A Cluster of Interferon-γ-Inducible p65 GTPases Plays a Critical Role in Host Defense against Toxoplasma gondii

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Abstract
Interferon-γ (IFN-γ) is essential for host defense against intracellular pathogens. Stimulation of innate immune cells by IFN-γ upregulates ~2,000 effector genes such as immunity-related GTPases including p65 guanylate-binding protein (Gbp) family genes. We show that a cluster of Gbp genes was required for host cellular immunity against the intracellular parasite Toxoplasma gondii. We generated mice deficient for all six Gbp genes located on chromosome 3 (Gbp(chr3)) by targeted chromosome engineering. Mice lacking Gbp(chr3) were highly susceptible to T. gondii infection, resulting in increased parasite burden in immune organs. Furthermore, Gbp(chr3)-deleted macrophages were defective in IFN-γ-mediated suppression of T. gondii intracellular growth and recruitment of IFN-γ-inducible p47 GTPase Irgb6 to the parasitophorous vacuole. In addition, some members of Gbp(chr3) restored the protective response against T. gondii in Gbp(chr3)-deleted cells. Our results suggest that Gbp(chr3) play a pivotal role in anti-T. gondii host defense by controlling IFN-γ-mediated Irgb6-dependent cellular innate immunity.

Reference

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A Cluster of Interferon-γ-Inducible p65 GTPases Plays a Critical Role in Host Defense against Toxoplasma gondii

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SUMMARY

Interferon-γ (IFN-γ) is essential for host defense against intracellular pathogens. Stimulation of innate immune cells by IFN-γ upregulates ~2,000 effector genes such as immunity-related GTPases including p65 guanylate-binding protein (Gbp) family genes. We show that a cluster of Gbp genes was required for host cellular immunity against the intracellular parasite Toxoplasma gondii. We generated mice deficient for all six Gbp genes located on chromosome 3 (Gbpchr3) by targeted chromosome engineering. Mice lacking Gbpchr3 were highly susceptible to T. gondii infection, resulting in increased parasite burden in immune organs. Furthermore, Gbpchr3-deleted macrophages were defective in IFN-γ-mediated suppression of T. gondii intracellular growth and recruitment of IFN-γ-inducible p47 GTPase Lrgb6 to the parasitophorous vacuole. In addition, some members of Gbpchr3 restored the protective response against T. gondii in Gbpchr3-deleted cells. Our results suggest that Gbpchr3 play a pivotal role in anti-T. gondii host defense by controlling IFN-γ-mediated Lrgb6-dependent cellular innate immunity.
reproducing parasites. Accumulation of Irgs eventually leads to disruption of the integrity of the PV membranes (Howard et al., 2011; Ling et al., 2006; Taylor et al., 2007; Zhao et al., 2008).

Not only Irgs but also Gbps are known to accumulate around the PV shortly after T. gondii invasion (Degrandi et al., 2007). Moreover, because virulent strains of T. gondii inhibit the recruitment of Gbps around the PV (Degrandi et al., 2007; Vireire Winter et al., 2011), Gbps are considered anti-T. gondii defensive factors. Among Gbps, Gbp1 and Gbp2 are reported to modulate cellular proliferation (Gorbacheva et al., 2002; Guenzi et al., 2001). In addition, Gbp1 is involved in the regulation of matrix metalloproteinase 1 in cancer cell lines (Guenzi et al., 2003; Li et al., 2011). Although in vitro studies have been reported, the physiological protective role of Gbps against T. gondii remains uncertain. The mouse genome carries 13 Gbp genes (11 active members and 2 pseudogenes) that are organized in clusters and share a high degree of homology (Kresse et al., 2008). Six and seven family members are tandemly aligned on chromosomes 3 and 5, respectively (Kresse et al., 2008). Such a complex configuration has hampered in vivo investigation of the Gbp genes through genetic approaches.

To elucidate the in vivo functional contribution of the Gbps to host defense against T. gondii, we have generated mice lacking the entire cluster of Gbps on chromosome 3 (Gbp chr3) by Cre-loxP-based chromosome engineering. Gbp chr3-deleted mice were highly susceptible to T. gondii infection with a considerably increased parasite burden in tissues. Furthermore, Gbp chr3-deleted macrophages showed defective suppression of parasite growth in response to IFN-γ. Although parasite infection-induced production of oxidants and proinflammatory cytokines as well as autophagy-related 4b (Atg4b) recruitment to the parasites were normal, IFN-γ-induced disruption of the PV membrane and localization of Irgs such as Irgb6 and Irgb10 to the PV were compromised in Gbp chr3-deleted cells. Moreover, endogenous Gbps colocalized and interacted with Irgb6. The reintroduction of Gbp1, Gbp5, or Gbp7 into Gbp chr3-deleted cells partially restored the IFN-γ-dependent anti-T. gondii response. Taken together, these results demonstrate that this cluster of Gbps has a defensive function against T. gondii by positively regulating IFN-γ-inducible Irgb6-dependent cellular innate immunity.

RESULTS

Generation of Mice Lacking the Entire Gbp Locus on Chromosome 3

To assess the anti-T. gondii immunity of Gbp chr3 in vivo, we generated embryonic stem (ES) cells possessingloxP sites at the most proximal and distal loci from the centromere in the gene cluster (Gbp5 and Gbp2ps, respectively) by sequential conventional gene targeting methods (Figure 1A and Figure S1 available online). Deletion of the entire Gbp chr3 locus spanning 173 kb was achieved by crossing the F1 mice with CAG-Cre transgenic mice and was confirmed by Southern blotting and PCR (Figures 1B and 1C). Gbp chr3-deleted mice were successfully obtained by intercrossing heterozygous mice, were born at the expected Mendelian ratio, and were healthy and normal in specific-pathogen-free conditions (Figure 1D). Under nonstimulated conditions, Gbp chr3-deleted mice showed normal parameters of cellular immunity (Figure S1H). To test whether the expression of Gbp chr3 was correctly ablated in Gbp chr3-deleted cells, we confirmed by quantitative RT-PCR that the mRNAs derived from Gbp chr3 (Gbp1, Gbp2, Gbp3, Gbp5, Gbp7, and Gbp2ps) were not induced in response to IFN-γ in Gbp chr3-deleted cells (Figure 1E). On the other hand, mRNAs for the Gbps on chromosome 5 (Gbp4, Gbp6, Gbp8, Gbp9, Gbp10, and Gbp11) were normally induced. We further analyzed Gbp chr3 expression by protein immunoblotting and found that Gbp1, Gbp2, and Gbp5 proteins were not detected. A weak signal was observed when we performed blotting with Gbp1-5 monoclonal antibodies that are raised against amino acids 1–300 mapping at the N terminus of human GBP1 and that recognize a shared epitope among Gbp1, Gbp2, Gbp3, Gbp4, and Gbp5. Expression of the gene encoding Gbp4, which is not disrupted in Gbp chr3-deleted cells, could account for this weak signal (Figure 1F).

Gbp chr3 Confers Resistance to T. gondii In Vivo

First, mice lacking Gbp2ps alone or deficient in both Gbp2ps and Gbp5 were infected intraperitoneally with the avirulent T. gondii strain ME49 and shown to exhibit survival rates similar to those of wild-type mice (Figures S1C and S1F). In contrast, infection of Gbp chr3-deleted mice revealed that these mutant mice were highly prone to die after T. gondii infection (Figure 2A). Because Gbp1 and Gbp5 were recently shown to be involved in host defense against Listeria monocytogenes (Kim et al., 2011; Shenoy et al., 2007), we challenged Gbp chr3-deleted mice with L. monocytogenes by intraperitoneal injection. Wild-type and Gbp chr3-deleted mice exhibited comparable bacterial burdens in tissues and survival rate (Figures S2A and S2B). To monitor the course of infection more accurately, we generated transgenic ME49 T. gondii expressing luciferase and monitored the kinetics by in vivo imaging in wild-type and Gbp chr3-deleted mice infected with 10⁶ parasites. Significant increases in parasite number were observed at days 7, 8, and 9 in Gbp chr3-deleted mice compared with those in wild-type mice (Figures 2B and S2C). We next compared parasite burdens in organs from the infected mice. The parasite load showed an excellent correlation with luciferase counts in vitro (Figures S2D and S2E). We collected the spleens and mesenteric lymph nodes from wild-type or Gbp chr3-deleted mice 9 days after a challenge with luciferase-expressing parasites and calculated the parasite numbers according to the luciferase signal. The parasite load in the tissues originating from Gbp chr3-deleted mice was markedly elevated in comparison with that from wild-type mice (Figure 2C). Taken together, these findings demonstrate that Gbp chr3 protects against the spreading and proliferation of T. gondii in vivo.

Next we examined immune responses during parasite infection in wild-type and Gbp chr3-deleted mice. IL-12 is important for the development of type I immunity, in which IFN-γ-producing CD4+ and CD8+ T cells play central roles for anti-T. gondii responses (Hunter et al., 1995). The concentrations of IL-12p40 and IFN-γ measured in sera were similarly increased in wild-type and Gbp chr3-deleted mice infected with T. gondii (Figure 2D). Furthermore, cellularity in spleens and IFN-γ production from splenic CD4+ and CD8+ T cells in response to anti-CD3 was comparable in wild-type and Gbp chr3-deleted mice (Figures 2E and S2F), suggesting that the high susceptibility to T. gondii in Gbp chr3-deleted mice was not due to defects in production of IL-12 or IFN-γ or in T cell responses.
Anti-Toxoplasma Role of Gbp by Regulating Irg

A

IFN-γ-inducible p65 GTPases on Chr.3 (Gbpchr3)

Wild-type (WT) locus

1st targeting

Neo deletion by Cre in ES cells

2nd targeting

2nd targeted locus

Germ-line transmission, and deletion of Gbpchr3 locus by crossing with CAG-Cre mice

Deleted (Δ) locus

B

Wild-type 2nd targeted (hetero)

IFN-γ

C

WT detection

Δ detection

D

No. of mice

106 192 99

Ratio

1 1.8 0.93

E

p65 GTPases on Chr.3

Gbp1

Gbp2

Gbp3

Gbp5

Gbp7

Gbp2ps

mRNA levels (relative)

WT KO

WT KO

WT KO

WT KO

WT KO

WT KO

F

p65 GTPases on Chr.5

Gbp4

Gbp6

Gbp8

Gbp9

Gbp10

Gbp11

mRNA levels (relative)

WT KO

WT KO

WT KO

WT KO

WT KO

WT KO

IFN-γ
**Gbp<sup>chr3</sup> Are Essential for IFN-γ-Induced Reduction of *T. gondii* Infection in Macrophages**

Macrophages play a vital role in IFN-γ-mediated innate immunity against *T. gondii* (Suzuki et al., 1988). Peritoneal macrophages represent a major cell type targeted by the parasite at the early stage after intraperitoneal infection (Jensen et al., 2011). To analyze the impact of Gbp<sup>chr3</sup> deficiency in this cell type, we infected wild-type or Gbp<sup>chr3</sup>-deleted peritoneal macrophages with *T. gondii* expressing luciferase in the presence of IFN-γ and assessed the luciferase units at 1, 12, 24, 36, or 48 hr postinfection (Figure 3A). We observed higher luciferase emissions in Gbp<sup>chr3</sup>-deleted cells than in wild-type cells at all time points tested except for 1 hr. Next, we analyzed the IFN-γ-dependent reduction of parasite burden by counting parasite numbers and the luciferase units in macrophages and mouse embryonic fibroblasts (MEFs) from wild-type or Gbp<sup>chr3</sup>-deleted mice. The dose-dependent reduction of parasite numbers and luciferase signals in both cell types lacking Gbp<sup>chr3</sup> was less pronounced than that in wild-type cells (Figures 3B and S3A). On the other hand, costimulation of tumor necrosis factor-α (TNF-α), which is known to strongly enhance antitoxoplasmal activity in macrophage in combination with IFN-γ (Sibley et al., 1991), abrogated the difference between wild-type and Gbp<sup>chr3</sup>-deleted cells (Figure S3B). These data suggested a selective impairment in IFN-γ-mediated reduction of parasite burden.

IFN-γ leads to inhibition of *T. gondii* proliferation and promotes its clearance from macrophages (Ling et al., 2006). To measure clearance, we next used confocal microscopy to compare the degree of parasite infection and growth in macrophages isolated from wild-type or Gbp<sup>chr3</sup>-deleted mice. At 5 hr postinfection, the percentage of cells infected with the parasites was comparable between wild-type and Gbp<sup>chr3</sup>-deleted mice (Figure 3C). In contrast, the infection rate of IFN-γ-stimulated macrophages from Gbp<sup>chr3</sup>-deleted mice was remarkably higher than that from wild-type cells after 20 hr (Figures 3C, 3D, and S3C). Next, we compared the rate of parasite replication in wild-type and Gbp<sup>chr3</sup>-deleted macrophages in the presence of IFN-γ by counting the parasite numbers per PV. In Gbp<sup>chr3</sup>-deleted cells, the number of parasites per PV was modestly increased compared with that in wild-type cells (Figures 3D and 3E), indicating that Gbp<sup>chr3</sup> are not only required for clearance but also inhibit parasite replication in IFN-γ-stimulated macrophages.

**Impact of Gbp<sup>chr3</sup> Deficiency on IFN-γ-Mediated Anti-*T. gondii* Response**

Among Gbp<sup>chr3</sup>, Gbp1 and Gbp7 have been shown to participate in antibacterial host defense by inducing an oxidative response and recruiting autophagy effectors such as Atg4b to L. monocytogenes and *Mycobacterium bovis* (Kim et al., 2011). To test whether these mechanisms also apply to anti-*T. gondii* defense, we first measured IFN-γ-induced oxide ion (O<sup>2-</sup>) production in wild-type or Gbp<sup>chr3</sup>-deleted cells infected with *T. gondii* (Figure 4A). The O<sup>2-</sup> production in Gbp<sup>chr3</sup>-deleted macrophages was normally enhanced. Next, we examined Atg4b recruitment to the parasite by confocal microscopy. As observed for pathogenic bacteria, Atg4b colocalized with intracellular *T. gondii* in wild-type cells. The increased recruitment of Atg4b to parasites in Gbp<sup>chr3</sup>-deleted macrophages was not altered during the course of infection (Figures 4B and S4A). Taken together, these results suggest that the anti-*T. gondii* action of Gbp<sup>chr3</sup> operates independently of O<sup>2-</sup> and Atg4b recruitment.

IFN-γ is also known to augment proinflammatory cytokine production in response to TLR ligands, and *T. gondii* possesses a profilin that can serve as a TLR11 ligand called profilin-like molecule (Plattner et al., 2008; Yarovinsky et al., 2005). We analyzed the production of proinflammatory cytokines in macrophages. Production of tumor necrosis factor α, IL-6, and IL-12p40 in IFN-γ-treated Gbp<sup>chr3</sup>-deleted cells in response to *T. gondii* infection or lipopolysaccharide (LPS) was normal (Figure S4B), indicating that Gbp<sup>chr3</sup> are dispensable for IFN-γ-mediated production of proinflammatory cytokines.

Because nitric oxide (NO) was previously reported to inhibit *T. gondii* replication in vitro (Adams et al., 1990), we assessed NO production in wild-type and Gbp<sup>chr3</sup>-deleted cells. Nonstimulated, *T. gondii*-infected, or LPS-treated macrophages from Gbp<sup>chr3</sup>-deleted mice produced similar concentrations of nitrite ion (NO<sub>2</sub>−) compared with that produced by wild-type cells (Figure S4C). Furthermore, hepatic expression of iNOS mRNAs was unchanged between wild-type and Gbp<sup>chr3</sup>-deleted mice in *T. gondii* infection (Figure S4D). In spite of normal NO production in Gbp<sup>chr3</sup>-deleted macrophages (Figure S4C), the inhibition of the parasite replication was modestly impaired by the Gbp<sup>chr3</sup> deficiency (Figure 3E). To examine whether NO is involved in inhibition of parasite replication in Gbp<sup>chr3</sup>-deleted cells, we compared the replication in Gbp<sup>chr3</sup>-deleted cells between those untreated and those treated with aminoguanidine (AG), an NO inhibitor (Figure 4C). Although the infection rate was not affected at all, AG-treated Gbp<sup>chr3</sup>-deleted cells contained significantly larger numbers of the parasites per vacuole than control cells, indicating that NO indeed plays a major role in inhibition of *T. gondii* replication in Gbp<sup>chr3</sup>-deleted macrophages in vitro. To further determine relative importance of Gbp<sup>chr3</sup> in the IFN-γ-dependent anti-*T. gondii* response in vivo, we compared parasite burdens and the survival rate of wild-type and...
**Impaired Disruption of the PV Membrane in Gbp<sup>chr3</sup>-Deleted Macrophages**

Gbp<sup>chr3</sup>-deleted mice treated with anti-IFN-γ (Figures 4D and 4E). Although the mortality was not increased by anti-IFN-γ treatment in Gbp<sup>chr3</sup>-deleted mice (Figure 4D), Gbp<sup>chr3</sup>-deleted mice treated with anti-IFN-γ displayed much higher parasite burdens than did control IgG-treated groups (Figure 4E). These results indicated that Gbpps do not fully account for the IFN-γ-dependent anti-T. gondii immunity in vitro and in vivo.

**Defective Irgb6 Recruitment to PVs in Gbp<sup>chr3</sup>-Deleted Macrophages**

A previous study demonstrated that macrophages lacking the autophagy protein Atg5 exhibit defective blebbing of the T. gondii PV (Zhao et al., 2008), which is reminiscent of the phenotype observed in Gbp<sup>chr3</sup>-deleted cells (Figure 5B). Atg5 has been reported to be required for the recruitment of Irg6 to the PV (Zhao et al., 2008). Irgs including Irg6 (also known as IIGP1), Irgb6 (TGTP), and Irgb10 were shown to be phosphorylated and dampened by T. gondii ROP18, a parasite-secreted kinase that acts as a virulence effector molecule (Fentress et al., 2010; Steinfeldt et al., 2010). The CTG strain of T. gondii produces an extremely low ROP18 mRNA expression (Boothroyd and Dubremetz, 2008; Saeij et al., 2006; Taylor et al., 2006); hence the CTG parasites as well as ME49 are permissive for Irg recruitment to the PV membrane (Saeij et al., 2006; Taylor et al., 2006). By using the CTG strain expressing luciferase, we found that the IFN-γ-dependent decrease in CTG parasites was impaired in Gbp<sup>chr3</sup>-deleted macrophages (Figure 6A), suggesting that Gbpps affect IFN-γ-induced clearance of T. gondii shortly after invasion. Moreover, 24 hr postinfection, a markedly increased number of dead parasites with damaged parasite membranes or no PV membranes were observed in wild-type cells compared with those in Gbp<sup>chr3</sup>-deleted cells (Figure 5C and data not shown).
saying that Irg-mediated immunity could be affected by Gbp<sup>chr3</sup>-deficiency. To test this possibility, we first analyzed the amount of Irgb6 protein expression in IFN-γ-treated macrophages. Similar amounts of Irgb6 protein were induced in wild-type and Gbp<sup>chr3</sup>-deleted cells upon stimulation by IFN-γ (Figure 6B). Next, we tested the recruitment of Irgb6 to the PV by confocal microscopy. At 6 hr postinfection, Irgb6 was detectable at the PV in wild-type cells. In contrast, the recruitment of Irgb6 was severely impaired in Gbp<sup>chr3</sup>-deleted macrophages (Figure 6C). The extent of Irgb6 recruitment was analyzed at 2, 4, and 6 hr postinfection. Compared with that in wild-type cells, the percentage of Irgb6-positive parasites in Gbp<sup>chr3</sup>-deleted cells was significantly lower at every time point analyzed (Figure 6D). In addition to Irgb6, we tested the recruitment of other Irgs such as Irgb10, Irga6, and Irgm3 (Figure S5). The recruitment of Irgb10 to the PV was also impaired in Gbp<sup>chr3</sup>-deleted cells (Figure S5A), whereas that of Irga6 was comparable between Gbp<sup>chr3</sup>-deleted wild-type and Gbp<sup>chr3</sup>-deleted cells (Figures S5B and S5C). We failed to observe the accumulation of Irgm3 to the PVs even in IFN-γ-stimulated wild-type cells (data not shown). Taken together, these results suggest that Gbp<sup>chr3</sup> are required for recruitment of Irgb6 and Irgb10 to the PV of <i>T. gondii</i> in IFN-γ-activated macrophages.

**Gbps Interact with Irgb6**

In a previous study, an overexpressed green fluorescent protein (GFP)-Gbp1 fusion protein was shown to colocalize with endogenous Irgb6 and <i>T. gondii</i> parasites (Virreira Winter et al., 2011). We confirmed these observations with endogenous proteins by costaining for anti-Gbp1-5 and anti-Irgb6 in wild-type macrophages infected with <i>T. gondii</i> (Figure 6E). Furthermore, intensity profile analysis revealed that the signals for both proteins were detected at almost the same sites (Figure 6F). Then, we assessed the interaction between Gbps and Irgb6 in endogenous settings. Immunoprecipitation with anti-Gbp1-5 coprecipitated Irgb6 in IFN-γ-stimulated macrophages, and the amount of Irgb6 coimmunoprecipitated was markedly increased upon <i>T. gondii</i> infection (Figure 6G). These results suggest that...
association of Irgb6 with Gbps is fundamental to the defective Irgb6 recruitment to T. gondii in Gbpchr3-deleted cells.

**Gbpchr3 Participate Differentially in Anti-T. gondii Defense**

Although the Gbps share a high degree of homology (Kresse et al., 2008), variation in the amino acid sequence among Gbpchr3 is greater than that among Gbpchr5. It is uncertain whether the five active members of Gbpchr3 (Gbp1, Gbp2, Gbp3, Gbp5, and Gbp7) similarly or differentially contribute to the anti-T. gondii cellular immunity. To address this question, we cloned each Gbp into drug-responsive retroviral expression vectors and expressed them in a tightly doxycycline-dependent manner in Gbpchr3-deleted primary MEFs (Figure 7A). Parasite clearance in the absence or presence of IFN-γ was then tested in these transduced cells. Reintroduction of Gbp1, Gbp5, or Gbp7 into Gbpchr3-deleted MEFs partially restored the IFN-γ-dependent clearance of T. gondii compared with wild-type cells (Figures 3B and 7B), indicating that these proteins possess common and compensatory functions in the anti-T. gondii immune response. Taken together, our results show that Gbpchr3 play a pivotal role in IFN-γ-mediated cellular innate immune response to T. gondii.

**DISCUSSION**

This study makes use of targeted chromosome engineering to provide genetic evidence for the critical role of Gbpchr3 in defense against T. gondii infection. Mice lacking Gbpchr3 were highly susceptible to T. gondii infection, and Gbpchr3-deleted macrophages are defective in IFN-γ-mediated inhibition of intracellular parasite growth.

The Gbp-mediated cellular immune mechanism was previously reported to be pleiotropic and implicated IFN-γ-inducible phagocytic oxidative killing and the trafficking of antimicrobial peptides to autophagolysosomes by their interaction with NADPH oxidase subunits and autophagy-related molecules such as Atg4b, respectively (Kim et al., 2011). In this previous
study. Gbp function was mainly tested with dominant-negative forms of Gbps or small interfering RNA-mediated knockdown (Kim et al., 2011). Here, we observed normal O$_2^-$ production in response to IFN-\(\gamma\) stimulation and Atg4b recruitment to PVs in Gbp$_{chr3}$-deleted macrophages. Instead, we found that Gbp$_{chr3}$ deficiency affected recruitment of some Irgs to \textit{T. gondii}-infected macrophages. The discrepancy between the two studies might be explained by the different pathogens, or alternatively, it is possible that the other cluster of Gbps on chromosome 5 (Gbp$_{chr5}$) may play a compensatory role in the oxidative and autophagy-related responses.

Blebbing of the PV membrane was not induced in IFN-\(\gamma\)-activated macrophages lacking Gbp$_{chr3}$, which is akin to that observed in Atg5-deficient cells (Zhao et al., 2008). The similar phenotypes of both mutant mice prompted us to examine the localization of Irgs during \textit{T. gondii} infection. Gbp$_{chr3}$ deficiency impaired accumulation of Irgb6 and Irgb10 but not of Irga6 around the parasite, suggesting that the Gbps play a major role in some Irg recruitment. This model contrasts with previous studies suggesting that Irgs control localization of Gbps based on the fact that IFN-\(\gamma\) prestimulation is required for the localization of overexpressed Gbps to \textit{T. gondii} (Degrandi et al., 2007), and that Gbp2 localization is altered in Irgm-deficient cells (Tra-\textit{ver et al., 2011}). Considering that Gbps and Irgs participate in the anti-\textit{T. gondii} response in close proximity, it is possible that the two families of IFN-\(\gamma\)-inducible GTPases mutually control their localization, at least in part, through physical association. Given that the number of Irgs and Gbps varies among species (2 Irgs and 7 Gbps in human versus 23 Irgs and 13 Gbps in mouse) (Kresse et al., 2008; Shenoy et al., 2007), evolutionary pressure to increase or decrease the number and variety of Irgs could influence the repertoire of Gbps. Furthermore, virulent \textit{T. gondii} strains such as RH are capable of evading recognition by Irgs via the action of the virulence factor ROP18 that phosphorylates the Irgs (Degrandi et al., 2007; Fentress et al., 2010; Steinfeldt et al., 2010; Virreira Winter et al., 2011). In addition, virulent \textit{T. gondii} also prevents the accumulation of Gbps (Virreira Winter et al., 2011). Taken together, ROP18 phosphorylation of Irgs could affect the interaction with Gbps, promoting their dissociation from the parasites. In terms of the kinetics of the recruitment of Irgb6, given that Gbp$_{chr3}$ deficiency affected the later phase rather than the early stage, Gbp$_{chr3}$ might play a role in persistence of Irgb6 on PVs in the later stage. Alternatively, other factors including Gbp$_{chr5}$ might also participate in the Irgb6 recruitment in the early phase of infection. Whether deficiency of either Gbp$_{chr3}$ or Gbp$_{chr5}$ (or both) also affects the localization of other Irgs such as Irgm and Irgd deserves further investigation. Despite the fact that Irgb6 and Irgb10 are shown to be phosphorylated by ROP18 in vitro (strongly indicating the defensive roles of these Irgs) (Fentress et al., 2010), their in vivo functions in immunity to \textit{T. gondii} should still be assessed and confirmed under physiological conditions via mice lacking these Irgs.

The impaired inhibition of \textit{T. gondii} replication in Gbp$_{chr3}$-deleted cells prompted us to investigate the effect of NO on the Gbp$_{chr3}$-dependent resistance to \textit{T. gondii} in vitro. We found that the presence or absence of NO did not affect parasite clearance; the rate of macrophages infected with \textit{T. gondii} was not altered by addition of the NO inhibitor. Given the collaboration
of Irg and Gbp, the NO-independent role of Gbpchr3 may be parallel to the NO-independent effect of Irg on Toxoplasma clear-
ance (Collazo et al., 2002). In contrast, the presence of Gbpchr3 had a modest effect on the inhibition of Toxoplasma replication, which critically involves NO, because Gbpchr3-deleted macro-
phages contained larger numbers of the parasites per PV. Because there is no direct evidence indicating a role of Irg in the suppression of Toxoplasma replication to date, these data may be indicative of an unknown Irg-independent mechanism(s) of Gbpchr3 to promote the NO-mediated inhibition of the parasite replication in vitro. Nevertheless, considering that INOS defi-
ciency has a minor effect on early resistance in vivo (Scharton-
Kersten et al., 1997), the high susceptibility in acute Toxoplasma infection in Gbpchr3-deleted mice might be due to the defective parasite clearance dependent on some Irgs. Given the relative significance of Gbpchr3 in IFN-γ-dependent anti-Toxoplasma responses, the fact that anti-IFN-γ treatment in Gbpchr3-deleted mice resulted in enhanced parasite burdens in vivo suggests that additional IFN-γ-inducible effector(s) controls early resistance to the parasite. On the other hand, Gbpchr3-deleted mice treated with anti-IFN-γ displayed similar survival rate of mice with control IgG. The apparent disparity might be because anti-
IFN-γ blocked IFN-γ-dependent anti-Toxoplasma response, leading not only to high parasite burdens but also to immune pathology mediated by massive and dysregulated doses of IFN-γ at the terminal phase of Toxoplasma infection (Nguyen et al., 2003). In addition, costimulation of TNF-α with IFN-γ resulted in incom-
parable inhibition of the parasite growth and abolished the Gbpchr3-mediated effect. Although NO concentration in TNF-α and IFN-γ-treated macrophages was markedly higher than that in cells stimulated with IFN-γ alone, further investigation is required to determine whether only the difference of NO
to restore, albeit not fully, the killing activity by IFN-γ 
Gbp
etically to the anti-
et al., 2011; Shenoy et al., 2012). In contrast, our 
tion of Gbp1 and Gbp5 in the antibacterial response ( Kim 
L. monocytogenes
recently been shown to play a key role in the host defense in 
L. monocytogenes
and augmentation of phagocytic activity accounts for the 
concentration or other NO-independent effects such as autoph-
(Table 3) Retroviral vectors encoding the indicated Flag-tagged Gbps were stably 
introduced in Gbpchr3-deleted (KO) primary MEFs. The transfected MEFs were 
treated with or without 1 μg/ml doxycycline (Dox) for 24 hr and lysed. The lysates were immunoprecipitated with anti-Flag and detected by protein 
immunoblot with the indicated antibodies. 
(B) The KO MEFs transfected with indicated Gbps were unstimulated or stimulated with 100 ng/ml of IFN-γ for 24 hr with or without 1 μg/ml Dox. Cells 
were infected with ME49 T. gondii parasites expressing luciferase (moi = 0.5) 
for 36 hr, and the luciferase units (LU) were analyzed. The percentages of the activities after each Gbp induction with Dox over those without Dox (no Gbp 
induction) in nonstimulated (left) or stimulated (right) cells were shown. Indi-
cated values are means ± SD of triplicates.

Data are representative of two independent experiments. * p < 0.01, ** p < 0.001.

In conclusion, our genetic study with Gbpchr3-deleted mice has established that Gbpchr3 are required for IFN-γ-mediated host defense against T. gondii by regulating Irg6 recruitment to the parasite. To understand the functions of this family of IFN-γ-inducible p65 GTPases fully, future studies will have to include the second cluster, Gbpchr5, and will need to investigate the effect of these proteins in defense against a broader range of parasites, as well as against other pathogens including viruses and bacteria.

**EXPERIMENTAL PROCEDURES**

**Cells, Mice, and Parasites**

CS7BL/6 mice were obtained from SLC. ME49 and CTG derivatives of T. gondii 
were maintained in Vero cells by biweekly passage in RPMI1640 (Nacalai 
Tesque) supplemented with 2% heat-inactivated fetal calf serum (URHBiosci-
ence), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (GIBCO). MyD88- 
deficient mice were kindly provided by S. Akira. Animal experiments were 
conducted with the approval of the Animal Research Committee of the 
Graduate School of Medicine and of the Research Institute for Microbial 
Diseases, Osaka University.

**Antibodies**

Antibodies against T. gondii (sc-73210), TGT6 (IgG6: sc-11079), Atg6b 
(sc-130968), Gbp1 (sc-10586), Gbp2 (sc-10588), Gbp5 (sc-160566), Gbp1- 
5 (sc-166966), and actin (sc-8432) were purchased from Santa Cruz. Anti-
CD11b (M1/70) was obtained from Becton Dickinson. Aminoguanidine 
hydrochloride, LPS (a TLR4 ligand) from Salmonella minnesota Re 595, 
and anti-Flag were purchased from Sigma. Anti-GAP45 rabbit and anti-
MIC2 mouse antibodies were as described previously (Frênil et al., 2010). 
Anti-Irg6α (165/3) and IgB10 (940/6) rabbit antibodies and anti-Irgm3 
(BD Transduction Laboratories) mouse antibodies were kindly provided by 
J. Howard. Anti-IFN-γ (BE0055) and control Rat IgG1 (BE0088) were ob-
tained from Bioxcell. Recombinant IFN-γ and TNF-α were obtained from 
Peprotech.
**In Vivo Measurement of Parasites by Imaging**

Mice were intraperitoneally infected with $1 \times 10^5$ freshly egressed ME49 tachyzoites expressing luciferase resuspended in 100 μl PBS, and bioluminescence was assessed on the indicated days after infection. Treatment of anti-IFN-γ and control IgG was performed by the intraperitoneal injection 1 day before *T. gondii* infection. For the detection of bioluminescence emission, mice were intraperitoneally injected with 3 μg of D-luciferin in 200 μl PBS (Promega), maintained for 5 min to allow adequate dissemination of the luciferin, then anesthetized with isoflurane (Dainippon Sumitomo Pharma). At 10 min postinjection of D-luciferin, abdominal photon emission was assessed during a 60 s exposure by an in vivo imaging system (IVIS 100; Xenogen) and analyzed as described previously (Yamamoto et al., 2011).

**Immunofluorescence**

Peritoneal macrophages ($1 \times 10^5$) infected with *T. gondii* (moi = 0.5) were fixed with PBS containing 0.1% Triton X-100 and then blocked with 8% fetal calf serum in PBS. Subsequently, cells were incubated with anti-CD11b rat antibody (1:200) and anti-GAP45 rabbit antibody (1:1,000) in Figure 3D; anti-Atg4b rabbit antibody (1:200) and anti-T. gondii mouse antibody (1:50) in Figure 4B; anti-GAP45 rabbit antibody (1:1,000), anti-lgb6 goat antibody (1:50), and anti-Gbp1-5 mouse antibody (1:200) in Figure 6; and anti-lgb10 or anti-lga6 rabbit antibody (1:1,000) and anti-MIC2 mouse antibody (1:250), and anti-GAP45 rabbit antibody (1:1,000) in Figure 5F, for 1 hr at 37°C, followed by incubation with donkey IgG antibodies (1:10,000); Alexa Fluor 488-conjugated anti-rabbit IgG, Alexa Fluor 594-conjugated anti-goat, or Alexa Fluor 647 or Alexa Fluor 594-conjugated antibodies (1:1,000), anti-Irgm3 mouse antibody (1:250), and anti-GAP45 rabbit antibody (1:1,000) in Figure 5S, stained with lead citrate and uranyl acetate, and observed with an H-7650 electron microscope (Hitachi). The images were analyzed with Fluoview (Olympus).

**Transmission Electron Microscopy**

Peritoneal macrophages ($1 \times 10^5$) untreated or treated with 100 ng/ml IFN-γ for 24 hr were infected with *T. gondii* (moi = 0.5) for 6 or 24 hr. After washing with PBS, the cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer at 4°C overnight. The cells were postfixed with 1% OsO4 in the same buffer at 4°C for 1 hr, dehydrated in a graded series of ethanol, and embedded in Quetol 812 (Nissin EM). Silver sections were cut with an ultramicrotome, stained with lead citrate and uranyl acetate, and observed with an H-7650 electron microscope (Hitachi).

**Statistical Analysis**

The unpaired Student’s t test was used to determine the statistical significance of the experimental data.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at [http://dx.doi.org/10.1016/j.immuni.2012.06.009](http://dx.doi.org/10.1016/j.immuni.2012.06.009).

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