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Immune Evasion Mechanisms of the Zoonotic Protozoan Parasite *Toxoplasma Gondii* in Mammalian Hosts

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Abstract

Toxoplasma gondii is a zoonotic protozoan pathogen that causes toxoplasmosis, an infectious disease that affects most mammals, including domestic animals, wild animals, and humans. Toxoplasmosis in domestic animals causes miscarriages or stillbirths, resulting in economic losses and posing a challenge in animal husbandry. *T. gondii* is thus an important pathogen that causes serious animal and public health issues, yet there is still no vaccine or preventative medicine. Therefore, efforts to develop novel treatments for toxoplasmosis and to understand the interaction between the host immune response and the parasite in host cells are essential. We know that interferon-γ (IFN-γ)-induced tryptophan degradation by indole-2,3-dioxygenase (IDO1) plays an important role in the IFN-γ-induced anti-*T. gondii* response. However, little is known about *T. gondii* virulence mechanisms targeting IDO1. Therefore, we focused on the *T. gondii* effector TgGRA15 and analyzed its virulence function and mechanism to antagonize the IDO1-mediated anti-*T. gondii* response. In this study, we demonstrate that inducible nitric oxide synthase is a key host factor for TgGRA15-dependent disruption of the IDO1-dependent anti-*T. gondii* response.

Introduction

Toxoplasma gondii is an obligate intracellular zoonotic protozoan parasite that causes toxoplasmosis in most mammals, including domestic animals, wild animals, and humans (Boothroyd, 2009; Dubey, 2010). The family *Felidae*, which includes domestic cats, is the definitive host of *T. gondii*. The parasite can easily spread infection through the accidental swallowing of food or water contaminated with oocysts. Accordingly, *T. gondii* is prevalent in most areas of the world (Montazeri *et al*., 2020). Toxoplasmosis in humans and domestic animals can cause congenital disease, miscarriages and stillbirths, leading to not only problems of animal hygiene and public health, but also economic losses to farmers (Stelzer *et al*., 2019). Yet, no effective vaccine or preventive drug has yet been developed. Recently, along with increased overlap of the living space between humans, domestic animals, and wild animals, the number of cases of toxoplasmosis has been increasing annually. In fact, in 2015, we reported that the number of *T. gondii* infected-wild animals is increasing in Japan (Bando *et al*., 2015). Furthermore, *T. gondii* has been ranked among the top five human pathogens that cause life impairment and economic losses in the United States (Batz *et al*., 2012). Therefore, to develop novel therapeutic methods or medicines against *T. gondii*, basic research on the interaction between *T. gondii* and its host is essential.

The host immune resistance responses to *T. gondii* rely on innate and adaptive immunity (Lee *et al*., 2015; Ma *et al*., 2014; MacMicking, 2012). Interferon-γ (IFN-γ), which is produced by $CD4^+$ T cells and natural killer cells and stimulates cell-autonomous responses in both immune and non-immune cells, is the most important molecule for anti-*T. gondii* responses (Suzuki *et al*., 1988). IFN-γ plays a role in the activation of the STAT1 transcription factor and induction of the expression of hundreds of genes (Platanias, 2005). Some studies have shown that IFN-γ-inducible GTPases mediate parasiticidal and parasitostatic responses in mice (Taylor *et al*., 2007; Zhao *et al*., 2009; Yamamoto *et al*., 2012), whereas other recent

studies have reported that these GTPases may not play major roles in IFN-γ dependent anti-*T. gondii* responses in human cells (Ohshima *et al*., 2015; Fisch *et al*., 2019). We have shown that IFN-γ stimulates the expression of indoleamine 2,3-dioxygenase (IDO) and has an essential role in the anti-*T. gondii* responses of various human cell types (Bando *et al*., 2018b). Thus, although IFN-γ has a critical role in the anti-*T. gondii* response of both humans and mice, the IFN-γ-inducible effector mechanisms may differ between these two species.

T. gondii secretes various effector molecules, called rhoptry proteins (ROPs) and dense granule proteins (GRAs), into host cells. These effectors are frequently used to promote parasite growth in host cells (Hakimi *et al*., 2017; Hunter and Sibley, 2012), and their virulence mechanisms, function, and significance have been extensively researched in mouse models (Behnke *et al*., 2011; Etheridge *et al*., 2014; Fentress *et al*., 2010; Reese *et al*., 2011; Rosowski *et al*., 2014; Rosowski and Saeij, 2012; Steinfeldt *et al*., 2010). The *Toxoplasma* effector TgGRA15, one of the dense granule proteins, is secreted into host cells to activate the host transcription factor NF-κB in mice (Gov *et al*., 2013; Jensen *et al*., 2011; Rosowski *et al*., 2011), although it should be noted that most virulence factors suppress the host immune responses (Olias *et al*., 2016; Gay *et al*., 2016). TgGRA15-deficient *T. gondii* has been shown to promote parasite proliferation *in vivo* in mice (Jensen *et al*., 2013; Rosowski *et al*., 2011), meaning that TgGRA15 can support host survival by preventing parasite growth. Thus, the significance of TgGRA15 as a virulence factor remains unclear. In this study, we introduce the virulent mechanism of TgGRA15 targeting the IDO1-dependent anti-*T. gondii* response in human cells.

TgGRA15 promotes *T. gondii* growth when co-cultured in the presence of IFN-γ

The function of TgGRA15 as a virulence factor is unclear; therefore, to explore it in human cells, we generated TgGRA15-deficient (TgGRA15-KO) *T. gondii* by using the CRISPR/Cas9 system. Then, we tested whether TgGRA15 has an important role in the suppression of host immune responses under human cell mono-culture conditions. However, we failed to find any advantageous effect of TgGRA15 on parasite growth in various human cell lines. When *T. gondii* infects its host, the parasite preferentially infects CD11b+ cells such as monocytes, and then the infected cells are carried by the bloodstream to various organs (Courret *et al*., 2006). Several kinds of co-culture models have been established to mimic complex cell-cell interactions by using human tissue or immune cell lines, one of which is the monocyte-hepatocyte co-culture model (Frenkel and Remington, 1980). Because one of the major symptoms of toxoplasmosis is hepatitis, we developed a *T. gondii* infection model using monocyte-hepatocyte co-culture conditions. Human acute monocytic leukemia cell line THP-1 cells were infected with wild-type or TgGRA15-KO parasite, and then both the culture supernatant and infected THP-1 cells were seeded onto human hepatoma cell line Huh7 cells with or without IFN-γ. Interestingly, the parasite numbers under the TgGRA15-KO parasite-infected co-culture condition were significantly reduced compared with the wild-type parasite-infected co-culture condition. These data indicate that TgGRA15 has an advantageous effect on *T. gondii* growth under human cell co-culture conditions.

NLRP3-dependent IL-1β secretion from monocytes is essential for the pro-parasitic effect of TgGRA15 in hepatocytes

We next attempt to reveal the mechanisms of the pro-parasitic effect of TgGRA15 under co-culture conditions. First, to test whether TgGRA15 has an effect on monocytes or hepatocytes, the culture supernatants were collected from wild-type and TgGRA15-KO *T. gondii*-infected THP-1 cells, and then both the parasites and THP-1 cells were removed by filtration. The filtered culture supernatants and newly prepared wild-type or TgGRA15-KO parasites were then added to Huh7 cells with IFN-γ. Then the number of parasites in the Huh7 cells was assessed. The presence of TgGRA15 in THP-1 cells, but not Huh7 cells, led to a reduction in parasite number, suggesting that the presence of TgGRA15 in monocytes and their supernatant is essential for the pro-parasitic effect. Therefore, we next focused on the components of the supernatant from the parasite infected-THP-1 cell culture. Previous studies have reported that *T. gondii* infection induces proinflammatory cytokine IL-1β secretion from THP-1 cells in a TgGRA15-dependent manner (Gov *et al*., 2013). It has also been reported that IL-1β production in monocytes is dependent on Caspase-1 and inflammasome activation (Gov *et al*., 2013; Gov *et al*., 2017). Therefore, to test whether TgGRA15-dependent Caspase-1 and inflammasome activation are important for IL-1β secretion from monocytes, we generated NLRP3-deficient (NLRP3-KO) or Caspase-1-deficient (CASP1-KO) THP-1 cells by using CRISPR/Cas9 systems, and then analyzed IL-1β secretion levels in the culture supernatant. We found that both NLRP3-KO- and CASP1-KO-infected THP-1 cells showed significantly reduced IL-1β secretion. Then, we examined whether IL-1β secretion in THP-1 cells is essential for suppressing the IFN-γ-dependent anti-*T. gondii* response under co-culture conditions. We found that the parasite number in both wild-type parasite-infected NLRP3-KO and CASP1-KO THP-1 cells was significantly reduced compared with that of wild-type THP-1 cells. These results indicate that IL-1β secretion through Caspase-1 and NLRP3 inflammasome activation in THP-1 cells has an important role in the TgGRA15-dependent suppression of the IFN-γ-dependent anti-*T. gondii* response.

The IFN-γ-induced IDO1-dependent anti-*T. gondii* response is downregulated by TgGRA15 in hepatocytes

We previously reported that IDO1-induced tryptophan degradation has an important role in the IFN-γ-dependent anti-*T. gondii* response in various human cell types including hepatocytes (Bando *et al*., 2018b; Bando *et al*., 2019) because tryptophan is an essential amino acid for parasite growth. In fact, we found that the IFN-γ-dependent reduction in parasite numbers in IDO1-deficient (IDO1-KO) Huh7 cells was abolished under TgGRA15-KO parasite-infected co-culture conditions. Therefore, we examined whether IL-1β affects IDO1 expression in Huh7 cells. We found that IL-

1β and IFN-γ co-stimulation severely inhibited IDO1 mRNA and protein levels in Huh7 cells. Then, to examine whether IL-1β-dependent impairment of the IFN-γ-dependent anti-*T. gondii* response was IDO1-dependent, we generated MyD88-deficient (MyD88-KO)— MyD88 is essential molecule for the IL-1 receptor signaling pathway (Adachi *et al*., 1998)—and IL1R1-deficient (IL1R1-KO) Huh7 cells by using CRISPR/Cas9 systems. We found that the pro-parasitic effect of IL-1β in IDO1-KO, MyD88-KO, and IL1R1-KO Huh7 cells was completely abolished. Then, we compared the protein levels of IDO1 under wild-type *T. gondii*- and TgGRA15-KO parasite-infected and non-infected co-culture conditions. We found that the protein levels of IDO1 were significantly reduced under wild-type parasite-infected conditions compared with non-infected conditions. Importantly, the protein levels of IDO1 under TgGRA15-KO parasite-infected conditions recovered to the same levels as those seen under non-infected conditions. These results indicate that the TgGAR15-induced IL-1β-dependent downregulation of IDO1 expression is important for the impairment of the IFN-γ-dependent anti-*T. gondii* response in hepatocytes.

iNOS is essential for TgGRA15-dependent inhibition of the IDO1-dependent anti-*T. gondii* response

Nitric oxide (NO) production is known to strongly downregulate IDO activity transcriptionally, translationally, and post-translationally (Thomas *et al*., 1994). In addition, inducible nitric oxide synthase (iNOS) has been shown to be an important factor for IFN-γ-mediated NO production (Nathan and Xie, 1994). Hence to explain the mechanism of the IL-1β-dependent IDO1 suppression, we focused on iNOS and NO-dependent downregulation of IDO1 activity in hepatocytes. First, we examined the expression level of iNOS mRNA in Huh7 cells. We found that IFN-γ and IL-1β co-stimulation enhanced the expression level of iNOS mRNA and strongly induced NO production in Huh7 cells. Then, to examine the role of iNOS, we generated iNOS-deficient (iNOS-KO) Huh7 cells by using the CRISPR/Cas9 system. We found that NO was not produced from iNOS-KO Huh7 cells upon IFN-γ and IL-1β co-stimulation and that IL-1β-dependent reduction of IDO1 protein levels did not occur in iNOS-KO Huh7 cells. Furthermore, the IL-1β-dependent pro-parasitic effect was completely abolished in the iNOS-KO Huh7 cells under mono-culture conditions, suggesting that IL-1β induces iNOS expression to inhibit the IDO1-dependent anti-*T. gondii* response. Then, we tested whether this mechanism occurs under co-culture conditions. We found that NO production and the reduction of IDO1 was not observed in iNOS-KO Huh7 cells co-cultured with wild-type parasite-infected wild-type THP-1 cells. Moreover, the TgGRA15-dependent pro-parasitic effect was abolished in iNOS-KO Huh7 cells under these co-culture conditions. Finally, we confirmed the GRA15-dependent virulence mechanism in primary human cells. Taken together, our results indicate that iNOS is an essential host factor for the TgGRA15-dependent virulence mechanism under monocyte-hepatocyte co-culture conditions.

Conclusion

In summary, here we showed that IL-1β is produced from monocytes in a *Toxoplasma* effector TgGRA15- and host NLRP3 inflammasome-dependent manner. We further showed that iNOS has an essential role in the *Toxoplasma* TgGRA15-dependent inhibition of the IDO1-induced anti-*T gondii* response in human cells (Bando *et al*., 2018a). Although immune responses in humans and domestic animals are not identical (Guzman and Montoya, 2018), the tryptophan-degrading enzyme IDO and nitric oxide synthases NOS have been found in most mammalian species (Yao *et al*., 2011). Hence, the TgGRA15-dependent virulence mechanism may contribute to *T. gondii* infection in not only humans but also domestic animals. Studies to examine whether the TgGRA15-dependent virulence mechanism has an important role in *T. gondii* infection of domestic animals, and to identify chemical compounds that block iNOS expression or NO production could contribute to the development of novel anti-toxoplasmosis therapies for humans and domestic animals.

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