterial effect is mediated

87 through the tryptophan-catabolizing enzyme, indoleamine 2,3-dioxygenase (IDO) (9).

88 Conflicting results are also reported in mouse, in which the decision as to whether mMSCs

89 increase the activity of phagocytes or not depends on the origin of these cells (11).

90   *Toxoplasma gondii* is an obligatory intracellular protozoan parasite that infects virtually all

91   warm-blooded vertebrates including humans. Clinical symptoms are rarely observed in most

92   *T. gondii*-infected immunocompetent individuals. However, the parasite can cause severe

93   disease and even death in immunosuppressed individuals, such as AIDS, cancer and organ

94   transplantation patients. *T. gondii* can actively invade host cells *in vitro* by dividing within a

95   nonfusogenic parasitophorous vacuole (PV), a membrane structure formed during invasion

96   that is maintained to surround the intracellular replicating parasites. However, this activity

97   may not be completed *in vivo* due to the innate resistance mechanisms in host cells and,

98   especially, in those that are naturally resistant to *T. gondii* (12).

99   During *T. gondii* infection, NK cells, neutrophils, CD4+ cells and CD8+ T cells can all

100   release IFN-γ, which is the central regulator of the immune response against *T. gondii* (12-14).

101   In mouse cells, the most important IFN-γ-inducible effectors against *T. gondii* are likely to

102   include inducible nitric oxide synthase (iNOS) (15), reactive oxygen species (ROS) (16),

103   immunity-related p47 GTPases (IRGs) (17), and GBPs (18). Mice lacking a fragment of

104   chromosome 3 that encodes GBP1, -2, -3, -5, -7, and -2ps were highly susceptible to *T. gondii*

105   infection even after stimulation of IFN-γ (18). This indicates the importance of GBPs in

106   immunity to *T. gondii* and providing insight into the antimicrobial effects of IFN-γ (18). It has

107   been confirmed that members of the GBP family, namely GBP1, -6, -7 and -10, all play a key

108   role in IFN-γ-mediated cell-autonomous immunity against bacterial infection and GBP1, in

109   particular, is essential for function in macrophage cell lines (19). However, IFN-γ-mediated

110   immunity to intracellular pathogens appears to be cell type-specific and occurs in a

111   species-specific manner. IFN-γ stimulated human monocytes and mouse macrophages are

112   able to produce high levels of ROS to kill the parasite (15, 16). However, ROS production is

113   not induced in *T. gondii*-infected human macrophages (20). Human fibroblasts can also

114   display IFN-γ-dependent cell-autonomous immunity against *T. gondii*, although the

115   involvement of IDO remains controversial (21, 22). Thus, data from animal models may not

116   directly apply to human toxoplasmosis and the nature/relevance of innate immunity against *T.*

117   *gondii* infection in humans is much less well understood. It is, therefore, useful to understand

118   the fate of hMSCs (an important cell source for tissue/organ recovery) in *T. gondii* infection

119   (23). In addition, the molecular mechanisms through which hMSCs augment

120   anti-toxoplasmosis remain unclear.

121   To elucidate the functional contribution of human MSCs to host defense against *T. gondii*,

122   we conducted a detailed analysis of gene expression in hMSCs before and after IFN-γ

123   stimulation using Genome-wide RNA-seq, and generated hGBP1-, hGBP2- and

124   hGBP5-knockdown hMSC cell lines. hGBP1, but not other hGBPs or IDO knockdown,

125   partially restored the IFN-γ-dependent anti-*T. gondii* response in hMSCs. Moreover, we

126   observed a significant increase in colocalization of endogenous hGBP1 in the PV membrane

127   of *T. gondii*-infected cells. These data clearly show that hGBP1 is associated with the

128   replication of intracellular *T. gondii* upon IFN-γ stimulation, suggesting an important function

129   for hGBP1 in the defense against *T. gondii*.

130

131   **Results**

132   **The growth of *T. gondii* in human MSCs is dose-dependently inhibited by IFN-γ**

133   **pretreatment.** First, we tested the effects of IFN-γ-stimulated human MSCs on *T. gondii*

134   growth. Human MSCs were pretreated with various concentrations of IFN-γ for 48 hr and

135   then infected with two *T. gondii* strains (type I strain RH/GFP and type II strain PLK/RED),

136   respectively. The fluorescence-labeled intracellular parasites were counted microscopically at

137   multiple time points post-infection, and the inhibition rates of *T. gondii* growth were

138   calculated. The growth of *T. gondii* was found to be dose-dependently inhibited by IFN-γ

139   pretreatment. In addition, along with IFN-γ incubation time, the inhibition rate also

140   significantly increased (Fig. 1*A*). Interestingly, the same concentrations of IFN-γ had stronger

141   inhibitory effects on the PLK/RED strain than on the RH/GFP strain (Fig. 1*B*). Furthermore,

142   the number of parasites per vacuole at 24 hr post-infection (PLK/RED, Fig. 1*C*) and the ratio

143   between parasite rosettes and single parasites at 48 hr post-infection (PLK/RED, Fig. 1*D*)

144   were significantly lower in IFN-γ-treated hMSCs than in untreated hMSCs (RH/GFP, SI Fig.

145   1*A* and *B*). Representative fluorescence images of intracellular *T. gondii* growth at 48 hr

146   post-infection are shown in Fig. 1*E* (PLK/RED) and SI Fig. 1*C* (RH/GFP). To exclude the

147   possibility that the presence of IFN-γ in the culture medium could have a direct effect on

148   parasites, we assessed the effect of conditioned medium (CM) obtained from

149   IFN-γ-stimulated MSCs (at 48 hr post-infection), on *T. gondii* infection. The CM from

150   pretreated cultures did not have any significant effect on the ability of *T. gondii* to invade

151   hMSCs or to replicate, as compared with CM from unstimulated hMSCs (RH/GFP, $p = 0.34$;

152   PLK/RED, $p = 0.45$; SI Fig. 2). These results strongly suggest that human MSCs act against *T.*

153   *gondii* using some intracellular products that can be induced by IFN-γ.

154

155   **The anti-*toxoplasma* activity of human MSCs is not associated with IDO.** Previous

8

156   studies showed that in some human cell types (e.g., HeLa cells), IFN-γ stimulation inhibits *T.*

157   *gondii* growth by depletion of tryptophan (Trp), via the activity of IDO (22, 24). To assess

158   whether inhibiting the IDO activity could affect the proliferation of *T. gondii* in

159   IFN-γ-stimulated MSCs, we tested the effects of three IDO-specific inhibitors (DL-1 MT,

160   D-1 MT and L-1 MT) or excess amounts of Trp, respectively. Our results revealed that both,

161   inhibiting the IDO activity by 1-methyl-D/L-tryptophan and adding Trp, significantly

162   decreased the antimicrobial effect against *T. gondii* in IFN-γ-stimulated HeLa cells ($p < 0.001$)

163   (Fig. 2*A*), confirming that Hela cells rely on IDO-mediated degradation of Trp for their ability

164   to resist *T. gondii* infection. In human MSCs, in contrast, Trp and all three different IDO

165   inhibitors failed to rescue both strains of the parasite from IFN-γ-stimulated growth inhibition

166   (Fig. 2*A* and 2*B*).

167   To further confirm these observations, we designed siRNAs which targeted three different

168   regions of the IDO mRNA. Based on the RT-PCR results, we confirmed that they

169   significantly decreased the expression of IDO stimulated by IFN-γ (by ~90% in hMSCs

170   treated with siIDO 1 and siIDO 3) (Fig. 2*C*). As shown in Fig. 2*D*, siRNA-mediated IDO

171   knockdown also could not rescue the parasite from IFN-γ-mediated growth inhibition. These

172   results suggest that the ability of human MSCs inhibiting the growth of *T. gondii* does not

173   depend on Trp deprivation or IDO activity. This is in contrast to the results presented from a

174   previously reported study (9).

175

176   **IFN-γ-inducible p65 guanylate-binding proteins (hGBPs) are significantly upregulated**

177   **in MSCs.** To examine the possible mechanism of activity of human MSCs against *T. gondii*

178 infection, we performed genome-wide RNA-seq to assess the changes in the expression of

179 host defense related genes of MSCs treated with recombinant IFN-γ (20 ng/mL). Six

180 sequencing libraries were constructed (three samples from each group). On average, more

181 than 18 million 50-bp reads were obtained, of which more than 70% of the reads were

182 aligned to unique locations in the genome, and included more than 17,000 coding genes from

183 each library. The global transcriptional profiling data will be published elsewhere. MSCs

184 from three different donors expressed a series of host defense related factors. Through gene

185 ontology (GO) analysis, we identified 106, 53, 12 and 9 expressed genes (Reads per kilobase

186 pairs per million (RPKM) value > 5) that were associated with defense responses to viral,

187 bacterial, protozoan and fungal infections, respectively (SI Fig. 3). Previous reports have

188 demonstrated that mouse GBPs are involved in *T. gondii* growth inhibition (18, 25, 26).

189 Accordingly, we analyzed the expression of GBP family members in human MSCs before

190 and after IFN-γ treatment. As shown in supplementary Figure S4, the RPKM values for

191 hGBP1, -2, -3, -4 and -5 significantly increased, qRT-PCR results further showed that the

192 expression levels of hGBP1, -2, -4 and -5 were increased up to 100-fold following IFN-γ

193 treatment (Fig. 3*A*). The relative abundances of the mRNAs for hGBP1, -2 and -5 compared

194 with β-actin were significantly higher than that of hGBP3 and hGBP4 (Fig. 3*B*). Western

195 blotting revealed that the protein expression levels of hGBP1, -2 and -5 were greatly

196 increased after IFN-γ stimulation (Fig. 3*C*). These top three most highly expressed hGBPs

197 (hGBP1, -2 and -5) were selected for further testing for their potential roles in human MSC

198 activity against *T. gondii* infection .

199   In mice, MSCs rely on nitric oxide (NO) produced by iNOS to protect themselves (9).

200　Interestingly, we found that the expression of iNOS (NOS2) and human IRG genes were

201　close to zero in MSCs both before and after stimulation with IFN-γ. This was also the case

202　for the constitutive NOS (NOS1 and NOS3) genes, further supporting the notion that iNOS

203　shows no increase in expression following stimulation. Using reverse transcriptase

204　quantitative PCR and western blotting, we also observed no iNOS expression in human

205　MSCs. To further confirm these results, using the iNOS-specific inhibitor (N^GMMA), we

206　revealed that inhibiting the iNOS activity did not influence the action of MSCs against *T.*

207　*gondii* (SI Fig. 5). Taken together, our results indicate that the ability of human MSCs to

208　inhibit the growth of *T. gondii* does not depend on iNOS.

209

210　**Generation of hGBP knockdowns in human MSCs.** Stable hGBP1-, hGBP2- and

211　hGBP5-knockdown hMSC cell lines were generated using specific short hairpin RNA

212　(shRNA) transcription cassettes and confirmed by RT-PCR (Fig. 4*A* and 4*B*) using

213　gene-specific primers (SI Table 1) and Western blotting (Fig. 4*C*). To test the characteristics

214　of the three kinds of shGBP-MSCs, FACS was used to analyze hMSC surface markers.

215　Compared with wild-type human MSCs, all transduced cells expressed the same panel of

216　surface markers, exhibiting positive expression of CD29, CD44, CD73, CD90, D166 and

217　CD105, while detectable expression of the hematopoietic stem cell markers CD34 and CD45

218　was not found. Representative FACS results for shCtrl- and shGBP1-MSCs are shown in SI

219　Fig. 6*A*. Our results clearly indicated that transduced cells maintained the cell-surface marker

220　phenotype of human MSCs.

221　　To demonstrate that these cells still maintain their multipotency, they were cultured under

222   conditions that could promote their differentiation into osteogenic or adipogenic lineages.

223   Following culture in osteogenic medium for 2 weeks, the shGBP-MSCs were induced to

224   differentiate into osteoblasts as confirmed by strong Alizarin Red S staining (SI Fig. 6*B*).

225   Similarly, oil red O staining revealed the presence of lipid droplets in the cytoplasm of

226   shGBP-MSCs on day 21 after adipogenic differentiation (SI Fig. 6*C*). The knockdown of

227   hGBPs did not alter the proliferative properties of human MSCs, which was verified by

228   CCK8 assays ($p = 0.37$, SI Fig. 6*D*). Cell survival analysis showed that the cells cultured with

229   serum-deprived conditions for 48 hours only produced a mild, non-significant, increase in the

230   death rate which was similar to that found in shGBP1-MSCs (7.82% ± 0.94%) and

231   shCtrl-MSCs (7.31% ± 0.73%; $p = 0.21$) (Fig. 4*D*). Similar features of sustained

232   multipotency were also observed in shGBP2- and shGBP5-MSCs. These results

233   demonstrated that the osteogenic and adipogenic differentiation, proliferation and survival

234   potential of shGBP-MSCs showed no observed differences from the control human MSCs.

235

236   **hGBP1 is a key factor for inhibition of *T. gondii* growth in human MSCs stimulated by**

237   **IFN-γ.** To investigate whether GBP family members contribute to the IFN-γ induced

238   antimicrobial effect of MSCs, we examined the effect of GBP knockdown in MSCs on the

239   growth of *T. gondii*. Remarkably, compared to control cells, inhibition of *T. gondii* growth

240   was rescued in shGBP1-MSCs (Fig. 5*A*, Upper, RH/GFP; Lower, PLK/RED) at 24 hr

241   post-infection. Specifically, using the RH/GFP and PLK/RED strains, we found the inhibition

242   rates of *T. gondii* growth in MSCs at 5 and 20 ng/ml of IFN-γ were significantly reduced by

243   shGBP1, compared with control MSCs (Fig. 5*B*). In contrast, there was no obvious difference

244   between control cells and shGBP2- or shGBP5-MSCs at 24 hr post-infection (Fig. 5*D*). Thus,

245   our studies demonstrate that hGBP1 may play a major role in inhibiting *T. gondii* growth in

246   IFN-γ stimulated human MSCs, in contrast to hGBP2 and hGBP5 that do not.

247

248   **Human GBP1 is recruited to the parasitovorous vacuole during *T. gondii* infection.**

249   Recruitment of mouse GBPs to the PV was reported to be essential for the replication control

250   of *T. gondii* (18, 25, 26). To explore the role of hGBP1 in human MSCs, we used

251   immunofluorescence to assess its intracellular localization after *T. gondii* infection.

252   Endogenous hGBP1 was observed in ~10% of vacuoles belonging to IFN-γ-stimulated MSCs

253   infected with RH/GFP strain at 3 hr post-infection, but only 3% in the corresponding

254   non-stimulated MSCs (Fig. 6*A*). A significantly higher percentage of PVs staining positive for

255   hGBP1 was found in the cells infected with PLK/RED strain (Fig. 6*B*). This could potentially

256   explain the stronger inhibitory effect of MSCs on PLK/RED versus RH/GFP strain.

257   Collectively, these data indicated that the accumulation of hGBP1 on the PV membranes of

258   IFN-γ-stimulated hMSCs could significantly inhibit the replication of *T. gondii*.

259

Discussion

260

261   As an obligatory intracellular protozoan parasite, *T. gondii* is protected by the plasma

262   membrane of the host cell, and is thus able to escape from most antibody-based immune

263   responses (12). Therefore, the host's innate immune response is largely responsible for

264   controlling *T. gondii* infection. Although numerous studies have demonstrated that *T. gondii*

265   can grow in various somatic cells from warm-blooded animals and humans *in vitro*, the

266   related disease condition is not commonly found *in vivo*. Previous studies have shown that an

267   IFN-γ-mediated response against *T. gondii* infection appears to act in a cell type-specific and

268   species-specific manner, e.g. non-lymphocytes shown in SI Fig. 7. In mice, astrocytes can use

269   IRGs to restrict *T. gondii* infection; mMSCs use iNOS to produce NO; and skeletal muscle

270   cells use both mechanisms (27). The mGBPs may also critically contribute to fighting *T.*

271   *gondii* replication *in vivo* (18).

272   However, many studies in animal models show significant differences from humans, and

273   vast differences have been observed between immune and somatic cells of patients infected

274   with *T. gondii* (14-22). High IDO activity which could result in the depletion of Trp is

275   considered a key weapon against *T. gondii* growth in human fibroblasts treated with IFN-γ,

276   but it does not appear to occur in mouse fibroblasts (20, 28, 29). Human MSCs treated with

277   IFN-γ were found to exhibit an IDO-mediated antimicrobial effector function against various

278   pathogens suggesting a similar mechanism which might be used to curtail *T. gondii* (9). Our

279   results, however, have indicated that IDO is not a main factor in the ability of human MSCs

280   to inhibit the growth of *T. gondii* (Fig. 2*D*).

281   The iNOS-mediated production of NO is a well-known molecular effector against

14

282   pathogens in the innate immune responses of various animals, including mMSCs (9), skeletal

283   muscle cells (27), and also *in vivo* (15, 30, 31). However, a previous study reported that iNOS

284   protein expression was not detectable in human MSCs (9). Consistent with these results, our

285   data also showed that MSCs derived from IFN-γ-stimulated human bone-marrow neither

286   express any detectable level of iNOS RNA nor inhibition by any iNOS activity. Therefore, it

287   is highly possible that NO is not the main molecular effector against replication of *T. gondii*

288   in human MSCs stimulated by IFN-γ.

289   It has been reported that an IRG protein-based resistance mechanism is essential to control

290   *T. gondii* replication in mice (17, 27). Numerous IRGs were found to converge on vacuoles

291   containing *T. gondii* in IFN-γ-activated mouse macrophages, fibroblasts, and astrocytes

292   (32-34). IRGm in mouse is a membrane marker for host cell self-recognition that is used to

293   regulate the IRG pathway (35) and other IRGs such as the glycine-lysine-serine motif

294   containing IRGs (also known as GKSs). Mouse IRGs were found to cooperate with mGBPs

295   in acting against *T. gondii* infection (13, 14, 18). These findings suggested that mGBPs and

296   mIRGs could play important roles in the IFN-γ-dependent, autonomous cellular control of

297   toxoplasmosis. Furthermore it can be predicted that these protein families could play broader

298   roles in the host defense systems of murine cells. To our surprise, however, only

299   one complete IRG (hIRGm) is present in the human genome and it lacks the necessary IFN-γ

300   induction element (33). In fact, based on our work, we also did not observe the up-regulation

301   of any hIRGs in our RNA-seq experiments. Our data provide further evidence to suggest that

302   hIRGm has little (if any) activity against *T. gondii* infection in humans at least in the case of

303   hMSCs. Without the cooperation of hIRGm, hGBPs, if present, may be sole contributors to

15

304 anti-toxoplasmosis activity.

305 In a similar manner to mIRGs, mGBPs belong to the IFN-inducible GTPase family. Kim et

306 al. reported that mGBPs (at least mGBP1 and mGBP7) participated in antibacterial responses

307 by recruiting host defense proteins, including phagocyte oxidase, antimicrobial peptides, and

308 autophagic effectors within macrophages and mGBP1$^{-/-}$ mice (19). A recent report showed

309 that mGBP2 and mGBP5 could promote bacteriolysis by controlling the activation of the

310 inflammasome protein, AIM2, in mouse bone marrow–derived macrophages (36). In human

311 cells, hGBP1 shows one of the highest induction levels in response to IFN-γ stimulation (37).

312 This has been shown to be associated with viral infections, inflammatory bowel diseases,

313 glioblastoma and cutaneous lupus erythematosus (38-41).

314 In the case of toxoplasmosis, previous studies demonstrated that mGBP1, -2, -3, -6, -7, and

315 -9 were recruited to *T. gondii*-containing PVs in IFN-γ-activated mouse embryonic fibroblasts

316 or RAW 264.7 macrophages (42). Recently, a mGBP cluster on chromosome 3 (comprising

317 the genes encoding mGBP1, -2, -3, -5, and -7) was found to cooperate with mIRGb6 and

318 mIRGb10 during toxoplasmosis (18). Mice defective in p65 GTPases were highly susceptible

319 to *T. gondii* infection and GBPs-deleted in chromosome 3 aneuploid macrophages were

320 defective in IFN-γ-mediated inhibition of intracellular parasite growth (18). The recruitment

321 of mGBP2 to *T. gondii*-containing PVs is essential for a cell's ability to control *T. gondii*

322 replication (25). Furthermore, recruitment of mGBP1 to PVs is associated with vesiculation

323 and rupture and thus also protects against *T. gondii* infection (26). Both mGBP1 and mGBP2

324 have been shown to form tetramers and may also hetero-oligomerize to cooperatively activate

325 GTPase activity (25).

326   The hGBP1, -2 and -3 proteins are paralogs of mGBP1 and mGBP2, and both hGBP1 and

327   mGBP2 show isoprenylation, which targets proteins to intracellular membranes and/or

328   facilitates protein/protein interactions (43). Such findings suggest that these GBPs may play

329   similar roles in protecting human and mouse cells against *T. gondii* infection. Our present

330   data clearly show that hGBP1 is recruited to *T. gondii* PVs (Fig. 6*A* and 6*B*) and provides

331   additional evidence that hGBP1 plays a role similar to that of mGBP1/2. Interestingly, our

332   RNA-seq and RNAi data showed that hGBP1, not hGBP2, is the main responder in

333   IFN-γ-stimulated human MSCs. Thus, hGBP1 is unlikely to interact with hGBP2 to execute

334   this protective function. The third paralog, hGBP3, is also not likely to be a main effector, as

335   its depletion showed little change. Thus, hGBP1 in human MSCs stimulated by IFN-γ could

336   be a functional homodimer in the innate immune response.

337   Recently, Haldar et al. reported that the deposition of mGBPs on *T. gondii*-containing

338   vacuoles needed IRG protein-dependent ubiquitination of PVs after priming their host cells

339   with IFN-γ (44). The recruitment of hGBPs to the membranes of *T. gondii*-containing PVs is

340   regulated by the autophagic protein, ATG16L1, in IFN-γ-stimulated human

341   fibroblast-like (HAP1) cells (45). Here, using both the non-cyst and cyst forming strains

342   (RH/GFP and PLK/RED) of *T. gondii* (46), we observed the recruitment of endogenous

343   hGBP1 to *T. gondii*-containing PVs at 3 hr post-infection in IFN-γ-stimulated human MSCs

344   (Fig. 6*A* and 6*B*). The signaling pathway that is responsible for recruiting hGBP1 to act

345   against *T. gondii* is not yet fully understood. Future studies are needed to examine whether

346   hGBPs contribute directly to membrane blebbing and the physical disruption of the PV

347   membrane and/or if they modulate the function of other effectors at this interface.

348   In summary, we herein define hGBP1 as an important effector molecule in the

349   IFN-γ-triggered autonomous resistance of human MSCs against *T. gondii* infection. Our

350   studies reveal that non-lymphocytes could apply various mechanisms of IFN-γ-triggered

351   autonomous resistance against *T. gondii* infection in a cell type-specific and species-specific

352   manner. Human MSCs offer an excellent example of a somatic cell type that plays a very

353   important role in the host defense system by providing protection against intracellular

354   pathogens such as *T. gondii*. The emerging involvement of MSCs in hematopoiesis and

355   antimicrobial responses suggest that they are worthy of further development in the context of

356   novel cellular antimicrobial therapies aimed at reducing infection-related morbidity and

357   mortality, particularly in immunocompromised patients.

358

359

Materials and Methods

Cell Culture and Parasite Infection. Human cells were isolated and/or cultured as described: MSCs (47), human foreskin fibroblasts (HFFs)(48) and HeLa cells (22). Cell proliferation and cell survival assays were performed as described previously (49). Tachyzoites of *T. gondii* RH/GFP (50) and PLK/RED (51) were allowed to naturally egress from the host cells, and were then quickly harvested using standard procedures (15). The effects of IFN-γ-treated human MSCs, non-treated human MSCs or conditioned medium (CM) on the growth of *T. gondii* (moi = 0.5) were assessed by microscopic counting at different time points post-infection (18).

Small Interfering RNA and Short Hairpin RNA Knockdown. *In vitro* silencing of IDO in hMSCs was performed as described (47), using three sets of IDO-siRNAs (SI Table 1). The short hairpin RNA (shRNA) expression vector, pLKOpuro1 (Addgene, USA) was used to express the control (shCtrl) and hGBP1, -2 and -5-specific shRNAs using specific oligos (SI Table 1). The helper plasmids, psPAX2 and pMD2.G, were used as lentiviral expression vectors (Addgene, USA). 293T host cells were used for packaging of the shRNAs. The MISSION Non-Target pLKOpuro1 control vector (Sigma-Aldrich, USA) was used as a control (shCtrl). Pools of stably transduced cells were selected using puromycin (2 μg/ml) for 5-7 days. All experiments were performed in puromycin-free media, and each knockdown was validated by RT-PCR and Western blotting.

Other methods used in this paper can be found in the Supplementary Information.

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522

Figure Legends

524 **Fig. 1. IFN-γ-stimulated human MSCs dose-dependently inhibit the growth of *T. gondii*.**

525 Human MSCs were pretreated for 48 hr with IFN-γ and then infected with *T. gondii*

526 (RH/GFP or PLK/RED). (*A* and *B*) *T. gondii* growth inhibition rates were calculated at 6, 12

527 and 24 hr post-infection (*A*, RH/GFP; *B*, PLK/RED). (*C*) At 24 hr post-infection, the number

528 of parasites per vacuole in IFN-γ (20 ng/ml)-stimulated hMSCs and unstimulated hMSCs

529 (controls) were calculated (PLK/RED). (*D*) At 48 hr post-infection, ratios of intracellular *T.*

530 *gondii* rosettes and PVs containing a single parasite were presented (PLK/RED). (*E*)

531 Representative fluorescence images of *D*. DNA was stained with DAPI. Scale bars represent

532 20 μm. All presented values are means ± SD, $n = 4$. Statistical significance was indicated by

533 comparison with controls. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$; N.S., not significant.

534

535 **Fig. 2. IFN-γ-mediated resistance in hMSCs does not require IDO.**

536 (*A* and *B*) Human MSCs or HeLa cells were stimulated with 20 ng/ml of IFN-γ and treated

537 with 1 mM Trp, or IDO inhibitor (DL-1 MT, D-1 MT, or L-1 MT), or the same volume of

538 solvent (0.1 M NaOH, control) for 48 h. The cells were then infected with *T. gondii* (*A*,

539 RH/GFP; *B*, PLK/RED) for 24 hours, and growth inhibition was calculated. (*C* and *D*)

540 Human MSCs were transfected with a control siRNA (siCtrl) or siRNAs targeting three

541 different regions of IDO (siIDO1, -2, -3) for 24 hr, and treated with IFN-γ and infected with *T.*

542 *gondii* as in *A* and *B*. The relative RNA levels of IDO in siIDO- and siCtrl MSCs were

543 examined by RT-PCR (*C*). *T. gondii* growth inhibition rates were calculated at 24 hr

544 post-infection (*D*). All results are expressed as the means ± SEM; **, $p < 0.01$; ***, $p <$

545 0.001; N.S., not significant; $n = 4$.

546

Fig. 3. The expression levels of hGBPs are significantly upregulated in IFN-γ-activated

MSCs.

Human MSCs were pretreated for 24 hr with IFN-γ (20 ng/ml). (*A*) qRT-PCR results show

the magnitude of change of hGBP1-5 mRNA expression in IFN-γ-stimulated MSCs

comparison to pre-stimulated cells. (*B*) Relative mRNA levels of hGBP1-5 in

IFN-γ-stimulated MSCs by RT-PCR with reference to beta-actin controls. (*C*) The protein

levels hGBP1, -2, and -5 in IFN-γ-stimulated and non-stimulated MSCs, as assessed by

Western blotting. All presented values are given as means ± SEM; $n = 3$.

555

Fig. 4. Characterization of shGBP-MSCs.

Lentiviral shRNAs were used to knock down hGBP1, -2, and -5 (shGBPs), and pLKOpuro1

was used as a control (shCtrl). (*A-C*) Human MSCs transduced with shCtrl or shGBPs were

stimulated with 20 ng/ml IFN-γ for 48 hr and the knockdown efficiencies of hGBP1 (*A*), -2

and -5 (*B*) were assessed by RT-PCR and Western blotting (*C*). $n = 4$. (*D*) MSCs transduced

with shGBPs and shCtrl were cultured in serum-free medium for 48 hours and cell survival

was tested using a LIVE/DEAD viability/cytotoxicity kit. $n = 4$. The results are expressed as

the means ± SEM; **, $p < 0.01$; ***, $p < 0.001$; N.S., not significant; PI, propidium iodide

staining. Scale bars represent 20 μm.

565

Fig. 5. IFN-γ-mediated resistance in MSCs requires hGBP1.

567 Human MSCs transduced with shCtrl or shGBPs were stimulated with IFN-γ for 48 h and

568 then infected with RH/GFP or PLK/RED. (*A*) Representative fluorescence images of

569 intracellular *T. gondii* growth at 24 hr post-infection. IFN-γ, 20 ng/ml; scale bars represent 10

570 μm. (*B* and *C*) The inhibition rates of *T. gondii* growth in the indicated knockdown and

571 control cells were calculated at 24 hr post-infection (RH/GFP; PLK/RED). The results are

572 expressed as the means ± SEM. $n = 4$. **, $p < 0.01$; ***, $p < 0.001$; N.S., not significant.

573

574 **Fig. 6. Localization of hGBP1 in *T. gondii*-containing parasitophorous vacuoles.**

575 The subcellular localization of hGBP1 in human MSCs was analyzed by immunostaining. (*A*

576 and *B*) Cells were stimulated with IFN-γ (20 ng/ml) for 48 h, infected with *T. gondii* for 3 hr,

577 stained with anti-hGBP1 and DAPI (*A*, RH/GFP; *B*, PLK/RED). $n = 4$. The results are

578 expressed as the means ± SEM. ***, $p < 0.001$. Scale bars represent 5 μm.