

Role of the Immune Modulator Programmed Cell Death-1 during Development and Apoptosis of Mouse Retinal Ganglion Cells

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PURPOSE. Mammalian programmed cell death (PD)-1 is a membrane-associated receptor regulating the balance between T-cell activation, tolerance, and immunopathology; however, its role in neurons has not yet been defined. The hypothesis that PD-1 signaling actively promotes retinal ganglion cell (RGC) death within the developing mouse retina was investigated.

METHODS. Mature retinal cell types expressing PD-1 were identified by immunofluorescence staining of vertical retina sections; developmental expression was localized by immunostaining and quantified by Western blot analysis. PD-1 involvement in developmental RGC survival was assessed in vitro using retinal explants and in vivo using PD-1 knockout mice. PD-1 ligand gene expression was detected by RT-PCR.

RESULTS. PD-1 is expressed in most adult RGCs and undergoes dynamic upregulation during the early postnatal window of retinal cell maturation and physiological programmed cell death (PCD). In vitro blockade of PD-1 signaling during this time selectively increases the survival of RGCs. Furthermore, PD-1-deficient mice show a selective increase in RGC number in the neonatal retina at the peak of developmental RGC death. Lastly, gene expression of the immune PD-1 ligand genes

Pdcd1lg1 and *Pdcd1lg2* was found throughout postnatal retina maturation.

CONCLUSIONS. These findings collectively support a novel role for a PD-1-mediated signaling pathway in developmental PCD during postnatal RGC maturation. (*Invest Ophthalmol Vis Sci*. 2009;50:4941-4948) DOI:10.1167/iovs.09-3602

Modulation of signaling elicited by cell-cell interactions is critical for the formation and remodeling of neuronal synaptic networks in development and learning as well as for the generation of immunity toward environmental and endogenous antigens.^{1,2} In some cases, molecules involved in such selection may be shared by the immune system and the central nervous system (CNS). For example, the major histocompatibility complex class I ligand and its receptor component CD3 ζ , a canonical receptor-ligand immune recognition pair, are required to establish functional connections between the retina and the brain during development,³ and the initiating complement protein C1q marks neural retina synapses for elimination in development and degenerative disease.⁴

In the immune system, cell-cell interaction molecules are critical for regulating lymphocyte function. PD-1 (CD279) is a key immunoregulatory receptor, inducibly expressed on T cells, B cells, NK T cells, activated monocytes, and dendritic cells.⁵ PD-1 transduces an inhibitory signal when engaged in combination with the T cell receptor (TCR). These immunoinhibitory signals regulate the extent of T cell activation, attenuate antimicrobial immunity, facilitate chronic viral infections, and provide inhibitory signals that regulate central and peripheral T cell tolerance.² During the establishment of central tolerance, PD-1 is expressed on developing thymocytes as they progress through several maturational stages in which PD-1 signaling modifies signaling thresholds in thymocytes during positive and negative selection stages of maturation.^{6,7} In addition, PD-1 regulates the induction and maintenance of peripheral T cell tolerance by limiting mature self-reactive T cell function.⁵

We recently observed constitutive neuronal expression of PD-1 in retinal ganglion cells (RGCs),⁸ suggesting that PD-1 may also provide inhibitory signals important for physiological loss of neurons during retinal maturation. In this study, we tested whether PD-1 has a parallel role in negative selection during neuronal network formation in the developing and adult retina, an organ with well-defined cytological architecture that has been an important model for investigating molecular mechanisms of neurogenesis.

MATERIALS AND METHODS

Animals

Mice were purchased from Charles River Laboratory (Wilmington, MA) unless otherwise noted. Embryonic and adult CD1 mice were used for PD-1 blocking experiments. C57BL/6 mice were used for PD-1 immu-

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noblotting and PD-1 ligand gene expression studies. For the PD-1^{-/-} characterization, PD-1^{-/-} mice were constructed in the C57BL/6 background, as previously described,⁹ and wild-type C57BL/6 age-matched mice were purchased from the Charles River Laboratory. All animal experiments were reviewed and approved by the University of California at Los Angeles Chancellor's Animal Research Committee and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Tissue Preparation

Eyes were rapidly enucleated from adult and embryonic mice. Posterior eyecups were fixed in 4% paraformaldehyde at room temperature for 1 hour, followed by cryoprotection in 30% sucrose/PBS and OCT embedding. Cryostat sectioning was performed at a thickness of 6 to 8 μ m. The sections were selected to represent the equivalent regions of the globes and were taken just adjacent to the optic nerve plane. To ensure the validity of analysis, sections were used for quantification only if the retinal architecture represented a vertical section through the retina.

RGC Isolation

Positive selection of RGCs was performed using magnetic beads coated with a Thy-1.1 monoclonal antibody (Millipore/Chemicon, Temecula, CA), as previously described.¹⁰

Retinal Explants

Neural retinas were dissected from newborn postnatal day (P) 0 mice and were cultured as previously described.¹¹ Briefly, retinas were placed on polycarbonate filter discs (Millipore) and were cultured for 24 hours at 37°C/5% CO₂ in Dulbecco modified Eagle medium/F12 (Mediatech, Herndon, VA) containing 10% fetal bovine serum (Omega Scientific, Tarzana, CA), and 1 \times N2 supplement (Gibco, Grand Island, NY). For the antibody blocking experiment, the following functional grade antibodies were used at a final concentration of 7 μ g/mL: anti-mouse PD-1 (clone J43, hamster IgG; eBioscience, San Diego, CA) for which specificity¹² and ability to block interaction with PD-L1 and PD-L2¹³ have previously been described, or isotype control hamster IgG (eBioscience). After 24 hours in culture, explants were fixed, cryoprotected, embedded in OCT, and cryosectioned, as described.

Immunostaining

Sections were incubated at 4°C overnight with the following primary antibodies: anti-mouse PD-1 (clone 29F.1A12, rat IgG2a) for which specificity has previously been described¹⁴; RGC markers anti-Brn3a (clone 5A3.2, mouse IgG1; Millipore/Chemicon)¹⁵ and anti-NeuN (mouse IgG1, clone A60, Millipore/Chemicon)¹⁶; amacrine cell marker anti-mouse AP2 α (3B5, mouse IgG2b; Developmental Studies Hybridoma Bank, Iowa City, IA)¹⁷; or apoptotic cell marker rabbit anti-mouse activated caspase-3 (R&D Systems, Minneapolis, MN).¹⁸ For immunohistochemistry, the rat IgG (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA), AEC substrate kit, and hematoxylin QS counterstain (Vector Laboratories) were used according to product instructions. For immunofluorescence staining, AlexaFluor 488- or AlexaFluor 594-conjugated secondary fluorescent antibodies and 1.4 μ M DAPI counterstain (Invitrogen, Carlsbad, CA) were used.

Quantification of Immunofluorescence Staining

Retinal sections were imaged at 200 \times magnification with a microscope (E800; Nikon, Tokyo, Japan) and a digital camera (SPOTII; Diagnostic Instruments, Sterling Heights, MD), yielding a retinal image width of 0.63 mm. Within the GCL, immunostained nuclei or cells and DAPI-positive nuclei were counted, for each retinal section, by two investigators masked to the identity of samples and experiment. Values are reported as the mean \pm SEM. The Mann-Whitney nonparametric *t*-test was used to analyze differences between experimental groups, and *P* < 0.05 was considered significant. Graphing and statistical analysis

was also performed (Prism 5; GraphPad, San Diego, CA). For the PD-1 blocking study, each of three experimental repeats examined four retinal explants; for each explant, five sections were costained with caspase-3, Brn3a, and DAPI. For the PD-1^{-/-} study, three animals were examined at each time point, for both PD-1^{-/-} and wild-type; at least three retinal sections (*n* \geq 3 animals) were stained with DAPI and either Brn3a or AP2.

Western Blot Analysis

Retinas were isolated from C57BL/6 and PD-1^{-/-} mice. Retinas were sonicated on ice in lysis buffer (30 mM Tris-HCl, 10 mM EGTA, 5 mM EDTA, 1% Triton X-100, 250 mM sucrose)¹⁹ with complete protease inhibitor cocktail (Roche, Nutley, NJ), for 2 minutes (4 \times 30-second pulses). Thirty micrograms of each lysate was analyzed by SDS-PAGE, as previously described.²⁰ These primary antibodies were used: rat anti-mouse PD-1 (clone 29F.9A2, rat IgG2a,k) or mouse anti- β -actin (clone 2A2.1, mouse IgG1; US Biological, Swampscott, MA). PD-1 and β -actin were detected with anti-rat IgG-HRP and anti-mouse IgG-HRP (Southern Biotechnology Associates, Birmingham, AL), respectively.

Reverse Transcription-Polymerase Chain Reaction

Neural retinas were dissected or primary thymocytes were isolated. Retinas from three animals were pooled for each time point; three experimental repeats were performed. Tissues or cells were stabilized (RNA Later; Qiagen, Valencia, CA). Total RNA was extracted (RNeasy Mini Kit; Qiagen) and purified with a cleanup kit (RNeasy MinElute Cleanup Kit; Qiagen). Total RNA (0.5 μ g) was used as a template for reverse transcription (SuperScript III First-Strand Synthesis SuperMix for qRT-PCR; Invitrogen) and were amplified (Platinum Quantitative PCR SuperMix-UDG; Invitrogen) on a platform (ABI 7500; Applied Biosystems, Foster City, CA). The following 20 \times assays (TaqMan; Applied Biosystems) were used: mouse GAPDH endogenous control, FAM-MGB (GenBank accession no. NM_008084.2); PD-L1-FAM (Mm00452055_m1, GenBank accession no. NM_021893.2); PD-L2-FAM (Mm01208507_m1, GenBank NM_021396.1).

RESULTS

PD-1 Expression in RGCs and Displaced Amacrine Cells of the Adult Mouse Retina

In the adult retina, PD-1 expression was detected primarily in the GCL, and a subpopulation of cells was detected in the inner nuclear layer.⁸ Given that the mouse retina consists of six functionally distinct neuronal cell types distributed in three cell layers, we were interested in determining the identity of the cell populations that express PD-1. Immunofluorescent double labeling with antibodies directed against specific retinal cell makers revealed colocalization of PD-1 with a specific RGC marker, Brn3a, and an amacrine cell marker, AP2 α , hereafter referred to as AP2 (Fig. 1A). To further validate RGC-specific PD-1 expression, we used another well-established RGC marker, Thy-1.²¹ Thy-1.1-conjugated magnetic beads were used to isolate RGCs, followed by PD-1 immunostaining (Fig. 1B), and our results confirm that Thy-1-positive cells are PD-1 positive. Quantification of costaining demonstrated that more than 95% of the Brn3a-positive cells were PD-1 positive. Among the PD-1-positive cells in GCL, 68% were Brn3a-positive RGCs, and 28% were AP2-positive amacrine cells (Fig. 1C). To assess the possibility that PD-1 expression is coupled to Brn3a expression, we assessed the colocalization of PD-1 with an independent RGC marker, NeuN¹⁶ (Fig. 2A). Among NeuN-positive RGCs, we found 99% \pm 0.2% PD-1-positive and 1.2% \pm 0.2% PD-1-negative cells (Fig. 2B). Thus, we conclude that authentic PD-1 is expressed by mature retinal neurons, predominantly in the RGC population.

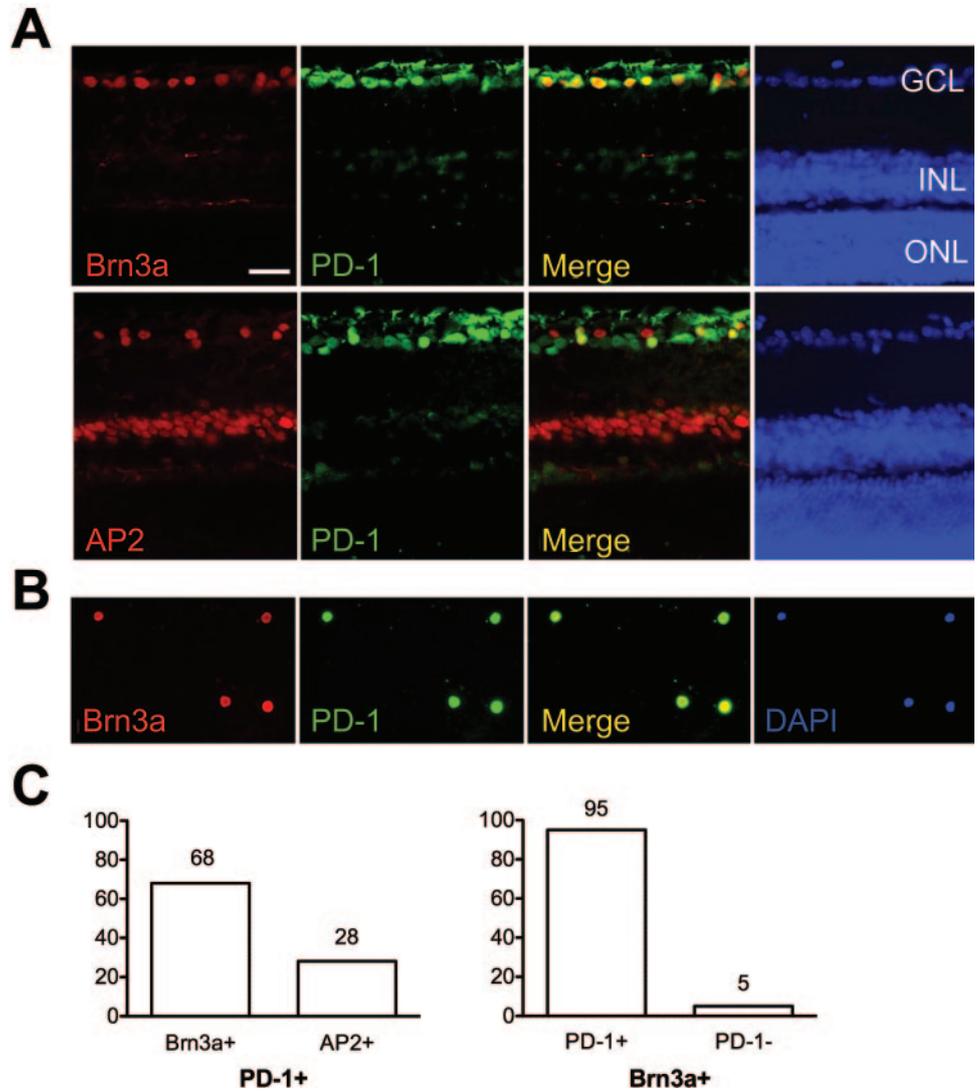


FIGURE 1. PD-1 expression in the adult mouse retina. **(A)** Double staining of PD-1 with Brn3a or AP2 on vertical retina sections. Brn3a or AP2 (red), PD-1 (green), merge (yellow), and nuclear DAPI (blue) staining of retinal sections. **(B)** Isolated RGCs using Thy-1-coated magnetic beads. Brn3a (red), PD-1 (green), and nuclear DAPI (blue). **(C)** Quantification of PD-1-positive population in vivo. Percentage of Brn3a (68% ± 11%) or AP2 (28% ± 7%) cells among PD-1-positive cells (left). Percentage of PD-1 positive (95% ± 3.4%) and PD-1 negative (5% ± 1.6%) cells among Brn3a-positive RGCs (right). Percentages represent mean ± SEM. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar, 50 μm. These findings are representative of four experiments.

Dynamic Developmental Expression of PD-1 in the Retina

We next characterized the regulation of PD-1 expression in developing retina. RGCs are the first retinal neurons, emerging after embryonic day (E) 11 in mice and completing maturation after a postnatal retinal maturation process.²² Therefore, we examined the spatiotemporal expression pattern of PD-1 at defined times during the period of retinal development. Low levels of PD-1 expression were initially detected in the inner and outer neuroblast layers by immunohistochemistry at E14

(Fig. 3A), around the peak period of RGC genesis. Weak expression of PD-1 appeared throughout the GCL, which contained RGCs, and the neuroblast layer, which contained migrating RGC precursors and retinal progenitors (Fig. 3A, E17). By P0, relatively high levels of PD-1 were present in the inner retina, where postmigratory RGC precursors undergo differentiation; substantial PD-1 expression was also detected in a subset of proliferating neuroblasts (arrows) occupying the ventricular zone (vz). In contrast to the embryonic and adult time points, PD-1 expression appeared greatest at P0 and P13 (Fig. 3A). In the adult retina, PD-1 expression appeared decreased,

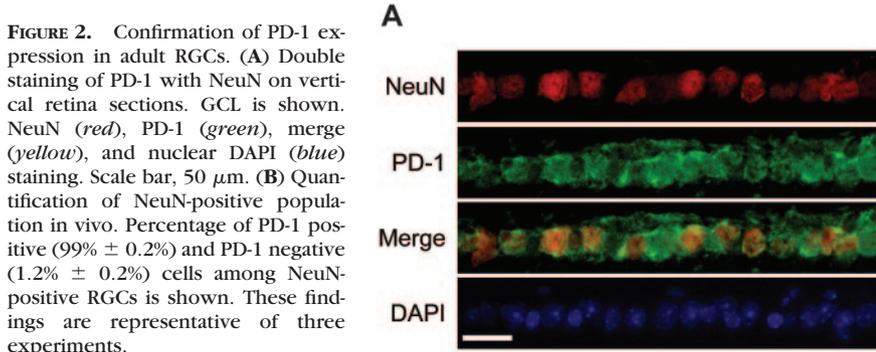
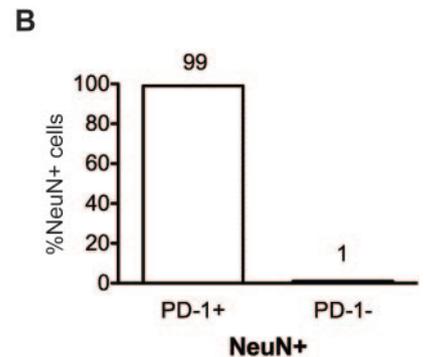


FIGURE 2. Confirmation of PD-1 expression in adult RGCs. **(A)** Double staining of PD-1 with NeuN on vertical retina sections. GCL is shown. NeuN (red), PD-1 (green), merge (yellow), and nuclear DAPI (blue) staining. Scale bar, 50 μm. **(B)** Quantification of NeuN-positive population in vivo. Percentage of PD-1 positive (99% ± 0.2%) and PD-1 negative (1.2% ± 0.2%) cells among NeuN-positive RGCs is shown. These findings are representative of three experiments.



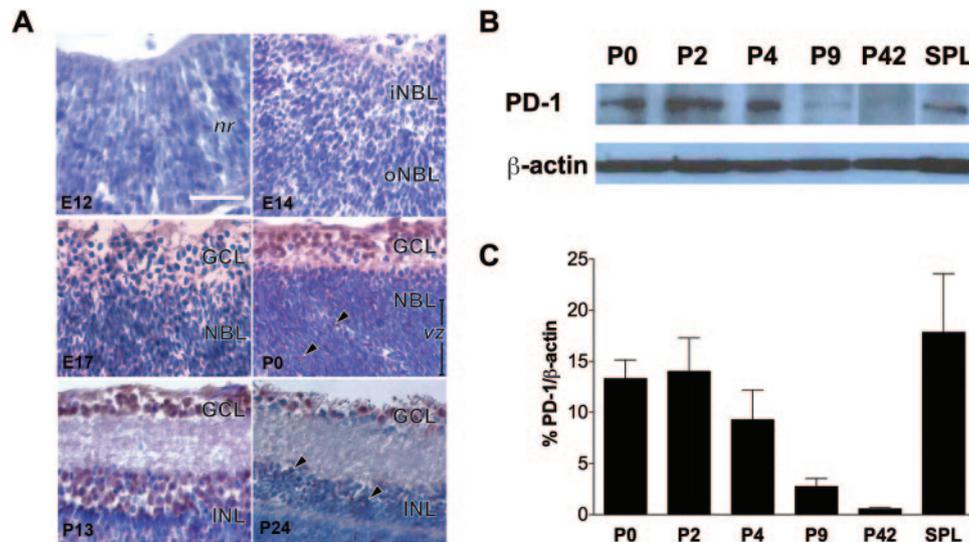


FIGURE 3. Dynamic regulation of PD-1 expression during retinal development. (A) Immunohistochemical staining on retinal sections from E12, E14, E17, P0, P13, and P24. PD-1 (red) and hematoxylin (blue). Scale bar, 50 μ m. These findings are representative of three experiments. (B) Western blot for PD-1 at P0, P2, P4, P9, P42, and spleen as a positive control. β -Actin was used as a loading control. (C) Quantification of PD-1 immunoblots. PD-1 expression decreased by 80% \pm 3.8% by P9 and 96% \pm 0.9% by P42 compared with P0 expression; values reported as mean \pm SEM. $P = 0.03$ by ANOVA analysis across all retina samples. Negative controls for reagent (secondary antibody only) and tissue (skeletal muscle) showed no detectable staining (data not shown). These findings are representative of three animals per time point. *nr*, neural retina; *iNBL*, inner neuroblast layer; *oNBL*, outer neuroblast layer; *vz*, ventricular zone; *SPL*, spleen.

with expression retained in the GCL and in a subset of INL neurons (Fig. 3A, P24, arrows). These observations by immunohistochemistry were confirmed through quantification of postnatal PD-1 protein expression by Western blot analysis (Fig. 3B). Retinal PD-1 expression was greatest from P0 to P4, coinciding with a very active time of neuronal culling²³; total PD-1 expression decreased dramatically, 85% by P9 and 96% by P42, compared with P0 (Fig. 3C). Together, these findings indicate that PD-1 is present in the developing retina, especially in the differentiating RGCs, and the dramatic increase in PD-1 expression coincides with the period of retinal cell maturation and synaptogenesis.

PD-1 Signaling Modulation of Neonatal RGC Apoptosis

During murine postnatal retina maturation, 50% of RGCs undergo PCD,²³ representing one of the key mechanisms regulating cell number homeostasis. To test whether functional PD-1 signaling is involved in the apoptosis of neonatal RGCs, we performed a study on postnatal retinal explants, a culture system that preserves neuronal connections and retinal cytological architecture and serves as an *in vitro* model to study the developing retina. Neonatal retinal explants were treated with an antibody that blocks PD-1 function and were compared with isotype control antibody treated and untreated explants. After 24-hour incubation, explants were evaluated for expression of cleaved caspase-3, Brn3a, and AP2 as markers for apoptosis, RGCs, and amacrine cells, respectively.

When retinal explants were cultured in the presence of a PD-1 blocking antibody, there was a significant decrease in caspase-3-mediated apoptosis in the GCL of retinal explants ($P < 0.0001$; Figs. 4A, B). In contrast, isotype control antibody and untreated controls had indistinguishable levels of apoptosis ($P = 0.25$; Fig. 4B). Brn3a, a specific marker for RGCs, was observed to be downregulated or absent during RGC apoptosis.²⁴ A significant increase in the number of Brn3a-positive cells was observed in the explants that were exposed to the

PD-1 functional blocking antibody ($P < 0.0001$; Figs. 4A, C), concordant with the decreased apoptosis in the GCL. We also assessed the effect of blocking PD-1 in amacrine cells, a subset of which expresses PD-1 (Fig. 4D). We considered amacrine cells as a specificity control because, unlike RGCs, amacrine cells are resistant to apoptosis during the first 2 days after birth.²⁵ Amacrine cells did not undergo apoptosis in our *in vitro* system (Fig. 4D), and there were no significant changes in numbers of amacrine cells after the administration of antibodies ($P = 0.48$; Fig. 4D). Taken together, the data suggest that PD-1 signaling is important in murine postnatal retinal maturation during which active signaling through the PD-1 axis may be critical for caspase-3-mediated RGC apoptosis during this developmental period.

Selective Increase in RGC Cellularity during Peak of Developmental RGC Apoptosis Due to Absence of PD-1

To test the physiological importance of PD-1 in eye development, we compared retinal structure and cellular composition in the developing PD-1^{-/-} mouse retina with those of age-matched wild-type C57BL/6 controls. To assess GCL composition, immunofluorescent staining was performed using the RGC- and amacrine cell-specific nuclear markers Brn3a and AP2, respectively (Fig. 5A). Total cell number in the GCL, as measured by the number of DAPI-positive cells per millimeter, was not significantly changed in the absence of PD-1 at P2 (Fig. 5B). However, during this critical developmental window, the PD-1^{-/-} retina experienced a significant increase in the fraction of Brn3a-positive RGCs ($P < 0.0001$) within the GCL (fraction refers to Brn3a/DAPI and will hereafter be referred to as RGC fraction). In contrast, there was no significant change in the fraction of AP2-positive amacrine cells ($P = 0.15$) within this layer (Fig. 5B). In the PD-1^{-/-} animals, at P2 and P4, the peak of naturally occurring PCD, there was an increase in the number of Brn3a-positive cells in the GCL ($P < 0.0001$ for

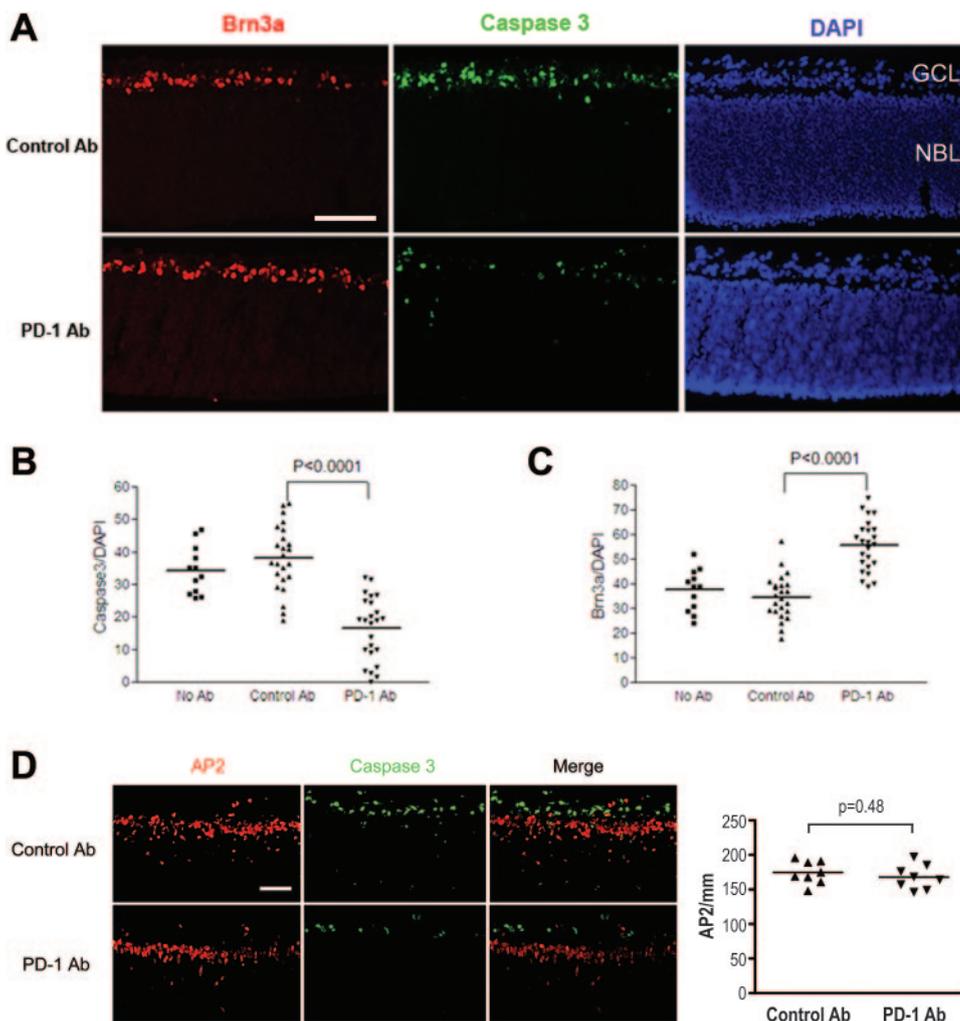


FIGURE 4. PD-1 blockade inhibits apoptosis of retinal ganglion cells in the developing retina. Neonatal retinal explants were incubated for 24 hours in basal medium containing isotype control antibody (*top*) or PD-1 blocking antibody (*bottom*) (**A**, **D**). Immunofluorescent staining was performed with antibodies against Brn3a (*red*, **A**) or AP2 (*red*, **D**), activated form of caspase-3 (*green*), and DAPI (*blue*). Quantification of percentage of Brn3a-positive cells among DAPI-positive cells (**B**) or caspase-3 positive cells among DAPI-positive cells (**C**) in the GCL in three experimental groups, as indicated in the figure. (**D**) Amacrine cell survival is not affected by PD-1 blockade. Immunofluorescence staining was performed, and the merged image supports lack of colocalization of cleaved caspase-3 and AP2-positive amacrine cells. Quantification of AP2-positive cells also revealed no difference between the anti-PD-1 and control antibody treatments. Scale bars, 100 μ m. All findings are representative of three experiments.

each, Fig. 5C); the RGC fraction was also significantly increased at P2 and P4 ($P \leq 0.0003$ for each, data not shown). The early developmental increase in RGC number did not persist: by P9 and subsequent time points, the PD-1^{-/-} retina showed no significant difference in RGC number (P9, $P = 0.62$; adult, $P = 0.052$, Fig. 5C) or RGC fraction (P9, $P = 0.40$; adult, $P = 0.29$; data not shown) compared with wild-type. These data show that the absence of PD-1 is associated with a transiently increased RGC number during the peak of postnatal neuronal culling in the mouse retina, providing further evidence for the importance of PD-1 function during the critical window of retinal maturation.

PD-1 Ligand Genes Expressed throughout Postnatal Retina Maturation

To investigate the cell surface interactions responsible for PD-1-mediated PCD in the developing mouse retina, we sought to characterize expression of the PD-1 ligands present during retina development. There are two known PD-1 ligands in the immune system, PD-1 ligand 1 (PD-L1) and PD-1 ligand 2 (PD-L2), which, on ligation with PD-1, function to regulate the balance between lymphocyte activation and suppression.⁵ In mice, PD-L2 expression is thought to be restricted to hematopoietic cell types, whereas PD-L1 protein is widely expressed in many organs, including immunoprivileged regions such as placenta,⁵ corneal endothelium and ciliary body of the eye,²⁶ and brain.²⁷ The PD-L1 gene, *Pdcd1lg1*, is transcribed in the mature mouse retina,²⁶ making it a strong candidate for PD-

ligand functioning during developmental culling. We examined PD-L1 and PD-L2 expression during postnatal development at P0, P2, P4, P7, and P30 by RT-PCR and found both ligands to be expressed at all ages studied (Fig. 6), supporting the idea that PD-L1 or PD-L2, or both, is the activating signal for PD-1-mediated PCD.

DISCUSSION

PCD regulates CNS cell-number homeostasis and formation of functional neuronal networks^{28,29} through a balance of survival factors and death signals.²² In particular, it has been well established that neurotrophic factors and electrical activity contribute to postnatal RGC survival.^{25,30-34} This study introduces a distinct hypothesis that PCD may also result from an active negative selection process through the PD-1 pathway. PD-1 protein is transiently upregulated in the GCL and INL during the critical postnatal period of RGC target-finding and synaptogenesis. This spatiotemporal pattern is consistent with an important role for PD-1 receptor function in RGC culling. Our functional perturbations, using a PD-1 receptor-neutralizing antibody in vitro and PD-1-deficient mice in vivo, have demonstrated selective increases in RGC survival, thus strongly supporting a role for PD-1 signaling during the peak period of RGC physiological PCD. PD-1 is also developmentally expressed in a subset of cells located within the ventricular zone of the neonatal retina, where retinal progenitors are localized, so it is possible that PD-1 ligation has an additional role in

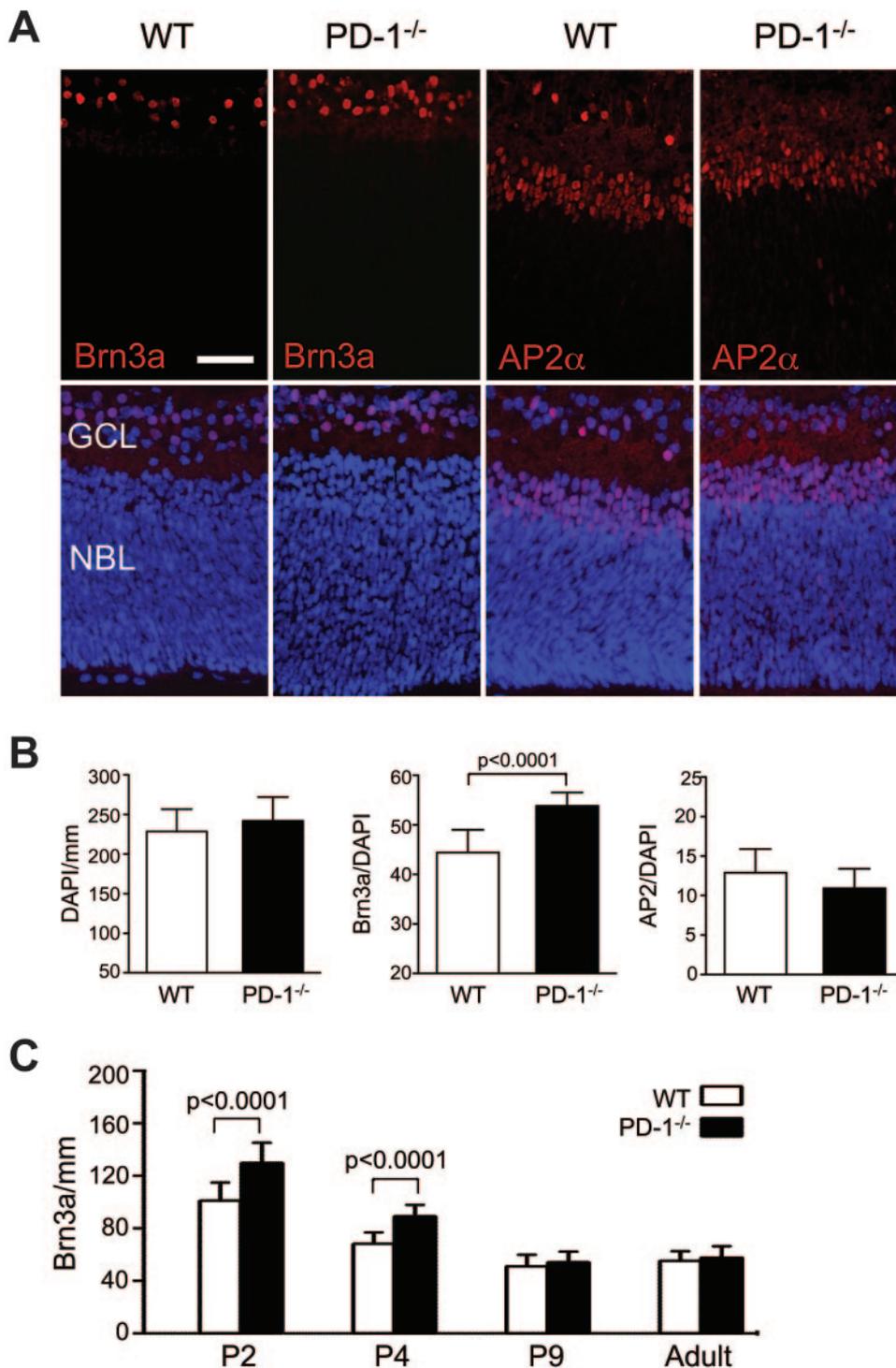


FIGURE 5. Increased RGCs during postnatal retinal development in the PD-1^{-/-} mouse. **(A)** Immunofluorescence staining was performed on wild-type and PD-1^{-/-} P2 retina cryosections using antibodies against either Brn3a (red) or AP2 (red). Nuclei were visualized with DAPI (blue). **(B)** Within the GCL, quantification of total cells (DAPI/mm), Brn3a⁺ cells (Brn3a/DAPI), and AP2⁺ cells (AP2/mm), at P2, in wild-type and PD-1^{-/-}. *P* values denote significant differences between wild-type and PD-1^{-/-}. **(C)** Quantification of Brn3a in wild-type and PD-1^{-/-} retinal sections from P2, P4, P9, and adult (8-week-old) animals was performed as noted. *P* values denote significant differences, at P2 and P4, between wild-type and PD-1^{-/-}. Scale bar, 50 μ m. NBL, neuroblast layer.

regulating the survival of progenitors. Whether the observed RGC protective effect functions directly through a blockade on RGCs or indirectly through an effect on progenitors is an important consideration. Because no overt altered phenotypes have been detected in other postnatally generated cell types, such as amacrine cells, in PD-1 null retinas, it is unlikely that PD-1 plays a significant developmental role in controlling amacrine cell number. The role of PD-1 expression and the definition of the amacrine cell subset expressing this molecule require additional investigation. Our results thus support a direct function for PD-1 signaling in RGC survival. We present the first evidence that PD-L1 and PD-L2 are transcribed

throughout postnatal maturation of the retina. PD-ligands may be expressed by neuronal, endothelial, or resident immune (e.g., macrophage or dendritic) cell types; spatiotemporal localization of these ligands will be key to understanding which cell compartments experience the consequences of PD-1-mediated signaling during development. However, we cannot rule out the possibility that, in addition to the known PD ligands, novel PD-ligand-like molecules could be involved in CNS-specific functions of PD-1 signaling.

Given that PD-1 ligation does not directly engage apoptotic pathways in T cells but, rather acts as a cosignaling event, reducing signal strength from the TCR during thymocyte se-

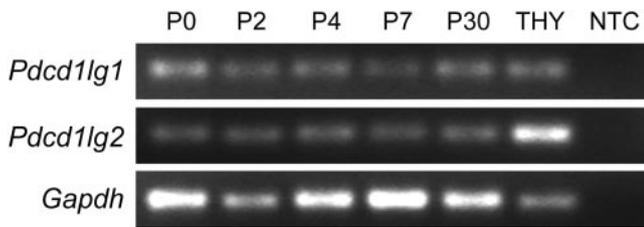


FIGURE 6. Expression of PD-L1 and PD-L2 throughout retina maturation. RT-PCR products showing *Pdccl1g1* (PD-L1 gene) and *Pdccl1g2* (PD-L2 gene) expression at various ages, with *Gapdh* as an endogenous control and thymus as a positive control. NTC, no template control.

lection,⁶ we propose a similar mechanism for PD-1 signaling during RGC culling: PD-1 may act to modulate the strength of neuronal survival signals. We have determined that the TCR subunits TCR β and CD3 ϵ are not expressed in the developing or adult retina by RT-PCR or immunostaining (data not shown). Thus, TCR is not the neuronal co-receptor, and identification of such molecules will be an important next step. It should be noted that the PD-1^{-/-} retina shows only a transient increase in RGCs, with normal cellularity after retinal maturation is complete. This implies a role for additional mechanisms that determine the overall outcome of neuronal culling in the retina, presumably including those previously delineated.^{25,30-34} Because immune PD-ligands are known to have functional interactions with molecules other than PD-1,³⁵ we cannot rule out the possibility that one or both PD-ligands could be necessary for RGC culling. Although normal RGC cellularity is eventually achieved in PD-1^{-/-} mice, it will be important to determine whether the temporal disorder of RGC formation has consequences for definitive retina organization, retinogeniculate synapse formation, and visual function.

Programmed cell death of RGCs is prominent during the first 11 days after birth, peaking during the first 5 days,^{28,36} with a key role for caspase-3.^{18,37} Our findings are consistent with a PD-1-mediated mechanism for physiological apoptosis through a caspase-dependent pathway as PD-1 blockade decreases caspase-3 induction. The retinal explant system used in this study involves experimental axotomy, but axotomy-induced RGC death is an unlikely explanation for our observations because it is independent of caspase activation in the neonatal retina.³⁸ Although the precise PD-1-mediated apoptotic mechanism has yet to be defined, two possible mediators are proapoptotic Bax³⁹ and anti-apoptotic Bcl-2,⁴⁰ both known to be critical intracellular effector molecules regulating developmental RGC apoptosis in mouse models.⁴¹ Furthermore, Bcl-2 is negatively regulated by PD-1-mediated signaling during immunologic thymocyte maturation.⁶

According to the trophic factor theory for neuronal survival, RGCs die after unsuccessful competition for target-derived trophic factors, with stable connectivity also influenced by neuronal activity.^{33,34} The present study provides evidence for an additional regulatory mechanism: RGC death can also involve an active process of negative selection. It is notable that developmental RGC apoptosis may be similar to the molecular pathogenesis of RGC degeneration.^{42,43} Intriguingly, the PD-1 receptor is constitutively expressed, and PD-1 ligands are transcribed in the adult retina, a state in which there is no active cell death, and we speculate that PD-1 ligation may trigger RGC death subsequent to optic nerve damage. In addition, since PD-1 is constitutively expressed across neuronal populations in many cerebral compartments,⁸ PD-1 signaling might also act to augment neuronal injury in cerebral disease. Accordingly, the present study gives impetus to investigation of the role of PD-1 in neurodegenerative diseases and in specific therapies for neurodegenerative disorders.

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