

Guanylate-binding protein 1 (GBP1) contributes to the immunity of human mesenchymal stromal cells against *Toxoplasma gondii*

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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) prestimulated with IFN- γ showed a significant and dose-dependent ability to inhibit the growth of two types of *Toxoplasma gondii* [type I RH strain with green fluorescent proteins (RH/GFP) or type II PLK strain with red fluorescent proteins (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 sequencing (RNA-seq) analysis revealed that IFN- γ increased the expression of the p65 family of human guanylate-binding proteins (hGBPs) in hMSCs, especially hGBP1. To analyze the functional role of hGBPs, stable knockdowns of hGBP1, -2, and -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 nontargeted 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.

human stem cells | parasitic protozoan | innate immunity | in vitro cultivation

Mesenchymal stromal cells (MSCs) comprise a heterogeneous cell population endowed with multilineage 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 seem 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 achieved this result 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 proinflammatory 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

Significance

Mesenchymal stem cells (MSCs) are thought to be derived from pericytes and exhibit a cellular, autonomous antimicrobial effector function that 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 *Toxoplasma 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.

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the innate resistance mechanisms in host cells and, especially, in those that are naturally resistant to *T. gondii* (12).

During *T. gondii* infection, natural killer (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 guanylate-binding proteins (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), which indicates the importance of GBPs in immunity to *T. gondii* and provides 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 that GBP1, in particular, is essential for function in macrophage cell lines (19). However, IFN- γ -mediated immunity to intracellular pathogens seems 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 sequencing (RNA-seq) and generated human GBP1 (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* growth. Human MSCs were pretreated with various concentrations of IFN- γ for 48 h and then infected with two *T. gondii* strains [type I strain RH with green fluorescent proteins (RH/GFP) and type II strain PLK with red fluorescent proteins (PLK/RED)], respectively. The fluorescence-labeled intracellular parasites were counted microscopically at multiple time points postinfection, and the inhibition rates of *T. gondii* growth were calculated. The growth of *T. gondii* was found to be dose-dependently inhibited by IFN- γ pretreatment. In addition, along with IFN- γ incubation time, the inhibition rate also significantly increased (Fig. 1A). Interestingly, the same concentrations of IFN- γ had stronger inhibitory effects on the PLK/RED strain than on the RH/GFP strain (Fig. 1B). Furthermore, the number of parasites per vacuole at 24 h postinfection (PLK/RED, Fig. 1C) and the ratio between parasite rosettes and single parasites at 48 h postinfection (PLK/RED) (Fig. 1D) were significantly lower in IFN- γ -treated hMSCs than in untreated hMSCs (RH/GFP) (Fig. S1 A and B). Representative fluorescence images of intracellular *T. gondii* growth at 48 h postinfection are shown in Fig. 1E

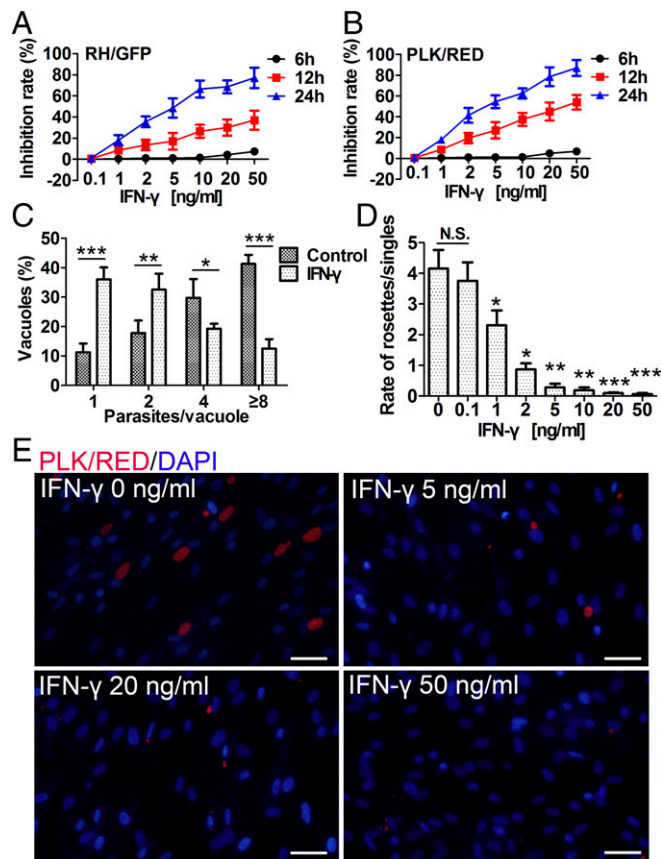


Fig. 1. IFN- γ -stimulated human MSCs dose-dependently inhibit the growth of *T. gondii*. Human MSCs were pretreated for 48 h with IFN- γ and then infected with *T. gondii* (RH/GFP or PLK/RED). (A and B) *T. gondii* growth inhibition rates were calculated at 6, 12, and 24 h postinfection (A, RH/GFP; B, PLK/RED). (C) At 24 h postinfection, the number of parasites per vacuole in IFN- γ -stimulated (20 ng/mL) hMSCs and unstimulated hMSCs (controls) were calculated (PLK/RED). (D) At 48 h postinfection, 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: 20 μ m.) All presented values are means \pm 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.

(PLK/RED) and Fig. S1C (RH/GFP). To exclude the possibility that the presence of IFN- γ in the culture medium could have a direct effect on parasites, we assessed the effect of conditioned medium (CM), obtained from IFN- γ -stimulated MSCs (at 48 h postinfection), on *T. gondii* infection. The CM from pretreated cultures did not have any significant effect on the ability of *T. gondii* to invade hMSCs or to replicate, compared with CM from unstimulated hMSCs (RH/GFP, $P = 0.34$; PLK/RED, $P = 0.45$) (Fig. S2). These results strongly suggest that human MSCs act against *T. gondii* using some intracellular products that can be induced by IFN- γ .

The Anti-Toxoplasma Activity of Human MSCs Is Not Associated with IDO. Previous studies showed that, in some human cell types (e.g., HeLa cells), IFN- γ stimulation inhibits *T. gondii* growth by depletion of tryptophan (Trp), via the activity of IDO (22, 24). To assess whether inhibiting the IDO activity could affect the proliferation of *T. gondii* in IFN- γ -stimulated MSCs, we tested the effects of three IDO-specific inhibitors (DL-1 MT, D-1 MT, and L-1 MT) or excess amounts of Trp, respectively. Our results revealed that both, inhibiting the IDO activity by 1-methyl-D/L-tryptophan and adding Trp, significantly decreased the antimicrobial effect against *T. gondii* in IFN- γ -stimulated HeLa cells ($P < 0.001$) (Fig. 2A), confirming

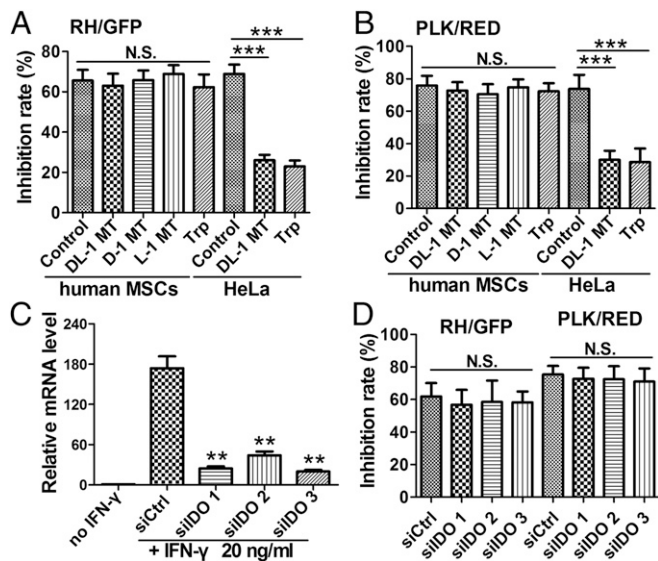


Fig. 2. IFN- γ -mediated resistance in hMSCs does not require IDO. (A and B) Human MSCs or HeLa cells were stimulated with 20 ng/mL IFN- γ and treated with 1 mM Trp, or IDO inhibitor (DL-1 MT, D-1 MT, or L-1 MT), or the same volume of solvent (0.1 M NaOH, control) for 48 h. The cells were then infected with *T. gondii* (A, RH/GFP; B, PLK/RED) for 24 h, 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 h 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 h post-infection (D). All results are expressed as the means \pm SEM; *** P < 0.001; N.S., not significant; n = 4.

that HeLa cells rely on IDO-mediated degradation of Trp for their ability to resist *T. gondii* infection. In human MSCs, in contrast, Trp and all three different IDO inhibitors failed to rescue both strains of the parasite from IFN- γ -stimulated growth inhibition (Fig. 2 A and B).

To further confirm these observations, we designed siRNAs that targeted three different regions of the IDO mRNA. Based on the RT-PCR results, we confirmed that they significantly decreased the expression of IDO stimulated by IFN- γ (by \sim 90% in hMSCs treated with siIDO 1 and siIDO 3) (Fig. 2C). As shown in Fig. 2D, siRNA-mediated IDO knockdown also could not rescue the parasite from IFN- γ -mediated growth inhibition. These results suggest that the ability of human MSCs inhibiting the growth of *T. gondii* does not depend on Trp deprivation or IDO activity, which is in contrast to the results presented from a previously reported study (9).

IFN- γ -Inducible p65 Guanylate-Binding Proteins Are Significantly Up-Regulated 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 per million (RPKM) value > 5] that were associated with defense responses to viral, bacterial, protozoan, and fungal infections, respectively (Fig. S3). 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 Fig. S4, the RPKM values for hGBP1, -2, -3, -4, and -5 significantly increased, and RT-PCR results further showed that the expression levels of hGBP1, -2, -4, and -5 were increased up to 100-fold after IFN- γ treatment (Fig. 3A). 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. 3B). Western blotting revealed that the protein expression levels of hGBP1, -2, and -5 were greatly increased after IFN- γ stimulation (Fig. 3C). 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 was close to zero in MSCs both before and after stimulation with IFN- γ , which was also the case for the constitutive NOS (NOS1 and NOS3) genes, further supporting the notion that iNOS shows no increase in expression after 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-monomethyl-L-arginine acetate (*N*^GMMA), we revealed that inhibiting the iNOS activity did not influence the action of MSCs against *T. gondii* (Fig. S5). 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 B) using gene-specific primers (Table S1) and Western blotting (Fig. 4C). To test the characteristics of the three kinds of shGBP-MSCs, FACS was used to analyze hMSC surface markers. Compared with WT human MSCs, all transduced cells expressed the same panel of surface markers, exhibiting positive expression of CD29, CD44, CD73, CD90, CD166, and CD105, whereas 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 Fig. S6A. 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. After culture in osteogenic medium for 2 wk, the shGBP-MSCs were induced to differentiate into osteoblasts as confirmed by strong Alizarin Red S staining (Fig. S6B). Similarly, oil red O staining revealed the

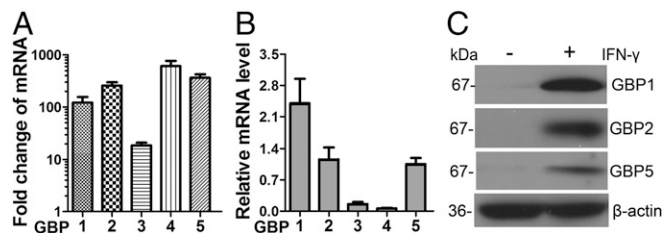


Fig. 3. The expression levels of hGBPs are significantly up-regulated in IFN- γ -activated MSCs. Human MSCs were pretreated for 24 h with IFN- γ (20 ng/mL). (A) RT-PCR results show the magnitude of change of hGBP1 to -5 mRNA expression in IFN- γ -stimulated MSCs in comparison with prestimulated cells. (B) Relative mRNA levels of hGBP1 to -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 nonstimulated MSCs, as assessed by Western blotting. All presented values are given as means \pm SEM; n = 3.

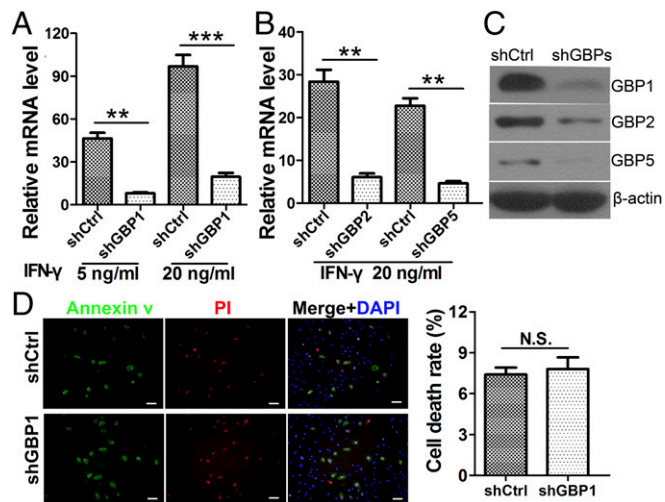


Fig. 4. Characterization of shGBP-MSCs. Lentiviral shRNAs were used to knock down hGBP1, -2, and -5 (shGBPs), and pLKOpuro1 was used as a control (shCtrl). (A–C) Human MSCs transduced with shCtrl or shGBPs were stimulated with 20 ng/mL IFN- γ for 48 h, and the knockdown efficiencies of hGBP1 (A) and hGBP2 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 h, and cell survival was tested using a LIVE/DEAD viability/cytotoxicity kit. $n = 4$. The results are expressed as the means \pm SEM; ** $P < 0.01$; *** $P < 0.001$; N.S., not significant; PI, propidium iodide staining. (Scale bars: 20 μ m.)

presence of lipid droplets in the cytoplasm of shGBP-MSCs on day 21 after adipogenic differentiation (Fig. S6C). The knockdown of hGBPs did not alter the proliferative properties of human MSCs, which were verified by CCK8 assays ($P = 0.37$) (Fig. S6D). Cell survival analysis showed that the cells cultured with serum-deprived conditions for 48 h produced only a mild, nonsignificant increase in the death rate that was similar to that found in shGBP1-MSCs ($7.82 \pm 0.94\%$) and shCtrl-MSCs ($7.31 \pm 0.73\%$; $P = 0.21$) (Fig. 4D). 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 with control cells, inhibition of *T. gondii* growth was rescued in shGBP1-MSCs [Fig. 5A, Upper (RH/GFP) and Lower (PLK/RED)] at 24 h postinfection. 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. 5B). In contrast, there was no obvious difference between control cells and shGBP2- or shGBP5-MSCs at 24 h postinfection (Fig. 5C). 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, which do not.

Human GBP1 Is Recruited to the Parasitophorous 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 $\sim 10\%$ of vacuoles belonging to IFN- γ -stimulated MSCs infected with RH/GFP strain

at 3 h postinfection, but only 3% in the corresponding non-stimulated MSCs (Fig. 6A). A significantly higher percentage of PVs staining positive for hGBP1 was found in the cells infected with PLK/RED strain (Fig. 6B), which 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 seems to act in a cell type-specific and species-specific manner: e.g., nonlymphocytes shown in Fig. S7. 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 seem 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 that might be used to curtail *T. gondii* (9). Our results, however, have

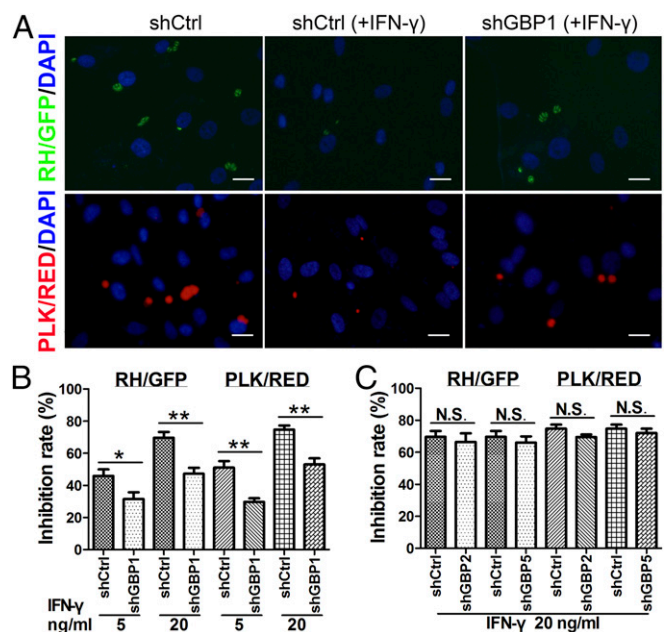


Fig. 5. IFN- γ -mediated resistance in MSCs requires hGBP1. Human MSCs transduced with shCtrl or shGBPs were stimulated with IFN- γ for 48 h and then infected with RH/GFP or PLK/RED. (A) Representative fluorescence images of intracellular *T. gondii* growth at 24 h postinfection. IFN- γ , 20 ng/mL. (Scale bars: 10 μ m.) (B and C) The inhibition rates of *T. gondii* growth in the indicated knockdown and control cells were calculated at 24 h postinfection (RH/GFP; PLK/RED). The results are expressed as the means \pm SEM $n = 4$. * $P < 0.05$; ** $P < 0.01$; N.S., not significant.

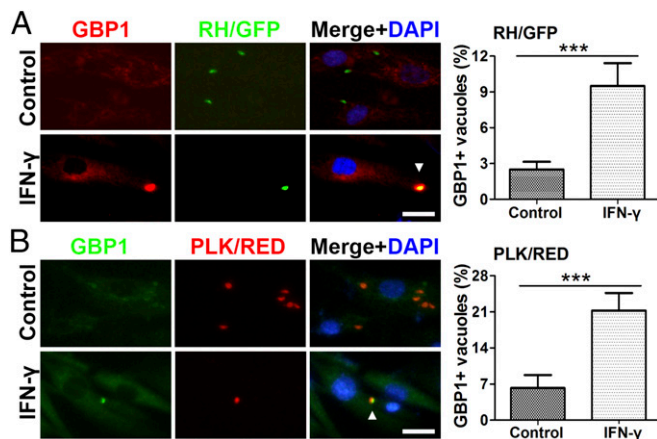


Fig. 6. Localization of hGBP1 in *T. gondii*-containing parasitophorous vacuoles. The subcellular localization of hGBP1 in human MSCs was analyzed by immunostaining. (A and B) Cells were stimulated with IFN- γ (20 ng/mL) for 48 h, infected with *T. gondii* for 3 h, stained with anti-hGBP1 and DAPI (A, RH/GFP; B, PLK/RED). $n = 4$. The results are expressed as the means \pm SEM. *** $P < 0.001$. (Scale bars: 5 μ m.)

indicated that IDO is not a main factor in the ability of human MSCs to inhibit the growth of *T. gondii* (Fig. 2D).

The iNOS-mediated production of NO is a well-known molecular effector against pathogens in the innate immune responses of various animals, including mMSCs (9) and skeletal muscle cells (27), and also in vivo (15, 30, 31). However, a previous study reported that iNOS protein expression was not detectable in human MSCs (9). Consistent with these results, our data also showed that MSCs derived from IFN- γ -stimulated human bone marrow neither expressed any detectable level of *iNOS* RNA nor displayed any inhibition of *T. gondii*'s growth via iNOS activity. Therefore, it is highly possible that NO is not the main molecular effector against replication of *T. gondii* in human MSCs stimulated by IFN- γ .

It has been reported that an IRG protein-based resistance mechanism is essential to control *T. gondii* replication in mice (17, 27). Numerous IRGs were found to converge on vacuoles containing *T. gondii* in IFN- γ -activated mouse macrophages, fibroblasts, and astrocytes (32–34). IRG subclass m (IRGm) in mouse is a membrane marker for host cell self-recognition that is used to regulate the IRG pathway (35) and other IRGs such as the glycine-lysine-serine motif containing IRGs (also known as GKs). Mouse IRGs were found to cooperate with mGBPs in acting against *T. gondii* infection (13, 14, 18). These findings suggested that mGBPs and mIRGs could play important roles in the IFN- γ -dependent, autonomous cellular control of toxoplasmosis. Furthermore, it can be predicted that these protein families could play broader roles in the host defense systems of murine cells. To our surprise, however, only one complete IRG (hIRGm) is present in the human genome, and it lacks the necessary IFN- γ induction element (33). In fact, based on our work, we also did not observe the up-regulation of any hIRGs in our RNA-seq experiments. Our data provide further evidence to suggest that hIRGm has little (if any) activity against *T. gondii* infection in humans, at least in the case of hMSCs. Without the cooperation of hIRGm, hGBPs, if present, may be sole contributors to antitoxoplasmosis 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), which 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, an mGBP cluster on chromosome 3 (comprising the genes encoding mGBP1, -2, -3, -5, and -7) was found to cooperate with mIRG6 and mIRG10 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 heterooligomerize 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) 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 mRNA level was low and of little capability of up-regulation (Fig. 3). Thus, hGBP1 in human MSCs stimulated by IFN- γ could be a functional homodimer in the innate immune response.

Recently, Halder 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 noncyst 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 h postinfection in IFN- γ -stimulated human MSCs (Fig. 6). 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 whether 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 suggests 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) used in this study have been approved by the Institutional Review Board of

Sun Yat-Sen University (2014064). 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, nontreated human MSCs, or conditioned medium (CM) on the growth of *T. gondii* [multiplicity of infection (moi) = 0.5] were assessed by microscopic counting at different time points postinfection (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 (Table S1). The short hairpin RNA (shRNA) expression vector pLKOpuo1 (Addgene) was used to express the control (shCtrl) and hGBP1, -2 and -5-specific shRNAs using specific oligos (Table S1). The helper plasmids pSPAX2 and pMD2.G were used as lentiviral expression vectors (Addgene). The 293T host cells were

used for packaging of the shRNAs. The MISSION Non-Target pLKOpuo1 control vector (Sigma-Aldrich) was used as a control (shCtrl). Pools of stably transduced cells were selected using puromycin (2 μ g/mL) for 5 to 7 d. 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 *SI Materials and Methods*.

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