The GRA15 protein from Toxoplasma gondii enhances host defense responses by activating the interferon stimulator STING

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ABSTRACT

Toxoplasma gondii (T. gondii) is an important neurotropic pathogen that establishes latent infections in humans that can cause toxoplasmosis in immunocompromised individuals. It replicates inside host cells and has developed several strategies to manipulate host immune responses. However, the cytoplasmic pathogen-sensing pathway that detects T. gondii is not well characterized. Here, we found that cGAS, a sensor of foreign dsDNA, is required for activation of anti–T. gondii immune signaling in a mouse model. We also found that mice deficient in STING (Sting−/− mice) are much more susceptible to T. gondii infection than wild type mice. Of note, the induction of inflammatory cytokines, type I IFNs, and interferon-stimulated genes in the spleen from
Sting<sup>−/−</sup> mice was significantly impaired. Sting<sup>−/−</sup> mice exhibited more severe symptoms than cGAS-deficient mice after T. gondii infection. Interestingly, we found that the dense granule protein GRA15 from T. gondii is secreted into the host cell cytoplasm and then localizes to the endoplasmic reticulum (ER), mediated by the second transmembrane motif in GRA15, which is essential for activating STING and innate immune responses. Mechanistically, GRA15 promoted STING polyubiquitination at Lys-337 and STING oligomerization in a TRAF protein–dependent manner. Accordingly, GRA15-deficient T. gondii failed to elicit robust innate immune responses compared with wild type T. gondii. Consequently, GRA15<sup>−/−</sup> T. gondii was more virulent and caused higher mortality of wild type mice but not Sting<sup>−/−</sup> mice upon infection. Together, T. gondii infection triggers cGAS/STING signaling, which is enhanced by GRA15 in a STING- and TRAF-dependent manner.

The protozoan parasite Toxoplasma gondii (T. gondii) can infect nearly all warm-blooded animals (1,2). As for humans, nearly 30% of the world’s population is infected with T. gondii (3). In healthy adults, T. gondii is controlled by the immune system and remains dormant in the brain. However, in immunocompromised individuals, the defect of the immune system leads to the reactivation of the T. gondii parasites and the development of toxoplasmosis. Reactivated parasite replication causes life-threatening brain damage with brain abscesses and necrotic areas (4). Thus, HIV/AIDS patients, cancer patients and organ transplant recipients are highly susceptible to T. gondii infection.

The infection of T. gondii parasites is recognized by pattern recognition receptors (PRRs). Previous studies showed that TLR11 is the PRR of T. gondii in murine cells. TLR11 is able to detect the actin-binding protein Profilin, which is required for entry of T. gondii during infection. TLR11 and TLR12 form a heterodimer in murine dendritic cells (DC) after sensing Profilin, and activate adaptor protein MyD88 to initiate downstream signaling for defense against T. gondii (5). Moreover, TLR7 and TLR9 are able to compensate for the loss of TLR11 by activating MyD88 in TLR11 deficient mice (6). Interestingly, TLR7 is activated by T. gondii RNA and triggers innate immune signaling only in TLR11 deficient cells (7). In addition, TLR7 expression is undetectable in CD9α+DCs, which is believed as the primary DC subset to surveillance the infection of T. gondii (8). Therefore, the importance of TLR7 in defense against T. gondii is unclear. TLR9 recognizes the methylated DNA of T. gondii (9,10). However, TLR9 expression is not always detected in the T. gondii infected DC, whose expression needs priming by IFNg (6). In addition to those receptors localized in endosomes, the cell plasma membrane receptors TLR2 and TLR4 are shown to recognize the glycosylphosphatidylinositol (GPI) from the surface of T. gondii and T. gondii-derived heat shock protein 70 (HSP70) (11,12). However, some studies showed that T. gondii suppressed innate immune responses via TLR4. Leng et al., found that chromatin remodeling in T. gondii infected macrophages inhibited cytokine production via TLR4 (13). Lee et al., also reported that T. gondii suppressed the production of pro-inflammatory cytokines after TLR4/ligand interactions (14). The well-studied murine PRRs of T. gondii are TLR11 and TLR12. However, the human genome does not encode TLR11 or TLR12 proteins. Nearly all the known T. gondii PRRs are membrane harbored, on plasma membrane or endosome. Cytoplasmic PPR pathways are also crucial for sensing invading pathogens and initiating host defense. The cytoplasmic sensors for virus and bacterium have been well characterized (15-18). Moreover, T.
GRA15 enhances STING activity

gondii is an intracellular pathogen. Previous study showed that NLRP1 is an inflammasome sensor for T. gondii (19), which influences susceptibility to human congenital toxoplasmosis (20). However, T. gondii was not able to activate NLRP1 signaling without pretreatment of LPS. Moreover, the inflammasome mainly mediated maturation of proinflammatory cytokines and cell pyroptosis, but not induction of immune genes. Therefore, the PRRs of T. gondii, particularly the cytoplasmic inflammasome sensor NLRP1, plays a critical role in the host immune response to T. gondii infection.

The classic anti-viral cytokine interferon (IFN)β can be induced during parasitic infection. It has been shown that IFNβ was able to inhibit replication of T. gondii (21,22). A recent study showed that a small group of atypical strains are able to induce type I IFN production in bone marrow-derived macrophages (BMDM) in a RIG-I and IRF3 dependent manner. The canonical Type I, II or III strain failed to trigger type I IFN induction (23). However, Robey et al. found inflammatory monocytes (IMs) produce IFNβ in response to type II T. gondii infection (24), suggesting different types of cells respond to T. gondii infection differently.

Cyclic GMP-AMP synthase (cGAS) is the cytoplasmic double-stranded DNA (dsDNA) sensor recognizing foreign dsDNA and abnormal host dsRNA, which is critical for defense against DNA viruses and regulating homeostasis of immune system (25,26). cGAS synthesizes secondary messenger cGAMP, which in turn activates STING. STING is a key signal molecule localized in ER. Activated STING recruits kinase TBK1 that phosphorylates IRF3/IRF7 to initiate transcription of type I IFNs. It is believed that posttranslational modification of STING modulates its activity and protein stability. TBK1 phosphorylates STING at serine 366, which is critical for IRF3 recruitment and type I IFN induction (27). E3 ligase TRIM56 is able to attach K63-linked polyubiquitin chain to STING and promotes dimerization and activation of STING (28). E3 protein complex autocrine motility factor receptor (AMFR) and insulin-induced gene 1 (INSIG1) promote K27-linked polyubiquitination of STING and direct its translocation from ER to perinuclear vesicle (29). RNF5 ubiquitinates STING at lysine 150 resulting in its degradation by the proteasome, which could be antagonized by RNF26 that attaches K11-linked polyubiquitin to the same lysine of STING (30,31). However, the role of cGAS/STING in the host defense against T. gondii has not been well characterized.

Accumulating studies have shown that T. gondii virulence is mediated by effector proteins secreted by parasite into the host cell. Dense granule proteins (GRAs) are produced by T. gondii, many of which are secreted into the parasitophorous vacuole (PV) and contribute to the maturation and structural integrity of the PV (32). GRA proteins also participate in the modulation of host defense during infection (33,34). GRA proteins mainly suppress host immune responses and facilitate parasite replication (35). However, GRA15 activates NFκB signaling in a TRAF6 dependent way, and in turn upregulates the production of IL12 (36). Moreover, GRA15 promotes the secretion of IL1β via inflammatory monocytes during infection (37).

In the present study, we found that cGAS is required for innate immune response against T. gondii. cGAS deficient mice are much more susceptible to lethal infection of T. gondii than wild type mice. Loss of STING results in a more severe phenotype during T. gondii infection. Mechanistically, we demonstrated that secreted dense granule protein GRA15 promotes oligomerization and activation of STING. GRA15 promotes polyubiquitination of STING at lysine 337 via TRAF proteins, which is required for
activation of STING. Together, GRA15 enhances cGAS/STING signaling by potentiating STING activity during T. gondii infection.

Results

**cGAS/STING axis is critical for anti-T. gondii immunity in vivo and in vitro**

The cGAS/STING axis recognizes cytoplasmic DNA and triggers innate immune responses. We here investigated the role of STING in host defense against the infection of T. gondii. The STING loss-of-function Tmelm173\textsuperscript{gt/gt} mice (also known as Golden ticket and here referred to Sting\textsuperscript{B/B} strain) was more susceptible to T. gondii infection than wild type animals (Fig. 1A). Consistent with the survival results, the parasite burden (as assessed by T. gondii B1 DNA) was significantly higher in the spleen of Sting\textsuperscript{B/B} mice than wild type mice after T. gondii infection (Fig. 1B). Furthermore, we detected less type I IFNs, including IFNβ and IFNa4 in the spleen of Sting\textsuperscript{B/B} mice than wild type mice during infection (Fig. 1C and Fig. S1A). Consequently, induction of interferon-stimulated genes, including ISG15 and CXCL10, were significantly impaired in STING mutant mice. In addition to type I IFNs, STING is also required for the induction of inflammatory cytokine interleukin 12 (IL12), which is believed critical for controlling T. gondii infection (Fig. 1C). Consistently, the induction of IFNg is modestly decreased in Sting\textsuperscript{B/B} mice (Fig. 1C). Therefore, STING is required for host defense against infection with T. gondii in vivo. At cellular level, we isolated peritoneal macrophages from wild type and Sting\textsuperscript{B/B} mice and infected these cells with T. gondii. In line with the animal experiments, macrophages with mutant STING failed to mount production of IFNβ, ISG15, CXCL10 and IL12 during T. gondii infection (Fig. 1D).

To further confirm these findings, we infected immortalized Bone Marrow-Derived Macrophages (iBMDM) with T. gondii after deleting endogenous STING by using CRISPR/Cas9. The lacking of STING resulted in more parasite replication in iBMDM, which was measured by immunofluorescence and T. gondii B1 DNA qRT-PCR (Fig. 1E and Fig. S1B). The induction of ISGs and IL12 was also significantly decreased in STING deficient iBMDM, although the induction of these cytokines in wild type iBMDM was not as robust as that in wild type primary peritoneal macrophages (Fig. 1F).

As cGAS is a cytoplasmic DNA sensor that functions upstream of STING (39), we reasoned that cGAS might also be involved in anti-T. gondii responses by sensing the cytoplasmic DNA. We then evaluated the involvement of cGAS in anti-T. gondii immune responses. The mouse survival experiment showed that cGAS was required for anti-T. gondii responses (Fig. 1G). In addition to DNA, RNA from the invading pathogen can also trigger innate immunity of the host. RLRs recognize foreign RNA in the cytoplasm and activate adaptor protein MAVS. To this end, we infected MAVS deficient mice, whose cytoplasmic RNA sensing pathway was blocked, or wild type mice with T. gondii. The survival and body weight results showed that MAVS was dispensable for anti-T. gondii immune responses (Fig. S1D). Together, cGAS and STING are crucial for innate immunity against T. gondii.
GRA15 is required for mounting innate immune response during T. gondii infection

During T. gondii infection in cGAS deficient mice, interestingly, we noticed that loss of cGAS resulted in less severe symptom and delayed death than STING deficiency, indicating additional mechanism contributing to STING mediated defense exists. We next focused on revealing this additional mechanism by which T. gondii activates STING signaling. In addition to nuclear acids, proteins from pathogens are also able to regulate host immune responses (35,40). It is believed that the effector proteins secreted by T. gondii are able to modulate host immune responses (41). Dense granule proteins (GRAs) have emerged as important determinants of T. gondii pathogenesis. To determine if the T. gondii proteins affect innate immune activation, we constructed plasmids encoding various dense granule proteins of T. gondii and transfected these plasmids together with cGAS, STING and IFNβ luciferase reporter plasmids. We found that only GRA15, which was reported to regulate NFκB activation (36), was able to significantly enhance cGAS/STING mediated induction of IFNβ (Fig. 2A). We then investigated the effect of GRA15 on parasite virulence. We generated GRA15 deficient T. gondii by using CRISPR/Cas9 (Fig. 2B). In contrast to the previous finding (36), C57BL/6 mice were more susceptible to GRA15−/− T. gondii than WT T. gondii (Fig. 2C). Consistent with the survival data, mice infected with GRA15−/− T. gondii showed significantly higher parasite burden in the spleen than mice infected with WT T. gondii (Fig. 2D). Moreover, replication of GRA15−/− T. gondii significantly increased in MEFs compared to wild type parasites (Fig. S2A). Furthermore, GRA15−/− T. gondii induced less IFNβ, ISG15, CXCL10, IFNg and IL12 than wild type T. gondii did in the spleen (Fig. 2E), although the decrease of IFNβ induction was not as significant as other cytokines. Consequently, GRA15−/− parasite infection caused more severe mortality of mice. These results suggested that GRA15 is required for competent induction of innate immune responses. To further confirm this, we infected iBMDM with wild type or GRA15−/− T. gondii. qRT-PCR assay showed that the production of cytokines was severely impaired during GRA15−/− T. gondii infection compared to wild type parasite infection (Fig. 2F). To examine if GRA15 is involved in STING signaling in vivo, we infected Sting−/− mice with wild type or GRA15−/− parasite. The infection of wild type or GRA15−/− parasite resulted in similar phenotypes in Sting−/− mice (Fig. 2G). Similarly, GRA15−/− parasite failed to cause more severe toxoplasmosis in Cgas−/− mice (Fig. S2B), suggesting that GRA15 might enhance innate immunity in a cGAS/STING dependent manner.

GRA15 enhances innate immunity via STING

To strengthen the point that GRA15 facilitates immune responses in a cGAS/STING dependent way, we co-transfected GRA15 and cGAS/STING into cells and performed IFNβ reporter assay. GRA15 significantly enhanced the induction of IFNβ by STING but not by MyD88 (Fig. S3A). Strikingly, a robust induction of IFNβ was observed in the presence of cGAS/STING and GRA15, which was confirmed by qRT-PCR (Fig. S3A). It is known that GAR15 is able to activate NFkB, which also contributes to transcriptional activation of IFNβ. To this end, we knocked out RELA (also termed as p65), a subunit of NFkB (Fig. S3B), from HEK293 cells and investigated the activation of IFNβ. The activation of NFkB reporter was abrogated in RELA deficient cells (Fig. S3C). However, GRA15 was still able to synergize IFNβ induction by cGAS/STING (Fig. 3A). Consistently, infection with wild type but not GRA15−/− T. gondii enhanced activation of IFNβ and IL-12 (Fig. 3B). Accordingly, IFNβ
enhancement was also observed in RELA deficient cells (Fig. S3D). These results suggested that GRA15 also activates a NFκB independent innate immune signaling, which contributes to type I IFN enhancement. To further determine the detailed mechanism by which GRA15 enhances innate immunity, we delivered GRA15 into iBMDM with lentivirus and performed genome-wide RNA-seq analysis. GRA15 induced expression of a group of ISGs, including Ddx58, Ifih1, Ifit1, Ifit2, Ifit3, Irf1, Irf7 and Isg15 (Fig. 3C and D). Ddx58 and Ifih1 encode RIG-I and MDA5 respectively, which are important PRRs recognizing RNA viruses in cytoplasm. Irf1 and Irf7 are key transcription factors that direct transcription of type I IFNs. The rest of these ISGs are also critical for host defense against different kinds of pathogens. Consistent with the above data that GRA15 enhances cGAS/STING signaling, the RNA-seq analysis showed that GRA15 induced the expression of the genes involved in innate immune responses, defense response to virus and herpes simplex infection (Fig. 3E). Moreover, induction of these ISGs by GRA15, including GBP3, was significantly attenuated in STING deficient macrophages (Fig. 3F). Some ISGs activation was impaired in cGAS deficient macrophages (Fig. S3E), suggesting that cGAS is also important for GRA15 mediated signaling.

To gain further insight into the mechanism by which GRA15 facilitates innate immunity, we constructed a series of GRA15 truncations and examined their function. Reporter assay showed that the first 100 amino acids were essential for the induction of both IFNβ and NFκB (Fig. 3G and Fig. S3F). We then analyzed these amino acids and found two transmembrane motifs within the first 100 amino acids (Fig. S3G). However, the role of these transmembrane motifs has not been characterized (42). We deleted these two transmembrane motifs and examined their function in activating IFNβ. We found that the first transmembrane motif was dispensable for the enhancement of IFNβ. However, lacking of the second transmembrane motif almost completely blocked the synergistic effect of GRA15 on IFNβ induction (Fig. 3H). The second transmembrane motif is also required for activation of NFκB by GRA15 (Fig. S3H), although we did not observe a synergistic effect of GRA15 and STING on NFκB activation (Fig. S3F). Taken together, GRA15 enhances the innate immune responses by cGAS/STING, which relies on the second transmembrane motif.

**GRA15 anchors to the endoplasmic reticulum (ER) of host cells and activates STING**

It is believed that GRA15 is secreted into host cells during *T. gondii* infection (36). We above found that the second transmembrane motif is critical for the function of GRA15, which prompted us to investigate the distribution of GRA15 in the host cell. Surprisingly, we observed that GRA15 mainly located on ER, as assessed by immunofluorescence (Fig. 4A and Fig. S4A). To confirm this, we performed subcellular fractionation to isolate the ER and detected the localization of GRA15. Consistent with the immunofluorescence results, GRA15 exclusively presented in the fraction of ER. However, most of the GRA15 translocated into cytosol when the second transmembrane motif was deleted, suggesting this transmembrane motif is responsible for anchoring GRA15 to the ER (Fig. 4B). It is known that STING localizes on the ER, which prompted us to examine the possibility that GRA15 might interact with STING. To this end, we transfected STING and GRA15 together into cells and performed co-immunoprecipitation. The result confirmed that GRA15 could interact with STING, but not cGAS (Fig. 4C). Moreover, ER localization was critical for the interaction between STING and
GRA15 (Fig. 4D). More interestingly, GRA15 promoted the oligomerization of STING, which is believed to be the active form of STING (Fig. 4E). To strengthen this point, we stained STING in HeLa cells transfected with GRA15 or T. gondii actin. The immunofluorescence results further confirmed that GRA15 promoted the oligomerization of STING (Fig. 4F). Furthermore, the translocation of STING to the Golgi apparatus and increased recruitment of TBK1 induced by GRA15 implied that STING was further activated by GRA15 (Fig. S4B and C). Therefore, GRA15 interacts with STING and promotes its oligomerization, translocation, interaction with TBK1 to activate immune response.

**GRA15 promotes ubiquitination and activation of STING**

The previous report showed that STING could be targeted by TRIM56 for lysine 63-linked ubiquitination, which induced STING dimerization. This dimerization is a prerequisite for the recruitment of antiviral kinase TBK1 and subsequent induction of IFNβ (28). Therefore, to determine if the posttranslational modification (PTM) of STING is affected by GRA15, we overexpressed GRA15 in HEK293 cells transfected with plasmids encoding STING and purified Flag-STING by immunoprecipitation (Fig. S5A and S5B). PTM analysis of STING by mass spectrometry identified lysine 337 of STING was ubiquitinated in 293T cells expressing GRA15 (Fig. 5A and Fig. S5C). Next, we constructed a Flag-STING mutant in which lysine 337 was substituted with an alanine residue (K337A). Notably, STING K337A was not able to potentiate IFNβ-luc activation effectively. Moreover, the synergistic effect between GRA15 and STING was dampened when lysine 337 of STING is replaced with alanine (Fig. 5B). We then mutated this alanine back to lysine (K337A re), which completely rescued the function of STING (Fig. 5B). Accordingly, GRA15 promotes ubiquitination of wild type STING but not the K337A mutant (Fig. 5C). As noted above, GRA15 promoted the oligomerization of STING. We then determined if this ubiquitination is related to the oligomerization of STING. The SDD-AGE assay showed lysine 337 is required for oligomerization of STING (Fig. 5D). It is well established that STING functions via IRF3 and NFκB (26). We examined the effect of this mutation on transcription activation. The K337A mutation was able to induce NFκB activation, which is comparable to wild type STING. However, luciferase activity induction of P561, which is believed as an IRF3 specific activating element (43), was significantly impaired by this mutant (Fig. 5E). Therefore, GRA15 can promote lysine 337 ubiquitination of STING, leading to oligomerization and activation. The activated STING stimulates downstream signals via IRF3.

**GRA15 relies on TRAFs to activate STING**

In order to more clearly understand GRA15-related immunity and to identify GRA15 interacting factors, we overexpressed Flag-GRA15 in HeLa cells and purified Flag-GRA15 by immunoprecipitation. Mass spectrometry analysis identified TRAF2 and TRAF6 as GRA15 interacting proteins (Fig. S6A). The interactions were confirmed by coimmunoprecipitation (Fig. S6B). To further evaluate the importance of TRAF2 and TRAF6, we knocked out TRAF2/TRAF6, we knocked out TRAF2/TRAF6 separately or together in HEK293 cells by using CRISPR/Cas9. NFκB activation was abrogated in TRAF6 deficient cells and decreased in TRAF2 deficient cells (Fig. S6C). However, we observed that GRA15 was still able to synergize with cGAS and STING in TRAF6−/−, TRAF2−/− or TRAF2/6−/− cells (Fig. 6A and Fig. S6D). This result suggests again there is a NFκB independent way that mediated GRA15/cGAS/STING immune response.
It is known that TRAF proteins form heterodimers and function redundantly (44). We thus did not observe complete abolishment of IFNβ enhancement by GRA15 in TRAF2 and TRAF6 deficient cells. To this end, we performed this experiment with TRAF2, TRAF3, TRAF5 and TRAF6 four genes deficient cells (TRAFs−/−). The results showed that GRA15 was not able to enhance IFNβ induction by cGAS/STING without TRAF proteins (Fig. 6B and 6C). Accordingly, the oligomerization of STING induced by GRA15 was abolished in TRAFs−/− 293T cells (Fig. 6D). In line with this, GRA15 was not able to interact with STING in TRAFs deficient cells (Fig. 6E), suggesting that TRAFs bridged the interaction between GRA15 and STING. To this end, we performed yeast two hybridization to confirm the interaction among these proteins. GRA15 directly binds to TRAF2 or TRAF6, but not STING (Fig. S6E). In line with that, the coimmunoprecipitation results indicated that TRAF2, 5, 6 could bind to STING significantly, while the interaction between TRAF3 and STING was much weaker (Fig. 6F). Accordingly, TRAF2 and TRAF6 can be recruited to ER by GRA15 (Fig. S6F). Taken together, TRAF proteins play a vital role in cGAS/STING/GRA15 mediated immune response by bridging the interaction between GRA15 and STING.

Discussion

*T. gondii* is an important intracellular pathogen that usually establishes latent infection in nervous system. As an intracellular pathogen, its cytoplasmic PRR has not been well characterized yet. We here identified that cGAS participates in anti-parasite immune responses during *T. gondii* infection. The source of the dsDNA activating cGAS, *T. gondii*-derived or host-derived, remains to be elucidated. ME49 used in this study is a type II strain of *T. gondii*, one of canonical strains. Different from the atypical *T. gondii* strains (23), canonical stains could not activate RLR signaling pathway to induce IFNβ production. MAVS deficient mice showed similar symptoms as wild type mice after challenge with *T. gondii* ME49 (Fig. S1D). In addition to cGAS, other cytoplasmic dsDNA sensors including DDX41, IFI16, DAI, IFI204 and AIM2 (45-48) may compensate for loss of cGAS or also function in activating immune response against *T. gondii*, which could explain the fact that the more mild phenotype for cGAS deficient mice compared to STING deficient mice. It will be interesting to investigate the function of these receptors in host defense against *T. gondii*.

After recognizing dsDNA, cGAS activates STING and downstream signaling. In addition to the signals from cGAS, *T. gondii* dense granule protein GRA15 promotes activation of STING in a TRAF proteins dependent way. We confirmed the direct interaction between TRAF2 or TRAF6 and GRA15 with coimmunoprecipitation and yeast two hybridization. However, we still observed enhancement of type I interferon induction by GRA15 in TRAF2 deficient, TRAF6 deficient or TRAF2 and TRAF6 double knockout cells. This enhancement is completely abolished in TRAF2, TRAF3, TRAF5 and TRAF6 four genes knockout cells. TRAF2/TRAF5 and TRAF6 are believed to play important roles in NFκB activation. It is known that GRA15 activates NFκB via TRAF6. TRAF6 deficiency abrogates NFκB activation but not IFNβ enhancement by GRA15. GRA15 activates IFNβ production, at least partially, in a NFκB independent way. Consistently, we still observed IFNβ upregulation by GRA15 in p65 deficient cells. On the other hand, TRAF3 is believed to play a role in activation of TBK1 and IRF3/IRF7. Deficiency of TRAF3 could not block the enhancement of IFNβ induction by GRA15 either. Each TRAF protein seems to be required for this enhancement of IFNβ induction by GRA15 (Fig. 6). Therefore, these data suggested that
GRA15 activated both NFκB and IRFs pathways. Our results suggested that STING activated by GRA15 mainly depends on IRF3 to induce target genes expression (Fig. 5E).

Consistent with a previous study (23), we did not observe type I IFN induction in iBMDM during *T. gondii* ME49 infection (Fig. S1C). However, IFNβ was significantly induced in peritoneal macrophages by *T. gondii* ME49 infection (Fig. 1D). Moreover, the induction of IL12, ISG15 and CXCL10 was also more significant in peritoneal macrophages than in iBMDM during *T. gondii* ME49 infection (Fig. 1D and 1F). We also observed type I IFN induction in mouse spleen during *T. gondii* infection (Fig. 1C and Fig. 2E). These results suggested that different types of cells develop different strategies to counteract *T. gondii* evasion. Therefore, *T. gondii* induces type I IFN in a cell type-dependent manner. Although we here found that type I IFNs and ISGs are upregulated via cGAS/STING in vitro and in vivo. The importance of type I IFNs in controlling *T. gondii* infection is still controversial (49,50). Sailen Barik group showed that induction of ISGs by IRF3 promotes replication of *T. gondii* when they infected mice with tachyzoite of type I *T. gondii* (RH strain) (51). Ellen A. Robey and colleagues found type I IFN is required for defense against *T. gondii* when they challenged mice with cysts of type II *T. gondii* (Prugniaud strain) (24). In the present study, we used type II *T. gondii* (ME49 strain) and observed that cGAS/STING/IRF3 axis is required for inhibiting the infection of this parasite.

We here found that GRA15 promoted ubiquitination of STING at lysine 337 in wild type cells but not in TRAFs deficient cells. TRAF proteins are known as Ring finger domain containing ubiquitin E3 ligase (52). Further studies should be done to clarify the mechanistic details of which TRAFs act with GRA15 to direct ubiquitination of STING, and which type of polyubiquitin has been attached to STING. Our yeast two hybridization assay showed that GRA15 directly interacted with TRAF2 and TRAF6 but not STING. Moreover, there was significant interaction between TRAF proteins and STING (Fig. 6). These results suggested that TRAF proteins function as an adaptor to bridge the association between GAR15 and STING. In line with this, we observed coimmunoprecipitation of GRA15 and STING in wild type cells but not in TRAFs deficient cell. However, the mechanism by which GRA15 activates TRAF proteins is unknown.

GRA15 is a secreted dense granule protein of *T. gondii*. Interestingly, we here found that the second transmembrane motif of GRA15 directed its localization on ER. The ER localization is essential for its activity to potentiate innate immune responses, including NFκB activation and type I IFN induction. Our immunofluorescence results suggested that GRA15 could recruit TRAF2 and TRAF6 to ER (Fig. S6F), allowing their engagement with STING. However, it is puzzling why ER localization is critical for GRA15 to activate NFκB by TRAF6.

In conclusion, we demonstrated that *T. gondii*-derived protein GRA15 promotes STING polyubiquitination at lys-337 to activate STING via TRAF proteins, which in turn enhances DNA-cGAS/STING pathway for host defense against *T. gondii* infection (Fig. S7). The ER-localized GRA15 enhances activation of innate immune signaling to suppress the propagation of *T. gondii*, which might be needed for moderate replication of the parasite and establishment of latent infection in host cells. Because excessive *T. gondii* replication would kill hosts before cysts formation. The present study found a cytoplasmic innate immune signaling modulator derived from invading microbe, and shed new insights on how a microbial protein modulates host immune activation.
Experimental procedures

Viruses, growth of T. gondii

VSV (Vesicular Stomatitis Virus, Indiana strain) was a gift from J. Rose (Yale University).

All experiments in this paper used the T. gondii ME49 strain, a type II strain provided by Dr. Yang Zhao (Yale University). T. gondii stain was grown in HFF cells.

Mice and T. gondii infection

All animals care and use adhered to the Guide for the Care and Use of Laboratory Animals of the Chinese Association for Laboratory Animal Science. All procedures of animal handling were approved by the Animal Care and Use Committee of Peking University Health Science Center (permit number LA 2016240). Sting<sup>gt/gt</sup> mice and Mavs<sup>−/−</sup> mice have been described previously (53). Wild type B6 mice were purchased from Department of Laboratory Animal Science of Peking University Health Science Center, Beijing. Mice were kept and bred in pathogen-free conditions.

Age- and sex-matched C57BL/6 littermates were produced and used in all the in vivo experiments. T. gondii was purified by filtration through 5 μm Syringe Filters and suspended in phosphate-buffered saline, counted in a hemocytometer under microscope. Six-week-old mice were infected with 10<sup>5</sup> wild type T. gondii or GRA15<sup>−/−</sup> T. gondii per mouse by intraperitoneal injection. For cytokine studies and T. gondii genome copy number measurement, mice were sacrificed at day 5 post-infection and spleens were harvested.

Cells

iBMDM cells were a gift from Dr. Feng Shao (National Institute of Biological Sciences, Beijing). Human foreskin fibroblast (HFF) cells were given by Dr. Guang Yang (Jinan University). HEK293 were purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 100U/ml Penicillin-Streptomycin.

 Luciferase reporter analysis

HEK293 cells seeded on 24-well plates were transiently transfected with 50ng of the luciferase reporter plasmid together with a total of equal amount of various expression plasmids or empty control plasmids. Then, 24 hours later, reporter gene activity was analyzed using the Dual-Luciferase Reporter 1000 Assay System (Promega) and measured with a TD-20/20 Luminometer (Turner Designs) according to the manufactures’ instruction.

 Regular reverse transcription PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR)

Total RNA from cells and homogenized tissues were isolated using the RNA simple Total RNA kit (TIANGEN). 1 μg RNA was reverse transcribed using a FastKing RT Kit (TIANGEN). Levels of the indicated genes were analyzed by RT-PCR for 28–35 cycles at 94°C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 20 seconds, or analyzed by qRT-PCR amplified using SYBR Green (Transgene). Data shown are the relative abundance of the indicated mRNA normalized to GAPDH or Tubulin. The primers were list in Table S1.

 Coimmunoprecipitation

HEK293 cells seeded on 10-cm<sup>2</sup> dishes (1 × 10<sup>7</sup> cells/dish) were transfected with a total of 10 μg of empty plasmid or various expression plasmids. At 24-36 h after transfection, cells were lysed in lysis buffer (0.5% Triton-X-100, 20 mM HEPES (PH 7.4), 150mM NaCl, 12.5 mM β-glycerolphosphate, 1.5 mM MgCl2, 2 mM EGTA, 10 mM NaF, 1 mM Na3VO4, 2 mM DTT) containing protease inhibitors. Lysates were
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centrifuged and incubated with anti-Flag antibodies at 4°C overnight. The next day, prewashed protein A/G beads (Pierce) were added and incubated at 4°C for 4 hours. The beads were washed with cold PBS 4 times and eluted with DTT-containing SDS sample buffer by boiling for 10 minutes for western blotting.

**Immunofluorescent microscopy**

HeLa and MEF cells on coverslips were washed once with pre-warmed phosphate buffered saline (PBS) and fixed in 4% (wt/vol) paraformaldehyde for 15 minutes. After three washes in PBS, cells were permeabilized with 0.2% (vol/vol) Triton X-100 for 5 minutes. After three washes in PBS, cells were blocked in PBS containing 5% (wt/vol) bovine serum albumin (BSA) for 30 minutes, and incubated with indicated antibodies in PBS containing 3% (wt/vol) BSA for 2 hours at 37°C. After three washes, cells were incubated with Alexa Fluor 488-conjugated secondary antibodies or Alexa Fluor 555-conjugated secondary antibodies for 1 hour at 37°C, and then with DAPI (4',6-Diamidino-2-phenylindole, Roche) for 15 minutes. The coverslips were washed extensively and mounted onto slides. Imaging of the cells was carried out using N-STORM5.0 (Nikon) microscope under a × 100 oil objective.

**CRISPR/Cas9 system**

Sting⁻/⁻, Cgas⁻/⁻ iBMDM and STING⁻/⁻, TRAF2⁻/⁻, TRAF6⁻/⁻, TRAF3⁻/⁻, TRAF5⁻/⁻, TRAF2/6⁻/⁻, p65⁻/⁻ HEK293 cells were constructed by CRISPR/Cas9 system. Specific guide RNA was ligated into the BsmB1 restriction site of the inducible lentiviral vector (lentiGuide-Puro). Lentivirus particles were produced by co-transfected HEK293 cells with guide RNA plasmids (2 μg), packaging plasmids pCMV-VSV-G (800 ng, AddGene 8454) and psPAX2 (800 ng, AddGene 12260). The medium was changed to fresh DMEM containing 10% FBS at 12 hours post transfection and viral supernatant was collected at 48-72 hours. Then, a total of 1×10⁵ iBMDMs or HEK293 cells were infected with 2 ml viral supernatant supplemented with 8 μg/ml polybrene and incubated for 48 hours. Possible knockout cells were screened by puromycin (1 μg) and each monoclonal was confirmed by sequencing. Cells were negative for mycoplasma.

HEK293 cells were transfected by standard calcium phosphate precipitation method. HeLa cells were transfected by lipofectamine 2000 (Invitrogen) according to procedures recommended.

GRA15⁺/⁺ T. gondii were constructed as previously described (54). The high efficiency gRNA was designed on http://grna.ctegd.uga.edu/. The forward primer is: GAAGCGACTTCTAAACACGTGGTTTTAGAGCTAGAAATAGC, reverse primer is: AACTTGACATCCCCATTAC. Then, the GRA15 gRNA replaced the target gRNA of parental plasmid pDHFR-SAG1::Cas9-U6::sgUPRT using the Q5® Site-Directed Mutagenesis Kit (New England BioLabs, E0552S). Next for transfection and selection of the mutated parasites, T. gondii were nucleofected with 10 μg of circular pDHFR-SAG1::Cas9-U6::sgGRA15 plasmid using the Basic Parasite Nucleofector® Kit 1 (Lonza, Basel, Switzerland) according to the manufacturer’s instructions. Briefly, 10⁶ to 10⁷ T. gondii were suspended in 100 μl of nucleofection buffer (82 μl Basic Parasite Nucleofector® solution 1 supplemented with 18 μl Supplement P1). The 100 μl of the parasite suspension was mixed with DNA solution and transferred to a cuvette (provided as a component of the kit). T. gondii were nucleofected using program U-033 and were immediately inoculated into confluent HFF cells. After two days post nucleofection, T. gondii were selected by 1μM pyrimethamine in the 10% FBS DMEM. The guiding RNAs were listed in Table S2.
Antibodies

The following antibodies were used: anti-HA (H3663), anti-Tubulin (AF7011); and anti-Flag (mouse, M183-3L), anti-Flag (rabbit, PM020), anti-Lamin B1 (66095) was from Proteintech. The antibodies were diluted 1,000 times for immunoblots, 200 times in immunofluorescence. The mouse anti-GRA15 polyclonal antibodies were raised against bacterial pET-28a (+)-GRA15 (full length) protein.

Mass spectrometry

To identify proteins that interacts with Flag-GRA15 and posttranslational modification of Flag-STING, samples pulled down with Flag antibody were send for mass spectrometry.

Plasmid constructs

The plasmids coding human STING, MAVS have been described previously(53). Genes of T. gondii, including GRA15, were amplified by PCR and were cloned into pCMV7.1 plasmids or pcDNA3.1 plasmids to generate Flag-tagged or His-tagged expression constructs.

RNA seq

We harvested iBMDM overexpressing GRA15 and control cells, and purified whole RNA by using RNeasy Mini Kit (Qiagen No. 74104). The transcriptome library for sequencing was generated using VAHTSTM mRNA-seq v2 Library Prep Kit for Illumina® (Vazyme Biotech Co., Ltd, Nanjing, China) following the manufacturer’s recommendations. After clustering, the libraries were sequenced on IlluminaHiseq X Ten platform using (2 × 150 bp) paired-end module. The raw images were transformed into raw reads by base calling using CASAVA (http://www. illumina.com/ support/documentation.ilmn). Then, raw reads in a fastq format were first processed using in-house perl scripts. Clean reads were obtained by removing reads with adapters, reads in which unknown bases were more than 5% and low quality reads (the percentage of low quality bases was over 50% in a read, we defined the low quality base to be the base whose sequencing quality was no more than 10). At the same time, Q20, Q30, GC content of the clean data were calculated (Vazyme Biotech Co., Ltd, Nanjing, China). The original data of the RNA-seq was uploaded to the GEO DataSets.

Semidenaturing Detergent Agarose Gel Electrophoresis (SDD-AGE)

Cells from six-well plate were lysed with 100 μl pre-cold lysis buffer (0.5% Triton-X-100, 50 mM Tris, 150 mM NaCl, 10% Glycerol, proteinase cocktail and phosphatase cocktail) on ice for 40 minutes. Supernatants were collected by centrifugation at 13,000 rpm for 10 minutes at 4 ℃. Protein loading buffer were added into supernatants and loaded to the gel, performed vertical Agarose electrophoresis for 35 minutes with a constant voltage of 100 V on ice, and followed standard Western blotting procedure.

Statistical Analysis

RNA-seq data was analyzed using Enrichr (http://amp.pharm.mssm.edu/Enrichr). For all the scatter plots, data were expressed as mean ± SD. Prism 7 software (graphic software) was used for survival curves, charts and statistical analyses. Survival curves were analyzed using a log-rank (mantel-cox) test. For the in vitro results and animal results, a standard two-tailed unpaired Student’s t-test was used. The results with p value < 0.05 were considered significant. The sample sizes (biological repeats), specific statistical tests used, and the main effects of our statistical analyses for each experiment are detailed in each Figure legend. All attempt at replication were successful. The investigators were blinded to group allocation during data collection and analyses.
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**Conflict of Interests:** The authors declare that they have no conflicts of interest with the contents of this article.

**Author Contributions:** F.Y. designed the study, analyzed the data and wrote or revised the paper. PY.W., S.L., Y.Z., S.L., H.D., L.C., X.Y. B.Z., P.L. performed most experiments. P.W. analyzed the data. F.S. provided technical support expertise. G.Y. provided technical help and expertise and contributed to experiment with *T. gondii* infection.
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FOOTNOTES
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**Figure 1. Sting<sup>gt/gt</sup> mice is susceptible to T. gondii infection.**

A, C57BL/6 wild type (WT, black lines, n=6) and Sting<sup>gt/gt</sup> (red lines, n=8) mice were infected with 10<sup>5</sup> T. gondii tachyzoites per mouse, survival and body weight were monitored. B, Mice (n=4) were infected with T. gondii as in panel A, spleens from live animals were harvested 5 days post-infection, followed by DNA isolation. Parasite growth was quantified by qRT-PCR of TgB1 primers and normalized against Tubulin. C, RNA was isolated after spleens were harvested as in panel B. Ifng, Il12a, Ifnb1, Isg15 mRNA was quantified by qRT-PCR. D, qRT-PCR analysis of Il12a, Ifnb1, Isg15, Cxcl10 in wild type or Sting<sup>gt/gt</sup> peritoneal macrophages after 12h of T. gondii infection. E, Parasite growth was qualified by qRT-PCR in wild type or Sting<sup>gt/gt</sup> iBMDM cells after 24h of T. gondii infection. F, qRT-PCR analysis of Il12a, Isg15, Cxcl10 mRNA in wild type or
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Sting<sup>-/-</sup> iBMDM cells after 24h of *T. gondii* infection. G, C57BL/6 wild type (WT, black lines, n=6) and *Cgas<sup>-/-</sup>* (red lines, n=7) mice were infected with 10<sup>5</sup> *T. gondii* tachyzoites per mouse, survival was monitored. Statistical analysis was performed by log rank test for (A, G) and *t* test for (B - F). Data were from three independent experiments. NS, not significant; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Error bars, S.D.
Figure 2. GRA15 is vital for T. gondii-stimulated immune response. A, In HEK293 cells overexpressing cGAS and STING, luciferase activity of IFNβ-luc was measured after transfection of plasmids encoding different genes of T. gondii. B, GRA15−/− T. gondii was constructed by CRISPR/Cas9.
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Mutation was checked by DNA sequencing of GRA15. Total infected cell proteins at 48 hours post-infection were analyzed by immunoblotting to detect *T. gondii* GRA15 and SAG1 proteins. C, Wild type mice were infected with $10^5$ wild type (black lines, n=6) or GRA15$^{-/-}$ (red lines, n=6) *T. gondii* tachyzoites per mouse, survival and body weight were monitored. D, Mice (n=3) were infected with *T. gondii* as in panel C, spleens from live animals were harvested 5-days post-infection, followed by DNA isolation. Parasite growth was quantified by qPCR of TgB1 primers and normalized against Tubulin. E, RNA was isolated after spleens were harvested as in panel D. *Il12a, Ifng, Ifnb1, Isg15, Cxcl10* mRNA was quantified by qRT-PCR. F, qRT-PCR analysis of *Il12a, Isg15, Cxcl10* mRNA in wild type iBMDM cells after 24h of wild type or GRA15$^{-/-}$ *T. gondii* infection. G, C57BL/6 *Sting$^{gt/gt}$* mice were infected with $10^5$ wild type (black lines, n=8) or GRA15$^{-/-}$ (red lines, n=8) *T. gondii* tachyzoites, survival and body weight were monitored. Statistical analysis was performed by log rank test for (C, G) and *t* test for (A, D - F). Data were from three independent experiments. NS, not significant; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Error bars, S.D.
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Figure 3. GRA15 enhances STING mediated anti-Toxoplasma gondii immunity. A, Luciferase activity of IFNβ-luc in p65−/− HEK293 cells 24h after transfection with indicated reporter plasmids and empty vector (Vec) or GRA15. B, Luciferase activity of IFNβ-luc, IL12-luc were measured 24h after wild type or GRA15−/− T. gondii infection in HEK293 cells overexpressing cGAS and STING for 24h. C,
GRA15 enhances STING activity

Selected activated genes in iBMDM cells overexpressing GRA15. D, RT-PCR experiments showing the expression of some ISGs, IRG and GBP was increased in iBMDM cells overexpressing GRA15. E, Histogram of the combined score (https://david.nei.gov/tools.jsp) for biological processes (up) and pathway (down) for the activated genes in iBMDM cells overexpressing GRA15. F, qRT-PCR experiments showing some genes were activated in wild type (WT) iBMDM cells overexpressing GRA15 but not in Sting−/− iBMDM cells. G, Luciferase activity of IFNβ-luc was measured in HEK293 cells overexpressing FL (GRA15 full length) and different deletions of GRA15 with STING. H, Luciferase assay of IFNβ-luc of HEK293 cells transfected with plasmids encoding empty vector (Vec), GRA15 full length (GRA15 FL), GRA15 without transmembrane domain 1 (GRA15 Δmem1) or GRA15 without transmembrane domain 2 (GRA15 Δmem2) with cGAS and STING. Statistical analysis was performed by t test for (A, B, F - H). Data were from three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001. Error bars, S.D.
Figure 4. GRA15 located on ER to activate immune response. A, Immortalized MEF cells were infected by *T. gondii*, and at 24h post-infection stained for nucleus (DAPI, blue), *T. gondii* (green), GRA15 (red) and endoplasmic reticulum (purple) and visualized by confocal microscopy. The scale bar length is 10 μm. B, HEK293 cells were transfected with plasmids encoding GRA15 FL or GRA15 Δmem2, following immunoblot analysis of whole cell lysis (WCL), isolated ER and cytosol. C, Immunoblot analysis of HEK293 cells transfected plasmids encoding His-GRA15, Flag-STING or Flag-cGAS, lysed and immunoprecipitated with anti-Flag. Whole cell lysates (WCL) were immunoblotted with antibodies to indicated proteins (* indicates non-specific band). D, Immunoblot analysis of HEK293 cells transfected plasmids encoding His-GRA15 ΔMEM2, Flag-STING, lysed and immunoprecipitated with anti-Flag. Whole cell lysates (WCL) were immunoblotted with antibodies to indicated proteins. E, SDD-AGE and SDS-PAGE of HEK293 cells expressing Flag-STING and His-GRA15. F, 2fTGH cells were transfected with plasmids encoding Flag-GRA15 or Flag-TgActin, and at 24h stained for STING (green) and Flag (red) and visualized by confocal microscopy. The scale bar length is 10 μm.
Figure 5. GRA15 promotes activation of STING. A, Diagram detailing STING domains (CBD and CTT) and ubiquitinated site. B, Left: Luciferase assay of IFNβ-luc in STING−/− HEK293 cells 24h after transfection with plasmids encoding wild type (WT), mutant STING (K337A) or STING recover (K337A re). Middle: Luciferase assay as left in STING−/− HEK293 cells overexpressing GRA15. Right: Immunoblot analysis of HEK293 cells transfected with plasmids encoding empty vector (Vec), wild type (WT), mutant STING (K337A) or STING recover (K337A re). C, Immunoblot analysis of HEK293 cells transfected plasmids encoding HA-ub, His-GRA15, Flag-STING wild type (WT) or Flag-STING mutant (K337A), lysed and immunoprecipitated with anti-Flag. Whole cell lysates (WCL) were immunoblotted with antibodies to indicated proteins. D, SDD-AGE and SDS-PAGE of STING−/− HEK293 cells expressing wild type STING, STING K337A, Flag-GRA15 or Flag-TgActin. E, Luciferase assay of NFκB-luc and p561-luc in STING−/− HEK293 cells 24h after transfection with plasmids encoding wild type or mutant
STING(K337A). Statistical analysis was performed by t test for (B, E). Data were from three independent experiments. *P < 0.05; **P < 0.01. Error bars, S.D.
Figure 6. GRA15 depends on TRAFs to activate STING. A, qRT-PCR analysis of IFNB1 mRNA in wild type, TRAF6<sup>−/−</sup>, TRAF2<sup>−/−</sup> HEK293 cells overexpressing GRA15, cGAS and STING. B, qRT-PCR analysis of IFNB1 mRNA in TRAFs<sup>−/−</sup> HEK293 cells overexpressing GRA15, cGAS and STING. C, Enhancement of IFNβ-luc activity in TRAF2<sup>−/−</sup>, TRAF6<sup>−/−</sup>, TRAF2/6<sup>−/−</sup>, TRAF3<sup>−/−</sup>, TRAF5<sup>−/−</sup> or TRAFs<sup>−/−</sup> HEK293 cells overexpressing GRA15, cGAS and STING. D, SDD-AGE and SDS-PAGE of wild type or TRAFs<sup>−/−</sup> HEK293 cells overexpressing Flag-STING and His-GRA15. E, Immunoblot analysis of wild type and TRAFs<sup>−/−</sup> HEK293 cells transfected with indicated plasmids, lysed and immunoprecipitated with anti-Flag antibody. Whole cell lysates (WCL) were immunoblotted with antibodies to indicated proteins. F, Immunoblot analysis of HEK293 cells transfected with plasmids encoding HA-STING, Flag-TRAF2, Flag-TRAF3, Flag-TRAF5 or Flag-TRAF6, lysed and immunoprecipitated with anti-Flag antibody. Whole cell lysates (WCL) were immunoblotted with antibodies to indicated proteins. Statistical analysis was performed by t test for (A - C). Data were from three independent experiments. NS, not significant; *P < 0.05; **P < 0.01; ***P < 0.001. Error bars, S.D.
The GRA15 protein from Toxoplasma gondii enhances host defense responses by activating the interferon stimulator STING
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