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The local immune response to intraocular *Toxoplasma* re-challenge: Less pathology and better parasite control through Treg/Th1/Th2 induction

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ABSTRACT

Ocular toxoplasmosis is a major cause of blindness world-wide. Ocular involvement is frequently seen following congenital infection. Many of these infections are quiescent but pose a life-time risk of reactivation. However, the physiopathology of ocular toxoplasmosis reactivation is largely unexplored. We previously developed a Swiss-Webster outbred mouse model for congenital toxoplasmosis by neonatal injection of *Toxoplasma gondii* cysts. We also used a mouse model of direct intraocular infection to show a deleterious local T helper 17 type response upon primary infection. In the present study, our two models were combined to study intravitreal re-challenge of neonatally infected mice, as an approximate model of reactivation, in comparison with a primary ocular infection. Using BioPlex proteomic assays in aqueous humour and reverse transcription-PCR for T helper cell transcription factors, we observed diminished T helper 17 type reaction in reinfection, compared with primary infection. In contrast, T helper 2 and T regulatory responses were enhanced. Interestingly, this was also true for T helper 1 markers such as IFN-γ, which was paralleled by better parasite control. Secretion of IL-27, a central cytokine for shifting the immune response from T helper 17 to T helper 1, was also greatly enhanced. We observed a similar protective immune reaction pattern in the eye upon reinfection with the virulent RH strain, with the notable exception of IFN-γ. In summary, our results show that the balance is shifted from T helper 17 to a less pathogenic but more effective anti-parasite Treg/T helper 1/T helper 2 pattern in a reactivation setting.

1. Introduction

Infection with the apicomplexan parasite *Toxoplasma gondii* is generally benign, but can have serious consequences in immunocompromised individuals and in cases of congenital infection. In the latter case ocular infection, mostly retinochoroiditis, is the most common consequence. Even if ocular toxoplasmosis (OT) is now known to also occur as a result of postnatally acquired infection (Delair et al., 2008), it is still an important medical issue for the follow-up of children who have been infected in utero. Many of these ocular infections are undetectable at birth but pose a life-time risk of reactivation, especially during infancy and adolescence (McAuley, 2008). However, there are very few data on the physiopathology and immunology of OT and even less is known about reactivation, which has to date prevented the introduction of an efficient treatment to avoid further reactivation (Holland, 2003, 2004; Garweg and Candolfi, 2009). Importantly, the eye is considered an immune privileged organ, where all inflammatory reaction has to be controlled with particular caution, as it may cause irreversible tissue damage such as has been shown for autoimmune uveitis (Caspi, 2008). The aim of our work was to elucidate the local immune response to a reactivation following congenital infection, compared with a primary infection.

One experimental problem is that ocular infection is not consistent in systemic infection, and much less in infection of the mother during pregnancy and subsequent congenital transmission. Therefore, we recently established a mouse model of neonatal infection of Swiss-Webster mice and showed that this infection results in similar ocular pathology as true congenital infection, but with much higher success rates and is therefore much easier to study (Lahmar et al., 2010). Secondly, we immunologically characterised a mouse model of direct intraocular injection of tachyzoites, which has the advantage that the local immune response can be followed in a homogeneous fashion (Sauer et al., 2009; Charles et al., 2010). We subsequently showed a general increase in immune mediators in the aqueous humour (AqH). Using neutralising antibodies, we demonstrated the deleterious role of IL-17A on pathology and on

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parasite control through suppression of IFN-γ production (Sauer et al., 2012). These central roles of IFN-γ and IL-17A in OT were also shown in another recent study (Kikumura et al., 2012). This again resembles the deleterious role of T helper (Th)17 cells in autoimmune uveitis (Amadi-Obi et al., 2007). Therefore, the cytokine network, which is measurable in AqH, plays a major role in the physiopathology of OT. For our study, we combined our two successful models. However, the mechanisms behind ocular *Toxoplasma* reactivation are still completely unknown. Evidently, neutralisation of crucial immune factors such as CD4+ and CD8+ cells or IFN-γ lead to strong parasite proliferation and pathology (Gazzinelli et al., 1994), but this does not reflect the clinical picture seen in patients with reactivation. Interestingly, one recent study showed the development of small, young cysts in the proximity of older cysts, in perfectly immunocompetent mice (Melzer et al., 2010). This could indicate transient extracellular parasites which then immediately infect new cells, multiplying locally before being controlled by the immune system. Therefore, we chose intravitreal injection of parasites into 4 week old neonatally infected mice as an approximate model of reactivation. In order to obtain mechanistic insights into protective and detrimental immune responses, we compared the reinfection with a primary intraocular infection without previous neonatal infection. Analysis by multiplex protein assay and reverse transcription-PCR to quantify intracellular transcription factors allowed us to draw a comprehensive picture of the local immune reaction during such re-challenge in young individuals.

2. Materials and methods

2.1. Mice and parasites

Outbred Swiss-Webster mice were originally obtained from Centre d'Elevage R. Janvier (Le Genest-Saint-Isle, France). Animals were bred under specific-pathogen-free conditions at our laboratory. All experiments were performed in accordance with ARVO (Association for Research in Vision and Ophthalmology, USA) Statement for the Use of Animals in Ophthalmic and Vision Research, as well as with national and local restrictions.

Cysts of the type II (avirulent) *T. gondii* strain, PRU, were obtained from brains of previously infected mice. Tachyzoites of the *T. gondii* PRU strain were maintained in human MRC5 fibroblast cultures. Tachyzoites of the type I (virulent) strain, RH, were maintained by weekly passages in mice. All strains were obtained from the French Biological Resource Centre *Toxoplasma* (CRB *Toxoplasma* – Laboratoire de Parasitologie, CHU Reims, France).

2.2. Experimental schedule

For the neonatal primary infection (mice of the reinfection group), animals were s.c. injected with five *T. gondii* PRU (type II) cysts in 100 μl of PBS/brain suspension during the first week after birth. Infection was verified for each mouse by *Toxoplasma*-specific IgG ELISA. Four weeks later, the mice were infected intravitreally in both eyes with 2,000 tachyzoites in 5 μl of PBS, using 30-Gauge needles.

Intraocular injections were done after a sedative procedure using isoflurane inhalation. Clinical staging of intraocular inflammation was done as previously described (Hu et al., 1999) at days 1, 3, 5 and 7: 0, normal; 1, apparent ciliary congestion around the cornea; 2, intense ciliary congestion with slight cornea oedema and anterior chamber clouding; 3, obvious intraocular inflammatory reaction such as iris vessel prominence, vitreous and retinal opacification; 4, endophthalmitis or obvious ophthalmia with systemic symptoms and/or death. Groups of mice were sacrificed at these time points by anaesthetic overdose. AqH was collected by means of anterior chamber paracentesis (approximately 5 μl/eye), pooled and stored in aliquots of 25 μl at −80 °C until analysis. The eyes were finally enucleated and retinas were dissected and stored at −80 °C. Each experimental group consisted of five animals (10 eyes). Every experiment was performed three times.

2.3. Cytokine measurement in AqH

The Bio-Plex mouse Cytokine Panel assay (Bio-Rad, Marne-la-Coquette, France) was used to simultaneously quantify the following cytokines and chemokines in AqH: IFN-γ, IL-2, TNF-α, MCP-1, IL-6, IL-17A, IL-13, IL-10. The cytokine and chemokine assay plate layout consisted of a standard series in duplicate (1–32,000 pg/ml), four blank wells and 20 μl duplicates of pooled AqH samples, diluted to 50 μl with BioPlex mouse serum diluent. The BioPlex method was performed as recommended by the manufacturer. Data were analysed with Bio-Plex Manager TM software V1.1.

2.4. Quantitative RT-PCR analysis

Retinal parasite loads at different time points were assessed at the mRNA level, using the transcript for the main surface molecule of the tachyzoite form, SAG1. Furthermore, the T-lineage-specific transcription factors RORγt, GATA3, FoxP3 and T-bet, as well as the cytokines TGF-β and IL-27, which were not included in the Bio-Plex kit, were similarly assessed, as described before (Sauer et al., 2012). Briefly, RNA was extracted from pooled retinas (RNeasy, Qiagen, Courtaboeuf, France) and reverse transcribed. Real-time PCR was performed on a capillary-based LightCycler system (Roche Diagnostics, Boulogne-Billancourt, France). Specific product was quantified by an external standard curve, normalised to the housekeeping gene, hypoxanthine–guanine phosphoribosyltransferase (HPRT), and expressed relative to the mRNA levels of the same gene in non-infected mice.

2.5. Statistical analysis

Values shown are means ± S.D. of three independent experiments. Statistical evaluation of differences between the primary infection and reinfection groups at each time point was performed using a Student's *t*-test. All statistical analysis and graphs were done using GraphPad Prism software version 5 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Intravitreal reinfection with the homologous PRU (type II) strain

We assessed the local ocular immune reaction following intraocular injection of an avirulent *T. gondii* strain in 4 week old mice, both as a primary infection and as reinfection following neonatal primary infection. As shown in Fig. 1A, primary infection rapidly induces ocular inflammation, compared with control PBS injection. A plateau seems to be attained at day 5. In contrast, visible inflammation was significantly lower upon reinfection at all time points. No mortality was noted in either group, even up to 30 days after termination of the experiment (data not shown).

Retinal SAG1 transcript levels, as a measure of parasite load, did not visibly increase at days 1 and 3 (Fig. 1B). Consequently, parasite load was not different between the two groups. However, at day 5, substantial parasite multiplication was observed in the primary infection group. In contrast, in the reinfection group levels were significantly lower.