CD4 T-Cell Suppression by Cells from Toxoplasma gondii-Infected Retinas Is Mediated by Surface Protein PD-L1

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In the inflamed retina, CD4+ T cells can cause retinal damage when they are not properly regulated. Since tissue expression of major histocompatibility complex (MHC) class II and costimulatory molecules is a key mechanism for regulating effector T cells, we tested the hypothesis that upregulation of these proteins in the retina contributes to the regulation of CD4 T cells. Here we report that in retinas infected with the protozoan parasite Toxoplasma gondii, MHC class II is upregulated on infiltrating leukocytes as well as on resident retinal cells, including photoreceptors. Flow cytometric analysis indicated that B7 costimulatory family members (CD80, CD86, ICOS-L, and programmed death ligand 2 [PD-L2]) were not expressed on class II+ cells. In contrast, PD-L1 (also named B7-H1 or CD274) was expressed on the majority of both hematopoietic and resident retinal MHC class II-expressing cells. Retinal cells from Toxoplasma-infected animals were able to suppress T-cell activation in a PD-L1-dependent manner. Finally, we demonstrate that the expression of MHC class II and PD-L1 was critically dependent on gamma interferon (IFN-γ) expression. These data suggest that retinal MHC class II and PD-L1 expression is a novel mechanism by which the retina protects itself from CD4 T-cell-mediated immune damage in ocular toxoplasmosis and other types of retinal immune responses.

Toxoplasma gondii is an obligate intracellular protozoan parasite that infects humans and animals either congenitally or postnatally (38, 42). There are no vaccines to prevent this infection, and currently prescribed therapies are poorly tolerated and have severe side effects. In addition, the parasite responds to these drugs as well as to the host’s immune response by transforming into quiescent, asymptomatic cysts in tissues such as the eye, brain, and muscle (54). These cysts occasionally reanimate, and the released parasites will cause disease unless a properly regulated immune response is mounted. The retina is a common site for cyst reactivation and causes a disease, called ocular toxoplasmosis, that is one of the most common infections of the posterior retina (21).

The ability of a host to control a reactivated Toxoplasma infection is dependent largely on CD8+ T cells and, to a lesser extent, on CD4+ T cells (13, 43, 52). However, CD4+ T cells must be tightly regulated, because these cells cause immunemediated tissue destruction in the eye and other tissues (23, 32, 36). Because damaged retinal cells cannot be repaired or regenerated, it is imperative to understand how CD4+ T cells are regulated in the retina in ocular toxoplasmosis. As central regulators of CD4+ T cells, major histocompatibility complex (MHC) class II-expressing cells are likely to be critical in controlling CD4-based T-cell responses.

MHC class II expression and function in the retina have been the source of some debate. This is because retinal MHC class II expression is low as a result of the high expression of immunosuppressive cytokines and the blood-ocular barrier (49). Regardless, retinal microglia, endothelial cells, retinal pigment epithelial (RPE) cells, and other, less well characterized cells can express MHC class II under certain conditions (18, 25, 57). But the identity and function of MHC class II-expressing cells in Toxoplasma-infected retinas remain unknown.

At sites of inflammation, CD4+ T cells are regulated by two distinct signals from their target cells. First, they must come in contact with a cell expressing peptide-loaded MHC class II. Second, costimulatory molecules dictate the response of the engaged T cells. One major group of costimulatory molecules is the B7 protein family, which includes CD80, CD86, and ICOS-L (16). These proteins, which bind CD28 (CD80 and CD86) or ICOS (ICOS-L), are important for resistance to Toxoplasma (51, 56). Two additional B7 family members, programmed death ligand 1 (PD-L1) and PD-L2, bind the T-cell surface protein PD-1 and downregulate activated T cells (16). While PD-L1 and PD-L2 are functionally redundant, they have distinct expression patterns; PD-L2 is expressed primarily by cells derived from hematopoietic cells, and PD-L1 is expressed on hematopoietic and nonhematopoietic cells. In mice and humans, PD-L1 but not PD-L2 is expressed in various regions of the eye, including the cornea, ciliary body, iris, and retina (22, 48, 58). Ocular PD-L1 expression is important for the prevention of corneal allograft rejection, suggesting that it is a key player in maintaining immune privilege in the anterior chamber of the eye (22, 48). However, the importance of PD-L1 in the posterior chamber in inflammatory events such as Toxoplasma infections is unknown.
Here we report that CD4 recall responses to Toxoplasma antigen are blocked by retinal cells isolated from intravitreally infected mice. Flow cytometric and immunohistochemical analyses demonstrate that both infiltrating leukocytes and resident retinal cells expressed MHC class II and that MHC class II expression was dependent on gamma interferon (IFN-γ). Surprisingly, an overwhelming majority of the MHC class II-expressing cells did not express positively acting costimulatory molecules but rather expressed PD-L1. Finally, the suppressive effect of parasite-infected retinal cells on T cells was PD-L1 dependent.

MATERIALS AND METHODS

Parasites. The RH and cpsII pyrimidine auxotrophic Toxoplasma strains were grown in human foreskin fibroblasts as previously described (7, 11). To prepare parasites for injections, infected monolayers were scraped and parasites were mechanically released from their host cells by being passed three times through a 27-gauge syringe needle. The parasite suspension was filtered through a 3-μm-pore-size polycarbonate filter to separate parasites from host cells. Parasites were extensively washed in phenol red-free Dulbecco’s modified Eagle medium (DMEM) and were then resuspended in phenol red-free DMEM. Soluble tachyzoite antigen (STAg) was prepared in the presence of a protease inhibitor cocktail (Calbiochem) as previously described (27), except that the lysate was centrifuged at 16,000 × g for 20 min at 4°C.

Mice and intravitreal infections. Injections were performed as described previously (7). Needles with a tip size of more than 50 μm were loaded with a parasite suspension using a pneumatic pump delivery system. Six- to 12-week-old C57BL/6 or IFN-γ−/− mice (purchased from Jackson Labs, Bar Harbor, ME) were anesthetized with ketamine-xylazine, and then the needle was inserted immediately behind the limbus-parallell conjunctival vessels. After the needle was inserted, 0.5 μl of the parasite suspension or phenol red-free DMEM (as a mock control) was injected. Mice were intraperitoneally vaccinated with 10^4 irradiated cpsII parasites. All protocols adhered to University of Oklahoma Health Sciences Center’s IACUC and to ARVO’s Statement on the Use of Animals in Ophthalmic and Vision Research.

Flow cytometry. Mice were sacrificed by CO2 asphyxiation, and their eyes were immediately harvested, placed in phosphate-buffered saline (PBS), and mechanically disrupted with a stomacher (Tekmar, Cincinnati, OH). The cell suspension was treated with ACK lysis buffer (Cambrex, East Rutherford, NJ) to lyse the red blood cells. The splenocytes were then incubated with magnetic microbeads (Miltenyi Biotec, Auburn, CA). Antigen-presenting cells (APCs) were loaded with 10^4 irradiated cpsII parasites. All protocols adhered to University of Oklahoma Health Sciences Center’s IACUC and to ARVO’s Statement on the Use of Animals in Ophthalmic and Vision Research.

Immunocytochemistry. Mock- and parasite-infected eyes were harvested and fixed for 30 min at room temperature in 5% paraformaldehyde in PBS. Eyes were then placed in increasing concentrations of sucrose (10 to 30%) in PBS at room temperature, followed by overnight incubation at 4°C in PBS containing 30% sucrose. The cryoprotected eyes were then embedded in Tissue-Tek OCT compound (Sakura Fintek, Torrance, CA) and were rapidly frozen in liquid nitrogen. Sectioning was performed using a cryostat (Arctomet, Oakville, ON) in Hanks balanced salt solution (HBSS) for 30 min at 37°C. The single-cell suspension was treated with ACK lysis buffer (Cambrex, East Rutherford, NJ) to lyse the red blood cells. The splenocytes were then incubated with magnetic bead-conjugated antibodies against TCR, CD3, and NK1.1 (Miltenyi Biotec, Auburn, CA). Antigen-presenting cells (APCs) used in the assay were enriched CD11c+ dendritic cells (DCs) prepared by digesting spleens from naive mice with 5 mg/ml collagenase IV (Worthington Biochemical, Lakewood, NJ) and 0.1 mg/ml DNase (Sigma Chemical, St. Louis, MO) in Hanks balanced salt solution (HBSS) for 30 min at 37°C. The single-cell suspension was treated with ACK lysis buffer (Cambrex, East Rutherford, NJ) to lyse the red blood cells. The splenocytes were then incubated with magnetic bead-conjugated antibodies against CD8, CD45, and NK1.1 (Miltenyi Biotec) and were passed through an AutoMACS cell sorter to negatively select for the CD11c+ DCs. The purity of enriched DCs and T cells was always >95% as determined by flow cytometry. Enriched APCs were loaded with STAg protein (1.0 mg/ml) in serum-free RPMI medium by incubation at 37°C for 1 h. T-cell proliferation was measured in each well of a 96-well plate by plating 10^4 purified T cells with 5 × 10^5 STAg-loaded APCs (or 200 μg of STAg alone as a negative control). In addition, retinal cells prepared from mice 6 days after they were either mock infected or intravitreally infected with 10^4 RH parasites were added to the T cells. Plates were incubated for 6 h, and then 1 μCi of [3H]thymidine was added to each well for 6 h. Cells were harvested on glass fiber filters (PhD Cell Harvester; Cambridge Technology, Inc., Cambridge, MA) and counted by liquid scintillation.

IFN-γ cytokine measurement. Mock-infected and parasite-infected eyes were harvested from euthanized mice. The eyes were then enucleated, and posterior
segments were dissected away from the rest of the eye. The posterior segments were then placed in lysis buffer and homogenized with a hand-held homogenizer. 

Singleplex mouse IFN-γ kits were purchased from Bio-Rad (Hercules, CA) and were used according to the manufacturer’s instructions in conjunction with Bio-Plex instrumentation (Bio-Rad). IFN-γ concentrations in tissue supernatants were measured in duplicate and determined by interpolation from standard curves using Bio-Plex software.

RESULTS

Three distinct populations of cells express MHC class II in *Toxoplasma*-infected eyes. Dysregulated CD4+ T cells can cause retinal tissue damage in a variety of instances, including *Toxoplasma* infections and experimental autoimmune uveitis. Since CD4+ T-cell binding to peptide-loaded MHC class II on the target cell surface is a key mechanism for the regulation of effector CD4+ T-cell activity, MHC class II expression was examined in the retinas of *Toxoplasma*-infected mice. Thus, mice were either mock infected or intravitreally infected with 10^4 tachyzoites, and 4 or 6 days later, eyes were isolated and processed for flow cytometry to detect MHC class II. While ~1% of cells in mock-infected eyes were MHC class II+ (and these were class IIlo), more than 10% of the cells were MHC class II+ 4 days postinfection (Fig. 1, left panels). By 6 days postinfection, the numbers of MHC class II+ cells in parasite-infected eyes increased to >46%, while the numbers of class II+ cells in the mock-infected samples remained constant (Fig. 1, right panels). Similar time-dependent increases in staining were observed when eyes were analyzed 6 and 8 days after they were intravitreally injected with 10^5 parasites (not shown).

Hematoxylin-and-eosin (H&E)-stained sections from parasite-infected eyes revealed that large numbers of leukocytes with nuclear morphologies typical of monocytes and neutrophils were present in the retinas and, to a lesser extent, in the vitreous of *Toxoplasma*-infected eyes (7). To determine whether the infiltrating leukocytes were MHC class II+, intravitreally infected eyes were harvested, processed for flow cytometry, and stained with antibodies against MHC class II, CD11b, Ly6G, and CD45. This cocktail of antibodies was used because it can discriminate between neutrophils (CD45+ CD11b+ Ly6G+Lum) and inflammatory monocytes (CD45+ CD11b+ Ly6G−) as well as between retinal microglia (CD45lo) and resident neuroretinal cells (CD45+). Three distinct populations of MHC class II-expressing cells were found in *Toxoplasma*-infected retinas: populations I (CD45+ CD11b+ Ly6G+), II (CD45+ CD11b+ Ly6G−), and III (CD45− CD11b− Ly6G−) (Fig. 2A and B). In addition, populations I and II stained strongly with anti-Ly6C, suggesting that the Ly6G− cells in population II are inflammatory monocytes (Fig. 2C). These data indicated that populations I, II, and III consisted of neutrophils, monocytes, and resident retinal cells, respectively.

The histograms in Fig. 1 indicate that MHC class II expression levels were widespread in *Toxoplasma*-infected retinas. We therefore tested whether all cells expressed heterogenous

**FIG. 2.** Three distinct populations of MHC class II-expressing cells in *Toxoplasma*-infected eyes. Mice were intravitreally injected with 10^4 parasites, and 6 days later, eyes were harvested and analyzed by using flow cytometry. (A) FACS plot of CD11b and Ly6G expression on MHC class II+ gated cells. The distinct populations are highlighted as I (CD11b+ Ly6G+), II (CD11b+ Ly6G−), and III (CD11b− Ly6G−). (B through D) Histograms of CD45 (B), Ly6C (C), and MHC class II (D) expression levels in each of the 3 populations. The dashed lines in panels B and C represent isotype control staining levels. MFI, mean fluorescence intensity of each population. Representative results from three independent experiments are shown.
amounts of MHC class II or whether each population expressed class II at specific levels. The data indicated that neutrophils (population I) and resident retinal cells (population III) expressed low levels of MHC class II, while monocytes (population II) expressed it at much higher levels (Fig. 2D).

Dendritic cell marker staining of MHC class II cells in Toxoplasma-infected retinas. DCs are a large group of APCs that, in general, can be placed into 1 of 3 major classes: conventional DCs (cDCs); inflammatory, blood-derived DCs (iDCs); and plasmacytoid DCs (pDCs). Because CD11c is expressed at high levels on cDCs and iDCs and at low levels on pDCs, we assessed CD11c expression on class II cells 6 days after intravitreal injection of mice with $10^4$ parasites. We found that CD11c was expressed on cells in population II at levels comparable to those of splenic CD11c DCs (not shown). CD11c expression was, however, undetectable on cells in populations I and III (Fig. 3A). In addition, none of the class II cells stained with anti-CD8α or with the dendritic cell marker 33D1 (not shown). These data, combined with the fact that population II cells were Ly6C+, suggested that in parasite-infected retinas, blood-derived monocytes differentiate into iDCs. In addition, the CD45lo MHC class II cells were CD11b+, suggesting that they were not retinal microglia, which are CD11b+ myeloid-derived cells (18, 25). To determine whether MHC class II+ CD45−/− cells were endothelial cells, expression of the CD31 endothelial cell marker was assessed by flow cytometry. The data indicated that class II+ cells were CD31+, indicating that in Toxoplasma-infected retinas, endothelial cells were not MHC class II+ (not shown).

Due to a lack of established flow cytometry markers for other types of retinal cells, we used immunohistochemistry to identify the CD45−/− MHC class II+ cells. Thus, frozen sections

FIG. 3. Dendritic cell staining in Toxoplasma-infected eyes. Mice were intravitreally injected with $10^4$ parasites. Six days later, eyes were harvested and analyzed using flow cytometry. (A) Histogram of CD11c expression on the three populations of MHC class II-expressing cells from Fig. 2A. (B) FACS plots of PDCA1 and Siglec-H staining of class II+ CD11b+ and class II+ CD11b+ cells.
from eyes harvested 6 days after they were intravitreally injected with $10^4$ parasites were stained with antibodies against MHC class II and the *Toxoplasma* tachyzoite surface protein SAG1, as well as with 4',6-diamidino-2-phenylindole (DAPI) to identify the retinal layers (Fig. 4). SAG1 staining, which was localized primarily to necrotic regions of the retina, indicated the presence of large numbers of parasites in parasite-infected retinas. MHC class II staining was not detectable in mock-infected retinas (not shown), which was consistent with our flow cytometry data as well as with other reports that retinas normally express very few class II cells (4). In contrast, there was widespread, strong MHC class II expression in all regions of the infected retina that were SAG1$^+$ (Fig. 4). High class II expression was noted in cells containing bean-shaped nuclei, suggesting that these were the class II$^{hi}$ inflammatory monocytes (Fig. 4, boxed area). We also noted class II staining, which was greater than that in isotype control-stained retinas (not shown), in regions with little SAG1 staining (Fig. 4, arrowheads). These included both the outer nuclear layer (ONL), which contains photoreceptors, and the inner nuclear layer (INL), which contains several neurons, such as bipolar and horizontal cells. Thus, MHC class II expression in *Toxoplasma*-infected retinas is not restricted to cells derived from hematopoietic cells but is also present on multiple types of resident retinal cells.

**Costimulatory-molecule expression on MHC class II$^+$ cells in parasite-infected retinas.** The outcome of an interaction between a T cell and its target cell is dependent on costimulatory molecules, which may either stimulate or inhibit a T cell. We therefore examined the expression of members of the B7 family of costimulatory molecules. Single-cell suspensions prepared from retinas infected with $10^4$ parasites were stained with antibodies against positive-acting (CD80, CD86, and ICOS-L) and negative-acting (PD-L1 and PD-L2) coinhibitory
STAg-loaded CD11c from infected retinas, we first tested what effect the addition of cells could have on T cells and lysis of T cells, since the addition of 1 μM pyrimethamine, a significant inhibitor of parasite replication, had no significant effect on the ability of parasite-infected retinal cells to suppress T-cell responses (Fig. 6B).

We next tested whether the inhibition of T-cell recall responses by parasite-infected retinal cells was PD-L1 dependent. Thus, the T-cell recall assay was repeated, but anti-PD-L1 or an isotype control antibody was added to the T-cell cultures incubated with retinal cells from parasite-infected mice. The data indicated that the isotype control antibody had no significant effect on T-cell inhibition by infected retinal cells (Fig. 6C). In contrast, the anti-PD-L1 antibody significantly abrogated the ability of the infected retinal cells to suppress T-cell proliferation. These data indicate that PD-L1 is functionally expressed in Toxoplasma-infected eyes.

**DISCUSSION**

Immune privilege is important for the retina, because most damaged neural retinal cells cannot be repaired or replaced. The retina maintains its immune-privileged state, in part, by a paucity of endogenous MHC class II expression in the retina (49, 50). Recently, however, cells that do express MHC class II in inflamed retinas have been identified, and the majority of these cells are infiltrating leukocytes, RPE cells, or resident microglia (17, 18, 25, 33, 34, 44, 57). In Toxoplasma-infected eyes, MHC class II was expressed on a much more heterogenous population of resident retinal cells, including retinal neurons such as photoreceptors. It is not clear why other models of inflammation have yet to detect MHC class II expression and PD-L1 expression in Toxoplasma-infected eyes are largely dependent on IFN-γ.
Our work, therefore, further highlights the need to study retinal inflammation with both autoimmune and infection models.

Toxoplasma infections result in the development of long-lived tissue cysts in numerous tissues, including immune-privileged sites such as the retina. Studying the activation and regulation of retinal anti-Toxoplasma immune responses after a cyst reactivates has been difficult for several reasons. First, the number of cysts that develop in the retina is highly variable (12, 39). Second, the timing and location of spontaneous cyst reactivation is difficult to predict as well as to detect. Finally, chronically infected mice develop retinal damage only after becoming immune compromised by either genetic or pharmacological intervention. For these reasons, we used the tachyzoite intravitreal infection model to mimic the events that take place after a cyst ruptures and parasites convert to tachyzoites. The utility of this model is highlighted by the observations in this report (functional MHC class II and PD-L1 expression in the retina). Intravitreal infections do, however, have some caveats, as do all infection models. These include the fact that intravitreal injection of tachyzoites bypass any impact that chronic Toxoplasma tissue cysts and bradyzoites may have on host surveillance and immunity. Nevertheless, the physiological relevance of our findings is supported by recent work demonstrating that PD-L1 mRNA is upregulated in the brains of mice chronically infected with Toxoplasma (20). Moreover, the PD-L1 receptor, PD-1, is expressed on T cells recruited to Toxoplasma-infected brains (55) and retinas (our unpublished results). Another potential caveat is that our experiments were performed primarily by injecting relatively high numbers (10⁴) of parasites. But class II and PD-L1 upregulation also occurred with lower doses of parasites (10³), approximating the number that would be released after as few as 1 to 2 cyst bursts (9).

Our data clearly establish IFN-γ as a critical regulator of MHC class II and PD-L1 expression on both infiltrating leukocytes and resident retinal cells. However, IFN-γ does not appear to be sufficient for class II expression, since intravitreal or subretinal IFN-γ injection upregulated MHC class II in the cornea, in the choroid, and on retinal pigment epithelial cells, but not on cells of the inner retina (4, 19). This suggests that Toxoplasma infection induces retinal cells to become responsive to IFN-γ, perhaps by upregulating IFN-γ receptor expression.

Another important question is what cells are the source for IFN-γ in intravitreally infected mice. During reactivated ocular
FIG. 7. IFN-γ is important for MHC class II and PD-L1 expression in Toxoplasma-infected eyes. (A) Mice were intravitreally injected with 10^6 parasites (Toxo). After 4 and 6 days, eyes were harvested and emulsified, and IFN-γ levels were measured in lysates prepared from posterior segments. P values were determined using an unpaired, nonparametric Student t test. (B) Wild-type and IFN-γKO mice were intravitreally injected with 10^6 parasites. Six days later, eyes were harvested and processed for flow cytometry. Shown are representative FACS plots of MHC class II and PD-L1 expression from three independent experiments.

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toxoplasmosis, CD8+ and CD4+ T cells are important IFN-γ-secreting cells. However, significant numbers of retinal T cells are not detectable 6 days after the injection of 10^6 parasites. Thus, a non-T-cell source for IFN-γ is most likely responsible for the early upregulation of MHC class II and PD-L1. Inflammatory monocytes and neutrophils are the two major types of leukocytes in parasite-infected eyes. The monocytes are not predicted to express significant amounts of IFN-γ in parasite-infected retinas, since a failure to recruit these cells to Toxoplasma-infected peritoneal cavities or mucosae did not result in reduced IFN-γ expression (10, 46). Although a failure to recruit neutrophils during an intraperitoneal infection leads to decreased IFN-γ release, this is most likely due to a loss of neutrophil-derived interleukin 12 (IL-12) (3). However, other studies have demonstrated that neutrophils can express and release IFN-γ (26, 30), indicating that in parasite-infected retinas, neutrophil-derived IFN-γ may contribute to MHC class II and PD-L1 expression. Brain microglia (which stain as CD45<sup>hi</sup> CD11b<sup>hi</sup> cells) are important early sources of IFN-γ after cyst reactivation in the brain (53), and it is therefore possible that retinal microglia respond similarly to Toxoplasma. Finally, a third minor population (<5%) of CD45<sup>+</sup> cells that were CD11b<sup>+</sup> and Ly6G<sup>-</sup> was present in parasite-infected eyes and could be expressing IFN-γ (not shown). The identity of these cells is unknown, since they did not stain with anti-NK1.1 or anti-B220 antibodies, suggesting that they were not NK cells or B cells, respectively.

The importance of IFN-γ in controlling Toxoplasma replication and in upregulating retinal PD-L1 expression suggests that IFN-γ triggers a negative feedback loop to downregulate recruited effector T cells in an effort to limit immune-mediated pathology. IL-10 is another cytokine demonstrated to downregulate T-cell responses to Toxoplasma in the periphery and the retina (14, 37). However, PD-L1 expression is not dependent on IL-10 (5). Moreover, PD-L1 and IL-10 act in parallel to regulate the immune response during chronic lymphocytic choriomeningitis virus infection (5). Similarly, we propose that in the retina, IL-10 and PD-L1 act in concert to prevent immune-mediated tissue damage. Such a model is supported by the well-documented role of PD-L1 in downregulating T-cell-based responses during viral, bacterial, and parasitic infections (see, e.g., references 5, 24, and 31) as well as in various autoimmune models (see, e.g., references 27, 40, 45, and 47). In addition, retinal-cell suppression of T-cell responses was not fully abrogated by the anti-PD-L1 antibody, suggesting the presence of other T-cell-suppressing factors. Our future work will focus on identifying and defining the contributions of these various factors to the immunological responses during ocular toxoplasmosis.

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