



# Diabetes-mediated IL-17A enhances retinal inflammation, oxidative stress, and vascular permeability

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## ABSTRACT

Diabetic retinopathy is a prevailing diabetes complication, and one of the leading causes of blindness worldwide. IL-17A is a cytokine involved in the onset of diabetic complications. In the current study, we examined the role of IL-17A in the development of retinal inflammation and long-term vascular pathology in diabetic mice. We found IL-17A expressing T cells and neutrophils in the retinal vasculature. Further, the IL-17A receptor was expressed on Muller glia, retinal endothelial cells, and photoreceptors. Finally, diabetes-mediated retinal inflammation, oxidative stress, and vascular leakage were all significantly lower in IL-17A<sup>-/-</sup> mice. These are all clinically meaningful abnormalities that characterize the onset of diabetic retinopathy.

## 1. Introduction

Approximately 500 million people will be diagnosed with diabetes within the next 20 years. Amongst these diabetics, more than 50% Type II and almost all Type I diabetics will develop diabetic retinopathy, which is the leading cause of blindness in the working-age population worldwide [1,2]. Reports indicate inflammatory processes play a key role in the onset of diabetic retinopathy [3]. Low-grade chronic inflammation elicits permeability across the blood-retinal barrier, which leads to vascular impairment and the onset of diabetic retinopathy. Murine models of diabetes have helped define the inflammatory events involved in the development of diabetic retinopathy [4], wherein retinal pathogenesis has been attributed to inflammation, immune cell leukostasis, and oxidative stress [3–6]. Further, recent studies have shown that intravitreal injections of IL-17A enhance blood-retinal breakdown, while the blockade of the IL-17RA portion of the IL-17A receptor decreased diabetes-mediated retinal pathogenesis [7,8]. In the current study, we expanded these studies by examining the role of IL-17A in the progression of diabetic retinopathy over an 8-month duration in streptozotocin (STZ)-induced C57BL/6 and IL17A<sup>-/-</sup> diabetic mice. By using mice that express green fluorescent protein (GFP) under the control of the gene encoding IL-17A, we identified IL-17A-

expressing T cells and neutrophils adhered to the vasculature of diabetic retinas. We extended these observations to show that Muller glia, photoreceptor cells, and retinal endothelial cells express the IL-17RC portion of the IL-17A heterodimer receptor. Finally, we determined that ablation of IL-17A production in IL-17A<sup>-/-</sup> diabetic mice impaired retinal inflammation, oxidative stress, and vascular leakage in the retina. These findings identify a pathologic role for IL-17A in the vasoregressive process of retinal inflammation and vascular permeability, which lead to the onset of diabetic retinopathy.

## 2. Materials and methods

### 2.1. Murine model of streptozotocin (STZ)-diabetes

CWRU IACUC and LSVAMC ACORP approved the animal protocols employed in this study. C57BL/6J, IL-17A-GFP, and IL-17A<sup>-/-</sup> mice were obtained from Jackson Laboratories. Diabetes was induced in 8–10 week old male mice by intraperitoneal injections of streptozotocin (60 mg/kg) on 5 consecutive days, as previously described [4]. Development of diabetes was defined by non-fasted blood glucose concentrations greater than 250 mg/dl and hyperglycemic hemoglobin A1c levels. Insulin (0–0.2 U) was administered as needed to maintain proper

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body weight.

## 2.2. Leukostasis of IL-17A-GFP retinal vasculature

Leukostasis was analyzed as previously described [4,5]. Saline was perfused into the aorta to clear non-adherent leukocytes, then 10 ml of Rhodamine labeled Concanavalin A lectin (1 mg/ml in PBS) was perfused to stain vasculature. Retina flat mounts were imaged by a fluorescent stereoscope and a Leica HyVolution confocal.

## 2.3. Flow cytometry

Retinas were digested using the Papain Dissociation System (Worthington), incubated for 2 h at 37 °C in collagenase (80 U/ml; Sigma-Aldrich), and cells collected. Six retinas of three IL17A-GFP mice were pooled for flow cytometry analysis of IL-17A. Cells were incubated with anti-mouse CD16/32 antibody (Fc block; eBioscience), and anti-mouse CD4 (T cells; GK1.5, eBioscience), anti-mouse Ly6G (neutrophils; NIMP-R14, Abcam), anti-mouse NK1.1 (NK cells; PK136, eBioscience), anti-mouse  $\gamma\delta$  TCR ( $\gamma\delta$  T cells; eBioGL3, eBioscience), or anti-mouse F4/80 (macrophages; 521204, R&D). Alternatively, cells were incubated with anti-mouse/human antibodies: Vimentin (Muller glia; EPR3776, Abcam), ROM-1 (Photoreceptor cone cells; Aviva), and IL-17RC (R&D). Cells were analyzed using a C6 Accuri flow cytometer (BD); gates were set to isotype controls, and compensated using FlowJo.

## 2.4. Muller glia cells

Viable Muller glia was obtained from the posterior section of human cadaver retinal globes (Eversight). Retinas were incubated 1 h in PBS containing 0.1% trypsin, mechanically disrupted, and incubated in DMEM/HAM F12 media at 37 °C with 5% CO<sub>2</sub>. Cell purity (> 95% GLAST<sup>+</sup>/Vimentin<sup>+</sup>) was confirmed by flow cytometry.

## 2.5. Murine retinal endothelial cells (mREC)

Murine retinal endothelial cells originally were a kind gift of Dr. Nader Sheibani and isolated from the retinas of C57BL/6 mice as previously described [9,10]. Retinas were digested in collagenase and VE-Cadherin positive cells were purified using magnetic beads coated with endothelial cell adhesion molecule-1 (> 99% purity).

## 2.6. Human retinal endothelial cells (hREC)

Primary human retinal endothelial cells of the microvasculature (hREC) were purchased from Cell Systems. For IL-17A stimulations, hREC were stimulated with 100 ng/ml of human recombinant IL-17A.

## 2.7. Photoreceptor cone cells (661 W)

Photoreceptor cone cells originated from a retinal tumor in transgenic mice that express Sv40T-antigen, and were characterized as previously described [11].

## 2.8. Quantitative PCR

RNA was extracted, cDNA generated, and qPCR was performed using SYBR green with *mActb* (NM\_001243262) and *mIL17RC* (NM\_178942) primers. Quantitative fold changes were calculated using CT scores, and qualitative images are PCR products run on a 2% agarose gel and visualized using ethidium bromide.

## 2.9. Quantification of reactive oxygen species (ROS)

Blood vessels were perfused, retinas were isolated and incubated in Krebs-HEPES buffer (with 5 mmol/L glucose) for 25 min at 37 °C in 5%

CO<sub>2</sub>. Luminescence was measured 5 min after the addition of 0.5 mmol/L lucigenin, as previously described [6]. Alternatively, 2',7'-dichlorodihydrofluorescein diacetate (CFDA), was used as an indicator for ROS by flow cytometry analysis as previously described [12]. Anti-IL-17RC was added 2 h prior to IL-17A stimulation.

## 2.10. Inflammation protein array

Protein lysates were pooled from 3 retinas, and analyzed using a Ray Biotech Mouse Inflammation Array C1 (AAM-INF-1) per manufacturer's directions.

## 2.11. ELISA analysis

Protein lysates were collected and pooled from 3 retinas, and analyzed using 2-site ELISA according to the manufacturer's directions (R&D Biosciences).

## 2.12. Vascular permeability in vitro assay

A homologous monolayer of retina endothelial cells was established on a transwell insert and incubated overnight with or without 100 ng/ml rIL-17A. Media was removed and a FITC-Dextran solution was added to the monolayer of cells for 20 min. The FITC-Dextran solution in the lower receiving well was collected and fluorescent intensity measured. The insert containing the monolayer of cells was stained with Cresyl violet permeabilization stain per manufacturer's instructions (ECM644, Millipore).

## 2.13. Vascular leakage in vivo

Retinal vascular leakage was quantified as previously described [4,13]. FITC-BSA (100  $\mu$ g/gram body weight) was injected intravenously, circulated for 20 mins, and quantified for plasma fluorescent intensity. Retinas were collected, fixed, embedded in OCT for 11  $\mu$ m retina cross-sections. Fluorescent intensity (FIU) of retinal cryosections was measured by computer assisted fluorescence microscopy (NIS-Elements), and then normalized to plasma.

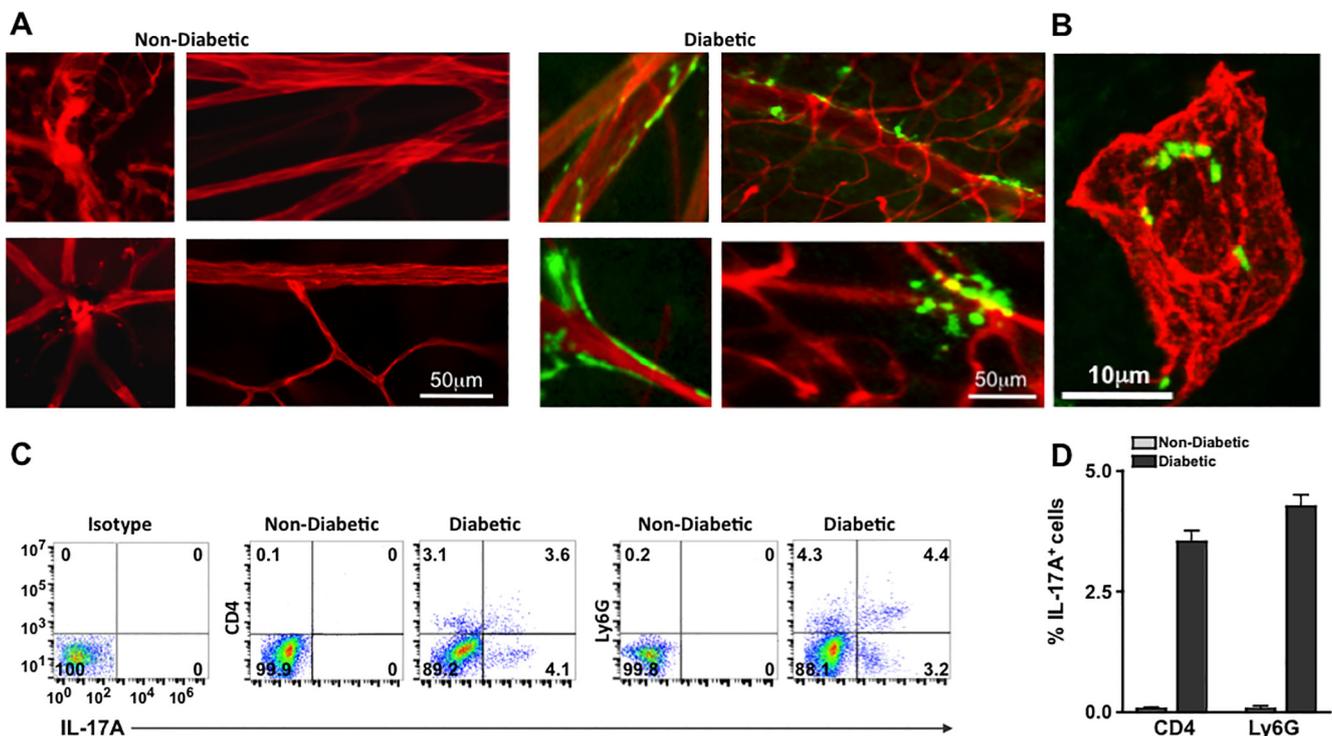
## 2.14. Statistical analysis

Statistical analysis was performed using a two-way ANOVA and an unpaired *t*-test with Tukey's post-hoc analysis (Prism); *p*-values < 0.05 are significant.

## 3. Results

### 3.1. IL-17A expressing cells in the retinal vasculature of diabetic mice

To detect cells that express IL-17A in the retinal vasculature, vessels were perfused, stained red with Rhodamine, and retina whole mounts were examined microscopically for the presence of IL17A-GFP cells. As shown in representative images, IL-17A/GFP<sup>+</sup> cells were adhered to the retinal vasculature of diabetic, but not non-diabetic mice (Fig. 1A, B). To identify these cells, retinas were collected, and incubated with anti-mouse CD4 (T helper cells), NK1.1 (NK cells),  $\gamma\delta$  TCR ( $\gamma\delta$  T cells), Ly6G (neutrophils), or F4/80 (macrophages) APC-conjugated antibody for flow cytometry analysis of all immune cells previously reported to produce IL-17A in other disease states. None of the IL-17A-GFP<sup>+</sup> cells were NK1.1,  $\gamma\delta$  TCR, or F4/80 positive (data not shown). However, 3.6% and 4.4% of total cells in the retina and retinal vasculature of diabetic mice were CD4<sup>+</sup>/IL17A-GFP<sup>+</sup> and Ly6G<sup>+</sup>/IL-17A-GFP<sup>+</sup> respectively, while no IL-17-GFP<sup>+</sup> cells were detected in non-diabetic mice (Fig. 1C). Similar results identifying Th17 cells and IL-17-producing neutrophils in diabetic retinas were observed in 3 separate samples (Fig. 1D).



**Fig. 1.** Diabetes-mediated IL-17A producing cells in the retinal vasculature. Fluorescent microscopy (A) and confocal (B) images of IL-17A-GFP<sup>+</sup> cells in retinas of non-diabetic and diabetic IL17A-GFP reporter mice following perfusion and vascular staining. Representative scatter plots (C) and flow cytometry quantification (D) of percent positive (of 10,000 events) IL-17A-GFP expressing CD4 and Ly6G cells in retinas of non-diabetic and diabetic mice. All data was collected 2-months after diabetes was confirmed, and is representative of 3 experiments.

### 3.2. Retina cells constitutively express IL-17A receptor

The receptors for IL-17A are comprised of IL-17RA and IL-17RC subunits [14,15]. IL-17RA is ubiquitously expressed on many different cell types, while expression of IL-17RC is much more limited [15]. Using flow cytometry analysis, IL-17RC was detected on photoreceptor cells (ROM-1<sup>+</sup>/IL-17RC<sup>+</sup>) and Muller glia (Vimentin<sup>+</sup>/IL-17RC<sup>+</sup>) in non-diabetic and diabetic mice (Fig. 2A). Although there was an increase in the number of IL-17RC<sup>+</sup> Muller glia in diabetic retinas, this increase was not significant (Fig. 2B). *IL-17RC* gene expression was also comparably detected in both non-diabetic and diabetic retinas (Fig. 2C). Further, IL-17RC protein was detected in non-diabetic retinas that slightly (non-significant) increased 2- and 8-months after diabetes was confirmed (Fig. 2D). Next, multiple retina cells lines were examined by flow cytometry to detect IL-17RC, wherein 50.9% human Muller glia, 61.8% murine retinal endothelial cells, 22.3% 661 W-photoreceptor cone cells, and 53.6% human retinal endothelial cells constitutively expressed IL-17RC (Fig. 2E, F). Although IL-17RC positivity did not significantly increase in any of these retina cells under hyperglycemic conditions (Fig. 2G), all of these results still provide evidence that photoreceptors, Muller glia, and retinal endothelial cells constitutively express IL-17RC.

### 3.3. Systemic ablation of IL-17A decreases retinal inflammation and oxidative stress

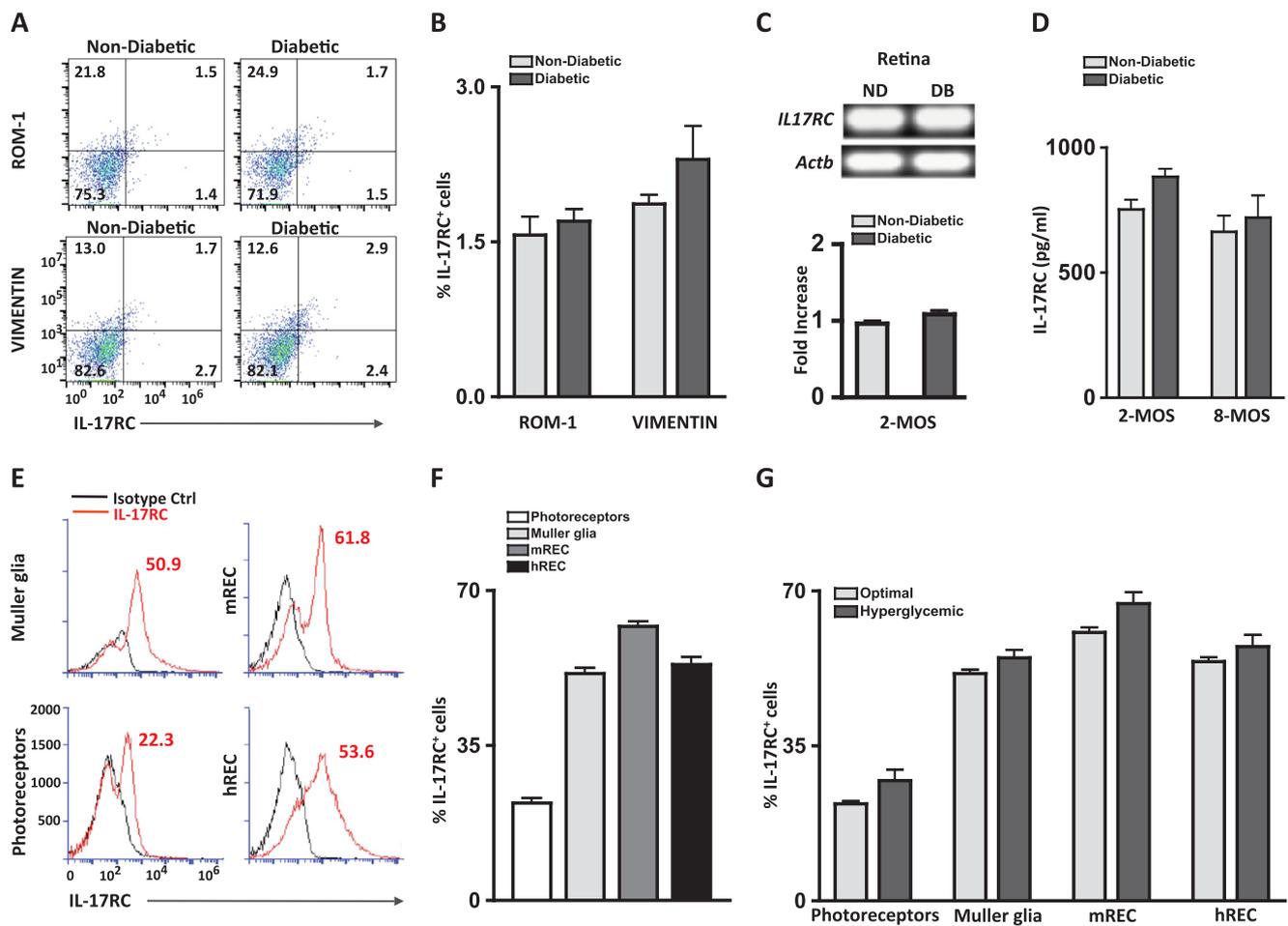
To ascertain the role of IL-17A in retinal oxidative stress during diabetes, reactive oxygen species (ROS) was quantified 2-months after diabetic conditions were confirmed in C57BL/6 and IL17A<sup>-/-</sup> mice. ROS was significantly increased in the retinas of diabetic compared with non-diabetic mice, which was significantly lowered in the diabetic IL-17A<sup>-/-</sup> mice (Fig. 3A). Previously it was determined that ROS is produced by photoreceptors in diabetic retinas [6]. To determine if IL-17A induces ROS production in photoreceptor cells, 661 W

photoreceptor cells were stimulated with 100 ng/ml of recombinant (r) IL17A for 1 h, and ROS-CFDA was measured by flow cytometry. ROS levels were significantly higher in the IL-17A stimulated than the unstimulated photoreceptor cells (Fig. 3B). Also, photoreceptor cells treated with anti-IL-17RC prior to IL-17A stimulation produced ROS levels similar to that of unstimulated cells (Fig. 3C), indicating that IL-17A-IL-17RC signaling can induce photoreceptor cells to produce ROS.

To further evaluate the effect of IL-17A on diabetes induced inflammation, protein lysates from the retinas of wild type C57BL/6 and IL17A<sup>-/-</sup> mice were collected 2-months after diabetic conditions were confirmed, and inflammatory proteins were evaluated using protein arrays. Results were quantified by densitometry, statistically analyzed (3 separate arrays), and displayed as fluorescent intensity (FIU) quantifications (Fig. 3D). Twenty-three inflammatory proteins were enhanced by diabetic conditions, while systemic deletion of IL-17A significantly decreased 9 inflammatory proteins and increased TIMP-1 (Fig. 3D). Further, per ELISA analysis chemokines (CCL2, CCL5, CX3CL1, CXCL1, and CXCL5), pro-inflammatory cytokines (TNF and IL-1), and growth factors (G-CSF and GM-CSF) were all significantly decreased, while MMP-9 inhibitor (TIMP-1) was significantly increased in the retinas of diabetic IL-17A<sup>-/-</sup> than diabetic wild type C57BL/6 mice (Fig. 3E). Taken together, these results signify a role for IL-17A in diabetes-mediated retinal inflammation and oxidative stress.

### 3.4. IL-17A enhances retinal vascular permeability

To further establish the independent role of IL-17A in vascular permeability, a homologous monolayer of murine (mREC) or human (hREC) retinal endothelial cells were established, and incubated for 18 h with rIL-17A. To quantify permeability, a FITC-Dextran solution was added to the top of the monolayer and relative fluorescent intensity was measured at the bottom of the monolayer. There was a significant increase in relative permeability in IL-17A stimulated mREC and hREC (Fig. 4A, B). Microscopy of stained mREC and hREC monolayers



**Fig. 2.** Neural and vascular retina cells express the IL-17A receptor. A-B) Flow cytometry analysis of IL17RC<sup>+</sup> photoreceptor cells (ROM-1<sup>+</sup>) and Muller glia (Vimentin<sup>+</sup>) in the retinas of C57BL/6 non-diabetic and diabetic mice (n = 6 retinas), numbers in quadrants indicate percent positive cells of 10,000 events (A), and graph quantifies the results of 3 separate experiments. Quantitative and qualitative qPCR (C), and ELISA (D) analysis of IL-17A receptor subunit IL-17RC in mRNA and protein lysates of retinas from non-diabetic and diabetic mice, 2- and 8- months after diabetic conditions were confirmed. *Actb* was used as a loading control. Representative flow cytometry (E) and quantification (F) of IL-17RC positivity (red) in human Muller glia, murine retinal endothelial cells (mREC), 661 W photoreceptor cells, and human retinal endothelial cells (hREC) that were gated to an isotype control (black). Numbers above overlays indicate percent positive cells. G) Flow cytometry quantification of IL-17RC<sup>+</sup> photoreceptors, Muller glia, mREC, and hREC cultured in normal (optimal) or high glucose (hyperglycemic) media for 24 h. Graph is percent positive cells of 30,000 events. Data are representative of three separate experiments with similar results.

corroborate these findings (permeable areas are circled in Fig. 4C). Collectively, these findings indicate that IL-17A is sufficient to induce vascular permeability in both human and murine retinal endothelial cells.

### 3.5. Ablation of IL-17A in vivo results in decreased retinal vascular leakage in diabetic mice

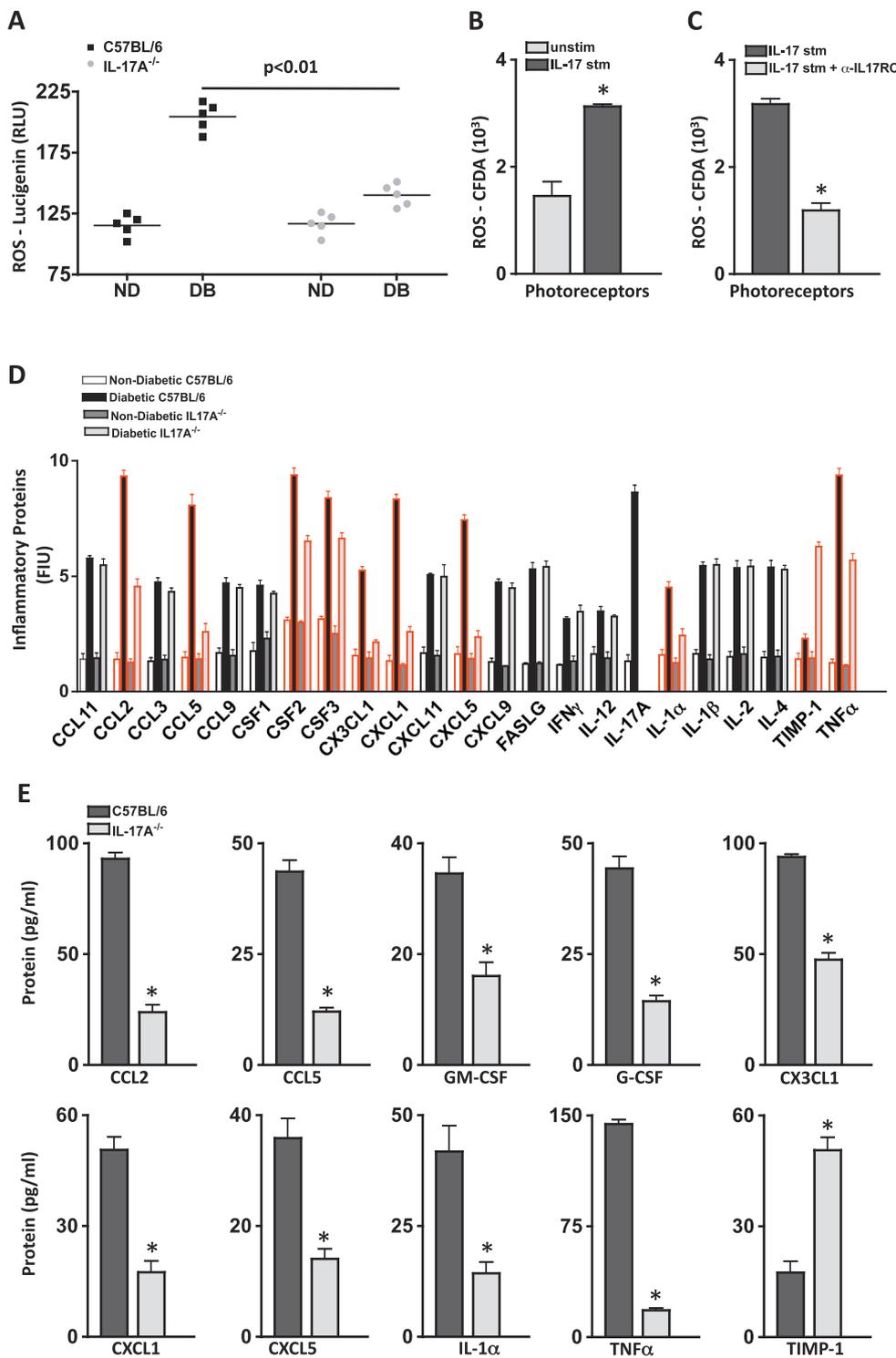
Diabetes-mediated chronic inflammation leads to vascular permeability and leakage in the retina [3]. To ascertain the role of IL-17A in the development and maintenance of chronic vascular leakage in the retina during diabetes, leakage was quantified 8-months after diabetic conditions were confirmed, by measuring intravenously injected FITC-BSA in C57BL/6 and IL17A<sup>-/-</sup> mice. Vascular leakage is indicated by a diffuse FITC-hyper-fluorescence in the inner nuclear layer (INL), outer plexiform layer (OPL), and the outer nuclear layer (ONL) of the retina, as shown in representative images (circled in Fig. 4D). Quantification of vascular leakage, using fluorescent intensity units (FIU), was significantly increased in all retinal layers of diabetic compared with non-diabetic mice, which was significantly lowered in the diabetic IL-17A<sup>-/-</sup> mice (Fig. 4E-G). While, the FIU in the retinal INL of diabetic IL17A<sup>-/-</sup> mice was similar to that of non-diabetic mice (Fig. 4G), indicating that diabetes-mediated IL-17A is required for vascular leakage

to occur in the inner nuclear layer of the retina.

## 4. Discussion

Several notable observations emerged in these studies. First this study provided evidence that diabetes mediates IL-17A production by Th17 cells and neutrophils, which is one of the first studies that have identified a role for IL-17-producing neutrophils in diabetes. Further, it was discovered that neural and vascular retinal cells constitutively express the IL-17RC subunit of the IL-17A receptor, with this being the first study to identify this receptor on photoreceptor cells. Both of these novel cellular discoveries further our understanding of retinal pathogenesis, and provide evidence that transcellular communication occurs between the IL-17A-producing immune cells and the IL-17-receptor expressing retina cells during the onset of diabetic retinopathy. Through IL-17A signaling, retinal inflammation and oxidative stress are enhanced, which has been previously shown to induce vascular impairment and vision loss [3,16–20].

Previous studies have demonstrated a role for IL-17A in retinal pathogenesis in 3-month diabetic mice. Diabetic conditions induced Muller glia to produce IL-17A, and enhanced the expression of the IL-17RA subunit of the IL-17A receptor on Muller glia. Neuronal apoptosis and vascular leukostasis was enhanced, while the early onset of

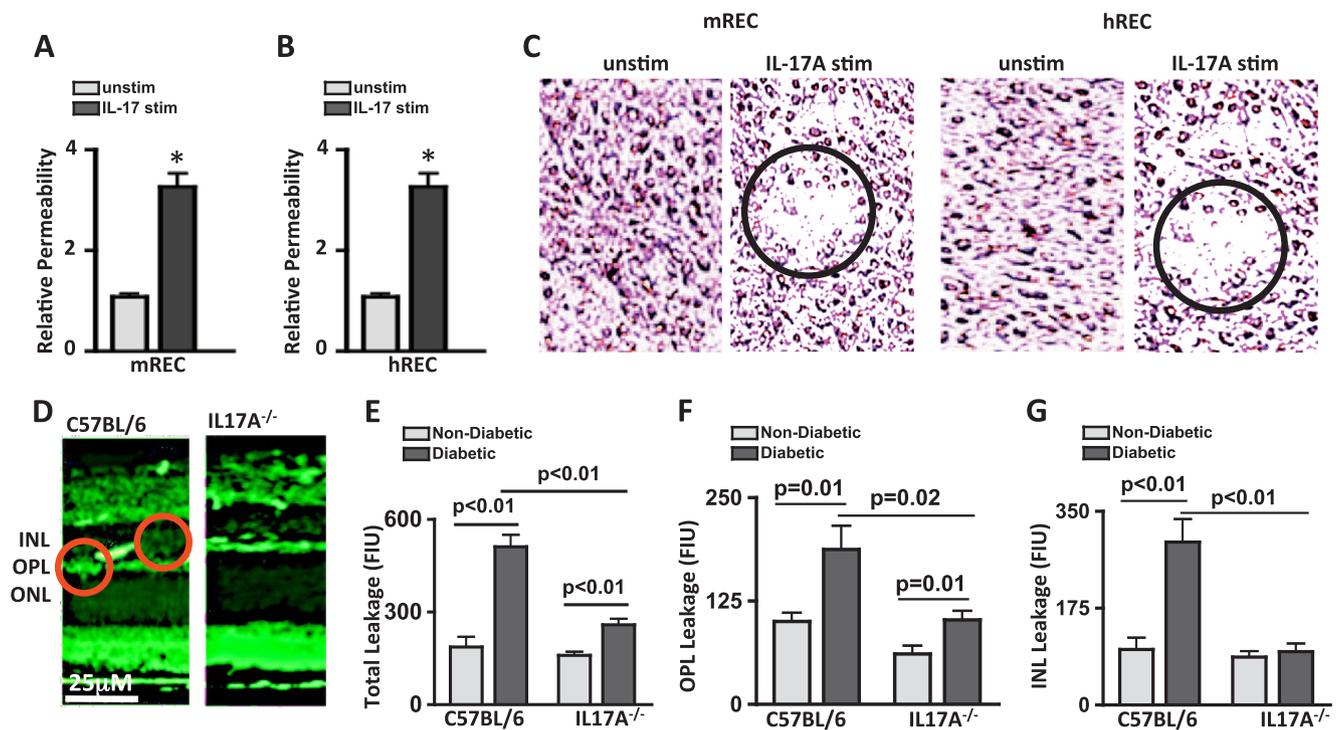


**Fig. 3.** Oxidative stress and retinal inflammation are significantly decreased in diabetic IL-17A<sup>-/-</sup> mice. **A)** Quantification of reactive oxygen species (ROS) in the retinas of non-diabetic (ND) and diabetic (DB) C57BL/6 (black squares) and IL-17A<sup>-/-</sup> (grey circles) mice; each data point represents an individual retina. Quantification of extracellular ROS of unstimulated (grey) and IL-17A stimulated (black) 661 W photoreceptor cells (**B**), or IL-17A stimulated 661 W photoreceptor cells (black) compared to cells treated with anti-IL17RC (grey) prior to IL-17A stimulation (**C**). ROS was analyzed 2-months after diabetic conditions were confirmed or 1 h after stimulation. **D)** Array analysis comparing the fluorescent intensity (FIU) of inflammatory proteins in retinas (n = 3/group) of diabetic C57BL/6 (black) and IL17A<sup>-/-</sup> (grey) mice. Diabetic values were normalized to non-diabetic C57BL/6 (white), diabetic C57BL/6 (black), non-diabetic IL-17A<sup>-/-</sup> (dark grey), and diabetic IL-17A<sup>-/-</sup> (light grey) mice 2-months after diabetic conditions were confirmed. Red outlined bars highlight the inflammatory proteins that were affected by IL-17A ablation. **E)** ELISA of inflammatory proteins detected in retinas (n = 3) of diabetic C57BL/6 and IL-17A<sup>-/-</sup> mice. \* = p < 0.01 per unpaired student's t-test.

vascular permeability was induced through an autocrine signaling cascade in Muller glia [7,8]. In this study we identified Th17 cells and neutrophils as a source of IL-17A in the diabetic retina. Additionally, we provided evidence that IL-17A enhances retinal inflammation and oxidative stress, which has been previously shown to lead to capillary non-perfusion, the onset of proliferative diabetic retinopathy, and vision loss [3,13,18,19]. Finally, we extended previous studies to determine that IL-17A plays a significant role in retinal vascular leakage. Since these studies demonstrate that diabetes-mediated IL-17A is sufficient to induce retinal vascular impairment, it is also possible that IL-17A plays a role in systemic vascular impairment in other diabetic complications

too.

Collectively, we have identified two new sources of diabetes-mediated IL-17A, identified multiple retina cells that constitutively express the IL-17 receptor, and have identified a role for diabetes-mediated IL-17A in retinal inflammation, oxidative stress, and vascular leakage. Since, all of these retinal pathologies are hallmarks for the onset of diabetic retinopathy, these findings may provide new therapeutic targets for diabetes mediated vision loss.



**Fig. 4.** Diabetes-mediated IL-17A enhances vascular permeability and leakage. Fluorescent quantification of permeability in unstimulated (grey) and IL-17A stimulated (black) mREC (A) and hREC (B); \* =  $p < 0.01$ . C) Representative images of permeability stained unstimulated and IL-17A stimulated mREC (right) and hREC (left) 18 h after stimulation. D) Representative fluorescent microscopy of FITC<sup>+</sup> vascular leakage in retina cross-sections of inner nuclear layer (INL), outer plexiform layer (OPL), and outer nuclear layer (ONL) from diabetic C57BL/6 and IL-17A<sup>-/-</sup> mice. Quantified vascular leakage in: total retina (E), OPL (F), and INL (G) of retinas from non-diabetic and diabetic C57BL/6 and IL17A<sup>-/-</sup> mice; 8 months after diabetic conditions were confirmed. p-values were calculated using a two-way ANOVA and an unpaired t-test with Tukey's post-hoc analysis. Data are representative of 2 experiments.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cellimm.2019.04.009>.

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