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

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Supporting Information

SI Materials and Methods

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

MSC Culture. MSCs were derived from bone marrow aspirates (10-30 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 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, 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 3-4 days 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 48h were co-incubated with freshly egressed *T. gondii* (moi = 0.5) for 2 hr, 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% 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, USA), 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, USA) following the manufacturer's protocol. The primer sequences are listed in SI Table 1. Quantitative real-time PCR was performed using a CFX96 Real-Time PCR System (Bio-Rad, USA). Relative mRNA levels were calculated after normalization with respect to β-actin.

MSCs were treated with IFN-γ (20 ng/mL) for 12 hours (n = 3). Then, RNA library preparation and sequencing were performed as recommended by the manufacturer (Genome Analyzer IIx; Illumina, USA). Sequencing data were processed using Consensus Assessment of Sequence and Variation (Illumina, USA) 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, hGBP5 (all at 1:500) or β-actin (1:5000). The bound primary antibodies were detected using peroxidase-coupled secondary antibodies and enhanced chemiluminescence (Cell Signaling Technologies, USA).

Immunofluorescence Analyses. Human MSCs (1×10^5) infected with *T. gondii* were fixed, permeabilized, blocked with 10% goat serum, incubated with anti-human GBP1 antibody (1:50) and then with Alexa Fluor 488/594-conjugated (green/red) IgG (Invitrogen, USA). 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, Japan) and an LSM710 confocal microscope (Zeiss, Germany), and analyzed using the ImageJ software (NIH, USA).

Statistical Analysis. Statistical analysis was performed using GraphPad Prism software (San Diego, USA), 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* values <0.05 were considered to be statistically significant.

SI Figure Legends

Fig. S1. The growth of *T. gondii* in IFN-γ-stimulated hMSCs. Human MSCs were pretreated for 48 hr with the indicated concentrations of IFN-γ and then infected with *T. gondii* strains (RH/GFP, moi = 0.5). (*A*) At 24 hr post-infection, 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 hr post-infection, 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. Abbreviations: N.S., not significant. (C) Representative fluorescence images of intracellular *T.gondii* growth were shown at 48 hr post-infection. Scale bars represent 20μm. Abbreviations: DAPI, 40,6-diamidino-2-phenylindole.

Fig. S2. 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 minutes and then infected with untreated MSCs. At 48hr post-infection, the ratio between the quantity of intracellular *T. gondii* rosettes and the quantity of PVs containing a single parasite was calculated. The results presented values are means ± SEM. $n = 4$.

Fig. S3. RNA-seq data analysis. Human MSCs from three different donors were pretreated for 12hr with IFN-γ (20 ng/ml). Then, RNA library preparation and sequencing were

performed as recommended by the manufacturer. Sequencing data were processed using Consensus Assessment of Sequence and Variation using the default settings (CASAVA, version 1.8.2; Illumina, USA). Genes with a RPKM value more than five were enrolled in the functional analysis according to the Gene Ontology (GO) database. Heat map displays 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 hGBP1-5 in hMSCs before and after IFN-γ stimulation. Human MSCs were pretreated for 12hr with IFN-γ (20 ng/ml). Mean reads per kilobase pairs per million (RPKM) values of hGBP1-5 in IFN-γ-stimulated MSCs, as assessed by RNA-seq. Indicated values are means ± SD. $n = 3$. *, data from non-stimulated hMSCs.

Fig. S5. iNOS activity on the effect of hMSCs against *T. gondii*. Human MSCs were stimulated with 20 ng/ml of IFN-γ and treated with 100ug/ml the iNOS-specific inhibitor N-methyl-L-arginine acetate (N^GMMA) for 48 h. The cells were then infected with the two strains of *T. gondii* (RH/GFP; PLK/RED) for 24 hours (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 shGBP-MSCs.

(*A*) Representative flow-cytometric analysis of shCtrl- and shGBPs-MSCs. CD29, CD34,

CD44, CD45, CD73, CD105, CD90, and CD166 expression in shCtrl- and shGBPs-MSCs were 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. The scale bar represents 50μm. (*D*) Cell proliferation was analyzed by CCK8 assay. CCK8, cell counting kit-8.

Fig. S7. IFN-γ stimulates protection in non-lymphocytes against *T. gondii* infection. (*A*) GKS and GBP pathways in mouse embryo fibroblast cells (MEF). IFN-γ stimulation upregulates the expression of glycine-lysine-serine motif containing immunity-related GTPase (GKS) proteins and guanylate binding proteins (GBP). GKS is activated via dimerization/oligomerization and translocated on the parasitophorous vacuole (PV) membrane (34), which induces ubiquitination (U) of the PV (44). Further, GKS recruits GBP on the PV membrane (26, 44), which inhibits growth of the parasite (18, 26, 42). Autophage proteins (ATG), are required for GKS and GBP recruitment although the mechanism is uncertain (45). (*B*) NO pathway in mouse mesenchymal stromal cells (mMSC). IFN-γ, tumor necrosis factor (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 (HEF). IFN-γ upregulates 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 (hMSC). The GBP pathway functions in a similar manner to that in MEF cells, but in the absence of GKS proteins in humans. An unknown factor (?) is yet to be revealed which may replace the GKS pathway. Alternatively, the IDO pathway may also be involved (9), but this is not observed in our study.