RESEARCH ARTICLE

Insights on the mechanisms of the protective immunity in the brain from the studies on infection with an intracellular microorganism, *Toxoplasma gondii*

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Abstract

The immune system operates the protection against infections by selecting efficient pathways depending on the pathogen. *Toxoplasma gondii*, an obligate intracellular protozoan parasite, has two lifecycle stages, tachyzoite and cyst, in intermediate hosts including humans. Tachyzoite is the acute stage form that quickly proliferates within host cells. Cyst is the chronic stage form that can slowly grow into more than 100 μm in diameter by containing hundreds to thousands of bradyzoites. Our studies on the IFN-γ-mediated protective immunity against cerebral tachyzoite growth revealed that IFN-γ production by brain-resident cells is not only required for upregulation of the innate protective immunity to limit cerebral tachyzoite proliferation during the early stage of the tachyzoite growth but also crucial for recruiting immune T cells from the periphery and activation of the recruited T cells to ultimately prevent the tachyzoite growth. Since IFN-γ is crucial for the protective immunity against various intracellular microorganisms in the brain, it is possible that IFN-γ produced by brain-resident cells plays a key first line defense role by orchestrating both the innate and T cell-mediated protective immunity to control not only *T. gondii* but also the other intracellular pathogens. Our studies on the protective immunity against *T. gondii* cysts uncovered the capability of cytotoxic T cells to penetrate into the target in a perforin-dependent manner for its elimination. After penetrating into the target, the cytotoxic T cells secrete granzyme B, which associates with an accumulation of phagocytes to eliminate the parasite. Since the presence of tumor-infiltrating $CD8^+$ T cells in solid cancers is an indicator of positive prognosis of cancer patients, the perforin-mediated penetration of $CD8^+$ T cells and an accumulation of phagocytes could function as a powerful protective mechanism against not only *T. gondii* cysts but also targets of large mass in general such as solid cancers.

Introduction

The immune system functions under collaborations among multiple cell populations in both innate and adaptive immunity. This collaboration includes innate immune cell populations that reside in tissues and the immune cells infiltrate into the tissues from the systemic circulation. The brain has the blood brain barrier that limits an infiltration of immune cells from the systemic circulation. Although the brain has tissue-resident innate immune cell populations such as microglia, T cells are usually required for ultimately controlling cerebral infections. On the other had, overly activated immune responses could cause unwanted and potentially serious tissue damages. Therefore, well-tuned interactions between brain-resident innate immune cells and infiltrating hematopoietic immune cell populations such as T cells are required for desirable host defense in the brain.

Toxoplasma gondii is an obligate intracellular protozoan parasite that can establish chronic infection in the brain. This chronic infection is widespread in humans worldwide including developed countries such as the United States and those in Europe[(1)]. One third of human populations are estimated to be infected with this parasite[(1)]. As far as the immune system functions appropriately, the parasite stays as a latent stage (tissue cysts) during the chronic stage of infection. However, when the immune system of the infected hosts become suppressed by various causes such as an infection with HIV and immunosuppressive treatments for organ transplants, chronic *T. gondii* infection can reactivate and cause serious and potentially life-threatening toxoplasmic encephalitis in those immunocompromised individuals[(1)]. Therefore, chronic cerebral infection with this parasite provides an excellent platform to analyze how the protective immunity functions in the interactions between brain-resident cells and infiltrating immune cell populations to effectively control the pathogen.

In this review, we summarize the two crucial insights generated by our recent studies on the mechanisms of the protective immunity against cerebral *T. gondii* infection using murine models. These two vital evidence are 1) a key coordinating role of IFN-γ production by brain-resident cells, most likely microglia, to orchestrate both innate and T cell-mediated protective immunity, and 2) a perforin-mediated invasion capability of $CD8^+$ T cells into a target of large mass to initiate its eradication in collaboration with an accumulation of phagocytes.

1. IFN-γ production by brain-resident cells orchestrates both innate and T cell-mediated immunity to prevent cerebral proliferation of *T. gondii* tachyzoites

1.1. Requirement of IFN-γ production by brain-resident cells to activate the cerebral innate protective immunity

During the acute stage of infection, *T. gondii* tachyzoites invade into a variety of nucleated cells in the hosts. Following the invasion into host cells, the tachyzoites form the parasitophorous vacuole (PV), which is formed from the plasma membranes of host cells and molecules derived from the parasite, and proliferate within the PV (Figure 1). IFN-γ is required for controlling the proliferation of the tachyzoites(2). Whereas IFN-γ-mediated protective immunity is able to control the tachyzoite growth, the parasite then forms the cysts within host cells in various organs, especially the brain, heart, and skeletal muscles, and establishes a chronic infection (Figure 1). The tissue cysts have the outer layer, the cyst wall, derived from the PV, and one cyst can contain hundreds to thousands of bradyzoites within the space

surrounded by the cyst wall (Figure 1). Appropriate immune responses are required to maintain the parasite as tissue cysts and the latency of the chronic infection. A reactivation of the chronic infection in immunocompromised individuals is initiated by a rupture of tissue cysts that releases bradyzoites located within the cysts. The released bradyzoites convert to tachyzoites, followed by proliferation of the tachyzoites. We analyzed the role of IFN-γ production by brain-resident cells in the protective immunity to control reactivation of cerebral *T. gondii* infection. We utilized bone marrow (BM) chimeras to distinguish the roles of IFN-γ produced by brain-resident cells and that produced by blood-derived innate immune cells that infiltrate into the brain. We generated two different types of BM chimeras by transferring BM cells from RAG1-knockout ($RAG1^{-/-}$) mice to two different recipients, irradiated $RAG1^{-/-}$ (namely $RAG1^{-/-}\rightarrow RAG1^{-/-}$) and IFN-γ-knockout ($IFN\text{-}\gamma^{-/-}$) mice (namely $RAG1^{-/-}\rightarrow IFN\text{-}\gamma^{-/-}$). Hematopoietic cells are irradiation-sensitive, and therefore both groups of the chimeras have hematopoietic cells derived only from the BM donor $RAG1^{-/-}$ mice, which lack T and B cells but have innate immune cells such as NK cells and neutrophils that can produce IFN-γ. The only difference in the brains of these two groups of animals is the presence ($RAG1^{-/-}\rightarrow RAG1^{-/-}$) or absence ($RAG1^{-/-}\rightarrow IFN\text{-}\gamma^{-/-}$) of IFN-γ production by brain-resident cells, which are irradiation-resistant. IFN-γ is the molecule required for preventing cerebral tachyzoite growth[3].

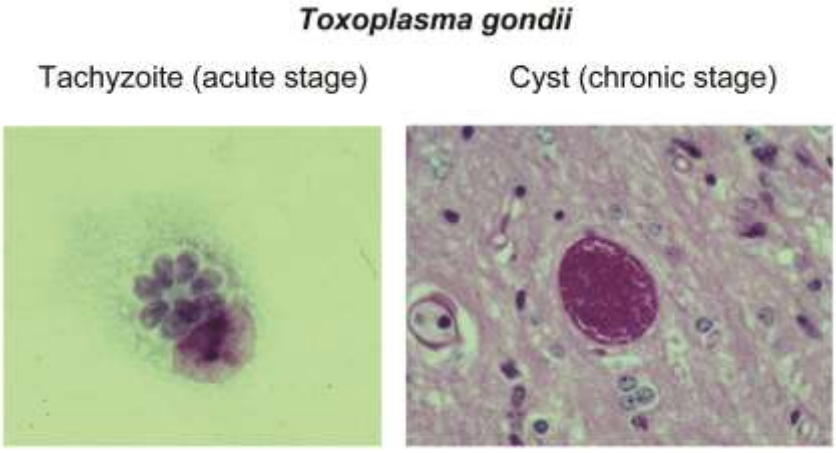

Figure 1. *T. gondii* has two different life cycle stages, tachyzoite and cyst, in intermediate hosts including humans. (A) Tachyzoites (the acute stage form) proliferating a murine macrophage (hematoxylin and eosin straining). (B) A cyst detected in the brain of a chronically infected mouse (hematoxylin and eosin staining).

Both $RAG1^{-/-}\rightarrow RAG1^{-/-}$ and $RAG1^{-/-}\rightarrow IFN\text{-}\gamma^{-/-}$ mice were infected with *T. gondii* and treated with sulfadiazine to control proliferation of tachyzoites and establish a chronic infection in their brains. Discontinuation of sulfadiazine initiates reactivation of the cerebral infection. We discovered that marked increases in cerebral expression of IFN-γ after initiation of the reactivation of infection occurs only in the $RAG1^{-/-}\rightarrow RAG1^{-/-}$ mice. Therefore, IFN-γ production by brain-resident cells is essential for upregulrating the innate IFN-γ expression in the brain (Figure 2A)[3]. On the other words, the presence of hematopoietic innate immune cells with the capability to produce IFN-γ in the periphery alone is not sufficient to

increase cerebral innate expression of IFN-γ following reactivation of the infection. Consistently, the "Reactivation index" which indicates the degree of an occurrence of reactivation of *T. gondii* infection in the brain (see the legend for Figure 2 for details) was approximately 10 times lower in the RAG1$^{-/-}$→RAG1$^{-/-}$ than RAG1$^{-/-}$→IFN-γ$^{-/-}$mice (Figure 2B)[3]. In agreement, upregulated cerebral expressions of effector molecules such as guanylate binding protein 1 and inducible nitric oxide synthase, which mediate the protective activities of the IFN-γ-mediated protective immunity against tachyzoites, were detected in the brains of only RAG1$^{-/-}$→RAG1$^{-/-}$ mice (3). These studies uncovered the essential role of IFN-γ production of the brain-resident cells to activate IFN-γ-mediated protective innate immunity to suppress cerebral proliferation of *T. gondii* tachyzoites during reactivation of the infection.

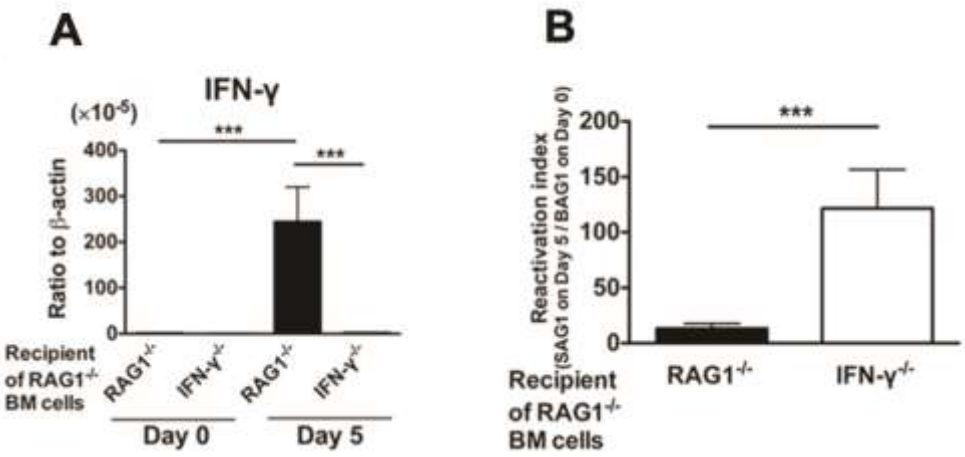

Figure 2. IFN-γ production by brain-resident cells is required for cerebral innate immunity to limit reactivation of *T. gondii* infection. RAG1$^{-/-}$→RAG1$^{-/-}$ and RAG1$^{-/-}$→IFN-γ$^{-/-}$ mice were infected with T. gondii and treated with sulfadiazine during the acute stage of infection to control tachyzoite proliferation and establish a chronic infection in their brains. Sulfadiazine was then discontinued to initiate reactivation of the infection. (**A**) Cerebral IFN-γ mRNA expression levels at 5 days after the initiation of reaction of the infection (Day 5). (**B**) "Reactivation index", which is a ratio of the amounts of tachyzoite-specific SAG1 mRNA at Day 5 versus the amounts of bradyzoite-specific BAG1 mRNA at Day 0 (the last day of sulfadiazine treatment). Mean ± SEM, n=7 or 8. **P*<0.05, ***P*<0.01, ****P*<0.001. These figures are a part of Fig. 1 of our article published in *The Journal of Immunology*, Sa, Q., R. Ochiai, A. Tiwari, S. Perkins, J. Mullins, M. Gehman, W. Huckle, W. H. Eyestone, T. L. Saunders, B. J. Shelton, and Y. Suzuki, 2015, Cutting Edge: IFN-γ Produced by Brain-Resident Cells Is Crucial to Control Cerebral Infection with *Toxoplasma gondii*, 195:756-800, Copyright ©[2015] The American Association of Immunologists, Inc.

The evidence described above does not essentially mean that IFN-γ detected in the brains of RAG1$^{-/-}$→RAG1$^{-/-}$ mice are solely derived from brain-resident cells. It is possible that IFN-γ produced by brain-resident cells initiates an infiltration of hematopoietic innate immune cells such as NK cells and macrophages from the periphery, and these infiltrated innate immune cells also contribute to producing IFN-γ. An important aspect is that this possible contribution of hematopoietic innate immune cells cannot occur in the absence of IFN-γ production by brain-resident cells.

In regard to the brain-resident cell population that produce IFN-γ during reactivation of cerebral infection with *T. gondii*, $CD11b^{+}CD45^{low}$ microglia and $CD11b^{+}CD45^{high}$ blood-derived macrophage purified from the brains of SCID mice deficient in both T and B cells during reactivation of the infection secreted IFN-γ into culture medium when these cell populations were cultured without any stimulation *in vitro*[3]. Furthermore, a microglia cell line (EOC20) and primary microglia purified from the brains of uninfected BALB/c mice both produce IFN-γ following stimulation with *T. gondii* antigens *in vitro*[3]. Therefore, microglia appear to be a key brain-resident cells that produce IFN-γ in response to cerebral proliferation of tachyzoites to activate IFN-γ-mediated protective innate immunity to suppress the pathogen growth, although a participation of the other brain-resident cell populations can not be excluded at this moment. Microglia and astrocytes purified from the brain have been shown to produce IFN-γ *in vitro* in response to various stimulations including lipopolysaccharide (LPS), which is a component of the outer membrane of gram-negative bacteria[4-6]. However, it was unknown whether IFN-γ produced by brain-resident cells including glial cells plays any roles in resistance to cerebral infections with microorganisms. Thus, our studies using cerebral *T. gondii* infection model provided a novel insight on an important first line defense role of brain-resident cells to sense proliferation of a pathogen and produce IFN-γ to activate cerebral innate immunity to limit the pathogen growth. Elucidating how brain-resident cells detect a pathogen growth and activate their IFN-γ production will further improve our understanding of the cerebral innate immune system against infection.

1.2. Importance of IFN-γ production by brain-resident cells to activate T cell-mediated protective immunity

Although IFN-γ-mediated cerebral innate immunity is able to limit tachyzoite proliferation, T cells are ultimately required to prevent the parasite growth and reactivation of the infection. CXCL9 chemokine plays a key role in mediating the recruitment of $CD4^{+}$ and $CD8^{+}$ T cells into the brain during reactivation of *T. gondii* infection and their migration to the areas in which tachyzoites are proliferating[7]. Notably, the studies using the $RAG1^{-/-}\rightarrow RAG1^{-/-}$ and $RAG1^{-/-}\rightarrow IFN\text{-}\gamma^{-/-}$ BM chimeric mice revealed that upregulation of cerebral CXCL9 expression in response to reactivation of the infection almost solely depends on IFN-γ production by brain-resident cells[3]. T cells not only need to migrate into the brain but also need to be activated by recognizing the parasite antigens presented by the MHC class I and II molecules to display their protective activities. IFN-γ production by brain-resident cells was identified to be required for the upregulation of cerebral expression of mRNA for both MHC class I and II molecules during the reactivation of *T. gondii* infection[3]. Therefore, IFN-γ production by brain-resident cells plays the critical roles in enhancing the expression of the molecules required for both the recruitment of the immune T cells and the activation of recruited T cells to prevent reactivation of cerebral *T. gondii* infection.

Eventually, when the immune T cells are transferred into BM chimeric mice with and without IFN-γ production in the brain-resident cells, 4-5 times larger numbers of both $CD4^{+}$ and $CD8^{+}$ immune T cells are recruited into the brains of the BM chimeras that have IFN-γ-producing brain-resident cells than those that do not have these brain-resident cells (Figures 3 A and 3B)[3]. Consistently, five times greater levels of IFN-γ expression are

detected in the brains of the former than the latter (Figure 3C), which is associated with much more efficient control of cerebral tachyzoite growth in the former than the latter (Figure 3D)[3]. These evidences along with the requirement of IFN-γ production by brain-resident cells for upregulating cerebral expression of CXCL9 and MHC class I and II molecules shed light on the key coordinating role of IFN-γ production by brain-resident cells to promote the migration and activation of immune T cells into the brain in response to reactivation of cerebral *T. gondii* infection and facilitate T cell-mediated protective immunity to prevent reactivation of the infection. These studies using murine models of reactivation of *T. gondii* infection all together uncovered the key first line defense role of IFN-γ produced by brain-resident cells to orchestrate both innate and T cell-mediated protective immunity to control the cerebral infection.

2. $CD8^+$ cytotoxic T cells penetrate into a target of large mass for its elimination in collaboration with phagocytes that accumulate to the T cell-invaded target.

T. gondii establishes chronic infection by forming tissue cysts as mentioned earlier. Since most of individuals chronically infected with *T. gondii* remain positive for IgG antibodies to this parasite for decades, it was generally considered that the immune system is unable to recognize or eliminate this chronic stage form of this parasite. However, our recent studies identified that $CD8^+$ T cells have the capability to remove *T. gondii* cysts from the brain of chronically infected hosts[8].

SCID mice were infected with *T. gondii* and treated with sulfadiazine to establish chronic infection in their brains. They thereafter received a systemic transfer of $CD8^+$ T cells (3.5×10^6 cells) from uninfected or chronically infected wild-type (WT) BALB/c mice. Sulfadiazine treatment was maintained even after the T cell transfer to keep the parasite in the cyst stage in the recipient animals. One week after the cell transfer, numbers of cysts in the brains of the recipients of the $CD8^+$ immune cells from the infected WT mice were approximately 11 times less than those of control mice that did not receive any T cells ($P<0.05$). In contrast, the cysts numbers in the recipients of $CD8^+$ normal T cells from uninfected WT mice did not differ from those of the controls[8]. In clear contrast to the protective immunity against tachyzoites, the deficiency of IFN-γ in the $CD8^+$ immune T cells do not affect their capability to remove the cysts from the brains of the recipient SCID mice[8]. On the other hand, the absence of perforin in the $CD8^+$ immune T cells totally ablates their capability to remove the cysts from the recipients[8]. These studies revealed that $CD8^+$ T cells have the capability to eliminate *T. gondii* cysts by utilizing a perforin-mediated effector mechanism(s).

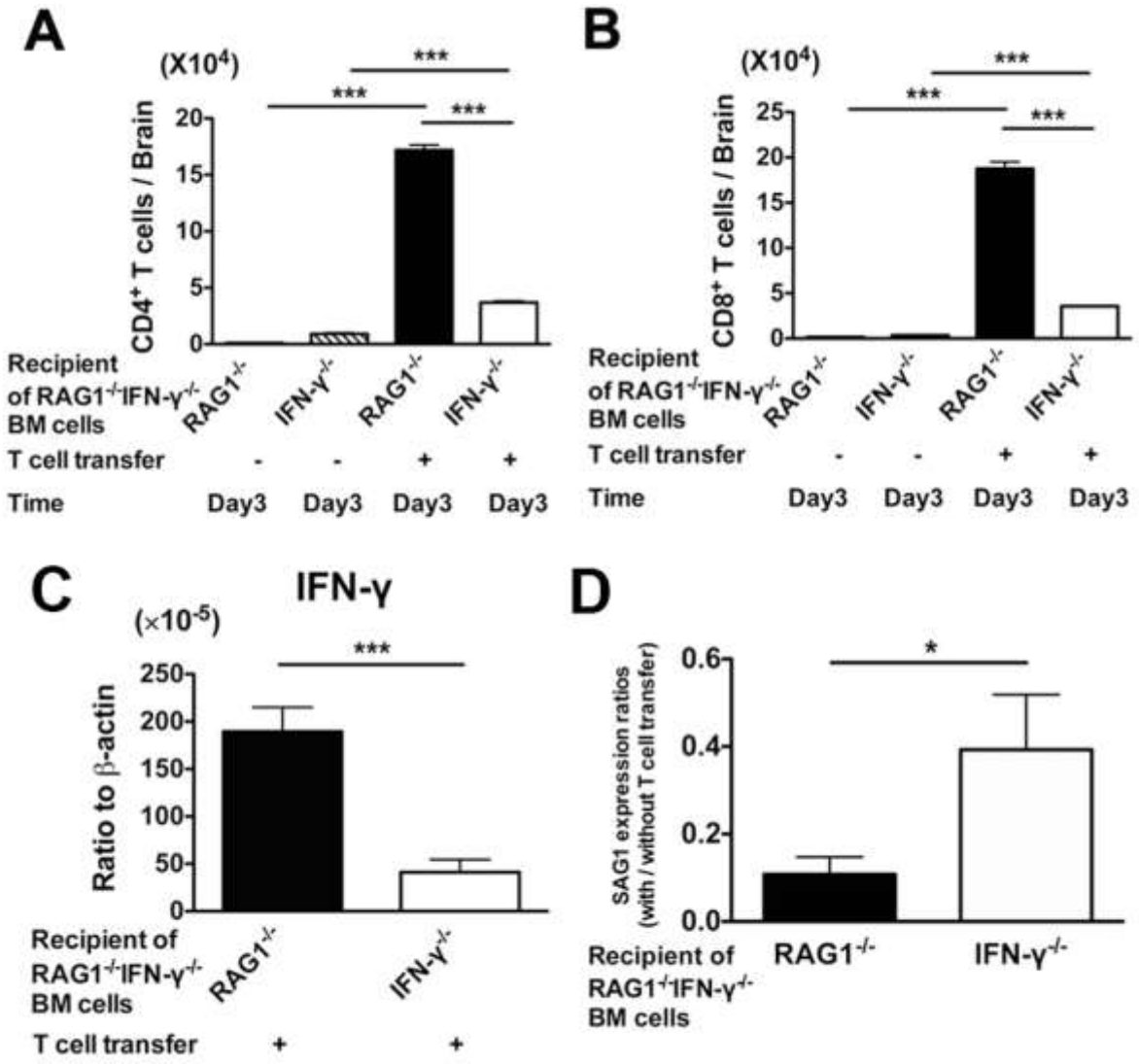

Figure 3. IFN-γ production by brain-resident cells is crucial for recruiting immune T cells into the brain and inducing IFN-γ-mediated protective T cell immunity to inhibit reactivation of *T. gondii* infection. Infected, sulfadiazine-treated RAG1$^{-/-}$IFN-γ$^{-/-}$→RAG1$^{-/-}$ and RAG1$^{-/-}$IFN-γ$^{-/-}$→IFN-γ$^{-/-}$ mice received an intravenous injection of immune T cells (1 x 10^7 cells) from a tail vein, and 4-5 days later, the sulfadiazine treatment was discontinued to initiate reactivation of the infection. (**A**) CD4$^+$ and (**B**) CD8$^+$ T cells that migrated into the brain in the gated area for lymphocytes. (**C**) Cerebral IFN-γ mRNA. (**D**) Ratios of tachyzoite-specific SAG1 mRNA in the brains of mice with the T cell transfer vs. those of animals without the T cell transfer. Mean ± SEM, n=4 (**A** and **B**) and n=8 (**C** and **D**) on Day 3-4. *P<0.05 and ***P<0.001. These figures are a part of Fig. 4 of our article published in *The Journal of Immunology*, Sa, Q., R. Ochiai, A. Tiwari, S. Perkins, J. Mullins, M. Gehman, W. Huckle, W. H. Eyestone, T. L. Saunders, B. J. Shelton, and Y. Suzuki, 2015, Cutting Edge: IFN-γ Produced by Brain-Resident Cells Is Crucial to Control Cerebral Infection with *Toxoplasma gondii*, 195:756-800, Copyright ©[2015] The American Association of Immunologists, Inc.

Cytotoxic $CD8^+$ T cells are appreciated to kill target cells by secreting perforin and other cytotoxic proteins through direct contact with the targets. Our immunohistochemical studies on interactions of $CD8^+$ immune T cells with *T. gondii* cysts in the brains of infected, T cell-deficient nude mice following a transfer of the T cells uncovered that the T cells not only attach on the surface of host cells harboring *T. gondii* cysts but also penetrate into the cysts through perforin-dependent manner[9]. The presence of the T cells (arrowed in Figure 4A) is clearly visible in the images taken at both the top and the bottom of the histological section of 4 μm thickness. Furthermore, the Z-stack 3-D images (Figure 4B) generated at the cut-line indicated in green on the top image (left panel of Figure 4A) show that those T cells are present within these cysts all the way through the thickness of the sections (arrowed in Figure 4B)[9], providing the definitive evidence that these $CD8^+$ T cells are located within the cyst. Notably, on the surface of cysts attached or invaded by $CD8^+$ T cells, no other cells, or only a few if any, are usually detected, strongly suggesting that $CD8^+$ cytotoxic T cells are the first immune cell population that initiates the attack on the cysts and penetrates into the target. $CD8^+$ T cells recognize targets by recognizing antigens presented by MHC class I molecules expressed on the surface of the targets. The cyst wall of *T. gondii* cysts derives from the PV, which is formed from the plasma membrane of the host cells and those from the parasite as mentioned earlier in section 1.1. Therefore, it is possible that *T. gondii* cyst wall maintains the MHC class I molecules derived from the plasma membrane of the host cells for antigen presentation.

T. gondii cysts penetrated by $CD8^+$ T cells often display morphological deterioration and destruction as seen in Figure 4 (9). Within a majority (80%) of morphologically denatured cysts, granular structures intensely positive for granzyme B (arrows in Figures 5A, 5B, and 5C) are detected (Figure 5F)[9]. In contrast, those granzyme B-positive structures are not detectable in morphologically intact cysts (Figure 5F and a representative image indicated by an arrowhead in Figure 5C)[9]. Granzyme B is a serine protease that cytotoxic T cells secrete during perforin-mediated cytotoxicity against target cells. Notably, many of these granzyme B-positive structures within the morphologically denatured cysts are detected in bound with bradyzoites (co-existing of green [granzyme B] and brown [*T. gondii* bradyzoites] indicated by white arrows) in comparison with existence of only granzyme B [orange arrows] seen as light green in Figures 5D and 5E)[9]. Furthermore, there is a tendency that fainter staining for the granzyme B is co-localized with fainter staining for *T. gondii*, suggesting that these granzyme B-bound bradyzoites are in the process of degradation.

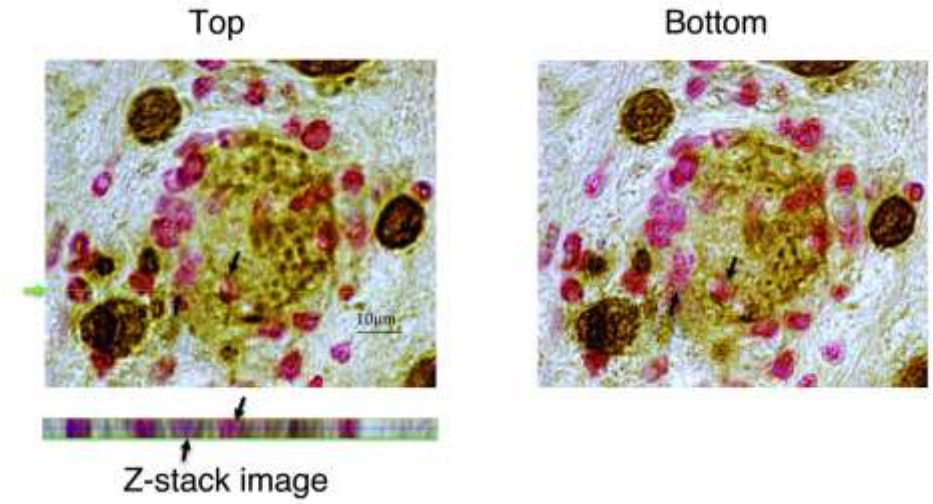

Figure 4. The images of CD8$^+$ T cells that had fully invaded into *T. gondii* cysts. Nude mice were infected and treated with sulfadiazine to establish a chronic infection by forming cysts in their brains. CD8$^+$ T cells (3.5 x 10^6 cells) purified from the spleens of infected BALB/c mice were injected intravenously from a tail vein, and 2-3 days later, their brains were applied for immunohistochemical staining for *T. gondii* (brown) and CD3 (red). A Z-stack image was obtained using light microscopy. (**A**) The images taken at the top and bottom of the histological section. The presence of the T cells (arrowed) can be seen in both images at the top and bottom of the sections. (B) The Z-stack images of the cysts at the cut-line indicated by a green arrow in panel A. The Z-stack image demonstrates the presence of the T cell (arrowed) all the way through the section. These images are a part of Figure 4 of our previous publication in The American Journal of Pathology, Tiwari, A, R Hannah, J Lutshumba, E Ochiai, L M Weiss, and Y Suzuki. Volume 189, Penetration of CD8$^+$ cytotoxic T cells into large target, tissue cysts of *Toxoplasma gondii*, leads to its elimination, pages 1594-1607, Copyright (2019), with permission from Elsevier.

Once the destruction process of *T. gondii* cysts mediated by CD8$^+$ cytotoxic T cells is initiated, Iba1$^+$ microglia and Ly6C$^+$ blood-derived macrophages accumulate to those cysts (Figures 5G and 5H)[9]. The majority of *T. gondii* organisms located within the destroyed cysts are detected within these microglia and macrophages (Figures 5G and 5H) (9). The *T. gondii*-positive materials located within the microglia and macrophages often do not maintain a clear morphology of the parasite (arrows in Figures 5G and 5H in comparison with the parasite that maintained a clear morphology [arrowheads]), suggesting that those organisms are destroyed within these phagocytes. Importantly, the accumulation of microglia and blood-derived macrophages are detected only on morphologically destroyed cysts (Figure 5I). As mentioned earlier, granzyme B is detectable within the majority of destroyed cysts but not morphologically intact cysts. Of interest, granzyme B has the activity to induce inflammation[10, 11]. Therefore, it would be possible that granzyme B contributes not only on killing of the bradyzoites but also inducing an accumulation of phagocytes. The microglia and macrophages are most likely the scavenger cells that eventually eradicate the bradyzoites once CD8$^+$ immune T cells invaded into the cysts and secreted granzyme B. This newly revealed effector mechanism initiated by perforin-mediated invasion of CD8$^+$ cytotoxic T cells, their secretion of

granzyme B, and an accumulation of phagocytes to eliminate *T. gondii* cysts appears to be a quite efficient effector mechanism against the targets of large mass.

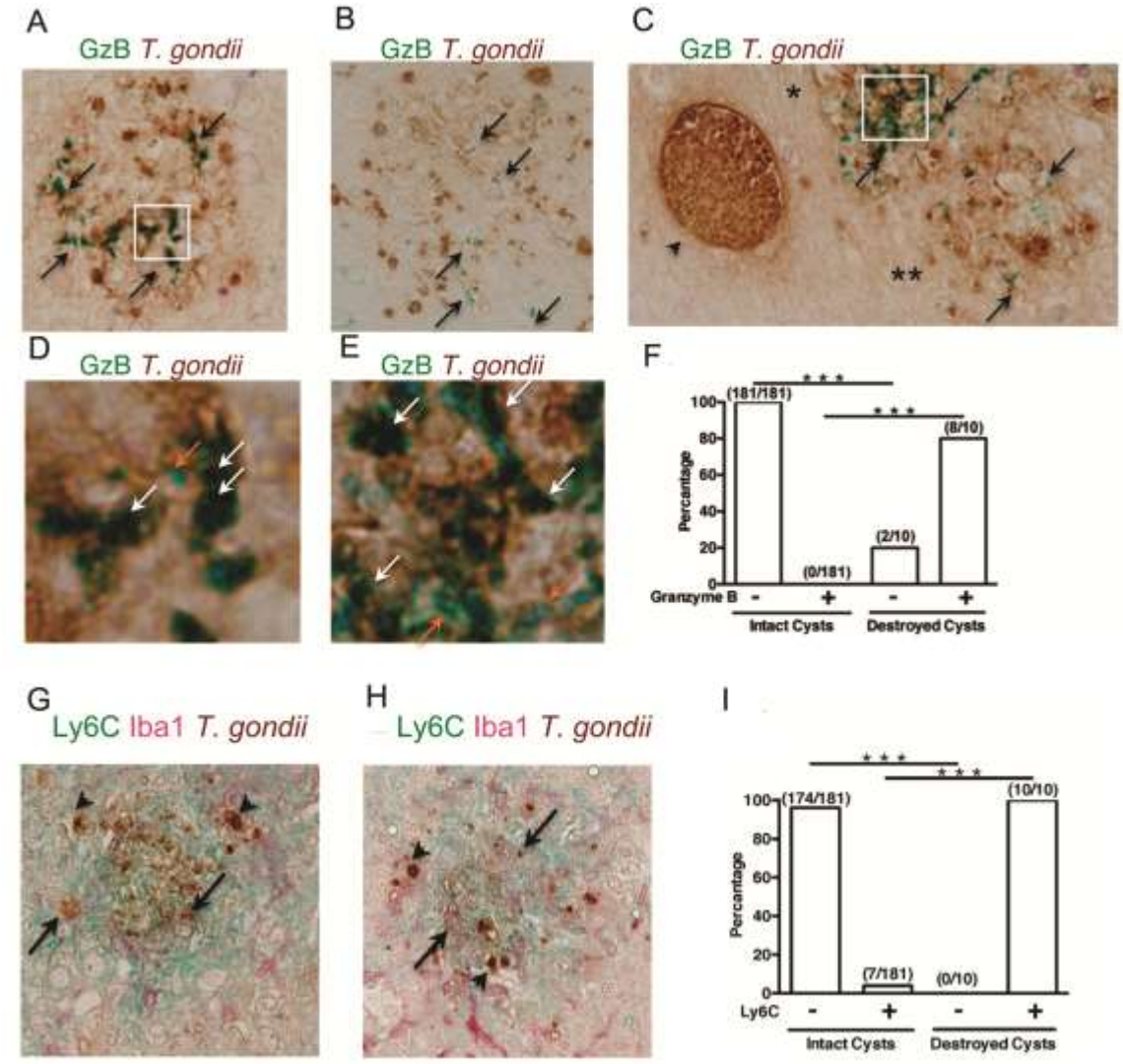

Figure 5. Penetration of CD8$^+$ immune T cells into *T. gondii* cysts induces morphological deterioration of the cysts and their destruction is associated with granular materials with high density of granzyme B and an accumulation of Iba1$^+$ microglia and Ly6C$^+$ inflammatory macrophages. Infected and sulfadiazine-treated nude mice received an intravenous injection of CD8$^+$ immune T cells (3.5 x 10^6 cells) purified from the spleens of infected BALB/c mice from a tail vein, and 2-3 days later, their brains were applied for immunohistochemical staining for *T. gondii*. (**A, B,** and **C**) The presence of granular staining of granzyme B (green, arrowed) in the areas of destroyed cysts (brown). An arrowhead in panel C indicates an intact cyst adjacent to the destroyed cysts. * and ** in panel C indicate two independent cysts destroyed with the presence of different amounts granular structures positive for granzyme B and different intensities of granzyme staining. (**D** and **E**) Enlarged images of the areas indicated by white squares in panel A (corresponding to panel D) and panel C (corresponding to panel E). Many of the granular structures positive for granzyme B within destroyed cysts were in dark green color (indicated by white arrows), resulting from

a mixture of green (the color used for staining granzyme B) and brown (the color used for staining *T. gondii*), when compared to staining showing only green color (indicated by orange arrows). (**F**) The frequencies of granular structures positive for granzyme B within destroyed cysts and morphologically intact cysts. ***$P<0.001$. (**G** and **H**) Accumulations of $Iba1^+$ microglia (red) and $Ly6C^+$ blood-derived macrophages (green) are associated with demolished cysts (brown). Arrowheads indicate *T. gondii* parasites that maintain a clear morphology. Arrows indicate the representatives of the parasites that had already been destroyed and lost a clear morphology of the parasite. (**I**) The differences in the frequencies of an accumulation of $Ly6C^+$ macrophages to morphologically destroyed and intact cysts. ***$P<0.001$. These images are a part of Figures 5 and 7 of our previous publication, in The American Journal of Pathology, Tiwari, A, R Hannah, J Lutshumba, E Ochiai, L M Weiss, and Y Suzuki. Volume 189, Penetration of $CD8^+$ cytotoxic T cells into large target, tissue cysts of *Toxoplasma gondii*, leads to its elimination, pages 1594-1607, Copyright (2019), with permission from Elsevier.

Conclusions

The immune system utilizes two distinct effector mechanisms, a secretion of a potent inflammatory cytokine, IFN-γ, to prevent proliferation of *T. gondii* tachyzoites and the perforin-mediated penetration of cytotoxic T cells to remove tissue cysts of the parasite. During the acute stage of infection, tachyzoites invade into host cells, quickly proliferate within the cells, and then destroys the host cells to invade into adjacent cells to proliferate. In this case, once T cells detect a tachyzoite-infected cell, it is high probable that many cells located nearby are also infected. Thus, secreting the powerful pro-inflammatory IFN-γ and activating the cells around the detected infected cells is an effective strategy to efficiently control this quickly proliferating stage of the parasite. In contrast, the situation in the chronic stage of the infection is different. *T. gondii* cysts are usually located in the tissues one by one in a scattered manner. Therefore, it is unlikely that multiple cysts are present nearby each other. In this case, destroying each of the cysts by direct cell-cell contact by cytotoxic T cells and their invasion into the target through a perforin-mediated mechanism, rather than secreting pro-inflammatory IFN-γ, is an effective approach to avoid unnecessary activation of cells located nearby that could cause unwanted tissue damages.

Notably, the analyses on each of the IFN-γ-mediated protective immunity against tachyzoites and the perforin-mediated elimination of the cysts generated important insights on the mechanisms of host defense system against infections in general. One is the crucial first line defense role of IFN-γ production by brain-resident cells to orchestrate both innate and T cell-mediated protective immunity to prevent cerebral proliferation of tachyzoites. In the absence of their IFN-γ production, hematopoietic innate immune cells circulating in the periphery is unable to activate IFN-γ-mediated innate protective immunity in the brain even though those hematopoietic innate immune cells such as NK cells and macrophages has the capability to produce IFN-γ. The production of this cytokine by brain-resident cells also plays a key role in inducing recruitment of immune T cells from the periphery and activating their IFN-γ production to prevent cerebral tachyzoite growth. Since IFN-γ is important for the protective immunity against cerebral infections with various intracellular pathogens[12, 13], it is possible that IFN-γ produced by brain-resident cells plays a key defense role in resistance against these pathogens as well. Thus, further elucidating the mechanisms by which the brain-resident cells orchestrate the cerebral innate and T cell-mediated protective immunity could provide valuable information to improve our

understanding on how the protective immunity functions to efficiently control cerebral infection in general.

Another notable insight is the capability of cytotoxic T cells to penetrate into a target of large mass for its elimination in collaboration with an accumulation of phagocytes. The studies on how $CD8^+$ cytotoxic T cells eliminate *T. gondii* cysts depicted a powerful capability of the $CD8^+$ T cells to invade into the target through a perforin-mediated mechanism(s) and secretion of granzyme B, which induces an accumulation of phagocytes for their eradication. The presence of tumor-infiltrating $CD8^+$ T cells in solid cancers is known as an indicator of positive prognosis of the patients[(14)]. It would be possible that at least a part of these $CD8^+$ T cells are cytotoxic T cells that invaded into the tumors through the perforin-mediated penetration mechanism. If this is the case, their invasion and secretion of granzyme B followed by an accumulation of phagocytes capable of attacking the cancer cells will most likely be quite effective to eliminate the tumors. Therefore, it would be possible that the perforin-mediated penetration of $CD8^+$ cytototoxic T cells followed by an accumulation of large numbers of phagocytes is a powerful effector function of the immune system to eradiate a target of large mass in general, which expresses the MHC class I molecules on its surface that allow $CD8^+$ T cells recognize the target for its elimination.

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