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.
A Cluster of Interferon-\(\gamma\)-Inducible p65 GTPases Plays a Critical Role in Host Defense against \textit{Toxoplasma gondii}

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SUMMARY

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

INTRODUCTION

Interferon-\(\gamma\) (IFN-\(\gamma\)) is an important T helper 1 (Th1) cell cytokine that strongly suppresses the growth and survival of intracellular pathogens (Boehm et al., 1997). Stimulation of innate immune cells such as macrophages and dendritic cells by IFN-\(\gamma\) results in robust gene expression of a number of effector molecules. These include immunity-related GTPases such as the Mx proteins, immunity-related p47 GTPases (Irgs), and p65 guanylate-binding proteins (Gbps) (Shenoy et al., 2007; Taylor et al., 2004). Mx proteins have been shown to participate in host defense against RNA viruses such as influenza and vesicular stomatitis virus (Sadler and Williams, 2008). Among the Irgs, mice deficient in Irgm1 (Lrg-47) are highly susceptible to Listeria, Salmonella, and mycobacteria (Deretic, 2006; MacMicking, 2004). Furthermore, Gbps have recently been shown to induce antibacterial responses involving phagocytic oxidases, autophagic effectors, and inflammasomes (Kim et al., 2011; Shenoy et al., 2012). Thus, IFN-\(\gamma\)-inducible immunity-related GTPases play pivotal roles in antiviral and antibacterial immune systems.

\textit{Toxoplasma gondii} is an obligatory intracellular protozoan parasite that infects virtually all warm-blooded vertebrates including human and mouse (Boothroyd, 2009; Israeliski and Remington, 1993). Infection of immunocompromised individuals such as those suffering from AIDS or those being treated with chemotherapy often leads to fatal toxoplasmosis encephalitis (Montoya and Remington, 2008). Innate immune cells, which recognize microbial components mainly via Toll-like receptors (TLRs) and the chemokine receptor CCR5, are essential in controlling \textit{T. gondii} infection via the production of proinflammatory cytokines such as interleukin-12 (IL-12) (Alberti et al., 2003; Hunter and Remington, 1995; Yarovinsky and Sher, 2006). IL-12 potentiates polarization of naïve T cells to Th1 cells, from which IFN-\(\gamma\) is produced in an antigen-dependent fashion (Trinchieri, 2003; Whitmarsh et al., 2011). IFN-\(\gamma\)-inducible GTPases are also important for the inhibition of \textit{T. gondii} growth by IFN-\(\gamma\). Mice lacking Irgm1, Irgd (Igtp), or Irga6 (Igtp1) are susceptible to acute and chronic infection (Collazo et al., 2001; Howard et al., 2011; Taylor et al., 2000). IRGs are recruited to the parasitophorous vacuole (PV), a membrane formed during invasion that is maintained to surround the intracellular...
replicating 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; Virreira 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 (Gbpchr3) by Cre-loxP-based chromosome engineering. Gbpchr3-deleted mice were highly susceptible to T. gondii infection with a considerably increased parasite burden in tissues. Furthermore, Gbpchr3-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 Gbpchr3-deleted cells. Moreover, endogenous Gbps colocalized and interacted with Irgb6. The reintroduction of Gbpchr3 Confer 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 Gbpchr3-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 Gbpchr3-deleted mice with L. monocytogenes by intraperitoneal injection. Wild-type and Gbpchr3-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 Gbpchr3-deleted mice infected with 10⁵ parasites. Significant increases in parasite number were observed at days 7, 8, and 9 in Gbpchr3-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 Gbpchr3-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 Gbpchr3-deleted mice was markedly elevated in comparison with that from wild-type mice (Figure 2C). Taken together, these findings demonstrate that Gbpchr3 protects against the spreading and proliferation of T. gondii in vivo.

Next we examined immune responses during parasite infection in wild-type and Gbpchr3-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 Gbpchr3-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 Gbpchr3-deleted mice (Figures 2E and S2F), suggesting that the high susceptibility to T. gondii in Gbpchr3-deleted mice was not due to defects in production of IL-12 or IFN-γ or in T cell responses.

RESULTS

Generation of Mice Lacking the Entire Gbp Locus on Chromosome 3

To assess the anti-T. gondii immunity of Gbpchr3 in vivo, we generated embryonic stem (ES) cells possessing loxP 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 Gbpchr3 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). Gbpchr3-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, Gbpchr3-deleted mice showed normal parameters of cellular immunity (Figure S1H). To test whether the expression
IFN-γ-inducible p65 GTPases on Chr.3 (Gbp<sup>chr3</sup>)

Wild-type (WT) locus

1<sup>st</sup> targeting

Neo deletion by Cre in ES cells

2<sup>nd</sup> targeting

Germ-line transmission, and deletion of Gbp<sup>chr3</sup> locus by crossing with CAG-Cre mice

Deleted (Δ) locus

WT KO WT KO WT KO WT KO WT KO WT KO WT KO

mRNA levels (relative)

IFN-γ-induced p65 GTPases on Chr.3 (Gbp<sup>chr3</sup>)

Wild-type 2<sup>nd</sup> targeted locus (hetero)

WT deletion

Δ detection

Δ detection primers

WT detection primers

M<br>Δ/+  +/+  Δ/Δ
1.5 kb 0.9 kb 0.8 kb 0.5 kb

No. of mice 106 192 99
Ratio 1 1.8 0.93

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 WT KO

Δ/+ +/+ Δ/Δ

Δ/Δ

WT KO WT KO WT KO WT KO WT KO WT KO WT KO

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 WT KO

WT KO WT KO WT KO WT KO WT KO WT KO WT KO

WCL IB: Gbp1

WCL IB: Gbp2

WCL IB: Gbp5

WCL IB: Gbp1-5

WCL IB: Actin

75 (kDa)

MW

+ + Δ/Δ

IFN-γ
**Gbpchr3 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 Gbpchr3 deficiency in this cell type, we infected wild-type or Gbpchr3-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 Gbpchr3-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 Gbpchr3-deleted mice. The dose-dependent reduction of parasite numbers and luciferase signals in both cell types lacking Gbpchr3 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 Gbpchr3-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 Gbpchr3-deleted mice. At 5 hr postinfection, the percentage of cells infected with the parasites was comparable between wild-type and Gbpchr3-deleted mice (Figure 3C). In contrast, the infection rate of IFN-γ-stimulated macrophages from Gbpchr3-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 Gbpchr3-deleted macrophages in the presence of IFN-γ by counting the parasite numbers per PV. In Gbpchr3-deleted cells, the number of parasites per PV was modestly increased compared with that in wild-type cells (Figures 3D and 3E), indicating that Gbpchr3 are not only required for clearance but also inhibit parasite replication in IFN-γ-stimulated macrophages.

**Impact of Gbpchr3 Deficiency on IFN-γ-Mediated Anti-*T. gondii* Response**

Among Gbpchr3, 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 (O²⁻) production in wild-type or Gbpchr3-deleted cells infected with *T. gondii* (Figure 4A). The O²⁻ production in Gbpchr3-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 Gbpchr3-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 Gbpchr3 operates independently of O²⁻ 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 Gbpchr3-deleted cells in response to *T. gondii* infection or lipopolysaccharide (LPS) was normal (Figure S4B), indicating that Gbpchr3 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 Gbpchr3-deleted cells. Nonstimulated, *T. gondii*-infected, or LPS-treated macrophages from Gbpchr3-deleted mice produced similar concentrations of nitrite ion (NO₂⁻) compared with that produced by wild-type cells (Figure S4C). Furthermore, hepatic expression of iNOS mRNAs was unchanged between wild-type and Gbpchr3-deleted mice in *T. gondii* infection (Figure S4D). In spite of normal NO production in Gbpchr3-deleted macrophages (Figure S4C), the inhibition of the parasite replication was modestly impaired by the Gbpchr3 deficiency (Figure 3E). To examine whether NO is involved in inhibition of parasite replication in Gbpchr3-deleted cells, we compared the replication in Gbpchr3-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 Gbpchr3-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 Gbpchr3-deleted macrophages in vitro. To further determine relative importance of Gbpchr3 in the IFN-γ-dependent anti-*T. gondii* response in vivo, we compared parasite burdens and the survival rate of wild-type and...
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 Gbps do not fully account for the IFN-γ-dependent anti-<i>T. gondii</i> immunity in vitro and in vivo.

Impaired Disruption of the PV Membrane in Gbp<sup>chr3</sup>-Deleted Macrophages

Gbp<sup>chr3</sup> deficiency resulted in compromised IFN-γ-induced clearance of <i>T. gondii</i> in macrophages (Figure 3). The killing by IFN-γ-activated macrophages is accompanied by blebbing of the PV membrane shortly after parasite entry (Ling et al., 2006; Zhao et al., 2008). Therefore, we examined by electron microscopy the morphology of the parasite PV membrane in wild-type and Gbp<sup>chr3</sup>-deleted macrophages 6 hr postinfection. Blebbing and vesiculation were easily detectable in the vicinity of the parasite PV membrane in wild-type cells (Figure 5A). In sharp contrast, in Gbp<sup>chr3</sup>-deleted cells the parasites were surrounded by a continuous intact PV membrane (Figure 5B), suggesting that Gbps affect IFN-γ-induced clearance of <i>T. gondii</i> 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).

**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 <i>T. gondii</i> 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 capped by <i>T. gondii</i> ROP18, a parasite-secreted kinase that acts as a virulence effector molecule (Fentress et al., 2010; Steinfeldt et al., 2010). The CTG strain of <i>T. gondii</i> produces an extremely low level of 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),
sugest 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 S5A). 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 and 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 Gbps are required for recruitment of Irgb6 and Irgb10 to the PV of T. gondii 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 T. gondii 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 T. gondii (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 T. gondii infection (Figure 6G). These results suggest that...
association of Irgb6 with Gbps is fundamental to the defective Irgb6 recruitment to *T. gondii* in Gbp<sup>chr3</sup>-deleted cells.

**Gbp<sup>chr3</sup> 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 Gbp<sup>chr3</sup> is greater than that among Gbp<sup>chr5</sup>. It is uncertain whether the five active members of Gbp<sup>chr3</sup> (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 Gbp<sup>chr3</sup>-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 Gbp<sup>chr3</sup>-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 Gbp<sup>chr3</sup> 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 Gbp<sup>chr3</sup> in defense against *T. gondii* infection. Mice lacking Gbp<sup>chr3</sup> were highly susceptible to *T. gondii* infection, and Gbp<sup>chr3</sup>-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
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 O2– production in response to IFN-γ stimulation and Atg4b recruitment to PVs in Gbpchr3-deleted macrophages. Instead, we found that Gbpchr3 deficiency affected recruitment of some Irgs to 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 (Gbpchr5) may play a compensatory role in the oxidative and autophagy-related responses.

Blebbing of the PV membrane was not induced in IFN-γ-activated macrophages lacking Gbpchr3, 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 T. gondii infection. Gbpchr3 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-γ prestimulation is required for the localization of overexpressed Gbps to T. gondii (Degrandi et al., 2007), and that Gbp2 localization is altered in Irgm-deficient cells (Tra-...
of Irg and Gbp, the NO-independent role of Gbp<sup>chr3</sup> may be parallel to the NO-independent effect of Irg on <i>T. gondii</i> clearance (Collazo et al., 2002). In contrast, the presence of Gbp<sup>chr3</sup> had a modest effect on the inhibition of <i>T. gondii</i> replication, which critically involves NO, because Gbp<sup>chr3</sup>-deleted macrophages contained larger numbers of the parasites per PV. Because there is no direct evidence indicating a role of Irg in the suppression of <i>T. gondii</i> replication to date, these data may be indicative of an unknown Irg-independent mechanism(s) of Gbp<sup>chr3</sup> to promote the NO-mediated inhibition of the parasite replication in vitro. Nevertheless, considering that INOS deficiency has a minor effect on early resistance in vivo (Scharton-Kersten et al., 1997), the high susceptibility in acute <i>T. gondii</i> infection in Gbp<sup>chr3</sup>-deleted mice might be due to the defective parasite clearance dependent on some Irgs. Given the relative significance of Gbp<sup>chr3</sup> in IFN-γ-dependent anti-<i>T. gondii</i> responses, the fact that anti-IFN-γ treatment in Gbp<sup>chr3</sup>-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, Gbp<sup>chr3</sup>-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-<i>T. gondii</i> 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 <i>T. gondii</i> infection (Nguyen et al., 2003). In addition, costimulation of TNF-α with IFN-γ resulted in incomparable inhibition of the parasite growth and abolished the Gbp<sup>chr3</sup>-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 the difference of NO
concentration or other NO-independent effects such as autophagy and augmentation of phagocytic activity accounts for the enhanced protective effect (Keller et al., 2011; Langermans et al., 1994).

Gbp1, Gbp5, and Gbp7, but not Gbp2 or Gbp3, were able to restore, albeit not fully, the killing activity by IFN-γ in Gbpchr3-deleted MEFs, indicating that Gbpchr3 contribute differentially to the anti-

T. gondii host defense mechanism. This is consistent with a previous finding that knockdown of some Gbps abrogates IFN-γ-dependent suppression of bacterial growth (Kim et al., 2011). In the context of the functional redundancy within Gbpchr3, we observed that mice lacking Gbp5, or Gbp5 and Gbp2ps, were resistant to T. gondii infection, suggesting that Gbp5 deficiency may be compensated for by other Gbpchr3-encoded proteins such as Gbp1 and Gbp7. On the other hand, mice lacking Gbp1 alone are susceptible to L. monocytogenes and M. bovis. Interestingly, Gbp5 has recently been shown to play a key role in the host defense in L. monocytogenes infection, indicating the nonredundant function of Gbp1 and Gbp5 in the antibacterial response (Kim et al., 2011; Shenoy et al., 2012). In contrast, our Gbpchr3-deleted mice displayed resistance to L. monocytogenes. This discrepancy may reflect different modes of infection (e.g., oral versus intraperitoneal infections in the previous and current studies, respectively) or utilization of different mouse strains (Gbp1 or Gbp5 singly deficient mice in previous studies and Gbpchr3-deleted mice in this study) (Kim et al., 2011; Shenoy et al., 2012). Moreover, we could not exclude the possibility that Gbpchr3 other than Gbp1 and Gbp5 play a negative role in Gbp1- and Gbp5-mediated anti-Listeria response. It remains to be seen whether deficiency of Gbp1 alone would be sufficient to disrupt the anti-

T. gondii defense system, but it is nevertheless plausible that each member of Gbpchr3 may play a differential role to combat distinct types of pathogens. Given that Gbp2 associates with Gbp1 (Virreira Winter et al., 2011), Gbp2 (and Gbp3) might play a role in host defense against other pathogens or have an additional anti-

T. gondii effect in the presence of other Gbpchr3. Considering that Gbpchr3-deleted cells lack a large genetic region, there is an inherent risk that the phenotype observed in Gbpchr3-deleted mice could be unrelated to Irg. Although we adopted in vitro retroviral transfection for the restoration of each Gbp in this study, none of Gbpchr3 failed to fully restore the effect of IFN-γ. It remains unclear whether the failure is due to limit of the transfection method or due to nonredundancy among Gbpchr3 except for Gbp5. In vivo transgenic reconstitution of the region by an artificial chromosome such as BAC and YAC should be utilized to reveal a role of each Gbp specifically participating in antiparasite in vivo responses in the future (Copeland et al., 2001).

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 Irgb6 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

CS7/BL/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 (JRH Biosciences), 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.

Reagents

Antibodies against T. gondii (sc-73210), TGGP (Irgb6; sc-11079), Atg4b (sc-10968), Gbp1 (sc-10586), Gbp2 (sc-10588), Gbp5 (sc-160356), Gbp1-5 (sc-166960), 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 (Fréнал et al., 2010). Anti-

Irga6 (165/3) and Irgb10 (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 obtained 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 × 10^6 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 mg 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 × 10^6) infected with T. gondii (moi = 0.5) were fixed for 10 min in PBS containing 3.7% formaldehyde. Cells were permeabilized 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-β1 integrin rabbit antibody (1:50) in Figure 4B; anti-GAP45 rabbit antibody (1:1,000), anti-Irgb6 goat antibody (1:50), and anti-Gbp1-5 mouse antibody (1:200) in Figure 6; and anti-Irgb10 or anti-Irga6 rabbit antibody (1:1,000) and anti-MIC2 mouse antibody (1:250), and anti-GAP45 rabbit antibody (1:1,000) in Figure 5S, 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 (1:10,000): Alexa Fluor 488-conjugated anti-rabbit IgG, Alexa Fluor 594-conjugated anti-goat, or Alexa Fluor 647 or Alexa Fluor 594-conjugated anti-mouse (Molecular Probes) for 1 hr at room temperature in the dark. Finally, the immunostained cells were mounted with PermaFluor (Thermo Scientific) on glass slides and analyzed by confocal laser microscopy (FV1000-D IX-81; Olympus); the images were analyzed with Fluoview (Olympus).

Transmission Electron Microscopy

Peritoneal macrophages (1 × 10^6) 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.

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REFERENCES

Immunity

Anti-Toxoplasma Role of Gbp by Regulating Irg


