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Cell Death of Gamma Interferon-Stimulated Human Fibroblasts upon *Toxoplasma gondii* Infection Induces Early Parasite Egress and Limits Parasite Replication

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The intracellular protozoan parasite *Toxoplasma gondii* is a major food-borne illness and opportunistic infection for the immunosuppressed. Resistance to *Toxoplasma* is dependent on gamma interferon (IFN-γ) activation of both hematopoietic and nonhematopoietic cells. Although IFN-γ-induced innate immunity in nonhematopoietic cells has been extensively studied in mice, it remains unclear what resistance mechanisms are relied on in nonhematopoietic human cells. Here, we report an IFN-γ-induced mechanism of resistance to *Toxoplasma* in primary human foreskin fibroblasts (HFFs) that does not depend on the deprivation of tryptophan or iron. In addition, infection is still controlled in HFFs deficient in the p65 guanylate binding proteins GBP1 or GBP2 and the autophagic protein ATG5. Resistance is coincident with host cell death that is not dependent on the necroptosis mediator RIPK3 or caspases and is correlated with early egress of the parasite before replication. This IFN-γ-induced cell death and early egress limits replication in HFFs and could promote clearance of the parasite by immune cells.

Innate immunity, in which immune cells recognize pathogen-associated molecular patterns and secrete proinflammatory cytokines to activate antimicrobial responses, is crucial in host defense against intracellular pathogens. For instance, the cytokine gamma interferon (IFN-γ) activates macrophages and many nonimmune cells to cell-autonomously fight infections of many intracellular organisms, including the protozoan parasite *Toxoplasma gondii* (1). *Toxoplasma* actively invades host cells, divides within a nonfusogenic parasitophorous vacuole (PV), and then destroys the cell upon active egress, making intracellular resistance mechanisms important for host defense (2).

Toxoplasma can infect all warm-blooded animals, including humans (3). It is estimated that a third of the global population is infected with *Toxoplasma*. Most infections in humans are asymptomatic, but *Toxoplasma* establishes a lifelong chronic infection by forming dormant cysts in brain and muscle tissue. However, *Toxoplasma* can cause severe disease and death in immunosuppressed individuals and in developing fetuses of pregnant women. It is also an important cause of ocular disease in both immunocompetent and immunosuppressed individuals (4, 5). In a recent study, *Toxoplasma* was among the top five pathogens responsible for the majority of economic losses and quality of life impairment due to food-borne illness in the United States (6).

Many resistance mechanisms effective against *Toxoplasma* have been identified in macrophages. For instance, in mouse and human macrophages, CD40 stimulation induces autophagic killing of the parasite by fusion of parasitophorous vacuoles with lysosomes (7). In addition, activation of the purinergic receptor P2X7R leads to killing of the parasite in murine and human macrophages, and killing is associated with fusion of the parasitophorous vacuole with lysosomes or apoptotic death in murine macrophages (8, 9). The NALP1 inflammasome receptor was also identified as a susceptibility locus for human congenital toxoplasmosis, and silencing NALP1 leads to uncontrolled parasite growth in human monocytes (10). Although IFN-γ-induced expression of nitric oxide synthase (NOS2) in macrophages is important for controlling the chronic stages of infection in mice (11), nitric oxide production does not appear to play a role in controlling *Toxoplasma* infection by human macrophages (12). However, IFN-γ not only activates macrophages but also induces anti-*Toxoplasma* activity in nonimmune cells (1). Indeed, in chimeric mice, IFN-γ receptors were shown to be necessary in both hematopoietic and nonhematopoietic cells to survive *Toxoplasma* infection (13). Although in mice the main IFN-γ-inducible effector mechanism against the acute phase of *Toxoplasma* infection is the p47 immunity-related GTPases (IRGs) that localize to and disrupt parasitophorous vacuoles (14), humans lack the multitude of IRGs present in mice (15). Indeed, ROP5 and ROP18, the virulence factors that allow *Toxoplasma* to evade the IRGs in mice, do not affect the ability of the parasite to survive in IFN-γ-activated human foreskin fibroblasts (HFFs) (16). Much less is known about the effector mechanisms of nonimmune cells in humans compared to mice.

The main characterized mechanism of resistance to *Toxoplasma* in nonimmune human cells is nutrient deprivation. For instance, *Toxoplasma* is auxotrophic for tryptophan, and the IFN-γ-inducible enzyme indoleamine 2,3-dioxygenase (IDO1) degrades tryptophan. Tryptophan supplementation has been shown

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to restore parasite growth in IFN-γ-stimulated human lung cells and fibroblasts (17–20). However, in human intestinal epithelial cells and umbilical vein endothelial cells, tryptophan supplementation was unable to reduce IFN-γ-induced inhibition of *Toxoplasma* growth (21, 22). Furthermore, IFN-γ was shown to inhibit *Toxoplasma* replication in rat enterocytes by limiting iron availability, and *Toxoplasma* growth was restored by addition of ferrous sulfate or holotransferrin (23). Although IFN-γ-activated human monocytes were shown to downregulate transferrin receptor expression to limit the growth of other microbes (24), iron supplement did not restore growth of *Toxoplasma* in IFN-γ-activated human macrophages (25). However, macrophages might have other mechanisms for resisting *Toxoplasma* growth that could make iron depletion unnecessary, and it is unknown whether iron deprivation plays a role in nonimmune cell resistance. Together, these studies suggest that methods of resistance vary by cell type and that other resistance mechanisms remain to be uncovered.

There are other antimicrobial effectors induced by IFN-γ that nonimmune cells can utilize in *Toxoplasma* resistance. For example, though humans do not possess the large family of IRGs present in mice and some other mammals, humans do have another family of large IFN-γ-induced GTPases called the p65 guanylate binding proteins (GBPs). In mice, it was shown that GBPs localize to the parasitophorous vacuole alongside the IRGs (26), and mice deficient in a cluster of six GBPs are susceptible to *Toxoplasma* and lack IRG localization to the parasitophorous vacuole (27). Humans have five IFN-inducible GBPs, and it is possible that they could play a similar role in *Toxoplasma* resistance in human cells.

Another resistance mechanism induced by IFN-γ is autophagy via phosphatidylinositol 3-kinase activation (28). Autophagosomes not only sequester organelles and cytoplasmic protein aggregates, but intracellular microbes as well, to deliver their contents to the lysosome for degradation. Autophagosome sequestration of *Toxoplasma* in human nonimmune cells has not been reported, but autophagy is also important for the regulation of some proteins, such as the IRGs and GBPs in murine cells (29, 30). Furthermore, some instances of excessive autophagy have been reported to correlate with cell death (31), and cell death, autophagic or otherwise, can also prevent parasite proliferation. Several cell death pathways have been implicated in immunity: caspase-dependent apoptosis, RIP kinase-dependent necroptosis, and caspase-1- and interleukin-1β (IL-1β)-dependent pyroptosis, which occurs only in inflammatory cells (32). It remains to be seen whether autophagy or host cell death plays a role in *Toxoplasma* resistance in nonimmune cells.

We report here that in IFN-γ-stimulated HFFs, neither tryptophan supplementation nor IDO1 inhibition can restore parasite growth. Furthermore, iron supplementation does not relieve IFN-γ-induced growth inhibition. In addition, *Toxoplasma* resistance is not significantly altered in cells deficient for GBP1, GBP2, or ATG5. Instead, we find that IFN-γ stimulation and *Toxoplasma* infection leads to increased host cell death that is unaffected by chemical inhibition of necroptosis or caspases or knockdown of the necroptosis mediator RIPK3. Interestingly, we find that IFN-γ and infection-induced host cell death is correlated with but not dependent on early egress of the parasite. Parasite proliferation is inhibited even through multiple rounds of reinvasion and egress without replication. Importantly, early egress of the parasite not only limits parasite burden by preventing growth but disrupts the intracellular niche, which could promote parasite clearance by immune cells *in vivo*.

MATERIALS AND METHODS

Reagents. A mouse monoclonal antibody against GBP1-5 (G-12[Santa Cruz], 1:100 dilution), a rabbit polyclonal antibody against LC3B (antibody 2775 [Cell Signaling]; 1:700 dilution), a rabbit polyclonal antibody against ATG5 (antibody 2630 [Cell Signaling]; 1:1,000 dilution), a rabbit polyclonal antibody against human HMGB1 (ab18256 [Abcam]; 1:900 dilution), a rat polyclonal antibody against GBP1 (1B1 [Santa Cruz]; 1:500 dilution), a goat polyclonal antibody against GBP2 (N-17 [Santa Cruz]; 1:500 dilution), a rabbit polyclonal antibody against *RIPK3* (M-2 [Santa Cruz]; 1:500 dilution), and a mouse monoclonal antibody against β-actin (ab8226 [Abcam]; 1:10,000 dilution) were used in immunofluorescence assays or Western blotting. Secondary antibodies were coupled with Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes) or horseradish peroxidase (Kirkegaard and Perry Laboratories). L-Tryptophan (MP Biochemicals) and 1-methyl-L-tryptophan (Sigma-Aldrich) were dissolved in 0.1 N NaOH before use. Indole (Sigma-Aldrich), ferric nitrate (MP Biomedicals), deferoxamine (CalBiochem), and dextran sulfate (Sigma-Aldrich) were dissolved in water before use. Necrostatin-1 (Sigma-Aldrich), 3-methyladenine (Sigma-Aldrich), z-VAD-FMK (Axxora), mycalolide B (Enzo Life Sciences), A23187 (Sigma-Aldrich), and 3-MB-PP1 (Calbiochem) were initially dissolved in dimethyl sulfoxide (DMSO) and further dissolved in Dulbecco modified Eagle medium (DMEM) before use. Hoechst 33342 (Invitrogen) was dissolved in DMSO. Human IFN-γ from AbD Serotec was dissolved in DMEM with 10% fetal bovine serum (FBS).

Parasites and cells. Parasites were maintained *in vitro* by serial passage on monolayers of human foreskin fibroblasts (HFFs) at 37°C in 5% CO_2. HFFs were grown as described previously (33), and HeLa cells were grown in HFF media supplemented with 1 mM sodium pyruvate. An RH strain engineered to express clickbeetle luciferase and green fluorescent protein (GFP; RH 1-1) was described previously (34). RH strains engineered to express $TgCDPK1^M$ and either $TgCDPK3^G$ or $TgCDPK3^M$ were gifts from S. Lourido and were grown as described previously (35).

Human umbilical vein endothelial cells (HUVEC) were grown on gelatin-coated dishes in 199 medium (Life Technologies) supplemented with 20% FBS, 50 μg of gentamicin/ml, 30 μg of ECGF/ml, and 10 U of heparin/ml and used before passage 6. Type I (RH) or type II (Prugniaud) strains of *Toxoplasma*, stably transfected with tdTomato or eGFP/luciferase, respectively, were used to infect HUVEC.

Immunofluorescence assays. Parasites were allowed to invade monolayers of HFF cells grown on coverslips previously incubated for 24 h with or without 100 U of IFN-γ/ml, and infection proceeded for 8 h. The cells were then fixed and prepared for immunofluorescence as described previously (33).

Plaque assay. For the plaque assay, 100 to 300 parasites per well were added to monolayers of HFFs seeded 2 days before and either previously stimulated with 100 U of human IFN-γ/ml or left unstimulated for 24 h before infection in a 24-well plate. Infections were then incubated for 4 days at 37°C, and the number of plaques was counted using a microscope.

PI staining. HFFs were seeded into a 24-well plate just as for the plaque assay. The medium was changed the next day, and cells were stimulated with 100 U of IFN-γ/ml. Syringe lysed parasites were passed through a Millipore 5-μm-pore-size filter to remove lysed nuclei before infection. A "parasite-only" well was used to ensure no host nuclei were added to the HFFs. After 8 or 24 h of infection, propidium iodide (PI) and Hoechst 33342 (Invitrogen) were added, and staining was imaged 15 min later using a fluorescence microscope.

Live microscopy. HFFs were plated on 24-well glass bottom plates; the next day the medium was changed, and the cells were stimulated with IFN-γ for 24 h before infection. Infection was synchronized by spinning at 900 rpm for 3 min and washing with phosphate-buffered saline five times after an hour of infection. Infected cells were then imaged every 10 min

over a 16-h period using a ×40 objective lens (NA 0.95) on a Nikon TE2000 inverted microscope equipped with an environmental chamber, Hamamatsu ORCA-ER digital camera, and NIS Elements Imaging Software.

HUVEC were transfected with eGFP-C1 and mCherry-C1 vectors by nucleofection to label the cytoplasmic compartment 48 h prior to infection, and the cells were plated on gelatin-coated, glass-bottom dishes. HUVEC were then stimulated and infected as for the HFF cells above, with Pru-GFP and RH-tomato parasites infecting the mCherry and eGFP-transfected HUVEC, respectively. Infected cells were imaged at 5-min intervals for up to 4 h using a ×60 objective lens on a DeltaVision microscope equipped with 37°C chamber and 5% CO_2.

shRNA knockdowns. HFFs were infected with lentivirally packaged short hairpin RNA (shRNA) vectors (Broad RNAi Consortium) in the presence of 8 μg of Polybrene (Sigma-Aldrich)/ml for 24 h (ATG5 target sequence, 5′-CCTTTCATTCAGAAGCTGTTT-3′; GBP1 target sequence, 5′-CCAGATGAGTACCTGACATAC-3′; GBP2 target sequence, 5′-ATTGAAGTGGAACGTATAAAG-3′; RIPK3 target sequence, 5′-GGCGACCGCTCGTTAACATAT-3′; LacZ target sequence, 5′-GTCGGCTTACGGCGGTGATTT-3′). The infection medium was removed, and the following day the cells were switched to and maintained in medium containing 2 μg of puromycin (Invitrogen)/ml. All experiments were performed in media without puromycin, and knockdown was reconfirmed at the end of the experiment. Knockdown was confirmed by reverse transcription-quantitative PCR (RT-qPCR) by comparison to β-actin and no shRNA control cells. Briefly, RNA was isolated with TRIzol (Invitrogen) and cleaned up with a Qiagen RNeasy kit. Reverse transcription was performed using the Superscript III reverse transcriptase system (Invitrogen) with oligo(dT). Quantitative real-time PCR was performed with the following primers: β-actin FW (forward), 5′-CATGTACGTTGCTATCCAGGC-3′, and RV (reverse), 5′-CTCCTTAATGTCACGCACGAT-3′; ATG5 FW, 5′-AGAAGCTGTTTCGTCCTGTGG-3′, and RV, 5′-AGGTGTTTCCAACATTGGCTC-3′; GBP1 FW, 5′-CTCTTAAACTTCAGGAACAGGAGC-3′, and RV, 5′-CATGATCATTGTACCACATGCC-3′; GBP2 FW, 5′-TTTCCAGCATTTGTGTGGACT-3′, and RV, 5′-GGGAAGAACTTTCGGATGCAC-3′; and RIPK3 FW, 5′-AATCCAGTAACAGGGCGACC-3′, and RV, 5′-GCCTCAGGATCTTTAGGGCC-3′.

Statistical analysis. All comparisons were analyzed for statistical significance by using two-tailed Student *t* tests.

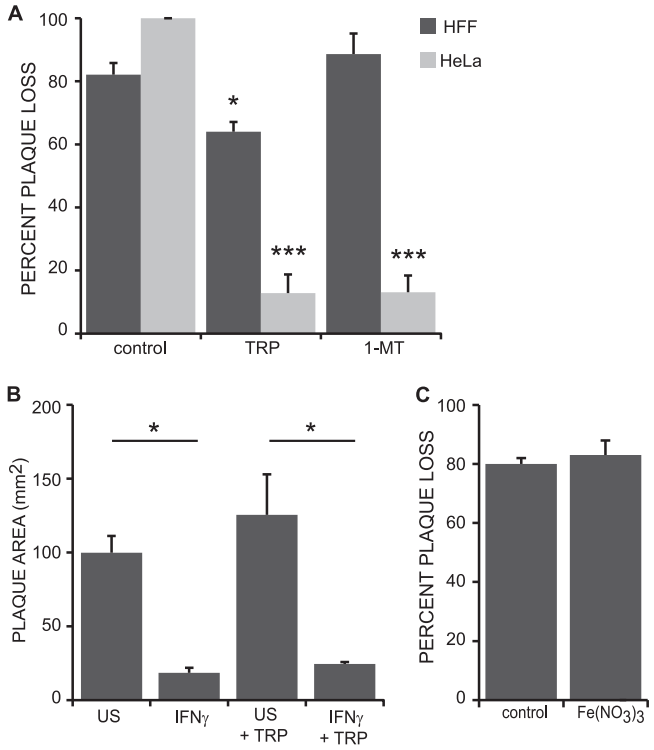

FIG 1 IFN-γ-mediated resistance in HFFs is not dependent on tryptophan or iron depletion. (A) The percent plaque loss on HFFs or HeLa cells previously stimulated with 100 U of IFN-γ/ml for 24 h was determined in the presence of 1 mM tryptophan (TRP) supplement added upon infection, 1 mM IDO1 inhibitor (1-MT) added at the time of IFN-γ stimulation or, as a control, the same volume of 0.1 N NaOH, the solvent used to dissolve both compounds. Means + the standard errors (SE) are shown ($n > 3$ experiments). *, $P < 0.05$; ***, $P < 0.001$ (Student *t* test). (B) Area of the plaques formed on IFN-γ-stimulated or unstimulated (US) HFFs in the presence or absence of tryptophan added upon infection. Means + the SE are shown ($n = 3$ experiments). *, $P < 0.05$ (Student *t* test). (C) The percent plaque loss on IFN-γ-stimulated HFFs was determined in the presence of 25 μM ferric nitrate [$Fe(NO_3)_3$] added upon infection. Means + the SE are shown ($n = 3$ experiments).

RESULTS

Tryptophan supplementation does not rescue *Toxoplasma* proliferation in IFN-γ-stimulated HFFs. To study intracellular resistance to *Toxoplasma* infection in primary nonimmune cells, we sought to measure *Toxoplasma* growth inhibition by IFN-γ in HFFs. Plaque formation includes all parts of the lytic cycle rather than measuring simply division, so the number and size of plaques can more accurately reflect *in vivo* parasite burden than other measures of growth such as parasite per vacuole counts. Therefore, we infected monolayers of cells either previously stimulated with IFN-γ for 24 h or left unstimulated and compared the number of plaques formed after 4 days of growth to determine the percent plaque loss due to IFN-γ stimulation. In HFFs, IFN-γ stimulation causes 82% plaque loss and 81% reduction in plaque area (Fig. 1A and B). Previous studies have shown that in some human cell types, IFN-γ stimulation inhibits *Toxoplasma* growth by depletion of tryptophan (19, 20, 36), while in other cell types tryptophan supplementation cannot restore parasite growth (21, 22). To test the role of tryptophan deprivation in the control of *Toxoplasma* proliferation in IFN-γ-stimulated primary HFFs, we measured the percent plaque loss due to IFN-γ when we supplemented the medium with L-tryptophan (L-Trp) simultaneously with infection or when we inhibited IDO1 by addition of 1-methyl-L-tryptophan (1-MT) (37) at the time of IFN-γ stimulation. As a positive control, we used HeLa cells that were shown to limit *Toxoplasma* growth by tryptophan depletion (36). Indeed, the percent plaque loss on IFN-γ-activated HFFs is only minimally reduced, from 82 to 64%, in the presence of tryptophan supplement ($P = 0.028$) and is unaffected by 1-MT (88% plaque loss) (Fig. 1A). Furthermore, although tryptophan supplementation results in larger plaques than control (mean of 125 mm^2 with tryptophan compared to a mean of 99.8 mm^2 without), it does not restore plaque size in IFN-γ-stimulated cells (mean of 24 mm^2 IFN-γ with tryptophan) (Fig. 1B). However, parasite survival is almost completely restored when IFN-γ-activated HeLa cells are supplemented with tryptophan or 1-MT (plaque loss of 12 and 13%, respectively; $P = 0.006$) (Fig. 1A), suggesting that the compounds are functional and that some cells do indeed solely rely on tryptophan degradation for *Toxoplasma* resistance. The inability of tryptophan to restore *Toxoplasma* growth in IFN-γ-stimulated HFFs indicates a different mechanism of resistance in these cells.

IFN-γ-induced *Toxoplasma* resistance in HFFs is not dependent on iron depletion. Because *Toxoplasma* is also auxotrophic for iron (23, 38), we wondered whether HFFs could use iron de-

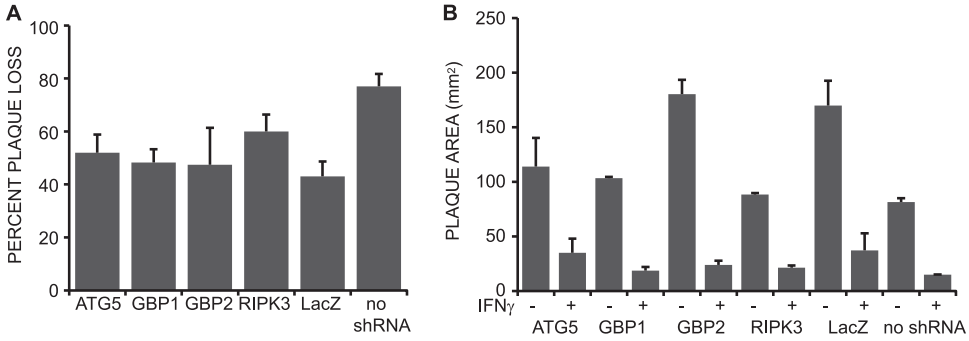

FIG 2 GBP1, GBP2, ATG5, and RIPK3 are not necessary for IFN-γ-mediated resistance in HFFs. Lentiviral shRNA was used to knock down *GBP1*, *GBP2*, *ATG5*, and *RIPK3*, or LacZ was used as a control. (A) Percent plaque loss on IFN-γ-stimulated HFFs for the indicated gene knockdown compared to a no-shRNA control. Means + the SE are shown ($n > 3$ experiments). (B) Area of the plaques formed on IFN-γ-stimulated or unstimulated HFFs with the indicated gene knocked down. Means + the SE are shown ($n = 3$ experiments).

pletion to curb parasite growth. To test this hypothesis, we performed the plaque assay in media supplemented with 25 μM ferric nitrate or 250 μM deferoxamine, an iron chelator. As expected, no plaques formed in the presence of deferoxamine because *Toxoplasma* requires iron to grow. However, iron supplementation did not restore growth in IFN-γ-induced HFFs (83% plaque loss compared to 80% without iron) (Fig. 1C), and indeed, we found that higher concentrations of iron inhibited parasite growth on unstimulated HFFs (data not shown). Thus, the observed inhibition of *Toxoplasma* growth in IFN-γ-stimulated HFFs is not dependent on iron depletion.

Autophagy is not necessary for IFN-γ-induced inhibition of *Toxoplasma* proliferation in HFFs. IFN-γ stimulation also induces autophagy (28), which could be important for inhibiting *Toxoplasma* replication in HFFs either by sequestration in autophagosomes or regulation of other effectors, as is the case for IRGs and GBPs in murine cells (30). To determine whether autophagosomes do sequester parasitophorous vacuoles, we used immunofluorescence to stain for LC3, a marker of autophagosomes, in IFN-γ-stimulated HFFs. We rarely observed (<1%) LC3 localized around the parasitophorous vacuole, making it unlikely that sequestration of PVs by autophagosomes is responsible for the inhibition of *Toxoplasma* growth in IFN-γ-stimulated HFFs. Because autophagy inhibitors also affect the parasite (39, 40; data not shown), we inhibited host autophagosome formation by creating stable *ATG5* knockdown HFF cell lines to test whether autophagy is necessary for IFN-γ-induced resistance to *Toxoplasma*. Knockdown was confirmed by RT-qPCR and Western blotting, and a limited amount of LC3 conversion to the lipidated form associated with autophagosomes was observed (see Fig. S1A and B in the supplemental material). IFN-γ-stimulated ATG5-deficient HFFs are not less able to resist *Toxoplasma* proliferation than LacZ shRNA control HFFs (52% plaque loss compared to 43% plaque loss for LacZ shRNA control; $P = 0.39$) (Fig. 2A). In addition, ATG5-deficient HFFs still limit plaque sizes on IFN-γ-stimulated monolayers (Fig. 2B). Because we do not see colocalization of parasitophorous vacuoles with autophagosomes or altered plaque loss in autophagy-deficient cells, it seems that autophagy is not necessary for IFN-γ-induced inhibition of parasite replication in HFFs.

GBP1 and GBP2 are not necessary for IFN-γ-induced *Toxoplasma* resistance in HFFs. Another possible cause for the inhibition of *Toxoplasma* growth in IFN-γ-stimulated HFFs is that host GBP proteins could localize to the parasitophorous vacuole and promote membrane remodeling or vacuolar destruction, as is observed in murine cells. To determine whether human GBPs colocalize with the PV, we stained IFN-γ-stimulated HFFs with an antibody that recognizes GBP1-5. At a very low frequency (<1%), we do observe vacuolar localization of GBPs, but it is unlikely that this low level of localization could explain the significantly decreased parasite survival in IFN-γ-stimulated HFFs. However, the GBPs were also shown to promote pyroptosis in *Salmonella*-infected macrophages and associate with autophagic machinery and components of the NADPH oxidase, so they could still play a role in resistance without localizing to the parasitophorous vacuole (41, 42). To test whether the GBPs are necessary for the observed IFN-γ-induced resistance in HFFs, we created stable *GBP1* and *GBP2* knockdown HFF cell lines, since these GBPs were shown to localize to chlamydial inclusions to inhibit their growth (43). After confirming knockdown by RT-qPCR (see Fig. S1A in the supplemental material), we performed the plaque assay with IFN-γ-stimulated HFFs in which GBP1 or GBP2 had been knocked down. IFN-γ-stimulated HFFs deficient in either GBP1 or GBP2 are not less able to resist *Toxoplasma* than a LacZ shRNA control, as measured by IFN-γ-induced plaque loss (48% plaque loss for GBP1 knockdown and 47% plaque loss for GBP2 compared to 43% LacZ shRNA controls; $P = 0.43$ and 0.83, respectively), and the plaque size in IFN-γ-stimulated GBP1 or GBP2 knockdown cells is also reduced compared to unstimulated knockdown cells (Fig. 2). Thus, GBP1 and GBP2 are not necessary for IFN-γ-induced *Toxoplasma* growth inhibition in primary HFFs.

Infected, IFN-γ-stimulated human fibroblasts undergo cell death independently of caspases, RIP kinases, autophagy, or purinergic receptor activation. In infected murine macrophages, $P2X_7R$ activation can induce cell death to prevent parasite replication (8, 10). In addition, in IFN-γ-activated murine embryonic fibroblasts (MEFs), infected host cells undergo necrotic cell death after IRG-mediated disruption of the parasitophorous vacuole (44). To investigate whether HFFs also undergo cell death during infection as a means to prevent parasite replication, we stained infected and IFN-γ-stimulated cells with propidium iodide (PI), a DNA dye that is excluded from viable cells but able to permeate dying cells (45). We compared the number of cells that were positive for PI to total number of cells, as measured by staining with the cell-permeable nuclear stain Hoechst 33342, which stains both viable and nonviable cells. We found that as early as 8 h postinfec-

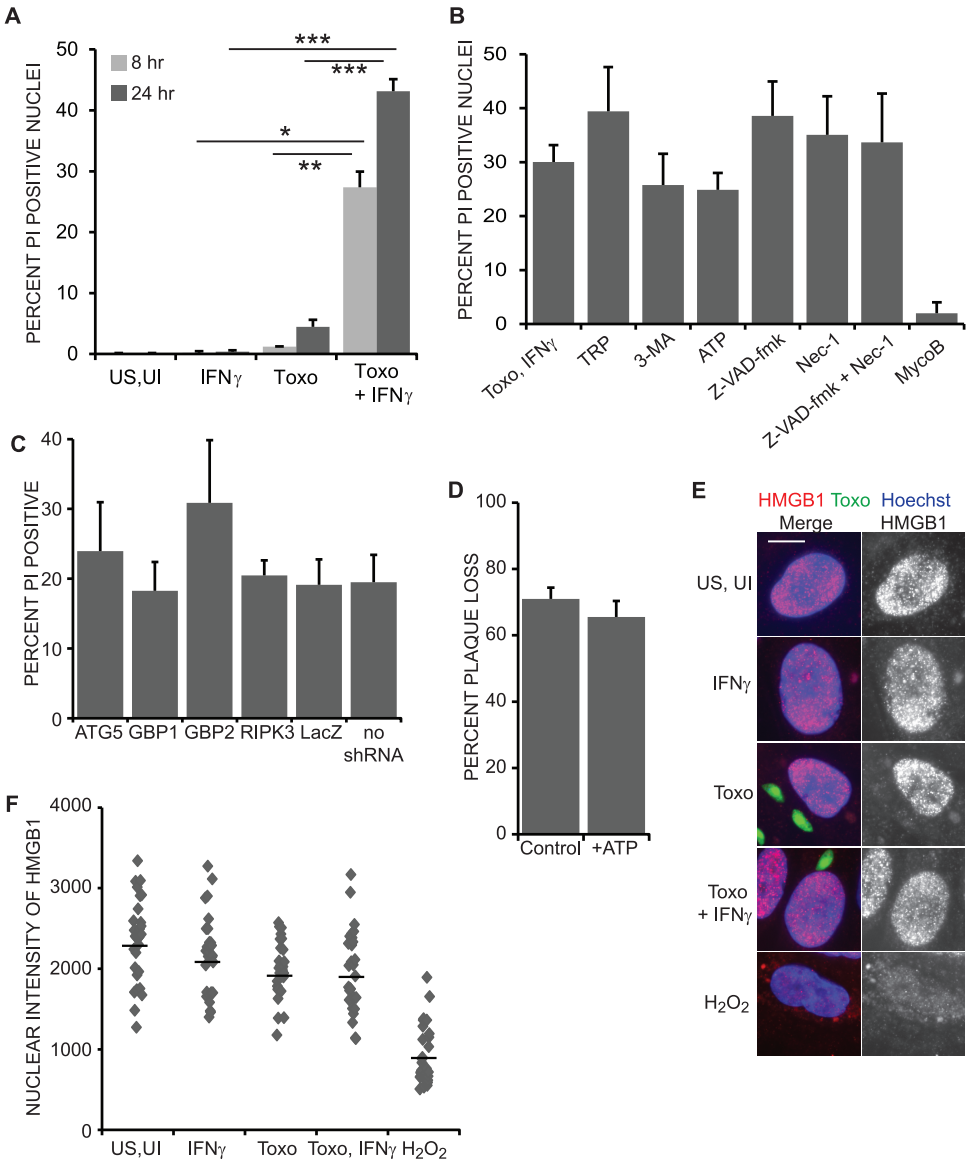

FIG 3 IFN-γ-stimulated, infected HFFs undergo cell death independently of apoptosis, necroptosis, or autophagy. (A) IFN-γ-stimulated or unstimulated (US) HFFs were infected for 8 or 24 h at an MOI of 3 or left uninfected (UI). Propidium iodide (PI) and membrane-permeable Hoechst were added for 15 min before visualization, and the percentage of PI-positive nuclei was determined. Means + the SE are shown ($n > 3$ experiments). *, $P < 0.05$; ***, $P < 0.001$ (Student t test). (B) Percentage of PI-positive nuclei in infected, IFN-γ-stimulated HFFs 8 h postinfection in the presence of 1 mM tryptophan (TRP), 3 mM ATP, or the indicated autophagy (10 mM 3-MA) or cell death (50 μM Nec-1 or 100 μM Z-VAD-fmk) inhibitors. Inhibitors were added 1 h prior to infection; ATP was added 2 h after infection. As a control, parasites were incubated with 3 μM mycalolide B for 15 min and washed thoroughly prior to infection to prevent invasion but not attachment or rhoptry secretion. Means + the SE are shown ($n = 3$ experiments). (C) Percentage of PI-positive cells at 8 h postinfection of IFN-γ-stimulated HFFs deficient in the indicated gene. Means + the SE are shown ($n = 3$ experiments). (D) The percent plaque loss on IFN-γ-stimulated HFFs was determined in the presence or absence of 3 mM ATP added 2 h after infection. Means + the SE are shown ($n = 3$ experiments). (E) IFN-γ-stimulated or unstimulated (US) HFFs were infected with parasites expressing GFP (green) for 8 h or left uninfected (UI), or necrosis was induced by 45 min of 1 mM hydrogen peroxide. Cells were fixed and stained for HMGB1 (red) and Hoechst (blue). Left, merged image; right, HMGB1 image. Scale bar, 10 μm. (F) Quantification of mean nuclear HMGB1 from panel C. Dots represent individual nuclei, and lines represent the mean (representative of three experiments).

tion, there is a significant increase in PI-positive nuclei in infected, stimulated cells (27% PI positive) compared to uninfected, stimulated HFFs (0.1%; $P = 0.04$) or unstimulated, infected (1%; $P = 0.004$) HFFs (Fig. 3A). As expected, this cell death in IFN-γ-stimulated, infected cells is multiplicity of infection (MOI) dependent but independent of tryptophan (Fig. 3B). After 24 h, the number of PI-positive nuclei reached 43% in infected, IFN-γ-stimulated HFFs, but only 4.5% in unstimulated, infected HFFs ($P = 0.001$). Thus, cell death is associated with IFN-γ-mediated resistance to *Toxoplasma* in HFFs, but it is unclear whether the observed cell death is related to parasite clearance.

Next, we wondered whether chemical inhibitors of cell death pathways could reduce IFN-γ-induced death of infected cells. We measured the percentage of PI-positive nuclei in IFN-γ-stimulated, infected HFFs in the presence of the caspase inhibitor Z-VAD-fmk to block apoptosis or the necroptosis inhibitor necro-

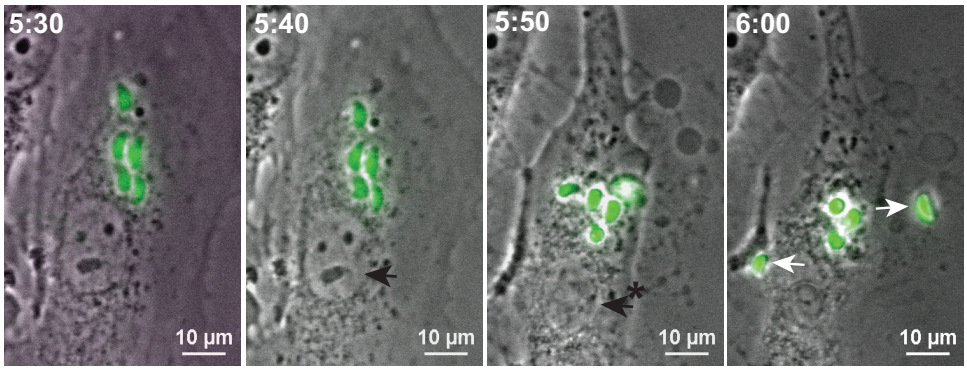

FIG 4 Parasites infecting IFN-γ-stimulated HFFs egress without replication. Live imaging of IFN-γ-stimulated HFFs infected with GFP-expressing parasites at the indicated time points (shown in hours and minutes) after infection was performed. White arrows point to egressed parasites, while some parasites remain in the host cell. Cell death is evident from the loss of nuclear integrity (black arrows indicate the nucleus; *, loss of integrity).

statin-1. We found no difference in PI-positive nuclei in the presence of either inhibitor or the combination of inhibitors (39% PI positive for Z-VAD-fmk, 28% PI positive for necrostatin-1 [Nec-1], and 34% PI positive for Z-VAD-fmk + Nec-1 compared to 31% control; $P = 0.30$, 0.61, and 0.73, respectively) (Fig. 3B). Accordingly, HFFs with the necroptosis signal transducer RIPK3 knocked down by lentiviral shRNA infection did not have less plaque loss due to IFN-γ (60% plaque loss compared to 43% LacZ shRNA control; $P = 0.12$) (Fig. 2) or reduced PI staining (20% PI positive compared to 19% for the LacZ control; $P = 0.57$) (Fig. 3C). This suggests that IFN-γ is not activating a programmed cell death pathway in infected cells, but because cell death pathways intersect, it remains possible that chemical inhibition cannot prevent previously initiated cell death from proceeding down another pathway.

High levels of autophagy often accompany cell death, so we also tested whether the inhibition of autophagy with the PI3K inhibitor 3-methyladenine (3-MA) or *ATG5* knockdown could prevent the observed cell death in IFN-γ-stimulated, infected HFFs. *ATG5*-deficient HFFs show similar cell death (24% PI positive) in infected stimulated cells to cells targeted with LacZ control shRNA (19% PI positive; $P = 0.53$) (Fig. 3C), and we found no difference in PI-positive nuclei in the presence of 3-MA (26% compared to 31% control; $P = 0.56$) (Fig. 3B). In addition, cell death in IFN-γ-stimulated infected *GBP1*- or *GBP2*-deficient HFFs is not significantly different than in LacZ control cells (18% PI positive for GBP1 and 30% PI positive for GBP2 compared to 19% PI positive for the LacZ control; $P = 0.91$ and 0.32, respectively) (Fig. 4C), indicating that GBP1 and GBP2 are not required to promote IFN-γ- and infection-induced cell death.

In murine macrophages, purinergic receptor activation leads to fusion of parasitophorous vacuoles with lysosomes and host cell death (9). Because human skin fibroblasts were shown to express P2RX7 (46), we tested whether purinergic receptor activation contributes to host cell death and/or parasite control in HFFs by measuring PI-positive nuclei and IFN-γ-induced plaque loss in the presence of 3 mM ATP added 2 h after infection. We found no significant differences in PI-positive infected, IFN-γ-stimulated cells in the presence of ATP (25% PI positive compared to 30% control; $P = 0.37$) (Fig. 3B) or percent plaque loss in the presence of ATP (66% loss compared to 71% control; $P = 0.48$) (Fig. 3D). This suggests that purinergic receptor activation does not induce host cell death or parasite clearance in HFFs.

Furthermore, we wondered whether parasite invasion was necessary to induce cell death in stimulated HFFs or whether a parasite secreted factor was sufficient. To test this, we preincubated parasites with the irreversible inhibitor of actin polymerization mycalolide B before infection to inhibit parasite invasion but not attachment or secretion of the contents of apical secretory organelles into the host. We did not observe cell death when parasites were pretreated with mycalolide B, indicating that invasion is necessary for cell death to occur (Fig. 3B).

Infected, IFN-γ-stimulated MEFs undergo necrosis after disrupting the parasitophorous vacuole (44). High-mobility group protein B1 (HMGB1) normally resides in the nucleus but is released into the supernatant by necrotic cells (47). To test whether infected HFFs die via necrosis, we analyzed the nuclear intensity of HMGB1 after 8 h of infection compared to 45 min of hydrogen peroxide-induced necrosis. Quantification of the mean nuclear fluorescence of HMGB1 in infected and uninfected HFFs indicated that HMGB1 levels are 15% lower in the nuclei of infected cells ($P = 0.004$) compared to uninfected, but in a manner independent of IFN-γ stimulation (16% lower in Toxo + IFN-γ than uninfected, unstimulated) (Fig. 3E and F). However, hydrogen peroxide-treated cells had 62% lower mean nuclear HMGB1 levels than did uninfected, unstimulated cells. This indicates that the observed cell death after infection and IFN-γ stimulation is not likely to be necrotic.

Parasites infecting IFN-γ-stimulated HFFs egress without replication. It is unclear whether cell death leads to parasite clearance or occurs as a result of it, so to clarify the order of events, we performed live imaging of infected IFN-γ-stimulated or unstimulated cells over the course of 16 h. Interestingly, in IFN-γ-stimulated HFFs we observed early egress without replication of 43 of the 56 (77%) parasites examined as early as 5 h after infection (Fig. 4; see Video S1 in the supplemental material). All 41 stimulated infected cells viewed died, and at least 7 parasites were observed to stay in a dying cell, but for the remainder of parasites, whether or not the parasite egressed could not be determined in the images. We witnessed only 1 of 90 parasites egress from the unstimulated cells that we imaged. We did observe 7 of the 56 unstimulated infected cells round up and peel off the tissue culture plate with parasites still inside. Although only rarely did the infecting *Toxoplasma* parasites remain within the dying IFN-γ-stimulated HFFs, the intracellular niche is disrupted and replication is prevented by early egress for the majority of parasites. Similarly, human umbil-

ical vein endothelial cells (HUVEC) stimulated with IFN-γ did not support the replication of *Toxoplasma*. Approximately 2 to 3 h postinvasion, infected cells started to die, and this was accompanied by the early egress of the parasite (see Fig. S2 and Videos S2 and S3 in the supplemental material). Cells appeared to round up just prior to parasite egress, suggesting cell death preceded the parasite leaving the cell. IFN-γ was shown to promote early egress without replication in murine astrocytes as well, but it was proposed that this was perhaps due to Irgm3-mediated fusion of the endoplasmic reticulum with the PV (48). Interestingly, this egress did not kill the host cell, and the parasite was able to glide away but was unable to reinvade a new monolayer. We predict that egress without replication could exhaust the parasite and explain the reduced plaque size and number on stimulated monolayers compared to the exponential amplification and spread of replicating parasites on unstimulated monolayers.

Inhibition of egress does not reduce cell death in IFN-γ-stimulated, infected fibroblasts. Cell membrane permeabilization leads to a loss of intracellular potassium, which can activate parasite motility (49). However, it is also possible that the parasite senses another signal that leads to egress, causing the observed host cell membrane permeabilization and death. To determine whether egress leads to cell death, we measured the percent PI-positive nuclei in IFN-γ-stimulated HFFs infected with parasites that were unable to egress. We used parasites that express TgCDPK3, which was shown to be necessary for egress, with either a glycine (G) or methionine (M) at the gatekeeper position that determines the sensitivity to the inhibitor 3-methyl-benzyl pyrazolo[3,4-*d*] pyrimidine (3-MB-PP1). The TgCDPK3^{G} strain cannot egress in the presence of the inhibitor, whereas TgCDPK3^{M} is uninhibited. As a positive control, we measured the percent PI-positive nuclei for these strains in the presence of 3-MB-PP1 and a calcium ionophore, A23187, which induces egress, killing the host cell. The inhibitor is able to prevent calcium ionophore induced egress of TgCDPK3^{G} but not TgCDPK3^{M}. However, there is no difference in PI positive nuclei in IFNγ-stimulated HFFs infected with either strain in the presence or absence of the inhibitor (Fig. 5). This indicates that cell death is not caused by egress but rather that the parasite egresses to escape a dying cell. Interestingly, cell death in infected, stimulated cells is similar to when egress is induced by calcium ionophore, suggesting that nearly all infected, stimulated cells die.

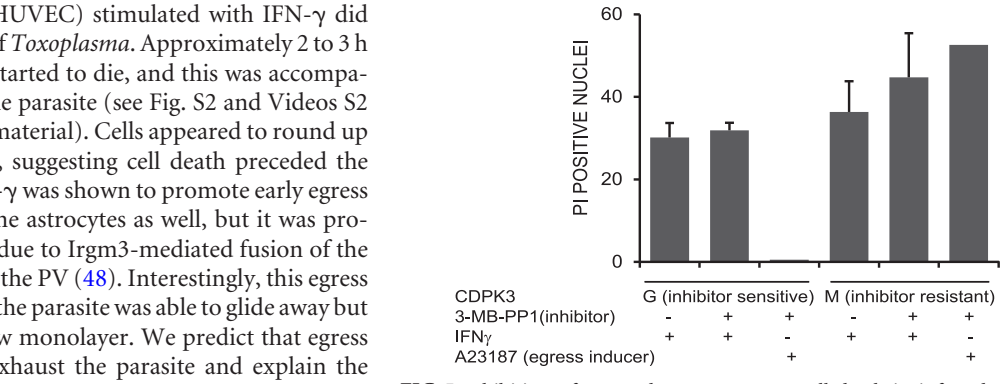

FIG 5 Inhibition of egress does not prevent cell death in infected IFN-γ-stimulated HFFs. IFN-γ-stimulated HFFs were infected with parasites expressing either TgCDPK3^{G} (sensitive to 3-MB-PP1) or TgCDPK3^{M} (insensitive to 3-MB-PP1) for 8 h, and a 5 μM concentration of inhibitor 3-MB-PP1 (or DMSO as a control) was added for the last 4 h to inhibit CDPK3-dependent egress. The percentage of PI-positive nuclei was determined. Means + the SE are shown ($n = 3$). As a positive control, unstimulated HFFs were infected with the indicated strains for 8 h and with 3-MB-PP1 for the last 4 h, and then a 2 μM concentration of the calcium ionophore A23187 was added for 20 min to induce egress.

DISCUSSION

Toxoplasma establishes a lifelong infection in hosts by forming cysts in brain and muscle tissue, and therefore cell-autonomous immunity in nonimmune cells is important for limiting parasite burden and cyst formation. Previously, the main characterized mechanism for controlling parasite growth in nonimmune human cells was IFN-γ-induced deprivation of tryptophan. We report here that tryptophan supplementation does not restore parasite growth in IFN-γ-stimulated primary HFFs. We did not find evidence of other reported anti-*Toxoplasma* mechanisms, such as iron deprivation or vacuolar destruction by p65 guanylate binding proteins (GBPs) or autophagy being involved in the observed resistance in HFFs. Instead, we observed that IFN-γ-stimulated HFFs undergo cell death upon *Toxoplasma* infection that induces parasites to egress as early as 5 h after infection, before replication occurs, leading to limited parasite proliferation and potentially promoting clearance by immune cells *in vivo*.

Previous studies showed that some nonimmune human cells, such as HeLa cells (36) and human fibroblasts (18, 19), controlled *Toxoplasma* infection by tryptophan degradation via induction of the IFN-γ-induced enzyme IDO1, while other cell types, such as intestinal epithelial cells and umbilical vein endothelial cells (21, 22), used a tryptophan-independent resistance mechanism. Our results confirm that HeLa cells do rely on tryptophan degradation to inhibit *Toxoplasma* replication, but in contrast to previous work with human fibroblasts, we find that tryptophan supplementation does not restore parasite growth in primary foreskin fibroblasts. The origin of the human fibroblasts from previous studies was not reported (18, 19), but it may be that differences in the specific tissue from which the fibroblasts were derived or the use of transformed rather than primary human fibroblasts could explain these differences in IFN-γ-mediated parasite clearance mechanisms.

Similarly, we find that iron supplementation does not abrogate resistance, but excess iron can inhibit parasite growth, even in unstimulated cells. Many cellular functions and immune mechanisms are sensitive to iron concentration in the cell (50). It is possible that the observed resistance mechanism in HFFs is in some way regulated by iron, but it would be difficult to differentiate that effect from other effects iron has on the cell. It is at least clear that iron deprivation is not responsible for limiting *Toxoplasma* growth in stimulated HFFs because iron supplementation does not restore growth.

We report that IFN-γ-stimulated HFFs limit *Toxoplasma* growth by dying before parasite replication can occur. It is not clear how cell death is induced, but it is unaffected in HFFs deficient for RIPK3, GBP1, GBP2, or ATG5 or in the presence of autophagy or cell death inhibitors. It is possible that, due to incomplete knockdown, the remaining protein expressed in these knockdowns is enough to function. It also remains possible that other GBPs aside from GBP1 or GBP2 promote resistance without vacuolar localization or that GBP1 and GBP2 are also involved, but that their functions are redundant. The fact that chemical inhibition of autophagy and cell death pathways is also unable to

prevent IFN-γ and infection induced cell death suggests that these pathways are either dispensable or redundant. Cell death pathways are so intertwined that inhibition of one pathway can cause cell death to proceed down another pathway. For instance, TNF activation can lead to apoptosis or necroptosis depending on caspase-8 activation (32). However, even chemical inhibition of both apoptosis and necroptosis simultaneously is also not sufficient to inhibit cell death. Thus, cell death is difficult to inhibit, and therefore determining the mediators involved will be a challenge. It will also be interesting to determine what factors of *Toxoplasma* infection contribute to this cell death, since parasites prevented from invading but not attaching or secreting factors into the host do not cause cell death.

Early egress and reinvasion have also been observed in murine peritoneal exudate cells (51). Egress was reported to be triggered externally by activated macrophages in a manner dependent on intracellular calcium and sensitive to a p38 mitogen-activated protein kinase inhibitor. Natural egress from a host cell is triggered by a reduction in cytoplasmic potassium concentration due to host membrane permeabilization (49). Egress can also be induced *in vitro* by calcium ionophores, dithiothreitol, and cell death inducers such as the *fas* ligand or perforin (49, 52–54). Furthermore, IFN-γ was shown to induce parasite egress in murine astrocytes, but this was deemed to be dependent on Irgm3-mediated fusion of the endoplasmic reticulum with the PVM (48). The fact that egress can be triggered externally by environmental cues suggests that the parasite may have adapted to be able to evacuate inhospitable cells. In the previous report, parasites that had egressed and reinvaded were preferentially restricted *in vivo* (51). It was suggested that the early egress triggered externally could reshuffle parasites to previously stimulated cells that are better able to restrict growth. Early egress from stimulated nonimmune cells may be similarly beneficial by promoting infection of immune cells with other clearance mechanisms or by depleting the contents of secretory organelles used for invasion and host cell manipulation. At the very least, even if egressed parasites are not able to invade an immune cell, the parasite burden is limited by the lack of or delay in replication and the death of some parasites with the host cell. Thus, even if human fibroblasts do not possess the vacuole-destroying abilities of immune cells or murine fibroblasts, they can still play an important role in limiting the course of *Toxoplasma* infection.

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