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Advances in imaging the innate and adaptive immune response to *Toxoplasma gondii*

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Abstract

Toxoplasma gondii is an intracellular protozoan parasite that infects a wide variety of warm-blooded hosts and can have devastating effects in the developing fetus as well as the immunocompromised host. An appreciation of how this organism interacts with the host immune system is crucial to understanding the pathogenesis of this disease. The last decade has been marked by the application of various imaging techniques, such as bioluminescent imaging as well as confocal and multiphoton microscopy to study toxoplasmosis. The ability to manipulate parasites to express fluorescent/bioluminescent markers or model antigens/enzymes combined with the development of reporter mice that allow the detection of distinct immune populations have been crucial to the success of many of these studies. These approaches have permitted the visualization of parasites and immune cells in real-time and provided new insights into the nature of host–pathogen interactions. This article highlights some of the advances in imaging techniques, their strengths and weaknesses, and how these techniques have impacted our understanding of the interaction between parasites and various immune populations during toxoplasmosis.

Keywords

bioluminescence; fluorescent protein; multiphoton microscopy; real-time imaging; *Toxoplasma gondii*

Toxoplasma gondii is an apicomplexan protozoan that can infect a wide range of hosts, including humans. As the third leading cause of death by foodborne illnesses in the USA, this is a pathogen of considerable public health concern [1]. Infection with *T. gondii* is characterized by two phases, which are distinct with regards to the developmental stage of the parasite and the sites of parasite persistence. Following ingestion, the parasite infects and replicates in intestinal epithelial cells before disseminating rapidly throughout the body. This process is poorly understood, but the parasite can cross several unique anatomical structures that normally restrict cellular access to tissues. Of particular relevance are the blood–brain barrier, blood–retina barrier and placenta because these are sites where the replication of

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parasites and associated cell death has the most adverse effects and can cause toxoplasmic encephalitis, chorioretinitis and congenital disease respectively [2,3]. As a consequence, the development of a rapid immune response is crucial to limiting parasite dissemination and tissue damage. The acute phase of this infection is characterized by the recruitment of dendritic cells (DCs), neutrophils and monocytes to sites of parasite invasion. These cells act as a source of IL-12, which in turn stimulates the production of IFN- γ , by natural killer and T cells, which is required to activate a variety of antimicrobial effector responses [4–7]. One consequence of an effective immune response is that the parasite transitions into a slow replicating bradyzoite stage, which forms tissue cysts throughout the body, but predominantly in muscle and nervous tissue. During this chronic phase of the infection there is good evidence, from clinical and experimental settings, that T cells and IFN- γ are required to prevent recrudescence, and when there is a loss of T cells, toxoplasmic encephalitis is a common manifestation [8,9].

Every step of the infection process is complex *in vivo*, and a full understanding of these events requires the ability to track parasites, parasite products and host immune cells efficiently. Advances in modern imaging techniques and the generation of various genetically modified strains of parasites and transgenic reporter mice have allowed the direct visualization of several relevant processes during toxoplasmosis as well as other infections [10–16]. While some studies have focused on parasite invasion and dissemination, others have examined the initiation of innate immune responses, antigen processing/presentation and activation of effector T cells and their migration *in vivo* (Figure 1) [11–14,17–22]. The aim of this review is to highlight the various approaches that have been used to visualize parasites and immune cells during toxoplasmosis, and discuss how these studies have improved our understanding of this specific host–pathogen interaction.

Use of reporter genes to identify & visualize parasites

Since the first description of *T. gondii* using light microscopy a century ago, there has been tremendous progress in the techniques used to visualize this parasite [23–28]. Ultrastructural studies by electron microscopy combined with the development of monoclonal antibodies against various parasite antigens and immunohistochemical techniques have helped to define the structure of the parasite and its localization within intracellular compartments *in vitro* and *in vivo* [25]. The availability of molecular tools to manipulate the parasite genome led to the generation of recombinant strains of parasites that expressed various fluorescent reporters (e.g., RFP, GFP, YFP). When these transgenic organisms were combined with techniques such as flow cytometry and fluorescent microscopy, it became possible to identify the specific cells that *T. gondii* infects [26–31]. The use of tagged parasites has also enabled differences in the dissemination of virulent and avirulent strains to be quantified, and were a key tool that led to the hypothesis that DCs can act as Trojan horses to transport parasites to distal sites [32,33]. The stage-specific expression of fluorescence proteins within the parasites has also been useful in understanding spatiotemporal details of the transition of the rapidly replicating tachyzoite to the slowly dividing bradyzoite stage (found within dormant cysts) during infection *in vivo* [34].

The genetic manipulation of *T. gondii* parasites to express bioluminescent markers, such as luciferase, in combination with bioluminescent imaging techniques has enabled the monitoring of infection in real-time. This approach has been used to assess the process of dissemination [35,36], as well as local recrudescence within the brain [15,37]. The clear advantage of this technology over the use of fluorescent parasites is that multiple time-points can be visualized noninvasively within the same animal over time. The disadvantages of the technique include its low-resolution limits and the difficulty in deciphering specific signals

over background noise at lower parasite numbers and in certain tissues (such as skeletal tissues).

While fluorescent and bioluminescent reporters have been useful in identifying infected cells, it is appreciated that not every contact between parasites and host cells results in a productive infection. A recently developed Cre-recombinase secreting strain of *Toxoplasma* (under the toxofillin promoter) has provided unexpected insights into how *Toxoplasma* interacts with individual cells [38]. As part of the normal events associated with invasion, the parasite secretes a number of proteins from specialized organelles termed 'rhoptries', which have been implicated in modifying host cell signaling and promoting parasite survival [39]. In this experimental system, the parasite is engineered to secrete Cre from the rhoptries and can be used to infect cell lines, which express floxed (lox-p flanked) versions of fluorescent/bioluminescent reporters (the expression of the reporter molecules in such a system can only occur after a Cre-lox site-specific recombination event) [40]. Using similar approaches *in vitro*, two groups have reported that parasites are able to cause Cre-mediated recombination in uninfected as well as infected cells [38,41]. Thus, the ability of *T. gondii* to inject rhoptry-derived proteins into host cells without productive infection could alter the local immune response. However, it should be noted that this technical approach to tagging cells affected by *T. gondii* has some disadvantages, which include the lag in time between the invasion, secretion of cre-recombinase, excision of the targeted gene and decay of the existing target gene product. This might limit the ability to detect the earliest events that are triggered during attachment and/or invasion.

Use of model antigens to study antigen-specific immune responses

Until quite recently there have been limited tools to study the process by which DCs process and present parasite-derived antigens for the generation of an adaptive immune response. One approach to this problem has been to use genetically modified organisms that express surrogate antigens, such as ovalbumin (OVA), for which there are numerous reagents, which include OVA-specific T-cell receptor transgenic models (OTI CD8⁺, OTII CD4⁺ and DO11.10 CD4⁺ T cells) and MHC class I tetramers (to detect OVA-specific CD8⁺ T cells). This strategy has been pioneered in other infection systems (*Salmonella enterica* serovar typhimurium, *Listeria monocytogenes* and *Trypanosoma cruzi*), to study the generation of antigen-specific T-cell responses [42–44]. The development of similar genetically modified strains of *T. gondii* that express model antigens (e.g., OVA, Eα and LacZ) has had a similar impact on the ability to reliably detect antigen-specific immune responses to this infection. The combination of parasites expressing fluorescent tags and model antigens has enabled the identification of intracellular antigen-processing pathways for MHC class I- and II-mediated antigen presentation [12,20,21,41]. Using these parasites, studies with DCs have indicated a dominant role for direct presentation by infected cells in priming CD8⁺ T-cell responses [12,20,26]. However, different DC subsets are known to be activated following challenge with *T. gondii*, including CD8α⁺ DCs [45], which are thought to have a prominent role in cross-presentation of antigens to CD8⁺ T cells. Thus, fundamental questions remain regarding the relative contributions of different antigen presentation pathways *in vivo*.

While the use of model antigens has provided a surrogate for parasite antigens, recently several immunodominant epitopes of *T. gondii*, including GRA6, GRA4, ROP7 and tgd057 have been identified by various groups [46–48]. In an attempt to develop a mouse model to study endogenous antigen-specific responses, epigenetic reprogramming by somatic cell nuclear transfer has been used [49]. This procedure involves the purification of CD8⁺ T cells specific for *T. gondii* epitopes (sorted with antigen-specific tetramers), followed by the isolation of their nuclei, which are placed into enucleated oocytes to create embryonic stem cells. These cell lines are then used to generate chimeric mice in which the majority of CD8⁺

T cells are specific for a single epitope of *T. gondii*. Development of such reagents to study endogenous antigen-specific responses will not only place the studies obtained using model antigens into perspective, but will also help us understand whether the repertoire of immunodominant antigens varies for the different strains of toxoplasma (Type I, II and III) and for distinct developmental stages (tachyzoite/bradyzoite), and how this influences the generation of the adaptive immune response.

Real-time imaging using multiphoton microscopy

The use of fluorescence microscopy in fixed-tissue and flow cytometry of isolated cell populations has provided snapshots of the immune response at various stages during toxoplasmosis. However, tachyzoites of *T. gondii* are motile and the ability of immune populations to traffic to sites of infection or within secondary lymphoid tissues is a key requirement for the control *T. gondii*. Due to the limitations of traditional light microscopy techniques, direct visualization of any of these events *in vivo* was not possible. While confocal microscopy has significantly improved the resolution in tissues compared with wide-field epifluorescence microscopy, high levels of scattering of visible light within tissues and an inability to achieve penetration depths beyond 70–80 μm , make imaging through thick tissue layers difficult. Many of these problems were solved with the advent of two photon laser scanning microscopy, which delivers lower energy (infrared light) photons in concentrated short pulses, thus reducing photobleaching and light scattering, and achieving greater depths of imaging. With the development of different techniques to label cells (using dyes or genetic tags), high-speed laser scanners and surgical procedures to expose tissues of interest, multiphoton microscopy has dramatically improved our ability to image immune cells *in situ*.

Some of the earliest studies to visualize the immune response *in situ* were performed using adoptively transferred DCs, and T- and B-cell receptor transgenic cells, as well as model antigens in noninfectious settings [10,50–52]. In this context, labeling purified cell populations with fluorescent dyes (several with non-overlapping emission spectra), such as CFSE, SNARF, CM-TMR and CMAC, has been useful for short-term trafficking and migration studies. However, cell division associated with T- and B-cell activation results in the dilution of dyes and makes it difficult to track these populations. The generation of various reporter mice expressing fluorescent proteins under promoters specific for different cell types (e.g., DCs, neutrophils, macrophages, and antigen-specific CD4^+ and CD8^+ T cells) has permitted long-term tracking of many different types of immune cells *in vivo*.

More recently, there has been increased focus on the use of real-time imaging by multiphoton microscopy to study host–pathogen interactions *in vivo* [11,13,14,53–56]. One of the first reports that used live imaging during experimental toxoplasmosis, by Robey and colleagues, used LysM^{GFP} reporter mice and showed rapid mobilization of neutrophils to the subcapsular sinus of the lymph node in response to this parasite [11]. These studies revealed the formation of transient and persistent neutrophil swarms; the formation of which coincided in time and location with the destruction of the subcapsular macrophages. As highlighted earlier, another innate immune cell type that has a critical role in coordinating the immune response to *T. gondii* is the DC, and these cells are required for resistance to acute infection [45,57]. Our recent studies, using an OVA-expressing strain of *T. gondii*, which examined priming *in vivo* of OVA- specific CD8^+ T cells during infection, revealed an early time-window when antigen-specific interactions between CD11c^+ cells and OT1^+ CD8^+ T cells could be visualized [13]. During the first 36–48 h after infection, the antigen-specific T cells and DCs were engaged in prolonged interactions, but once the T cells were activated (~48 h after infection), there was a transition to short-lived contacts between the T cells and DCs (Figure 2). Whether this represents effectors searching for infected target cells

or indicates a reduced need for co-stimulation by effector cells is not clear. Work by Robey and colleagues has similarly visualized prolonged interaction between infected cells and memory T cells during recall responses within the lymph node [18]. As part of these studies, they also observed the transfer of parasites from the infected DC to activated T cells. Other studies by Barragan and colleagues using *in vitro* imaging and flow cytometry have similarly noted transfer of parasites between DCs and natural killer cells [58]. It has been proposed that this ability of parasites to infect cytolytic effector cells acts as one mechanism by which the parasites subvert the host immune response and disseminate *in vivo*.

While the studies that have examined the interactions between DCs and T cells have highlighted the kinetics of *in vivo* antigen presentation during active infection, a number of questions still remain to be answered. With the increased focus on understanding the parasite genetics that underlie the differences in virulence, there is a need to better understand how different strains of parasites interact with the immune system. Type I strains are lethal in mice, and studies from this laboratory have indicated a defective DC recruitment and subsequent CD8⁺ T-cell responses during infection with a type I strain in comparison with avirulent type II strains [59]. Whether this is a consequence of a defect in DC–T-cell interactions and subsequent priming of an effector response remains to be tested.

Recently, several fluorescent reporters have been developed for cytokines that include IL-2, IL-10, IL-12 and IFN- γ , making it possible to visualize the production of cytokines *in vivo* and characterize how these cytokine-producing populations interact with other cell types. DCs are one of the dominant sources of early IL-12 during toxoplasmosis, and appear to have a critical role in the first 3 days to prime protective T-cell responses. It is currently unclear from DC depletion studies whether the role of DCs is to present antigen, produce IL-12 or whether they can simultaneously perform both functions. Using IL-12 reporter mice, it is possible to begin to address these questions about the source of IL-12 and whether it is produced by the same cell that presents antigen to T cells [60].

While imaging acute events during infection has helped to understand the initiation of a protective immune response, much less is known about the immune populations activated in the context of toxoplasmic encephalitis. More conventional approaches have identified the presence of T cells and antigen-presenting cell populations within the brain during toxoplasmic encephalitis [9,61]. Recent work performed by our group and by Robey and colleagues has characterized the movement and behavior of antigen-specific T cells within the brain during the chronic phase of this infection [14,18]. Future studies will be aimed at addressing the role of various signals, including adhesion molecules and chemokines in the recruitment and movement of these cells within the brain. In addition, understanding the interactions between distinct cellular populations, such as DCs, microglia and astrocytes with T cells and parasites, will help in better understanding their function *in vivo*.

While multiphoton microscopy has provided an unprecedented view of how the immune system behaves during toxoplasmosis, interpretations drawn from such studies must be viewed with caution. Often, the process of surgically exposing tissues for intra-vital imaging or removing tissues from their natural environment for explants can affect the processes that are being imaged. In addition, while fluorescent tagging of cells and parasites helps to visualize their movements *in vivo*, they move against a background of cells and tissue structures that cannot be seen, and how they influence the behavior of the cells being imaged is unclear. Consequently, there is a need to simultaneously visualize different cell types, and this requires the ability to detect and distinguish multiple colors. The development of fluorescent reporters with non-overlapping emission spectra and exciting these fluorophores (which have different excitation wavelengths) using a single tunable laser source has been a difficult task. One technical advance that has helped deal with this issue is the use of

multiphoton microscopes with dual lasers and multiple photomultiplier tubes for detection, which should make imaging of up to eight colors feasible. Future challenges for this technology include understanding the details of protein–protein interactions *in vivo*, with the application of techniques such as fluorescence resonance energy transfer, which will require increasing resolution and speed of imaging, so that such intracellular events can be better visualized *in vivo*.

Future perspective

The application of real-time imaging techniques to the study of infectious diseases in combination with traditional techniques, such as flow cytometry and immunohistochemistry, has provided a better understanding of the complex and dynamic interplay between pathogens and the immune system. Similar imaging studies in other infection systems, including *Leishmania major*, *Mycobacterium bovis*, *S. enterica*, *Leishmania donovani* and lymphocytic choriomeningitis virus [55,56,62–64], have already fundamentally changed how we think about the immune responses to these pathogens. Nevertheless, real-time imaging of the cellular responses to infection using the various techniques outlined in this report is still at an early stage, and the initial applications have largely been descriptive. While advances in the technology will help visualize many different cell types and structures simultaneously *in vivo*, future challenges include using these approaches to understand the factors that actually influence the behavior of the parasite and immune populations in real-time. The development of uniform analysis software and better methods of quantitation are some of the areas that need focus. Using imaging as a tool to test hypotheses rather than developing questions around the technique of imaging will become the norm as this field matures.

Executive summary

- *Toxoplasma gondii* is a protozoan parasite that can infect a wide range of hosts, including humans, and is potentially life-threatening in immunocompromised individuals.
- Recent advances in imaging techniques and the generation of transgenic parasites and fluorescent reporter mice have improved our understanding of many aspects of the pathogenesis of toxoplasmosis:
 - Recombinant strains of parasites that express fluorescent or bioluminescent reporters (GFP, RFP, YFP, luciferase) have enabled the tracking of parasites *in vitro* and *in vivo* by various techniques, including flow cytometry, bioluminescent imaging and confocal microscopy.
 - Expression of model antigens (ovalbumin, Ea and Lac Z) has enhanced the study of antigen-specific responses.
 - Real-time imaging using multiphoton microscopy has provided a dynamic view of several processes, including parasite invasion, recruitment of innate immune cells (neutrophils and dendritic cells), antigen presentation and migration of effector T cells to sites of inflammation such as the brain.
- The imaging techniques described in this article have been used in other infection systems and provided novel insights into host–pathogen interactions. In addition to technical improvements for deep-tissue imaging of different cell types simultaneously, future challenges include the use of these approaches to

address the role of various host and parasite factors in modulating the immune responses *in vivo*.

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Figure 1. Some of the key stages of the infection process where real-time imaging can provide useful insights

The studies that have addressed some of these specific questions using the imaging techniques outlined in this report are listed (as reference citations).

Figure 2. Example image obtained by multiphoton microscopy within an intact lymph node
The interaction between antigen-specific CD8⁺ T cells (OT1^{GFP}) and dendritic cells (CD11c^{YFP}) during infection with a *Toxoplasma gondii* strain expressing ovalbumin can be seen.