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http://dx.doi.org/10.1073/pnas.1619665114

# Guanylate-binding Protein 1 (GBP1) Contributes to the Immunity of Human Mesenchymal Stromal Cells against *Toxoplasma gondii*

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The authors indicate no potential conflicts of interest.

Author contributions:

AQ, DHL, ZRL, and APX designed the experiments and AQ, DHL, WH, YPW, XC and SY carried out the experiments. AQ, DHL, QL, HX, GH, ZRL, FJA and APX analyzed the data. AQ, DHL, ZRL, FJA and APX wrote the manuscript. All co-authors approved the final version.

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

**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.

## Significance

Mesenchymal stem cells (MSCs) are thought to be derived from pericytes and exhibit a cellular, autonomous antimicrobial effector function which provides therapeutic potential against infectious diseases. However, the molecular mechanism remains unknown. Here, we demonstrate that human guanylate-binding protein 1 (hGBP1) is a key protective factor against *T. gondii* infection in human MSCs (hMSCs). The recruitment of hGBP1 to the parasitophorous vacuole membrane in IFN-γ-stimulated hMSCs significantly inhibited *T. gondii* replication. Thus, our current study reveals an important function of hGBP1 in the defense against *T. gondii* and may shed new light on clarifying the mechanism of host defense properties of hMSCs.

**/body**

Mesenchymal stromal cells (MSCs) comprise a heterogeneous cell population endowed with multi-lineage differentiation potential and extensive immunomodulatory properties. MSCs have been successfully used to prevent and treat immune disorders, such as graft-versus-host disease, and emerging preclinical studies suggest that they might also protect against infectious challenges (1, 2). Recent studies showed that MSCs are located in the perivascular niche and constitute a subset of pericytes that are involved in both pathogen recognition and early inflammatory events (3). MSCs appear to impede pathogen growth and reduce the microbial burden by inhibiting growth through soluble factors or by enhancing the antimicrobial function of immune cells, as shown both *in vitro* and *in vivo* (2-5). For example, Nemeth et al. reported that mouse MSCs (mMSCs) prolonged the survival of septic mice and improved their organ (kidney, liver, and pancreas) functions (5). They did this by enhancing IL-10 production from murine alveolar macrophages via MSC-secreted cyclooxygenase-2 (COX2) and prostaglandin E2 (PGE2) (5). Data from murine colitis models have shown that human adipose-derived MSCs protect against dextran-induced colitis by decreasing the secretion of pro-inflammatory cytokines and chemokines (6). However, the antimicrobial effector molecules in vertebrate MSCs are not universally the same (4-11). The antimicrobial effect of unstimulated hMSCs is mediated by the cathelicidin, LL-37 (4), as shown both *in vitro* and *in vivo*. In IFN-γ-stimulated hMSCs, by contrast, the antibacterial effect is mediated through the tryptophan-catabolizing enzyme, indoleamine 2,3-dioxygenase (IDO) (9). Conflicting results are also reported in mouse, in which the decision as to whether mMSCs increase the activity of phagocytes or not depends on the origin of these cells (11).

*Toxoplasma gondii* is an obligatory intracellular protozoan parasite that infects virtually all warm-blooded vertebrates including humans. Clinical symptoms are rarely observed in most *T. gondii*-infected immunocompetent individuals. However, the parasite can cause severe disease and even death in immunosuppressed individuals, such as AIDS, cancer and organ transplantation patients. *T. gondii* can actively invade host cells *in vitro* by dividing within a nonfusogenic parasitophorous vacuole (PV), a membrane structure formed during invasion that is maintained to surround the intracellular replicating parasites. However, this activity may not be completed *in vivo* due to the innate resistance mechanisms in host cells and, especially, in those that are naturally resistant to *T. gondii* (12).

During *T. gondii* infection, NK cells, neutrophils, CD4+ cells and CD8+ T cells can all release IFN-γ, which is the central regulator of the immune response against *T. gondii* (12-14). In mouse cells, the most important IFN-γ-inducible effectors against *T. gondii* are likely to include inducible nitric oxide synthase (iNOS) (15), reactive oxygen species (ROS) (16), immunity-related p47 GTPases (IRGs) (17), and GBPs (18). Mice lacking a fragment of chromosome 3 that encodes GBP1, -2, -3, -5, -7, and -2ps were highly susceptible to *T. gondii* infection even after stimulation of IFN-γ (18). This indicates the importance of GBPs in immunity to *T. gondii* and providing insight into the antimicrobial effects of IFN-γ (18). It has been confirmed that members of the GBP family, namely GBP1, -6, -7 and -10, all play a key role in IFN-γ-mediated cell-autonomous immunity against bacterial infection and GBP1, in particular, is essential for function in macrophage cell lines (19). However, IFN-γ-mediated immunity to intracellular pathogens appears to be cell type-specific and occurs in a species-specific manner. IFN-γ stimulated human monocytes and mouse macrophages are

able to produce high levels of ROS to kill the parasite (15, 16). However, ROS production is not induced in *T. gondii*-infected human macrophages (20). Human fibroblasts can also display IFN-γ-dependent cell-autonomous immunity against *T. gondii*, although the involvement of IDO remains controversial (21, 22). Thus, data from animal models may not directly apply to human toxoplasmosis and the nature/relevance of innate immunity against *T. gondii* infection in humans is much less well understood. It is, therefore, useful to understand the fate of hMSCs (an important cell source for tissue/organ recovery) in *T. gondii* infection (23). In addition, the molecular mechanisms through which hMSCs augment anti-toxoplasmosis remain unclear.

To elucidate the functional contribution of human MSCs to host defense against *T. gondii*, we conducted a detailed analysis of gene expression in hMSCs before and after IFN-γ stimulation using Genome-wide RNA-seq, and generated hGBP1-, hGBP2- and hGBP5-knockdown hMSC cell lines. hGBP1, but not other hGBPs or IDO knockdown, partially restored the IFN-γ-dependent anti-*T. gondii* response in hMSCs. Moreover, we observed a significant increase in colocalization of endogenous hGBP1 in the PV membrane of *T. gondii*-infected cells. These data clearly show that hGBP1 is associated with the replication of intracellular *T. gondii* upon IFN-γ stimulation, suggesting an important function for hGBP1 in the defense against *T. gondii*.

## Results

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

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*

infection, we performed genome-wide RNA-seq to assess the changes in the expression of host defense related genes of MSCs treated with recombinant IFN-γ (20 ng/mL). Six sequencing libraries were constructed (three samples from each group). On average, more than 18 million 50-bp reads were obtained, of which more than 70% of the reads were aligned to unique locations in the genome, and included more than 17,000 coding genes from each library. The global transcriptional profiling data will be published elsewhere. MSCs from three different donors expressed a series of host defense related factors. Through gene ontology (GO) analysis, we identified 106, 53, 12 and 9 expressed genes (Reads per kilobase pairs per million (RPKM) value > 5) that were associated with defense responses to viral, bacterial, protozoan and fungal infections, respectively (SI Fig. 3). Previous reports have demonstrated that mouse GBPs are involved in *T. gondii* growth inhibition (18, 25, 26). Accordingly, we analyzed the expression of GBP family members in human MSCs before and after IFN-γ treatment. As shown in supplementary Figure S4, the RPKM values for hGBP1, -2, -3, -4 and -5 significantly increased, qRT-PCR results further showed that the expression levels of hGBP1, -2, -4 and -5 were increased up to 100-fold following IFN-γ treatment (Fig. 3*A*). The relative abundances of the mRNAs for hGBP1, -2 and -5 compared with β-actin were significantly higher than that of hGBP3 and hGBP4 (Fig. 3*B*). Western blotting revealed that the protein expression levels of hGBP1, -2 and -5 were greatly increased after IFN-γ stimulation (Fig. 3*C*). These top three most highly expressed hGBPs (hGBP1, -2 and -5) were selected for further testing for their potential roles in human MSC activity against *T. gondii* infection .

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

Interestingly, we found that the expression of iNOS (NOS2) and human IRG genes were close to zero in MSCs both before and after stimulation with IFN-γ. This was also the case for the constitutive NOS (NOS1 and NOS3) genes, further supporting the notion that iNOS shows no increase in expression following stimulation. Using reverse transcriptase quantitative PCR and western blotting, we also observed no iNOS expression in human MSCs. To further confirm these results, using the iNOS-specific inhibitor ($N^G$MMA), we revealed that inhibiting the iNOS activity did not influence the action of MSCs against *T. gondii* (SI Fig. 5). Taken together, our results indicate that the ability of human MSCs to inhibit the growth of *T. gondii* does not depend on iNOS.

**Generation of hGBP knockdowns in human MSCs.** Stable hGBP1-, hGBP2- and hGBP5-knockdown hMSC cell lines were generated using specific short hairpin RNA (shRNA) transcription cassettes and confirmed by RT-PCR (Fig. 4*A* and 4*B*) using gene-specific primers (SI Table 1) and Western blotting (Fig. 4*C*). To test the characteristics of the three kinds of shGBP-MSCs, FACS was used to analyze hMSC surface markers. Compared with wild-type human MSCs, all transduced cells expressed the same panel of surface markers, exhibiting positive expression of CD29, CD44, CD73, CD90, D166 and CD105, while detectable expression of the hematopoietic stem cell markers CD34 and CD45 was not found. Representative FACS results for shCtrl- and shGBP1-MSCs are shown in SI Fig. 6*A*. Our results clearly indicated that transduced cells maintained the cell-surface marker phenotype of human MSCs.

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

conditions that could promote their differentiation into osteogenic or adipogenic lineages. Following culture in osteogenic medium for 2 weeks, the shGBP-MSCs were induced to differentiate into osteoblasts as confirmed by strong Alizarin Red S staining (SI Fig. 6*B*). Similarly, oil red O staining revealed the presence of lipid droplets in the cytoplasm of shGBP-MSCs on day 21 after adipogenic differentiation (SI Fig. 6*C*). The knockdown of hGBPs did not alter the proliferative properties of human MSCs, which was verified by CCK8 assays ($p = 0.37$, SI Fig. 6*D*). Cell survival analysis showed that the cells cultured with serum-deprived conditions for 48 hours only produced a mild, non-significant, increase in the death rate which was similar to that found in shGBP1-MSCs (7.82% ± 0.94%) and shCtrl-MSCs (7.31% ± 0.73%; $p = 0.21$) (Fig. 4*D*). Similar features of sustained multipotency were also observed in shGBP2- and shGBP5-MSCs. These results demonstrated that the osteogenic and adipogenic differentiation, proliferation and survival potential of shGBP-MSCs showed no observed differences from the control human MSCs.

**hGBP1 is a key factor for inhibition of *T. gondii* growth in human MSCs stimulated by IFN-γ.** To investigate whether GBP family members contribute to the IFN-γ induced antimicrobial effect of MSCs, we examined the effect of GBP knockdown in MSCs on the growth of *T. gondii*. Remarkably, compared to control cells, inhibition of *T. gondii* growth was rescued in shGBP1-MSCs (Fig. 5*A*, Upper, RH/GFP; Lower, PLK/RED) at 24 hr post-infection. Specifically, using the RH/GFP and PLK/RED strains, we found the inhibition rates of *T. gondii* growth in MSCs at 5 and 20 ng/ml of IFN-γ were significantly reduced by shGBP1, compared with control MSCs (Fig. 5*B*). In contrast, there was no obvious difference

between control cells and shGBP2- or shGBP5-MSCs at 24 hr post-infection (Fig. 5*D*). Thus, our studies demonstrate that hGBP1 may play a major role in inhibiting *T. gondii* growth in IFN-γ stimulated human MSCs, in contrast to hGBP2 and hGBP5 that do not.

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

Recruitment of mouse GBPs to the PV was reported to be essential for the replication control of *T. gondii* (18, 25, 26). To explore the role of hGBP1 in human MSCs, we used immunofluorescence to assess its intracellular localization after *T. gondii* infection. Endogenous hGBP1 was observed in ~10% of vacuoles belonging to IFN-γ-stimulated MSCs infected with RH/GFP strain at 3 hr post-infection, but only 3% in the corresponding non-stimulated MSCs (Fig. 6*A*). A significantly higher percentage of PVs staining positive for hGBP1 was found in the cells infected with PLK/RED strain (Fig. 6*B*). This could potentially explain the stronger inhibitory effect of MSCs on PLK/RED versus RH/GFP strain. Collectively, these data indicated that the accumulation of hGBP1 on the PV membranes of IFN-γ-stimulated hMSCs could significantly inhibit the replication of *T. gondii*.

## Discussion

As an obligatory intracellular protozoan parasite, *T. gondii* is protected by the plasma membrane of the host cell, and is thus able to escape from most antibody-based immune responses (12). Therefore, the host's innate immune response is largely responsible for controlling *T. gondii* infection. Although numerous studies have demonstrated that *T. gondii* can grow in various somatic cells from warm-blooded animals and humans *in vitro*, the related disease condition is not commonly found *in vivo*. Previous studies have shown that an IFN-γ-mediated response against *T. gondii* infection appears to act in a cell type-specific and species-specific manner, e.g. non-lymphocytes shown in SI Fig. 7. In mice, astrocytes can use IRGs to restrict *T. gondii* infection; mMSCs use iNOS to produce NO; and skeletal muscle cells use both mechanisms (27). The mGBPs may also critically contribute to fighting *T. gondii* replication *in vivo* (18).

However, many studies in animal models show significant differences from humans, and vast differences have been observed between immune and somatic cells of patients infected with *T. gondii* (14-22). High IDO activity which could result in the depletion of Trp is considered a key weapon against *T. gondii* growth in human fibroblasts treated with IFN-γ, but it does not appear to occur in mouse fibroblasts (20, 28, 29). Human MSCs treated with IFN-γ were found to exhibit an IDO-mediated antimicrobial effector function against various pathogens suggesting a similar mechanism which might be used to curtail *T. gondii* (9). Our results, however, have indicated that IDO is not a main factor in the ability of human MSCs to inhibit the growth of *T. gondii* (Fig. 2*D*).

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-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

anti-toxoplasmosis activity.

In a similar manner to mIRGs, mGBPs belong to the IFN-inducible GTPase family. Kim et al. reported that mGBPs (at least mGBP1 and mGBP7) participated in antibacterial responses by recruiting host defense proteins, including phagocyte oxidase, antimicrobial peptides, and autophagic effectors within macrophages and mGBP1$^{-/-}$ mice (19). A recent report showed that mGBP2 and mGBP5 could promote bacteriolysis by controlling the activation of the inflammasome protein, AIM2, in mouse bone marrow–derived macrophages (36). In human cells, hGBP1 shows one of the highest induction levels in response to IFN-γ stimulation (37). This has been shown to be associated with viral infections, inflammatory bowel diseases, glioblastoma and cutaneous lupus erythematosus (38-41).

In the case of toxoplasmosis, previous studies demonstrated that mGBP1, -2, -3, -6, -7, and -9 were recruited to *T. gondii*-containing PVs in IFN-γ-activated mouse embryonic fibroblasts or RAW 264.7 macrophages (42). Recently, a mGBP cluster on chromosome 3 (comprising the genes encoding mGBP1, -2, -3, -5, and -7) was found to cooperate with mIRGb6 and mIRGb10 during toxoplasmosis (18). Mice defective in p65 GTPases were highly susceptible to *T. gondii* infection and GBPs-deleted in chromosome 3 aneuploid macrophages were defective in IFN-γ-mediated inhibition of intracellular parasite growth (18). The recruitment of mGBP2 to *T. gondii*-containing PVs is essential for a cell's ability to control *T. gondii* replication (25). Furthermore, recruitment of mGBP1 to PVs is associated with vesiculation and rupture and thus also protects against *T. gondii* infection (26). Both mGBP1 and mGBP2 have been shown to form tetramers and may also hetero-oligomerize to cooperatively activate GTPase activity (25).

The hGBP1, -2 and -3 proteins are paralogs of mGBP1 and mGBP2, and both hGBP1 and mGBP2 show isoprenylation, which targets proteins to intracellular membranes and/or facilitates protein/protein interactions (43). Such findings suggest that these GBPs may play similar roles in protecting human and mouse cells against *T. gondii* infection. Our present data clearly show that hGBP1 is recruited to *T. gondii* PVs (Fig. 6*A* and 6*B*) and provides additional evidence that hGBP1 plays a role similar to that of mGBP1/2. Interestingly, our RNA-seq and RNAi data showed that hGBP1, not hGBP2, is the main responder in IFN-γ-stimulated human MSCs. Thus, hGBP1 is unlikely to interact with hGBP2 to execute this protective function. The third paralog, hGBP3, is also not likely to be a main effector, as its depletion showed little change. Thus, hGBP1 in human MSCs stimulated by IFN-γ could be a functional homodimer in the innate immune response.

Recently, Haldar et al. reported that the deposition of mGBPs on *T. gondii*-containing vacuoles needed IRG protein-dependent ubiquitination of PVs after priming their host cells with IFN-γ (44). The recruitment of hGBPs to the membranes of *T. gondii*-containing PVs is regulated by the autophagic protein, ATG16L1, in IFN-γ-stimulated human fibroblast-like (HAP1) cells (45). Here, using both the non-cyst and cyst forming strains (RH/GFP and PLK/RED) of *T. gondii* (46), we observed the recruitment of endogenous hGBP1 to *T. gondii*-containing PVs at 3 hr post-infection in IFN-γ-stimulated human MSCs (Fig. 6*A* and 6*B*). The signaling pathway that is responsible for recruiting hGBP1 to act against *T. gondii* is not yet fully understood. Future studies are needed to examine whether hGBPs contribute directly to membrane blebbing and the physical disruption of the PV membrane and/or if they modulate the function of other effectors at this interface.

In summary, we herein define hGBP1 as an important effector molecule in the IFN-γ-triggered autonomous resistance of human MSCs against *T. gondii* infection. Our studies reveal that non-lymphocytes could apply various mechanisms of IFN-γ-triggered autonomous resistance against *T. gondii* infection in a cell type-specific and species-specific manner. Human MSCs offer an excellent example of a somatic cell type that plays a very important role in the host defense system by providing protection against intracellular pathogens such as *T. gondii*. The emerging involvement of MSCs in hematopoiesis and antimicrobial responses suggest that they are worthy of further development in the context of novel cellular antimicrobial therapies aimed at reducing infection-related morbidity and mortality, particularly in immunocompromised patients.

## 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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383 **ACKNOWLEDGMENTS**

384 This work was supported by grants from the China Postdoctoral Science Foundation
385 (2015A030310428), National Basic Research Program of China (973 Program,
386 No.:2010CB350000), the National Natural Science Foundation of China (81425016,
387 31472058) and the Key Scientific and Technological Projects of Guangdong Province
388 (2014B020226002). We also thank the members in the authors' laboratories who provided
389 great help during the work.

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## References


1. Balan A, *et al.* (2014) Mesenchymal stromal cells in the antimicrobial host response of hematopoietic stem cell recipients with graft-versus-host disease-friends or foes? *Leukemia* 28(10)**:** 1941–1948.

2. Auletta JJ, Deans RJ, Bartholomew AM (2012) Emerging roles for multipotent, bone marrow-derived stromal cells in host defense. *Blood* 119(8)**:** 1801–1809.

3. Paquet-Fifield S, *et al.* (2009) A role for pericytes as microenvironmental regulators of human skin tissue regeneration. *J Clin Invest* 119(9):2795–2806.

4. Krasnodembskaya A, *et al.* (2010) Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. *Stem Cells* 28(12)**:** 2229–2238.

5. Németh K, *et al.* (2008) Bone marrow stromal cells attenuate sepsis via prostaglandin E2–dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 15(1)**:** 42–49.

6. Gonzalez-Rey E, *et al.* (2009) Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. *Gut* 58(7)**:** 929–939.

7. Wong JH, *et al.* (2011) Antifungal action of human cathelicidin fragment (LL13-37) on *Candida albicans*. *Peptides* 32(10)**:** 1996–2002.

8. Gupta N, *et al.* (2012) Mesenchymal stem cells enhance survival and bacterial clearance in murine *Escherichia coli* pneumonia. *Thorax* 67(6)**:** 533–539.

9. Meisel R, *et al.* (2011) Human but not murine multipotent mesenchymal stromal cells exhibit broad-spectrum antimicrobial effector function mediated by indoleamine 2,3-dioxygenase. *Leukemia* 25(4)**:** 648–654.

10. Hall SR, *et al.* (2013) Mesenchymal stromal cells improve survival during sepsis in the absence of heme oxygenase-1: the importance of neutrophils. *Stem Cells* 31(2)**:** 397–407.

11. Mei SH, *et al.* (2010) Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. *Am J Respir Crit Care Med* 182(8)**:** 1047–1057.

12. Yarovinsky F. (2014) Innate immunity to *Toxoplasma gondii* infection. *Nat Rev Immunol*

14(2)**:** 109–121.

13. Suzuki Y, Orellana MA, Schreiber RD, Remington JS (1988) Interferon-gamma: the major mediator of resistance against *Toxoplasma gondii*. *Science* 240(4851)**:** 516–518.

14. Sturge CR, Yarovinsky F (2014) Complex Immune Cell Interplay in the Gamma Interferon Response during *Toxoplasma gondii* Infection. *Infect Immun* 82(8):3090–3097.

15. Li Z, *et al.* (2012) Differences in iNOS and arginase expression and activity in the macrophages of rats are responsible for the resistance against *T. gondii* infection. *PLoS One* 7(4)**:** e35834.

16. Murray HW, Juangbhanich CW, Nathan CF, Cohn ZA (1979) Macrophage oxygen-dependent antimicrobial activity. II. The role of oxygen intermediates. *J Exp Med* 150(4)**:** 950–964.

17. Howard JC, Hunn JP, Steinfeldt T (2011) The IRG protein-based resistance mechanism in mice and its relation to virulence in *Toxoplasma gondii*. *Curr Opin Microbiol* 14(4)**:** 414–421.

18. Yamamoto M, *et al.* (2012) A cluster of interferon-gamma-inducible p65 GTPases plays a critical role in host defense against *Toxoplasma gondii*. *Immunity* 37(2)**:** 302–313.

19. Kim BH, *et al.* (2011) A family of IFN-gamma-inducible 65-kD GTPases protects against bacterial infection. *Science* 332(6030)**:** 717–721.

20. Murray HW, Teitelbaum RF (1992) L-arginine-dependent reactive nitrogen intermediates and the antimicrobial effect of activated human mononuclear phagocytes. *J Infect Dis* 165(3)**:** 513–517.

21. Pfefferkorn ER (1984) Interferon gamma blocks the growth of *Toxoplasma gondii* in human fibroblasts by inducing the host cells to degrade tryptophan. *Proc Natl Acad Sci U S A* 81(3)**:** 908–912.

22. Niedelman W, Sprokholt JK, Clough B, Frickel EM, Saeij JP (2013) Cell death of gamma interferon-stimulated human fibroblasts upon *Toxoplasma gondii* infection induces early parasite egress and limits parasite replication. *Infect Immun* 81(12)**:** 4341–4349.

23. Ribitsch I, *et al.* (2010) Basic science and clinical application of stem cells in veterinary medicine. *Adv Biochem Eng Biotechnol* 123: 219–263.

24. Schmidt SK, *et al.* (2009) Antimicrobial and immunoregulatory properties of human

450 tryptophan 2,3-dioxygenase. *Eur J Immunol* 39(10)**:** 2755–2764.
451 25. Degrandi D, *et al.* (2013) Murine guanylate binding protein 2 (mGBP2) controls
452 *Toxoplasma gondii* replication. *Proc Natl Acad Sci U S A* 110(1)**:** 294–299.
453 26. Selleck EM*, et al.* (2013) Guanylate-binding protein 1 (Gbp1) contributes to
454 cell-autonomous immunity against *Toxoplasma gondii*. *PLoS Pathog* 9(4)**:** e1003320.
455 27. Takacs AC, Swierzy IJ, Luder CG (2012) Interferon-gamma restricts *Toxoplasma gondii*
456 development in murine skeletal muscle cells via nitric oxide production and immunity-related
457 GTPases. *PLoS One* 7(9)**:** e45440.
458 28. Dai W, Pan H, Kwok O, Dubey JP (1994) Human indoleamine 2,3-dioxygenase inhibits
459 *Toxoplasma gondii* growth in fibroblast cells. *J Interferon Res* 14(6)**:** 313–317.
460 29. Gazzinelli RT, Mendonca-Neto R, Lilue J, Howard J, Sher A (2014) Innate resistance
461 against *Toxoplasma gondii*: an evolutionary tale of mice, cats, and men. *Cell Host Microbe*
462 15(2)**:** 132–138.
463 30. Zhao ZJ, *et al.* (2013) Lower expression of inducible nitric oxide synthase
464 and higher expression of arginase in rat alveolar macrophages are linked to
465 their susceptibility to *Toxoplasma gondii* infection. *PLoS One*. 15;8(5):e63650.
466 31**.** Gao JM, *et al.* (2015) Investigation of infectivity of neonates and adults from
467 different rat strains to *Toxoplasma gondii* Prugniaud shows both variation which correlates
468 with iNOS and Arginase-1 activity and increased susceptibility of neonates to infection. *Exp*
469 *Parasitol.* 149:47–53.
470 32. Martens S, *et al.* (2005) Disruption of *Toxoplasma gondii* parasitophorous vacuoles by
471 the mouse p47-resistance GTPases. *PLoS Pathog* 1(3)**:** e24.
472
473 33. Bekpen C, *et al.* (2005) The interferon-inducible p47 (IRG) GTPases in vertebrates: loss
474 of the cell autonomous resistance mechanism in the human lineage. *Genome Biol* 6(11):R92.
475 34. Khaminets A, *et al.* (2010) Coordinated loading of IRG resistance GTPases on to the
476 *Toxoplasma gondii* parasitophorous vacuole. *Cell Microbiol* 12(7)**:** 939–961.
477 35. Haldar AK, *et al.* (2013) IRG and GBP host resistance factors target aberrant, “non-self”
478 vacuoles characterized by the missing of “self” IRGM proteins. *PLoS Pathog* 9(6):e1003414.

36. Meunier E, *et al.* (2015) Guanylate-binding proteins promote activation of the AIM2 inflammasome during infection with *Francisella novicida*. *Nat Immunol* 16(5)**:** 476–484.
37. Prakash B, Praefcke GJ, Renault L, Wittinghofer A, Herrmann C (2000) Structure of human guanylate-binding protein 1 representing a unique class of GTP-binding proteins. *Nature* 403(6769)**:** 567–571.
38. Li M, *et al.* (2011) Guanylate binding protein 1 is a novel effector of EGFR-driven invasion in glioblastoma. *J Exp Med* 208(13)**:** 2657–2673.
39. Naschberger E, *et al.* (2011) Increased expression of guanylate binding protein-1 in lesional skin of patients with cutaneous lupus erythematosus. *Exp Dermatol* 20(2)**:** 102–106.
40. Britzen-Laurent N, *et al.* (2013) GBP-1 acts as a tumor suppressor in colorectal cancer cells. *Carcinogenesis* 34(1)**:** 153–162.
41. Itsui Y, *et al.* (2009) Antiviral effects of the interferon-induced protein guanylate binding protein 1 and its interaction with the hepatitis C virus NS5B protein. *Hepatology* 50(6)**:** 1727–1737.
42. Degrandi D, *et al.* (2007) Extensive characterization of IFN-induced GTPases mGBP1 to mGBP10 involved in host defense. *J Immunol* 179(11)**:** 7729–7740.
43. Nantais DE, Schwemmle M, Stickney JT, Vestal DJ, Buss JE. (1996) Prenylation of an interferon-gamma-induced GTP-binding protein: the human guanylate binding protein, huGBP1. *J Leukoc Biol* 60(3)**:** 423–431.
44. Haldar AK, *et al.* (2015) Ubiquitin systems mark pathogen-containing vacuoles as targets for host defense by guanylate binding proteins. *Proc Natl Acad Sci U S A* 112(41):E5628–E5637.
45. Ohshima J, *et al.* (2014) Role of mouse and human autophagy proteins in IFN-gamma-induced cell-autonomous responses against *Toxoplasma gondii*. *J Immunol* 192(7)**:** 3328–3335.
46. Lun ZR, *et al.* (2015) Cancer in the parasitic protozoans *Trypanosoma brucei* and *Toxoplasma gondii*. *Proc Natl Acad Sci U S A* 21;112(29):8835–8842.
47. Peng Y, *et al.* (2015) Mesenchymal stromal cells infusions improve refractory chronic graft versus host disease through an increase of CD5+ regulatory B cells producing interleukin 10. *Leukemia* 29(3)**:** 636–646.

509 48. Takahashi K, Yamanaka S. (2006) Induction of pluripotent stem cells from mouse
510 embryonic and adult fibroblast cultures by defined factors. *Cell* 126(4)**:** 663–676.
511 49. Liu J, *et al.* (2014) Islet-1 overexpression in human mesenchymal stem cells promotes
512 vascularization through monocyte chemoattractant protein-3. *Stem Cells* 32(7)**:** 1843–1854.
513 50. Nishikawa Y, *et al.* (2003) Characterisation of *Toxoplasma gondii* engineered to express
514 mouse interferon-gamma. *Int J Parasitol* 33(13)**:** 1525–1535.
515 51. Takashima Y, *et al.* (2008) Detection of the initial site of *Toxoplasma gondii* reactivation
516 in brain tissue. *Int J Parasitol* 38(5)**:** 601–607.
517 52. Dominici M, *et al.* (2006) Minimal criteria for defining multipotent mesenchymal stromal
518 cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8(4)**:**
519 315–317.
520 53. Yan X, Ji Y, Liu X and Suo X (2015) Nitric oxide stimulates early egress of *Toxoplasma*
521 *gondii* tachyzoites from Human foreskin fibroblast cells. *Parasit Vectors* 13(8): 420.
522

**Figure Legends**

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

Human MSCs were pretreated for 48 hr with IFN-γ and then infected with *T. gondii* (RH/GFP or PLK/RED). (*A* and *B*) *T. gondii* growth inhibition rates were calculated at 6, 12 and 24 hr post-infection (*A*, RH/GFP; *B*, PLK/RED). (*C*) At 24 hr post-infection, the number of parasites per vacuole in IFN-γ (20 ng/ml)-stimulated hMSCs and unstimulated hMSCs (controls) were calculated (PLK/RED). (*D*) At 48 hr post-infection, ratios of intracellular *T. gondii* rosettes and PVs containing a single parasite were presented (PLK/RED). (*E*) Representative fluorescence images of *D*. DNA was stained with DAPI. Scale bars represent 20 μm. All presented values are means ± SD, $n = 4$. Statistical significance was indicated by comparison with controls. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$; N.S., not significant.

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

(*A* and *B*) Human MSCs or HeLa cells were stimulated with 20 ng/ml of IFN-γ and treated with 1 mM Trp, or IDO inhibitor (DL-1 MT, D-1 MT, or L-1 MT), or the same volume of solvent (0.1 M NaOH, control) for 48 h. The cells were then infected with *T. gondii* (*A*, RH/GFP; *B*, PLK/RED) for 24 hours, and growth inhibition was calculated. (*C* and *D*) Human MSCs were transfected with a control siRNA (siCtrl) or siRNAs targeting three different regions of IDO (siIDO1, -2, -3) for 24 hr, and treated with IFN-γ and infected with *T. gondii* as in *A* and *B*. The relative RNA levels of IDO in siIDO- and siCtrl MSCs were examined by RT-PCR (*C*). *T. gondii* growth inhibition rates were calculated at 24 hr post-infection (*D*). All results are expressed as the means ± SEM; **, $p < 0.01$; ***, $p <$

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

**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; $n = 3$.

**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.

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

Human MSCs transduced with shCtrl or shGBPs were stimulated with IFN-γ for 48 h and then infected with RH/GFP or PLK/RED. (*A*) Representative fluorescence images of intracellular *T. gondii* growth at 24 hr post-infection. IFN-γ, 20 ng/ml; scale bars represent 10 μm. (*B* and *C*) The inhibition rates of *T. gondii* growth in the indicated knockdown and control cells were calculated at 24 hr post-infection (RH/GFP; PLK/RED). The results are expressed as the means ± SEM. $n = 4$. **, $p < 0.01$; ***, $p < 0.001$; N.S., not significant.

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

The subcellular localization of hGBP1 in human MSCs was analyzed by immunostaining. (*A* and *B*) Cells were stimulated with IFN-γ (20 ng/ml) for 48 h, infected with *T. gondii* for 3 hr, stained with anti-hGBP1 and DAPI (*A*, RH/GFP; *B*, PLK/RED). $n = 4$. The results are expressed as the means ± SEM. ***, $p < 0.001$. Scale bars represent 5 μm.