$\gamma\delta$ T Cells as a Major Source of IL-17 Production During Age-Dependent RPE Degeneration

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PURPOSE. Chronic inflammation is a key factor contributing to the progression of age-related macular degeneration (AMD). The goals of the current study were to develop an improved mouse model with retinal pathologic features similar to those of AMD and to characterize the immunoreactive cells in the outer retina and choroid during degeneration of the retinal pigment epithelium (RPE).

METHODS. Mice deficient in nuclear erythroid 2-related factor 2 (Nrf2) at 12 months of age were fed a high-fat, cholesterol-rich diet for up to 16 weeks. Ocular phenotype was monitored by optical coherence tomography (OCT) and scanning laser ophthalmoscopy (SLO) in live animals, and was further validated by retinal histopathology. Immunofluorescence staining of either cryosections or RPE flat mounts was used to define immunoreactive cells. Flow cytometry analyses were further performed to define the subsets of intraocular T lymphocytes.

RESULTS. After 16 weeks on a high-fat (HF) diet, 58% of the eyes from *Nrf2*^{−/−} mice had progression of retinal lesions. Major histocompatibility complex class II (MHC II)-positive microglia, FoxP3⁺ regulatory T cells (Tregs), and CD3⁺ IL-17-producing T cells were detected in either the retina or sub-RPE space. Flow cytometry analyses further revealed that most of the IL-17-producing cells were CD3⁺ CD4[−] TCR $\gamma\delta$ ⁺ cells.

CONCLUSIONS. The results suggest that the T cell-mediated immune responses played important roles in controlling the progression of AMD-like phenotype in *Nrf2*-deficient mice.

Keywords: age-related macular degeneration, IL-17, inflammation, T lymphocyte

The incidence of age-related macular degeneration (AMD) and the prevalence of patients at late stages of the disease increase exponentially with aging.^{1–4} The majority of AMD patients have the atrophic (dry) form of the disease, which results from degeneration of the RPE and loss of photoreceptor cells in the central retina. The molecular mechanisms of disease progression from initial RPE degeneration to the more severe and vision-threatening conditions of geographic atrophy and choroidal neovascularization (CNV) are largely unknown. Chronic inflammation and immune responses in the outer retina can be key mechanisms that amplify the initial pathologic lesions and accelerate the otherwise linear decline of visual function with age.^{5,6} Genetic variations in complement gene are major risk factors, and the innate immune responses contribute to wet AMD.^{7,8} Whether adaptive immune responses play a role in AMD has not been clearly established.

The back of the eye has immune privilege. The RPE not only provides a barrier to shield the retinal antigens from entering systemic circulation, but also actively eliminates infiltrated T cells by Fas-mediated apoptosis^{9,10} or triggers their anergy.¹¹ However, the immune suppressive functions of the RPE become compromised in AMD; and retinal infiltrating T lymphocytes^{12,13} and autoantibodies against retinal antigens¹⁴ have been identified in AMD patients at different stages. A recent study by Cruz-Guilloty et al.¹⁵ reported that CD8⁺ T cells

caused RPE degeneration induced by active immunization with carboxyethylpyrrole (CEP)-modified albumin in Freund's adjuvant. Carboxyethylpyrrole-protein adducts are generated from oxidation of docosahexaenoate (DHA)-containing lipid and are potential biomarkers of AMD.¹⁶ Immunizing and boosting mice with CEP-modified albumin resulted in degenerative pathology of the RPE,¹⁷ indicating that when the acquired immune responses persist, particularly with adjuvant, activated T lymphocytes can penetrate the blood-retinal barrier and attack cells bearing the immunogenic epitope of DHA oxidation product. In addition to the cytotoxic T cells, IL-17-producing T cells have gained significant interest recently.

Interleukin-17 is a proinflammatory cytokine that plays causal roles in a number of age-related or autoimmune diseases.^{18,19} Mice deficient in IL-17A were less susceptible to developing experimental autoimmune encephalomyelitis and collagen-induced arthritis,^{20,21} and had smaller lesions of laser-induced CNV.²² The receptor for IL-17A is a heterodimer composed of IL-17 receptor A and C (RA and RC). The mRNA and protein levels of IL-17 RC were found to be elevated in macular tissues from AMD patients.^{23,24} The source of IL-17 in the outer retina has not been defined. Th17 cells, a subtype of activated CD4⁺ T cells, are often deemed the major producers of IL-17. On the other hand, $\gamma\delta$ T cells can produce a large

TABLE. Summary of Phenotypic Findings

	WT ND	WT HF	KO ND	KO HF	HF vs. ND, <i>Nrf2</i> ^{-/-}
Total eyes, <i>n</i>	28	36	34	64	
OCT					
<i>n</i>	18	18	26	38	
Subretinal drusenoid deposits	1	3	9	22	* <i>P</i> < 0.05
Lesion progression	1	0	2	12	* <i>P</i> < 0.05
Fundus examination					
<i>n</i>	8	6	12	16	
RPE mottling	2	4	7	14	
Drusen-like deposits	1	5	8	13	
Patchy lesions	1	2	7	12	
Histology					
<i>n</i>	7	10	8	20	
Pigmentary changes	2	8	6	18	
Vacuoles	1	6	5	19	
Drusen-like deposits	0	1	5	10	
CNV	0	0	1	3	

WT, wild type; ND, normal diet; KO, knockout.

* χ^2 test between *Nrf2* knockout mice on either control or high-fat diet.

amount of IL-17 in tissue-specific pathogen defense and autoimmune responses.^{25,26}

The $\gamma\delta$ T cells are characterized by their unique T cell receptor (TCR), a dimer constituted by γ and δ chains. Compared to the more commonly studied $\alpha\beta$ T cells, $\gamma\delta$ T cells respond to antigens with lower specificity but much faster kinetics, with minimal requirement for clonal expansion.²⁵ Activated $\gamma\delta$ T cells can produce effector cytokines, such as IL-17 and IFN- γ , independent of TCR ligation, and they form a primary line of defense against invading pathogens.²⁷⁻³⁴ The unique properties of $\gamma\delta$ T cells make them ideal candidates to mediate immune responses in ocular tissue, which is otherwise immune privileged.

A main goal of the current study was to define the identity of IL-17-producing cells in the posterior segment during RPE degeneration. We previously reported that nuclear erythroid 2-related factor 2 (*Nrf2*) knockout mice had age-dependent retinal pathology.³⁵ In the current study we further optimized the model by feeding the mice a high-fat (HF) diet. Our results showed that the HF diet promoted the development and progression of AMD-like pathology in *Nrf2*-deficient mice. During RPE degeneration, IL-17-positive T cells were identified as infiltrating the RPE layer. Using combined immunofluorescence staining and flow cytometry approaches, we concluded that tissue-specific $\gamma\delta$ T cells were the main source of IL-17 in the outer retina.

METHODS

Animals

Protocols for animal breeding, housing, and handling were approved by the University of Texas Medical Branch (UTMB) Institutional Animal Care and Use Committee (IACUC). All procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The *Nrf2*^{-/-} mice of C57BL6/SV129 background have been described previously,³⁶ and were housed at a pathogen-free vivarium of the UTMB Animal Resource Center.

Animals were fed normal rodent chow for the first 12 months and were then changed to either HF diet or control diet for up to an additional 4 months before being euthanized. The HF Brelsow Western diet (TestDiet, St. Louis, MO, USA) contained 20% fat and 0.15% cholesterol, while control rodent chow had 5% fat. The absence of rd8 mutation was verified by retinal histopathology, gene-specific PCR amplification,³⁷ and sequencing of the PCR products.

Clinical Examination of Mouse Fundus Pathology

Optical coherence tomography (OCT) and scanning laser ophthalmoscopy (SLO) were performed on a Spectralis spectral-domain (SD)-OCT multimodality imaging system (Heidelberg Engineering, Carlsbad, CA, USA). A 20-diopter (D) lens (Edmund Optics, Inc., Barrington, NJ, USA) was used to adapt for the refraction of rodent eye. Color fundus images and fundus fluorescein angiography were taken by a Micron III mouse fundus imaging system (Phoenix Research Labs, Pleasanton, CA, USA). During image acquisition, animals were anesthetized by isoflurane inhalation from a precision vaporizer (Harvard Apparatus, Holliston, MA, USA), with cornea kept moisturized and pupil fully dilated by Tropicamide Ophthalmic Solution (Bausch & Lomb, Rochester, NY, USA).

Animals were examined by fundus imaging before the start of the HF diet to create a baseline record for each mouse. Optical coherence tomography and SLO images were routinely taken every 4 to 6 weeks. On SLO images, areas of 1 × 1 mm² centered on the optic disc were used to count the autofluorescent spots. Data for each mouse were maintained and analyzed with Heidelberg Eye Explorer software (Heidelberg Engineering). Images were reviewed retrospectively for lesion grading and progression. A total of 50 mice were included in the in vivo imaging studies, and the details are elaborated in the Table.

Histology and Immunofluorescence Staining

For paraffin sections, eyes from euthanized mice in each experimental group were enucleated and postfixed in 4% paraformaldehyde for 24 hours before processing and embedding. For each eye, approximately 250 sagittal sections of 4- μ m thickness were cut from cornea to the optic nerve and stained with either hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS). Every section was reviewed for RPE and retina pathology; and 10 slides from the periopic nerve area spanning a distance of approximately 200 μ m were picked from every eye for pathology scoring and quantification of subretinal/sub-RPE cell infiltration.

A pathology scoring system with a scale of 0 to 4, similar to the one reported by Cruz-Guilloty et al.,¹⁵ was used to document the progression of histology findings (Supplementary Table S1). For subretinal cell infiltration, only cells located underneath the photoreceptor outer segment in direct contact with the apical side of the RPE were included for quantification. Cells within a distance of 60 μ m from the Bruch's membrane (BrM) were quantified for sub-RPE cellularity. Endothelial cells with a spindle or crescent shape were excluded.

For cryosections, mice were terminally anesthetized and subjected to whole-body perfusion with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Enucleated eyes were postfixed in the same fixative overnight before being embedded in Tissue-Tek Cryomold (Electron Microscopy Sciences, Hatfield, PA, USA). Sagittal cryosections of 8- μ m thickness were prepared from cornea to optic nerve and stained for various antigens of interest. To block nonspecific binding, tissue sections were incubated with normal serum

appropriate to the species of secondary antibodies diluted in 0.5% Triton X-100/PBS. They were then incubated with primary antibodies followed by Alexa Fluor-conjugated secondary antibodies (Life Technologies, Grand Island, NY, USA).

For RPE flat mounts, enucleated eyeballs were briefly fixed in 4% PFA for 30 minutes before dissection. After separating the retina and RPE, tissues were further fixed in the same fixative for 3 hours before blocking with 5% goat serum/0.1% Triton X-100/1× PBS overnight at 4°C. Primary antibodies were diluted with blocking buffer and incubated overnight at 4°C, followed by appropriate secondary antibodies. Nuclei were stained by Hoechst 33342 (Life Technologies), and tissues were mounted with mounting medium (Electron Microscopy Sciences). Alexa Fluor 647 phalloidin (Life Technologies) was used to delineate boundaries of the RPE. Fluorescence images were acquired by confocal microscopy (LSM510; Carl Zeiss, Thornwood, NY, USA). Detailed information on primary antibodies is presented in Supplementary Table S2.

Flow Cytometry Analyses of Lymphocytes in the Posterior Segment

Lymphocytes were enriched by discontinuous gradient centrifugation.³⁸ Briefly, eyes were enucleated and dissected to discard the anterior segment and the lens. Retina and RPE-choroid complex were isolated and digested with 0.05% collagenase D (Roche Applied Science, Indianapolis, IN, USA) in RPMI 1640 medium. Tissues were incubated at 37°C for 30 minutes and were dislodged into single cell suspension by repeated pipetting. After passing through a 40- μ m nylon cell strainer, cells were collected by centrifugation at 300g for 3 minutes. The cell pellet was resuspended in 30% Percoll/RPMI 1640 solution and laid over a 30%/70% discontinuous Percoll gradient (Sigma-Aldrich Corp., St. Louis, MO, USA), then centrifuged at 400g for 30 minutes. The enriched lymphocytes were visualized as a single band located at the interphase of the lower one-third of the gradient and were carefully collected for further analyses.

Staining for cell surface markers and intracellular antigens was performed according to our previously published methods.^{38,39} The enriched lymphocytes were first treated for 4 hours with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (750 ng/mL) in the presence of GolgiStop (BD Bioscience, San Jose, CA, USA). Cells were then collected and blocked with Fc γ R blocker (anti-mouse CD16/32; eBioscience, San Diego, CA, USA) and stained for specific surface molecules. Following surface staining, cells were processed with a fixation/permeabilization kit (eBioscience) and stained for intracellular cytokines. After stringent washes, samples were analyzed on an LSRII FACSFortessa flow cytometer (Becton Dickinson, San Jose, CA, USA), and the data were processed with FlowJo software (TreeStar, Ashland, OR, USA).

RT-PCR Analyses of $\gamma\delta$ T Cell Subset

Mouse lymphocytes were enriched from either RPE/choroid preparations or spleen as described above. Total RNA was extracted with Trizol reagent (Life Technologies) and reverse transcribed for PCR amplification with subset-specific primers.^{40,41} The primers used were V γ 1, 5'-CCGGCAAAAAGCAAAA AAGT-3' and 5'-AAGGAGACAAAGGTAGTCCAGC-3'; V γ 2, 5'-TTGGTACCGGCAAAAACAAATCA-3' and 5'-CAATACACCCTTA TGACATCG-3'; V γ 4, 5'-CTTGCAACCCCTACCATAT-3'; V γ 5, 5'-GAGGATCCCGCTTGGAATGGAT GAGA-3'; V γ 6, 5'-GATCCAA GAGGAAGGAAAGACGGC-3'; V γ 7, GATCCAACTTCGTGAGTT CCACAAC-3'. The reverse primer for V γ 4 to V γ 7 was 5'-CCACCA CTCGTTTCTTTAGG-3'. Polymerase chain reaction products were analyzed by 1% agarose gel electrophoresis and imaged

with an Ultralum Omega fluorescence gel analysis system (Biovision Technologies, Claremont, CA, USA).

RESULTS

Accelerated RPE Degeneration in *Nrf2*^{-/-} Mice Fed High-Fat, Cholesterol-Rich Diet

Age-dependent RPE pathology occurred in *Nrf2* knockout mice around 12 months, which is approximately 50% of their expected life span.³⁵ To further optimize this in vivo model of RPE aging and degeneration, we fed the 12-month-old *Nrf2*^{-/-} mice the HF diet and monitored the progression of their ocular pathology for additional 4 months. At 16 months, *Nrf2*^{-/-} mice on regular rodent chow showed RPE hypertrophy, vacuolation, and pigmentary changes, which are consistent with our previous observations.³⁵ The HF diet led to deposition of Oil Red O-positive particles in samples from both wild-type and *Nrf2*^{-/-} mice (Fig. 1H). Wild-type mice on the HF diet showed moderate RPE pathology such as vacuoles and pigmentary changes (Fig. 1A). In *Nrf2*^{-/-} mice on the HF diet, histology and OCT examinations identified severe RPE and retinal pathology, including RPE hyper- and hypopigmentation (Fig. 1F), RPE hyperplasia (Fig. 1G), and occasionally eosinophilic, PAS-positive deposits in sub-RPE (Fig. 1E) and subretinal space (Figs. 1B, 1C). Choroidal neovascularization was found in 3 of the 20 eyes examined in the *Nrf2*^{-/-} HF group, but not in the wild-type HF group. The vascular lesions presented a hypercellular structure with RPE proliferation and infiltration of melanin-ingested macrophages and cell debris (Fig. 1D), similar to the structure of CNV membranes excised from AMD eyes.^{42,43} The retinal pathology data are summarized in the Table. Overall, the HF-fed *Nrf2*^{-/-} mice had the most significant RPE pathology; and both wild-type and knockout mice treated with the HF diet had an exacerbated RPE pathology as compared to their normal chow-fed counterparts (Fig. 1J). Other pathologic features related to AMD, such as increased RPE autofluorescent granules, deposition of complement protein, and accumulation of oxidative-damaged protein in the RPE, were also observed in *Nrf2*^{-/-} mice (Supplementary Figs. S1–S3).

Subretinal drusen-like deposit (SDD) was recently reported as closely correlated to different stages of RPE degeneration in AMD patients.^{44–46} In addition to histopathology findings, OCT scan identified that 58% (22 out of 38) eyes in the HF *Nrf2*^{-/-} group developed SDD, versus 35% (9 out of 26 eyes) in normal chow-fed *Nrf2*^{-/-} mice (Table, $P < 0.05$, χ^2 test). By analyzing the OCT data from the same animal at the same region of the retina (Fig. 1D), progression in both size and number of SDD lesions was noted in *Nrf2* knockout mice. The HF diet increased the risk of progression by 5.5-fold (95% confidence interval ranging from 1.1 to 27.4) (Table). Subretinal drusen-like deposits of the *Nrf2*^{-/-} model were highly similar to various-stage SDDs as reported in previous human clinical studies.⁴⁷ Thus, by combining aging, oxidative stress, and dietary manipulation, we have further optimized our animal model and achieved markedly accelerated RPE degeneration in *Nrf2*-deficient mice at an advanced age.

Subretinal Accumulation of Microglia/Macrophages

Under SLO, a significant increase of autofluorescent spots was detected in *Nrf2*^{-/-} mice after the HF diet (Fig. 2A) (67 ± 11.4 spots/graph) as compared to knockout mice on normal chow (35 ± 6.4 spots/graph, $P < 0.001$, Student's t -test). Because the imaging focal plane was on the RPE, the RPE autofluorescence could contribute to the signals. On the other hand, in human

FIGURE 1. Accelerated RPE degeneration in *Nrf2*^{-/-} mice fed HF diet at advanced age. (A) Representative histology section from a 16-month-old wild-type mouse, showing normal outer retinal structure with intact RPE and BrM (white arrows). RPE vacuoles and pigmentary changes were observed in an age-matched wild-type mouse on HF diet, whereas photoreceptor layer was spared. Severe degenerative changes were observed in HF diet-treated *Nrf2*^{-/-} mouse with large area of RPE lysis, thickening of BrM (black arrows), dilation of choroidal vessels (asterisks), and loss of photoreceptors. Stage 2 (C) and stage 3 (B) SDDs were found on both OCT and histology sections in HF *Nrf2*^{-/-} mice. (D) Follow-up OCT scans of the same *Nrf2*^{-/-} mouse before and after HF diet, showing lesion progression in both size (arrows) and number (arrowheads). Other AMD-like pathologies included PAS-positive deposit (E), RPE hypopigmentation (F), and double-layer RPE proliferation (asterisk in [G]) in the knockout HF group. (H) Oil Red O-stained lipid droplets were accumulated in the BrM area in knockout HF mice, whereas only little staining was observed in wild-type counterparts. (I) CNV lesion was observed in one *Nrf2*^{-/-} HF mouse, which showed discontinuous BrM (arrow), RPE loss (asterisk), and subretinal cell infiltration (arrowheads). (J) Histology grading on paraffin sections showed that HF diet aggravated the retinal pathology in both wild-type and *Nrf2*^{-/-} mice, and that *Nrf2*^{-/-} HF mice had the most significant RPE pathology among all four groups (**P* < 0.05, one-way ANOVA and Bonferroni post hoc test). Scale bars: 100 μ m (A); 50 μ m (B–H).

AMD eyes, retinal microglia and infiltrated macrophages are frequently found at the sites of lesion.^{42,48} The dome shape along with the lattice distribution of the autofluorescent spots on SLO was suggestive of subretinal lipid-ingested microglia/macrophages.^{49,50} To test this possibility, we stained retinal and RPE whole mounts with microglial/macrophage markers Iba-1 and CD45.^{51,52} The number of double-positive cells in the subretinal space was significantly higher in *Nrf2*^{-/-} mice on the HF diet (Fig. 2B) compared to HF wild-type mice. The result was also confirmed by the histology approach, which identified significantly more subretinal cell infiltration in *Nrf2* knockouts on the HF diet (Fig. 2D).

Further scrutiny of these subretinal cells in *Nrf2*^{-/-} HF mice revealed heterogeneous morphology varying from those with thin ramified processes to those in amoeboid shapes, representing microglia at different functional stages (Fig. 2B, inset). Moreover, CD68 (also known as ED1), a lysosomal membrane protein in myeloid cells, was found in some of the subretinal

microglia/macrophages (Fig. 2C), indicating their activation with increased phagocytic activity.^{53,54}

IL-17-Producing $\gamma\delta$ T Cells in Posterior Segment

Increased expression of major histocompatibility complex class II (MHC II) molecule on residential retinal microglia has been reported to occur in aging retina as well as in AMD eyes.^{48,55,56} In the retina of HF diet-treated mice, MHC II⁺ microglial cells were detected exclusively in the *Nrf2*^{-/-} mice (Fig. 3A). The presence of antigen-presenting microglia prompted us to search for other immunocompetent cells in the eye. By staining retinal flat mounts, CD3⁺ lymphocytes were detected in HF diet-treated mice, both wild type and *Nrf2* deficient (Fig. 3B). The CD3⁺ cells displayed large round nuclei and scanty cytoplasm, with an average diameter of $12.9 \pm 2.1 \mu$ m. Most of the retinal T cells were located in the outer plexiform layer of the retina (data not shown). Further

FIGURE 2. Fundus autofluorescence and subretinal microglia accumulation in *Nrf2*^{-/-} mice on HF diet. (A) SLO images showing fundus autofluorescence in mice fed either control or HF diet. Digitally magnified area shown in the red box. (B) Immunofluorescent staining of Iba-1 and CD45 on RPE/choroid flat mount prepared from *Nrf2*^{-/-} mouse on HF diet. Boxed area in (B) is magnified on the right. (C) Immunostaining of cryosections, showing Iba-1 and CD68 double-positive microglia in the subretinal space. (D) Quantification of subretinal cells on histology sections (**P* < 0.05, one-way ANOVA and Bonferroni post hoc test). Scale bars: 200 μm (A); 1000 μm (B); 20 μm (C, D).

characterization of these cells revealed a subset of forkhead box P3 (FoxP3) and CD3 double-positive lymphocytes (Fig. 3C), indicating the presence of regulatory T cells (Treg). CD3⁺ lymphocytes were barely detectable in the retina of mice on normal chow.

During the histopathologic examination of paraffin sections, we frequently spotted cells in the choriocapillaris underneath the BrM (Figs. 4E, 1F). By quantifying the cells within 60-μm distance from the BrM, we found a significant increase of sub-RPE cellularity in eyes from HF diet-treated *Nrf2*^{-/-} mice (Fig. 4F). On cryosections, we detected CD3⁺ lymphocytes in the sub-RPE space, often aggregated near RPE cells with signs of degeneration (Fig. 4E). By immunostaining of RPE flat mounts and three-dimensional reconstruction of confocal optic sections, we identified CD3⁺ cells intercalating in the RPE layer at different depth (Fig. 4D). Some of the infiltrated lymphocytes were close to retinal microglia, suggesting possible antigen presentation to T cells (Figs. 4A–

FIGURE 3. Activated retinal microglia and lymphocyte infiltration in *Nrf2*^{-/-} mice on HF diet. (A) Immunofluorescent staining of Iba-1 and MHC II on retina flat mounts prepared from either wild-type or *Nrf2*^{-/-} mouse on HF diet. MHC II-positive microglia (arrowheads) were found exclusively in retina of *Nrf2*^{-/-} mice. (B) CD3 staining of retina flat mounts. T lymphocytes were found in retina of both wild-type and knockout mice on the HF diet. (C) CD3 and FoxP3 double staining of retina flat mount for identifying Tregs. Scale bars: 100 μm (A, B); 20 μm (C).

C). Furthermore, in *Nrf2* knockout mice on the HF diet, some of the sub-RPE lymphocytes were positively stained for IL-17 (Figs. 4D, 4E).

To provide an independent measure of infiltrated ocular lymphocytes, we next performed flow cytometry analyses of these cells. Consistent with our findings on tissue immunostaining, CD3⁺ lymphocytes were noted in both wild-type and *Nrf2* knockout mice fed the HF diet (Fig. 5A). Among those cells there were two subpopulations, CD3⁺ CD4⁻ cells and CD3⁺ CD4⁺ cells (8.8% and 6.3%, respectively, in HF *Nrf2*^{-/-} mice). However, those two groups of cells were either diminished or absent in eyes from *Nrf2*^{-/-} mice on the normal diet (1.1% and 0.4%, respectively). CD4⁺ FoxP3⁺ Tregs were identified in both wild-type and *Nrf2* knockout mice on the HF diet (Fig. 5B), and the percentage of Tregs in the total CD3⁺ population was higher in wild-type mice. In contrast, the number of IL-17-producing lymphocytes was significantly higher in *Nrf2*^{-/-} mice (Fig. 5C, 10.5% ± 0.9 vs. 4.1% ± 0.6, mean ± SEM, *P* < 0.01, Student's *t*-test). Further analyses revealed that the majority of IL-17 producers were $\gamma\delta$ T cells (Fig. 5F); and there was a significant increase of IL-17⁺ and TCR $\gamma\delta$ ⁺ cells in HF-fed *Nrf2*^{-/-} mice (Fig. 5D) (5.6 ± 1.5 vs. 1.9 ± 0.1, mean ± SEM, *P* < 0.05, Student's *t*-test). The number of Th17 cells (CD4⁺ IL-17⁺) was minimal in the eyes from either

FIGURE 4. Infiltration of IL-17-producing lymphocytes in the RPE/choroid complex of *Nrf2*-deficient mice on the HF diet. (A) Immunostaining of cryosections for MHC II and CD45. CD45 and MHC II double-positive cells (arrowheads) were mostly observed in *Nrf2* knockout mice. (B–D) Immunostaining of RPE flat mounts from *Nrf2*^{−/−} mice for lymphocyte markers. (B) CD45⁺ cells observed close to Iba-1⁺ microglia. Reconstruction of Z stack of the yellow boxed area showed CD45⁺ cells near the RPE defect as indicated by the discontinued tight junction (arrowheads in sidebars), surrounded by a microglia cell (red signal). (C) Adjacent CD3⁺ lymphocytes (arrowheads) and Iba-1⁺ microglia (red) separated by the RPE. (D, E) CD3 and IL-17 staining of RPE flat mount and cryosections, respectively. IL-17-producing lymphocytes (arrowheads) were identified on apical, in middle, or on basal side of the RPE layer (D). (F) Quantification of sub-RPE cells within 60-μm distance to the BrM on histology slides, showing significant increase of cells in knockout HF group compared to either normal chow-fed *Nrf2*^{−/−} mice or wild-type mice on HF diet (**P* < 0.05, one-way ANOVA and Bonferroni post hoc test). Scale bars: 20 μm.

wild-type or *Nrf2*^{−/−} mice (Fig. 5E). Thus, $\gamma\delta$ T cells are the major source of IL-17 production in the degenerating retina/RPE. We further characterized the subsets of $\gamma\delta$ T cells in the RPE/choroid in HF diet-fed *Nrf2*^{−/−} mice. As shown in Figure 6, the profile was different between spleen and RPE/choroid, and V γ 6 was the major subset in the eye.

DISCUSSION

We previously reported that mice deficient in *Nrf2* developed age-dependent RPE degeneration.³⁵ In the present study we showed that *Nrf2*^{−/−} mice fed high-fat, cholesterol-rich chow displayed more severe RPE pathology and accelerated progression of retinal lesions (Fig. 1). By monitoring the subretinal drusenoid deposits with SD-OCT, accelerated disease progression was observed in 58% of eyes (Supplementary Table S1) in

mice aged between 12 and 16 months. The pathologic features of the retinal and RPE lesions highly resemble those of human AMD.^{45–47} Thus, with noninvasive imaging technique and dietary control, we were able to show the dynamics of the disease progression within a narrowed time frame. While it should be kept in mind that rodents do not have macula and that the clinical manifestations of human AMD are not limited to the pathologic features presented by the *Nrf2*-deficient mice, this improved animal model of age-dependent RPE degeneration can provide a new tool for future interventional studies designed to control the disease progression in early AMD.

Strategies that increase dietary fat intake and/or disruption of lipid metabolism have often been employed to produce animal models related to AMD.^{57–63} In *Nrf2*^{−/−} mice on the HF diet, RPE cells were positively stained by Oil Red O (Fig. 1H)

FIGURE 5. Flow cytometry analyses of retinal and choroidal lymphocytes. **(A)** CD3⁺ and CD3⁺/CD4⁺ lymphocytes in both wild-type and knockout mice fed the HF diet. **(B)** CD4⁺/FoxP3⁺ Tregs in wild-type and knockout mice on the HF diet. **(C)** IL-17-producing T cells (CD3⁺) in wild-type and knockout mice on the HF diet. **(D)** Increased percentage of IL17⁺ TCRγδ⁺ cells in *Nrf2*^{-/-} mice on the HF diet. **(E)** No significant difference in Th17 cells (CD3⁺ IL-17⁺) between two strains of mice on the HF diet. **(F)** Percentage of TCRγδ⁺ cells in IL-17⁺ T cell population. The majority of IL-17-producing cells in *Nrf2*^{-/-} HF mice were TCR-γδ⁺. Data presented are representative of four to six separate experiments (mean ± SEM, **P* < 0.05; ***P* < 0.01, Student's *t*-test). Each plot contains pooled intraocular lymphocytes from three animals.

FIGURE 6. Characterization of $\gamma\delta$ T cell subset of intraocular lymphocytes. Enriched lymphocytes from RPE/choroid or spleen were used for RNA extraction and RT-PCR analyses. PCR products were analyzed by agarose gel electrophoresis. bp, base pair; M, 100-bp molecular weight marker.

and showed signs of degeneration such as increased vacuolation, disturbance of pigment, sub-RPE deposit formation, and RPE atrophy (Figs. 1A–F). It is likely that the chronic oxidative stress in *Nrf2*^{−/−} mice had additive effects with the hyperlipidemia, which changed the lipid composition of the BrM and retina. Although the underlying mechanisms remain to be explored, they are likely related to the increased lipid peroxidation products, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) equivalents,^{64,65} that are implicated in the etiology of AMD.^{66,67}

Activation of multiple lines of immune cells within the otherwise immunoprivileged intraocular environment (Figs. 3, 4) was observed in HF diet-fed *Nrf2*^{−/−} mice. Infiltrated T cells were detected in areas with RPE degeneration (Fig. 4B); and microglia activation and transition to antigen-presenting cells can be a key immune modulating event (Fig. 4B). Intraocular microglia can be divided into three populations: perivascular, parenchymal, and subretinal. Previous studies by Xu et al.⁶⁸ and Luhmann et al.⁶⁹ demonstrated that during aging and pathologic conditions in the outer retina, the parenchymal microglia migrated into the subretinal space to facilitate the removal of subretinal debris derived from shedding photoreceptor outer segments. Ingestion of these lipid-rich components rendered the microglia autofluorescent. The findings are consistent with our data showing the dome-shape look and autofluorescent cells on SLO (Fig. 2A). The subretinal microglia in HF-fed *Nrf2*^{−/−} mice can have in situ effects to promote the formation of SDD and drusen⁷⁰ and may secrete complement regulatory proteins, such as complement factor H (CFH) and complement factor B (CFB), which have been described in human SDD.^{71,72}

In this study, we observed a population of Iba-1⁺ microglia that expressed MHC II exclusively in the retina of HF diet-fed *Nrf2*^{−/−} mice (Fig. 3). In light of the finding that aberrant intraocular lymphocyte infiltration was discovered in this model, it is reasonable to suggest that the appearance of these cells was involved in T cell recruitment and activation. Under physiological conditions, retinal microglia are MHC II^{low} and are immunosuppressive with limited function to activate T lymphocytes.^{3,74} However, under extensive aging⁵⁶ or disease conditions, MHC II^{hi} microglia are observed in rodent retina correlating to RPE structural changes.⁷⁵ These cells are capable of activating T lymphocytes and are necessary for developing autoimmunity in the eye.^{73,76} The origin of these cells still remains elusive. While our current study is in favor of activated residential microglia,^{77,78} it can also be infiltrating macrophages from the breakdown of the blood-retinal barrier.⁷⁵

One of the novel findings of our study is the discovery of $\gamma\delta$ T cells in the sub-RPE space during age-dependent RPE degeneration (Fig. 4). The retina and choroid do not have well-developed lymphatic drainage.^{79,80} Conventional clonal expansion of $\alpha\beta$ T cells in local lymph nodes may have only limited efficiency during adaptive immune response of the outer retina and choroid. The tissue-specific $\gamma\delta$ T cells can effectively recognize a wide range of antigens and respond quickly in a way similar to the innate immune cells. Upon pathogen infection, $\gamma\delta$ T cells produce a large amount of effector cytokines including IL-17.^{27–34} Similarly, when RPE cells degenerate, they may release either antigens or cytokines that can activate $\gamma\delta$ T cells. Future studies will be needed to further determine how the local microenvironment and cytokines produced by retinal cells control the differentiation of T cell lineages. In mice, IL-17⁺ $\gamma\delta$ T cells are restricted to the V γ 4 and V γ 6 subsets, and V γ 6 was confirmed in the *Nrf2* model (Fig. 6). It should be noted that the approach of staining RPE flat mounts gives an underestimate of infiltrating T cells, as the RPE pigments interfere with the fluorescence imaging. And our flow cytometry analyses used pooled samples and did not

distinguish between retinal and choroidal lymphocytes. Consistent with our findings, in laser-induced CNV, $\gamma\delta$ T cells were recruited to the site of laser lesion and were responsible for the production of IL-17.²²

Increased IL-17 production and upregulation of its receptor have been reported in macular tissue prepared from human AMD eyes.^{23,24} Interleukin-17 was cytotoxic to cultured RPE cells.^{24,81} A recent study by Ardeljan and colleagues²⁴ showed that overexpression of a soluble decoy receptor of IL-17 prevented retinal and RPE degeneration in *Ccl2* and *Cx3cr1* double-knockout mice with *Crb1*^{md8} mutation. The results from their preclinical study suggest that intervening IL-17-mediated signaling pathways can be a novel therapeutic approach to treat retinal and/or RPE diseases including AMD.

In summary, in the current study we have developed an improved model of age-dependent RPE degeneration with pathologic features similar to those of human AMD. We have identified the activation and infiltration of multiple lines of immunoreactive cells in the degenerating retina. Lymphocytes infiltrated in the sub-RPE space were mainly $\gamma\delta$ T cells, which produced IL-17 as a proinflammatory signal. Deciphering mechanisms bridging between innate and acquired immune responses elicited by RPE degeneration can reveal new insights into the pathobiology of AMD.

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