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

Aiping Qin1,2,3, De-Hua Lai3,1, Qifa Liu3,1, Weijun Huang2,4, Ya-Ping Wu3, Xiaoyong Chen2,4, Sunxing Yan2,4, Huimin Xie4, Geoff Hidee5,6,7, Zhao-Rong Lun1,2, Francisco J. Ayala2, and Andy Peng Xiang2,4,7,8

1Department of Hematology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China; 2Center for Stem Cell Biology and Tissue Engineering, The Key Laboratory of Stem Cells and Tissue Engineering, Ministry of Education, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou 510080, China; 3Center for Parasitic Organisms, State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, China; 4Program of Stem Cells and Regenerative Medicine, Affiliated Guangzhou Women and Children’s Hospital, Guangzhou 510018, China; 5Ecosystems and Environment Research Centre, School of Environment and Life Sciences, University of Salford, Salford M5 4WT, United Kingdom; 6Biomedical Research Centre, School of Environment and Life Sciences, University of Salford, Salford M5 4WT, United Kingdom; 7Department of Ecology and Evolutionary Biology, University of California, Irvine, CA 92697; and 8Department of Biochemistry, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou 510080, China

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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 hGBP5 knockdowns had no effect. Moreover, the hGBP1 accumulation on the parasitophorous vacuole membrane (PV) in hMSCs is not affected by the presence of hGBP2 or -5. 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 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 authors declare no conflict of interest.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. SRP095307).

1A.Q., D.-H.L., Q.L., and W.H. contributed equally to this work.
2To whom correspondence may be addressed. Email: fayala@uci.edu, lszljr@mail.sysu.edu.cn, or xiangp@mail.sysu.edu.cn.

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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 -2p 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. L4). Interestingly, the same concentrations of IFN-γ had stronger inhibitory effects on the PLK/RED strain than on the RH/GFP strain (Fig. L8). 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. S1A and B). Representative fluorescence images of intracellular *T. gondii* growth at 48 h postinfection are shown in Fig. 1E (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* growth. 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...
that HeLa cells rely on IDO-mediated degradation of Trp for their ability to resist *T. gondii* infection. In human MSCs, in contrast, Trp-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.

### **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 ± SEM; **p < 0.01; ***p < 0.001; N.S., not significant; n = 4.

**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 ± SEM; n = 3.
Thus, our studies demonstrate that hGBP1 may play a major role in significantly reduced by shGBP1, compared with control MSCs (Fig. 6A).}

**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
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–activated mouse 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 *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 GSKs). 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 hGBP1 and hGBP2 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, 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 noncyt 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 nonlymphocytes 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 (14). Tachyzoites of T. gondii RH/GFP (50) and P. LK/R/GFP (51) were allowed to naturally express 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 293T cells was performed as described (47), using three sets of siRNAs (Table S1). The short hairpin RNA (shRNA) expression vector pLPKopuro1 (Addgene) was used to express the control (shCtrl) and hGBP-1, G, and 5’-specific shRNAs using specific oligos (Table S1). The helper plasmids pSAX2 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 pLKOpuro1 control vector (Sigma-Aldrich) was used as a control (shCtrl). Pools of stably transduced RhoA and p14 were stably transduced into the cell culture using a lentiviral expression vector pLPKopuro1 (Addgene) that was used to express the control (shCtrl) and hGBP-1, G, and 5’-specific shRNAs using specific oligos (Table S1). The helper plasmids pSAX2 and pMD2 G were used as lentiviral expression vectors (Addgene). The 293T host cells were

Supporting Information

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SI Materials and Methods

Chemicals and Reagents. Recombinant human IFN-γ was purchased from R&D Systems. L-tryptophan (Trp), 1-methyl-DL-tryptophan (DL-1 MT), D-1 MT, and L-1 MT were purchased from Sigma-Aldrich. The primary antibodies against hGBP1, hGBP2, hGBP5, and β-actin, plus the relevant HRP-conjugated secondary antibodies, were all purchased from Santa Cruz. Monoclonal antibodies against CD29, CD34, CD44, CD45, CD73, CD90, CD105, and CD166 were purchased from BD Biosciences Pharmingen.

MSC Culture. MSCs were derived from bone marrow aspirates (10 to 30 mL) of healthy voluntary donors after obtaining informed consent and according to guidelines approved by the Ethics Committee of Sun Yat-Sen University. Heparin-treated bone marrow was obtained by iliac crest aspiration from healthy donors who had provided informed consent. The MSCs were cultured and characterized as described previously and met all of the classification criteria for MSCs, as defined by the International Society of Cellular Therapy (52). Briefly, the unattached bone marrow cells were removed, and the cells that were positive for CD29, CD44, CD73, CD90, CD105, and CD166, but not CD34 or CD45, were collected by flow cytometry. These cells were confirmed as MSCs by validating that they could differentiate into osteoblasts and adipocytes. All of the MSCs used in this study were collected at passages 3 to 5. Each experiment was performed in triplicate using cells from at least three different donors.

Parasites and Infection. Toxoplasma gondii culture supernatants were harvested 3 to 4 d after infection, host cell debris was removed by differential centrifugation (5 min, 50 × g; 15 min, 500 × g), and the obtained parasites were resuspended in medium, counted, and immediately used to inoculate host cells. All parasite strains and cell lines were routinely checked for Mycoplasma contamination, which was not detected.

Human MSCs stimulated with IFN-γ for 48 h were coincubated with freshly egressed T. gondii (moi = 0.5) for 2 h, and extracellular parasites were removed. Cells were counted in 28 viewing fields (40×) of tangential circles aligned horizontally across the center of each well, which represented 3.6% of the area of each well. This counting method yielded consistent results (variation < 8%) across duplicate wells and between different observers.

RT-PCR and RNA-seq. RNA was isolated from human MSCs, using an RNeasy kit (Qiagen), and treated with DNase I to remove contaminating DNA. One microgram of total RNA was reverse transcribed to cDNA, and each target gene was amplified using the SYBR Green Master Mix (Bio-Rad) following the manufacturer’s protocol. The primer sequences are listed in Table S1. Quantitative real-time PCR (RT-PCR) was performed using a CFX96 Real-Time PCR System (Bio-Rad). Relative mRNA levels were calculated after normalization with respect to β-actin.

MSCs were treated with IFN-γ (20 ng/mL) for 12 h (n = 3). Then, RNA library preparation and sequencing were performed as recommended by the manufacturer (Genome Analyzer IIx; Illumina). Sequencing data were processed using Consensus Assessment of Sequence and Variation (Illumina) using the default settings. Genes with RPKM (reads per kilobase of exon per million mapped reads) values of more than five were enrolled in the functional analysis according to the Gene Ontology (GO) database. Genes related to host defense (GO:0051607 defense response to virus, GO:0042742 defense response to bacterium, GO:0042832 defense response to protozoan, GO:0050832 defense response to fungus) were selected to draw a heatmap with HemI (Heatmap Illustrator, version 1.0).

Western Blot Analyses. Cell lysates were analyzed on a 12% SDS/PAGE gel, blotted, and probed with antibodies against hGBP1, hGBP2, and hGBP5 (all at 1:500) or β-actin (1:5,000). The bound primary antibodies were detected using peroxidase-coupled secondary antibodies and enhanced chemiluminescence (Cell Signaling Technologies).

Immunofluorescence Analyses. Human MSCs (1 × 10^5) infected with T. gondii were fixed, permeabilized, blocked with 10% (vol/vol) goat serum, and incubated with anti-human GBP1 antibody (1:50) and then with Alexa Fluor 488/594-conjugated (green/red) IgG goat serum, and incubated with anti-human GBP1 antibody (1:50) and then with Alexa Fluor 488/594-conjugated (green/red) IgG goat serum, followed by secondary antibodies and Alexa Fluor 488 and 594 conjugated (green/red) IgG antibodies (Invitrogen). The samples were then washed three times with 0.01% Triton X-100 in PBS before the nuclei were stained with DAPI. The images were obtained using an IX71 fluorescence microscope (Olympus) and an LSM710 confocal microscope (Zeiss) and analyzed using ImageJ software (National Institutes of Health).

Statistical Analysis. Statistical analysis was performed using GraphPad Prism software, and the data are given as the means ± SD or means ± SEM of at least three independent experiments. The data were compared using the Student’s paired t test, and P < 0.05 were considered to be statistically significant.
The growth of T. gondii in IFN-γ–stimulated hMSCs. Human MSCs were pretreated for 48 h with the indicated concentrations of IFN-γ and then infected with T. gondii strains (RH/GFP, moi = 0.5). (A) At 24 h postinfection, the number of parasites per vacuole in IFN-γ–stimulated hMSCs (IFN-γ, 20 ng/mL) and unstimulated hMSCs (controls) were calculated. Indicated values are means ± SD, n = 4. (B) At 48 h postinfection, the numbers of intracellular T. gondii rosettes and parasitophorous vacuoles (PVs) containing a single parasite were counted microscopically, and ratios were calculated. Indicated values are means ± SD, n = 4. *P < 0.05, **P < 0.01, ***P < 0.001, compared with the controls. N.S., not significant. (C) Representative fluorescence images of intracellular T. gondii growth were shown at 48 h postinfection. (Scale bars: 20 μm.) DAPI, 4′,6-diamidino-2-phenylindole.

The effect of conditioned media (CM) from IFN-γ–treated MSCs on T. gondii growth of RH/GFP and PLK/RED strains. Toxoplasma gondii were incubated in the CM of IFN-γ–activated (20 ng/mL) MSCs for 30 min and then infected with untreated MSCs. At 48 h postinfection, the ratio between the quantity of intracellular T. gondii rosettes and the quantity of PVs containing a single parasite was calculated. The presented values are means ± SEM, n = 4.
Fig. S3. RNA-seq data analysis. Human MSCs from three different donors were pretreated for 12 h with IFN-γ (20 ng/mL). Then, RNA library preparation and sequencing were performed as recommended by the manufacturer. Sequencing data were processed using the default settings (CASAVA, version 1.8.2; Illumina). Genes with an RPKM value more than five were enrolled in the functional analysis according to the Gene Ontology (GO) database. Heat map display of genes related to host defense to virus (A), bacterium (B), protozoan (C), and fungus (D) are shown. The heatmaps of these genes were drawn with the RPKM value taking the logarithm of two.
Fig. S4. RPKM values of hGBP1 to -5 in hMSCs before and after IFN-γ stimulation. Human MSCs were pretreated for 12 h with IFN-γ (20 ng/mL). Mean reads per kilobase per million (RPKM) values of hGBP1 to -5 in IFN-γ–stimulated MSCs, as assessed by RNA-seq. Indicated values are means ± SD, n = 3. *, data from nonstimulated hMSCs.

Fig. S5. iNOS activity on the effect of hMSCs against T. gondii. Human MSCs were stimulated with 20 ng/mL IFN-γ and treated with 100 μg/mL the iNOS-specific inhibitor N⁵-monomethyl-L-arginine acetate (N⁵MMA) for 48 h. The cells were then infected with the two strains of T. gondii (RH/GFP; PLK/RED) for 24 h (moi = 0.5), and growth inhibition rates were calculated for the parasites (Left, RH/GFP; Right, PLK/RED). The results are expressed as the means ± SEM (n = 4).
Fig. S6. The phenotype and differentiation capacity of shCtrl- and shGBP1-MSCs. (A) Representative flow-cytometric analysis of shCtrl- and shGBP1-MSCs. CD29, CD34, CD44, CD45, CD73, CD105, CD90, and CD166 expression in shCtrl- and shGBP1-MSCs was analyzed by fluorescence-activated cell sorting, which showed that transduced cells maintained the phenotype of human MSCs. (B) Alizarin Red S staining of osteogenic-differentiated shGBP1-MSCs. (C) Oil red O staining of adipogenic-differentiated shGBP1-MSCs. (Scale bar: 50 μm.) (D) Cell proliferation was analyzed by cell counting kit-8 (CCK8) assay.
Fig. S7. IFN-γ stimulates protection in nonlymphocytes against T. gondii infection. (A) GKS and GBP pathways in mouse embryo fibroblast cells (MEFs). IFN-γ stimulation up-regulates the expression of the glycine-lysine-serine motif containing immunity-related GTPase (GKS) proteins and guanylate-binding proteins (GBPs). GKS is activated via dimerization/oligomerization and translocated on the parasitophorous vacuole (PV) membrane (34), which induces ubiquitination (Ub) of the PV (44). Further, GKS recruits GBP on the PV membrane (26, 44), which inhibits growth of the parasite (18, 26, 42). Autophagy proteins (ATG) are required for GKS and GBP recruitment although the mechanism is uncertain (45). (B) NO pathway in mouse mesenchymal stromal cells (mMSCs). IFN-γ, tumor necrosis factor α (TNFα), and interleukin 1β (IL1β) could induce inducible nitric oxide synthase (iNOS), but IFN-γ alone is not sufficient (9). iNOS consumes L-arginine and produces nitric oxide (NO), which inhibits the growth of parasites. (C) IDO pathway and an unknown pathway in human foreskin fibroblast cells (HFF). IFN-γ up-regulates indoleamine 2,3-dioxygenase 1 (IDO), which results in depletion of L-tryptophan (Trp) and thus causes nutrition restriction of the parasites (21). Alternatively, an unknown pathway stimulates host cell death in response to the early egress of parasite (22, 53). (D) GBP pathway and IDO pathway in human mesenchymal stromal cells (hMSCs). The GBP pathway in hMSCs is functional in a similar manner to that in MEF cells, but GKS proteins were not identified in humans. An unknown factor (?) is yet to be revealed that may replace the GKS pathway. Alternatively, the IDO pathway may also be involved (9), but this involvement was not observed in our study.
<table>
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<th>Table S1. Oligonucleotides used in this study</th>
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<td><strong>Assay</strong></td>
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| Quantitative real-time primers | β-actin | F: GATTACTGCTCTGCTCTCTAGC  
R: GACTGATCTACCTCTGTGTG |
| | IDO | F: AAGAAGTGGCTTTTGGCTCG  
R: CGCTGGACCTTGGTCTG |
| | hGBP1 | F: GTGGAGAAGGGGAAAGCTGA  
R: CAACCTGGACCTTGGTCCTT |
| | hGBP2 | F: GATTTCACCCTGAGACCTGGA  
R: GGATTGACCTTCTCTCCTT |
| | hGBP3 | F: TTAATCTGCCCAGACTCTGT  
R: CATGGACCCTTGATGCTCT |
| | hGBP4 | F: GCCCAAAATGAGAAGAAGT  
R: AGGCCCGATGAGTGACCA |
| | hGBP5 | F: AGGCGAAAGCATGAGTGA  
R: ATGATGACCCTGGAAGAGT |
| siRNA | siIDO1 | Sense: TGTTACAATGGGTAATGACAG  
Antisense: GCATTACCACATTGTAACAGA |
| | siIDO2 | Sense: TATAATCTACACTATACCT  
Antisense: GATATTACCTATTTCTACCT |
| | siIDO3 | Sense: TTAATCTGCCCACCTTGG  
Antisense: GAAAGATTGTTGAAAGAC |
| Nontargeting control (siCtrl) | Sense: AACGGATGAGAGACCAAC  
Antisense: TTGGTGCTCTTCATCTGTT |
| Short hairpin RNA primers | shGBP1 | AAATCCAACAGGAACTGACTGACATACCTCGAGCTGGATGTATCTCCTCT  
CCGGCCGATGAGGCTCCTGTAGACTCTCTCTCCTTGGT |
| | shGBP2 | AAATCAAACAGGAACTGACTGACATACCTCGAGCTGGATGTATCTCCTCTCTCCTTGGT |
| | shGBP5 | AAATCAAACAGGAACTGACTGACATACCTCGAGCTGGATGTATCTCCTCTCTCCTTGGT  
CCGGCCGATGAGGCTCCTGTAGACTCTCTCTCCTTGGT |

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