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# Blockade of Tumor Necrosis Factor Alpha Prevents Complications of Diabetic Retinopathy

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## Abstract

Tumor necrosis factor alpha (TNFα) plays an important role in the pathogenesis of diabetic retinopathy (DR). The objective of this study is to investigate the effect of TNFα blockade on complications of DR. Experimental models of diabetes were induced with streptozotocin (STZ) injection or insulin 2 gene point mutation (Akita) in mice. Intravitreal (IVT) and intraperitoneal (IP) injections were used to deliver anti-TNFα antibody and saline control. TUNEL and activated caspase-3 staining were used to examine apoptotic cell death. Transcardially-perfused FITC-ConA and fluorescence microscopy were used to monitor leukocyte adhesion. Trypsin digestion was used to prepare retinal vasculature and quantify acellular capillary. The leakage of $^{3}$H-mannitol into the retina was used to quantify the breakdown of blood-retinal barrier (BRB). TNFα blockade significantly prevented diabetes-related retinal leukostasis. The numbers of caspase 3-positive and TUNEL-positive cells were significantly increased in diabetic retina, but reduced due to anti-TNFα treatment. The increased acellular capillary by diabetes was significantly prevented by anti-TNFα treatment. Diabetes-caused BRB breakdown was prevented by antibody treatment at 3 and 6 months. IVT and IP routes of antibody delivery had similar efficacy and dose response curve. Among the examined dose ranges (1-10 μg/eye for IVT injection and 2-25 mg/kg for IP injection), the antibody inhibited complications of DR in a dose-dependent manner. These results suggest that anti-TNFα therapy is a potential therapeutic treatment for DR.

**Keywords:** Blood-etinal barrier; Diabetic retinopathy; Inflammation; Tumor necrosis factor-alpha

## Abbreviation

Akita: Insulin 2$^{Akita/+}$; BRB: Blood-Retinal Barrier; DME: Diabetic Macular Edema); DR: Diabetic Retinopathy; KO: Knockout; IVT: Intravitreal; IP: Intraperitoneal; PDR: Proliferative Diabetic Retinopathy; STZ: Streptozotocin; TNFα: Tumor Necrosis Factor-Alpha; TUNEL: Terminal dUTP Nick-end Labeling.

## Introduction

Diabetic retinopathy (DR) is a leading cause of visual loss in the working age population with with approximately 93 million people suffering from DR worldwide [1]. Diabetic macular edema (DME) and retinal neovascularization (NV), resulting from blood-retinal barrier (BRB) breakdown and hypoxia, are the two major causes of blindness in patients with DR. Inflammation has been shown an important contributor to these complications of DR [2-5]. The common features of inflammation in DR include leukocyte adhesion and infiltration, microglia activation, and cytokines/chemokines expression. Targeting these inflammatory elements represents an attractive therapeutic strategy for the prevention and treatment of DME and proliferative DR (PDR).

TNFα is a potent pro-inflammatory cytokine and implicated in the pathogenesis of DR. TNFα protein level is increased in diabetic rats relative to non-diabetic controls [6,7]. In PDR, TNFα protein is presented in the fibrovascular membranes of PDR [8] and increased in the vitreous fluid and plasma of patients with DR [9,10]. TNFα deficiency in mice attenuates diabetes-related retinal leukostasis, retinal cell apoptosis and breakdown of BRB [11]. TNFα inhibition by compounds inhibits the activity of caspase-3/-8, acellular capillary and vascular permeability in the rat models of diabetes [12,13]. TNFα is associated with insulin resistance [14,15] and its polymorphism is associated with type 2 diabetic patients [12,16].

Anti-TNFα therapy provides benefits to a variety of inflammatory disorders. For example, several TNFα inhibitors, including infliximab, etanercept, adalimumab, golimumab and certolizumab-pegol, have been developed and approved for the treatment of autoimmune disorders such as rheumatoid arthritis, inflammatory bowel disease, and psoriasis. However, whether TNF inhibitors can effectively inhibit complications of DR is unclear. In the present study, we evaluated the effect of an anti-murine TNFα antibody called CNTO5048 on complications of DR and hoped to provide insight into the potential application of this strategy to prevention and treatment DR and DEM.

## Methods

### Mouse models of diabetes

All animals were used in accordance with the approved protocols by the Institutional Animal Care and Use Committee of The Johns

Hopkins University School of Medicine and the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Two mouse models of diabetes were used: streptozotocin (STZ) treatment and insulin2 gene point mutation, both of which were described in our previous reports [11].

STZ is an alkylating antibiotic and is highly toxic to insulin-producing pancreatic β-cells. It has long been used to generate experimental models of diabetes. The $Ins2^{Akita/+}$ (Akita) mice are heterozygous for the insulin 2 gene with a transition from cysteine to tyrosine at the seventh amino acid of the protein. This mutation leads to the disruption of the disulfide bond between chains A and B of insulin, the intracellular accumulation of misfolded insulin protein, and consequently the death of pancreatic β-cells. Both models were used in our study because each model has its own advantages and disadvantages. The mutant Akita mice serve as an excellent animal model of diabetes with the phenotypes of hypoinsulinemia and hyperglycemia, with symptoms similar to those observed in diabetic patients. Retinal complications have been well characterized in these diabetic mice. The STZ-induced mouse is a more acute diabetic mouse model because STZ is toxic to many other cells, as well as to pancreatic β-cells. For this reason, Akita diabetic mice are thusly considered to be more relevant to diabetic patients than the STZ-induced diabetic mice. The Akita mouse strain is maintained by the breeding of a male Akita diabetic mouse and a non-diabetic C57BL6 female mouse; only half of the offspring become diabetic. Furthermore, only the diabetic male mice are suitable for experimentation because the hyperglycemia status of the female diabetic mice is not as consistent as in the male diabetic mice. So the method of STZ induction is more efficient at obtaining a large number of diabetic mice and non-diabetic controls than the Akita.

## Anti-TNFα treatment cohorts

For the STZ-induced diabetic mouse model, blood glucose levels were measured at one week after STZ injection. For the Akita model, glucose levels were measured at the age of one month. The blood glucose of over 250 mg/dL was considered diabetes. Prior to analysis (3 or 6 months), blood glucose was measured again. Diabetic and non-diabetic mice were separated and randomized for the treatment of anti-TNFα antibody CNTO5048, a rat-mouse chimeric monoclonal antibody that neutralizes murine TNFα [17]. Doses of 2, 8, and 25 mg /kg mouse body weight were used for IP injection, and 1, 5 and 10 μg antibody/eye for IVT injection. These dose ranges were chosen based on the antibody's clinical applications. The measurements were performed at 3 or 6 months, which were the time frames used in our previous mouse knockout (KO) study [11]. The treatment cohorts were described below and illustrated in Figure 1.

**Cohort 1-retinal leukostasis:** 20 STZ-induced diabetic C57BL6 mice were IVT injected with PBS, 1, 5, or 10μg antibody biweekly for 3 months. 5 age-matched non-diabetic mice were used as control.

**Cohort 2-apoptosis:** (A) 20 STZ-induced diabetic mice were IVT injected with PBS, 1, 5, or 10 μg antibody biweekly for 3 months. 5 age-matched non-diabetic mice were used as control. (B) 20 STZ-induced diabetic C57BL6 mice were IP injected with PBS, 2, 8, or 25 mg/kg weekly for 3 months. 5 age-matched non-diabetic mice were used as control.

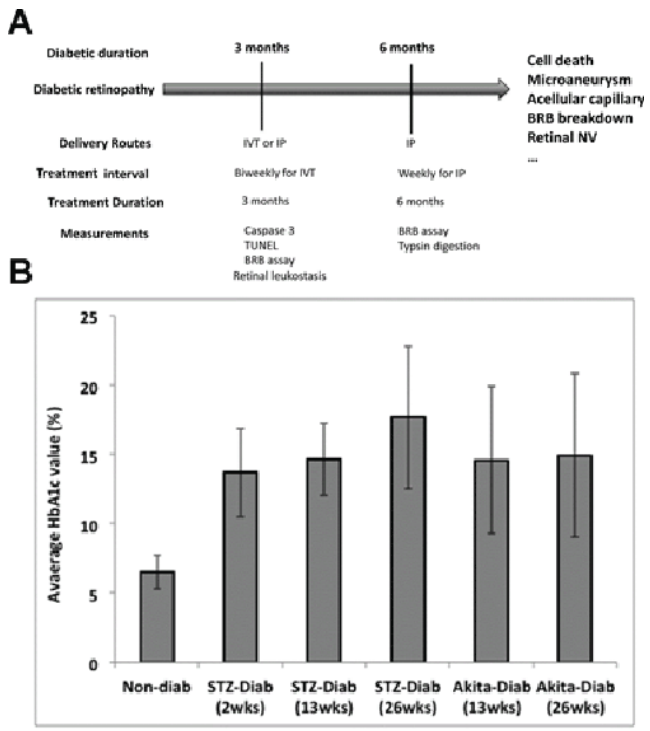



**Figure 1:** Treatment scheme and blood glucose. Separate cohorts of mice were used to evaluate the effect of anti-TNFα antibody on the complications of DR. (A) The line indicated the development of DR. Diabetes duration, delivery routes, measurements were shown. IVT: Intravitreal. IP: Intrapertonal. (B) Average HbA1c value of the mice that were used for assays. Blood samples used to test blood glucose concentrations were always collected in the afternoon between 2-4pm. The level of average glucose (AG, mg/gL) was converted into HbA1c value (%) using the equation HbA1c (%)=[AG(mg/dl)+77.3]/35.6, as suggested at http://www.ngsp.org/A1ceAG.asp. The results were expressed as mean ± SD (n=10).

**Cohort 3-acellular capillary:** 20 STZ-induced diabetic C57BL6 mice were IP injected with PBS, 2, 8, or 25 mg/kg weekly for 6 months. 5 age-matched non-diabetic control mice were used as control.

**Cohort 4- BRB assay:** (A) 20 Akita diabetic C57BL6 mice were IVT injected with PBS, 1, 5 or 10 μg antibody/eye biweekly for 3 months. 5 age-matched non-diabetic control mice were used as control. (B) 20 STZ-induced diabetic C57BL6 mice were IP injected with PBS, 2, 8 or 25 mg/kg for 3 months. 5 age-matched non-diabetic mice were used as control. (C) 20 STZ-induced diabetic mice were IP injected with PBS, 2, 8 or 25 mg/kg mouse body weight weekly for 6 months. 5 age-matched non-diabetic mice were used as control.

## Intravitreal (IVT) injections

IVT injections were performed with a Harvard pump microinjection apparatus and pulled glass micropipettes. Each micropipette was calibrated to deliver 1 μl of solution upon depression of a foot switch. Mice were anesthetized with 25 mg/kg of ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 4 mg/kg xylazine (Vedco, St. Joseph, MO). Pupils were dilated with 1% tropicamide.

Under a dissecting microscope, the sharpened tip of a micropipette was passed through the sclera just behind the limbus into the vitreous cavity, and the foot switch was depressed.

### Retinal leukostasis

Mice were first anesthetized as described above, then the descending aorta was clamped, and the right atrium was cut. The mice were perfused with 5 mL PBS to remove erythrocytes but not adherent leukocytes, followed by perfusion with fluorescein-conjugated con A to label adherent leukocytes. Another PBS perfusion was used to flush out unbound fluorescein. Retinal flat mounts were prepared to assess leukostasis. The eyes were removed and fixed for more than 1 hour with phosphate-buffered formalin. The cornea and lens were removed and, under a stereomicroscope (Stemi 2000C; Carl Zeiss Meditec, Inc., Thornwood, NY), the entire retina was carefully dissected from the eye cup, rapidly cut from the edge to the equator in all four quadrants, and flat-mounted with the photoreceptors facing upward. Leukocytes adherent to the vessel walls were labeled with fluorescein, and leukocytes within the vessels of each retina were counted under an epifluorescence microscope (Axio- pan2; Carl Zeiss Meditec, Inc.) by an investigator masked to the nature of the specimen. The counting began at the optic disc. The vessel nearest the 12 o'clock position and its branches were followed all the way to the periphery, with the focus changed as necessary to include all of the arteries, veins, and capillaries in the field. This process was repeated in a clockwise direction for each vessel radiating from the optic disc, so the total number of adherent leukocytes in all of the vessels of the retina was counted.

### TUNEL assay for detection of apoptotic nuclei

The terminal dUTP nick-end labeling (TUNEL) assay was performed with the ApopTag Red In Situ Apoptosis Detection Kit (Millipore, Temecula, CA) according to the manufacturer's instructions. In brief, eye cryosections were fixed in 1% paraformaldehyde (PFA) at room temperature (RT) for 10 min and in ethanol/acetic acid (2:1) at −20°C for 5 min and then washed twice with 1x PBS (pH 7.4) for 5 min. After the tailing of digoxigenin-dNTP catalyzed by the TdT enzyme, the sections were incubated with the anti-digoxigenin-rhodamine antibody at RT for 30 min. For negative controls, deionized water was substituted for the TdT enzyme. Processed sections were mounted with antifade mounting medium for fluorescence-containing DAPI (Vectashield; Vector, Burlingame, CA) and were viewed with a fluorescence microscope (Axiopan2; Carl Zeiss Meditec, Inc).

### Activated caspase-3 staining

Cryosections of eyes were fixed in ice-cold methanol/acetone (1:1) for 10 minutes at −20°C, washed with 0.01 M PBS (pH 7.4), and blocked with 4% normal goat serum for 90 minutes. They were incubated overnight at 4°C with polyclonal antibodies against activated caspase-3 (1:200; Cell Signaling Technology, Boston, MA). Negative control sections were similarly treated, but the normal IgG was added. Sections were rinsed and incubated for 1 hour with Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:1000; Invitrogen, Carlsbad, CA). Fluorescence microphotography was performed on the epifluorescence microscope (Axiopan2; Carl Zeiss Meditec, Inc.). Each section was scanned systematically from the temporal to the nasal side for fluorescent cells indicative of cells undergoing apoptosis, by an investigator masked to the nature of the specimens. The number and locations of positive cells were counted and photographed.

### Preparation of retinal vasculature and quantification of acellular capillary

Isolation of retinal vasculature and quantification of acellular capillaries were performed as previously described [18,19] with minor modifications. Briefly, the enucleated eyes were fixed with 2% PFA for 24 h. After removing the cornea, the eye-cups were fixed with 2% PFA for 24 h and dissected to isolate the retina. The retinas were rinsed by tap water overnight and then were incubated with 40 unit/ml elastase (Calbiochem, MA) in 100 mM sodium phosphate buffer with 150 mM sodium chloride and 5.0 mM EDTA, pH 6.5 at 37°C for 2 h. Retinas were transferred to a 100 mM Tris-HCl (8.5) solution at RT for 3 h. Nonvascular tissues were removed by gentle brushing, and the isolated vasculature was mounted on the slide. After drying overnight, the retinal blood vessels were subjected to the periodic acid–Schiff and hematoxylin stain. To quantify the acellular capillaries, the retinal blood vessels were analyzed for 20–25 random fields in masked fashion (200x magnification). Acellular capillaries were identified as capillary-sized vessel tubes without nuclei along their length. The numbers were normalized by the counting area (degenerated vessels/mm$^2$).

### The quantitative BRB assay

The quantitative BRB assay was performed according to a previously described technique [11,20] with some modifications. Mice were sedated as above and given an IP injection of 1μCi/gram body weight of 3H-mannitol. One hour after injection, the mice were sedated and retinas from the experimental and control eyes were rapidly removed. The posterior portion of the globe was firmly grasped with forceps and a razor blade was used to cut across the cornea and extrude the lens, vitreous, and retina. Retinas were dissected free from the lens, vitreous, and any RPE that was extruded, and were placed within pre-weighed scintillation vials within 30 seconds of sacrifice. The thoracic cavity was opened and the left superior lobe of the lung was removed, blotted free of excess blood and placed in another pre-weighed scintillation vial. A left dorsal incision was made and the retroperitoneal space was entered without entering the peritoneal cavity. The renal vessels were clamped with forceps and the left kidney was removed, cleaned of fat, blotted, and placed into a pre-weighed scintillation vial. Superficial liquid was allowed to evaporate over 20 min from the open vials. The vials containing the tissue were weighed and tissue weights were calculated and recorded. One ml of NCSII solubilizing solution was added to each vial and the vials were incubated overnight in a 50°C water bath. Solubilized tissue was brought to RT and decolorized with 20% benzoyl peroxide in toluene in a 50°C water bath. The vials were brought to RT and 5 ml of Cytoscint ES and 30 μl of glacial acetic acid were added. The vials were stored for several hours in darkness at 4°C to eliminate chemoluminescence. Radioactivity was counted with a LS 6500 Liquid Scintillation Counter (Beckman, Brea, CA). The CPM/mg tissue was measured for the lung, kidney, and experimental and control retinas. Retina/lung and retina/kidney ratios were calculated and compared.

### Statistical analysis

Statistical comparisons were made using analysis of variance (ANOVA) or a linear mixed model. *P*-values for comparison of treatments were adjusted for multiple comparisons by the Dunnett method. For data sets with two groups, statistical analyses were performed with the Mann-Whitney test. $P<0.05$ was designated as being statistically significant.

## Results

### Anti-TNFα treatment prevents retinal leukostasis in DR

In order to investigate whether anti-TNFα treatment prevents retinal leukostasis, the STZ-induced diabetic mice were IVT injected anti-TNFα antibody or PBS weekly. At 3 months, the mice were transcardially perfused with FITC-ConA to stain the static leukocytes in the retinal vasculatures; the stained leucocytes were visualized and counted under fluorescence microscopy. Consistence with our previous observations, diabetes caused an increase of adherent leukocyte in the retinal vasculatures. The number of leukocytes was significantly lower in the anti-TNFα treatment groups than in the PBS control group. Quantification showed the treatment inhibited retinal leukostasis in a dose-dependent manner (Figure 2).

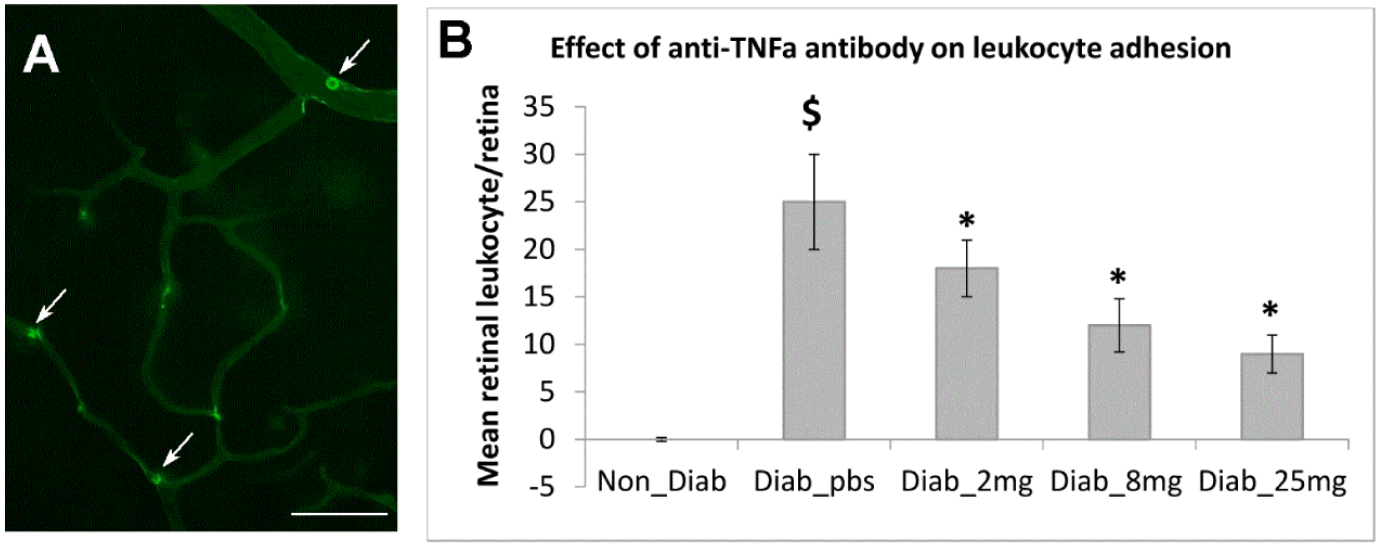



**Figure 2:** Prevention of retinal leukostasis by Anti-TNFα treatment. The STZ-induced diabetic mice were IVT injected antibody biweekly for 3 months. (A) The representative image showed leukocyte adhesion. (B) The quantitative results were expressed as mean ± SD (n=10). $ $p<0.05$ compared to non-diabetes. *$p<0.05$ compared to diabetes with PBS treatment. Scale bar: 100 μm.

### Anti-TNFα treatment prevents apoptotic cell death in DR

The STZ-induced diabetic mice were IVT or IP injected with anti-TNFα antibody or PBS weekly. At 3 months, the effect on the apoptotic cell death was evaluated by caspase-3 or TUNEL staining. Both caspase-3 (+) and TUNEL (+) cells were increased in the diabetic mice compared to the non-diabetic mice. The number of the caspase-3 (+) or TUNNEL (+) cells were significantly decreased in the antibody treatment groups compared to the PBS control group. The inhibition potency was enhanced with the increased dose of antibody. Both routes of delivery showed similar response curves (Figures 3 and 4).

### Anti-TNFα treatment prevents acellular capillary in DR

As aclleular capillary is one of the most important characteristics of DR, we investigated whether anti-TNFα antibody is effective at preventing this complication. The STZ-induced diabetic mice were IP injected with anti-TNFα antibody or PBS weekly. At 6 months, the eyes were subjected trypsin digestion; and the isolated retinal vasculatures were used for the quantification of aclleular capillary. Consistent with the previous reports, diabetes caused a significant increase of acellular capillary. Interestingly, the number of acellular capillary was largely reduced in the treatment groups compared to PBS group (Figure 5).

### Anti-TNFα treatment prevents BRB breakdown in DR

The effect of anti-TNFα treatment on BRB breakdown was examined with both STZ-induced and Akita diabetic mice. IV injection was made to the Akita mice biweekly; IP injections to the STZ-induced diabetic mice weekly. At 3 or 6 months, the mice were subjected the quantitative BRB assay, as described in Methods. Diabetes caused a significant BRB breakdown compared to non-diabetic controls. Anti-TNFα treatment consistently prevented BRB breakdown in either STZ-induced or Akita diabetic mice at 3 and 6 months (Figures 6-8).

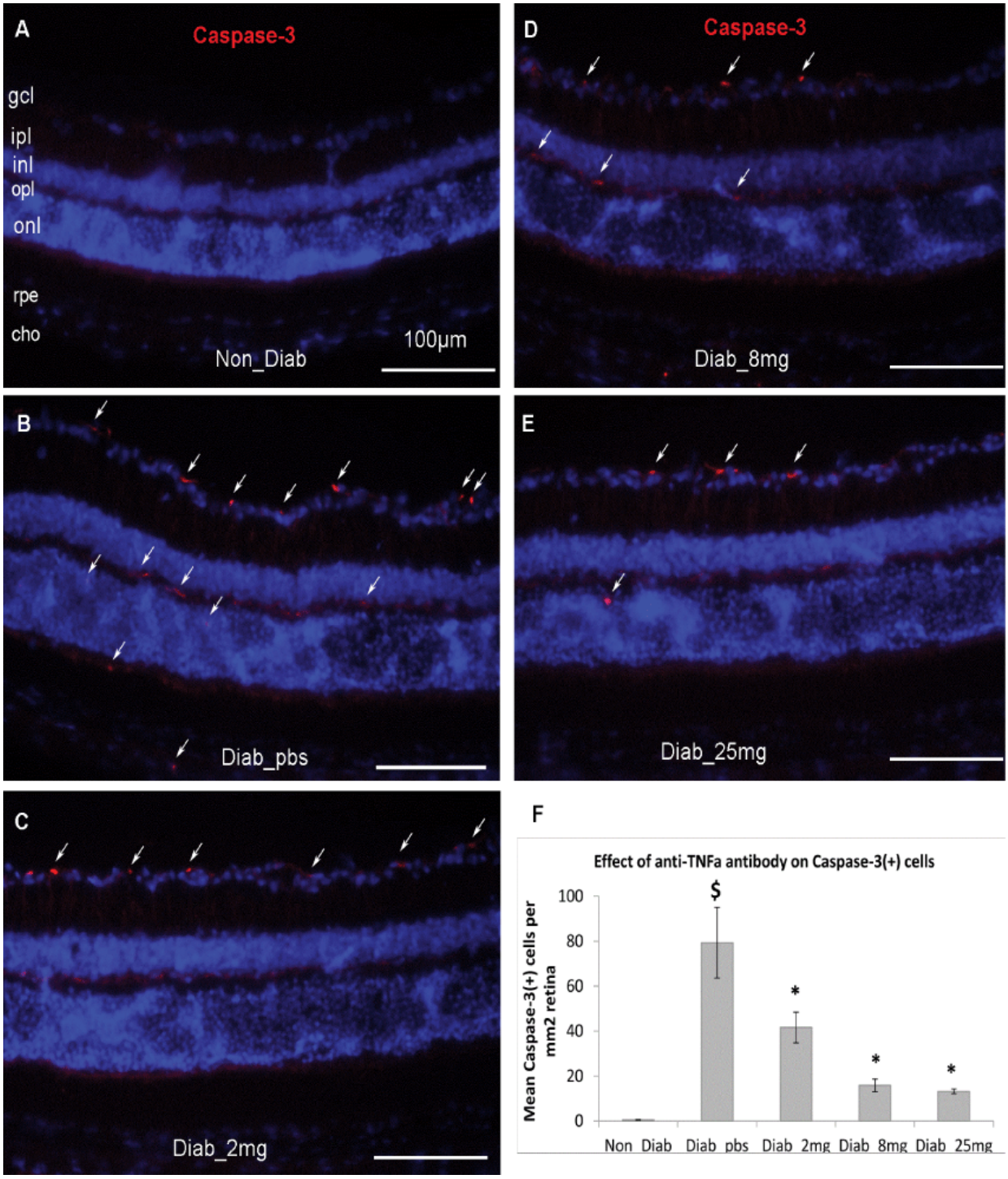



**Figure 3:** Prevention of caspase-3 (+) cells by anti-TNFα treatment. The STZ-induced diabetic mice were IVT injected antibody biweekly for 3 months. (A-E) The sample images of activated caspase-3 staining for non-diabetic (A), diabetic with the treatments of PBS (B), 1 μg (C), 5 μg (D) and 10 μg (E). (F) Quantitative results were expressed as mean ± SD (n=10). $p<0.05 compared to non-diabetes. *p <0.05 compared to diabetes with PBS treatment. Scale bar: 100 μm.

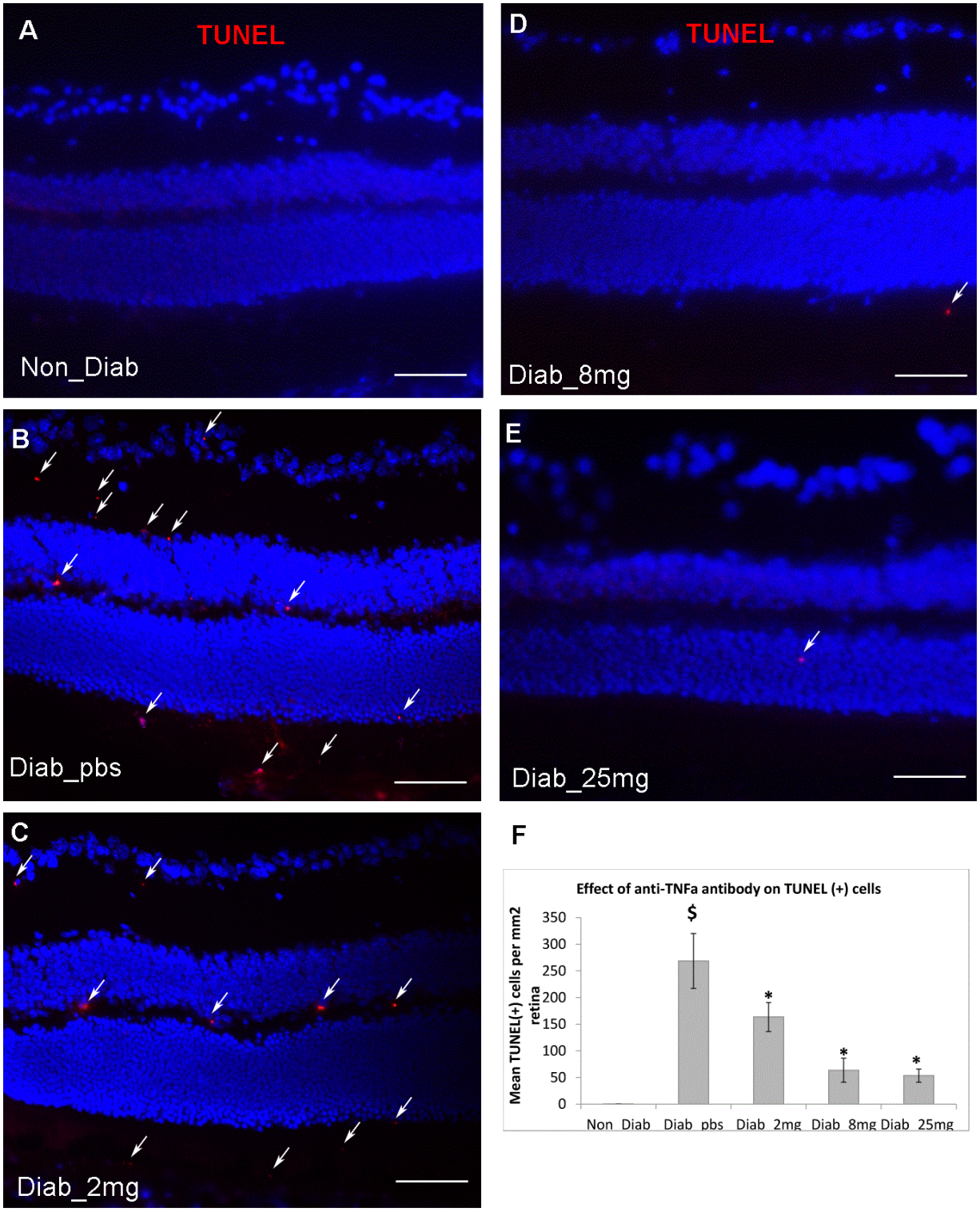



**Figure 4:** Prevention of TUNEL (+) cells by Anti-TNFα treatment. The STZ-induced diabetic mice were IP injected antibody weekly for 3 months. (A-E) The sample images of TUNEL staining for non-diabetic (A), diabetic with the treatments of PBS (B), 2 mg/kg (C), 8 mg/kg (D) and 25 mg/kg (E). (F) Quantitative results were expressed as mean ± SD (n=10). $ $p<0.05$ compared to non-diabetes. * $p<0.05$ compared to diabetes with PBS treatment. Scale bar: 100 μm.

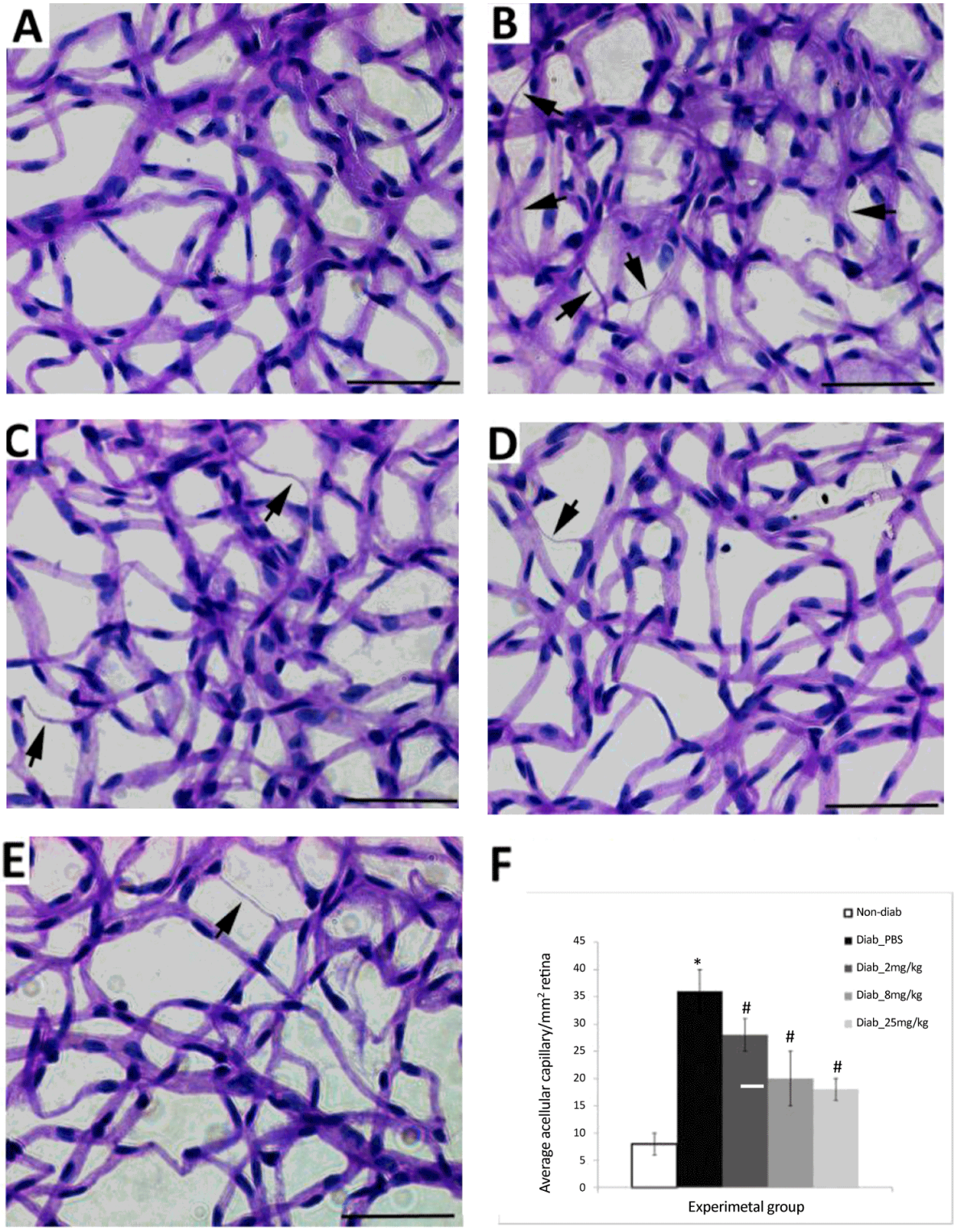
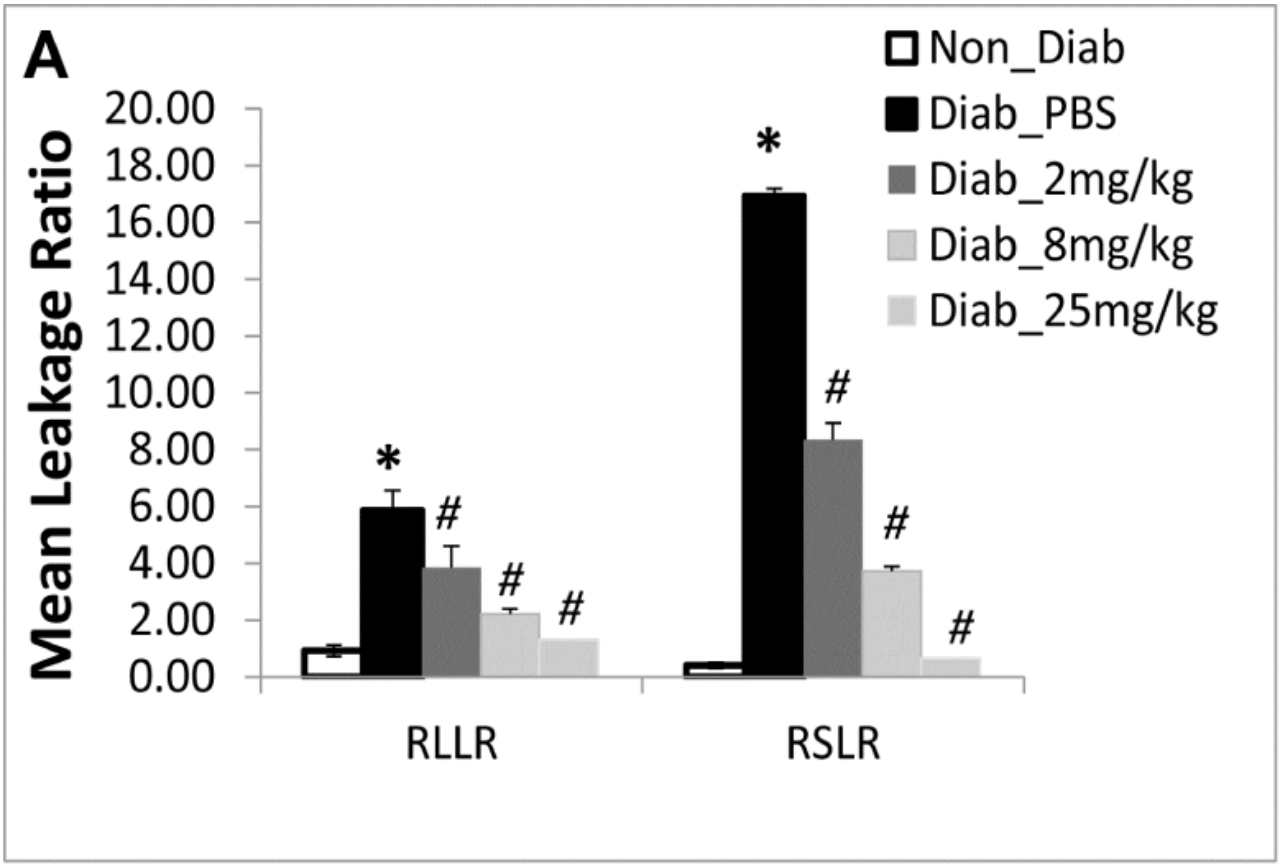



**Figure 5:** Prevention of acellular capillary by anti-TNFα treatment. The STZ-induced diabetic mice were IP injected antibody weekly for 6 months. (A-E) The example images of isolated retinal vasculatures for non-diabetic mice (A), PBS (B), the antibody doses of 2 mg/kg (C), 8 mg/kg (D), and 25 mg/kg (E). Arrows indicated acellular capillaries. (F) Quantitative results. The results were the averaged acellular capillary/mm$^2$ retina (n=6). *p<0.05 compared to non-diabetes. #p<0.05 compared to diabetes with PBS treatment. Scale bar: 50 μm.

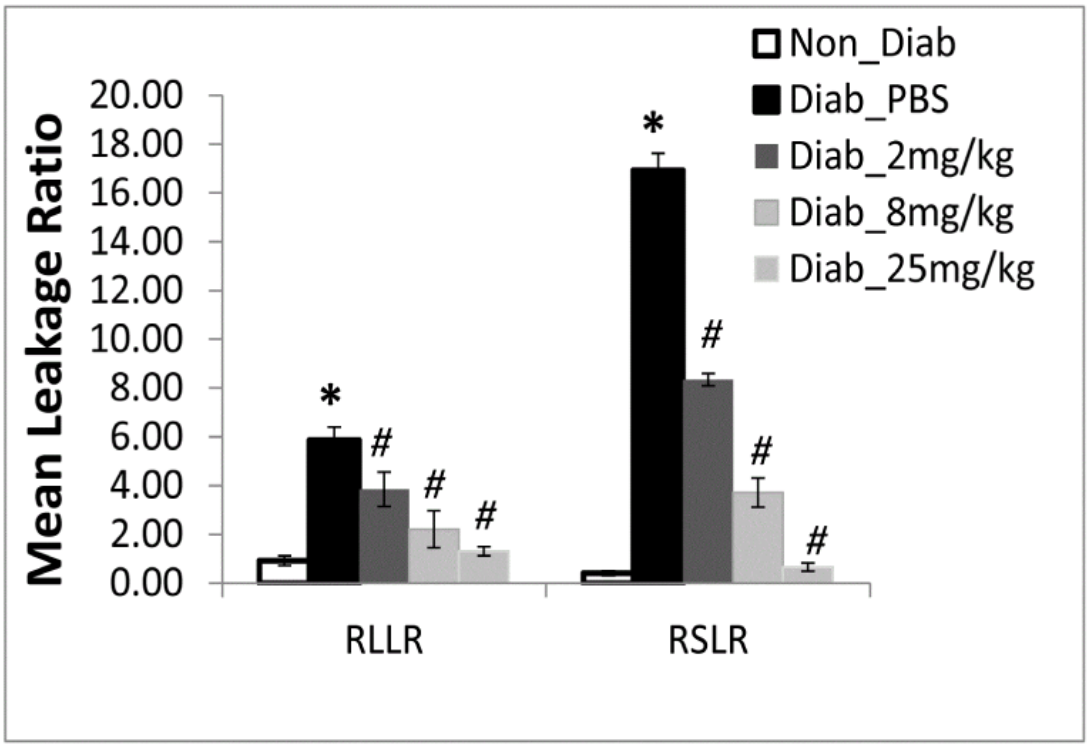



**B**

| 3 monhts/IVT | Average | | sd | | p value (vs. Ctrl) | |
|---|---|---|---|---|---|---|
| Akita diabetic | RLLR | RSLR | RLLR | RSLR | RLLR | RSLR |
| Non_Diab | 0.92 | 0.41 | 0.20 | 0.10 | | |
| Diab_PBS | 5.85 | 16.94 | 0.56 | 0.70 | 0.00 | 0.01 |
| Diab_2mg/kg | 3.85 | 8.34 | 0.70 | 0.25 | 0.46 | 0.04 |

Figure 6: Prevention of BRB breakdown by anti-TNFα treatment in Akita mice. Akita diabetic mice were IVT injected biweekly for 3 months. (A) The histogram shows the graphic view of inhibitory effect. (B) The table shows the value of quantitative BRB, which were expressed as mean ± SD (n=10). 1: RLLR (retina to lung leakage ratio); 2: RSLR (retina to serum leakage ratio). $^{*}p<0.05$ compared to non-diabetes; $^{\#}p<0.05$ compared to diabetes with PBS treatment.

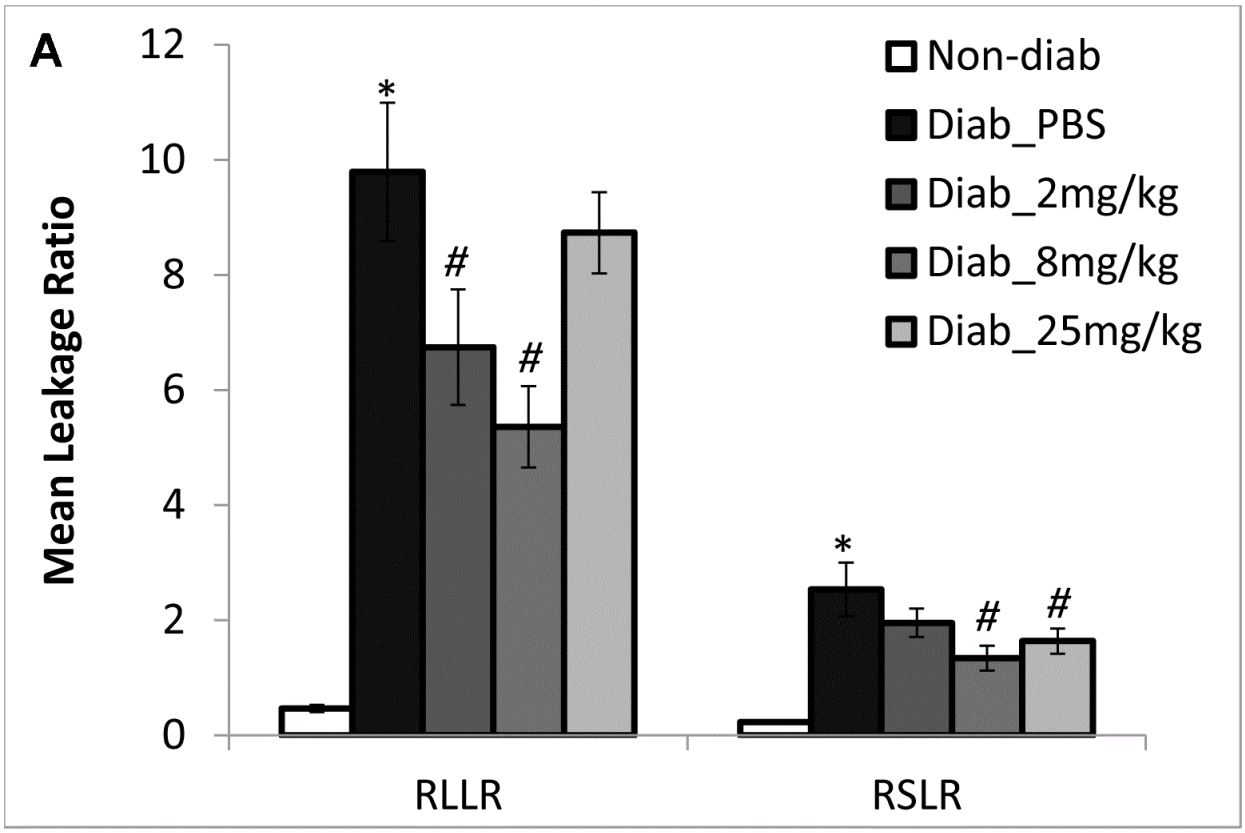
A




B

| 3 months/IP injection | Average | | SD | | p-value | |
|---|---|---|---|---|---|---|
| STZ-induced | RLLR | RSLR | RLLR | RSLR | RLLR | RSLR |
| Non-diab | 0.287 | 0.141 | 0.015 | 0.030 | | |
| Diab_PBS | 0.677 | 0.360 | 0.014 | 0.015 | 0.006 | 0.003 |
| Diab_2mg/kg | 0.510 | 0.225 | 0.043 | 0.025 | 0.182 | 0.041 |
| Diab_8mg/kg | 0.389 | 0.150 | 0.008 | 0.006 | 0.048 | 0.034 |
| Diab_25mg/kg | 0.274 | 0.034 | 0.021 | 0.024 | 0.044 | 0.004 |

Figure 7: Prevention of BRB breakdown by anti-TNFα treatment in STZ-induced mice (3 months). The STZ-induced diabetic mice were IP injected antibody weekly for 3 months. (A) The histogram was the graphic view. (B) The table was the value of quantitative BRB, which were expressed as mean ± SD (n=10). Lung and serum were used for reference. RLLR: retina to lung leakage ratio; RSLR: retina to serum leakage ratio. *p<0.05 compared to non-diabetes; #p<0.05 compared to diabetes with PBS treatment.




B

| 6 months/IP injection | Average | | SD | | p-value | |
|---|---|---|---|---|---|---|
| STZ-induced | RLLR | RSLR | RLLR | RSLR | RLLR | RSLR |
| Non-diab | 0.463 | 0.228 | 0.061 | 0.034 | | |
| Diab_PBS | 9.791 | 2.531 | 1.200 | 0.465 | 0.002 | 0.000 |
| Diab_2mg/kg | 6.745 | 1.953 | 1.002 | 0.245 | 0.030 | 0.071 |
| Diab_8mg/kg | 5.360 | 1.339 | 0.454 | 0.247 | 0.042 | 0.012 |
| Diab_25mg/kg | 8.734 | 1.635 | 0.707 | 0.217 | 0.359 | 0.025 |

**Figure 8:** Prevention of BRB breakdown by anti-TNFα treatment in STZ-induced diabetic mice (6 moths). The STZ-induced diabetic mice were IP injected weekly for 6 months. (A) The histogram was the graphic view. (B) The table was the value of quantitative BRB, which were expressed as mean ± SD (n=10). RLLR: retina to lung leakage ratio. RSLR: retina to serum leakage ratio. *p<0.05 compared to non-diabetes; #p<0.05 compared to diabetes with PBS treatment.

## Discussion

In this study, we investigated the effect of anti-TNFα antibody (CNTO5048) on complications of DR in the experimental models of diabetes. We found that this antibody effectively prevented diabetes-related retinal leukostasis, cell death, acelluar capillary, and BRB breakdown. Both IVT injection and IP injection showed similar efficacy and dose response curve. Among the three examined IP doses, the 8mg/kg appeared to be the most appropriate dose considering the efficacy and safety profiles, because 2 mg/kg did not show consistence sufficiency and 25 mg/kg were occasionally associated with some abnormalities. Although the abnormalities might be due to the long-term repeated treatments of such dose, we could not rule out the possibility of drug formula itself. IVT injection, which requires much less antibody amount (1, 5 or 10 μg/eye) and elicits minimal systemic impact, is a good strategy for drug delivery into the retina. Based on the previous report that the half-life (t1/2) of clearing Infliximab from rabbit vitreous was 6.5 days [21], we performed IVT injection of CNTO5048 into the mice vitreous cavity biweekly for 3 months. This treatment interval and duration consistently prevented complications of DR in either STZ-induced or Akita diabetic mice. The next step

would divert these results to the other models and translated to human clinical trials.

Several small clinical trials have been reported for anti-TNFα antibody in the treatment of DR. The results did not appear consistent. For example, two studies showed that the intravenous (IV) injection of infliximab increased the visual acuity of DME patients [22,23]. However, some others reports the IVT injection of this anti-TNF antibody had not any benefits and was even deleterious to the eyes of the patients with DME [24,25]. It is worthy of noting that infliximab's formulation was optimized for IV use, and not IVT injection. So the harmful effect could be due to other excipients in the formulation. Despite the inconsistent reports, the overall outcomes are encouraging for the potential application of anti-TNFα in the treatment of DR, but the treatment conditions, such as dosage, formulation, and delivery routes, may need to be further optimized.

Inflammation is an important mechanism that TNFα is implicated in the pathogenesis of DR [11,12]. TNFα is expressed by the activated microglia, which is a notable inflammatory response in human diabetic patients [26] and animal models of diabetes [27,28]. TNFα-mediated apoptotic cell death is another mechanism that is implicated in the pathogenesis of DR. Nuclear factor kappa B (NF-κB) is a mediator of TNFα inflammatory and death signaling pathways [29,30]. It can be hypothesize that TNFα contributes to the cascade events in DR: the death of vascular pericytes and endothelial cells microaneurysm and acellular capillary ischemic retina increased HIF-1α and VEGF expression vascular permeability and retinal neovascularization. In addition, TNFα is involved in the DNA binding activity and nuclear translocation of transcription factor FOXO1 in diabetic retina [31]. In retinal endothelia cell cultures, TNFα can regulate expression and cellular localization of ZO-1 and claudin-5, which is mediated by NF-κB and protein kinase (PKC)-delta [32]. TNFα was shown to be a potent downstream molecule of VEGF-, PAF- and IL-mediated retinal leukostasis [33]. How these signaling pathways and regulatory mechanisms are modulated by TNFα in the background of DR needs further elucidation.

In summary, the results of this study suggest that anti-TNFα treatment can be potentially applied to treat complications of DR, but the treatment conditions and underlying mechanisms need further elucidation.

## Acknowledgments

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## References

1. Yau JW, Rogers SL, Kawasaki R, Lamoureux EL, Kowalski JW, et al. (2012) Global prevalence and major risk factors of diabetic retinopathy. Diabetes Care 35: 556-564.
2. Powell ED, Field RA (1964) Diabetic retinopathy and rheumatoid arthritis. Lancet 2: 17-18.
3. Joussen AM, Poulaki V, Le ML, Koizumi K, Esser C, et al. (2004) A central role for inflammation in the pathogenesis of diabetic retinopathy. FASEB J 18: 1450-1452.
4. El-Asrar AM (2012) Role of inflammation in the pathogenesis of diabetic retinopathy. Middle East Afr J Ophthalmol 19: 70-74.
5. Tang J, Kern TS (2011) Inflammation in diabetic retinopathy. Prog Retin Eye Res 30: 343-358.
6. Joussen AM, Poulaki V, Mitsiades N, Kirchhof B, Koizumi K, et al. (2002) Nonsteroidal anti-inflammatory drugs prevent early diabetic retinopathy via TNF-alpha suppression. FASEB J 16: 438-440.
7. Zhang SX, Wang JJ, Gao G, Shao C, Mott R, et al. (2006) Pigment epithelium-derived factor (PEDF) is an endogenous antiinflammatory factor. FASEB J 20: 323-325.
8. Limb GA, Chignell AH, Green W, LeRoy F, Dumonde DC (1996) Distribution of TNF alpha and its reactive vascular adhesion molecules in fibrovascular membranes of proliferative diabetic retinopathy. Br J Ophthalmol 80: 168-173.
9. Zorena K, MyÅ›liwska J, MyÅ›liwiec M, Balcerska A, Hak Å, et al. (2007) Serum TNF-alpha level predicts nonproliferative diabetic retinopathy in children. Mediators Inflamm 2007: 92196.
10. Demircan N, Safran BG, Soylu M, Ozcan AA, Sizmaz S (2006) Determination of vitreous interleukin-1 (IL-1) and tumour necrosis factor (TNF) levels in proliferative diabetic retinopathy. Eye (Lond) 20: 1366-1369.
11. Huang H, Gandhi JK, Zhong X, Wei Y, Gong J, et al. (2011) TNFalpha is required for late BRB breakdown in diabetic retinopathy, and its inhibition prevents leukostasis and protects vessels and neurons from apoptosis. Invest Ophthalmol Vis Sci 52: 1336-1344.
12. Joussen AM, Doehmen S, Le ML, Koizumi K, Radetzky S, et al. (2009) TNF-alpha mediated apoptosis plays an important role in the development of early diabetic retinopathy and long-term histopathological alterations. Mol Vis 15: 1418-1428.
13. Behl Y, Krothapalli P, Desta T, DiPiazza A, Roy S, et al. (2008). Diabetes-enhanced tumor necrosis factor-alpha production promotes apoptosis and the loss of retinal microvascular cells in type 1 and type 2 models of diabetic retinopathy. Am J Pathol 172: 1411-1418.
14. Hotamisligil GS1 (1999) Mechanisms of TNF-alpha-induced insulin resistance. Exp Clin Endocrinol Diabetes 107: 119-125.
15. Borst SE (2004) The role of TNF-alpha in insulin resistance. Endocrine 23: 177-182.
16. Shiau MY, Wu CY, Huang CN, Hu SW, Lin SJ, et al. (2003) TNF-alpha polymorphisms and type 2 diabetes mellitus in Taiwanese patients. Tissue Antigens 61: 393-397.
17. Charles KA, Kulbe H, Soper R, Escorcio-Correia M, Lawrence T, et al. (2009) The tumor-promoting actions of TNF-alpha involve TNFR1 and IL-17 in ovarian cancer in mice and humans. J Clin Invest 119: 3011-3023.
18. Laver NM, Robison WG Jr, Pfeffer BA (1993) Novel procedures for isolating intact retinal vascular beds from diabetic humans and animal models. Invest Ophthalmol Vis Sci 34: 2097-2104.
19. Huang H, He J, Johnson D, Wei Y, Liu Y, et al. (2015) Deletion of Placental Growth Factor Prevents Diabetic Retinopathy and Is Associated With Akt Activation and HIF1Î±-VEGF Pathway Inhibition. Diabetes 64: 200-212.
20. Derevjanik NL, Vinores SA, Xiao WH, Mori K, Turon T, et al. (2002) Quantitative assessment of the integrity of the blood-retinal barrier in mice. Invest Ophthalmol Vis Sci 43: 2462-2467.
21. Giansanti F, Ramazzotti M, Giuntoli M, Virgili G, Vannozzi L, et al. (2009) Intravitreal infliximab clearance in a rabbit model: different sampling methods and assay techniques. Invest Ophthalmol Vis Sci 50: 5328-5335.
22. Sfikakis PP, Grigoropoulos V, Emfietzoglou I, Theodossiadis G, Tentolouris N, et al. (2010) Infliximab for diabetic macular edema refractory to laser photocoagulation: a randomized, double-blind, placebo-controlled, crossover, 32-week study. Diabetes Care 33: 1523-1528.
23. Sfikakis PP, Markomichelakis N, Theodossiadis GP, Grigoropoulos V, Katsilambros N, et al. (2005) Regression of sight-threatening macular

edema in type 2 diabetes following treatment with the anti-tumor necrosis factor monoclonal antibody infliximab. Diabetes Care 28:445-447.

24. Giganti M, Beer PM, Lemanski N, Hartman C, Schartman J, et al. (2010) Adverse events after intravitreal infliximab (Remicade). Retina 30: 71-80.
25. Wu L, Hernandez-Bogantes E, Roca JA, Arevalo JF, Barraza K, et al. (2011) Intravitreal tumor necrosis factor inhibitors in the treatment of refractory diabetic macular edema: a pilot study from the Pan-American Collaborative Retina Study Group. Retina 31: 298-303.
26. Zeng HY, Green WR, Tso MO (2008) Microglial activation in human diabetic retinopathy. Arch Ophthalmol 126: 227-232.
27. Ibrahim AS, El-Remessy AB, Matragoon S, Zhang W, Patel Y, et al. (2011) Retinal microglial activation and inflammation induced by amadori-glycated albumin in a rat model of diabetes. Diabetes 60: 1122-1133.
28. Kezic JM, Chen X, Rakoczy EP, McMenamin PG (2013) The effects of age and Cx3cr1 deficiency on retinal microglia in the Ins2(Akita) diabetic mouse. Invest Ophthalmol Vis Sci 54: 854-863.
29. Beg AA, Baltimore D (1996) An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. Science 274: 782-784.
30. Lee EG, Boone DL, Chai S, Libby SL, Chien M, et al. (2000) Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice. Science 289: 2350-2354.
31. Behl Y, Krothapalli P, Desta T, Roy S, Graves DT (2009) FOXO1 plays an important role in enhanced microvascular cell apoptosis and microvascular cell loss in type 1 and type 2 diabetic rats. Diabetes 58: 917-925.
32. Aveleira CA, Lin CM, Abcouwer SF, Ambrósio AF, Antonetti DA (2010) TNFα signals through PKCζ/NF-Kb to alter the tight junction complex and increase retinal endothelial cell permeability. Diabetes 59: 2872-2882.
33. Vinores SA, Xiao WH, Shen J, Campochiaro PA (2007) TNF-alpha is critical for ischemia-induced leukostasis, but not retinal neovascularization nor VEGF-induced leakage. J Neuroimmunol 182: 73-79.