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Immunity and *Toxoplasma* retinochoroiditis

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Summary

Toxoplasma infection accounts for up to 50% of all cases of posterior uveitis worldwide. In this review the control of _Toxoplasma_ infection generally, and specific in the eye, by the immune system is discussed.

Keywords: inflammation, ocular, *Toxoplasma*

Introduction

Toxoplasma gondii is an obligate intracellular parasite and an important opportunistic pathogen in humans. The life cycle of *T. gondii* has been well characterized. In brief, the parasite has three stages: tachyzoites responsible for active infection, bradyzoites found in tissue cysts and sporozoites found in environmentally resistant oocysts formed after the sexual stage of the life cycle. *T. gondii* relies upon the definitive host, members of the Felidae family, including domestic and feral cats, for the sexual reproductive stage, which takes place in their intestinal mucosa. Sporozoites excreted in the faeces of the cat enter the tachyzoite stage following ingestion by an intermediate host, which may include virtually any warm-blooded animal including humans. On entry into host cells the parasite forms the bradyzoite containing parasitophorus vacuole (PV) that protects it from destruction by cell components. *Toxoplasma* infection can be acquired either in food or by vertical transmission from the mother (congenital toxoplasmosis) leading to severe defects if contracted in the first trimester of pregnancy, or post-natally, which in immunosuppressed individuals can lead to fatal encephalitis [1]. The most common manifestation of the disease in both immunocompetent and immunocompromised individuals is retinochoroiditis, where inflammation in response to the organism causes intra-ocular inflammation (uveitis) and may result in a full-thickness scar with loss of retinal tissue and subsequent loss of vision [2].

Genotype

Three main genotypes of *T. gondii* are responsible for infection in humans: types I, II and III, which differ at the DNA sequence level by 1% or less [3]. Emerging evidence suggests that while the three types are found ubiquitously, the pattern of disease that each produces may differ. Type II is the most common cause of systemic *Toxoplasma* infection in Europe and the United States [4], yet it is the type I strains, long recognized to be most virulent in murine models [5], which appear to cause the greatest ocular morbidity. Type I *T. gondii* has been associated with particularly severe cases of ocular toxoplasmosis [6], and in Brazil type I was the only genotype found in retinochoroidal specimens of patients with ocular toxoplasmosis [7]. The severity of infection between strains is due in part to molecules such as ROP16, that is expressed in type II, but not type 1 or type III strains, and is a potent inducer of interleukin (IL)-12 [8].

Control of acute infection

Most of our current understanding of the control of *T. gondii* infection comes from murine models [9]. Control of acute infection in mice is mediated by IL-12-induced interferon (IFN)-γ production by natural killer (NK) cells [10]. *In vitro* studies have demonstrated that NK cells could not produce IFN-γ following exposure to parasite antigen when cultured alone. However, their ability to produce IFN-γ

was upregulated on the addition of adherent spleen accessory cells [11]. Anti-IL-12 antibodies abrogated the production of IFN-γ by NK-enriched spleen cells from SCID mice that had been stimulated by exposure to *T. gondii* tachyzoites. Conversely, treatment of infected SCID mice with recombinant IL-12 prolonged survival of these animals. IL-1β is involved in this process, as the addition of anti-IL-1β antibodies to *T. gondii* stimulated splenocyte cultures halted the production of IFN-γ completely [12].

Dendritic cells (DC) are the first cell type to produce IL-12 in the spleen in response to soluble tachyzoite antigen (STAg), without a requirement for priming by IFN-γ or by T cell signalling [13]. However, IL-12 production was transient and returned to baseline levels 24 h after intravenous injection of mice with live tachyzoites or STAg. IL-12 production could not be resumed for up to 1 week post-infection, termed DC paralysis. Paralysed DC could produce IL-12 following stimulation with STAg *in vitro*, and it was concluded that failure to produce IL-12 following restimulation *in vivo* was not caused by DC death or unresponsiveness, but that the splenic microenvironment may affect the ability of DC to produce IL-12, and thus play an important role in both the early immune response to parasite infection and protection from immunopathology [14].

Macrophages have also been shown to be an important source of IFN-γ. *In vitro* studies have demonstrated that challenge of mice with *T. gondii* resulted in increased NF-κB activity and resistance to infection, mediated by production of IFN-γ. The NF-κB family member RelB was shown to be essential to this process [15,16]. Macrophages are also a source of IL-12, which was shown by exposure of peritoneal macrophages to either live parasites or STAg. Treatment with anti-IL-12 antibodies reduced the production of IFN-γ and enhanced T helper 2 (Th2) cytokine synthesis [17].

Neutrophils are also involved in the initial response to *T. gondii* infection. Mice injected intraperitoneally with the virulent recombinant human strain of *T. gondii* responded with an influx of neutrophils into the peritoneum, which peaked at 8 h post-infection [18]. Both human and murine neutrophils have been shown to produce the cytokines IL-12 and tumour necrosis factor (TNF) in response to STAg in the absence of IFN-γ *in vitro*.

Parasite-induced neutrophil IL-12 and chemokine ligand 2 (CCL2) induction was shown to be myeloid differentiation primary response protein 88 (MyD88)-dependent, a common adaptor molecule for Toll-like receptor (TLR) signalling. However, only CCL2 was controlled by TLR-2 signalling, while IL-12 production utilized a separate pathway [19]. This may be TLR-11, which also uses MyD88, and responds to profilin, a potent stimulator of IL-12 by murine DC. Interestingly, profilin is also an immunodominant antigen for $CD4^+$ T lymphocytes [20,21].

Recent studies on novel IL-12 family members have shown that *T. gondii* infection of IL-27 receptor-deficient mice led to severe encephalitis associated with a strong IL-17 response as well as inhibition of IL-2 [22,23]. IL-17 receptor-deficient mice showed increased mortality following infection with *T. gondii* because of failure to attract neutrophils into infected sites during the early response [24]. IL-23 can, in the absence of IL-12, mediate infection with *T. gondii*, although it cannot substitute fully and any effects are secondary to IL-12 [25]. The potential role of these cytokines in control of *Toxoplasma* retinochoroiditis in humans needs to be investigated.

Mechanisms of immune control

Nitric oxide

In vivo studies have been carried out in mice, which showed that the injection of an anti-IFN-γ monoclonal antibody resulted in the death of intraperitoneally infected animals [26]. Murine microglial cells, following stimulation by IFN-γ and lipopolysaccharide, synthesized nitric oxide (NO) from L-arginine and the addition of N^Gmonomethyl-L-arginine (N^GMMA), a competitive inhibitor of the L-arginine-dependent NO pathway, prevented destruction of the parasite within these cells [27]. However, investigations in inducible NO synthase (iNOS) knock-out (KO) mice ($iNOS^{-/-}$) have demonstrated that although macrophages from these animals have impaired microbicidal mechanisms, the mice are able to survive acute infection by *T. gondii*. The use of anti-IFN-γ and anti-IL-12 antibodies abrogated this resistance to early infection, which indicated that NO-independent mechanisms may be involved in the control of acute disease [26,28].

Cytokines

While IFN-γ-mediated killing is important in control of infection, the rates of mortality in both $IL\text{-}4^{-/-}$ and $IL\text{-}10^{-/-}$ infected animals were significantly greater than immunocompetent controls. When infected with *T. gondii*, IL-10 KO mice show a massive generalized lymphocytic infiltration with extensive hepatic necrosis, but no inflammation seen in the central nervous system. Protection of IL-10-deficient mice against *T. gondii* challenge could be induced when STag was administered 24 h before challenge. This was accompanied by reduced IL-12 production and CCR5 expression [29].

Spleen cell culture supernatants from $IL\text{-}4^{-/-}$ mice exhibited greater levels of IL-12 and IFN-γ. Post-infection the brains of wild-type (WT) mice contained many tissue cysts and non-encysted organisms within necrotic lesions. However, $IL\text{-}4^{-/-}$ mice exhibited few cysts and no necrotic lesions, which indicated that the long-term effects of IL-4 may be damaging to the host by inhibiting the action of proinflammatory cytokines [30].

Novel pathways

In addition to NO and cytokines, several novel pathways have been identified recently that may protect against *T. gondii* infection. STag induced the production of lipoxinA4 (LXA_4), via the arachdonic acid pathway, that inhibited DC migration and IL-12 production. Levels of LXA_4 increased steadily with time during acute infection, and remained high during chronic disease [31,32]. To date, this pathway has not been studied in human *T. gondii* infection.

Evidence of different effector pathways has been found in murine models. After IFN-γ stimulation of infected murine astrocytes, p47GTPases accumulate in the PV leading to PV deterioration and parasite death. The p47 resistance system has also been shown to be involved in the control of *Salmonella typhimurium*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *M. avium*, *Leishmania donovani* and *Trypanosoma cruzi*; however, this system is absent in man [33]. Similarly, lysosomal-mediated degradation of *T. gondii* occurred after invasion of macrophages activated *in vivo* by IFN-γ. Pathogen elimination was dependent upon p47GTPase. As well as PV destruction, the parasite plasma membrane was stripped away and denuded parasites enveloped in autophagosome-like vacuoles that fused with lysosomes [34]. A role for autophagy has also been suggested. CD40 ligation induced *T. gondii* killing independent of iNOS, IFN-γ, p47GTPase, oxygen intermediates or tryptophan starvation. Vacuole/lysosome fusion mediated via CD40 caused co-localization of PV and LC3, a marker of autophagy, and anti-*T. gondii* activity was shown to be dependent upon autophagy [35]. Whether or not autophagy is important in human cells has yet to be identified.

Available evidence therefore suggests that stimulation of DC, neutrophils and macrophages by invading *T. gondii* leads to the production of IL-12, TNF and IL-1β. These cytokines stimulate the production of IFN-γ by NK cells, which in turn activates killing of *T. gondii* within infected macrophages by the production of NO, lipoxin production and other mechanisms.

Control of chronic infection

During chronic infection T cells have an important role in the control of toxoplasmosis and two subsets of T cells, $CD4^+$ and $CD8^+$, contribute to this protective role. Depletion of both these subsets is required to reactivate the disease, whereas treatment with either anti-CD4 antibody or anti-CD8 antibody alone did not result in increased pathology or mortality. Both T cell subsets were capable of producing IFN-γ and it was suggested that $CD4^+$ and $CD8^+$ cells act together to prevent disease reactivation. It is possible that $CD4^+$ T cells provide help to assist the $CD8^+$ effector cell function [36]. *In vitro* studies demonstrated that $CD8^+$ cells from mice exposed previously to *T. gondii* were capable of killing both infected macrophages and macrophages which had been exposed to soluble parasite antigen. Killing was target-specific, as stimulated $CD8^+$ cells did not kill uninfected macrophages included in the experimental cell population. Although $CD8^+$ cells from mice vaccinated with *T. gondii* produce IFN-γ, killing of infected macrophages by these cells could occur in its absence as addition of anti-IFN-γ antibodies to the culture supernatant did not affect the lysis of infected macrophages [37].

Recent experiments have demonstrated that persistent cytokine production is required in chronic as well as acute infection. Exogenous IL-12 was administered to IL-12p40$^{-/-}$ mice during the first 2 weeks of infection by *T. gondii*. Treated animals survived the acute phase of disease and established chronic infections, whereas all untreated animals died. Following withdrawal of IL-12, IL-12p40$^{-/-}$ animals began to exhibit symptoms of reactivation with increased brain cyst burdens and succumbed to toxoplasma encephalitis. Reactivation of infection was associated with a loss of T cell IFN-γ production, which was not due to an increase in cytokines such as IL-10 [38].

Toxoplasma retinochorooriditis

Aetiology

The most common clinical manifestation of *Toxoplasma* infection in man is subsequent acute necrotizing retinochoroiditis, which accounts for 28–50% of all cases of posterior uveitis worldwide [2]. Ocular disease is self-limiting in immunocompetent individuals and in most cases lesions heal within 6–8 weeks whether treated or not. Recurrent lesions appear to be a frequent feature of *Toxoplasma* retinochoroiditis, although the exact lifetime risk is unknown [39,40]. Recurrence can lead to permanent loss of visual acuity if the lesion is located in the macula. The cause of recurrent lesions has not yet been elucidated and it is not clear whether it is due to reactivation of tissue cysts within the retina or as a result of damage caused by the immune response. It was thought previously that intra-ocular involvement was rare in post-natally acquired infection, the prevalence being in the range of 1–3%. However, recent studies have suggested that up to two-thirds of patients with ocular toxoplasmosis acquired the infection after birth [41]. Mechanisms for persistence within brain and retina following acute infection hinge upon the stage conversion to bradyzoites which encyst within the tissues [42].

Pathogenesis

The eye is not a favoured site for parasite encystment. In man, cysts in the retina are rare, compared with those found in brain, in patients with acquired immune deficiency syndrome (AIDS) [43,44]. Moreover, post-mortem ocular examination of 277 eyes showed no incidence of *Toxoplasma*

cysts [45]. Whether this reflects a protective mechanism or merely the difference in volume of neural tissue is not known but the extent, if any, of encystment in human eyes remains unclear.

Two potential mechanisms for parasite dissemination from the intestinal lumen to brain and retina have been considered, both involving the bloodstream: first by parasites travelling freely within plasma and secondly by leucocyte transfer. Although *T. gondii* parasites are obligate intracellular parasites, they are motile and may survive in serum-containing medium for hours *in vitro.* Their invasion of host cells is entirely parasite-driven and as such it is conceivable that they might disseminate and cross endothelia at distant sites as free parasites [46]. Smith *et al.*, comparing *T. gondii* infection of various human endothelial cell lines *in vitro*, have demonstrated a predilection for retinal tissue, with proliferation significantly greater within retinal vascular endothelial (RVE) cells compared with the other cell lines [47]. Courret *et al.* has demonstrated the 'hijacking' of cells of $CD11b^+$ monocyte/macrophage lineage by *T. gondii* in mesenteric lymph nodes following ingestion and subsequent transfer of parasites within these host immune cells to the parenchyma of the brain. Depletion of CD11b-bearing cells reduced significantly the number of tachyzoites entering the brain [48]. Moreover, *T. gondii* infection of DC leads to hypermotility of these cells and migration into tissues. Adoptive transfer of *T. gondii*-infected DC caused more rapid dissemination of the parasite to tissues and exacerbation of infection [49]. While *T. gondii* tachyzoites clearly have the ability to cause tissue destruction through lysis of the cells they inhabit, active retinochoroidal lesions produce a brisk inflammatory response which is evident clinically and in experimental models. Such immune activation is important in the control of *Toxoplasma* infection, but some evidence from murine models suggests that a substantial amount of the retinal necrosis and tissue destruction seen with active *Toxoplasma* retinochoroiditis is attributable to the aggressive T lymphocyte response. *Toxoplasma* retinal infection in $CD4^{-/-}$ mice evokes little inflammatory response and an absence of retinal necrosis when compared with immunocompetent animals [50]. This is surprising, as *Toxoplasma* retinochoroiditis in the setting of human $CD4^+$ deficiency such as AIDS is severe and progressive, presumably because of unhindered spread of the parasites causing widespread retinal necrosis.

In animal studies tissue cysts have been observed in parts of the normal retina surrounding necrotic areas, yet such infected retinal cells attract a limited inflammatory response. Rupture of these cysts may lead to reinfection of retinal cells and their subsequent destruction. Mechanical rupture following multiplication of *T. gondii* bradyzoites, cell-mediated defence reactions, hormonal effects and products of inflammation have been postulated as mechanisms by which tissue cyst rupture may be initiated [51]. Recent studies found a qualitative difference in antibody responses between the intra-ocular and the systemic environments. Serum responses were predominantly against the major surface antigen, SAG-1, while the intra-ocular antibody response was against the Gra-2 antigen, which is expressed by both the tachyzoite and bradyzoite forms of the parasite, and may reflect an immune response against antigens from rupturing tissue cysts [52]. Damage to the retina in *Toxoplasma* retinochoroiditis may also occur as a result of damage by inflammatory cell products, such as lysosomal enzymes [53].

Autoimmune tissue destruction following retinal infection with *T. gondii* may cause retinal damage. Animal models of toxoplasmic retinochoroiditis have demonstrated that, although tissue cysts were present in the neural retina, there was consistent selective loss of the photoreceptor layer possibly through mechanisms of secondary autoimmunity [54,55]. Furthermore, lymphocytes from patients with *Toxoplasma* retinochoroiditis show enhanced proliferative responses to retinal soluble antigen *in vitro*. It was suggested that the initial infection may release a large amount of this antigen, which has been shown to induce experimental autoimmune uveitis in primates and humans [56,57]. In support of the autoimmune theory, several studies have investigated anti-retinal antibody responses in patients with *Toxoplasma* retinochoroiditis. When tested against bovine retinal antigens, sera from patients with *Toxoplasma* retinochoroiditis demonstrated a higher prevalence and titre of antibody compared with healthy controls [58]. In a second study the majority of sera from patients with clinical *Toxoplasma* retinochoroiditis showed activity against human retinal tissue, and titres of anti-photoreceptor antibody were significantly higher in *Toxoplasma* retinochoroiditis patients compared with healthy controls [59].

Cellular responses

Peripheral blood mononuclear cells from patients with postnatally acquired *Toxoplasma* retinochoroiditis showed significantly greater proliferation as well as IL-1, TNF and IL-10 production in response to STag compared with seropositive asymptomatic patients, patients with congenital *Toxoplasma* retinochoroiditis or seronegative controls. Conversely, seropositive asymptomatic patients produced significantly more IFN-γ in response to STag than either of the other groups. The data suggested that patients with congenital disease show peripheral tolerance to infection, while resistance to ocular lesions is associated with IL-12 and IFN-γ production and susceptibility is associated with IL-1 and TNF production [60].

Role of NO

In vivo murine studies have demonstrated a significant increase in inflammation in the choroid, retina and vitreous following treatment of chronically infected mice with the NO inhibitor, aminoguanidine [61]. An *in vitro* investigation

of *T. gondii* infection of human retinal pigment epithelial cells (RPE) found that NO did not appear to be the mechanism by which these cells controlled parasite replication. The pretreatment of human RPE cells with IFN-γ resulted in a dose-dependent inhibition of parasite growth. Addition of an anti-IFN-γ antibody to the culture medium ablated this toxoplasmostatic activity. NO production was not demonstrated in these cultures and the addition of N^GMMA did not affect parasite replication [62]. IFN-γ can induce toxoplasmostasis in human fibroblasts dependent upon the concentration of L-tryptophan in the culture medium [63]. The amino acid L-tryptophan is essential for the growth of *T. gondii*. Subsequent work showed that IFN-γ can induce the degradation of L-tryptophan to kyneurenine by upregulating the enzyme indoleamine-2-3-dioxygenase [64]. Growth of *T. gondii* within human RPE cells could be restored following addition of L-tryptophan to the culture medium. These results indicated that L-tryptophan starvation may be the mechanism by which IFN-γ induces toxoplasmostasis in human RPE cells [65]. Just as the RPE forms the blood–retinal barrier at the choroidal interface, so RVE cells form the inner blood–retinal barrier. Using cultured rat RVE we have shown that, similar to RPE cells, *T. gondii* infection did not induce a NO response in RVE cells but toxoplasmostasis was observed when stimulated with IFN-γ, TNF or IL-1β [66]. Again, this inhibition could be overcome by the addition of L-tryptophan.

Fas–Fas ligand (FasL) interactions

The importance of the Fas–FasL interaction during ocular toxoplasmosis was demonstrated using *lpr* and *gld* mice that lack functional Fas and FasL respectively. Infected *lpr* and *gld* mice demonstrated a much more severe inflammatory response to the parasite than WT animals. This inflammation was associated with the infiltration of large numbers of inflammatory cells, neovascularization, necrotic retinitis and uveitis and greatly reduced apoptosis of infiltrating inflammatory cells. Furthermore, the expression of both Fas and FasL was increased significantly in the ocular tissues of *T. gondii* infected WT mice. Thus the Fas–FasL interaction may serve to protect the eye from potentially harmful immune responses during parasite infection [67].

Conclusion

Toxoplasma retinochoroiditis is a potentially sight-threatening condition with a complex aetiology. The presence of parasites in retinal tissue sets up an inflammatory response which, in addition to controlling the parasite, causes necrotic damage to the tissue. The evidence for persistence of *T. gondii* cysts in the eye is sparse and in virtually all cases has been reported in patients with active disease. It is clear that if *T. gondii* encysts in the eye such a state is maintained without an obvious lymphoid cell infiltrate, as no evidence of persistent inflammation is seen. Neither RVE nor RPE is activated persistently, as would be the case if IFN-γ was produced constantly. Moreover, all the data point to the requirement for T cells being the prime producers of IFN-γ during chronic *T. gondii* infection and there is no evidence for constant recruitment of such cells into retinal tissue. Therefore, either other resident cells in the retina produce the appropriate signals to maintain encystment or other mechanisms besides cytokines are involved. As stated above, barrier RVE and RPE cells can control *T. gondii* growth when primed with proinflammatory cytokines but did not produce IFN-γ or IL-12 directly. Similarly, Muller cells can be infected readily with *Toxoplasma* and produce chemokines that would attract lymphoid cells but did not produce cytokines that would support encystment. Other cell types such as retinal microglia could fulfil the function, but to date no retinal cell appears capable of replacing the function of infiltrating lymphoid cells. Novel pathways of control of *Toxoplasma* retinochoroiditis including lipoxin production, autophagy and IL-23/IL-17/IL-27 production have also to be explored. A second possibility is that *Toxoplasma* retinochoroiditis is due to systemic cysts reactivating at another site in the body such as skeletal muscle causing fresh lesions in the eye through haematogenous spread. Finally, it remains possible that recurrent *Toxoplasma* retinochoroiditis is all acquired from fresh infections. The questions of whether *Toxoplasma* retinochoroiditis is caused by reactivation of ocular cysts or new infection with parasites by haematogenous spread and whether such parasites enter alone or are carried in via infected cells still need to be addressed.

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