

UCLA

UCLA Previously Published Works

Title

Choroidal $\gamma\delta$ T cells in protection against retinal pigment epithelium and retinal injury

Permalink

<https://escholarship.org/uc/item/6df002tz>

Journal

The FASEB Journal, 31(11)

ISSN

0892-6638

Authors

Zhao, Zhenyang
Liang, Yuejin
Liu, Yin
et al.

Publication Date

2017-11-01

DOI

10.1096/fj.201700533r

Peer reviewed

Choroidal $\gamma\delta$ T cells in protection against retinal pigment epithelium and retinal injury

Zhenyang Zhao,* Yuejin Liang,[†] Yin Liu,[‡] Pei Xu,* Miles J. Flamme-Wiese,[§] Deming Sun,[¶] Jiaren Sun,[†] Robert F. Mullins,[§] Yan Chen,* and Jiyang Cai^{*1}

*Department of Ophthalmology and Visual Sciences and [†]Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas, USA; [‡]Department of Neurobiology and Anatomy, University of Texas Health Science Center at Houston, Houston, Texas, USA; [§]Stephen A. Wynn Institute for Vision Research, University of Iowa, Iowa City, Iowa, USA; and [¶]Doheny Eye Institute, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, California, USA

ABSTRACT: $\gamma\delta$ T cells located near the epithelial barrier are integral components of local inflammatory and innate immune responses. We have previously reported the presence of choroidal $\gamma\delta$ T cells in a model of chronic degeneration of the retinal pigment epithelium (RPE). The goals of the current study were to further define the functions of choroidal $\gamma\delta$ T cells and to explore the underlying mechanisms of their action. Our data demonstrate that choroidal $\gamma\delta$ T cells are activated by RPE injury in response to NaIO_3 treatment, and that they express genes that encode immunosuppressive cytokines, such as IL-4 and IL-10. $\gamma\delta$ T cell-deficient mice developed profound RPE and retinal damage at doses that caused minimal effects in wild-type mice, and adoptive transfer of $\gamma\delta$ T cells prevented sensitization. Intravitreal injection of IL-4 and IL-10 ameliorated RPE toxicity that was induced by NaIO_3 . *Ex vivo* coculture of $\gamma\delta$ T cells with RPE explants activated the production of anti-inflammatory cytokines *via* an aryl hydrocarbon receptor (AhR)-dependent mechanism. AhR deficiency abolished the protective effects of $\gamma\delta$ T cells after adoptive transfer. Collectively, these findings define important roles for choroid $\gamma\delta$ T cells in maintaining tissue homeostasis in the outer retina.—Zhao, Z., Liang, Y., Liu, Y., Xu, P., Flamme-Wiese, M. J., Sun, D., Sun, J., Mullins, R. F., Chen, Y., Cai, J. Choroidal $\gamma\delta$ T cells in protection against retinal pigment epithelium and retinal injury. *FASEB J.* 31, 000–000 (2017). www.fasebj.org

KEY WORDS: inflammation · choriocapillaris drop out · immune modulation · AhR

The epithelial barrier of skin, lung, intestine, and the reproductive tract is part of the innate immune system and separates the core of the body from the environment. Tissue-associated T cells near the epithelium are part of the first line of defense that encounters pathogens and plays major roles in immune surveillance (1–3). A majority of these cells are $\gamma\delta$ T cells, which can display tissue-specific and unconventional functions, such as producing neurotrophic (4) and keratinocyte growth factors, to support epithelial cells and maintain the integrity of the barrier (5). Deficiency in $\gamma\delta$ T cells has been associated with an increased vulnerability of epithelial or mucosal tissues to injury and aggravated autoimmune responses (6, 7). Retinal pigment epithelium (RPE) is part of the blood-retinal

barrier that lines between the neural sensory retina and choroid. $\gamma\delta$ T cells in the choroid have not been well characterized, but likely share some of the distinct properties of the intraepithelial lymphocytes (IELs) in other organs (8).

RPE controls the immune responses of the posterior eye in a number of ways, such as producing cytokines and complement proteins, and interacts with T lymphocytes, macrophages, and microglia (9–11). Conditions of RPE degeneration, including age-related macular degeneration (AMD) and retinitis pigmentosa, are believed to involve dysregulated immune responses (12, 13). Although extensive studies have been performed to demonstrate how activated immune cells and complement proteins contribute to the inflammatory and angiogenic responses in the outer retina (14–17), it has not been established whether the innate immune cells of the choroid can exert protective effects against RPE injury.

In our previous studies on the basis of a chronic RPE degeneration model, we identified choroidal $\gamma\delta$ T cells that can produce IL-17 (18). The goals of the current study were to further define the functions of choroidal $\gamma\delta$ T cells and to explore the underlying mechanism that controls their properties in genetic and chemical toxicity models of RPE

ABBREVIATIONS: AhR, aryl hydrocarbon receptor; AMD, age-related macular degeneration; Dil, 1,1'-diiodo-3,3',3'-tetramethylindocarbocyanine perchlorate; IEL, intraepithelial lymphocyte; ONL, outer nuclear layer; RPE, retinal pigment epithelium; TCR, T cell receptor

¹ Correspondence: Department of Ophthalmology and Visual Sciences, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555, USA. E-mail: jicai@utmb.edu

doi: 10.1096/fj.201700533R

This article includes supplemental data. Please visit <http://www.fasebj.org> to obtain this information.

injury. Our results demonstrate that RPE damage elicited the expansion and activation of protective choroidal $\gamma\delta$ T cells. RPE modulated the functions of $\gamma\delta$ T cells and promoted their production of immunosuppressive cytokines *via* mechanisms that are dependent on aryl hydrocarbon receptors (AhRs).

MATERIALS AND METHODS

Mice

Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch. T cell receptor (TCR) δ chain knockout mice (Tcrd^{tm1Mom}) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). AhR knockout mice (Ahr^{tm1.2Arte}) were purchased from Taconic (Hudson, NY, USA). All mice were housed under cyclic 12-h light/dark conditions and fed *ad libitum*. The Rd8 mutation in Crb1 gene was routinely screened (19, 20) and was not detected. Euthanasia was performed by exposing mice to carbon dioxide that was delivered at 2 L/min *via* a 3-stage gas regulator. All procedures were conducted in accordance with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. All study mice were age 3–5 mo.

Abs and chemicals

Ab against glial fibrillary acidic protein was purchased from Cell Signaling Technology (Danvers, MA, USA). All other Abs were obtained from eBioscience (San Diego, CA, USA), and their detailed information is listed in Supplemental Table 1. NaIO₃ was purchased from Sigma-Aldrich (St. Louis, MO, USA). Peanut agglutinin, DAPI and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

In vivo examination of mouse fundus

Spectral-domain optical coherence tomography was performed on a Spectralis multimodality imaging system (Heidelberg Engineering, Carlsbad, CA, USA) as described previously (18). A 20-diopter lens (Edmund Optics, Barrington, NJ, USA) was used to adapt for the refraction of the mouse eye. Color fundus images were obtained by using a Micron III mouse fundus imaging system (Phoenix Research Labs, Pleasanton, CA, USA) (18). During image acquisition, animals were anesthetized by isoflurane inhalation from a Precision vaporizer (Harvard Apparatus, Holliston, MA, USA), with the cornea kept moisturized and the pupil fully dilated by Tropicamide Ophthalmic Solution (Bausch & Lomb, Rochester, NY, USA).

Histopathology and immunofluorescence staining

Paraffin sections of eyes were prepared as described previously (18). For each mouse eye, ~250 4- μ m-thick sagittal sections were cut from the cornea to the optic nerve and stained with hematoxylin and eosin. Every section was reviewed for RPE and retina pathology. To count the number of photoreceptor nuclei in the outer nuclear layer (ONL), serial sections were cut along the horizontal meridian. Ten slides from the periopic nerve area that spanned a distance of ~200 μ m were picked for quantification of the number of ONL nuclei (21). Cryosections of posterior eyes were used for immunostaining. Sagittal cryosections (8- μ m

thick) were prepared from the cornea to the optic nerve and stained for various antigens of interest (18). To block nonspecific binding, tissue sections were incubated with normal serum that was diluted in PBS with 0.5% Triton X-100. They were then incubated with primary Abs followed by Alexa Fluor-conjugated secondary Abs (Thermo Fisher Scientific). Nuclei were counterstained with DAPI. Images were obtained with a Carl Zeiss AxioVision microscope equipped with ApoTome (Zeiss, Jena, Germany). Cryosections of human donor eyes were obtained from the Iowa Lions Eye Bank (Iowa City, IA, USA) (22). Among the 10 AMD eyes that were examined, two showed lesions of choroidal neovascularization.

Quantitative RT-PCR analyses of RNA isolated from retina or RPE/choroid tissues

Eyes were enucleated from euthanized mice. After removal of the anterior segment, the neural retina and RPE/choroid tissues were harvested as described previously (20), and lysed in Trizol reagent (Thermo Fisher Scientific). Total RNA was isolated according to manufacturer instructions and treated with DNase (Thermo Fisher Scientific) to remove the trace amount of genomic DNA (23). cDNA was synthesized by using M-MLV Reverse Transcriptase and oligo(dT)₁₅ primer (Promega, Madison, WI, USA). Primers used for gene-specific PCR amplifications were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Primer sequences are listed in Supplemental Table 2.

Adoptive $\gamma\delta$ T-cell transfer

Splenic $\gamma\delta$ T cells were isolated by using paramagnetic beads according to the manufacturer's protocol (Miltenyi Biotec, San Diego, CA, USA). In brief, cells from the spleen were liberated by collagenase digestion and filtered through a 70- μ m nylon mesh. Red blood cells were removed by hypotonic lysis (Sigma-Aldrich). Remaining cells were labeled with FITC-conjugated anti-TCR $\gamma\delta$ Ab (GL3; eBioscience), followed by a secondary labeling with bead-conjugated anti-FITC Ab. The resultant cell suspension was loaded onto a separation column within a magnetic field that trapped the bead-bound $\gamma\delta$ T cells. Purified $\gamma\delta$ T cells were counted, and a total of 1×10^6 cells were transferred into the TCR $\delta^{-/-}$ mouse by intraperitoneal injection (24). For cell-tracing studies, isolated $\gamma\delta$ T cells were labeled with 5 μ M carboxyfluorescein succinimidyl ester (Thermo Fisher Scientific) before adoptive transfer (24, 25).

Optokinetic tracking

Visual function assessment was performed by using previously described methods (26). In brief, the testing mouse was placed in the center of a square box with 4 sides that were composed of LED monitors. A 3-dimensional virtual rotating drum was created to trigger the optokinetic reflex by displaying vertical sine wave grating, the frequency and direction of which were controlled by a computer program (OptoMotry; CerebralMechanics, Lethbridge, AB, Canada). A positive response was regarded as a smooth pursue movement of the mouse's head and neck. Responses were recorded when the spatial frequency of the grating was gradually increased until an optomotor movement could not be elicited.

Flow cytometry analyses of lymphocytes in the posterior eye

Ocular lymphocytes in single-cell suspension were prepared as described previously (18). In brief, retina and RPE/choroid

tissues were collected from enucleated eyes and digested with 0.05% collagenase D at 37°C for 30 min. At the end of the incubation, tissues were gently grinded and pipetted to release cells. After passing through a 40- μ m nylon cell strainer, cells were collected by centrifugation at 400 g for 4 min. The cell pellet was resuspended in 30% Percoll/RPMI 1640 solution and laid over a 30%/70% discontinuous Percoll gradient (Sigma-Aldrich), then centrifuged at 400 g for 30 min at room temperature. Enriched lymphocytes at the interphase of the lower one third of the gradient were carefully collected for additional analyses. Staining for cell-surface markers and intracellular antigens was performed according to our previously published methods (18). In brief, cells were stained with fixable viability dye eFluor 506 (eBioscience), blocked with Fc γ R blocker (CD16/32), and stained for specific surface molecules. For intracellular staining, cells were incubated for 4 h with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (750 ng/ml) in the presence of GolgiStop (BD Bioscience, Brea, CA, USA). After surface staining, cells were fixed, permeabilized, and stained for intracellular cytokines by using intracellular fixation buffer (eBioscience). Samples were analyzed on an LSRII fluorescence-activated cell sorting Fortessa flow cytometer (Becton Dickinson, San Jose, CA, USA), and data were processed with FlowJo software (TreeStar, Ashland, OR, USA).

Indirect immunofluorescent staining of choroid/sclera flat mount

Mice were terminally anesthetized and subjected to whole-body perfusion with PBS followed by 10 ml Dil dye (0.12 mg/ml in PBS) at a rate of 1–2 ml/min (27). At the end of the perfusion, eyes were enucleated and whole-mount tissues of RPE/choroid were prepared (20). RPE was removed by a brief exposure to hypertonic saline during tissue preparation (28) and by a gentle brush with a silicon-tipped cannula needle (Becton Dickinson). Tissues were fixed in 4% paraformaldehyde. After blocking in 10% fetal bovine serum, 0.5% Triton X-100, and 1 $\times$ PBS, choroid flat mount was stained with various Abs as described. To quantify TCR δ^+ cells on the flat mounts, 5 images were obtained randomly in the midperipheral region using a $\times 40$ lens for each flat mount, and 6 samples were used for each experimental group.

RNA sequencing and bioinformatics analyses

Twelve-week-old wild-type mice were injected with 20 mg/kg NaIO₃ to induce eye injury. Five day postinjection, eyes were enucleated and $\gamma\delta$ T cells were isolated by using the magnetic-activated cell-sorting method described in Materials and Methods. For each experimental condition, 16 eyes were used to collect $\gamma\delta$ T cells for RNA isolation and sequencing. Splenic $\gamma\delta$ T cells from the same animals were harvested as control. Altogether 6 samples were generated, with 3 biologic replicates—a total of 48 eyes—for choroidal $\gamma\delta$ T cells and the other 3 for splenic $\gamma\delta$ T cells. All RNA samples were processed at the same time and sequenced on an Illumina HiSeq 2500 platform (LC Sciences, Houston, TX, USA). Raw reads were trimmed and aligned to the mouse genome according to a previously described method (29). Differential gene expression was analyzed on the basis of gene counts by using DESeq2 3.4 in R (version 3.3.2; Bioconductor, Buffalo, NY, USA).

For gene ontology analysis, differentially expressed gene lists were generated by comparing choroidal $\gamma\delta$ T cells with splenic samples. Up- or down-regulated genes were defined as those with at least a 2-fold change in either direction with $P_{\text{adj}} < 0.01$. Biologic process gene ontology over-representation was analyzed with InnateDB (30), using the hypergeometric algorithm with Benjamini-Hochberg correction. Most pertinent processes

were picked from the top entries. Canonical pathway analyses were carried out by using the Ingenuity pathway analysis software (winter release, December 2016; Qiagen, Germantown, MD, USA). Representative genes that were related to T lymphocyte functions were selected and a heat map of normalized expression was drawn by pheatmap package in R (The R Foundation, Vienna, Austria).

For analyzing downstream genes of AhR, we compiled a list of target genes by using 2 approaches. First, by searching the mouse genome, we obtained the list of genes with promoter regions that contained AhR binding motifs that are annotated in TRANSFAC, a database of experimentally determined transcription factor binding sites (31). Second, we downloaded the literature-curated AhR target genes from the Transcriptional Regulatory Relationships Unravelling by Sentence-based Text-mining (TRRUST) database (32). Combining these 2 approaches, we obtained a list of 41 AhR targets. Among those, we only kept the genes with at least 1 read/million in all the samples according to our RNA sequencing data. This reduced the list to 31 genes, among which there were 11 and 2 genes significantly up- and down-regulated, respectively (false discover rate < 0.01).

Statistics

Data were composed of at least 3 independent experiments. Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Differences were determined by using a Student's *t* test in 2 group comparisons or 1-way ANOVA followed by Bonferroni's *post hoc* test when comparing more than 2 conditions.

RESULTS

Characterization of choroidal $\gamma\delta$ T lymphocytes and their responses to RPE injury

To visualize tissue-associated $\gamma\delta$ T cells in the choroidal stroma, we performed immunofluorescence staining of the choroidal flat mount (Fig. 1A). RPE was removed by a brief exposure to hypertonic saline during tissue preparation (28) to avoid the interference of its pigment on optical imaging of the choroid. Choroidal blood vessels were labeled with a lipophilic dye, Dil (27), so that cells that were trapped in residual blood during tissue processing could be excluded. The results (Fig. 1A) displayed the distribution of TCR $\gamma\delta^+$ cells in the extravascular space of the choroid. NaIO₃ treatment is a well-established approach to induce selective lesions of the RPE and retina in mouse models without causing apparent systemic toxicities (33–35). The number of choroidal $\gamma\delta$ T cells increased significantly at 5 d in mice that were treated with NaIO₃ at 20 mg/kg body weight *via* tail vein injection (Fig. 1A, B).

We next analyzed the lineage-specific markers of mononuclear cells from both retina and RPE/choroid fractions by using flow cytometry. Under normal conditions, CD3⁺ lymphocytes in the retina were barely detectable (Fig. 1C), and the number remained low after NaIO₃ injection. The percentage of lymphocytes in total mononuclear cells that were isolated from the RPE/choroidal fraction was approximately 50% (Fig. 1C). Consistent with data from flat-mount staining (Fig. 1A), RPE damage that was caused by NaIO₃ treatment led to an

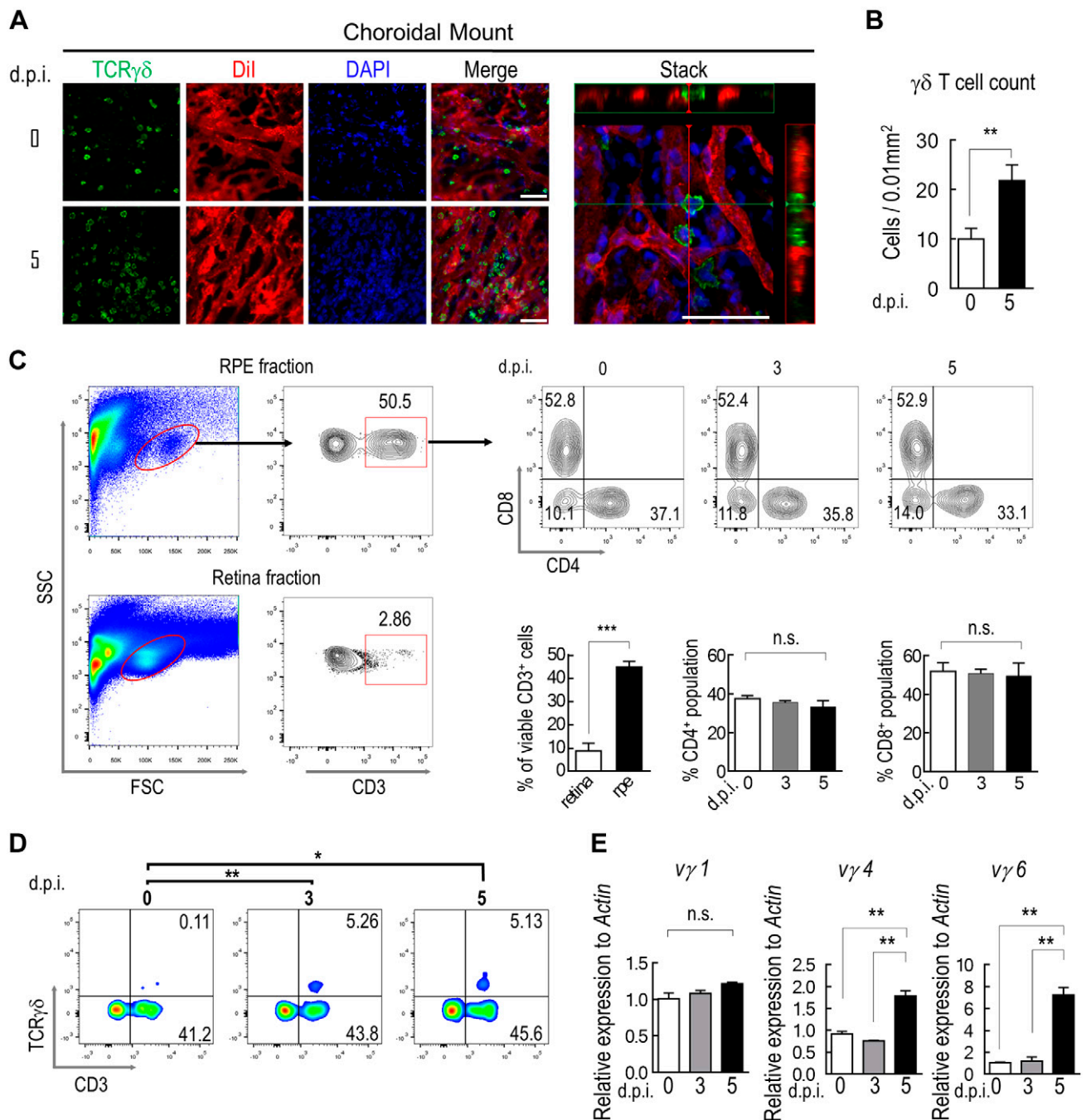


Figure 1. Choroidal $\gamma\delta$ T-cell infiltration after RPE injury. **A**) Immunostaining of TCR $\gamma\delta$ (green) on whole mounts of choroid/sclera prepared from mice that were perfused with endothelial cell dye, Dil (red). Nuclei were counterstained with DAPI (blue). A 3-dimensional reconstructed image was generated from a z-stack scan to show extravascular distribution of $\gamma\delta$ T cells (stack). **B**) Quantification of the average number of $\gamma\delta$ T cells per unit area on choroidal whole mount ($n = 6$). **C, D**) Flow cytometry analyses of enriched mononuclear cells that were isolated from RPE/choroid or retina for expression of CD3, CD4, CD8 (**C**) and TCR $\gamma\delta$ (**D**). **E**) RT-PCR measurement of choroidal $\gamma\delta$ T-cell subtype. $\gamma\delta$ T cells were purified by Ab-conjugated magnetic beads at indicated times after NaIO₃ treatment. N.s., not significant. Data presented are averages from 3 separate experiments (means $\pm$ SEM). $^{***}P < 0.01$. Scale bars, 20 μ m.

increase in the number of choroidal $\gamma\delta$ T cells (Fig. 1D), whereas the numbers of choroidal CD8⁺ or CD4⁺ lymphocytes were not altered by NaIO₃ (Fig. 1C). We then used the RT-PCR approach to define the subsets of choroidal $\gamma\delta$ T cells (Fig. 1E). After NaIO₃ treatment, both V γ 4 and V γ 6 cells were markedly increased after 5 d, and both subsets are likely to be tissue residential (36–40). The amount of $\gamma\delta$ T cells in the V γ 1 group was not changed.

Gene expression profiles of choroidal $\gamma\delta$ T cells were further analyzed by RNA sequencing. $\gamma\delta$ T cells were isolated by magnetic bead-conjugated anti-TCR $\gamma\delta$ Ab from the RPE/choroid of mice that were treated with NaIO₃. The purity of cells was approximately 94% as determined by flow cytometry (Supplemental Fig. 1). Comparisons of the transcriptomes of choroidal and splenic $\gamma\delta$ T cells revealed more than 2900 gene transcripts with highly

significant differences in either up- or down-regulation (fold change >2; P_{adj} <0.01, Benjamini-Hochberg procedure). Gene ontology analyses revealed key biologic processes and signaling pathways that are likely involved in the recruitment, migration, and activation of choroidal $\gamma\delta$ T cells (Fig. 2A, B). mRNA levels of IFN- γ and other proinflammatory cytokine genes were markedly lower in choroidal $\gamma\delta$ T cells than in splenic $\gamma\delta$ T cells (Fig. 2C). Expression of the IL-17a transcript was consistent with the enrichment of the V γ 4 and V γ 6 subsets in the choroid (8).

Selected sets of RNA sequencing data were further validated by quantitative RT-PCR. Sequencing data suggested that choroidal $\gamma\delta$ T cells expressed immunosuppressive cytokines, including IL-10 and IL-4, but not IFN- γ or TNF- α . Increased expression of IL-10 and IL-4 in isolated $\gamma\delta$ T cells was evident 3 d after NaIO₃ treatment and remained high at 5 d (Fig. 2D). CCR6 is a marker of IL-17-producing $\gamma\delta$ T cells (36, 41) and functions as a chemokine receptor that mediates $\gamma\delta$ T-cell migration (42). Its expression was up-regulated after 3 d. Furthermore, we observed a significantly elevated expression of AhR at both 3 and 5 d after NaIO₃ challenge, which was consistent with *in silico* pathway analyses (Fig. 2B, D). No significant changes were detected for the expression of TGF- β and Foxop3 in choroidal $\gamma\delta$ T cells that were prepared from NaIO₃-treated mice. Cytokine profiles of choroidal $\gamma\delta$ T cells were further verified by immunostaining and flow cytometry. On choroidal flat mounts, IL-10- and IL-4-positive $\gamma\delta$ T cells were readily detectable at 5 d after NaIO₃ treatment, whereas their expression in control samples was low (Fig. 2F). Flow cytometry analyses (Fig. 2E) also confirmed the increase of both IL-4⁺ and IL-10⁺ $\gamma\delta$ T cells in the choroid of NaIO₃-treated animals. Taken together, these data suggest that choroidal $\gamma\delta$ T cells are activated by RPE injury.

$\gamma\delta$ T-cell deficiency exacerbated NaIO₃-induced retinal inflammation

To characterize the function of choroidal $\gamma\delta$ T cells, we measured the responses of wild-type and TCR $\delta^{-/-}$ mice to RPE injury in response to NaIO₃ treatment. Consistent with previous reports (33), NaIO₃ caused minimal retinal pathology in wild-type mice at the dose of 15 mg/kg body weight *via* tail vein injection. In contrast, TCR $\delta^{-/-}$ mice that received this dose demonstrated extensive fundus changes at 3 d after NaIO₃ injection, and the lesions progressed to diffused pigment loss at d 7 (Fig. 3A). RPE injury was confirmed by both optical coherence tomography and histopathology (Fig. 3B). Quantification of the atrophic RPE sites (Fig. 3D, arrowheads) revealed significantly more RPE lesions in TCR $\delta^{-/-}$ mice than in wild-type animals (Fig. 3C). Undulating lesions in the ONL (Fig. 3D, blue-tinted area) of the neural retina were noted in areas with RPE pathology. Substantial losses of ONL nuclei and cone photoreceptor cells were observed only in $\gamma\delta$ T cell-deficient mice and were more prominent in the superior retina (Fig. 3E, G). Widespread Müller cell activation was observed in the retina of knockout mice

(Fig. 3F), which indicated aggravated neuronal injury. Expression levels of genes that encode proinflammatory cytokines and chemokines, including IFN- γ , TNF- α , IL-6, IL-1 β , and CXCL10, were found to be markedly elevated in the retina of TCR $\delta^{-/-}$ mice that were treated with NaIO₃ compared with wild-type mice that received the same treatment (Fig. 3H).

To confirm that the susceptibility of TCR $\delta^{-/-}$ mice to NaIO₃ treatment was a result of deficiency in $\gamma\delta$ T cells, we measured whether replenishing the ocular $\gamma\delta$ T-cell pool would restore the resistance to damage by NaIO₃ *via* transferring purified splenic $\gamma\delta$ T cells from wild-type mice to TCR $\delta^{-/-}$ mice (Fig. 4A). To verify the presence of transferred cells in the eye, we labeled purified $\gamma\delta$ T cells with carboxyfluorescein succinimidyl ester and examined the homing of labeled cells by using flow cytometry. Results (Fig. 4B) show that transferred $\gamma\delta$ T cells started to appear in the eyes of recipient animals 3 d after the procedure, and the population was further increased after 5 d; therefore, in subsequent functional studies, we waited for 5 d after adoptive transfer before NaIO₃ treatment (Fig. 4A). Adoptive transfer of wild-type $\gamma\delta$ T cells conferred marked protection in TCR $\delta^{-/-}$ mice. As shown on fundus images, mice that received $\gamma\delta$ T-cell transfer displayed mitigated retinal swelling 3 d after NaIO₃ treatment and RPE lesions were mostly recovered at day 7 (Fig. 4C). Consistent with clinical examinations, histopathology data demonstrated that $\gamma\delta$ T-cell transfer induced TCR $\delta^{-/-}$ mice to respond to NaIO₃ in a way that was similar to wild-type animals (Fig. 4D), whereas the knockout mice without transfer exhibited significantly more RPE lesions than the other 2 groups (Fig. 4E).

We further tested the visual functional changes by optokinetic tracking. Similar to the previous report (43), low-dose NaIO₃ caused a transient decrease of visual acuity in wild-type mice, followed by a recovery phase 3 d after injection (Fig. 4F, blue line). TCR $\delta^{-/-}$ mice demonstrated progressive deterioration of visual function, which became significantly different from wild-type animals at 4 d postinjection. Adoptive transfer of $\gamma\delta$ T cells before NaIO₃ treatment facilitated the recovery of visual acuity in a pattern that was similar to wild-type mice (Fig. 4F). Collectively, our data demonstrate a previously unidentified role for $\gamma\delta$ T cells in protecting against structural and functional damages to the RPE and retina.

Functional interactions between $\gamma\delta$ T cells and the RPE

The RPE has strong immunomodulatory functions. We assessed interactions between the RPE and $\gamma\delta$ T cells in an *ex vivo* coculture system (Fig. 5A). Naïve $\gamma\delta$ T cells were isolated from the mouse spleen and incubated with eye cups after removal of the retina. Consistent with *in vivo* cytokine profiles (Fig. 2D), RPE explants activated IL-4 and IL-10 expression in cocultured splenic $\gamma\delta$ T cells. Proinflammatory cytokines, such as IL-6 and IFN- γ , were down-regulated (Fig. 5B).

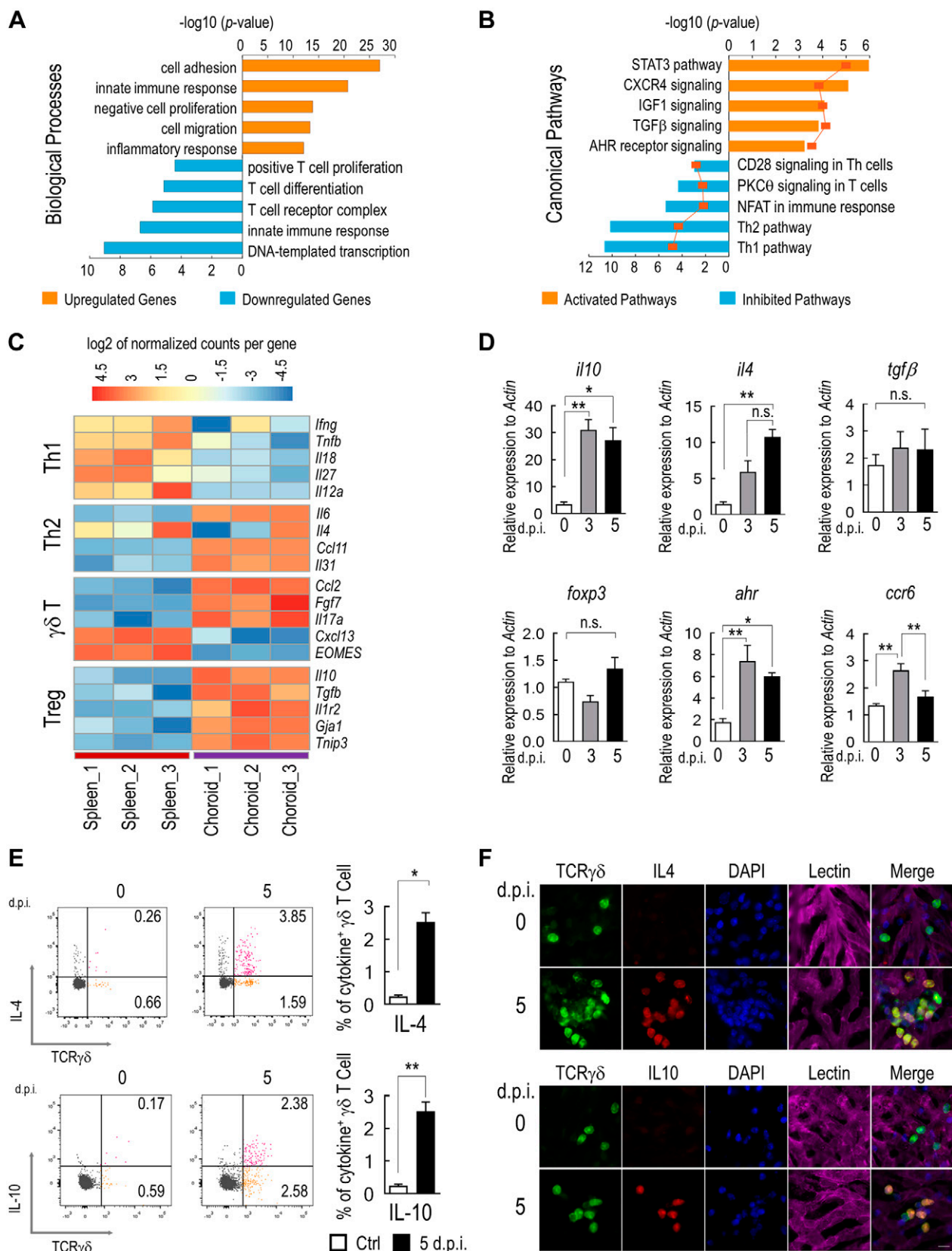


Figure 2. Production of immunosuppressive cytokines by choroidal $\gamma\delta$ T cells. **A)** Top over-represented biologic processes as determined by gene ontology analysis among differentially expressed genes (fold change >2 ; $P_{adj} < 0.01$, Benjamini-Hochberg procedure) between choroidal and splenic $\gamma\delta$ T cells. **B)** Significantly enriched immunology canonical pathways ($P < 0.01$; Fisher's exact test) were illustrated with respective percentages of overlapped genes shown on the line graph. Pathway activation and inhibition were determined by z-score. **C)** Heat map of representative genes related to distinct T-cell functions. (continued on next page)

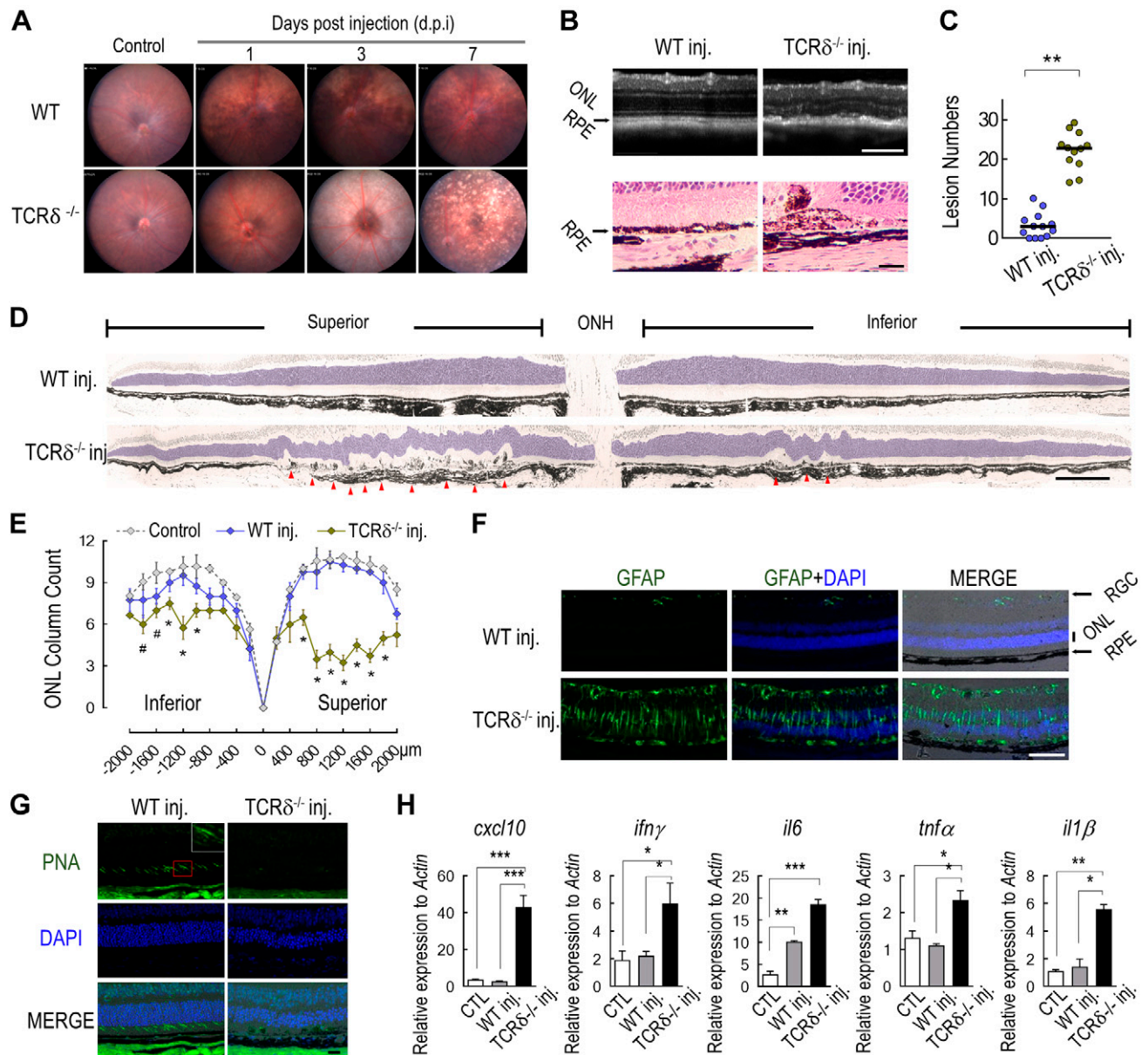


Figure 3. Ocular phenotype of TCR $\delta^{-/-}$ mice to NaIO₃. **A**) Fundus photos of wild-type (WT) and TCR $\delta^{-/-}$ mice at indicated times after NaIO₃ treatment. **B**) Optical coherence tomography scan (top) and hematoxylin and eosin staining of posterior eye sections (bottom) of mice 5 d after NaIO₃ injection. **C**) Quantification of the number of RPE and subretinal lesions on histology sections from NaIO₃-injected mice. ****** $P < 0.01$, Student's t test. **D**) Panoramic images of posterior eye sections from mice that were treated with NaIO₃ for 5 d. Sections were stained with toluidine blue. Arrowheads indicate RPE lesion sites. **E**) Numbers of rows of photoreceptor nuclei in mice with or without NaIO₃ treatment. **F**) Immunostaining of glial fibrillary acidic protein on frozen sections of posterior eyes. **G**) Peanut agglutinin (PNA) staining for cone cells in WT or $\gamma\delta$ T-deficient mice that were treated with NaIO₃. **H**) Quantitative RT-PCR analyses of retinal proinflammatory cytokine gene expression. Data were from 3 independent experiments and are presented as means $\pm$ SEM. *** $P < 0.05$** , **** $P < 0.01$** , ***** $P < 0.001$** . Scale bars: 200 μ m (**B**, upper; **D**; **F**); 20 μ m (**B**, lower; **G**).

We next tested the potential therapeutic effects of IL-4 and IL-10 against NaIO₃-induced injury. TCR $\delta^{-/-}$ mice first received an intravenous injection of NaIO₃ at 15 mg/kg per body weight. Immediately after the procedure, animals were treated with 1 μ l of a recombinant protein

cocktail of IL-4 (100 ng) and IL-10 (500 ng) *via* intravitreal injection (44). RPE injury was assessed 5 d thereafter. As shown by immunostaining of the RPE/choroid flat mount (Fig. 5C), IL-4 and IL-10 treatment decreased the lesion size by NaIO₃. The number of

D) Quantitative RT-PCR analyses of genes that are indicative of $\gamma\delta$ T-cell function. $\gamma\delta$ T cells were purified from mice that were treated with NaIO₃ at indicated times ($n = 5$, 1-way ANOVA with Bonferroni test). **E**) Flow cytometry analyses and quantification of IL-4 and IL-10 expression by choroidal $\gamma\delta$ T cells ($n = 3$, Student's t test). **F**) Representative images of costaining of IL-4 or IL-10 with TCR $\gamma\delta$ on choroidal flat mounts. Data are presented as mean $\pm$ SEM. *** $P < 0.05$** , **** $P < 0.01$** . Scale bar, 20 μ m.

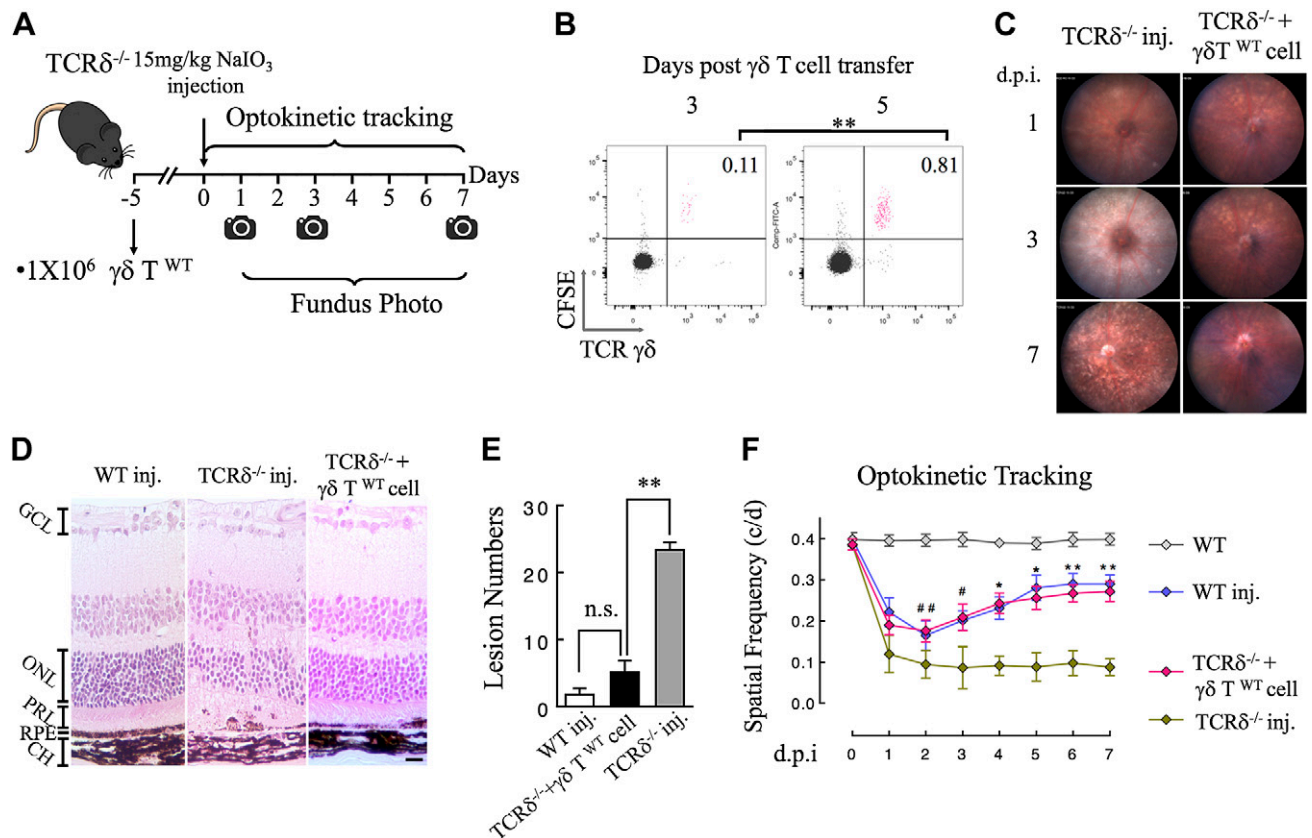


Figure 4. Responses of TCR $\delta^{-/-}$ mice to NaIO₃ after adoptive transfer of $\gamma\delta$ T cells. **A)** Schematic illustration of the experimental protocol for $\gamma\delta$ T-cell adoptive transfer and NaIO₃ treatment. **B)** Flow cytometry analysis of $\gamma\delta$ T cells in the RPE/choroid tissues of recipient mice after homing of transferred cells. Purified splenic $\gamma\delta$ T cells were prelabeled with fluorescent dye, carboxyfluorescein succinimidyl ester (y-axis). **C, D)** Representative fundus images and histopathology of NaIO₃-treated mice with or without $\gamma\delta$ T-cell transfer. **E)** Quantification of the number of RPE and subretinal lesions on histology sections ($n = 6$ for each group). **F)** Time-dependent changes of visual acuity after NaIO₃ treatment. Daily measurements of optokinetic tracking were performed in different groups of mice for up to 7 d ($n = 6$ for each group). CH, choroid; PRL, photoreceptor layer. Data are presented as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ (1-way ANOVA and Tukey's *post hoc* test). Scale bar, 20 μ m.

ionized calcium-binding adapter molecule 1 microglia in the subretinal space was also reduced. It was noticeable that the distribution of activated microglia was mainly located in areas with RPE lesions.

All $\gamma\delta$ T cells express AhR, which is a key transcription factor that controls their tissue-specific properties (8, 45). RNA sequencing data revealed that a number of AhR downstream genes were differentially expressed between choroidal and splenic $\gamma\delta$ T cells (Supplemental Fig. 2), which indicated that the transcriptional regulation by AhR is tissue specific. In *ex vivo* coculture, the induction of IL-4 and IL-10 production was abolished in $\gamma\delta$ T cells that were isolated from AhR-deficient mice (Fig. 5B), and was suppressed by an AhR antagonist, CH223191 (Fig. 6A), which indicated the importance of AhR in controlling $\gamma\delta$ T-cell function. Levels of IFN- γ or IL-6 were not influenced by CH223191.

To determine whether the protective effects of choroidal $\gamma\delta$ T cells were dependent on AhR, we used $\gamma\delta$ T cells from AhR knockout mice for adoptive transfer into TCR $\delta^{-/-}$ mice and measured the response of recipient animals to NaIO₃. Results show that $\gamma\delta$ T cells that lacked AhR expression ($\gamma\delta$ T ^{Δ AhR}) failed to protect the RPE and retina from NaIO₃-induced injury (Fig. 6B). After adoptive

transfer, recipient mice exhibited severe RPE hypertrophy and degeneration 7 d after injection (Fig. 6B, C), to the same extent as that observed in TCR $\delta^{-/-}$ mice. Significant loss of photoreceptor nuclei (Fig. 6D) and decreased optokinetic responses (Fig. 6E) were observed in TCR $\delta^{-/-}$ mice that received $\gamma\delta$ T ^{Δ AhR} cells in response to NaIO₃ injection. Taken together, our data suggest that the microenvironment of the outer retina, RPE, and choroid exhibited a general anti-inflammatory tone to maintain ocular immune homeostasis *via* $\gamma\delta$ T- and AhR-dependent mechanisms.

Choroidal $\gamma\delta$ T lymphocytes in human samples

To assess $\gamma\delta$ T lymphocytes in human choroid, we performed immunostaining of CD3 or TCR δ antigens on macular sections that were prepared from 10 eyes with AMD and 6 age-matched control eyes. Mean age was 85.6 y (95% CI, 80–91) for the AMD group, and 85.2 (95% CI, 80–90) for the control group. In the choroid of control eyes, CD3⁺ T lymphocytes and $\gamma\delta$ T cells were observed in the choriocapillaris, larger blood vessels, and the stroma, with focal areas of dense distribution of CD3⁺ T cells in the stroma (Fig. 7A). Choroid of AMD eyes displayed the

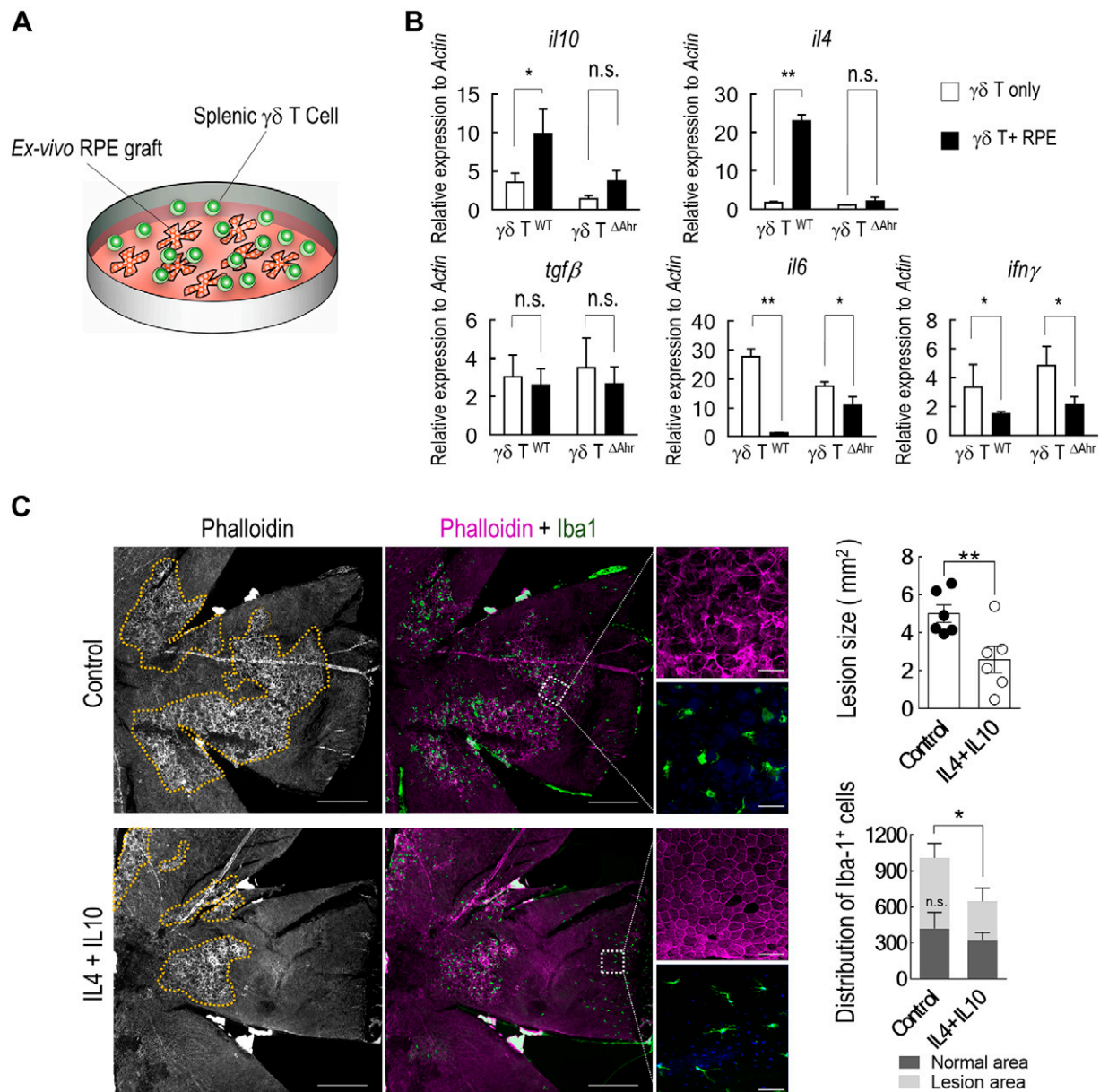


Figure 5. RPE promotes the suppressive phenotype of $\gamma\delta$ T cells in an AhR-dependent manner. **A)** Schematic illustration of cocultured splenic $\gamma\delta$ T cells with RPE explants. **B)** Cytokine gene expressions of $\gamma\delta$ T cells were measured from either wild-type or AhR^{-/-} mice after coculturing with RPE explants for 16 h. **C)** RPE flat mounts of NaIO₃-injected mice with intravitreal delivery of either IL-4 plus IL-10 cocktail or vehicle control. RPE lesions are outlined in yellow. Subretinal microglia infiltration were measured by ionized calcium-binding adapter molecule 1 staining ($n = 6$). Scale bars: 200 μ m; insert, 20 μ m.

same pattern of T-cell distribution (Fig. 7A), with the exception that $\gamma\delta$ T cells in the choriocapillaris frequently showed local expansion. $\gamma\delta$ T cells accounted for more than 50% of total T cells in the choriocapillaris and small choroidal vessels (Fig. 7B–D). Over-representation of $\gamma\delta$ T cells, which is a feature of IELs (46), suggested that their presence on tissue sections was not merely caused by the residual blood of postmortem tissue. We did not find statistical differences in the number of CD3⁺ lymphocytes of $\gamma\delta$ T cells between AMD and age-matched control groups (Fig. 7B–D); however, in areas with choriocapillaris dropout (47, 48), there was an apparent loss of interaction between the RPE and $\gamma\delta$ T cells (Fig. 7A).

DISCUSSION

IELs in epidermis, lung, and intestinal epithelium perform immune surveillance functions to protect against pathogen invasion. A majority of IELs are $\gamma\delta$ T cells (8). Cytokines that are produced by IELs and surrounding cells in the local microenvironment can facilitate the recovery and repair of damaged epithelium (49, 50). The RPE is part of the blood-retinal barrier, which separates neuronal tissue from the circulation and limits the accessibility of immune cells to the intraocular site (51). Similar to their topology in other organs (52), $\gamma\delta$ T cells reside on the mesenchymal side with choroidal blood vessels (Fig. 1). We found that choroidal $\gamma\delta$ T cells produce various modulatory cytokines

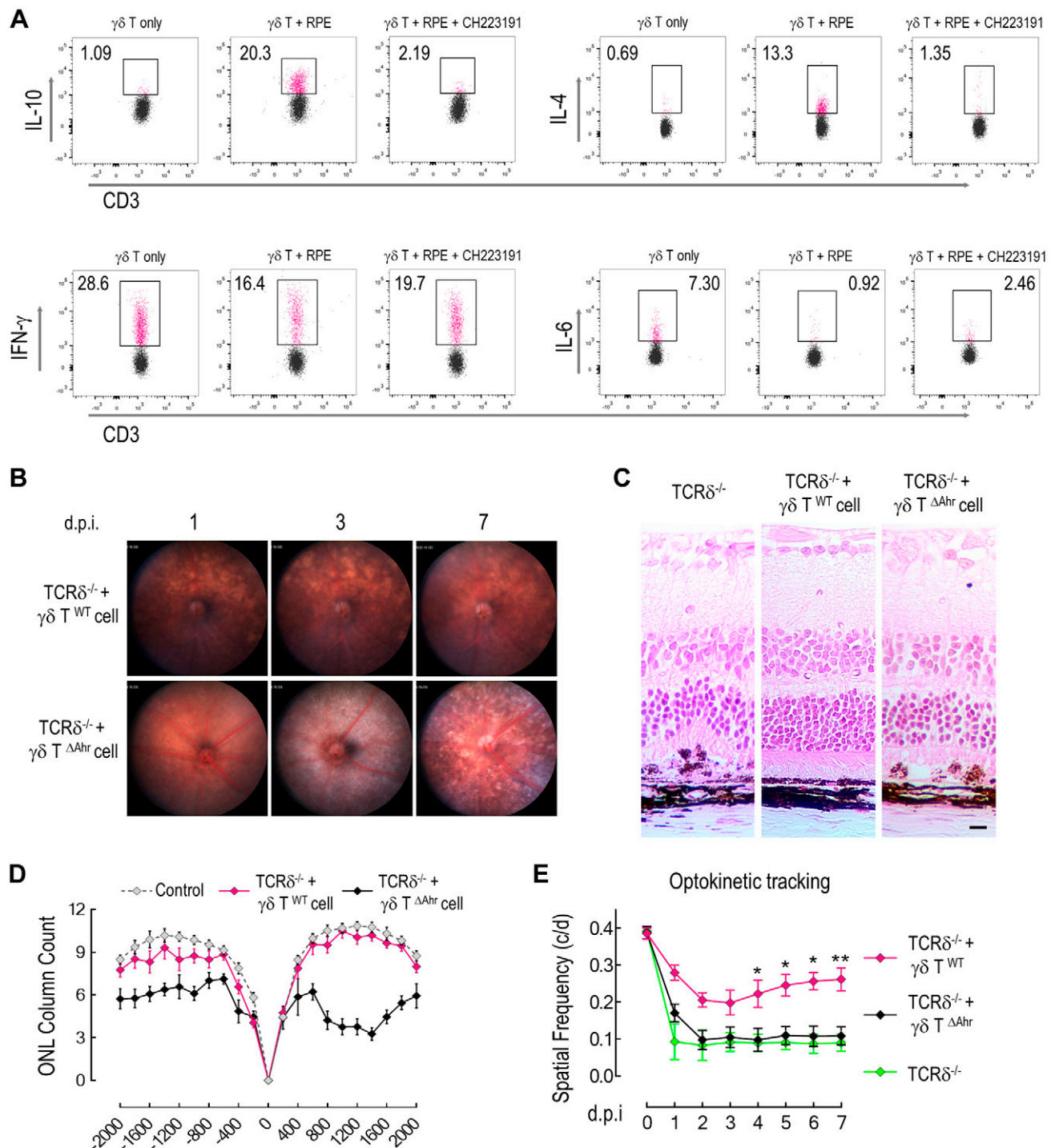


Figure 6. AhR is required for the protective function of $\gamma\delta$ T cells against RPE injury. **A**) Flow cytometry analysis of cytokine expression from $\gamma\delta$ T cells that were cocultured with RPE explants in the presence or absence of AhR inhibitor, CH223191. **B**) Fundus photos from $\gamma\delta$ T cell-deficient mice that were treated with NaIO_3 at indicated times after adoptive transfer of purified $\gamma\delta$ T cells from either wild-type or AhR-deficient mice. **C**) Histopathology of $\gamma\delta$ T cell-deficient mice after receiving adoptive transfer of wild-type or AhR-deficient $\gamma\delta$ T cells and being treated with NaIO_3 for 7 d. **D**, **E**) Counting of photoreceptor outer nuclei layers in $\gamma\delta$ T cell-transferred mice ($n = 3$) and visual function examination by optokinetic tracking ($n = 5$). Data are presented as means $\pm$ SEM. Statistical differences are determined by either 1-way ANOVA with Bonferroni test (**D**) or Student's t test (**E**). * $P < 0.05$, ** $P < 0.01$. Scale bars, 20 μm .

to suppress inflammation and maintain epithelial cell integrity and support their recovery (Figs. 2 and 4). These effects are consistent with the roles of $\gamma\delta$ T cells in skin wound healing (4) and protection against experimental colitis (53).

Our findings from imaging, histopathologic, and functional studies demonstrate that mice that are deficient in $\gamma\delta$ T cells are more susceptible to RPE and retinal degeneration induced by NaIO_3 (Figs. 3 and 4). Adoptive

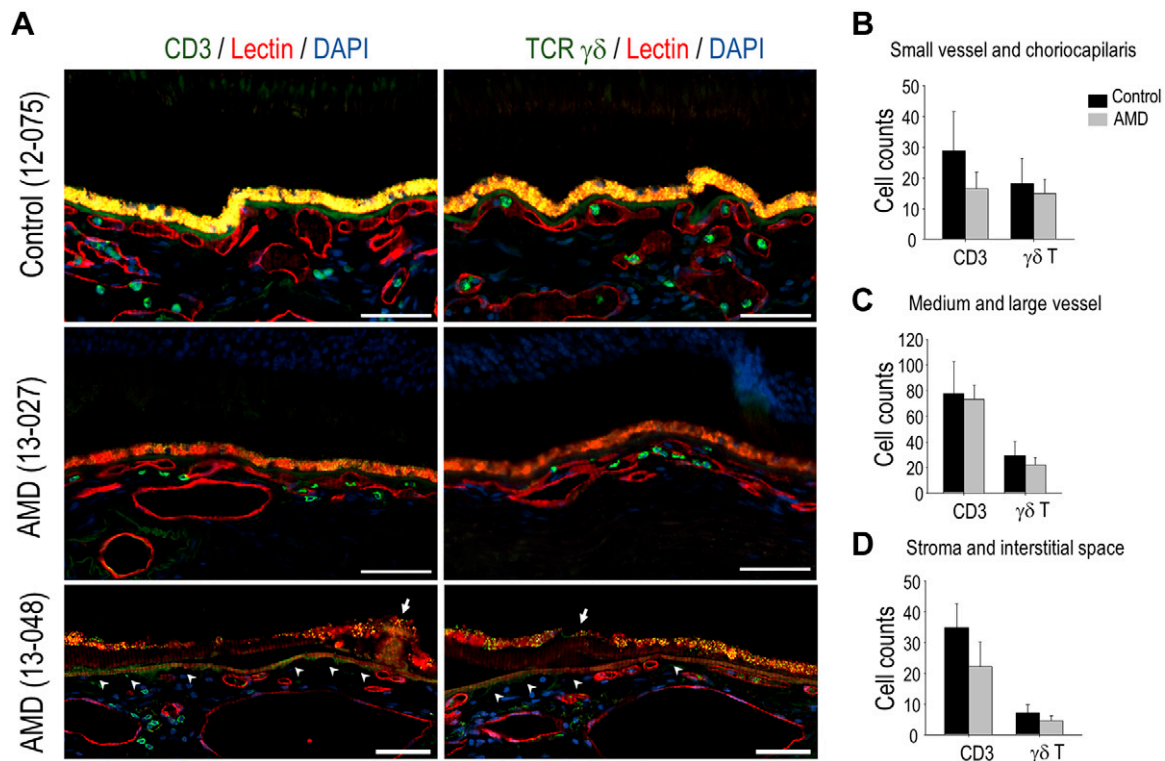


Figure 7. Choroidal $\gamma\delta$ T cells in human donor eyes. **A)** Representative images of immunolabeling of CD3 and $\gamma\delta$ TCR on adjacent sections that were prepared from human donor eyes from either patients with AMD or age-matched controls. Vascular endothelium was labeled with lectin (red fluorescence). Arrow indicates drusen; arrowhead indicates areas with choriocapillaris dropout. **B–D)** Quantification data showing the number of CD3⁺ cells and $\gamma\delta$ T cells in small and larger choroidal vessels and stroma on either control or AMD sections. Scale bars, 50 μ m.

transfer of wild-type $\gamma\delta$ T cells conferred protection, and these cells demonstrated homing to the choroid (Fig. 4). $\gamma\delta$ T cells in the choroidal tissue may directly promote tissue repair by secreting growth factors and activating cell survival pathways, as reported in skin tissue (4). Conversely, choroidal $\gamma\delta$ T cells produce immunosuppressive cytokines, IL-4 and IL-10 (Fig. 2), which may act on subretinal microglia to augment the resolution of inflammation and tissue repair (54–56). Previous studies have reported critical roles for IL-10 in the regulation of immune senescence of the retinal microglia and infiltrated macrophages, as well as in controlling their functions in choroidal neovascularization (57, 58). Our results suggest that $\gamma\delta$ T cells can be a source of IL-10 production near sites of RPE injury and degeneration. As a proof of concept, our data (Fig. 5C) demonstrate the *in vivo* protective effects of IL-4 and IL-10 against NaIO₃ toxicity. Detailed molecular mechanisms of their activation and protection remain to be explored in future studies and likely extend beyond IL-4 and IL-10.

We observed $\gamma\delta$ T cells on human posterior pole sections. Although their presence in large choroidal blood vessels can be a result of residual blood, $\gamma\delta$ T cells in the choriocapillaris and surrounding areas have close interactions with the RPE (Fig. 7). One of the functional consequences of choriocapillaris dropout is the loss of support from protective $\gamma\delta$ T cells. These findings are consistent with a recent report on choroidal $\gamma\delta$ T cells in eyes with geographic atrophy (59). Compared with age-matched controls, there seemed to be a decrease in the percentage of

$\gamma\delta$ T cells in the total number of CD3⁺ T lymphocytes in the choroid of AMD eyes, although the difference was not statistically significant (Fig. 7). Future studies are needed to further quantify the changes in both the number and properties of choroidal $\gamma\delta$ T cells during different stages of RPE degeneration in AMD. In a skin wound healing model, epidermal $\gamma\delta$ T cells in aged mice show decreased activation and supportive functions for re-epithelialization (60). Similar mechanisms and signaling pathways can be applicable to choroidal $\gamma\delta$ T cells.

The RPE is known to modulate T-cell function. RPE-conditioned medium can differentiate naïve T cells to T_{regs} (61, 62). Cocultured RPE can suppress T-cell activation (11) by producing both soluble and membrane-associated immunomodulatory factors (63–66). Results from our current study show that the *ex vivo* coculture of RPE with splenic $\gamma\delta$ T cells directed the differentiation toward regulatory $\gamma\delta$ T cells that produced suppressive cytokines, such as IL-4 and IL-10 (Fig. 5). The transcriptome of choroidal $\gamma\delta$ T cells that were isolated from NaIO₃-treated mice has similarities to T_{regs}, although they do not express *Foxp3* (Fig. 2C, D). The RPE-mediated induction of $\gamma\delta$ T cells was dependent on AhR (Fig. 5). Recent studies with AhR knockout mice observed age-dependent RPE degeneration and retinal inflammation (67, 68), and AhR deficiency potentiated experimental choroidal neovascularization (69). Future studies can be performed to further explore the functional changes of $\gamma\delta$ T cells and their roles in the degeneration phenotype in AhR-deficient

mice. AhR is a nuclear receptor protein that can be activated by a variety of ligands, including tryptophan derivatives (70). A major tryptophan-metabolizing enzyme, indoleamine 2,3-dioxygenase, is expressed by the RPE and is likely involved in the immune modulation of RPE on lymphocytes (71). AhR-mediated gene expression can be stimulated by IL-1 β (72), which can be produced by the RPE inflammasome under conditions of stress (73–75). These previously reported findings further support the role of choroidal $\gamma\delta$ T cells as an integral component of the innate immune system at the RPE barrier.

Ocular $\gamma\delta$ T cells have important regulatory roles during experimental autoimmune uveitis (24, 76, 77) and anterior chamber-associated immune deviation (78, 79). Experimental autoimmune uveitis and anterior chamber-associated immune deviation are adaptive immune responses that are experimentally induced by specific antigens. Activation of choroidal $\gamma\delta$ T cells in the RPE injury model that we used in the current study is an innate response that occurred within 3 d after NaIO₃ injection in nonprimed mice. The number of V γ 4 and V γ 6 cells markedly increased after 5 d (Fig. 1E). Both subsets are likely to be tissue residential (36–40). $\gamma\delta$ T cells have moderate effects in laser-induced choroidal neovascularization (14, 80). Depending on the interactions with other immune cells and the conditions of activation, they can either enhance or suppress the inflammatory and angiogenic responses. Thus, deciphering the mechanisms of their activation and the condition-specific downstream effects will be essential for future translational applications.

There are several limitations in our current study. The number of human eye samples examined was small and we did not have sufficient statistical power to distinguish between dry and wet forms of AMD. Splenic $\gamma\delta$ T cells were used for adoptive transfer and they do not represent the tissue specificity and unique subsets of choroidal $\gamma\delta$ T cells. Intravitreal injection of IL-4 and IL-10 was performed as a preventive treatment that was delivered as a single shot at a high dose. Other cytokines that were produced by choroidal $\gamma\delta$ T cells, such as IL-17, will likely contribute to their interactions with RPE.

In summary, choroidal $\gamma\delta$ T cells are a special group of immune cells with properties that resemble those of IELs, and that exert protective functions under conditions of sterile inflammation and RPE degeneration. $\gamma\delta$ T cells produce an optimized combination of protective and immunosuppressive cytokines. $\gamma\delta$ T-cell therapy is potentially a novel interventional strategy for treating retinal diseases that involve RPE degeneration. **[F]**

ACKNOWLEDGMENTS

This work was supported by U.S. National Institutes of Health, National Eye Institute Grants EY021937, EY026999, EY022403, and EY024605; the Ted Nash Long Life Foundation; Carl Marshall Reeves and Mildred Almen Reeves Foundation; BrightFocus Foundation; and International Retina Research Foundation. The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

D. Sun, J. Sun, R. F. Mullins, Y. Chen, and J. Cai designed research; Z. Zhao, Y. Liang, P. Xu, M. J. Flamme-Wiess, Y. Chen, and J. Cai performed the experiments; Z. Zhao, Y. Liu, Y. Chen and J. Cai analyzed data; and Z. Zhao, Y. Liu, Y. Chen, and J. Cai wrote the manuscript.

REFERENCES

- O'Brien, R. L., and Born, W. K. (2010) Gammadelta T cell subsets: a link between TCR and function? *Semin. Immunol.* **22**, 193–198
- Hayday, A., and Tigelaar, R. (2003) Immunoregulation in the tissues by gammadelta T cells. *Nat. Rev. Immunol.* **3**, 233–242
- Chien, Y. H., Meyer, C., and Bonneville, M. (2014) $\gamma\delta$ T cells: first line of defense and beyond. *Annu. Rev. Immunol.* **32**, 121–155
- Gay, D., Kwon, O., Zhang, Z., Spata, M., Plikus, M. V., Holler, P. D., Ito, M., Yang, Z., Treffeisen, E., Kim, C. D., Nace, A., Zhang, X., Baraton, S., Wang, F., Ornitz, D. M., Millar, S. E., and Cotsarelis, G. (2013) Fgf9 from dermal $\gamma\delta$ T cells induces hair follicle neogenesis after wounding. *Nat. Med.* **19**, 916–923
- Havran, W. L., Chien, Y. H., and Allison, J. P. (1991) Recognition of self antigens by skin-derived T cells with invariant gamma delta antigen receptors. *Science* **252**, 1430–1432
- Born, W. K., Lahn, M., Takeda, K., Kanehiro, A., O'Brien, R. L., and Gelfand, E. W. (2000) Role of gammadelta T cells in protecting normal airway function. *Respir. Res.* **1**, 151–158
- Kühl, A. A., Pawlowski, N. N., Grollich, K., Loddenkemper, C., Zeitz, M., and Hoffmann, J. C. (2007) Aggravation of intestinal inflammation by depletion/deficiency of gammadelta T cells in different types of IBD animal models. *J. Leukoc. Biol.* **81**, 168–175
- Stange, J., and Veldhoen, M. (2013) The aryl hydrocarbon receptor in innate T cell immunity. *Semin. Immunopathol.* **35**, 645–655
- Detrick, B., and Hooks, J. J. (2010) Immune regulation in the retina. *Immunol. Res.* **47**, 153–161
- Zhou, R., Horai, R., Silver, P. B., Mattapallil, M. J., Zárate-Bladés, C. R., Chong, W. P., Chen, J., Rigden, R. C., Villasmil, R., and Caspi, R. R. (2012) The living eye “disarms” uncommitted autoreactive T cells by converting them to Foxp3⁺ regulatory cells following local antigen recognition. *J. Immunol.* **188**, 1742–1750
- Gregerson, D. S., Heuss, N. D., Lew, K. L., McPherson, S. W., and Ferrington, D. A. (2007) Interaction of retinal pigmented epithelial cells and CD4 T cells leads to T-cell anergy. *Invest. Ophthalmol. Vis. Sci.* **48**, 4654–4663
- Patel, M., and Chan, C. C. (2008) Immunopathological aspects of age-related macular degeneration. *Semin. Immunopathol.* **30**, 97–110
- Ambati, J., Atkinson, J. P., and Gelfand, B. D. (2013) Immunology of age-related macular degeneration. *Nat. Rev. Immunol.* **13**, 438–451
- Coughlin, B., Schnabolk, G., Joseph, K., Raikwar, H., Kunchithapatham, K., Johnson, K., Moore, K., Wang, Y., and Rohrer, B. (2016) Connecting the innate and adaptive immune responses in mouse choroidal neovascularization via the anaphylatoxin C5a and $\gamma\delta$ T-cells. *Sci. Rep.* **6**, 23794
- Zhang, S., Yu, N., Zhang, R., Zhang, S., and Wu, J. (2016) Interleukin-17A induces IL-1 β secretion from RPE cells via the NLRP3 inflammasome. *Invest. Ophthalmol. Vis. Sci.* **57**, 312–319
- Chen, S., Shen, D., Popp, N. A., Ogilvy, A. J., Tuo, J., Abu-Asab, M., Xie, T., and Chan, C. C. (2015) Responses of multipotent retinal stem cells to IL-1 β , IL-18, or IL-17. *J. Ophthalmol.* **2015**, 369312
- Zhu, Y., Tan, W., Demetriades, A. M., Cai, Y., Gao, Y., Sui, A., Lu, Q., Shen, X., Jiang, C., Xie, B., and Sun, X. (2016) Interleukin-17A neutralization alleviated ocular neovascularization by promoting M2 and mitigating M1 macrophage polarization. *Immunology* **147**, 414–428
- Zhao, Z., Xu, P., Jie, Z., Zuo, Y., Yu, B., Soong, L., Sun, J., Chen, Y., and Cai, J. (2014) $\gamma\delta$ T cells as a major source of IL-17 production during age-dependent RPE degeneration. *Invest. Ophthalmol. Vis. Sci.* **55**, 6580–6589
- Mattapallil, M. J., Wawrousek, E. F., Chan, C. C., Zhao, H., Roychoudhury, J., Ferguson, T. A., and Caspi, R. R. (2012) The Rd8 mutation of the Crbl gene is present in vendor lines of C57BL/6N mice and embryonic stem cells, and confounds ocular induced mutant phenotypes. *Invest. Ophthalmol. Vis. Sci.* **53**, 2921–2927
- Yu, B., Xu, P., Zhao, Z., Cai, J., Sternberg, P., and Chen, Y. (2014) Subcellular distribution and activity of mechanistic target of

- rapamycin in aged retinal pigment epithelium. *Invest. Ophthalmol. Vis. Sci.* **55**, 8638–8650
21. Hollyfield, J. G., Bonilha, V. L., Rayborn, M. E., Yang, X., Shadrach, K. G., Lu, L., Ufret, R. L., Salomon, R. G., and Perez, V. L. (2008) Oxidative damage-induced inflammation initiates age-related macular degeneration. *Nat. Med.* **14**, 194–198
 22. Chirco, K. R., Tucker, B. A., Stone, E. M., and Mullins, R. F. (2016) Selective accumulation of the complement membrane attack complex in aging choriocapillaris. *Exp. Eye Res.* **146**, 393–397
 23. Chen, Y., Cai, J., Murphy, T. J., and Jones, D. P. (2002) Overexpressed human mitochondrial thioredoxin confers resistance to oxidant-induced apoptosis in human osteosarcoma cells. *J. Biol. Chem.* **277**, 33242–33248
 24. Liang, D., Zuo, A., Zhao, R., Shao, H., Born, W. K., O'Brien, R. L., Kaplan, H. J., and Sun, D. (2016) CD73 expressed on $\gamma\delta$ T cells shapes their regulatory effect in experimental autoimmune uveitis. *PLoS One* **11**, e0150078
 25. Cong, Y., Feng, T., Fujihashi, K., Schoeb, T. R., and Elson, C. O. (2009) A dominant, coordinated T regulatory cell-IgA response to the intestinal microbiota. *Proc. Natl. Acad. Sci. USA* **106**, 19256–19261
 26. Prusky, G. T., Alam, N. M., Beekman, S., and Douglas, R. M. (2004) Rapid quantification of adult and developing mouse spatial vision using a virtual optomotor system. *Invest. Ophthalmol. Vis. Sci.* **45**, 4611–4616
 27. Li, Y., Song, Y., Zhao, L., Gaidosh, G., Laties, A. M., and Wen, R. (2008) Direct labeling and visualization of blood vessels with lipophilic carbocyanine dye DiI. *Nat. Protoc.* **3**, 1703–1708
 28. Cheruvu, N. P., and Kompella, U. B. (2006) Bovine and porcine transcleral solute transport: influence of lipophilicity and the Choroid-Bruch's layer. *Invest. Ophthalmol. Vis. Sci.* **47**, 4513–4522
 29. Anders, S., McCarthy, D. J., Chen, Y., Okoniewski, M., Smyth, G. K., Huber, W., and Robinson, M. D. (2013) Count-based differential expression analysis of RNA sequencing data using R and bioconductor. *Nat. Protoc.* **8**, 1765–1786
 30. Lynn, D. J., Winsor, G. L., Chan, C., Richard, N., Laird, M. R., Barsky, A., Gardy, J. L., Roche, F. M., Chan, T. H., Shah, N., Lo, R., Naseer, M., Que, J., Yau, M., Acab, M., Tulpan, D., Whiteside, M. D., Chikatarla, A., Mah, B., Munzner, T., Hokamp, K., Hancock, R. E., and Brinkman, F. S. (2008) InnateDB: facilitating systems-level analyses of the mammalian innate immune response. *Mol. Syst. Biol.* **4**, 218
 31. Matys, V., Kel-Margoulis, O. V., Fricke, E., Liebich, I., Land, S., Barre-Dirrie, A., Reuter, I., Chekmenev, D., Krull, M., Hornischer, K., Voss, N., Stegmaier, P., Lewicki-Potapov, B., Saxel, H., Kel, A. E., and Wingender, E. (2006) TRANSFAC and its module TRANSCOMP: transcriptional gene regulation in eukaryotes. *Nucleic Acids Res.* **34**, D108–D110
 32. Han, H., Shim, H., Shin, D., Shim, J. E., Ko, Y., Shin, J., Kim, H., Cho, A., Kim, E., Lee, T., Kim, H., Kim, K., Yang, S., Bae, D., Yun, A., Kim, S., Kim, C. Y., Cho, H. J., Kang, B., Shin, S., and Lee, I. (2015) TRRUST: a reference database of human transcriptional regulatory interactions. *Sci. Rep.* **5**, 11432
 33. Wang, W., Noel, J., Kaplan, H. J., and Dean, D. C. (2011) Circulating reactive oxidant causes apoptosis of retinal pigment epithelium and cone photoreceptors in the mouse central retina. *Ophthalmol. Eye Dis.* **3**, 45–54
 34. Kannan, R., and Hinton, D. R. (2014) Sodium iodate induced retinal degeneration: new insights from an old model. *Neural Regen. Res.* **9**, 2044–2045
 35. Balmer, J., Zulliger, R., Roberti, S., and Enzmann, V. (2015) Retinal cell death caused by sodium iodate involves multiple caspase-dependent and caspase-independent cell-death pathways. *Int. J. Mol. Sci.* **16**, 15086–15103
 36. Haas, J. D., Ravens, S., Düber, S., Sandrock, I., Oberdörfer, L., Kashani, E., Chennupati, V., Föhse, L., Naumann, R., Weiss, S., Krueger, A., Förster, R., and Prinz, I. (2012) Development of interleukin-17-producing $\gamma\delta$ T cells is restricted to a functional embryonic wave. *Immunity* **37**, 48–59
 37. Sumaria, N., Roediger, B., Ng, L. G., Qin, J., Pinto, R., Cavanagh, L. L., Shklovskaya, E., Fazekas de St Groth, B., Triccas, J. A., and Weninger, W. (2011) Cutaneous immunosurveillance by self-renewing dermal gammadelta T cells. *J. Exp. Med.* **208**, 505–518
 38. Sim, G. K., Rajaserkar, R., Dessing, M., and Augustin, A. (1994) Homing and *in situ* differentiation of resident pulmonary lymphocytes. *Int. Immunol.* **6**, 1287–1295
 39. Misiak, A., Wilk, M. M., Raverdeau, M., and Mills, K. H. (2017) IL-17-producing innate and pathogen-specific tissue resident memory $\gamma\delta$ T cells expand in the lungs of bordetella pertussis-infected mice. *J. Immunol.* **198**, 363–374
 40. Akitsu, A., Ishigame, H., Kakuta, S., Chung, S. H., Ikeda, S., Shimizu, K., Kubo, S., Liu, Y., Umemura, M., Matsuzaki, G., Yoshikai, Y., Saijo, S., and Iwakura, Y. (2015) IL-1 receptor antagonist-deficient mice develop autoimmune arthritis due to intrinsic activation of IL-17-producing CCR2^{hi}V γ 6^{hi} $\gamma\delta$ T cells. *Nat. Commun.* **6**, 7464
 41. Haas, J. D., González, F. H., Schmitz, S., Chennupati, V., Föhse, L., Kremmer, E., Förster, R., and Prinz, I. (2009) CCR6 and NK1.1 distinguish between IL-17A and IFN- γ -producing gammadelta effector T cells. *Eur. J. Immunol.* **39**, 3488–3497
 42. Yamazaki, T., Yang, X. O., Chung, Y., Fukunaga, A., Nurieva, R., Pappu, B., Martin-Orozco, N., Kang, H. S., Ma, L., Panopoulos, A. D., Craig, S., Watowich, S. S., Jetten, A. M., Tian, Q., and Dong, C. (2008) CCR6 regulates the migration of inflammatory and regulatory T cells. *J. Immunol.* **181**, 8391–8401
 43. Carido, M., Zhu, Y., Postel, K., Benkner, B., Cimalla, P., Karl, M. O., Kurth, T., Paquet-Durand, F., Koch, E., Münch, T. A., Tanaka, E. M., and Ader, M. (2014) Characterization of a mouse model with complete RPE loss and its use for RPE cell transplantation. *Invest. Ophthalmol. Vis. Sci.* **55**, 5431–5444
 44. Rosenbaum, J. T., and Angell, E. (1995) Paradoxical effects of IL-10 in endotoxin-induced uveitis. *J. Immunol.* **155**, 4090–4094
 45. Li, Y., Innocentin, S., Withers, D. R., Roberts, N. A., Gallagher, A. R., Grigorieva, E. F., Wilhelm, C., and Veldhoen, M. (2011) Exogenous stimuli maintain intraepithelial lymphocytes *via* aryl hydrocarbon receptor activation. *Cell* **147**, 629–640
 46. Cheroutre, H., Lambollez, F., and Mucida, D. (2011) The light and dark sides of intestinal intraepithelial lymphocytes. *Nat. Rev. Immunol.* **11**, 445–456
 47. McLeod, D. S., Grebe, R., Bhutto, I., Merges, C., Baba, T., and Luty, G. A. (2009) Relationship between RPE and choriocapillaris in age-related macular degeneration. *Invest. Ophthalmol. Vis. Sci.* **50**, 4982–4991
 48. Mullins, R. F., Johnson, M. N., Faidley, E. A., Skeie, J. M., and Huang, J. (2011) Choriocapillaris vascular dropout related to density of drusen in human eyes with early age-related macular degeneration. *Invest. Ophthalmol. Vis. Sci.* **52**, 1606–1612
 49. Song, X., Dai, D., He, X., Zhu, S., Yao, Y., Gao, H., Wang, J., Qu, F., Qiu, J., Wang, H., Li, X., Shen, N., and Qian, Y. (2015) Growth factor FGF2 cooperates with interleukin-17 to repair intestinal epithelial damage. *Immunity* **43**, 488–501
 50. Maxwell, J. R., Zhang, Y., Brown, W. A., Smith, C. L., Byrne, F. R., Fiorino, M., Stevens, E., Bigler, J., Davis, J. A., Rottman, J. B., Budelsky, A. L., Symons, A., and Towne, J. E. (2015) Differential roles for interleukin-23 and interleukin-17 in intestinal immunoregulation. *Immunity* **43**, 739–750
 51. Wenkel, H., and Streilein, J. W. (2000) Evidence that retinal pigment epithelium functions as an immune-privileged tissue. *Invest. Ophthalmol. Vis. Sci.* **41**, 3467–3473
 52. Nowarski, R., Jackson, R., and Flavell, R. A. (2017) The stromal intervention: regulation of immunity and inflammation at the epithelial-mesenchymal barrier. *Cell* **168**, 362–375
 53. Chen, Y., Chou, K., Fuchs, E., Havran, W. L., and Boismenu, R. (2002) Protection of the intestinal mucosa by intraepithelial gamma delta T cells. *Proc. Natl. Acad. Sci. USA* **99**, 14338–14343
 54. Dace, D. S., Khan, A. A., Kelly, J., and Apte, R. S. (2008) Interleukin-10 promotes pathological angiogenesis by regulating macrophage response to hypoxia during development. *PLoS One* **3**, e3381
 55. Wohl, S. G., Schmeer, C. W., Friesen, T., Witte, O. W., and Isenmann, S. (2011) *In situ* dividing and phagocytosing retinal microglia express nestin, vimentin, and NG2 *in vivo*. *PLoS One* **6**, e22408
 56. Gordon, S., and Taylor, P. R. (2005) Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.* **5**, 953–964
 57. Kelly, J., Ali Khan, A., Yin, J., Ferguson, T. A., and Apte, R. S. (2007) Senescence regulates macrophage activation and angiogenic fate at sites of tissue injury in mice. *J. Clin. Invest.* **117**, 3421–3426
 58. Apte, R. S., Richter, J., Herndon, J., and Ferguson, T. A. (2006) Macrophages inhibit neovascularization in a murine model of age-related macular degeneration. *PLoS Med.* **3**, e310
 59. Camelo, S., Lavelette, S., Guillonnet, X., Raoul, W., and Sennlaub, F. (2016) Association of choroidal interleukin-17-producing T lymphocytes and macrophages with geographic atrophy. *Ophthalmologica* **236**, 53–58
 60. Keyes, B. E., Liu, S., Asare, A., Naik, S., Levorso, J., Polak, L., Lu, C. P., Nikolova, M., Pasolli, H. A., and Fuchs, E. (2016) Impaired epidermal to dendritic T cell signaling slows wound repair in aged skin. *Cell* **167**, 1323–1338.e1314

61. Kawazoe, Y., Sugita, S., Keino, H., Yamada, Y., Imai, A., Horie, S., and Mochizuki, M. (2012) Retinoic acid from retinal pigment epithelium induces T regulatory cells. *Exp. Eye Res.* **94**, 32–40
62. Imai, A., Sugita, S., Kawazoe, Y., Horie, S., Yamada, Y., Keino, H., Maruyama, K., and Mochizuki, M. (2012) Immunosuppressive properties of regulatory T cells generated by incubation of peripheral blood mononuclear cells with supernatants of human RPE cells. *Invest. Ophthalmol. Vis. Sci.* **53**, 7299–7309
63. Kaestel, C. G., Lovato, P., Ødum, N., Nissen, M. H., and Röpke, C. (2005) The immune privilege of the eye: human retinal pigment epithelial cells selectively modulate T-cell activation *in vitro*. *Curr. Eye Res.* **30**, 375–383
64. Wallace, C. A., Moir, G., Malone, D. F., Duncan, L., Devarajan, G., and Crane, I. J. (2013) Regulation of T-lymphocyte CCL3 and CCL4 production by retinal pigment epithelial cells. *Invest. Ophthalmol. Vis. Sci.* **54**, 722–730
65. Clemson, C. M., Yost, J., and Taylor, A. W. (2017) The role of alpha-MSH as a modulator of ocular immunobiology exemplifies mechanistic differences between melanocortins and steroids. *Ocul. Immunol. Inflamm.* **25**, 179–189
66. Green, D. R., and Ferguson, T. A. (2001) The role of Fas ligand in immune privilege. *Nat. Rev. Mol. Cell Biol.* **2**, 917–924
67. Hu, P., Herrmann, R., Bednar, A., Saloupis, P., Dwyer, M. A., Yang, P., Qi, X., Thomas, R. S., Jaffe, G. J., Boulton, M. E., McDonnell, D. P., and Malek, G. (2013) Aryl hydrocarbon receptor deficiency causes dysregulated cellular matrix metabolism and age-related macular degeneration-like pathology. *Proc. Natl. Acad. Sci. USA* **110**, E4069–E4078
68. Kim, S. Y., Yang, H. J., Chang, Y. S., Kim, J. W., Brooks, M., Chew, E. Y., Wong, W. T., Fariss, R. N., Rachel, R. A., Cogliati, T., Qian, H., and Swaroop, A. (2014) Deletion of aryl hydrocarbon receptor AHR in mice leads to subretinal accumulation of microglia and RPE atrophy. *Invest. Ophthalmol. Vis. Sci.* **55**, 6031–6040
69. Choudhary, M., Kazmin, D., Hu, P., Thomas, R. S., McDonnell, D. P., and Malek, G. (2015) Aryl hydrocarbon receptor knock-out exacerbates choroidal neovascularization *via* multiple pathogenic pathways. *J. Pathol.* **235**, 101–112
70. Nguyen, L. P., and Bradfield, C. A. (2008) The search for endogenous activators of the aryl hydrocarbon receptor. *Chem. Res. Toxicol.* **21**, 102–116
71. Park, C. Y., Yang, S. H., Chuck, R. S., Gehlbach, P. L., and Park, C. G. (2010) The role of indoleamine 2,3-dioxygenase in retinal pigment epithelial cell-mediated immune modulation. *Ocul. Immunol. Inflamm.* **18**, 24–31
72. Sutton, C. E., Lalor, S. J., Sweeney, C. M., Brereton, C. F., Lavelle, E. C., and Mills, K. H. (2009) Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* **31**, 331–341
73. Tseng, W. A., Thein, T., Kinnunen, K., Lashkari, K., Gregory, M. S., D'Amore, P. A., and Ksander, B. R. (2013) NLRP3 inflammasome activation in retinal pigment epithelial cells by lysosomal destabilization: implications for age-related macular degeneration. *Invest. Ophthalmol. Vis. Sci.* **54**, 110–120
74. Kauppinen, A., Niskanen, H., Suuronen, T., Kinnunen, K., Salminen, A., and Kaarniranta, K. (2012) Oxidative stress activates NLRP3 inflammasomes in ARPE-19 cells—implications for age-related macular degeneration (AMD). *Immunol. Lett.* **147**, 29–33
75. Doyle, S. L., Campbell, M., Ozaki, E., Salomon, R. G., Mori, A., Kenna, P. F., Farrar, G. J., Kiang, A. S., Humphries, M. M., Lavelle, E. C., O'Neill, L. A., Hollyfield, J. G., and Humphries, P. (2012) NLRP3 has a protective role in age-related macular degeneration through the induction of IL-18 by drusen components. *Nat. Med.* **18**, 791–798
76. Liang, D., Zuo, A., Shao, H., Born, W. K., O'Brien, R. L., Kaplan, H. J., and Sun, D. (2013) IL-23 receptor expression on $\gamma\delta$ T cells correlates with their enhancing or suppressive effects on autoreactive T cells in experimental autoimmune uveitis. *J. Immunol.* **191**, 1118–1125
77. Nian, H., Shao, H., O'Brien, R. L., Born, W. K., Kaplan, H. J., and Sun, D. (2011) Activated gammadelta T cells promote the activation of uveitogenic T cells and exacerbate EAU development. *Invest. Ophthalmol. Vis. Sci.* **52**, 5920–5927
78. Skelley, M. E., Mellon, J., and Niederkorn, J. Y. (2001) Gamma delta T cells are needed for ocular immune privilege and corneal graft survival. *J. Immunol.* **166**, 4327–4333
79. Ashour, H. M., and Niederkorn, J. Y. (2006) Gammadelta T cells promote anterior chamber-associated immune deviation and immune privilege through their production of IL-10. *J. Immunol.* **177**, 8331–8337
80. Hasegawa, E., Sonoda, K. H., Shichita, T., Morita, R., Sekiya, T., Kimura, A., Oshima, Y., Takeda, A., Yoshimura, T., Yoshida, S., Ishibashi, T., and Yoshimura, A. (2013) IL-23-independent induction of IL-17 from $\gamma\delta$ T cells and innate lymphoid cells promotes experimental intraocular neovascularization. *J. Immunol.* **190**, 1778–1787

Received for publication June 10, 2017.

Accepted for publication July 5, 2017.

Choroidal $\gamma\delta$ T cells in protection against retinal pigment epithelium and retinal injury

Zhenyang Zhao, Yuejin Liang, Yin Liu, et al.

FASEB J published online July 20, 2017

Access the most recent version at doi:[10.1096/fj.201700533R](https://doi.org/10.1096/fj.201700533R)

Supplemental Material

<http://www.fasebj.org/content/suppl/2017/07/20/fj.201700533R.DC1>

Subscriptions

Information about subscribing to *The FASEB Journal* is online at
<http://www.faseb.org/The-FASEB-Journal/Librarian-s-Resources.aspx>

Permissions

Submit copyright permission requests at:
<http://www.fasebj.org/site/misc/copyright.xhtml>

Email Alerts

Receive free email alerts when new an article cites this article - sign up at
<http://www.fasebj.org/cgi/alerts>



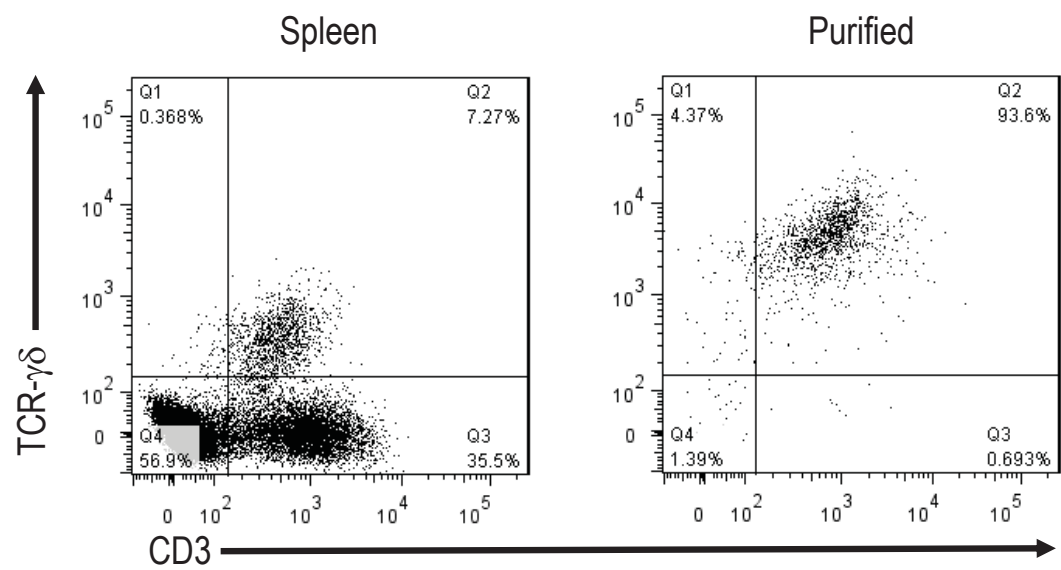
Avanti[®]
POLAR LIPIDS, INC.

Dive into lipidomics with
SPLASH[™] Lipidomix[®] Mass Spec Standard*

*Includes all major lipid classes in ratios relative to human plasma

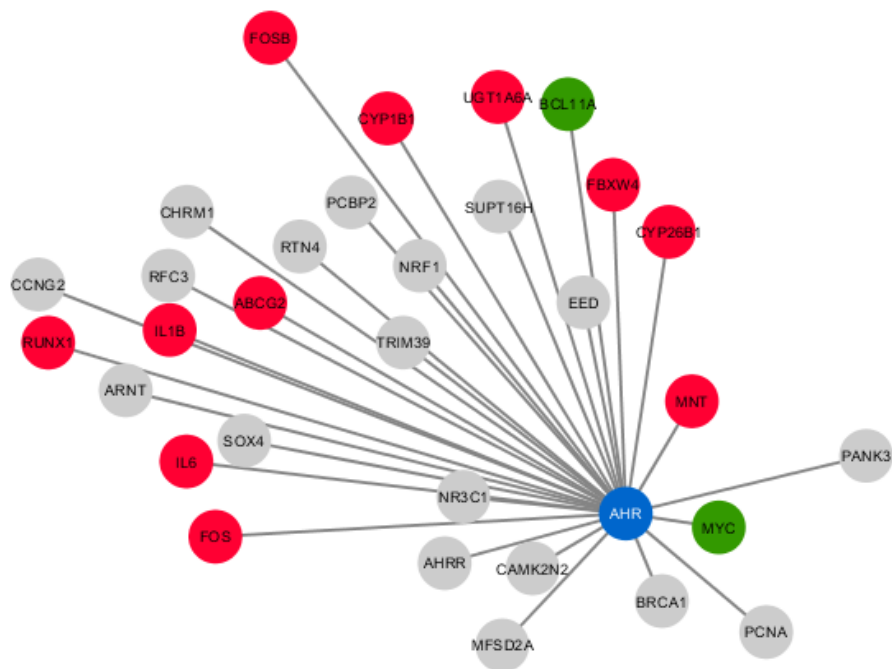


Supplementary Figure 1



Spleen $\gamma\delta$ T cells were labelled before and after magnetic bead purification.. Isolated $\gamma\delta$ T cell purity was confirmed on flow cytometry.

Supplementary Figure 2



Gene transcripts that showed differential expression in choroidal and splenic $\gamma\delta$ T cells, and are likely to be under the transcriptional regulation of AhR