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p47 GTPases Regulate *Toxoplasma gondii* Survival in Activated Macrophages

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The cytokine gamma interferon (IFN-γ) is critical for resistance to *Toxoplasma gondii*. IFN-γ strongly activates macrophages and nonphagocytic host cells to limit intracellular growth of *T. gondii*; however, the cellular factors that are required for this effect are largely unknown. We have shown previously that IGTP and LRG-47, members of the IFN-γ-regulated family of p47 GTPases, are required for resistance to acute *T. gondii* infections in vivo. In contrast, IRG-47, another member of this family, is not required. In the present work, we addressed whether these GTPases are required for IFN-γ-induced suppression of *T. gondii* growth in macrophages in vitro. Bone marrow macrophages that lacked IGTP or LRG-47 displayed greatly attenuated IFN-γ-induced inhibition of *T. gondii* growth, while macrophages that lacked IRG-47 displayed normal inhibition. Thus, the ability of the p47 GTPases to limit acute infection in vivo correlated with their ability to suppress intracellular growth in macrophages in vitro. Using confocal microscopy and sucrose density fractionation, we demonstrated that IGTP largely colocalizes with endoplasmic reticulum markers, while LRG-47 was mainly restricted to the Golgi. Although both IGTP and LRG-47 localized to vacuoles containing latex beads, neither protein localized to vacuoles containing live *T. gondii*. These results suggest that IGTP and LRG-47 are able to regulate host resistance to acute *T. gondii* infections through their ability to inhibit parasite growth within the macrophage.

Toxoplasma gondii is an intracellular protozoan parasite that causes severe and often fatal disease in immunocompromised hosts (for a review, see references 7 and 11). Infection with the parasite is characterized by an acute phase in which the rapidly proliferating tachyzoite disseminates throughout the host. If this phase is suppressed, chronic infection ensues in which the slowly dividing bradyzoite inhabits mainly the central nervous system and muscle. Should acute infection proceed unchecked, however, extensive and often lethal tissue damage can result. The host effectively suppresses acute infection by initiating strong cell-mediated immune responses that restrain growth and spread of the parasite, thereby initiating latency. Dormancy is then maintained for the life of the host unless an immunosuppressive event occurs, such as the acquisition of AIDS (7, 11).

The immune mechanisms through which acute *T. gondii* infection is controlled are poorly understood. The cytokine gamma interferon (IFN-γ) plays a central role in resistance, as it strongly activates macrophages and nonphagocytic host cells to limit intracellular growth of tachyzoites (18, 22). Consequently, mice that do not produce IFN-γ quickly succumb to normally avirulent strains of *T. gondii*, with mortality accompanied by rampant tachyzoite replication in macrophages and other cells (22). The critical intracellular effectors that are activated by IFN-γ and that limit early *T. gondii* growth during the acute phase remain to be identified.

Recently, a new family of IFN-γ-induced genes has been discovered that includes important mediators of host resistance to *T. gondii*, as well as other pathogens (23). This family comprises at least six members in the mouse, including IGTP (26), LRG-47 (21), IRG-47 (9), TGTP/Mg21 (2, 12), IIGP (1), and GTPI (1). While their precise functions are unknown, each encodes a 47-to 48-kDa protein that possesses inherent GTPase activity (25, 28) and binds to subcellular membranes, including the endoplasmic reticulum (ER) and Golgi (15, 25, 30). Following parasitic and bacterial infection, the GTPases are highly expressed in an IFN-dependent manner in a variety of hematopoietic and nonhematopoietic cells including macrophages. We have shown previously that three of the proteins—IGTP, LRG-47, and IRG-47—play essential, though distinct, roles in resistance to various bacteria and protozoa (4, 24). In response to acute *T. gondii* infection, knockout (KO) mice that lack either IGTP or LRG-47 die rapidly, with similar kinetics to that seen in IFN-γ KO mice. The mechanism that underlies the antitoxoplasma activity of IGTP and LRG-47 has not been determined. IGTP is also required for resistance to chronic *T. gondii* infection (3), possibly by limiting intracellular

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growth of the pathogen in the brain, as astrocytes which lack IGTP expression display a reduced capacity to kill *T. gondii* in vitro (10). In contrast to IGTP and LRG-47, IRG-47 seems to play a minor role in *T. gondii* resistance, with IRG-47 KO mice having normal control of infection at early time points (4).

Other lines of study have shown that the p47 GTPases are important for resistance to intracellular bacteria including *Listeria monocytogenes* (4, 24), *Mycobacterium tuberculosis* (14), and *Mycobacterium avium* (6). For each of these bacteria, LRG-47 is essential for IFN-γ-regulated defense, while IGTP and IRG-47 are dispensable. Regarding *M. tuberculosis*, LRG-47 has been found to associate with the bacterial phagosome in macrophages; furthermore, LRG-47 is necessary for IFN-γ-promoted acidification and maturation of the phagosome and ultimately for bacterial clearance from the macrophage (14). While these results suggest that LRG-47 traffics to the phagosome and regulates its maturation, the mechanism through which this occurs has not been defined. Concerning *M. avium*, LRG-47 deficiency causes a profound lymphopenia following bacterial infection, which may, in turn, contribute to decreased host resistance. Thus, LRG-47 and possibly the other GTPases may have multiple activities that are differentially required for resistance to pathogens.

In the present work, we addressed the roles of IGTP and LRG-47 in acute resistance to *T. gondii* by assessing parasite growth and survival in KO macrophages. We also analyzed the subcellular localization of the GTPases in infected wild-type macrophages. Our data suggest that IGTP and LRG-47 function as key effectors of parasite killing in macrophages, a critical step for successful early control of the pathogen.

MATERIALS AND METHODS

Cell culture and mice. Primary murine bone marrow macrophages (BMM) were isolated from the tibia and femurs of 2-to 4-month-old mice that were deficient in IGTP, LRG-47, or IRG-47 production, or were wild-type (C57BL/6 × 129SvImJ background) (4, 24). Bone marrow was flushed from the bones using a 27-gauge syringe filled with Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Gaithersburg, MD); the marrow was dispersed by being drawn through the syringe a few times, and red cells were lysed with ammonium chloride. Adherent cells were cultured in DMEM supplemented with 10% (vol/vol) fetal bovine serum (FBS) (HyClone, Logan, UT) and 30% (vol/vol) L929 cell-conditioned medium. The cells were cultured for 5 to 6 days, after which they were replated following scraping and then cultured an additional 2 days before use in the infection assays. The cells used for RNA preparation were grown for the same length of time but were not replated. The genetic background of the mice that were used in these experiments was C57BL/6 × 129 SvImJ.

J774 cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM supplemented with 10% (vol/vol) FBS.

***T. gondii* culture.** Parasites of the low virulence ME49 strain were maintained by serial passage on HS27 human foreskin fibroblasts (American Type Collection CRL-1634) grown in DMEM supplemented with 10% FBS. Parasites were harvested after 3 to 5 days in culture when the fibroblast host cell layer reached 80 to 90% lysis. Suspensions of parasites were cleared of fibroblast debris by centrifugation at 50 × *g* for 5 min, and parasites were enumerated using a hemocytometer. For studies requiring heat-killed parasites, they were heated to 65°C for 15 min.

Northern blotting. For Northern blot analysis, 15-μg samples of total RNA were separated on 1.2% agarose-formaldehyde gels that were transferred to nylon filters and probed as described previously (26). Probes included a 1.2-kb fragment of human glyceraldehyde-phosphate dehydrogenase cDNA pHcGAP (27), an EcoRI fragment of the IGTP cDNA encompassing bases 1645 to 1927 (GenBank accession no. U53219), a BglII fragment of the LRG-47 cDNA encompassing bases 1726 to 2041 (GenBank accession no. U19119), and a PCR-generated fragment of the IRG-47 cDNA encompassing bases 1374 to 1625 (GenBank accession no. M63630). The portions of the IGTP, LRG-47, and IRG-47 cDNAs that were used as probes were derived from the 3′ untranslated regions of the cDNAs that are not conserved among the cDNAs.

In vitro assessment of *T. gondii* killing in BMM. This procedure is a modification of a previously published technique (18). Cells from 5-to 6-day-old BMM cultures were plated in 24-well plates at 0.35×10^6 cells per well. After 24 h, the medium was changed, and the cells were cultured for another 24 h in the presence or absence of 100 U/ml murine IFN-γ (Calbiochem, San Diego, CA). To infect the cells, tachyzoites were added at a 2:1 (tachyzoite:macrophage) ratio and incubated for 2 h. Extracellular tachyzoites were removed by washing the cells twice with phosphate-buffered saline (PBS), and 1 ml of BMM growth medium containing 2.5 μCi [H^3]uracil was added. After 48 h of incubation, metabolically incorporated uracil was determined by washing the cells three times with PBS and then lysing the cells in 500 μl of 0.1% (wt/vol) sodium dodecyl sulfate in PBS for 10 min at room temperature with shaking. RNA and other macromolecules were precipitated from the lysates by the addition of 481 μl of 100% (wt/vol) trichloroacetic acid, followed by 15 min of incubation on ice. The samples were then filtered through glass fiber filters, which were used for scintillation counting. By this method, the majority of [^{3}H]uracil that was incorporated into RNA resulted from parasite metabolism; in comparison, the amount that occurred in uninfected cells was negligible.

Measurement of NO and NOS activity. NO (measured as nitrite and nitrate) was determined in cell supernatants spectrophotometrically using the Greiss reagent, as described previously (29). NOS activity was determined in cell lysates as the ability to convert [^{14}C]arginine to [^{14}C]citrulline (29).

Subcellular membrane fractionation. Membrane fractions were prepared using a modified version of a previously published procedure (25). Four confluent 150-mm plates of cells were incubated overnight in the presence of 50 U/ml IFN-γ. The cells were collected by scraping into ice-cold PBS, followed by washing with a hypotonic buffer (8.5% [wt/vol] sucrose, 20 mM HEPES, pH 7.2, and protease inhibitors [539134; Calbiochem]). Cell lysates were then prepared by disruption of the cells in 1 ml of hypotonic buffer using 25 to 30 strokes in a Dounce homogenizer with a Teflon pestle. The postnuclear supernatant, obtained by centrifugation of the homogenate (1,000 × *g* for 5 min), was fractionated on a sucrose gradient consisting of 20%, 30%, and 38% sucrose solutions prepared in 20 mM HEPES, pH 7.2. After centrifugation, (140,000 × *g* for 2 h), four membrane fractions were isolated from the gradient at the 8.5/20% interface, 20/30% interface, and 30/38% interface and from the resuspended pellet. Each fraction was further processed by diluting with PBS and pelleting by centrifugation at 55,000 × *g* for 15 min; fractions were then resuspended in 1% (wt/vol) NP-40–PBS. The fractions were used for Western blotting as previously described (26) with polyclonal anti-IGTP antibodies (26), polyclonal anti-LRG-47 antibodies (4), monoclonal anti-GM130 antibodies (G65120; BD-Transduction Labs, Lexington, KY), and monoclonal anti-GRP78 antibodies (G73320; BD Transduction Labs).

Latex bead vacuole isolation. J774 cells were stimulated with 100 U/ml IFN-γ for 24 h and were then allowed to phagocytose 1-to 2-μm paramagnetic latex beads (no. 18190; Polysciences, Warrington, PA) diluted into the culture medium at 1:200. After 20 min, the cells were rinsed three times with PBS to remove extracellular beads, and fresh tissue culture medium was added. Following incubation times ranging from 0 to 180 min, the cells were rinsed three times with ice-cold PBS and then scraped into PBS and pelleted. The cells were then rinsed one time with homogenization buffer (HB) (8.56% [wt/vol] sucrose, 20 mM HEPES, pH 7.2, 1 mM $MgCl_2$), followed by incubation in HB for 10 min. The cells were disrupted with 35 strokes in a Teflon-pestle Dounce homogenizer. Residual whole cells, nuclei, and large cellular debris were removed with three 5-min centrifugations at 250 × *g*. The latex bead vacuoles were separated from the lysate with a magnet and then washed three times with HB, and each time the magnet was used to collect the vacuoles. The purified latex bead vacuoles were solubilized in a buffer containing 1% (vol/vol) NP-40.

Confocal and deconvolution microscopy. BMM were prepared for microscopy by plating at about 50% confluency on uncoated glass coverslips. The cells were allowed to adhere for 24 h, after which they were exposed to 100 U/ml IFN-γ for another 24 h to stimulate production of the p47 GTPases. The cells were then infected with *T. gondii* by adding tachyzoites at a multiplicity of infection (MOI) of 2:1 and spinning parasites onto the coverslips at 400 × *g* for 2 min at room temperature, followed by an additional 10-min incubation at 37°C; the coverslips were washed three times with PBS. Alternatively, the cells were allowed to phagocytose 2-μm latex beads (no. 18327; Polysciences) that were added to the culture medium at 5 beads per cell. Following incubations for various times in BMM medium, the cells were prepared for immunocytochemistry by fixing them with 4% (wt/vol) formaldehyde in PBS for 15 min, permeablizing with 0.2% (wt/vol) saponin in PBS for 15 min, blocking with 10% FBS–0.2% saponin in PBS for 1 h, and then incubating with primary and secondary antibodies in blocking

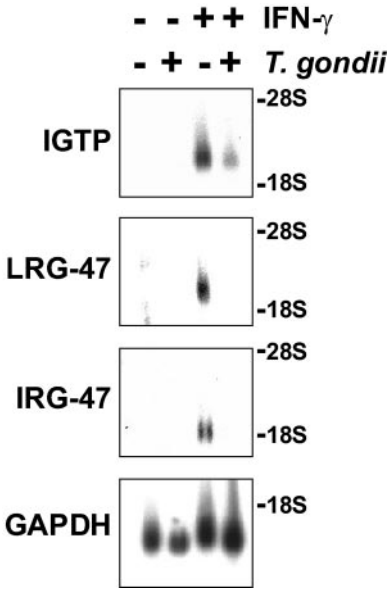

FIG. 1. IFN-γ-stimulated expression of IGTP, LRG-47, and IRG-47 in BMM. WT BMM were cultured in the presence or absence of IFN-γ or were infected with *T. gondii* at an MOI of 2:1. After 24 h, total cellular RNA was prepared and used for Northern blotting with the indicated probes.

buffer for 1 h each. The antibodies included a mouse monoclonal anti-IGTP antibody (I68120; BD-Transduction Labs), a mouse monoclonal anti-LRG-47 antibody that was raised against the peptide CEAAPLLPNMAETHY, a rat monoclonal anti-LAMP-1 antibody (1D4B; Developmental Studies Hybridoma Bank, Iowa City, IA), a rabbit polyclonal antitoxoplasma antibody (PU125-UP; BioGenex, San Ramon, CA), and various Alex Fluor 488- and Alexa Fluor 594-conjugated anti-mouse, anti-rat, and anti-rabbit immunoglobulin G antibodies (Molecular Probes, Eugene, OR).

RESULTS

IGTP, LRG-47, and IRG-47 expression in macrophages. Bone marrow macrophages were generated from wild-type (WT) and IGTP, LRG-47, and IRG-47 knockout mice according to standard protocols. The morphology of the BMM from each background appeared essentially identical, with greater than 95% being positive for the macrophage markers F4/80 and CD11b as assessed by flow cytometry (data not shown). Expression of IGTP, LRG-47, and IRG-47 RNA was examined in WT BMM and found to be low in resting cells but increased to high levels following exposure to IFN-γ (Fig. 1). These results are in agreement with those published previously using similar BMM cultures (14). Infection of the cells with *T. gondii* alone, however, did not increase expression of the GTPases. Interestingly, when *T. gondii* and IFN-γ were added to the cells at the same time, induced expression of all three GTPases was significantly attenuated (Fig. 1 and data not shown). This is likely because *T. gondii* blocks translocation to the nucleus of STAT-1 (13), a transcription factor that is required for expression of the p47 GTPases (3, 8).

BMM that lacked expression of IGTP, LRG-47, or IRG-47 were also examined for their capacity to produce nitric oxide, a key mediator of IFN-γ-induced resistance to many pathogens including *T. gondii* (18). It was found that the BMM produced normal amounts of NO after stimulation with IFN-γ and lipopolysaccharide, regardless of their p47 GTPase status (data not shown). These studies included direct measurement of nitric oxide synthase activity in cell lysates by quantifying the conversion of L-arginine to L-citrulline, as well as indirect measurement of nitric oxide levels in the BMM culture medium using the Greiss assay (data not shown). These results are in agreement with published studies (14). In addition, it has been shown previously that LRG-47 KO BMM also produce normal amounts of tumor necrosis factor alpha and indolamine-2,3-dioxygenase, two other IFN-γ-induced proteins that are important mediators of host resistance (14).

***T. gondii* growth suppression in IGTP KO macrophages.** To determine whether IGTP deficiency affected the ability of macrophages to suppress *T. gondii* growth, we used a standard method for assessing *T. gondii* growth in cultured cells. WT and IGTP KO BMM were exposed to IFN-γ for 24 h to induce anti-*T. gondii* activity, after which the cells were infected with the ME49 strain of *T. gondii* and incubated in the presence of [^{3}H]uracil for 48 h. This allowed the parasites sufficient time to multiply within the host cells without cell lysis. The amount of uracil that was incorporated into RNA was then determined and used as a measure of parasite growth within the host cell. Wild-type cells that were pretreated with IFN-γ were able to limit the amount of *T. gondii* growth, displaying about 31% of the growth seen in the same cells that were not exposed to IFN-γ (Fig. 2A). In contrast, IGTP KO BMM displayed little or no IFN-γ-induced growth suppression (Fig. 2A). The effect that IGTP deficiency had on the antitoxoplasma activity of BMM varied somewhat over several experiments (data not shown), but this activity was always attenuated in cells lacking IGTP relative to WT cells (Fig. 2 and 3 and data not shown). Similar experiments were performed with a virulent *T. gondii* strain (RH) (18), and essentially the same effect on parasite growth suppression was noted in IGTP KO BMM (data not shown). As an additional method of assessing a productive infection, *T. gondii* infections were allowed to progress until >90% of the BMM were lysed; the parasites that had exited the host cells were then enumerated in the culture medium. By this measure, the ability of IFN-γ to limit a *T. gondii* growth was also greatly attenuated in the absence of IGTP (Fig. 2B).

Toxoplasma growth was also assessed microscopically by staining infected BMM with antitoxoplasma antibodies at 1 and 24 h postinfection to determine survival and growth of the parasites. The results using this approach were more variable; however, in the majority of the cases, the effectiveness of IFN-γ in decreasing parasite survival at 24 h postinfection was reduced in IGTP KO cells. For instance, the number of parasite-positive cells in IFN-γ-treated groups was 38% ± 9% that of control-treated groups for WT BMM, versus 82% ± 28% of control in IGTP KO cells (average percent ± standard deviation based on six experiments.) Nevertheless, at 1 h postinfection about equal numbers of IFN-γ-stimulated IGTP KO and WT cells were infected. In addition, at 24 h the WT and IGTP KO BMM contained about the same number of parasites. Taken together, these data suggest that IGTP deficiency affects initial parasite survival, but infectivity and the later growth phase are unaffected.

Differing effects of IGTP, LRG-47, and IRG-47 on *T. gondii* growth in macrophages. Next, we compared the consequences of IGTP, LRG-47, or IRG-47 deficiency on *T. gondii* growth in

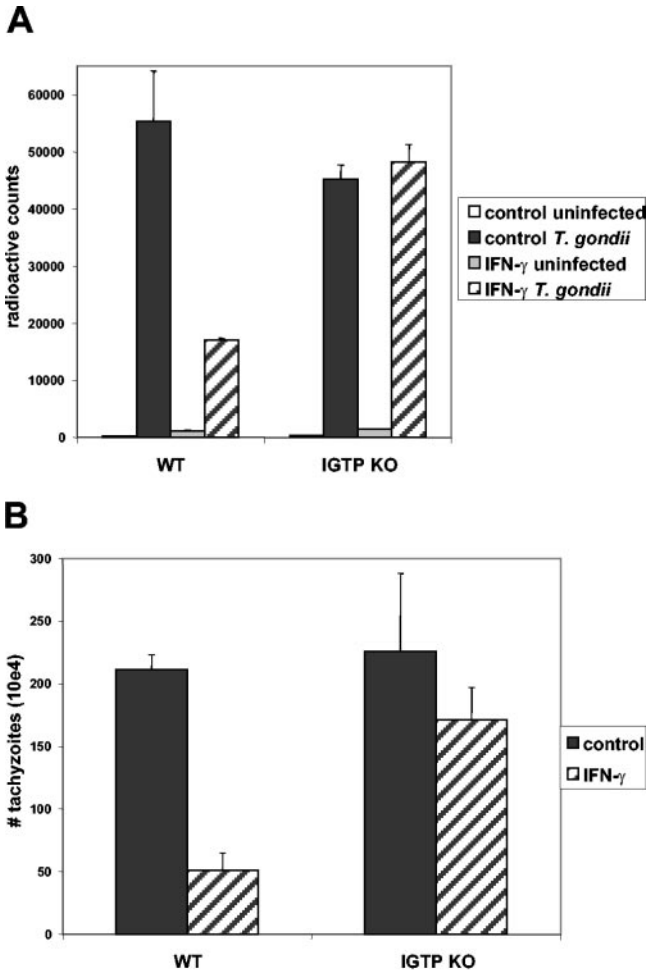

FIG. 2. Loss of IFN-γ-mediated *T. gondii* growth suppression in IGTP KO macrophages. (A) Wild-type or IGTP KO BMM were cultured in the presence or absence of IFN-γ for 24 h. The cultures were subsequently infected with *T. gondii* at an MOI of 2:1 for 2 h or were left uninfected, and then cultures were pulsed with [^{3}H]uracil. After 48 h, incorporated radioactivity was determined and expressed as cpm ± standard deviation. These results are representative of four independent experiments. (B) Wild-type or IGTP KO BMM that had been cultured in the presence or absence of IFN-γ for 24 h were infected for 2 h with *T. gondii* at an MOI of 2:1. When the host cells reached almost complete lysis, the resulting tachyzoites were enumerated microscopically. These results are representative of two independent experiments.

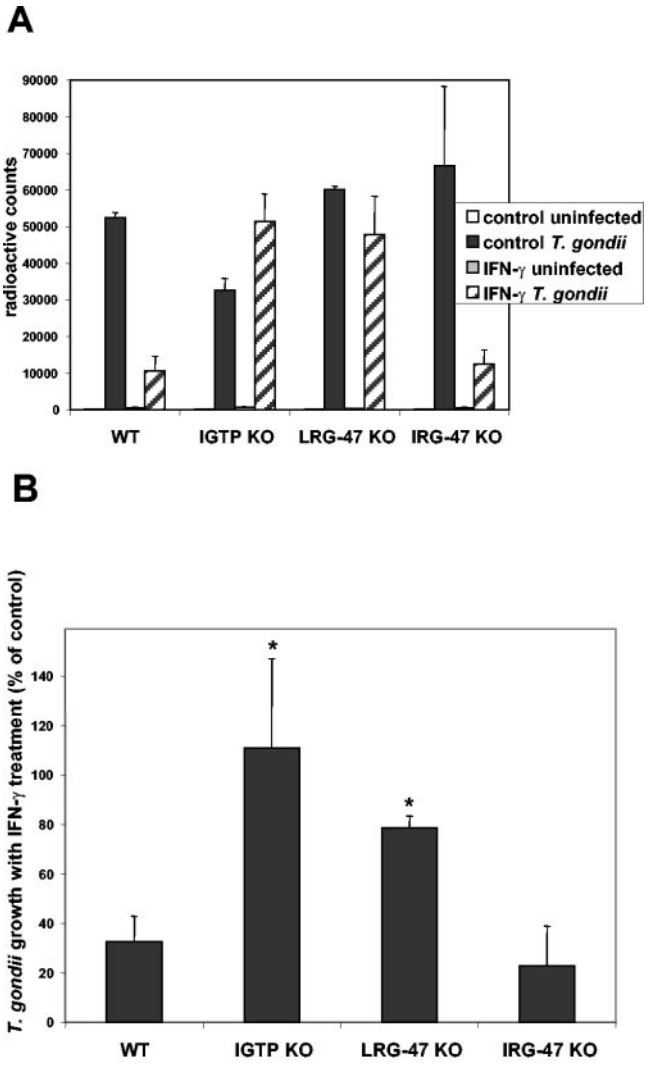

FIG. 3. Differential effects of IGTP, LRG-47, and IRG-47 deficiency on IFN-γ-mediated *T. gondii* growth suppression in macrophages. WT BMM or cells that lacked the indicated GTPases were cultured in the presence or absence of IFN-γ for 24 h. The cultures were subsequently infected with *T. gondii* at an MOI of 2:1 for 2 h and then were pulsed with [^{3}H]uracil. After 48 h, incorporated radioactivity was determined. (A) Shown is a representative experiment with the results expressed as cpm ± standard deviation. (B) Also shown is the average of four independent experiments in which the results indicate the effect of IFN-γ pretreatment on *T. gondii* growth, relative to growth under control conditions (no IFN-γ pretreatment). The asterisks (*) indicate statistically significant differences ($P < 0.05$).

BMM. Given that all three GTPases are expressed in these cells, it seemed plausible that each could be involved in IFN-γ-induced growth suppression, but that was not the case. Based on results of the tritiated-uracil incorporation assay, the ability to limit *T. gondii* growth in an IFN-γ-dependent manner was greatly attenuated in LRG-47 KO BMM, much as it was in IGTP KO BMM (Fig. 3). In contrast, IRG-47 KO BMM displayed robust IFN-γ-induced inhibition of *T. gondii* growth (Fig. 3). Parasitic survival and growth were also assessed microscopically. The results of these studies were variable, but on average the ability of IFN-γ to decrease parasite survival at 24 h was reduced in LRG-47 KO BMM. The percent of parasite-positive cells in IFN-γ-treated groups was 48% ± 10% of that in control-treated groups for WT BMM, versus 91% ± 37% for LRG-47 KO cells (average of four experiments). In contrast, the initial number of parasite-infected cells at 1 h postinfection was essentially the same for WT and LRG-47 KO BMM, and the numbers of parasites per infected cell at 24 h postinfection were comparable (data not shown). Contrasting with the results for LRG-47 KO BMM, the ability of IFN-γ to reduce intracellular parasite survival and growth at 24 h postinfection was not diminished in IRG-47 KO BMM. The percent of parasite-positive cells in IFN-γ-treated groups was 47% ± 8% in control-treated groups for WT BMM, versus 45% ± 8%

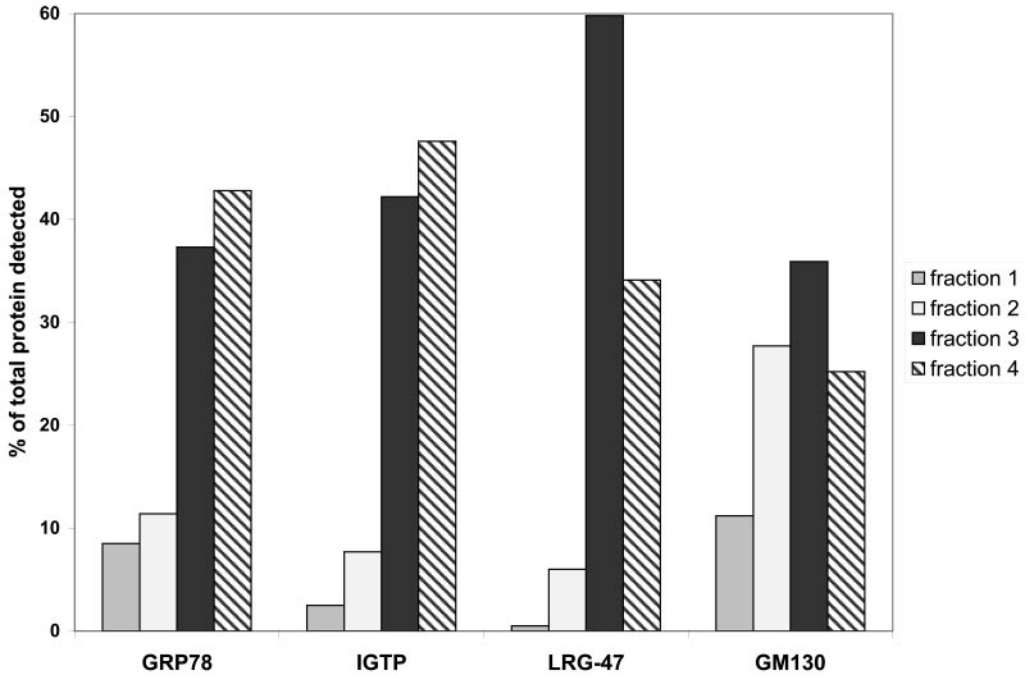

FIG. 4. Distribution of IGTP and LRG-47 among sucrose gradient fractions. Postnuclear supernatants were prepared from J774 macrophages that had been stimulated with IFN-γ. The supernatants were then applied to sucrose step gradients and separated into four fractions, with fraction 1 being the most buoyant and fraction 4 the densest. Equal amounts of protein from each fraction were used for Western blot analysis with the indicated probes. Densitometry was applied to the autoradiographs to quantify the protein detected in each fraction. These results are representative of four experiments.

for IRG-47 KO cells (average of four experiments). In summary, the ability of each GTPase to regulate *T. gondii* growth in macrophages correlates with the effect that each has on acute *T. gondii* infection in vivo: IGTP and LRG-47 are each essential for antitoxoplasma effects in macrophages and acute resistance in vivo, while IRG-47 is required for neither.

Localization of IGTP and LRG-47 in *T. gondii*-infected macrophages. The p47 GTPases have been found to localize to subcellular membrane compartments in uninfected cells, but the percentage of protein that is membrane bound and the particular membrane compartment to which the protein binds vary for individual GTPases (15). For instance, IRG-47 has been found to be <10% membrane bound, although the target compartment has not been identified, while in contrast, IGTP and LRG-47 are greater than 90% membrane bound (15). We have localized IGTP to the ER previously (25), and LRG-47 has recently been localized to the Golgi (15). In addition, LRG-47 has been found in macrophages in *M. tuberculosis*-containing phagosomes (14), as well as in latex bead-containing phagosomes (15). These results raise the intriguing possibility that LRG-47, and possibly other p47 GTPases, may traffic to pathogen-containing phagosomes and affect phagosome processing in a manner that undermines pathogen survival. In the current study, therefore, we examined whether IGTP and LRG-47 are found in *T. gondii* parasitophorous vacuoles in activated macrophages.

We first verified the localization of LRG-47 in our primary macrophage system by biochemical fractionation of subcellular membranes and by immunocytochemistry. Regarding the former, sucrose gradients were used to fractionate membranes from various types of cells including primary BMM, J774 macrophages, RAW264.7 macrophages, and NIH 3T3 fibroblasts. The distribution of LRG-47 among the lipid fractions was essentially the same regardless of the cell source for the lipid, but it varied markedly from that of IGTP (Fig. 4 and data not shown). Overall, the distribution of LRG-47 was comparable to that of the Golgi marker GM130, suggesting that a portion of LRG-47 localized to the Golgi, while the distribution of IGTP was very similar to that of the ER marker GRP78 (Fig. 4). When macrophages were stained simultaneously with LRG-47 and IGTP antibodies, the localization of the two proteins was largely exclusive, except for slight IGTP reactivity in the areas of intense LRG-47 staining. Further immunofluorescence staining revealed a tight perinuclear staining pattern for LRG-47 which colocalized with the Golgi marker, GM130 (Fig. 5 and data not shown). In contrast, IGTP staining colocalized with that of the ER marker, TRAPα (Fig. 5).

We next examined the distribution of LRG-47 and IGTP in cells containing latex bead vacuoles. It has been shown previously by confocal microscopy that LRG-47 localizes to nascent latex bead vacuoles and remains associated with the vacuoles (15), but the localization of IGTP to vacuoles containing latex beads or pathogens has not been examined. In the present studies, localization of the two GTPases to latex bead vacuoles was first addressed by biochemical purification of latex bead vacuoles from macrophage cell lysates (Fig. 6). Latex vacuoles have typically been isolated from cell lysates by taking advantage of their relative buoyancy on sucrose gradients (5). However, using this technique it was found for both GTPases, and IGTP in particular, that ER/Golgi-associated protein copuri-

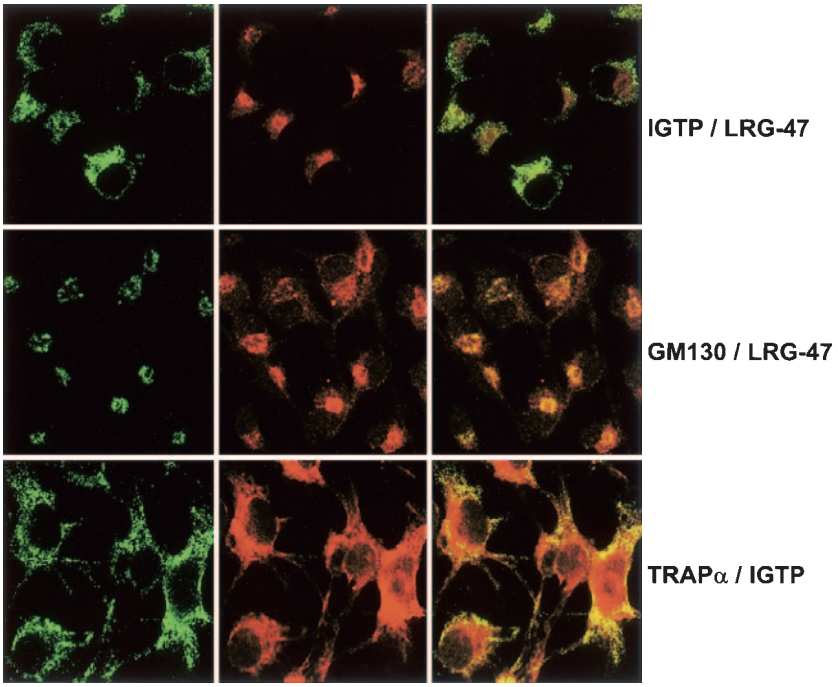

FIG. 5. LRG-47 and IGTP immunofluorescence in macrophages. BMM that had been activated with IFN-γ for 24 h were stained with the indicated primary antibodies and Alexa Fluor 488 (green)- or Alexa Fluor 594 (red)-conjugated secondary antibodies, as described in the text. The staining was then analyzed by confocal microscopy (magnification, ×900). The LRG-47 (red) and IGTP (green) localizations are largely exclusive. LRG-47 (red) colocalizes with the Golgi marker, GM130 (green). IGTP (red) and TRAPα (green) display colocalization within the endoplasmic reticulum. In the merged images (right column), colocalization of green and red staining appears yellow.

fied with vacuole-associated protein due to the similar buoyancies of the latex bead vacuoles and the ER and Golgi membranes (data not shown). To circumvent this problem, a method was formulated to purify latex bead vacuoles using paramagnetic latex beads, which effectively separated the vacuole-associated GTPases from the ER or Golgi-associated proteins. Using this technique LRG-47 and IGTP were both found at high levels in latex bead vacuole preparations (Fig. 6 and data not shown). The amount of protein associated with the vacuoles modulated to some extent following formation of the vacuole, with LRG-47 levels peaking around 30 min following vacuole formation and decreasing thereafter, while IGTP levels increased modestly over 3 h following vacuole formation (Fig. 6). In contrast, the prototypical lysosomal marker, LAMP1, showed more dynamic fluctuations in its level of association with the vacuoles, with amounts increasing substantially over the 3-h time course (Fig. 6). These data suggest that the GTPases do, in fact, traffic to latex bead vacuoles, but they do not show the dynamic association with maturing vacuoles that is typical of many endosomal and lysosomal proteins. To verify the vacuole fractionation data, cells were stained with LRG-47, IGTP, and LAMP1 antibodies. Staining for both LRG-47 and IGTP colocalized with LAMP1 on latex bead vacuoles (Fig. 7). Apparent levels of LRG-47 and IGTP on the vacuoles as assessed microscopically did not vary to a great extent for the first 3 h following vacuole formation (not shown).

To determine whether LRG-47 or IGTP was present in vacuoles containing *T. gondii*, BMM were given heat-killed or live parasites, and localization was examined by immunofluorescence microscopy at various times after infection. Typical results from the 60-min time point are shown in Fig. 8. Neither IGTP nor LRG-47 was found to localize to the *T. gondii* vacuole at any of the examined times. Where the parasite was present in the periphery of the cell, there was no appreciable IGTP or LRG-47 staining near the vacuole. In cases in which the parasite was located in the ER or Golgi region of the cell, the vacuole was surrounded by areas that were positive for IGTP and LRG-47 staining, but the proteins did not seem to concentrate on the vacuole. In parallel studies, *T. gondii* that had been heat killed and opsonized with antibody were phagocytosed by BMM that were then examined for GTPase localization (Fig. 8). The majority of phagosomes containing heat-killed *T. gondii* displayed little IGTP or LRG-47 reactivity, although in a minority of the phagosomes, some LRG-47 staining was noted. LAMP-1 staining confirmed that the heat-killed parasites had indeed been phagocytosed and were within late endosomes/lysosomes.

DISCUSSION

We have shown here that IGTP and LRG-47 are required for IFN-γ-induced inhibition of *T. gondii* growth in macrophages, while the related p47 GTPase, IRG-47, is not required. The roles of the proteins in regulating parasite growth in the macrophage in vitro, therefore, correlate with their roles in controlling acute *T. gondii* in vivo: IGTP and LRG-47 are essential for IFN-γ-regulated host resistance, while IRG-47 is not. These data underscore the critical role that macrophages play in controlling acute infection, and the data define a gen-

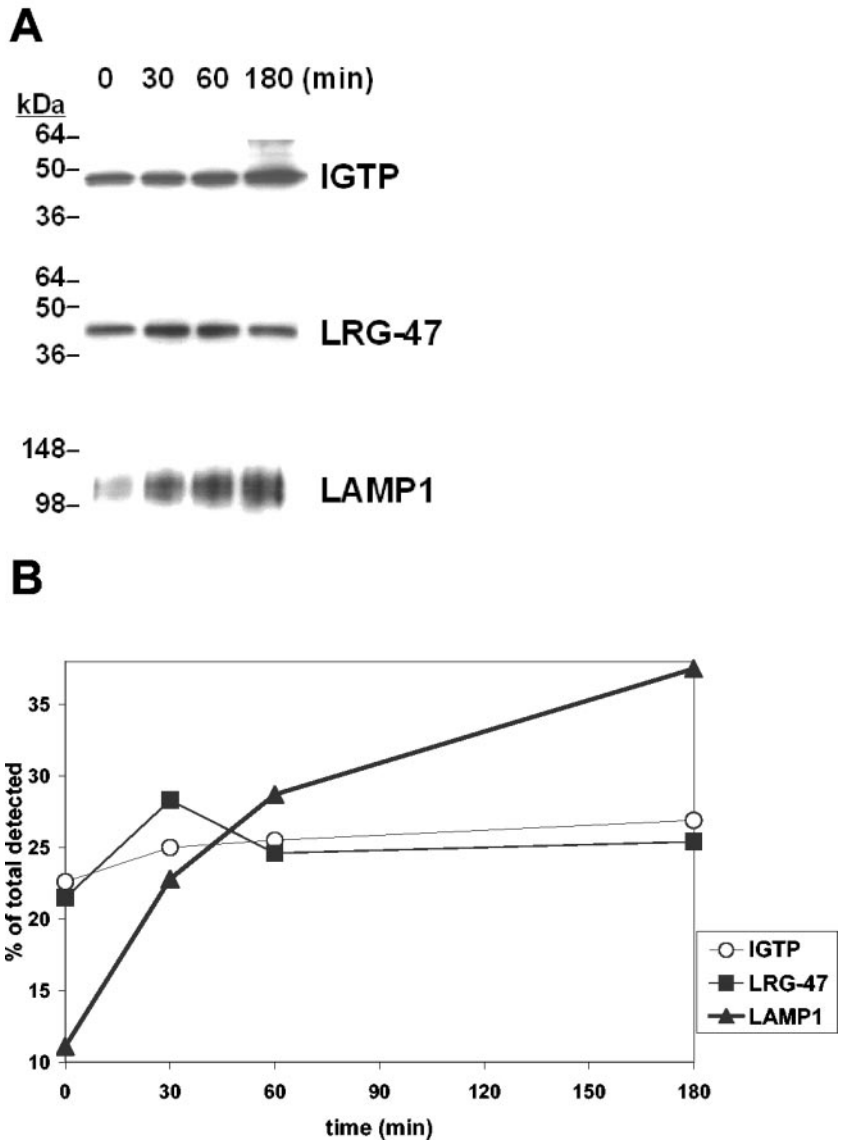

FIG. 6. Distribution of LRG-47 and IGTP in macrophages containing latex bead vacuoles. J774 macrophages were stimulated with IFN-γ for 24 h and were then allowed to phagocytose latex beads for 20 min; they were then incubated for the indicated times in the absence of beads. Latex bead vacuoles were then isolated, as described in Materials and Methods, and equal amounts or vacuole-associated protein were used for Western blotting with LRG-47, IGTP, and LAMP1 antibodies. Shown are a representative Western blot (A) and the relative signals for each protein as assessed by densitometry (B), with the levels detected at each time point expressed as a percentage of the total protein detected (average levels from four separate experiments).

eral mechanism through which IGTP and LRG-47 provide host resistance in this context. It is not apparent why IRG-47 has a distinct effect on *T. gondii* growth compared to the effects of IGTP and LRG-47. Our data indicate that it is not lack of expression, as all three GTPases were expressed in the BMM cultures. However, IRG-47 does show a very different distribution pattern within the cells, with <10% of the protein being membrane bound, compared with >90% membrane-bound protein for IGTP and LRG-47 (15). This would suggest a distinct role in regulating membrane trafficking, a function that has been hypothesized for the p47 GTPases, as we discuss below. Further studies will be required to determine the unique aspect of IRG-47 that leads to its differential function.

It has been demonstrated previously that IGTP regulates IFN-γ-induced *T. gondii* killing in astrocytes (10). This suggests that the effects of the p47 GTPases are not specific to macrophages and may well extend to multiple cell types and be a generalized cell-based host defense mechanism. In fact, there is no precedent for cell-specific expression of the GTPases; rather, they seem to be broadly expressed in many types of cells, including macrophages, lymphocytes, astrocytes, and fibroblasts, in response to infection in vivo or IFN-γ stimulation in vitro. In addition, p47 GTPase expression does not seem to be coordinately regulated, as IGTP KO cells express normal levels of LRG-47, and vice versa (G. Taylor, unpublished data).

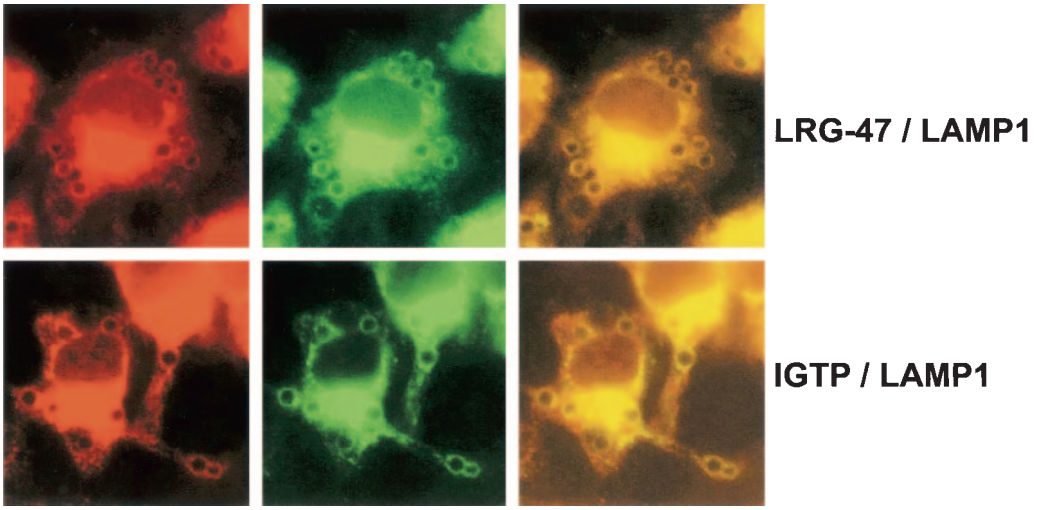

FIG. 7. LRG-47 and IGTP immunofluorescence in macrophages containing latex bead vacuoles. BMM that had been activated with IFN-γ for 24 h were allowed to phagocytose latex beads for 20 min and then were incubated in the absence of beads for 30 min. The cells were then fixed and stained with the indicated antibodies. Staining was assessed by deconvolution microscopy (magnification, ×600).

A model for p47 GTPase action has been proposed in which the proteins traffic from the ER or Golgi to the pathogen-containing vacuoles, where they affect genesis or processing of the vacuole and undermine survival of the pathogens. This model is clearly supported by two studies that focused on LRG-47. In the first, the protein was found to be present in the *M. tuberculosis* phagosome, where it was required for normal phagosomal acidification and eradication of the bacterium from macrophages (14). In the second, LRG-47 was found to be recruited to the plasma membrane of macrophages during phagocytosis of latex beads and then to remain with the phagosome as it matured (15). Furthermore, in the current work we demonstrate that IGTP also traffics to latex bead vacuoles, with kinetics that vary somewhat from the trafficking of LRG-47 to the vacuoles. Despite these provocative results, in the present studies, IGTP and LRG-47 did not concentrate in phagosomes containing live *T. gondii* in IFN-γ-activated cells. In one sense this result is not unexpected, as vacuoles that contain live *T. gondii* are known to avoid contact with the host cell's endocytic machinery, effectively circumventing acquisition of classical endosomal and lysosomal markers (16, 17, 19). In contrast, vacuoles that contain heat-killed or opsonized *T. gondii* are known to interact with the endosomal/lysosomal machinery of the cell, actively acquiring host cell markers (16, 17, 19). Consequently, it is perhaps more significant that IGTP and LRG-47 were not present in phagosomes that contained heat-killed *T. gondii*. It remains possible that IGTP and LRG-47 could function by influencing genesis or the processing of parasitic vacuoles, albeit via a mechanism that does not appear to involve their presence in the vacuole. It is notable that *T. gondii* vacuoles are often found proximal to the ER and Golgi where IGTP and LRG-47 reside (17, 20). Future studies should address the role of IGTP and LRG-47 in the formation and processing of the *T. gondii* vacuole.

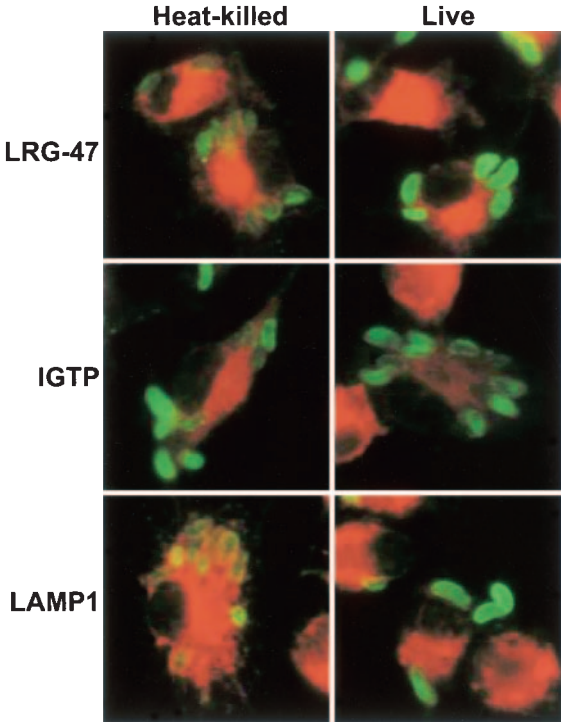

FIG. 8. LRG-47 and IGTP immunofluorescence in *T. gondii*-infected macrophages. BMM that had been activated with IFN-γ for 24 h were infected with live or heat-killed *T. gondii*, as indicated in the figure, for 1 h. Parasites appear green, and the GTPases and LAMP-1 appear red. Colocalization of heat-killed parasites with LAMP-1, indicating late endosomal/lysosomal localization, appears yellow.

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