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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.

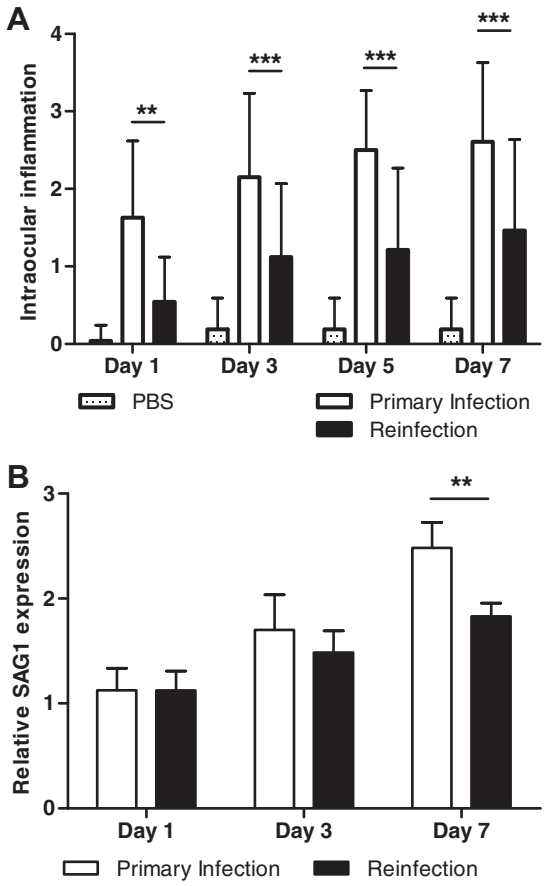

Fig. 1. Intravitreal injection of 2,000 tachyzoites of the *Toxoplasma gondii* type II strain, PRU, into naïve mice (Primary Infection) or mice infected neonatally with five PRU cysts (Reinfection). (A) Evolution of intraocular inflammation. Control infection with PBS injected into naïve mice is also shown. Values are means ± S.D. of 30 eyes (five mice per experiment, three experiments). (B) Retinal parasite load, determined by quantitative reverse transcription-PCR for the main *T. gondii* surface antigen, SAG1. Values are means ± S.D. of three independent experiments ($^{**}P < 0.01$, $^{***}P < 0.001$).

Bioplex assay of cytokine levels in the AqH showed that most cytokines were still somewhat upregulated 4 weeks after neonatal PRU cyst infection (Fig. 2, day 0). However, intravitreal parasite injection led to considerably higher cytokine levels as early as 1 day following injection. The observed cytokine production could thus be nearly exclusively attributed to the intravitreal re-challenge. A clear pattern is visible when considering the different categories of immune mediators. The levels of the inflammatory (Th17) cytokines IL-6 and IL-17A, as well as the inflammatory chemokine MCP-1, were significantly lower in the reinfection group, compared with the primary infection group. In contrast, the Th1 cytokine IFN-γ was present at higher concentrations at all time points. Similarly, the Th2 type cytokine IL-13 was significantly more expressed in the reinfection group, as was the downregulatory IL-10. Levels of TNF-α did not differ between the two groups at all time points after intraocular infection. This shift in the cytokine balance was further confirmed by an increased level of the central Th17 downregulatory and Th1 inducing cytokine IL-27 in the reinfection group at the mRNA level (Fig. 3A).

To dissect the underlying cellular regulation of this difference in retinal cytokine pattern, we assessed expression of the central transcription factors for the different Th cell lineages (Zhu and Paul, 2008) (Fig. 3B). Despite the rapid difference in IL-17A levels, RORγt, the master regulator of Th17 cells, showed no difference until day 3 and only a slight, but significant, diminution at day 7 in the reinfection group. In contrast, FoxP3, the central transcription factors for regulatory T (Treg) cells, was enhanced in the reinfection group from day 3 onwards, confirming the IL-27 mRNA data. The regulators of Th1 and Th2, T-bet and GATA3, were also enhanced in the reinfection group, compared with the primary infection group.

3.2. Intraocular reinfection with the heterologous virulent RH (type I) strain

Next, we wanted to know whether neonatal infection can also provide an anti-inflammatory and protective response following intravitreal injection of the more aggressive type I strain, RH. As with PRU re-challenge, we first looked at the clinical presentation by macroscopic eye examination (Fig. 4A). During primary infection, clinical evolution was similar to PRU injection but the scores, especially at day 3, were somewhat higher. All mice in this group died between days 3 and 7 due to a generalised infection. In the reinfection group, mice were again partially protected, the clinical score being significantly lower and similar to the PRU reinfection group (Fig. 1A) throughout the experiment. All mice in this group were alive at day 7 but all of them died afterwards, before day 14, contrary to PRU infection.

Parasite multiplication, as assessed by SAG1 transcript quantification, was faster than following PRU injection (Fig. 4B). SAG1 transcript levels tended to be lower in the reinfection group at days 1 and 3, but differences were not statistically different. Due to the early mortality of the primary infected mice, no comparison was possible at day 7.

The cytokine expression in the two groups was again analysed by a BioPlex assay (Fig. 5). Only the cytokine levels at day 3 could be assessed, due to mortality in the primary infection group between days 3 and 7. As with PRU re-challenge, inflammatory cytokine levels (IL-6, IL-17A) were lower in the reinfection group. However, contrary to the PRU injection experiment, IFN-γ levels were lower in this group. The Th2 cytokine IL-13 and the downmodulatory cytokine IL-10 showed the same pattern as with PRU infection, being elevated in the reinfection group. At the mRNA level (Fig. 6A), IL-27 expression was only augmented in the reinfection group, again similar to the PRU experiments.

When we looked at the Th cell transcription factors (Fig. 6B), we again observed the same patterns as in the PRU experiments (Fig. 3B). However, activation markers tended to be visible earlier. RORγt levels were already significantly lower in the reinfection group at day 3 and T-bet and FoxP3 levels showed clearer differences at day 3 than following PRU infection. Of note, T-bet levels were again higher in the reinfection group, despite lower IFN-γ levels in the BioPlex study.

4. Discussion

Despite the medical importance of reactivation of ocular toxoplasmic lesions, especially during childhood and adolescence, little is known about its physiopathology. Given the lack of mouse models for reactivation of existing retinal lesions, mice were infected neonatally, which is a validated model of congenital OT (Lahmar et al., 2010) and tachyzoites were injected directly into the vitreous humour 4 weeks later. There is broad mechanistic insight into peripheral protection against reinfection, mainly relying on CD8+ cells and IFN-γ (Parker et al., 1991; Abou-Bacar et al., 2004). However, given the immunoprivileged situation of the eye (Streilein, 2003; Niederkorn, 2006), different mechanisms are certainly at play.

In our study, macroscopic examination indicated a less severe inflammation upon reinfection as early as 1 day following injec-

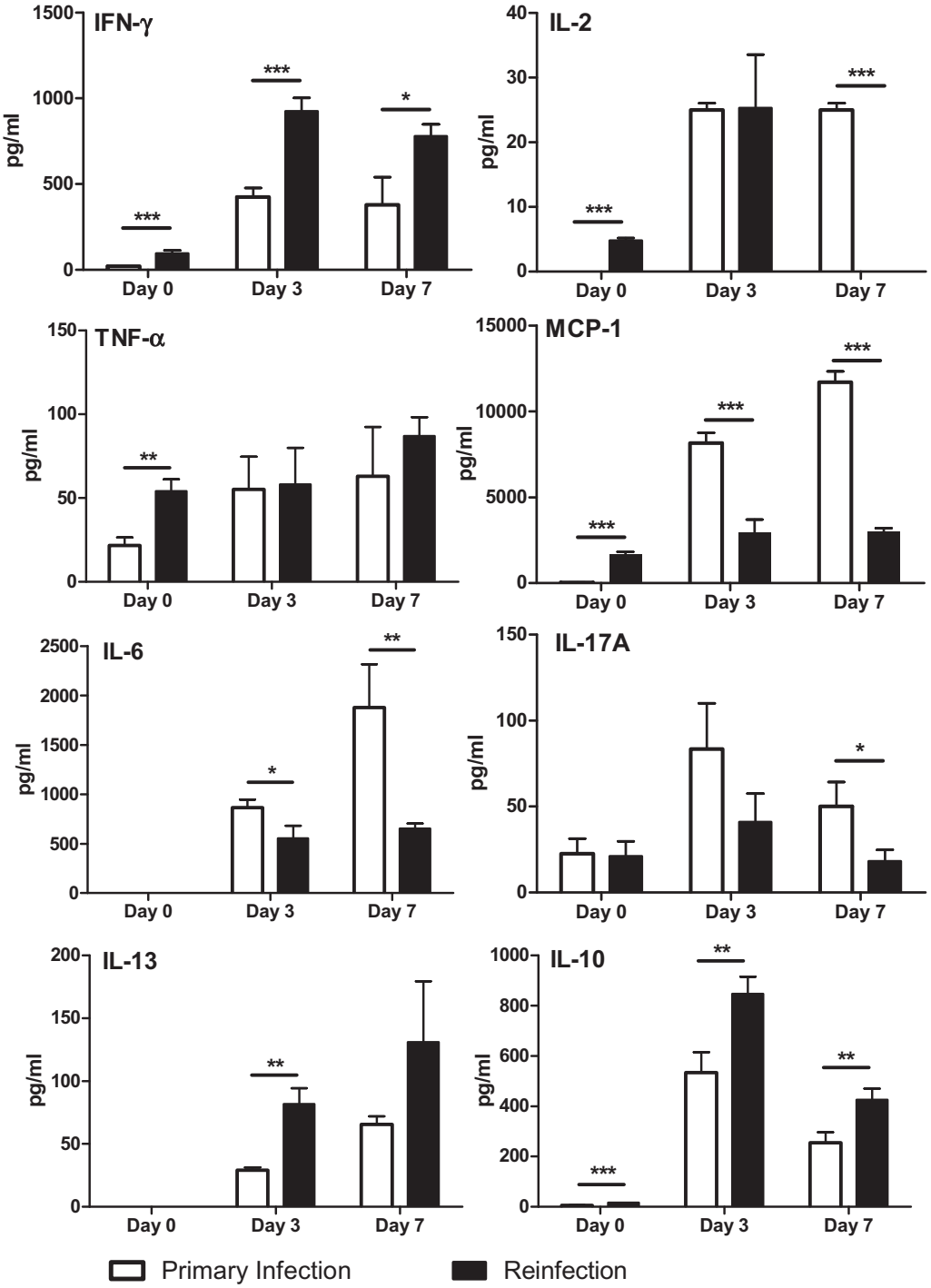

Fig. 2. Cytokine levels in pools of aqueous humours following intravitreal injection of 2,000 tachyzoites of the *Toxoplasma gondii* type II strain, PRU, into naïve mice (Primary Infection) or mice infected neonatally with five PRU cysts (Reinfection). Cytokine levels in aqueous humour were measured by BioPlex assay. Values are means ± S.D. of three independent experiments ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

tion, proving the development of a local protective mechanism by primary systemic infection. Our immunological results proved that some of the injected parasites infected retinal cells and persisted there, even if this parasite multiplication was barely measurable, and that the retina responded with a detectable immune reaction. It is therefore likely that the infection of a few cells rapidly initiated a network of protective immune responses, implying various cell types.

Immunological analysis using multiplex and mRNA assays of cytokines and central transcription factors produced a comprehensive picture of the immune reaction in the retina upon reinfection. Moreover, the comparison of primary infection with a clinically less pathogenic secondary infection allowed some mechanistic insight into these complex processes, in particular to distinguish protective and deleterious mediators. The inflammatory mediators IL-6 and IL-17A were clearly correlated with the pathological primary response. IL-17A is the signature cytokine of the recently described Th17 response and IL-6 is necessary to induce IL-17A production (Stumhofer et al., 2006; Guiton et al., 2010). Numerous studies have confirmed the central role of Th17-type cytokines in autoim-

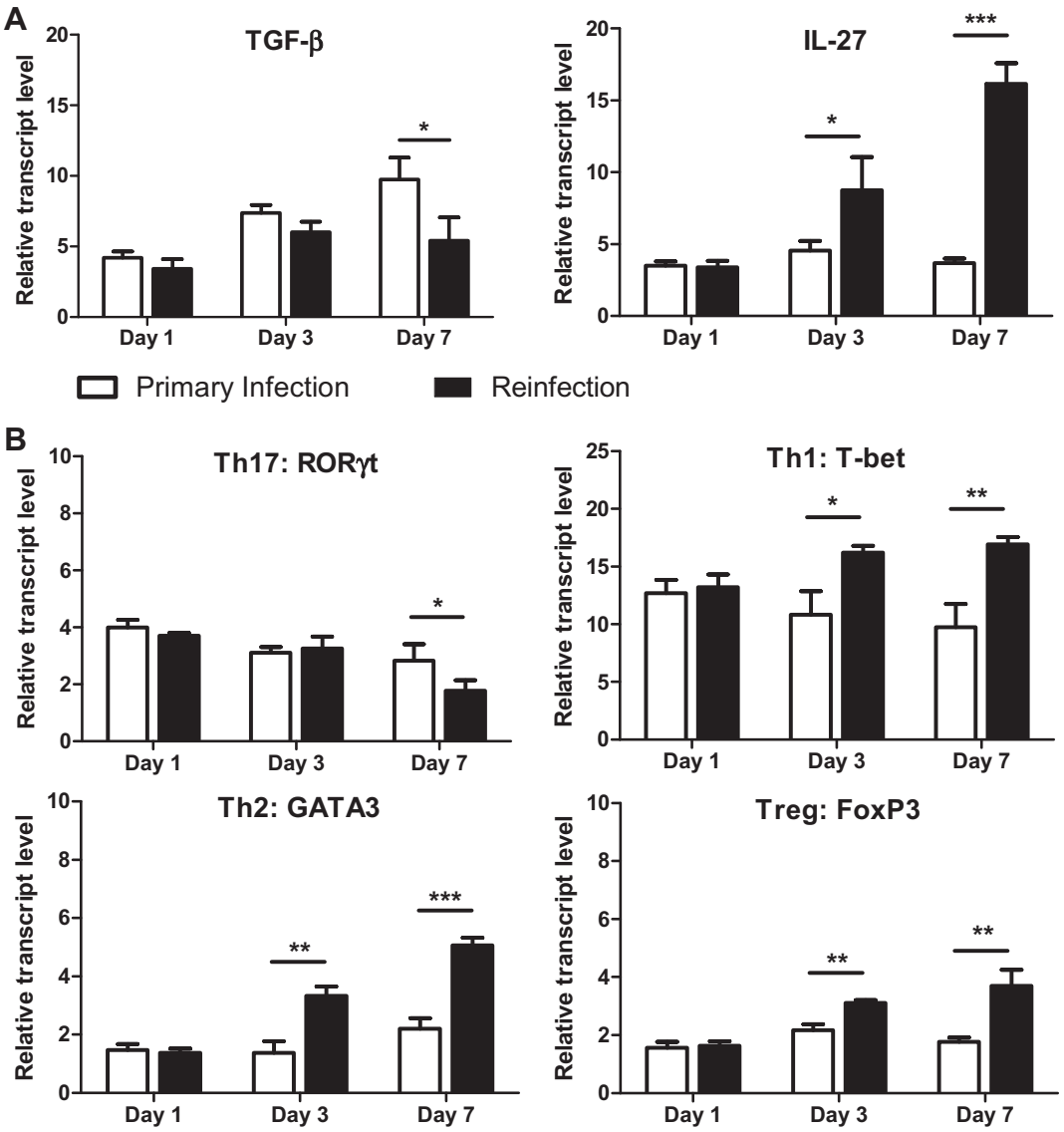

Fig. 3. Retinal gene transcript levels of selected cytokines (A) and T helper cell transcription factors (B) following intravitreal injection of 2,000 tachyzoites of the *Toxoplasma gondii* type II strain, PRU, into naïve mice (Primary Infection) or mice infected neonatally with five PRU cysts (Reinfection). Quantitative reverse transcription-PCR analysis was done using pools of the retinas from five mice per group. Values are means ± S.D. of three independent experiments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

mune pathology (Weaver et al., 2006; Amadi-Obi et al., 2007). The role of IL-17A in infectious diseases is more ambiguous, between anti-pathogenic activity and tissue destruction. While IL-17 is certainly responsible for tissue destruction during *T. gondii* infection, there is considerable discrepancy in results in terms of its anti-parasitic effect, possibly due to a focus on different organs (Kelly et al., 2005; Stumhofer et al., 2006; Guiton et al., 2010). For our model system, we had to account, additionally, for the particular immunosuppressive environment in the eye (Streilein, 2003). Retinal pigment epithelium and probably other cell types confer tolerance to activated T cells either locally or via the systemic induction of Treg cells (Sugita et al., 2008; Horie et al., 2010). Using our intravitreal infection model, we recently demonstrated that IL-17A neutralisation clearly ameliorates pathology (Sauer et al., 2012). The present results show that the 'natural' protection during reinfection also implies diminished IL-17A expression.

The Th1 response and its signature cytokine, IFN-γ, have long been acknowledged to be protective and necessary to control *T. gondii* infection (Suzuki et al., 1988), which was more recently extended to the IFN-γ induced transcription factor, STAT1 (Gavrilescu et al., 2004). Our results show that retinal cells are more primed to this protective response in a reinfection setting, thus ameliorating parasite control. The mRNA assays suggested that increased IL-27 levels could be at the origin of the observed shift from pathogenic Th17 to a protective Th1 type response. IL-27, expressed by antigen presenting cells, directly inhibits Th17 cell commitment and development during autoimmune encephalitis and cerebral toxoplasmosis (Stumhofer et al., 2006; Diveu et al., 2009). While IL-27 was initially described as a Th1 inducing factor in *T. gondii* infection studies (Villarino et al., 2006), more recent works showed pleiotropic effects on Th subset regulation (Hall et al., 2012; Wojno and Hunter, 2012).

Higher IL-10 levels in the reinfection group also point to a better control of inflammation, which could explain the ameliorated pathology. Interestingly, levels of the Th2 cytokine IL-13 are also enhanced in the reinfection group. Even if Th2 cytokines are not directly involved in anti-*T. gondii* responses, recent results indicate a Th17 downregulating role of IL-13 (Newcomb et al., 2012), together with other Th2 cytokines, IL-25 and IL-33 (Kleinschek et al., 2007; Jones et al., 2010). Further studies focusing on these

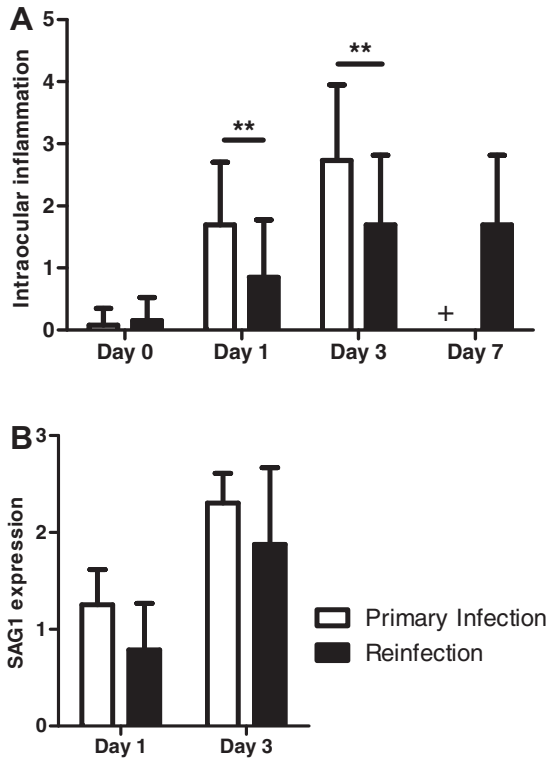

Fig. 4. Intravitreal injection of 2,000 tachyzoites of the *Toxoplasma gondii* type I strain, RH, into naïve mice (Primary Infection) or mice infected neonatally with five PRU (type II) cysts (Reinfection). (A) Evolution of intraocular inflammation. Control infection with PBS injected into naïve mice is also shown. Values are means ± S.D. of 30 eyes (five mice per experiment, three experiments). (B) Retinal parasite load, determined by quantitative reverse transcription-PCR for the main *T. gondii* surface antigen, SAG1. Values are means ± S.D. of three independent experiments. [†]No mouse survived until day 7 in the primary infection group; $^{**}P < 0.01$, $^{***}P < 0.001$.

cytokines will give us new insights into the complex interplay between Th cell subsets.

Quantification of Th cell transcription factor transcripts allowed a mechanistic insight into T cell development during OT. The most striking feature was the late and only slight diminution of RORγt, the master regulator of Th17 cell commitment, which contrasted with the clear and more rapid suppression of IL-17A in the reinfection group. In contrast, the Treg factor FoxP3 was enhanced in the reinfection group. Treg upregulation could explain the striking absence of Il-2 at day 7 in the reinfection group, as some studies suggest IL-2 deprivation by CD25 as a means of T effector cell silencing (Letourneau et al., 2009). The master regulators of Th1 and Th2, T-bet and GATA3, were also rapidly enhanced in the reinfection group, confirming the BioPlex data on IFN-γ and IL-13. These results suggest that suppression of the Th17 inflammatory response during reinfection relies mainly on the activation of counterbalancing Th lineages, at least at early time points. Such a direct IL-17 suppressing effect has been described for IFN-γ (Irmler et al., 2007), as well as for Th2 cytokines, as discussed above (Newcomb et al., 2012). The diminution or inactivation of Th17 cells could then be a delayed secondary effect.

We also reinfected mice with tachyzoites of the virulent type I strain, RH, to see whether the local, ocular immune system can cope with this more aggressive challenge. The overall patterns of parasitology, pathology and immune response were similar to a type II strain re-challenge. Clinical scores were slightly higher and the retinal parasite load rose faster but, even at day 3, the differences between groups were not significant. This is astonishing because all mice of the primary infection group died before day 7, much earlier than the mice in the reinfection group. This result shows that protection by a previous infection is also effective for a subsequent infection with a heterologous virulent strain but this protection is not absolute. It would be interesting to study reinfections with genetically distant, atypical *T. gondii* strains to assess the strain specificity of our protective response. Our results also dem-

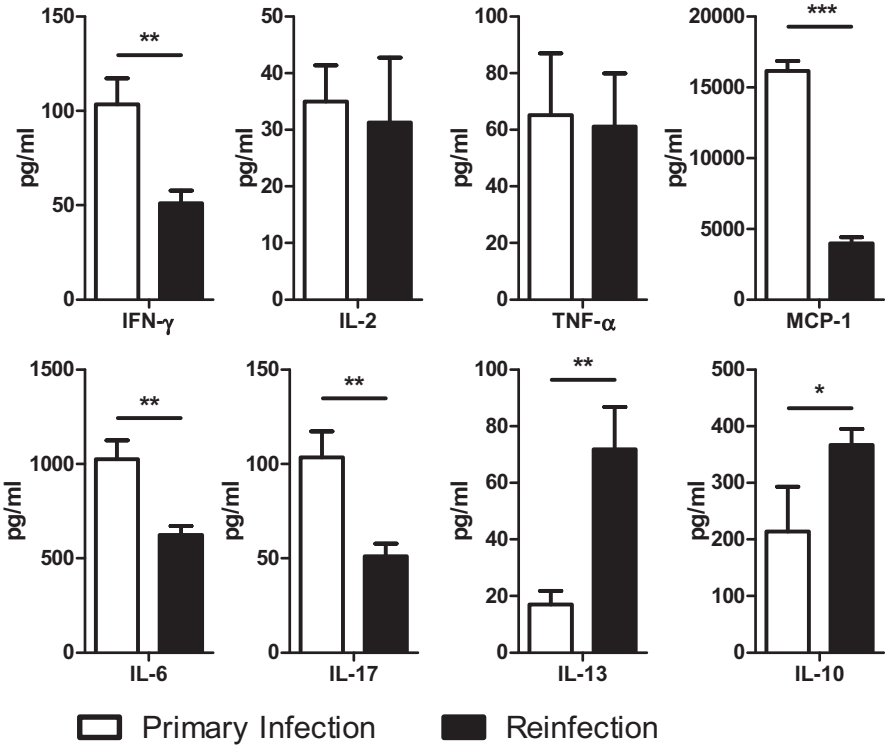

Fig. 5. Cytokine levels in pools of aqueous humours 3 days after intravitreal injection of 2,000 tachyzoite of the *Toxoplasma gondii* type I strain, RH, into naïve mice (Primary Infection) or mice infected neonatally with five PRU (type II) cysts (Reinfection). Cytokine levels in aqueous humours were measured by BioPlex assay. Values are means ± S.D. of three independent experiments ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

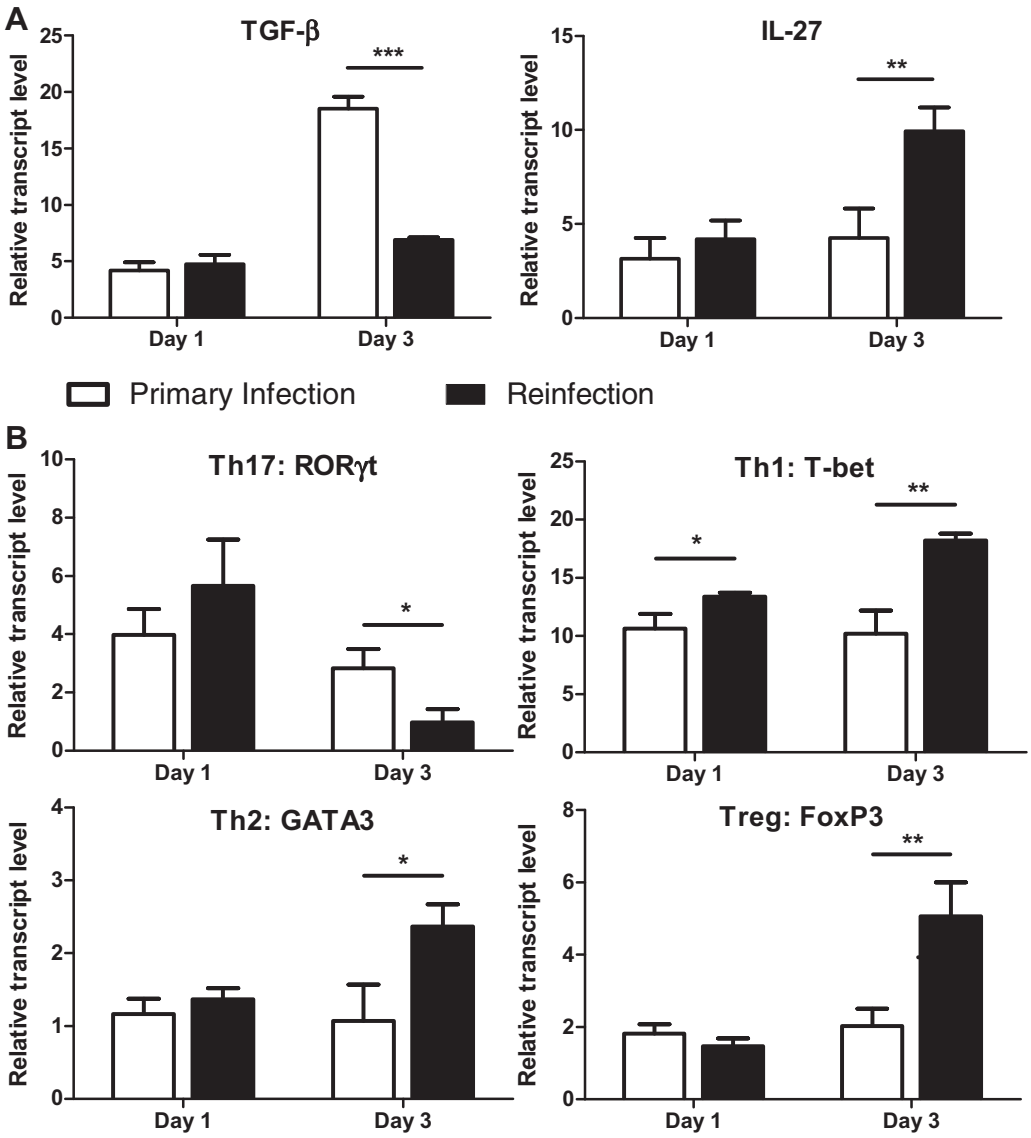

Fig. 6. Retinal gene transcript levels of selected cytokines (A) and T helper cell transcription factors (B) following intravitreal infection with 2,000 tachyzoites of the *Toxoplasma gondii* type I strain, RH, into naïve mice (Primary Infection) or mice infected neonatally with five PRU (type II) cysts (Reinfection). Quantitative reverse transcription-PCR analysis was done using pools of the retinas of five mice per group. Values are means ± S.D. of three independent experiments ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

onstrate again the particular environment within the eye. Apparently, while causing only moderate ocular inflammation, the parasites provoked lethal systemic damage. The only striking difference compared with type II reinfection was that IFN-γ levels were lower in the reinfection group. However, IFN-γ levels were much lower in both groups than following type II reinfection, which is in line with previous in vitro studies demonstrating that infection with type I strains blocks production of IL-12 and, consequently, an efficient IFN-γ and Th1 response (Saeij et al., 2007; Rosowski et al., 2011). While the exact mechanisms in our in vivo system in the ocular environment is yet to be determined, our results do not show a general inhibition of the Th1 phenotype, as the Th1 determining transcription factor, T-bet, was still enhanced in the reinfection group. Possibly, factors other than IFN-γ can drive a global Th1 response in mice already primed by the previous neonatal infection.

In summary, our results showed in detail the mechanisms of the immunological response during reinfection within the particular immune privileged ocular setting, as an approximate model for reactivation of ocular toxoplasmosis following congenital infection. Moreover, due to diminished pathology and parasite proliferation during reinfection, our study also enabled mechanistic insight into protective and detrimental immune reactions in the eye. The understanding of immunopathological OT events may open new and targeted approaches to immune therapy.

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