Comprehensive analysis of a mouse model of spontaneous uveoretinitis using single-cell RNA sequencing

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Autoimmune uveoretinitis is a significant cause of visual loss, and mouse models offer unique opportunities to study its disease mechanisms. \textit{Aire}\textsuperscript{−/−} mice fail to express self-antigens in the thymus, exhibit reduced central tolerance, and develop a spontaneous, chronic, and progressive uveoretinitis. Using single-cell RNA sequencing (scRNA-seq), we characterized wild-type and spontaneous, chronic, and progressive uveoretinitis. Using single-cell RNA sequencing (scRNA-seq), we characterized wild-type and Aire\textsuperscript{−/−} retinas, to define, in a comprehensive and unbiased manner, the cell populations and gene expression patterns associated with disease. Based on scRNA-seq, immunostaining, and in situ hybridization, we infer that 1) the dominant effector response in Aire\textsuperscript{−/−} retinas is Th1-driven, 2) a subset of monocytes convert to either a macrophage/microglia state or a dendritic cell state, 3) the development of tertiary lymphoid structures constitutes part of the Aire\textsuperscript{−/−} retinal phenotype, 4) all major resident retinal cell types respond to interferon gamma (IFNγ) and may act as antigen-presenting cells.

Aire knockout | mouse model | ocular immunology | single-cell RNAseq | autoimmune uveitis

Noninfectious posterior uveitis, an autoimmune and/or auto-inflammatory disease of the pars plana of the ciliary body, vitreous, choroid, and retina, affects ~0.01% of the population (1) and is estimated to be the fourth leading cause of severe vision loss in the industrialized world (2). When the disease encompasses the retina, it is often associated with autoreactivity to retinal antigens such as photoreceptor Arrestin and Interphotoreceptor Retinol Binding Protein (IRBP), and this is referred to as uveoretinitis (3).

Mouse models of autoimmune uveoretinitis offer unique opportunities to study the mechanisms of disease pathogenesis. The most widely used and best-characterized mouse model is referred to as experimental autoimmune uveitis/uveoretinitis (EAU) (4, 5). In the original EAU model, uveoretinitis is initiated by immunizing with a retinal antigen—typically IRBP or a peptide derived from IRBP—that has been emulsified in complete Freund’s adjuvant (CFA). One limitation of this EAU model is the use of mineral oil supplemented with heat-killed mycobacteria, the active ingredient in CFA, which causes nonphysiological stimulation of the innate immune response. Inflammation is further enhanced by injection of pertussis toxin, which is required for efficient disease production in most rodent strains. These treatments may alter the adaptive immune response, which is considered to be the central driver of uveoretinitis. In contrast, adjuvant-free models of uveitis rely on genetic backgrounds that increase the number of auto-reactive T cells and have a more protracted clinical course relative to the original EAU model (6).

Loss of central tolerance to retinal antigens has been shown to mediate the development of spontaneous uveoretinitis (7). Many retinal antigens, including IRBP, are expressed in the thymus under the control of the Autoimmune Regulator (AIRE) transcription factor (8), and the susceptibility of mouse strains to EAU has been shown to correlate inversely with the amount of IRBP expressed in the thymus (9). Aire knockout (Aire\textsuperscript{−/−}) mice develop a spontaneous and chronic-progressive uveoretinitis as part of a multiorgan autoimmune phenotype, and, therefore, Aire\textsuperscript{−/−} mice represent an adjuvant-free model of uveoretinitis secondary to a loss of central tolerance to retinal antigens (10–12). The Aire\textsuperscript{−/−} mouse phenotype resembles autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (13, 14), a human condition caused by loss-of-function mutations in the human AIRE gene. At present, the Aire\textsuperscript{−/−} uveoretinitis model is relatively unexplored in terms of the immune cell types involved, the dominant effector response, and the response of resident retinal cells.

Droplet-based single-cell RNA sequencing (scRNA-seq) has emerged as a powerful and unbiased method for characterizing cell types in complex tissues in both normal and disease contexts (15, 16). Thus far, scRNA-seq has not yet been used to characterize uveoretinitis. To define the full range of cell types and

Significance

Autoimmune inflammatory diseases of the retina represent a major source of vision loss worldwide. Single-cell RNA sequencing has been used as a comprehensive and unbiased approach to investigate cell types and gene expression patterns in the retinas of a mouse model of spontaneous, chronic, and progressive autoimmune uveoretinitis. This work defines the dominant immune effector cell types involved, reveals the development of tertiary lymphoid structures within the diseased retina, characterizes the conversion of monocytes to a macrophage/microglia state or a dendritic cell state, shows that essentially all resident retinal cell types respond to interferon gamma as part of the disease process, and suggests that Muller glia may act as antigen-presenting cells.


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Data deposition: scRNA-seq data reported in this paper have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (accession nos. GSE132229 and GSM3854512–GSM3854519). The annotated datasets can be viewed at https://jacobheng.shinyapps.io/uveoretinitis/ and http://loom.gofflab.org. Supplementary code for processing and visualizing the scRNA-seq data can be found in an R package, cellwrangler, available at https://github.com/jacobheng/cellwrangler.

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cellular responses in a mouse model of uveoretinitis, we have characterized the neural retinas of Aire−/− mice on a C57BL/6J background using scRNA-seq, with additional analyses using immunostaining and in situ hybridization (ISH).

**Results**

**scRNA-seq Reveals a Diverse Immune Cell Infiltrate in Uveoretinitis.** To characterize uveoretinitis in Aire−/− mice on a C57BL/6J background, we performed retinal imaging on mice between 5 and 25 wk of age and graded disease severity according to the published EAU clinical grading scale (SI Appendix, Fig. S1A) (17). We used mice between 10 and 16 wk of age for scRNA-seq and mice between 8 and 25 wk of age for immunohistochemistry. As previously described (6), Aire−/− mice develop a chronic-progressive spontaneous uveoretinitis, as seen in the fundus images and fluorescein angiograms obtained from one Aire−/− mouse at 5 time points over 20 wk (SI Appendix, Fig. S1B). Fig. 1A shows representative images of retina cross-sections with their corresponding fundus images for each clinical grade. In general, there was progressive retinal thinning and an increase in the number of infiltrating CD45-positive leukocytes with higher disease grade, consistent with previous descriptions of Aire−/− eyes on a B10.RII background (6). In a minority of mice, the uveoretinitis was largely limited to one eye (SI Appendix, Fig. S1C).

Using a droplet-based scRNA-seq platform (10x Genomics), we characterized 64,196 dissociated retinal cells from 8 samples (one neural retina per sample): 2 grade 2 Aire−/− mice (16,884 cells), 2 grade 3 Aire−/− mice (12,640 cells), and their wild-type (WT) littermate controls (34,672 cells) (Fig. 1B). The mean number of genes detected was 928 per cell (median: 584; interquartile range: 383 to 1,289). To control for sex effects, only female mice were studied. By principal component analysis, the samples were clearly segregated by disease grade (SI Appendix, Fig. S1D). On a Uniform Manifold Approximation and Projection (UMAP) (18) plot, the single-cell transcriptomes segregated predominantly by cell type (Fig. 1C), based on known markers for the 12 major retinal cell types (SI Appendix, Table S1), rather than by disease grade or batch (SI Appendix, Fig. S1E). (In this UMAP plot, microglia constitute a subset of the cell clusters labeled “Immune cells.”) A total of 1,266 presumed multiplets were excluded from subsequent analyses.

In Aire−/− retinas, scRNA-seq revealed multiple immune cell types. When visualized on a separate UMAP plot, these cells formed distinct clusters representing microglia, cells of monocyte lineage, T and Natural Killer (NK) cells, B cells, and plasma cells (Fig. 1D and SI Appendix, Table S2). In WT retinas, a small number of monocyte-derived cells that are transcriptionally distinct from microglia were present (Fig. 1E, Left), but B cells, T and NK cells, and plasma cells were not detected. The absence or near-absence of lymphoid cells in WT retinas may be a feature of ocular immune privilege (5). In Aire−/− retinas, there were many more nonresident immune cells, and there was a preponderance of T and NK cells and monocyte-derived cells, accompanied by a smaller number of B cells and plasma cells (Fig. 1E, Right). The proportion of B cells and plasma cells increased with disease severity ($\chi^2 = 37.855$, degree of freedom (df) = 6, $P$ value = 1.199 × 10−4).
Fig. 2. Analysis of T cell diversity in Aire−/− retinas. (A) UMAP plot of Cd3-expressing cells from Aire−/− retinas showing different T cell and NK cell subtypes. (B) Heatmap showing, for each T and NK cell subtype (horizontal axis), the scaled mean unique molecular identifiers (UMIs) of transcripts for known cell-type markers (vertical axis). (C) Immunostaining of cross-sections of WT vs. Aire−/− retinas showing T-BET (green), IFNG (red), CD4 (magenta), and nuclei (DAPI; blue). T-BET+ IFNG+ CD4+ Th1 cells are indicated by arrows. In this and other figures, the regions in the square Insets are enlarged below. (D) UMAP reploting of Th1 cells from the UMAP plot in A enclosed in dashed lines showing 2 distinct clusters of Th1 cells: a Cd40-ligand (Cd40Lg)-positive cluster and an IL10-positive cluster (SI Appendix, Fig. S2B). (E) Immunostaining of cross-sections of WT vs. Aire−/− retinas showing T-BET (green), IL10 (red), CD4 (magenta), and nuclei (DAPI; blue). T-BET+ IL10+ CD4+ Tfh cells are indicated by arrows. (F) Heatmap showing 40 genes that are differentially expressed between the 2 Th1 clusters. (G, Left) Bar plots showing total UMIs for Tgfb1, Tgfb2, and Tgfb3. (G, Right) Heatmap showing mean expression per cell of Tgfb1, Tgfb2, and Tgfb3 for each cell type. (H) Retinal cross-sections showing fluorescent ISH for Tgfb2 (orange) and immunostaining for COL4A (green) and CD45 (magenta); nuclei are marked by DAPI (blue). (Scale bars in C, E, and H, 50 μm.)
We note the lack of significant expression of *Aire* transcripts in resident retinal cells in the WT mouse retina, implying that the phenotype associated with *Aire* loss of function reflects *Aire* expression in nonretinal cells, presumably medullary thymic epithelial cells (8, 11, 12). In a previous study, transfer of thymi from *Aire*−/− mice into *Aire*-sufficient athymic mice was sufficient to induce autoantibodies to eye antigens (8).

**Analysis of T Cell Diversity Reveals Th1 Cells as the Main Effector T Cells.** Since experimental uveoretinitis has been characterized as a T cell-driven disease (5), we analyzed the cluster of cells that express *Cd3e* or *Krai1* transcripts or both—representing T cells and NK cells—by first embedding these cells on a separate UMAP plot (Fig. 2A). By classifying cells according to known markers (Fig. 2B and SI Appendix, Table S2), we identified the following 6 classes: Th1 cells (T-bet+, *Ifng*+, *Ccxr6*+, *Cd4*+, *Cd8a−, Krai1−, *Cd8a+* T cells (*Cd8a*+, *Cd4*+, *Krai1−*)), T follicular helper (Th9) cells (*Bcl6*+, *Ccxr5*+, *Cd4*+, *Cd8a−*), regulatory T (Treg) cells (*Fapx3*+, *Cd4*+, *Il10*+), NK cells (*Krai1+,* Cd3e−, Cd4−, Cd8a−), and NK T cells (*Krai1+*, *Cd3e−*, *Cd8a+*). We also found a population of apparently undifferentiated *Cd4*+ *T* cells that were enriched for *Sip1* but did not express any of the classical effector T cell markers (*Sip1*+, *Cd4*+, *Cd8a−*). Only 3 of 714 T and NK cells in *Aire*−/− retinas were classified as Th17 cells, as defined by *Il17a* expression (Fig. 2A and B). These presumed Th17 cells do not form a distinct cluster on the UMAP plot, but this could reflect a combination of the small number of cells and/or variation in their gene expression profiles.

The classification of the different T cell populations revealed Th1 cells as the predominant class of T helper cells in *Aire*−/− retinas. Direct quantification of Th1 and Th17 markers in *Cd4*+ T cells revealed a predominance of Th1 markers in both grade 2 and grade 3 disease (SI Appendix, Fig. S2A). Immuno- staining of *Aire*−/− retinas for T-BET, interferon gamma (IFNG), and CD4 verified the presence of Th1 cells, which express all of these markers (Fig. 2C). The predominance of Th1 cells as effector T cells in the *Aire*−/− retina is consistent with previous reports that the main effector response in nonocular *Aire* tissues is Th1 dominant (19). Closer examination of the scRNA-seq data derived from the Th1 population revealed 2 distinct clusters—one that is *Il10+* (Interleukin-10+) and another that is *Cd40ligand+* (*Cd40ligand*) (Fig. 2D). These clusters appear to represent the previously described activated (*Cd40ligand*) and self-regulatory (*Il10*) states, respectively (20). These 2 states were correlated with the differential expression of multiple genes (Fig. 2F) that may underlie the dynamics of Th1 cell self-regulation. Notably, both of these Th1 subpopulations express the IFN gamma (*Ifng*) gene and the Th1 markers *Ccxr6* and *Thx21*, the latter coding for T-BET (SI Appendix, Fig. S2B). The IL10-expressing Th1 cells do not express *Fapx3*, indicating that they are not regulatory T cells (SI Appendix, Fig. S2B). Fig. 2E shows accumulation of IL10 in a subset of T-BET+ and CD4+ T cells in the *Aire*−/− retina.

Th1 cells are sensitive to transforming growth factor-beta (TGF-β), which has been shown to promote either an effector state or a self-regulatory state, depending on the context (21). Promoting a self-regulatory state associated with induction of *Il10* appears to be the predominant response for mature Th1 cells, as seen, for example, in a mouse model of experimental autoimmune encephalitis (22). Our scRNA-seq data show that *Tgfb2* is the principal TGF-β family member expressed in both WT and *Aire*−/− retinas and that it is expressed by multiple retinal cell types, predominantly in the inner nuclear layer (INL) (Fig. 2G), which was confirmed by fluorescent ISH (Fig. 2H). There was no difference in the mean abundance of *Tgfb2* transcripts between WT and *Aire*−/− retinas (SI Appendix, Fig. S2 C and D). *Tgfb1* transcripts were present at a lower level and were detected principally in infiltrating immune cells, especially in cells of the monocyte lineage.

**Th2 Cells and the Formation of Tertiary Lymphoid Structures.** The cluster of *Bcl6*+, *Ccxr5*+, *Cd4*+, and *Cd8a−* cells observed with scRNA-seq of *Aire*−/− retinas match the profile of Th2 cells (23, 24). This raises the possibility that tertiary lymphoid structures (TLSs) could have formed within *Aire*−/− retinas, a phenomenon that has been reported in the R161H mouse line, a mouse model of spontaneous uveoretinitis that is driven by transgenic expression of a T cell receptor (TCR) that recognizes IRBP (25).

By immunostaining of *Aire*−/− retinas, we observed aggregates of CD4+ T cells, CD8+ T cells, and CD19+ B cells (Fig. 3A). There appeared to be a predominance of CD4+ T cells over CD8+ T cells, as reflected in the scRNA-seq data, where 61.3% (*χ^2~(df=1)=4.27×10^-3~* and 76.3% (*χ^2~(df=1)=255.12, df=1, P value < 2.2×10^-16~*) of all T cells detected in grade 2 and grade 3 disease, respectively, were CD4+ T cells (SI Appendix, Fig. S2E). To assess whether these cell aggregates might resemble TLSs, we assessed markers indicative of TLSs. This analysis revealed the close proximity of BCL6+;CD4+ presumptive Th1 cells and CD19+ B cells in these cell aggregates (Fig. 3B). We also detected the classical germinal center marker Peripheral Lymph Node Addressin (PNAd) in close proximity to aggregates of CD19+ B cells and CD4+ T cells (Fig. 3C). PNAd is a glycoprotein that promotes the homing of T and B cells to TLSs and is a marker for high endothelial venules (HEVs), which are postcapillary venous structures important for lymphocyte homing and trafficking (26). We found PNAd+ blood vessels in close proximity to the cell aggregates (Fig. 3C). The aggregates also exhibited close apposition of CCR7+;CD4+ T cells and CCR7+ antigen-presenting cells (APCs), characterized by the expression of the major histocompatibility complex (MHC) class II proteins and visualized by immunostaining with an antibody directed against I-A and I-E MHC class II proteins (Fig. 3D). CCR7 has been shown to be important for organizing the T cell zone in lymph nodes and is necessary for the formation of TLSs (27). Finally, the detection of phosphorylated ZAP70 in CD4+ T cells and the presence of the costimulatory molecule CD80 indicate that TCR activation was occurring in these presumed TLSs (Fig. 3E). Consistent with CD4+ T cell activation and signaling, the TLSs also contained CD19+, SDC1+ plasmablasts, and CD19- and SDC1+ plasma cells (Fig. 3F).

**Monocyte Transitional States: Microglia-Like Macrophages and Dendritic Cells.** As shown in Fig. 1 C and D, retinal microglia and dendritic cells of the monocyte lineage form transcriptionally distinct clusters when visualized on a UMAP plot. Based on recent scRNA-seq studies of microglia and macrophages in the brain and retina (28–30), we were able to further resolve the cells of the monocyte lineage into monocytes, monocyte-derived macrophages (mo-Mφs), monocyte-derived dendritic cells (mo-DCs), replicating monocytes, perivascular macrophages, and plasmacytoid dendritic cells (Fig. 4A). Each of these populations of cells expressed distinct markers (SI Appendix, Fig. S3A). Two populations of cells, termed macrophage-committed monocytes (Mφ-committed Mo) and dendritic cell-committed monocytes (DC-committed Mo), expressed macrophage and dendritic markers, respectively, in addition to monocyte markers (SI Appendix, Fig. S3B).

Since monocytes, Mφ-committed Mo, and mo-Mφs, DC-committed Mo, and mo-DCs formed a continuum on a UMAP plot from *Aire*−/− retinas (Fig. 4A), we constructed a cell trajectory map for these cell types. Starting with *Ccr2*+ monocytes, this trajectory bifurcates into mo-Mφs (branch 1) and mo-DCs (branch 2) (Fig. 4B). The cell trajectory analysis shows that, as monocytes progress toward Mo, they reduce expression of the monocyte markers *Anxa8*, *Ly6c*, and *Ccr2*, while increasing expression of macrophage...
markers, including \textit{ Trem}2 and \textit{ Hexb} (Fig. 4C). Interestingly, the mo-M\textPhi s also increase expression of classical microglial markers such as \textit{Tmem119} and \textit{P2ry12}. This observation is consistent with the findings of a previous study indicating that bone-marrow–derived monocytes can migrate into both the normal and the injured retina to assume a microglia-like ramified morphology with expression of microglial markers (31). Similarly, as monocytes progress toward mo-DCs, they increase expression of DC markers, including \textit{Clec9a} and \textit{Zbtb46} (Fig. 4C). Fig. 4D illustrates, on a cell-by-cell basis, the changing patterns of expression for 5 markers: \textit{Cd14}, which is enriched in monocytes, M\Phi-committed Mo, and mo-M\Phi s; \textit{Mrc1}, which codes for CD206 and is expressed in monocytes and M\Phi-committed Mo; \textit{P2ry12}, which is enriched in mo-M\Phi s (and microglia); \textit{Smad7}, which is a marker of TGF-\beta signaling and is up-regulated as monocytes convert to mo-M\Phi s and to mo-DCs; and \textit{Zbtb46}, a marker of dendritic cells, which is enriched in mo-DCs.

A recent study has shown that, in mouse models of photoreceptor degeneration, resident microglia migrate to the subretinal space and adhere to the apical retinal pigment epithelium (RPE), whereas infiltrating monocytes and mo-M\Phi s remain in the neural retina (29). In a similar fashion, immunostaining of \textit{Aire}^{-/-} retinas showed P2RY12\textsuperscript{+};CD14\textsuperscript{+} mo-M\Phi s and IBA1\textsuperscript{+};MRC1\textsuperscript{+} M\Phi-committed-Mo predominantly in the neural retina, with only occasional
Fig. 4. Divergent fates of monocyte lineage cells in Aire−/− retinas. (A) UMAP plot from Aire−/− retinas showing different subtypes of monocyte lineage cells. (B) Cell trajectory of monocyte lineage cells showing a bifurcation into Mo-MΦs and Mo-DCs. (C) Heatmap showing branch expression analysis modeling of the branch point between Mo-MΦs (Left) and Mo-DCs (Right). (D) Expression of Cd14, Mrc1, P2ry12, Smad7, and Zbtb46 along the cell trajectory shown in B. (E) Immunostaining of cross-sections of WT vs. Aire−/− retinas showing P2RY12 (green), CD14 (red), IBA1 (magenta), and nuclei (DAPI; blue). A P2RY12+CD14+ cell is indicated by the arrows. (F) Immunostaining as in E for P2RY12 (green), MRC1 (red), IBA1 (magenta), and nuclei (DAPI; blue). Most IBA1+P2RY12+ cells are MRC1− (indicated by arrowheads) with a minority being MRC1+ (indicated by arrows). (G) Barplots showing mean log10 normalized expression per cell for Cd68, Aif1, and Lgals. *P value < 0.05 and **P value < 0.01. (H) Immunostaining as in E for CD68 (green), IBA1 (red), Galectin 3 (magenta), and nuclei (DAPI; blue). (Scale bars in E, F, and H, 50 μm.)
P2RY12+;CD14+ cells in the subretinal space (Fig. 4E and F). Interestingly, colonization of the microglial niche by mo-MΦs has been shown to be TGF-β dependent in the brain and retina (32, 33).

In previous studies, it has been difficult to distinguish infiltrating mo-MΦs from resident microglia because the 2 cell types exhibit similar patterns of gene expression and undergo similar gene expression changes in response to disease (34). Therefore, we examined gene expression patterns and disease-associated changes in those patterns in mo-MΦs and resident microglia in Aire−/− retinas. Some genes, such as Hexb and Trem2, are expressed at similar levels in both mo-MΦs and resident microglia (SI Appendix, Fig. S4A). Other genes are more specific to one or the other population. In particular, we examined Galectin-3 (Lgals3)

Fig. 5. IFN gamma response in resident cells in Aire−/− retinas. (A and B) Statistically significant MsigDB Hallmark pathways in 3 or more cell types in GSEA reveal pathways enriched in Aire−/− over WT retinas across multiple retinal cell types in grade 2 and grade 3 disease. *P value < 0.05. (C and D) Heatmaps showing GSEA for the IFN gamma response gene set in grade 2 (A) and grade 3 (B) Aire−/− retinas. (Left) Single column showing normalized enrichment score (NES) for each cell type. (Right) Heatmap showing normalized regression coefficients for each cell type (rows represent cell types; columns represent genes). (E) Immunostaining of cross-sections of WT vs. Aire−/− retinas showing IRF1 (green), CD45 (red), COL4A1 (magenta), and nuclei (DAPI; blue). (F) Immunostaining as in E for PSMB9 (green), CD45 (red), COL4A1 (magenta), and nuclei (DAPI; blue). (Scale bars in E and F, 50 μm.)
and Cd68, as these 2 genes are enriched and up-regulated in subretinal microglia in a light damage model (29). In Aire−/− retinas, Lgals3 was up-regulated in both mo-MΦs and resident microglia (P value < 0.01 for both), but Cd68 was unchanged (Fig. 4G).

Interestingly, we observed increased immunostaining for both Galectin-3 and CD68 in IBA1+ cells in Aire−/− retinas (Fig. 4H), suggesting that CD68 may be regulated post-transcriptionally. Both Galectin-3 and CD68 were detected in IBA1+ cells in the neural retina and the subretinal space, in contrast to their predominant subretinal localization in a light damage model (29). Transcripts coding for IBA1 (Aif1), a commonly used marker for microglia and macrophages, were only modestly up-regulated (P value < 0.05 for microglia and P value < 0.01 for mo-MΦs) (Fig. 4G), whereas IBA1 immunostaining was substantially increased in Aire−/− retinas in both mo-MΦs and microglia (Fig. 4H). Lgals3 and Aif1 are representative of the 145 genes that are up-regulated in both mo-MΦs and microglia in Aire−/− retinas. Thirty of these genes are shown in SI Appendix, Fig. S4B.

Taken together, these findings show that, although mo-MΦs and microglia express similar markers and undergo similar gene expression changes in Aire−/− retinas, they still maintain distinct transcriptional profiles.

Widespread Responses to IFN Gamma Among Resident Retinal Cells. We next examined the gene expression changes in resident retinal cell types in Aire−/− retinas. Cross-sample normalization was first performed via mean-scaling of the raw transcript copies per cell in each cell type independently. Using Monocle2 (35, 36), we generated a negative binomial regression model that included parameters for genotype, cell type, sequencing depth, and batch effects. This permitted the calculation of a z-scored genotype regression coefficient for each gene and the identification of differentially expressed genes on a per-cell type basis. To identify gene sets with differential representation in WT vs. Aire−/− datasets, genes were ranked based on the z-scored genotype regression coefficient for each gene in each cell type, and this ranking was used for a preranked Gene Set Enrichment Analysis (GSEA) (37). This preranked GSEA was performed with the Hallmark gene sets curated by the Molecular Signatures Database (MSigDB) (38) to identify gene sets that were enriched in Aire−/− vs. WT retinas in both grade 2 and grade 3 disease.

To examine gene expression changes that may underlie a common disease process across multiple cell types, we examined MSigDB Hallmark gene sets that were significantly enriched (q-value < 0.05) in 3 or more cell types and found multiple enriched gene sets in both grade 2 and grade 3 disease (Fig. 5A and B). Notably, there were more enriched gene sets in grade 3 disease compared to grade 2 disease, which is consistent with the increased disease severity. Of these gene sets, the IFN-responsive gene sets appeared to be the most significantly enriched across all resident retinal cell types. As only IFN gamma, but not -alpha or -beta, transcripts were detected in the Aire−/− retina dataset, we presume that these gene expression changes were driven by IFN gamma, consistent with the predominant Th1 response. By examining the regression coefficients of IFN gamma stimulated genes as defined in the Hallmark Interferon Gamma Response gene set, we identified genes that were differentially expressed across multiple cell types, as well as those that were differentially expressed in specific cell types (Fig. 5C and D).

One example of a gene that was significantly up-regulated across all retinal cell types is the transcription factor Ifi1, a major target of IFN gamma signaling (SI Appendix, Fig. S5A). Additionally, expression of Pbmb9, an IFI1 target gene, was increased in all retinal cell types (SI Appendix, Fig. S5B). PSMB9 is a subunit of the immunoproteasome, which is responsible for the processing of peptides for display by class I MHC proteins. Consistent with scRNA-seq data, immunostaining for IFI1 and PSMB9 showed widespread up-regulation in Aire−/− retinas (Fig. 5E and F). These observations imply that resident retinal cells exhibit enhanced antigen presentation in Aire−/− retinas, thereby further stimulating the autoimmune process.

The Muller Glial Response to IFN Gamma. Although all resident retinal cell types appear to be responsive to IFN gamma, the transcript heatmaps in Fig. 5C and D show the greatest responses in Muller glia. Consistent with this transcriptomic analysis, immunostaining of Aire−/− retinas showed that IRF1 was most significantly up-regulated in Muller glia (marked by nuclear SOX9 immunostaining in the INL) (Fig. 6A and SI Appendix, SS A and E).

Using a specificity score of 20.4 (Monocle2) as a threshold for Muller glia, we identified IFN gamma target genes that were up-regulated and enriched in Muller glia in Aire−/− retinas. Examples are shown in Fig. 6B (see also SI Appendix, Fig. SS C and D). The increase in expression of known IFN gamma target genes Cd4b and Cd274 was confirmed by fluorescent ISH (Fig. 6 C and D). Cd4b is a complement factor that promotes inflammation, as deletion of Cd4b is protective in EAU (39). Cd274 codes for PD-L1, a checkpoint regulator that dampens the adaptive immune response and has been implicated in suppressing EAU (40). In addition, the up-regulation of Vcam1 in Muller glia is consistent with an earlier study on EAU, which showed the up-regulation of VCAM1 in Muller glial radial processes by immunostaining (41). Vcam1 codes for Vascular Cell Adhesion Molecule 1, a protein involved in leukocyte migration into sites of inflammation.

Interestingly, multiple MHC class II genes are preferentially up-regulated in Muller glia in Aire−/− retinas (Fig. 6E). These genes are typically expressed in APCs including macrophages and dendritic cells. By immunostaining, MHC class II proteins were found to be enriched in Muller glial cell bodies (marked by arrow indicating nuclear SOX9 in Fig. 6F) and in their radial processes (Fig. 6F). Some of these MHC class II+ radial processes appeared to be in direct apposition to CD4+ T cells (arrowhead in Fig. 6F), suggesting that Muller glia may be functioning as APCs in Aire−/− retinas.

Discussion. This paper describes the application of scRNA-seq to characterize spontaneous uveoretinitis in Aire−/− mice. Based on the abundances of different immune cell types, the patterns of gene expression in immune and retinal cells, and the results of a parallel analysis by immunostaining and ISH, we infer that 1) the dominant effector response in Aire−/− retinas is Th1-driven, 2) the development of tertiary lymphoid structures constitutes an integral part of the Aire−/− retinal phenotype, 3) a subset of monocytes convert to either a macrophage/microglia state or a dendritic cell state, 4) all major classes of resident retinal cells respond to IFN gamma as evidenced by changes in their patterns of gene expression, and 5) Muller glia up-regulate specific genes in response to IFN gamma and may act as APCs. This study adds to a growing body of evidence showing that ocular inflammation is context-specific in terms of the molecular mechanisms and immune cell types involved (5).

The dominant effector response in uveitis is known to differ among animal models. In the classical EAU model, the use of killed mycobacteria in CFA promotes a Th17-dominant response that requires IL-17 for both the induction and the progression of uveitis (42). In contrast, in the R161H transgenic mouse line, which develops spontaneous uveitis through the expression of an IRBP-specific TCR transgene, Th1 cells appear to play a central role based on the large IFN gamma response (43). The present work supports a similarly central role for Th1 cells in Aire−/− uveoretinitis based on the abundance of Th1 cells, the presence of IFN gamma transcripts, and an IFN gamma-response gene expression signature in both immune and resident retinal cell types. These findings are consistent with previous work showing...
that many nonocular tissue pathologies in Aire−/− mice are Th1 dependent (19).

By immunostaining of Aire−/− retinas, we observed aggregates of T follicular helper cells, B cells, plasmablasts, plasma cells, and APCs that resemble TLSs. TLSs can be defined as unencapsulated, but structurally and functionally organized, aggregates of lymphoid cells that form in tissues other than primary lymphoid organs (i.e., thymus and bone marrow) or secondary lymphoid organs (i.e., lymph nodes, spleen, and Peyer’s patches) in the context of chronic inflammation (26, 44, 45). We use the term TLSs, instead of tertiary lymphoid organs (TLOs) because we have demonstrated some but not all of the features of TLOs (44). TLO features include 1) anatomically distinct yet adjacent B and T cell compartments, 2) the presence of PNAd-positive HEVs in the

![Fig. 6](image_url)
T cell compartment, and 3) the presence of germinal center re-
actions as evidenced by the presence of plasmablasts and plasma
cells. In contrast to the diffuse inflammatory cellular infiltrates seen
in the EAU model, the spatial organization of the retinal TLSs
studied here more closely resembles that of lymphoid tissues. Similar
TLSs have been described in the R161H transgenic mouse line
(25), another model of spontaneous uveitis (described in the pre-
ceding paragraph). In R161H mice, retinas with TLSs initially have
lower clinical and histological disease scores as well as slower loss
of visual function (as measured by the electroretinogram) com-
pared to retinas that lack TLSs, but the presence of well-developed,
late-stage TLSs with abundant plasma cells was associated with increased
visual loss and disease progression, presumably due to autoantibody
production (25). Similarly, the presence of plasma
cells in TLSs in Aire−/− retinas suggests that autoantibodies may also contribute to disease progression in Aire−/− uveoretinitis.

There is evidence that autoantibodies play a pathogenic role in the
EAU model (46) and in human uveitis (47, 48).

Retinal microglia, which arise from the yolk sac, are difficult
to distinguish from mo-MΦs as the 2 cell populations share many of
the same markers and both microglia and mo-MΦs up-regulate
many of the same genes and can occupy new tissue niches in re-
sponse to disease. A previous study that utilized irradiated mice
injected with bone marrow (BM) cells marked by enhanced green
fluorescent protein showed that BM-derived monocytes can popu-
late the normal retina and assume microglial-like morphology with
expression of classical microglial markers (31). The migration of BM-
derived cells was enhanced in a model of N-methyl-N-nitosourea-
induced retinal injury, and the BM-derived microglial-like cells were
restricted to the juxtapapillary and peripheral regions of the retina
(31). More recently, fate-mapping studies in mouse models of
photoreceptor injury and degeneration have shown that resident
microglia migrate to the subretinal space where they adhere to the
apical RPE, while mo-MΦs occupy the vacated microglial niche in
the neuroretina and do not migrate to the subretinal space (29,
34). By immunostaining of Aire−/− retinas, we observed CD14+ and
P2ry12-positive cells (i.e., mo-MΦs) mainly in the neural retina,
although some were found in the subretinal space adhering to the
apical RPE. The differences across retinal disease models imply
that the tissue niches occupied by microglia and mo-MΦs can vary
depending on the disease context.

Our analysis expands on this dynamic picture by implying that many monocytes transition from a state charac-
terized by markers such as Ccr2 and Ly6c2 to a mo-MΦ state
characterized by markers such as Hexb and Trem2. The rapid
down-regulation of Ccr2 in monocytes infiltrating the retina has
also been described in the context of photoreceptor degeneration
(49). Mo-MΦs are difficult to distinguish from resident retinal
microglia as they also express conventional microglial markers
such as P2ry12 and Tnem119, a finding that is consistent with
recent scRNA-seq studies of fluorescence-activated cell sorting-
purified microglia and mo-MΦs isolated from murine retina and
brain (28–30, 50). The down-regulation of genes such as Ccr2
and the up-regulation of genes such as Tnem119 may account for
the previously observed heterogeneity in gene expression of monocyte-derived cells at early time points after these cells have
infiltrated the retina in the context of light-induced photore-
ceptor degeneration (29, 30). The pseudotime analysis also im-
plies that a distinct subset of monocytes transitions to a dendritic
cell state characterized by markers such as Clec9a and Zbtb46.
Interestingly, both the mo-MΦ and mo-DC states were associated
with an up-regulation of Siam7, a marker of TGF-β signaling.

The scRNA-seq and ISH analyses reveal TGF-β2 expression in
the INL in Aire−/− retinas. TGF-β2 has potent immunosuppres-
sive properties (21, 51) and is thought to contribute to ocular
immune privilege (5). In the context of Aire−/− uveoretinitis,
TGF-β2 may mitigate against excessive immune-mediated de-
struction through its effects on Th1 cells and microglia/mo-MΦs.

Consistent with this idea, the induction of IL10 expression in
Th1 cells has been shown to be TGF-β dependent (22), and
the Th1 cells in the Aire−/− retina exhibit distinct Cd40l+ and IL10+
states that most likely represent activated and self-regulatory
states, respectively (20). Also consistent with this idea, genetic
ablation of Tgfbr2 in spinal cord mo-MΦs (32) and in retinal microglia
(33) resulted in down-regulation of classical microglial markers such as Tmem119 and conversion to a more proinflammatory phenotype.

The IFN γ response gene expression signature that we observe in all major classes of resident retinal cells is charac-
terized by widespread IRF1 up-regulation and up-regulation of
putative IFN γ target genes across retinal cell types. The role of IFN γ in uveoretinitis appears to be multifaceted. Retinal production of IFN γ, either by expression of a
transgene (52) or by infusing an IRBP-specific Th1-like T cell
line that produces large amounts of IFN γ (53), appears to be
uveitogenic. Moreover, disease induction in an EAU model
based on injection of antigen-exposed dendritic cells requires
host production of IFN γ (54). However, removing IFN γ, either by systemic administration of a neutralizing an-
tibody (55) or by genetic deletion (56), exacerbated uveoretinitis in
EAU instead of conferring a protective effect. These differ-
ences in the effects of IFN γ in the context of uveoretinitis have been attributed to differences in the stage of the disease at
which IFN γ exposure occurs, with early exposure to IFN γ eliciting an antinflammatory effect and late exposure eliciting a proinflammatory effect (57, 58).

One intriguing instance of a cell type-specific up-regulation of
putative IFN γ target genes is the increased production of
MHC class II messenger RNAs and proteins in Muller glia, with evidence of direct contact between Muller glia processes and
CD4+ T cells. Muller glia are the major glial component of the
retina, and their processes densely ramify throughout the en-
tirety of the neural retina between the nerve fiber layer and the
outer limiting membrane. Thus, any immune cell infiltrating the
retina will come into contact with Muller glia processes. Under
normal conditions, Muller glia are thought to contribute to oc-
ular immune privilege by inhibiting the proliferation and acti-
vation of lymphocytes through a direct-contact mechanism, as
demonstrated in cultured Muller glia (59). The addition of IFN γ in vitro has been shown to induce production of MHC
class II proteins in cultured Muller glia, with the expression of IFN γ eliciting an antiinflammatory effect and late exposure
eliciting a proinflammatory phenotype (57, 58).

For the previously observed heterogeneity in gene expression of
monocyte-derived cells at early time points after these cells have
infiltrated the retina in the context of light-induced photore-
ceptor degeneration (29, 30). The pseudotime analysis also im-
plies that a distinct subset of monocytes transitions to a dendritic
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TGF-β2 may mitigate against excessive immune-mediated de-
struction through its effects on Th1 cells and microglia/mo-MΦs.

Materials and Methods
All animal experiments were approved by and conducted in accordance with
the regulations of the Institutional Animal Care and Use Committee at Johns
Hopkins University School of Medicine (protocol MO16M367). Mouse hus-
bandry, scRNA-seq, in situ hybridization, immunostaining, and computational
methods are described in SI Appendix. scRNAseq data were submitted to the
National Center for Biotechnology Information Gene Expression Omnibus
database under accession numbers GSE132229 and GSM385412–385419.
The annotated datasets can be viewed at https://jacobheng.shinyapps.io/
uveoretinitis/ and http://loom.gofflab.org. Supplementary code for processing

and visualizing the scRNA-seq data can be found in an R package, cell-