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Use and abuse of dendritic cells by *Toxoplasma gondii*

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Abbreviations: APC, antigen presenting cell; BBB, blood-brain barrier; DCs, dendritic cells; i.p., intraperitoneal; IFNγ, interferon gamma; LN, lymph node; LP, lamina propria; MLN, mesenteric LN; NK cell, natural killer cell; p.o., per oral; pDC, plasmacytoid DC; PP, Peyer's patches; PV, parasitophorous vacuole; STAg, soluble parasite extract; TLR, Toll like receptor

The ubiquitous apicomplexan parasite *Toxoplasma gondii* stimulates its host's immune response to achieve quiescent chronic infection. Central to this goal are host dendritic cells. The parasite exploits dendritic cells to disseminate through the body, produce pro-inflammatory cytokines, present its antigens to the immune system and yet at the same time subvert their signaling pathways in order to evade detection. This carefully struck balance by Toxoplasma makes it the most successful parasite on this planet. Recent progress has highlighted specific parasite and host molecules that mediate some of these processes particularly in dendritic cells and in other cells of the innate immune system. Critically, there are several important factors that need to be taken into consideration when concluding how the dendritic cells and the immune system deal with a Toxoplasma infection, including the route of administration, parasite strain and host genotype.

Introducing *Toxoplasma gondii*

Imagine you are *Toxoplasma gondii*, arguably the most successful parasite on this planet.[1-3] Your ultimate goal is to sexually replicate in a feline, whether it be an Asian leopard, an African lion, a South American puma or maybe the common European pet cat.[4] The way you achieve this is to efficiently infect, yet not kill, an intermediate host and persist to chronicity. The immune system of your intermediate host presents challenges, but also opportunities. At the forefront of what you encounter are dendritic cells (DCs): secretors of defense molecules, mediators of crosstalk to T cells, but also potential shuttle rides to various locations within your host. The consequences of these interactions most likely affect human infections, for example in terms of the prevalence of particular parasite strains, their clinical impact and the way in which the parasite has evolved to manipulate an intermediate host.[1,5-7]

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All warm-blooded mammals including humans and birds are potential intermediate hosts for Toxoplasma and the parasite exists in two inter-convertible stages: the lytic, invasive and active tachyzoites and the slow-growing, encysted bradyzoites. In the definitive host, the feline, the parasite presents as oocysts, which are shed for a limited period in the feces and are highly infective and long-lived.[4] Natural infection usually proceeds by direct contact with oocysts or by ingesting undercooked meat containing bradyzoite cysts. Bradyzoite cysts convert to tachyzoites in the small intestine of the intermediate host and can infect almost all nucleated cells. Here they replicate within a parasitophorous vacuole (PV), egress by lysing the cell and infect neighboring cells. Tachyzoites elicit a potent immune response that eliminates most parasites. However, some tachyzoites can evade this response, convert back to bradyzoites and persist mostly in non-replicative cells such as those in the brain or heart of their intermediate host. Toxoplasma-infected intermediate hosts will present with a chronic infection of bradyzoite cysts for the rest of their lives. Tachyzoites that grow in the absence of a functioning immune system cause tissue destruction, which can be fatal. Alternatively, an overstimulation of the immune system can lead to hyperinflammation with equally fatal consequences to the host. Thus, Toxoplasma needs to carefully strike a balance between inducing and evading the immune response to reach its ultimate goal of quiescent chronic infection in the brain. Clinically, immunocompromised individuals are most at risk of developing encephalitic, ocular or pneumatic toxoplasmosis by reactivation of bradyzoite cysts to tachyzoites in neural or muscle cells.[3,8] Moreover, vertical transmission of an acute infection from a mother to her unborn child can lead to spontaneous abortion, stillbirth or severe birth defects in the form of ocular or neurological deficits.[9] To date no human vaccine is available, the chronic phase of infection is refractory to all anti-toxoplasmotic drugs and diagnosis of a recent infection remains challenging.[10]

Furthermore, it is important to note that Toxoplasma exists as strains of varying genotypes, resulting virulence and potential disease outcome. Isolates from humans and livestock in Europe and North America mostly fall within three clonal lineages, type I, II and III. Of these, type I is highly virulent in mice (lethal to mice at just one parasite), while type II and III Toxoplasma are much less virulent (lethal to 50% of a mouse colony at 10^3–10^5

parasites). Recent progress in sample collection from wildlife and more advanced genotyping methods have securely placed atypical strains on the Toxoplasma population map (reviewed in ref. 11). Currently, it is unclear how and why this population structure has evolved, what the natural hosts for different strains are and how this has impacted parasite selection by hosts' immune responses. Moreover, distinct Toxoplasma protein products, such as Rop16, Rop18, Rop5, Gra2 and Gra15 have been identified as some of the causes of these differences in virulence at least in mice.[12-17] In the future it will become increasingly important to assess studies of the immune response to Toxoplasma by carefully noting the strain of Toxoplasma utilized, its dose and potential attenuation state and the route of administration to which strain of mice.

Immune Control of *Toxoplasma gondii*

Toxoplasma is promiscuous and can infect virtually any nucleated host cell.[18] Asymptomatic infection is achieved by the rapid induction of a strong cell-mediated immune response, which elicits production of high levels of gamma interferon (IFNγ) by natural killer (NK) cells, $CD4^+$ and $CD8^+$ T cells during the acute and chronic phase of infection. Interleukin 12 (IL-12) is the major cytokine inducing IFNγ production by lymphocytes and is derived mainly from dendritic cells, macrophages, neutrophils and monocytes.[19] These two cytokines drive the strong Th1-biased phenotype of $CD4^+$ and $CD8^+$ T cells. Early in the acute phase of infection, NK cell-derived IFNγ is triggered by IL-12 production leading to protection against the infection.[20,21] Essential in both the acute and chronic phase of infection is the IFNγ-producing capability of $CD8^+$ T cells, ultimately aiding in the establishment of chronic infection.[22,23] Eventually, the anti-inflammatory cytokines IL-10, TGFβ and IL-27 are responsible for dampening the inflammatory response and minimizing damage caused by inflammation.[24-28] Toxoplasma seemingly has the ability to determine its own destiny by maximizing its persistence and minimizing host immunopathology, and all of this in the presence of one of the most powerful pro-inflammatory responses known. It is becoming increasingly clear that different types of Toxoplasma elicit different innate immune responses and in mice, virulent Toxoplasma fails to establish a life-long chronic infection, killing the host prematurely due to hyperinflammation or heavy parasite burden depending on mouse genotype.[29-32]

IFNγ activates different intracellular anti-parasitic defense mechanisms within infected cells. In both mice and humans the production of reactive nitrogen intermediates by NK and T cells, macrophages, antigen presenting cells (APC) and neutrophils leads to metabolic poisoning of the parasite.[33-36] IFNγ activates indolamine 2,3-deoxygenase that in turn induces tryptophan degradation and thus inhibits parasite growth.[37-39] The p47 GTPases, a class of large GTPases present in the mouse genome are transcriptionally upregulated in response to IFNγ in cells such as macrophages, astrocytes and fibroblasts, and confer resistance to Toxoplasma by mediating vacuolar degradation.[40,41] In humans and in mice, IFNγ can also upregulate guanylate binding proteins, that are implicated in Toxoplasma vacuolar recognition[42,43] and mediation of bacterial defense mechanisms, such as autophagy, control of reactive oxygen bursts and control of ubiquitinated cargo, reminiscent of potentially important anti-Toxoplasma measures.[44]

In this review, we focus on how DCs are manipulated by the apicomplexan parasite *Toxoplasma gondii* in its natural host to achieve a state of chronic infection. For a brief visual summary please refer to **Figure 1**.

Molecular Recognition of *Toxoplasma gondii* by Dendritic Cells

Toxoplasma orchestrates a carefully balanced string of events between various cell types including neutrophils, DCs and macrophages upon first encountering the host's innate immune defense. A complex network of molecular signaling pathways leads to the activation and regulation of cytokines and ultimately to the production of effector molecules. Here, we focus on the parasite molecules that stimulate or manipulate host responses in DCs. A more global view of the parasites interaction with other cells of the innate immune system has been expertly reviewed previously.[19,45-47]

IL-12 production by DCs is often used as a measure of Toxoplasma recognition by these immune cells. It had been found that the IL-12 response of splenic DCs to soluble parasite extract (STAg) exceeded that of lipopolysaccharide (LPS) and CpG oligonucleotides.[48] In a seminal study, it was recognized that the Toll-like receptor (TLR) adaptor protein MyD88 is a molecule of major importance in host defense to Toxoplasma, with STAg being capable of mediating the induction of IL-12 production by DCs either in vivo or ex vivo (see **Fig. 1**, Infection Site).[49] In the search for which TLR would be the major player in DC activation, TLR11 was identified to signal upon binding a Toxoplasma profilin-like molecule.[50] The resulting IL-12 production was selective to the $CD8\alpha^+$ subset of DCs.[50,51] In a more recent study, TLR11 was localized intracellularly in association with the nucleic acid-sensing TLR trafficking protein UNC93B1.[52] Mice carrying a single point mutation in UNC93B1, retaining the protein in the endoplasmic reticulum thus preventing intracellular TLR trafficking, are highly susceptible to Toxoplasma and produce less IL-12 upon intraperitoneal (i.p.) Toxoplasma bradyzoite infection.[52,53] As direct infection of DCs by Toxoplasma was not required, but in fact very low levels of Toxoplasma profilin were sufficient to induce cytokine production in a transwell assay, it can be speculated that the intracellular location of TLR11 is a very sensitive way to sense Toxoplasma products after phagocytosis.[52] However, $TLR11^{-/-}$ mice survive acute Toxoplasma infection in contrast to the severe lethality seen for $MyD88^{-/-}$ animals, but display increased cyst burden in the chronic phase.[50]

Albeit not demonstrated specifically in DCs, other TLRs, such as TLR2, can also be activated in response to Toxoplasma.[54] TLR2 and TLR4 both signal after binding Toxoplasma glycosylphosphatidylinositol (GPI) anchors,[55] however single absence of either TLR2 or TLR4 in DCs did not reduce the production of IL-12 in response to STAg.[49]

The route of infection plays an important role in TLR recognition of Toxoplasma. It has long been established that

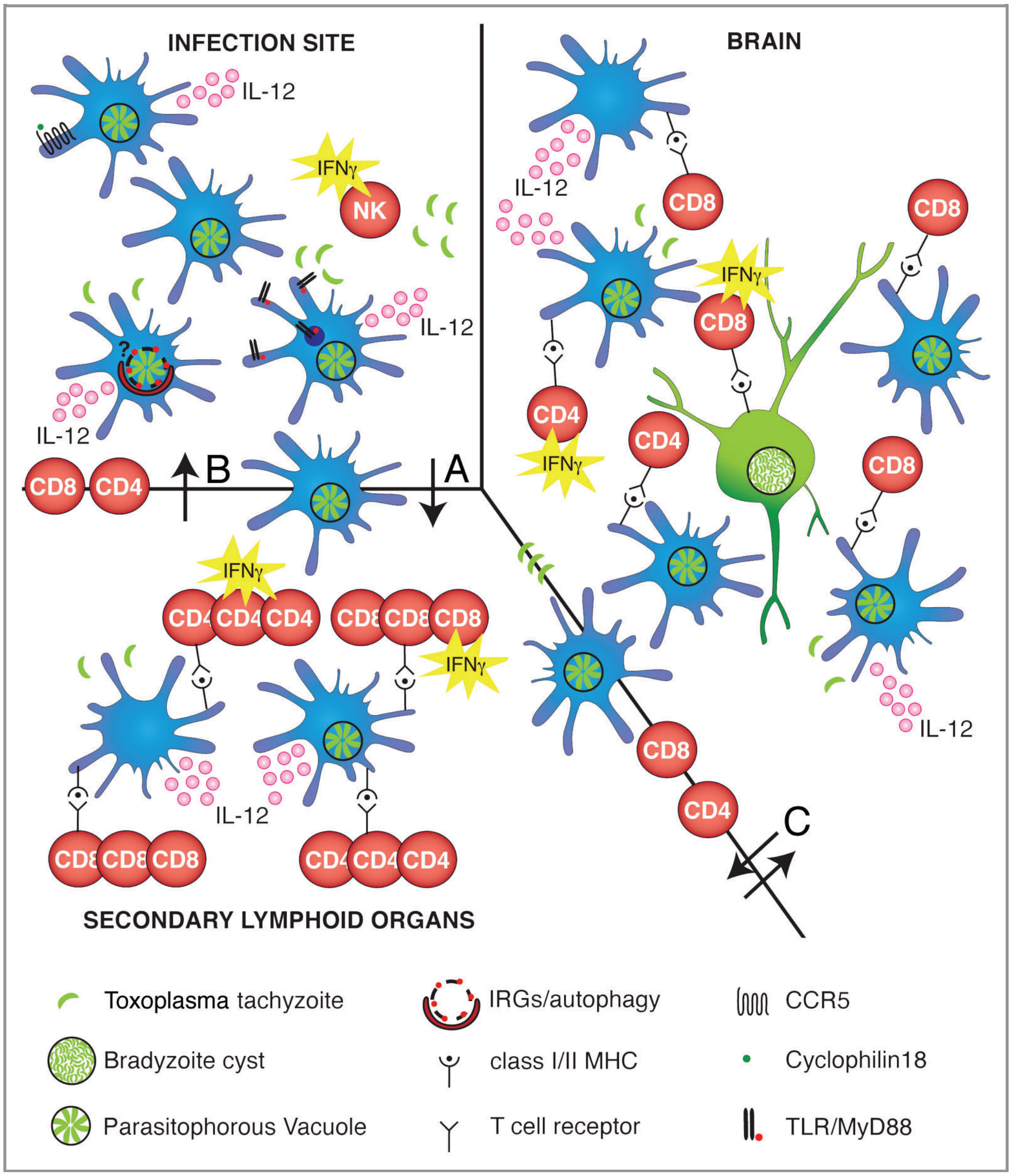

Figure 1. Complexity of dendritic cells interactions with *Toxoplasma gondii* on its way to achieve persistence in the host. Infection site: Toxoplasma enters an intermediate host's body either via the natural route of infection in the gut or as in numerous studies covered here, after being intraperitoneally injected as tachyzoites. Regardless, dendritic cells (DCs) present the first line of defense. Toxoplasma infected and bystander DCs secrete the cytokine IL-12, which in turn stimulates the production of IFNγ by natural killer (NK) cells. Molecular recognition of Toxoplasma products by DCs proceeds via CCR5 sensing Toxoplasma cyclophilin18, TLR-mediated sensing of Toxoplasma profilin or other yet unknown parasite products. It remains to be investigated if IFNγ-upregulated GTPases and autophagy contribute to parasite elimination in DCs as already shown in macrophages. Arrow (**A**): Infected and activated bystander DCs travel from the infection site to the secondary lymphoid organ. Secondary lymphoid organ: Infected and activated bystander DCs produce IL-12 and activate $CD4^+$ and $CD8^+$ T cells to proliferate and produce IFNγ to activate effector molecules and mechanisms. Arrow (**B**): Proliferated and activated $CD4^+$ and $CD8^+$ T cells travel back to the infection site. Arrow (**C**): Infected DCs/monocytes are used as a Trojan horse by Toxoplasma to cross the blood brain barrier. Next activated $CD4^+$ and $CD8^+$ T cells cross the blood-brain barrier. Brain: Toxoplasma-infected and bystander DCs, astrocytes and microglia can present antigens to $CD4^+$ and $CD8^+$ T cells which secrete IFNγ. IFNγ secretion by $CD8^+$ T cells is the dominant and necessary immune response for the parasite to be maintained in the bradyzoite stage and to avoid recrudescence to tachyzoites.

C57BL/6 mice infected per oral (p.o.) with Toxoplasma develop severe pathology in the small intestine due to pro-inflammatory cytokines.[56] DC maturation and migration to the draining lymph node (LN), as well as resulting $CD8^+$ and $CD4^+$ T cell activation are impaired in $TLR9^{-/-}$ mice infected orally with Toxoplasma.[57,58] Parasite-induced damage of the intestinal mucosa is decreased in $TLR4^{-/-}$ mice[59] and in mice treated with broad-spectrum antibiotics[60] in association with decreased pro-inflammatory cytokines. In contrast, $TLR2^{-/-}$, $TLR4^{-/-}$ and $TLR9^{-/-}$ mice infected systemically i.p. with Toxoplasma demonstrate limited susceptibility and no appreciable defect in IL-12 production in response to the infection as opposed to the same animals receiving the parasite orally.[58] Germ-free mice fail to produce IL-12 upon p.o. Toxoplasma infection, an ability that can be rescued by co-administering LPS.[58] The resulting model proposes that parasitic infection causes damage to the intestinal epithelium resulting in the translocation of microflora and subsequent MyD88-dependent signaling and IL-12 production.

DC mobilization and IL-12 production by DCs are moreover mediated by a MyD88-independent mechanism via the chemokine receptor CCR5 (see **Fig. 1**, Infection Site). Following Toxoplasma infection, increased parasite cyst numbers correlate with lower levels of serum IL-12 and IFNγ in CCR5$^{-/-}$ animals.[61] Secreted parasitic cyclophilin (C18) was shown to trigger IL-12 production by DCs albeit to a lesser extent than STAg itself.[62] It is important to note that both of these studies were performed by i.p. injection of STAg. When tachyzoites of type I vs. type II were injected i.p., different panels of chemokines were produced by macrophages at the site of infection possibly leading to the recruitment and retention of different cell populations.[63,64] Natural Toxoplasma infection usually occurs by ingesting infectious oocysts or bradyzoite cysts. Few studies have addressed the role of DCs and how they sense parasite products after oral infection. Gr-1$^+$ inflammatory monocytes were found to be required to mediate mucosal resistance of Toxoplasma after oral infection of B57BL/6 mice, a property not dependent on CD11c$^+$ DCs, but on the presence of the chemokine receptor CCR2.[65]

It is possible that DCs can directly act as effector cells to eliminate Toxoplasma as suggested by their ability to display oxygen-dependent microbicidal activity after IFNγ activation.[66] Moreover, plasmacytoid DCs (pDCs) have been shown to be efficient at autophagy,[67] a process known to eliminate Toxoplasma in primed macrophages[68-71] and to involve the family of p47 GTPases (see **Fig. 1**, Infection Site).[69] The various subsets of DCs possibly recognize either direct infection with Toxoplasma or sense parasite products differently, and are thus important mediators of parasitic elimination and facilitators for the development of an efficient adaptive immune response. We will discuss which DC subsets are responding with IL-12 production after sensing the parasite in the next section.

Toxoplasma gondii Stimulates IL-12 Production by Dendritic Cells

Toxoplasma is a powerful inducer of DC-derived IL-12. IL-12 critically drives Th1 cell development.[72] In the first report linking this cytokine to mouse DCs, splenic CD8α$^+$ DCs stimulated in vivo intravenously with STAg produced IL-12 without priming by IFNγ.[73] Subsequently, CD11c$^+$ DCs, both of the CD8α$^+$ or CD8α$^-$ type, and pDCs have all been shown to play an important role in host resistance to Toxoplasma through their capacity to produce IL-12. Additionally, besides DCs, inflammatory monocytes, neutrophils and macrophages are all implicated in IL-12 production during early phases of infection.[20,74-76] Which of these cellular sources confer protection against the infection in vivo is currently being investigated and debated.

IL-12 is a heterodimer consisting of a p40 subunit that is also shared with the cytokine IL-23. The p40 subunit is covalently linked to a light chain p35 subunit to make biologically active IL-12, also known as IL-12p70. IL-12p40 deficient mice are more susceptible to Toxoplasma infection than IL-12p35 deficient mice, but both are more sensitive than wild-type mice.[77] Mice deficient in IL-23p19, the subunit specific to IL-23, develop normal T cell responses upon Toxoplasma infection and can control parasite replication.[77] Several cell types produce high levels of the heterodimer IL-12p70 and its production depends upon parasite genotype. In macrophages, it has been shown that acute type II infections induce both IL-12p40 and IL-12p70 production while type I infections primarily induce high levels of IL-12p40.[76,78] An attenuated type I parasite in contrast to replicating type I Toxoplasma led to the production of IL-12p70 systemically and in peritoneal cells.[79]

Conventional CD11c$^+$ DCs have been shown to play key roles in host resistance to Toxoplasma bradyzoite cysts administered i.p.[80,81] In the first study, a lineage ablation approach was used by transgenic expression of simian diphtheria toxin receptor under control of the CD11c promoter. Diphtheria toxin administration to these mice causes transient deletion of CD11c-expressing cells and renders these animals more susceptible to i.p. Toxoplasma bradyzoite infection. In the second study, MyD88 was exclusively deleted by Cre recombinase in CD11c-expressing cells. This decreased early IL-12 production, again after i.p. infection with Toxoplasma bradyzoite cysts, and delayed the IFNγ response by NK cells, rendering the mice more susceptible to infection. While both elegant studies, they do not formally exclude the possibility that IL-12 is produced by CD11c-expressing macrophages. Besides conventional DCs, pDCs have been shown to expand after p.o. or i.p. infection with type II parasites.[82] In vitro infected pDCs were shown to produce IL-12p40, a phenomenon dependent on TLR11.[82] Upon deletion of the transcription factor interferon regulatory factor 8 (Irf8), mice infected i.p. with type II bradyzoite cysts failed to transcribe IL-12p40, a property ascribed to either macrophages and/or dendritic cells and rendering the mice susceptible to the infection.[83] Batf3$^{-/-}$ mice are specifically defective in generating CD8α$^+$ DCs and exhibited decreased IL-12 and IFNγ production and succumbed during the peak of acute infection to Toxoplasma type II tachyzoite administered i.p.[84] Splenic CD8α$^+$ DCs expanded from 2.5% to 17% of the total DC compartment after infection in wild-type mice, and interestingly the resulting CD8$^+$ T cell response to two endogenous Toxoplasma antigens (Gra4 and Gra6) investigated was defective. Another report finds circulating Ly6C$^+$ monocytes to be recruited to the site of Toxoplasma infection and to differentiate into macrophages and IL-12 producing CD11b$^+$ CD8α$^-$ DCs. NK cell-derived IFNγ was deemed to be crucial for monocyte differentiation at the site of infection.[85] This study was performed using i.p. infection with Toxoplasma type II bradyzoite cysts. Both studies reciprocally infect with bradyzoite vs. tachyzoites i.p. and confirm that this changes the major IL-12 producing DC subset originally described. Thus it seems imperative to correlate the original question asked with the type and route of Toxoplasma infection chosen.

It has been shown that injection of STAg renders DCs unresponsive to further IL-12 production triggered by subsequent

Toxoplasma infection.[86,87] This phenomenon of DC paralysis is induced by lipoxin A4, an arachidonate inhibitor of inflammation.[86-88] Lipoxin A4 activates the receptors AhR and LXAR in DCs and thus triggers the expression of SOCS-2, a suppressor of cytokine signaling.[89] Consequently, SOCS-2 partially dampens the pro-inflammatory IL-12 mediated response to Toxoplasma, in part by downregulating CCR5.[89]

Even though systemic administration of STAg alone induces rapid splenic DC-derived IL-12,[73,86] maximal levels of bioactive IL-12p70 are produced only after receiving a second signal via CD40 ligation on DCs.[90] Infection with type II Toxoplasma can also induce splenic $CD8\alpha^+$ and $CD8\alpha^-$ DCs to become activated and produce IL-12 dependent upon CD40 cross-linking.[91] CD40L knockout mice produce lower levels of IL-12, however, this is enough to induce IFNγ production to ensure the survival of the mice through acute infection.[92] In human DCs, CD40-CD40L interaction is required for IL-12 production in response to Toxoplasma infection.[93,94] Interestingly, this may explain why patients defective in CD40L expression are more susceptible to intracellular infection linked to T cell mediated immunity.[95]

Once Toxoplasma reaches the brain it encysts as bradyzoites. IL-12 production by $CD11c^+$ DCs isolated from the brain has been found to persist for one year post-infection.[96] Continued production of IL-12 in the chronic phase of infection prevents parasite recrudescence.[97]

What are the long-term consequences of an intact IL-12 response mostly mediated by DCs for the outcome of a Toxoplasma infection? In bacterial listeriosis, IL-12 via IL-12p35 is thought to promote the generation of effector memory $CD8^+$ T cells, but dampen the differentiation of long-term central memory $CD8^+$ T cells.[98] For a replication attenuated type I Toxoplasma strain, similar results were found as in IL-12p35 deficient animals $CD62L^{low}KLRG1^+$ $CD8^+$ effector T cells did not develop.[99] Also, IL-12 appeared dispensable or maybe even slightly negative for central memory $CD8^+$ T cell differentiation.[100] Nevertheless, IL-12p40 is required for protective immunity elicited by vaccination with the replication deficient strain and re-challenge with type I replicative RH type I Toxoplasma.[101] Thus, replication deficient type I Toxoplasma probably induces enough IL-12p70 to mediate long-lasting $CD8^+$ T cell-mediated immunity. Mice infected i.p. with type I vs. type II Toxoplasma tachyzoites both expressing and secreting ovalbumin develop fewer DCs at the site of infection and fewer antigen-specific $CD8^+$ T cells. In this study, IL-12p70 administration during type I infection moderately rescued this deficiency.[64] It remains to be investigated how different strains of Toxoplasma induce varying levels of IL-12 and what the consequences for long-term protective immunity are.

Toxoplasma gondii Modulates Dendritic Cell Interactions with T Cells

Dendritic cells are known as professional APCs that are specialized in loading peptides derived from exogenous and endogenous sources onto both MHC class I and II molecules for presentation to $CD8^+$ and $CD4^+$ T cells respectively.[102] Toxoplasma is controlled in the acute and chronic phase of infection by $CD8^+$ T cells[22,23,103,104] which means that its antigens are effectively presented in the context of MHC class I (see **Fig. 1**, Secondary Lymphoid Organs). Potential problems arise when thinking about Toxoplasma antigen presentation from infected DCs. First, the PV has long been believed to be a nondegradative and nonfusogenic compartment.[105] Thus, potential antigens contained in this compartment need to escape and with a pore limit of 1300 daltons, this seems an inexplicable task.[106] Second, infection of DCs and macrophages by Toxoplasma interferes with several signaling pathways that are crucial to develop protective immunity.[107] Toxoplasma can replicate in nonhematopoietic cells as well as professional APCs. It is not clear which cell type in general primes T cells in a Toxoplasma infection in vivo.

Most studies to date have been undertaken with model antigens such as ovalbumin expressed and secreted into the PV by type I or type II parasites.[64,108-113] Recently, four endogenous Toxoplasma MHC class I epitopes were identified, restricted to two separate class I MHC alleles.[100,114,115] The $H\text{-}2L^d$ MHC locus expressed by BALB/c mice has been ascribed to mediate resistance to toxoplasmic encephalitis in the chronic phase of infection in H-2d mice,[116-118] thus BALB/c mice were used for the two former studies. Epitopes from the two Toxoplasma's dense granule proteins Gra4 and Gra6 were identified, as well as one from the inactive rhoptry kinase Rop7. In order to be able to use basic immunological tools confined to C57BL/6 mice, the last study identified another epitope from an unidentified Toxoplasma protein called T57 on this background. Moreover, using somatic cell nuclear transfer, antigen-specific transnuclear $CD8^+$ T cell mice for all of these epitopes were generated and are easily maintained (ref. 119 and unpublished results).

Bone marrow-derived DCs infected in vitro with Toxoplasma tachyzoites expressing and secreting ovalbumin have been shown to induce $CD8^+$ T cell proliferation dependent on the transporter associated with antigen processing (TAP).[112] Additionally, the generation of the endogenous epitope GRA6 in DCs is dependent on the ER-associated aminopeptidase.[114] Cross-presentation of dead parasite material out of uninfected DCs or general splenocytes was ruled out as a presentation pathway in a number of studies employing ovalbumin-secreting tachyzoites.[108-110] However, two reports show cross-presentation by bystander DCs both in the LN early in infection as well as during toxoplasmic encephalitis.[120,121] When investigating which antigens targeted to intra-parasitic and intra-vacuolar locations would be efficiently presented by bone-marrow DCs or macrophages ex vivo to OT I T cells, it was determined that only antigen secreted into the vacuole would be appropriate.[113] Employing this strain of transgenic Ova-secreting parasites, the ER was speculated to fuse with the vacuole[108] to enhance antigen presentation, a process dependent on Sec22b.[122]

The role of DCs in presenting Toxoplasma antigens to $CD4^+$ T cells is less clear. It has been proposed that Toxoplasma profilin is a major immunodominant antigen that can simultaneously activate DCs and be processed to be presented to $CD4^+$ T cells dependent upon TLR11.[51] Active invasion by Toxoplasma tachyzoites blocks LPS-induced bone marrow-derived DC maturation in vitro and their subsequent capacity to activate $CD4^+$ T cells.[123] In contrast, pDCs expand during acute i.p. tachyzoite infection with Toxoplasma, upregulate MHC class II and co-stimulatory molecules and prime $CD4^+$ T cells.[82] IL-12 production and pDC maturation was dependent on TLR11 which suggests that this DC subset is important to control the infection in vivo.[82] Recently, a Toxoplasma 15-mer epitope presented on I-A^b MHC molecules in C57/BL6 mice has been identified and immunization with this peptide was shown to confer significant protection against parasite challenge.[124] A peptide-specific T cell response was observed by these authors even with heat-killed parasites, while another study found enhanced presentation of the secreted version of the model antigen ovalbumin.[111] Further studies with this newly identified immunogenic CD4 Toxoplasma epitope will facilitate the understanding of the CD4 antigen processing pathway and the exact role $CD4^+$ T cells play in controlling the infection.

After natural oral infection with Toxoplasma, lamina propria DCs are hampered in their ability to induce regulatory $CD4^+$ T cells in vitro. Consequently, IL-2 production in the gastrointestinal tract and in the periphery is reduced leading to immunopathogenesis via heightened IFNγ-producing effector T cells.[125] Also, gut DCs exposed to Toxoplasma antigen in vitro induce fewer regulatory T cells.[125]

It will be important to revisit some of the specifics of antigen presentation to $CD8^+$ T cells using the knowledge of the true endogenous epitopes and their associated tools, as there may be crucial differences depending on parasite strain and epitope under study, antigen expression level, mode of infection and time-point post-infection. Moreover, antigen presentation to $CD4^+$ T cells remains virtually uncharacterized, yet activated $CD4^+$ T cells are found equally numerous as $CD8^+$ T cells in a chronically Toxoplasma-infected mouse brain. Knowledge of how DCs manipulate the generation of this effector T cell population and control the levels of regulatory T cells may have profound influence on the generation of vaccine-mediated immunity.

Dendritic Cells are Hijacked by *Toxoplasma gondii*

Commonly, Toxoplasma infects its intermediate host via the oral route or in the case of a congenital infection it passes through the placenta. Oocysts or bradyzoites can be ingested by an intermediate host in contaminated water, soil or meat and will end up in the gut. The dissemination out of the gastrointestinal tract before activation of an immune response is crucial for the establishment of a chronic infection and Toxoplasma must cross the intestinal epithelium to achieve this. Bradyzoites and sporozoites released from oocysts infect cells of the small intestine where they convert to fast-replicating and highly invasive tachyzoites.[18,126] This is a rapid process. Already one hour after oral infection with bradyzoites, parasites can be found in the lamina propia (LP).[126,127] Within two hours, parasites are transported to the LNs and they are able to reach the brain within six days of initial contact with the host.[126] To travel quickly Toxoplasma uses highways within the host's body, namely the bloodstream and the lymphatic system. As extracellular parasites are more vulnerable to elimination from the blood than intracellular ones,[128] Toxoplasma hijacks host cells and uses them as means of transportation (see **Fig. 1A and C**).

Toxoplasma can infect any nucleated cell, but it has a preference for cells of the immune system, mainly DCs.[129-131] DCs are present in many tissues, scanning the body for invading pathogens. As described above, upon detection of an intruder, they raise an alarm by producing cytokines that attract and activate other cells of the immune system, and migrate to LNs to activate pathogen-specific T cells.[132] It may seem paradoxical that Toxoplasma chooses to target the cell type that predominantly fights infections. However, the parasite does not want to kill its intermediate host. Hence, triggering the immune system in order to be kept under control while hitching a ride may thus be of interest to the parasite. Because of their motile properties, DCs are likely candidates to act as Trojan horses to disseminate Toxoplasma to other tissues. DCs infected with Toxoplasma exhibit a hypermotility phenotype.[131,133-136] Type II tachyzoites are superior to type I at inducing migration of human DCs in vitro,[131] and murine DCs in vivo.[134] Lambert et al.[133] showed that only live Toxoplasma can induce a migratory phenotype in DCs, suggesting that it is not simply the effect of recognition of the pathogen and maturation of the DCs, but active manipulation of the DCs by Toxoplasma. In contrast to mouse DCs, human DCs migrate in response to soluble antigens produced by both type I and type II strains of Toxoplasma without maturation.[137] Toxoplasma is not the only pathogen that manipulates migratory function of DCs as *Neospora caninum*-infected DCs exhibit the same phenotype.[135] Importantly, type II Toxoplasma use DCs more effectively as a shuttle, while type I parasites are predominantly using the extracellular route.[134] This serotype difference in the infection/migration route may dictate by the greater ability of type I tachyzoites to cross the epithelial barriers as an extracellular parasite than type II tachyzoites.[127] Toxoplasma hidden inside DCs can travel to the secondary lymphoid tissue and to other organs of the body away from the inflammatory site and into the circulation (see **Fig. 1A**).

Toxoplasma infects different subtypes of DCs including pDCs.[131,134,138] Bierly et al.[138] showed that in an i.p. infection with the type I Toxoplasma tachyzoites, $CD11c^+GR1^+$ DCs expressing pDC markers B220 and PDCA-1 were preferentially infected and responsible for shuttling Toxoplasma from the peritoneum to the spleen. Additionally, using $CCR2^{-/-}$ mice they demonstrated that this receptor unlike CCR5 was important for migration.[138] Nevertheless, first contact of Toxoplasma with DCs in the course of a natural oral infection will occur in the small intestine, where Toxoplasma invades epithelial cells.[127] Resident intestinal DCs are likely to be among the first leukocytes to be infected by Toxoplasma. Many different subtypes of conventional and pDCs residing in the intestinal mucosa have been described (reviewed in refs. 139 and 140). However, the question of which DC subsets are important for Toxoplasma dissemination in early mucosal infection has not been fully addressed. In vitro $CD11c^+MHCII^+$ DCs isolated from LP of the small intestine and Payer's patches (PP) can be effectively infected by type I and type II Toxoplasma tachyzoites.[134] When Courret et al.[130] orally infected mice with type II Toxoplasma bradyzoite cysts, mimicking a natural infection, two days post-infection the majority of CD11c$^+$CD11b$^{+/-}$ cells in the LP were parasitized. This suggests that CD11c$^+$CD11b$^{+/-}$ cells present in the LP are likely to be used by the parasite to travel from the intestine to the mesenteric lymph nodes (MLN) and PP.

It is not clear whether Toxoplasma transported in DCs to the LNs needs to change its vehicle to disseminate further into the bloodstream and to other organs. It is generally accepted that DCs do not leave the secondary lymphoid organs once they have entered. However, there is indirect evidence for DCs leaving LNs to act as Trojan horses.[141] It is conceivable that the hyper-migratory phenotype of DCs infected with Toxoplasma would allow them to exit the LNs and via the thoracic duct enter the bloodstream. This hypothesis still awaits verification. Another, more probable scenario is that the DCs carrying Toxoplasma to the LNs are being lysed, and released tachyzoites can in turn infect neighboring cells that enter the blood stream. As reported by Courret et al.[130] parasitized leukocytes found in the blood are of a different phenotype (CD11c$^-$CD11b$^+$) than those in LNs (CD11c$^+$CD11b$^{+/-}$).

Toxoplasma gondii Employs Dendritic Cells to Enter Immune-Privileged Organs

The chronic phase of Toxoplasma infection is characterized by cysts of the bradyzoite stage localized in different body tissues of the intermediate host. However, preferential target organs for Toxoplasma are immune privileged sites like the brain or the eye.[3,8] In these organs, as well as in the developing fetus (targeted by the parasite during acute infection of a pregnant female) immune responses are limited or prevented. This enables Toxoplasma to hide from surveillance by the cells of the immune system as well as from circulating antibodies. To reach these organs Toxoplasma has to pass barriers protecting them from exaggerated immune responses. In the case of the brain this involves crossing the blood-brain barrier (BBB) while to infect the fetus Toxoplasma must cross the placenta.

Entering the placenta. In the case of acute infection during pregnancy, Toxoplasma is able to pass the placental barrier and infect the fetus.[142,143] The mechanism by which this happens is poorly understood. One possible route is directly via the maternal blood to the cells forming the fetal part of the placenta. Another option is that infected maternal leukocytes bring Toxoplasma to the decidua—the maternal part of the placenta that participates in the exchange of oxygen, nutrients and waste with the developing fetus as well as protecting the fetus from the maternal immune system.[144] The infected maternal leukocytes will be killed by residing NK cells or lysed by the multiplying parasites. Released extracellular tachyzoites may cross to the fetus by infecting cells of the fetal part of the placenta. A number of in vitro studies have shown that placental cells can be infected by Toxoplasma; however, no strain differences were noted in infection capability.[145]

An alternative mechanism for Toxoplasma to traverse the placenta is to again use host cells as Trojan horses. Maternal leukocytes rarely travel to the fetus. However, it has been suggested that maternal APC, possibly decidual DCs cross the placenta to reside in fetal LNs where they induce the development of regulatory T cells.[146] This type of DC could give Toxoplasma the opportunity to shuttle across the placenta to infect the fetus.

In an in vitro system, Toxoplasma type II exhibited a higher dependency on DC-mediated transmigration for efficient translocation across polarized cellular monolayers in contrast to type I parasite, which transmigrated as extracellular tachyzoites.[135] These findings are consistent with the notion that Toxoplasma type I parasites preferentially disseminate extracellularly,[127] whereas type II parasites preferentially exploit the shuttling-function of DCs.[134] As Toxoplasma type II causes more vertical infections in comparison to type I,[5] it is likely that the Trojan horse mechanism is more effective in crossing the placenta and infecting the fetus than extracellular transmigration.

Crossing the blood-brain barrier. Toxoplasma can invade endothelial cells, but its ability to cross the BBB as extracellular parasites in vivo needs clarification. Only few Toxoplasma tachyzoites injected i.v. in mice were observed in the brain in contrast to those injected i.v. as intracellular parasites.[128] Thus, Toxoplasma most likely uses leukocytes as Trojan horses to enter the brain. Access of immune cells to the brain is limited but it does occur, not only during neuroinflammation, but also as an immune surveillance mechanism (reviewed in ref. 147). It is therefore reasonable to hypothesize that DCs transporting Toxoplasma from the infection site to the LNs could play the role of Trojan horses sneaking it into the brain (see **Fig. 1C**). However, a study by Courret et al.[130] suggests CD11c$^-$CD11b$^+$ cells, most likely monocytes, play this role. They showed that both CD11c$^+$ and CD11b$^+$ cells circulating in blood are able to cross the BBB and can be detected in the brain of infected mice seven days after p.o. infection with type II Toxoplasma cysts. Nevertheless, at day seven post-infection (the earliest time point for parasite detection in the brain) the majority of brain mononuclear cells containing parasites were of CD11c$^-$CD11b$^-$ or CD11b$^+$ phenotype and only at day 15 post-infection more CD11c$^+$ cells were found to be parasitized. That would suggest that CD11c$^-$CD11b$^+$ cells are carrying Toxoplasma across the BBB. CD11c$^-$CD11b$^+$ cells are in general considered to be monocytes or macrophages, however DCs of that phenotype have also been reported.[148] In contrast, Lachenmaier et al.[136] using i.v. injection of type I Toxoplasma-infected cells showed that there is no difference between the ability of CD11b$^+$ and CD11c$^+$ cells in crossing the BBB, suggesting that both macrophages and DCs are used by Toxoplasma as Trojan horses. Discrepancies between these two studies can probably be explained by the different strains of Toxoplasma used (type I vs. type II) and different routes of infection (i.v. vs. p.o.) where the model used by Courret et al. most closely reflects the natural course of infection.

To fully characterize which leukocytes are important for dissemination of Toxoplasma to the brain during natural oral infection additional studies should be performed taking into account different parasite strains, stage of infection and infection route.

DCs in the Infected Brain Facilitate Persistence of *Toxoplasma gondii*

Upon entry to the brain tachyzoites infect astrocytes, neurons and microglial cells (see **Fig. 1**, Brain). The rapidly replicating tachyzoites transform into the very slowly replicating bradyzoites, which form cysts that can persist throughout the lifetime of the host. Infiltration of the parasite is followed by expansion and recruitment of mononuclear cell populations in the brain. There are multiple reports indicating substantial increase in the number of DCs in the brain upon infection with Toxoplasma.[96,149-151] Different sources of these DCs have been reported. Fischer at al.[96] showed expansion of a population of brain DCs that originates from bone marrow precursors and expands in the brain upon i.p. infection with cysts of Toxoplasma type II. This occurs relatively late, four weeks after infection and is dependent on GM-CSF production.[96] However, occurrence of this DC expansion is not only specific for an infection with Toxoplasma as a similar population arises in the brain upon induction of experimental autoimmune encephalomyelitis (EAE).[152] Additionally, John et al.[151] reported that DCs can be recruited to the Toxoplasma infected brain from the circulation and that this recruitment is dependent on $G\alpha_i$-coupled receptor signaling and engagement of LFA-1.

What is the role of DCs in the brain in a Toxoplasma infection? DCs isolated from Toxoplasma-infected brains were shown to be the main producers of IL-12 and in vivo IL-12 production was associated with dividing parasites (see **Fig. 1**, Brain).[96] IL-12 is important for maintaining IFNγ production by T cells.[72] Regulated IL-12 production by DCs in the Toxoplasma-infected brain is a well-balanced mechanism essential to eliminate rapidly dividing tachyzoites that may be released from sporadically bursting cysts, but not responding to the latent bradyzoite form of the parasite thus preventing encephalitis.

With the development of new imaging techniques including two-photon microscopy, it is now possible to visualize real-time DC-T cells interactions in the brain.[149,152] It has been shown that DCs in Toxoplasma-infected brain interact with T cells and that many of the DCs are localized proximal to infected cells or free tachyzoites.[149,151] Schaeffer et al.[149] demonstrated that DCs and $CD11c^-CD11b^+$ cells in Toxoplasma-infected brain form aggregates around isolated, mainly extracellular parasites, but not intact cysts suggesting that DCs can sense free parasites released from bursting cysts and shape a barrier around them to prevent the infection spreading.

Moreover, DCs isolated from the Toxoplasma-infected brains were shown to have a mature phenotype and to be able to trigger antigen-specific T cell responses.[96,151] Aggregating DCs observed by Schaeffer et al.[149] were surrounded by antigen-specific T cells suggesting their role in antigen presentation and T cell activation. These DCs were not parasitized by Toxoplasma, thus cross-presentation of the Toxoplasma-derived antigens to the $CD8^+$ T cells is conceivable.[149]

Taken together, brain-infiltrating DCs may be crucial for local restimulation of Toxoplasma antigen specific effector T cells during Toxoplasma infection and may contribute to the chronicity of the host response.

Concluding Remarks and Outlook

Toxoplasma has learned to exploit and subvert DCs of its intermediate host's immune system to achieve persistent chronic infection. It is becoming clear that Toxoplasma can stir cytokine production by DCs, use DCs to mediate interactions with T cells and employ DCs to circumnavigate the host. Identification of further molecular players of Toxoplasma that can differentially modulate DC function will be key to understanding the link between innate immune recognition and protective adaptive Th1-mediated immunity. Equally important is to identify host effector molecules and mechanisms that elicit defined immune responses to the parasite in DCs and other effector cells. Host-pathogen interactions are like two sides of the same coin and cannot be investigated without taking both into account. Careful dissection of parasite and host genotype, route of infection and stage of the parasite are essential to properly address and answer questions of how Toxoplasma became the most successful human parasite. The completion of several Toxoplasma strain genomes of different virulence combined with host genomes will facilitate identification of new host-pathogen interaction mechanisms.[153,154] Furthermore, Toxoplasma might transcriptionally modify DCs to serve its desired purpose, which can now easily be studied using tools for epigenetic gene regulation. As it is becoming clear that there are major differences in how different strains of Toxoplasma exert their different virulence phenotypes, it will be imperative to distinguish between their ability to subvert the immune response in general and DCs in particular. This knowledge will be important in designing effective counter-measures, particularly vaccines.

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