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# Guanylate-binding Protein 1 (GBP1) contributes to the immunity of human mesenchymal stromal cells against toxoplasma gondii

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

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

3

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30

31 Author contributions:

32 AQ, DHL, ZRL, and APX designed the experiments and AQ, DHL, WH, YPW, XC and SY

33 carried out the experiments. AQ, DHL, QL, HX, GH, ZRL, FJA and APX analyzed the data.

34 AQ, DHL, ZRL, FJA and APX wrote the manuscript. All co-authors approved the final

35 version.

36

37 **Key Words:** Human stem cells; Parasitic protozoan; Innate immunity; In vitro cultivation

38

39 **Abstract**

40 Mesenchymal stromal cells (MSCs) have recently been shown to play important roles in  
41 mammalian host defenses against intracellular pathogens, but the molecular mechanism still  
42 needs to be clarified. We confirmed that human MSCs (hMSCs) pre-stimulated with IFN- $\gamma$   
43 showed a significant and dose-dependent ability to inhibit the growth of two types of  
44 *Toxoplasma gondii* (type I strain RH/GFP or type II strain PLK/RED). However, in contrast  
45 to previous reports, the anti-*T. gondii* activity of hMSCs was not mediated by indoleamine  
46 2,3-dioxygenase (IDO). Genome-wide RNA-seq analysis revealed that IFN- $\gamma$  increased the  
47 expression of the p65 family of guanylate-binding proteins (hGBPs) in hMSCs, especially  
48 hGBP1. To analyze the functional role of hGBPs, stable knockdowns of hGBP1, -2, -5 in  
49 hMSCs were established using a lentiviral transfection system. hGBP1 knockdown in hMSCs  
50 resulted in a significant loss of the anti-*T. gondii* host defense property, compared with  
51 hMSCs infected with non-targetted control sequences. hGBP2 and -5 knockdowns had no  
52 effect. Moreover, the hGBP1 accumulation on the parasitophorous vacuole (PV) membranes  
53 of IFN- $\gamma$ -stimulated hMSCs might protect against *T. gondii* infection. Taken together, our  
54 results suggest that hGBP1 plays a pivotal role in anti-*T. gondii* protection of hMSCs and  
55 may shed new light on clarifying the mechanism of host defense properties of hMSCs.

56

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- $\gamma$ -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- $\gamma$ -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- $\gamma$ , which is the central regulator of the immune response against *T. gondii* (12-14).  
101 In mouse cells, the most important IFN- $\gamma$ -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- $\gamma$  (18). This indicates the importance of GBPs in  
106 immunity to *T. gondii* and providing insight into the antimicrobial effects of IFN- $\gamma$  (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- $\gamma$ -mediated cell-autonomous immunity against bacterial infection and GBP1, in  
109 particular, is essential for function in macrophage cell lines (19). However, IFN- $\gamma$ -mediated  
110 immunity to intracellular pathogens appears to be cell type-specific and occurs in a  
111 species-specific manner. IFN- $\gamma$  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- $\gamma$ -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- $\gamma$   
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- $\gamma$ -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- $\gamma$  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- $\gamma$**

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



134 growth. Human MSCs were pretreated with various concentrations of IFN- $\gamma$  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- $\gamma$   
139 pretreatment. In addition, along with IFN- $\gamma$  incubation time, the inhibition rate also  
140 significantly increased (Fig. 1A). Interestingly, the same concentrations of IFN- $\gamma$  had stronger  
141 inhibitory effects on the PLK/RED strain than on the RH/GFP strain (Fig. 1B). Furthermore,  
142 the number of parasites per vacuole at 24 hr post-infection (PLK/RED, Fig. 1C) and the ratio  
143 between parasite rosettes and single parasites at 48 hr post-infection (PLK/RED, Fig. 1D)  
144 were significantly lower in IFN- $\gamma$ -treated hMSCs than in untreated hMSCs (RH/GFP, SI Fig.  
145 1A and B). Representative fluorescence images of intracellular *T. gondii* growth at 48 hr  
146 post-infection are shown in Fig. 1E (PLK/RED) and SI Fig. 1C (RH/GFP). To exclude the  
147 possibility that the presence of IFN- $\gamma$  in the culture medium could have a direct effect on  
148 parasites, we assessed the effect of conditioned medium (CM) obtained from  
149 IFN- $\gamma$ -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- $\gamma$ .

154

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

156 studies showed that in some human cell types (e.g., HeLa cells), IFN- $\gamma$  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- $\gamma$ -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- $\gamma$ -stimulated HeLa cells ( $p < 0.001$ )  
163 (Fig. 2A), 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- $\gamma$ -stimulated growth inhibition  
166 (Fig. 2A and 2B).

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- $\gamma$  (by ~90% in hMSCs  
170 treated with siIDO 1 and siIDO 3) (Fig. 2C). As shown in Fig. 2D, siRNA-mediated IDO  
171 knockdown also could not rescue the parasite from IFN- $\gamma$ -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- $\gamma$ -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- $\gamma$  (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- $\gamma$  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- $\gamma$   
193 treatment (Fig. 3A). The relative abundances of the mRNAs for hGBP1, -2 and -5 compared  
194 with  $\beta$ -actin were significantly higher than that of hGBP3 and hGBP4 (Fig. 3B). Western  
195 blotting revealed that the protein expression levels of hGBP1, -2 and -5 were greatly  
196 increased after IFN- $\gamma$  stimulation (Fig. 3C). 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- $\gamma$ . 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<sup>G</sup>MMA), 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. 4A and 4B) using  
213 gene-specific primers (SI Table 1) and Western blotting (Fig. 4C). 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. 6A. 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. 6B).  
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. 6C). 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. 6D). 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 ( $7.82\% \pm 0.94\%$ ) and  
231 shCtrl-MSCs ( $7.31\% \pm 0.73\%$ ;  $p = 0.21$ ) (Fig. 4D). 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- $\gamma$ .** To investigate whether GBP family members contribute to the IFN- $\gamma$  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. 5A, 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- $\gamma$  were significantly reduced by  
243 shGBP1, compared with control MSCs (Fig. 5B). In contrast, there was no obvious difference

244 between control cells and shGBP2- or shGBP5-MSCs at 24 hr post-infection (Fig. 5D). Thus,  
245 our studies demonstrate that hGBP1 may play a major role in inhibiting *T. gondii* growth in  
246 IFN- $\gamma$  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- $\gamma$ -stimulated MSCs  
253 infected with RH/GFP strain at 3 hr post-infection, but only 3% in the corresponding

254 non-stimulated MSCs (Fig. 6A). A significantly higher percentage of PVs staining positive for  
255 hGBP1 was found in the cells infected with PLK/RED strain (Fig. 6B). 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- $\gamma$ -stimulated hMSCs could significantly inhibit the replication of *T. gondii*.

259

260 **Discussion**

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- $\gamma$ -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- $\gamma$ ,  
276 but it does not appear to occur in mouse fibroblasts (20, 28, 29). Human MSCs treated with  
277 IFN- $\gamma$  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. 2D).

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

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- $\gamma$ -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- $\gamma$ .

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- $\gamma$ -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- $\gamma$ -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- $\gamma$   
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



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<sup>-/-</sup> 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- $\gamma$  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- $\gamma$ -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- $\gamma$ -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. 6A and 6B) 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- $\gamma$ -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- $\gamma$  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- $\gamma$  (44). The recruitment of hGBPs to the membranes of *T. gondii*-containing PVs is  
340 regulated by the autophagic protein, ATG16L1, in IFN- $\gamma$ -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- $\gamma$ -stimulated human MSCs  
344 (Fig. 6A and 6B). 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- $\gamma$ -triggered autonomous resistance of human MSCs against *T. gondii* infection. Our  
350 studies reveal that non-lymphocytes could apply various mechanisms of IFN- $\gamma$ -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

360 **Materials and Methods**

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

369

370 **Small Interfering RNA and Short Hairpin RNA Knockdown.** *In vitro* silencing of IDO in  
371 hMSCs was performed as described (47), using three sets of IDO-siRNAs (SI Table 1). The  
372 short hairpin RNA (shRNA) expression vector, pLKOpuro1 (Addgene, USA) was used to  
373 express the control (shCtrl) and hGBP1, -2 and -5-specific shRNAs using specific oligos (SI  
374 Table 1). The helper plasmids, psPAX2 and pMD2.G, were used as lentiviral expression  
375 vectors (Addgene, USA). 293T host cells were used for packaging of the shRNAs. The  
376 MISSION Non-Target pLKOpuro1 control vector (Sigma-Aldrich, USA) was used as a  
377 control (shCtrl). Pools of stably transduced cells were selected using puromycin (2  $\mu$ g/ml) for  
378 5-7 days. All experiments were performed in puromycin-free media, and each knockdown  
379 was validated by RT-PCR and Western blotting.

380

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

382

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390

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- 522

523 **Figure Legends**

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

525 Human MSCs were pretreated for 48 hr with IFN- $\gamma$  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- $\gamma$  (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  $\mu$ m. All presented values are means  $\pm$  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- $\gamma$ -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- $\gamma$  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- $\gamma$  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  $\pm$  SEM; \*\*,  $p < 0.01$ ; \*\*\*,  $p <$

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

546

547 **Fig. 3. The expression levels of hGBPs are significantly upregulated in IFN- $\gamma$ -activated**  
548 **MSCs.**

549 Human MSCs were pretreated for 24 hr with IFN- $\gamma$  (20 ng/ml). (A) qRT-PCR results show  
550 the magnitude of change of hGBP1-5 mRNA expression in IFN- $\gamma$ -stimulated MSCs  
551 comparison to pre-stimulated cells. (B) Relative mRNA levels of hGBP1-5 in  
552 IFN- $\gamma$ -stimulated MSCs by RT-PCR with reference to beta-actin controls. (C) The protein  
553 levels hGBP1, -2, and -5 in IFN- $\gamma$ -stimulated and non-stimulated MSCs, as assessed by  
554 Western blotting. All presented values are given as means  $\pm$  SEM;  $n = 3$ .

555

556 **Fig. 4. Characterization of shGBP-MSCs.**

557 Lentiviral shRNAs were used to knock down hGBP1, -2, and -5 (shGBPs), and pLKOpurol  
558 was used as a control (shCtrl). (A-C) Human MSCs transduced with shCtrl or shGBPs were  
559 stimulated with 20 ng/ml IFN- $\gamma$  for 48 hr and the knockdown efficiencies of hGBP1 (A), -2  
560 and -5 (B) were assessed by RT-PCR and Western blotting (C).  $n = 4$ . (D) MSCs transduced  
561 with shGBPs and shCtrl were cultured in serum-free medium for 48 hours and cell survival  
562 was tested using a LIVE/DEAD viability/cytotoxicity kit.  $n = 4$ . The results are expressed as  
563 the means  $\pm$  SEM; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; N.S., not significant; PI, propidium iodide  
564 staining. Scale bars represent 20  $\mu$ m.

565

566 **Fig. 5. IFN- $\gamma$ -mediated resistance in MSCs requires hGBP1.**

567 Human MSCs transduced with shCtrl or shGBPs were stimulated with IFN- $\gamma$  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- $\gamma$ , 20 ng/ml; scale bars represent 10  
570  $\mu$ m. (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  $\pm$  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- $\gamma$  (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  $\pm$  SEM. \*\*\*,  $p < 0.001$ . Scale bars represent 5  $\mu$ m.