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## Nuclear factor of activated T-cells (NFAT) regulation of IL-1 $\beta$ -induced retinal vascular inflammation

Meredith J. Giblin<sup>1</sup>, Taylor E. Smith<sup>2</sup>, Garrett Winkler<sup>2</sup>, Hannah A. Pendergrass<sup>2</sup>, Minjae J. Kim<sup>2</sup>, Megan E. Capozzi<sup>3</sup>, Rong Yang<sup>2</sup>, Gary W. McCollum<sup>2</sup>, John S. Penn<sup>1,2</sup>

<sup>1</sup>Department of Cell and Developmental Biology, Vanderbilt University

<sup>2</sup>Department of Ophthalmology and Visual Sciences, Vanderbilt University Medical Center

<sup>3</sup>Duke Molecular Physiology Institute, Duke University

### Abstract

Chronic low-grade retinal inflammation is an essential contributor to the pathogenesis of diabetic retinopathy (DR). It is characterized by increased retinal cell expression and secretion of a variety of inflammatory cytokines; among these, IL-1 $\beta$  has the reputation of being a major driver of cytokine-induced inflammation. IL-1 $\beta$  and other cytokines drive inflammatory changes that cause damage to retinal cells, leading to the hallmark vascular lesions of DR; these include increased leukocyte adherence, vascular permeability, and capillary cell death. Nuclear factor of activated T-cells (NFAT) is a transcriptional regulator of inflammatory cytokines and adhesion molecules and is expressed in retinal cells. Consequently, it may influence multiple pathogenic steps early in DR. We investigated the NFAT-dependency of IL-1 $\beta$ -induced inflammation in human Müller cells (hMC) and human retinal microvascular endothelial cells (hRMEC). Our results show that an NFAT inhibitor, Inhibitor of NFAT-Calcineurin Association-6 (INCA-6), decreased IL-1 $\beta$ -induced expression of IL-1 $\beta$  and TNF $\alpha$  in hMC, while having no effect on VEGF, CCL2, or CCL5 expression. We also demonstrate that INCA-6 attenuated IL-1 $\beta$ -induced increases of IL-1 $\beta$ , TNF $\alpha$ , IL-6, CCL2, and CCL5 (inflammatory cytokines and chemokines), and ICAM-1 and E-selectin (leukocyte adhesion molecules) expression in hRMEC. INCA-6 similarly inhibited IL-1 $\beta$ -induced increases in leukocyte adhesion in both hRMEC monolayers *in vitro* and an acute model of retinal inflammation *in vivo*. Finally, INCA-6 rescued IL-1 $\beta$ -induced permeability in both hRMEC monolayers *in vitro* and an acute model of retinal inflammation *in vivo*. Taken

#### Credit Author Statement

**Meredith J. Giblin:** Conceptualization, Formal analysis, Investigation, Writing - original draft. **Taylor E. Smith:** Methodology, Formal analysis, Investigation, Writing - review and editing. **Garrett Winkler:** Methodology, Investigation. **Hannah A. Pendergrass:** Methodology, Investigation. **Minjae J. Kim:** Investigation. **Megan E. Capozzi:** Conceptualization, Investigation. **Rong Yang:** Investigation. **Gary W. McCollum:** Writing- Review and editing. **John S. Penn:** Conceptualization, Writing- Review and editing, Project administration, Funding acquisition.

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#### Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

together, these data demonstrate the potential of NFAT inhibition to mitigate retinal inflammation secondary to diabetes.

## 1. Introduction

Diabetic retinopathy (DR) is the leading cause of blindness in working-age Americans.<sup>1</sup> DR has two clinically defined stages, the non-proliferative (NPDR) and the proliferative (PDR). Once a patient has reached the proliferative stage of DR, the retina has sustained irreparable damage which places complex demands on potential therapeutics. Therefore, the identification and blockade of therapeutic targets relevant to early NPDR could halt disease onset and progression, preempting PDR and its vision-threatening consequences.<sup>2-4</sup> Current therapies that target vascular endothelial growth factor (VEGF) are invasive and only effective in about half of DR patients.<sup>5</sup> These unsatisfactory outcomes have motivated the exploration of therapeutic alternatives, perhaps targeting other inflammatory mediators in addition to VEGF. Chronic low-grade inflammation is a component of early NPDR and, in recent years, its potential as a therapeutic target has received considerable attention. Elevated retinal or vitreous levels of cytokines, including IL-1 $\beta$ , TNF $\alpha$ , IL-8, IL-6, CCL2, and CCL5, have been observed early in DR in both animal models<sup>6-10</sup> and patients.<sup>5-7,11-18</sup> These cytokines not only initiate retinal vascular damage but also activate autocrine and paracrine signaling cascades that promote chronic retinal inflammation, all of which contributes to the development of vision-threatening pathology.<sup>3,5</sup> Numerous studies have identified IL-1 $\beta$  as a primary driver of inflammation in a wide-range of pathological conditions, including neuro-inflammatory diseases, diabetes, and DR.<sup>3,19-23</sup> Increased levels of IL-1 $\beta$  are observed in the serum and vitreous of diabetics and, unlike VEGF, correlate closely with DR severity and progression.<sup>3,24,25</sup> Abundant data suggest that IL-1 $\beta$  plays a causative role in several hallmark DR pathologies, including glial activation,<sup>26</sup> cell death,<sup>27</sup> vascular permeability,<sup>28</sup> leukocyte adhesion,<sup>29,30</sup> and vasoregression.<sup>27</sup> Furthermore, inhibition of the IL-1 $\beta$ -activating enzyme caspase 1 reduced retinal IL-1 $\beta$  levels and retinal capillary regression in both STZ and galactose-fed mice; deletion of the IL-1 $\beta$  receptor IL-1R1 also protected against capillary loss in both models.<sup>31</sup> We became particularly interested in IL-1 $\beta$  as a driver of retinal inflammation after RNA sequencing experiments comparing human Müller cell (hMC) responses to treatment with IL-1 $\beta$ , TNF $\alpha$ , IL-8, and IL-6 demonstrated that IL-1 $\beta$  caused the greatest and longest lasting inflammatory response in hMC (manuscript in preparation). Interestingly, these data also identified IL-1 $\beta$  itself as one of the most highly upregulated genes in IL-1 $\beta$ -treated hMC, supporting existing evidence that increased IL-1 $\beta$  levels via auto-amplification may exacerbate and sustain retinal inflammation in DR.<sup>23</sup> In aggregate, this evidence suggests that IL-1 $\beta$  acts as a “master regulator” of inflammation in the pathological cascade of DR, and that preventing its downstream effects in retinal cells may therefore significantly slow inflammation-induced damage.<sup>19,23,32</sup>

IL-1 $\beta$  mediates downstream inflammatory signaling via the activation of several transcription factors, including nuclear factor of activated T-cells (NFAT).<sup>33,34</sup> The NFAT family consists of five proteins, four of which (c1-c4) are regulated by the calcium-dependent phosphatase calcineurin (CN).<sup>35-37</sup> In quiescent cells, NFAT exists in the

cytosol in a hyperphosphorylated state. Upon challenge with an inflammatory stimulus, CN dephosphorylates NFAT, causing it to shuttle to the nucleus where it increases the transcription of inflammatory genes.<sup>38-40</sup> The small molecule inhibitor, Inhibitor of NFAT-Calcineurin Association-6 (INCA-6), blocks CN-NFAT association, thereby preventing the dephosphorylation/activation of the four CN-dependent NFAT isoforms.<sup>41,42</sup> NFAT is known to play a role in the regulation of inflammatory mediators, extracellular matrix proteins, vascular permeability, and adhesion molecules,<sup>35,38,43-54</sup> and as such may control multiple pathogenic steps early in DR. Additionally, published evidence demonstrates that NFAT inhibition is a successful strategy in preventing pathogenic retinal cell behaviors downstream of both hyperglycemia and TNF $\alpha$ .<sup>43,50</sup> Combined, these data demonstrate that NFAT mediates a variety of characteristic pathogenic changes that arise in response to multiple components of the diabetic environment; therefore, we hypothesized that NFAT may likewise influence the response of retinal cells to elevated IL-1 $\beta$  in the diabetic milieu.

The diabetic retina is a complex environment of multiple cell types, including endothelial cells, pericytes, astrocytes, and Müller cells.<sup>55</sup> Pathogenic behaviors by each of these cell types do not exist in isolation, rather they can produce both autocrine and paracrine factors that potentiate disease progression in surrounding cells. For instance, VEGF, a major target of DR therapeutics, is secreted predominantly by Müller cells and subsequently causes downstream pathogenic responses in endothelial cells.<sup>4,56,57</sup> Since multiple retinal cell types are involved in the DR cascade and cell-specific targeting is difficult *in vivo*, it would be ideal to identify a therapeutic target with the capacity to inhibit pathological responses in more than one retinal cell type. Therefore, in this study, we sought to investigate the role of NFAT in regulating key cell-specific responses to IL-1 $\beta$ , specifically Müller cell auto-amplification of IL-1 $\beta$  and endothelial cell inflammation, leukocyte adhesion, and permeability. We found that inhibition of NFAT not only attenuates Müller cell amplification of inflammation but also inhibits the downstream pathologic response of human retinal microvascular endothelial cells (hRMEC) to IL-1 $\beta$  challenge. These results suggest that targeting NFAT has significant therapeutic potential for slowing early inflammation and subsequent endothelial cell dysfunction in DR.

## 2. Methods

### 2.1. Human Müller cell (hMC) isolation and culture

Primary hMC were isolated from human donor tissue (NDRI) within 24 hours post-mortem, using an adapted protocol from previously developed methods.<sup>58</sup> Briefly, the retina was dissected from the eye cup and dissociated in Dulbecco's Modified Eagle Medium (DMEM; Gibco; Carlsbad, CA) supplemented with trypsin and collagenase. After incubation in dissociation medium, hMC were cultured in DMEM supplemented with 10% FBS (R&D Systems; Minneapolis, MN) and 1x antibiotic/antimycotic solution (Gibco). Passages 4-6 were used for all experiments. Cultures were incubated at 37 °C, 5% CO<sub>2</sub>, and 20.9% O<sub>2</sub> and 95% relative humidity.

## 2.2. hMC qRT-PCR

hMC were cultured in 6-well plates until 70% confluence, then cultured in serum-reduced conditions (2% FBS) for 12 hours before treatment. hMC were treated in serum-reduced media containing 50pg/mL IL-1 $\beta$  (Sino Biological; Wayne, PA) with or without 1 or 2.5 $\mu$ M INCA-6 (Tocris; Minneapolis, MN) for 8 hours. Total RNA was collected using an RNeasy Mini kit (Qiagen; Valencia, CA), according to the manufacturer's protocol. The High-Capacity cDNA Archive Kit (Applied Biosystems; Waltham, MA) was used to reverse transcribe the total RNA. Quantitative RT-PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems). Using technical duplicates, the co-amplification of human *IL1B*, *TNFA*, *VEGF*, *CCL2*, and *CCL5* cDNA was compared with *TBP* (*TATA-binding protein*) as the normalization control. Data were analyzed using the comparative Ct method. Experiments were performed using a minimum of 3 biologically independent samples as well as technical replicates for each sample.

## 2.3. Human retinal microvascular endothelial cell (hRMEC) culture

Primary hRMEC (Cell Systems; Kirkland, WA) were cultured in phenol red-free endothelial basal medium (EBM; Cell Systems) supplemented with 10% FBS (R&D Systems) and SingleQuots (Lonza, Inc.; Allendale, NJ) and grown on attachment factor- (Cell Systems) coated culture dishes. Passages 5-8 were used for all experiments.

## 2.4. hRMEC qRT-PCR

hRMEC were plated in 6-well plates and grown in 10% EBM to 75-85% confluency before treatment. Cells were treated for 2 hours with 1ng/mL IL-1 $\beta$  in the presence or absence of 1, 2.5, or 5 $\mu$ M INCA-6 in serum-reduced media (2% FBS EBM supplemented with antibiotic). Subsequently, cells were lysed and qRT-PCR analysis for human *IL1B*, *TNFA*, *IL8*, *IL6*, *CCL2*, *CCL5*, *VCAM1*, *ICAM1*, or *SELE* (E-selectin) expression was completed as described above for hMC. Experiments were performed using a minimum of 3 biologically independent samples as well as technical replicates for each sample.

## 2.5. Parallel plate flow chamber assay (PPFC)

hRMEC were plated onto attachment factor-coated glass slides (Thermo Fisher; Waltham, MA). Once cells formed a complete monolayer, slides were treated with 1ng/mL IL-1 $\beta$  with or without 5 $\mu$ M INCA-6 in serum-reduced media. After 4 hours, slides were used to assemble the PPFC (GlycoTech; Gaithersburg, MD) and leukocyte adhesion was assayed as previously described.<sup>43</sup> Briefly, human peripheral blood mononuclear cells (PBMC; Precision for Medicine; Fredrick, MD) were resuspended at a concentration of  $5 \times 10^5$  cells/mL in Hank's Buffered Salt Solution (HBSS; Gibco). PBMC were flowed at a shear stress of 1 dyn/cm<sup>2</sup> over treated monolayers for 7 minutes. Non-adherent cells were then washed away by flowing HBSS over the monolayers at 2 dyn/cm<sup>2</sup> for 2 minutes. Eight fields per slide were randomly selected prior to PBMC flow and images were captured before and after flow. A masked observer compared the two sets of images to count adherent leukocytes in the after images. Each data point represents the average number of adherent leukocytes across the eight captured fields per slide divided by the count area (mm<sup>2</sup>).

## 2.6. Retinal leukostasis

All experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committee and were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Six- to eight-week-old C57BL/6J mice (Charles River; Wilmington, MA) received intravitreal injections of vehicle, IL-1 $\beta$  (50pg), INCA-6 (150ng), or IL-1 $\beta$  (50pg) + INCA-6 (150ng). Retinal leukostasis was performed as described previously.<sup>43,59</sup> Briefly, 12 hours after treatment mice were anesthetized with ketamine/xylazine and then perfused with 0.9% saline for 2.5 minutes followed by FITC-conjugated concanavalin-A (40 $\mu$ g/mL in 5.0mL PBS; Vector Laboratories; Burlingame, CA). Saline was then perfused for 2.5 minutes to remove any non-adherent leukocytes. Retinas were immediately dissected into 4% paraformaldehyde, flat-mounted, and images were captured with a Zeiss LSM710 Confocal Microscope (Zeiss; Pleasanton, CA) at 20x magnification. Using ImageJ (NIH; Bethesda, MD), a masked observer selected 4 regions per retina and adherent leukocytes in the superficial plexus of the retinal vasculature were counted. Each data point represents the average number of adherent leukocytes across the 4 regions divided by the count area (mm<sup>2</sup>).

## 2.7. Transendothelial electrical resistance (TEER) measurements

hRMEC were plated at a density of 40,000 cells/well in 96W10idf plates (Applied Biophysics; Troy, NY) in 10% FBS EBM. Per the manufacturer's instructions, hRMEC were allowed to settle onto plates for 15 minutes at room temperature before insertion into the Electric Cell-substrate Impedance Sensing (ECIS) Z-Theta instrument (Applied Biophysics). After resistance plateaued between 24 and 48 hours, monolayers were treated with 1ng/mL IL-1 $\beta$  with or without 5 $\mu$ M INCA-6. Resistance was monitored for 24 hours after treatment and normalized resistance was calculated using Applied Biophysics software.<sup>60,61</sup> Each well was normalized to its resistance 1 hour prior to treatment.

## 2.8. Quantitative fluorescein angiography (qFA)

Six- to eight-week-old C57BL/6J mice (Charles River) received intravitreal injections of vehicle, IL-1 $\beta$  (50pg), INCA-6 (150ng), or IL-1 $\beta$  (50pg) + INCA-6 (150ng). Eight hours after treatment, vascular permeability was assessed using qFA. Mice were anesthetized with an intraperitoneal injection of ketamine/xylazine and their pupils were dilated with tropicamide and phenylephrine before receiving an intraperitoneal injection of sodium fluorescein (NDC 17478-250-20; Akorn; Lake Forest, IL) at 10 $\mu$ L/g body weight. At 2 and 4 minutes after injection, fluorescent fundus images were captured with the Micron IV retinal imaging system (Phoenix Research Labs; Pleasanton, CA). A masked observer checked all images for quality, ensuring proper visualization of the vasculature was achieved in each. ImageJ software was then used to quantify the fluorescence intensity of the images using the integrated density function, an algorithm that measures the mean gray value in a selected area of the image and then multiplies it by the selected area. The integrated density was measured for both the 2 minute and 4 minute images. The integrated density at 2 minutes (baseline) was then subtracted from the integrated density at 4 minutes, with the difference in integrated density between 2 and 4 minutes employed as a readout of vascular leakage.

## 2.9. Statistical analysis

All data were analyzed with Prism software (GraphPad; La Jolla, CA). Analysis of variance (ANOVA) with Tukey's multiple comparisons post hoc analysis was used; values of  $p < 0.05$  were considered statistically significant. Grubbs' test was utilized to identify outliers. For ECIS, two-way ANOVA with Tukey's multiple comparisons post hoc analysis was used.

## 3. Results

### 3.1. INCA-6 decreases IL-1 $\beta$ -induced inflammatory cytokine expression in hMC

Based on Müller cells' known role as major propagators of retinal inflammation,<sup>55,57,62,63</sup> we first sought to determine if NFAT is involved in the regulation of hMC inflammatory responses in an autocrine and paracrine manner. IL-1 $\beta$  increased the expression of IL-1 $\beta$  (152-fold,  $p < 0.0001$ ), TNF $\alpha$  (86-fold,  $p < 0.0001$ ), VEGF (1.3-fold,  $p = 0.0132$ ), CCL2 (15-fold,  $p < 0.0001$ ), and CCL5 (31-fold,  $p < 0.0001$ ) in hMC. IL-1 $\beta$  and TNF $\alpha$  expression levels were decreased 34% ( $p = 0.0367$ ) and 36% ( $p = 0.0303$ ), respectively, in hMC co-treated with IL-1 $\beta$  and 2.5  $\mu$ M INCA-6, compared to IL-1 $\beta$  alone (Figure 1A-B). INCA-6 had no effect on the IL-1 $\beta$ -induced expression of VEGF, CCL2, or CCL5 at either of the concentrations tested (Figure 1C-E).

### 3.2. INCA-6 decreases IL-1 $\beta$ -induced inflammatory cytokine expression in hRMEC

Similar to hMC, hRMEC respond to cytokine treatment by increasing expression and secretion of inflammatory cytokines, thereby propagating and sustaining local inflammation.<sup>5,23</sup> Therefore, we examined the NFAT-dependency of the hRMEC inflammatory response to IL-1 $\beta$ . IL-1 $\beta$  increased the expression of IL-1 $\beta$  (80-fold,  $p < 0.0001$ ), TNF $\alpha$  (3479-fold,  $p < 0.0001$ ), IL-8 (179-fold,  $p < 0.0001$ ), and IL-6 (66-fold,  $p = 0.0001$ ) in hRMEC. IL-1 $\beta$ , TNF $\alpha$ , and IL-6 expression decreased 81% ( $p < 0.0001$ ), 83% ( $p < 0.0001$ ), and 59% ( $p = 0.0051$ ), respectively, in hRMEC co-treated with IL-1 $\beta$  and 5 $\mu$ M INCA-6 compared to IL-1 $\beta$  alone. However, INCA-6 had no significant effect on IL-1 $\beta$ -induced IL-8 expression (Figure 2A-D). Additionally, we examined the chemokines, CCL2 and CCL5. IL-1 $\beta$  increased the expression of CCL2 (53-fold,  $p < 0.0001$ ) and CCL5 (122-fold,  $p < 0.0001$ ) in hRMEC. CCL2 and CCL5 expression decreased 60% ( $p = 0.0003$ ) and 87% ( $p < 0.0001$ ), respectively, in hRMEC co-treated with IL-1 $\beta$  and 5 $\mu$ M INCA-6, compared to IL-1 $\beta$  alone (Figure 2E-F).

### 3.3. INCA-6 mitigates IL-1 $\beta$ -induced leukocyte adhesion to hRMEC

We sought to investigate the efficacy of INCA-6 in preventing inflammation-induced leukocyte-endothelial cell adhesion, a characteristic pathogenic event in NPDR.<sup>5</sup> We performed a brief dose response experiment to determine the optimal concentration of INCA-6 to mitigate IL-1 $\beta$ -induced adhesion molecule expression in hRMEC. IL-1 $\beta$  increased VCAM-1, ICAM-1, and E-selectin expression 920- ( $p = 0.0003$ ), 229- ( $p < 0.0001$ ), and 1417-fold ( $p < 0.0001$ ), respectively, in hRMEC. Statistically significant decreases in ICAM-1 (51%;  $p = 0.0002$ ) and E-selectin (32%,  $p = 0.0103$ ) expression were achieved in hRMEC co-treated with IL-1 $\beta$  and 5 $\mu$ M INCA-6 (highest dose) compared to IL-1 $\beta$  alone (Figure 3A-C).

Next, we sought to determine if these changes in adhesion molecule expression translated into similar trends in PBMC adhesion to hRMEC monolayers using the parallel plate flow chamber (PPFC) assay. IL-1 $\beta$  caused an 11-fold ( $p < 0.0001$ ) increase in PBMC adhesion to hRMEC monolayers. When monolayers were co-treated with IL-1 $\beta$  and 5 $\mu$ M INCA-6, the optimal dose identified in Figure 3, PBMC adhesion to hRMEC monolayers decreased 41% ( $p = 0.0485$ ) compared to monolayers treated with IL-1 $\beta$  alone (Figure 4).

### 3.4. INCA-6 attenuates IL-1 $\beta$ -induced leukostasis in mice

Next, we investigated whether the efficacy of INCA-6 for inhibiting leukocyte adhesion *in vitro* could be translated *in vivo*. We used a mouse model of acute cytokine-induced retinal inflammation to test the efficacy of INCA-6 against retinal leukostasis. Retinal leukostasis increased 3.72-fold ( $p < 0.0001$ ) in C57BL/6J mice receiving intravitreal injections of IL-1 $\beta$  compared to vehicle. When mice received intravitreal injections of an IL-1 $\beta$ /INCA-6 cocktail, retinal leukostasis decreased to near vehicle levels ( $p < 0.0001$ ) (Figure 5).

### 3.5. INCA-6 rescues IL-1 $\beta$ -induced permeability

Finally, we were interested in determining whether INCA-6 was similarly efficacious in attenuating inflammation-induced vascular hyperpermeability, another characteristic pathology of NPDR.<sup>5</sup> Transendothelial electrical resistance (TEER) measurements were performed to assess whether INCA-6 has the capacity to correct IL-1 $\beta$ -induced defects in hRMEC barrier function. IL-1 $\beta$  decreased the normalized resistance of hRMEC monolayers 40% ( $t = 2-23$  hours,  $p < 0.0001$ ) compared to vehicle. This decreased barrier function was essentially reversed in hRMEC monolayers co-treated with IL-1 $\beta$  and INCA-6 ( $t = 2-23$  hours,  $p < 0.0001$ ) (Figure 6). This is clearly demonstrated during the last 6 hours of monitoring, during which there were no significant differences between the normalized resistance measures of hRMEC monolayers treated with vehicle compared to those co-treated with IL-1 $\beta$  and INCA-6; control phenotype was completely restored. Interestingly, INCA-6 alone caused an increase in resistance over vehicle ( $t = 2-23$  hours,  $p < 0.0001$ ).

Finally, we tested the capacity of INCA-6 to rescue retinal vascular hyperpermeability *in vivo*, assessed by fluorescein angiography, in our acute model of retinal inflammation. The fluorescein intensity in retinal fundus images nearly doubled ( $p = 0.0296$ ) in mice receiving intravitreal injections of IL-1 $\beta$ , indicating pronounced leakage from retinal capillaries. The fluorescein intensity decreased 84% ( $p = 0.0351$ ) in mice receiving intravitreal injections of an IL-1 $\beta$ /INCA-6 cocktail (Figure 7A-B).

## 4. Discussion

Our findings demonstrate for the first time that NFAT controls multiple IL-1 $\beta$ -induced inflammatory responses in both Müller cells and retinal microvascular endothelial cells. We had previously demonstrated that NFAT inhibition prevents TNF $\alpha$ -induced retinal leukostasis.<sup>43</sup> However, since the diabetic retinal environment contains a complex mix of inflammatory cytokines, it is desirable to identify a therapeutic target that can universally slow pathogenic responses to multiple stimuli. Additionally, mounting evidence indicates that IL-1 $\beta$  acts as a master regulator of the retinal inflammation consequent to

diabetes. Therefore, we tested the potential of INCA-6 to inhibit IL-1 $\beta$ -induced Müller cell and endothelial cell inflammation, leukocyte-endothelial cell adhesion, and vascular hyperpermeability in order to investigate the importance of CN/NFAT signaling in multiple pathogenic events of relevance to DR.

The current clinical approach to targeting the CN/NFAT signaling axis utilizes drugs (cyclosporin A and Tacrolimus) that block CN's ability to phosphorylate any substrate.<sup>39,40,42</sup> Therefore, they indiscriminately prevent downstream signaling, including important signaling hubs like MAP-K, contributing to severe side effects. Recent efforts have focused on identifying small molecule inhibitors that can specifically inhibit NFAT activation with minimal effects on CN's other substrates.<sup>40</sup> INCA-6 prevents the protein-protein interaction between CN and NFAT at the NFAT docking site and was identified based on its ability to compete out the high affinity VIVIT peptide. INCA-6 not only inhibited NFAT activation – as measured by phospho-NFAT Western blot, NFAT nuclear translocation staining, and expression levels of known NFAT target mRNA – but also showed no inhibition of CN phosphatase activity, no changes in MAP-K activation, and no changes in non-NFAT controlled mRNA expression.<sup>42</sup> Based on this favorable specificity profile, we chose to utilize INCA-6 for our studies, selecting dose ranges in the lowest concentrations conventionally used *in vitro*.<sup>43,64-68</sup>

Müller cells are retina-specific macroglia that act as sensors of the local environment, and it is well established that Müller cells become activated in the diabetic milieu.<sup>63,69</sup> We have previously demonstrated that, among non-neuronal retinal cells, Müller cells have the most potent inflammatory response to a variety of diabetes-relevant stimuli.<sup>57</sup> Furthermore, we have also observed that IL-1 $\beta$  potently induces its own expression in hMC, supporting published findings that suggest IL-1 $\beta$  auto-amplification propagates and sustains retinal inflammation.<sup>23</sup> Although NFAT has been previously implicated in the control of inflammatory responses in other glial cell types,<sup>53,70-72</sup> a potential role in Müller cell inflammation has not been established. To determine whether NFAT inhibition could attenuate Müller cell inflammatory responses, we examined hMC expression of key representative inflammatory mediators: IL-1 $\beta$ , TNF $\alpha$ , VEGF, CCL2 (also MCP-1), and CCL5 (also RANTES). Analogous to findings in other investigations of glial cell responses,<sup>53,70-72</sup> our data demonstrated that INCA-6 effectively inhibits the potent IL-1 $\beta$ -induced expression of IL-1 $\beta$  and TNF $\alpha$  in hMC. Recent evidence, in microglia, has indicated that NFAT can signal not only via calcium-dependent nuclear translocation but also via a calcium-independent mechanism involving mitochondrial translocation.<sup>73</sup> However, since INCA-6 acts by blocking the binding of CN and NFAT, we believe that, here, NFAT is acting in hMC via its canonical calcium-dependent mechanism, not this novel calcium-independent mechanism. INCA-6 was not efficacious in preventing the modest IL-1 $\beta$ -induced increases in VEGF, CCL2, or CCL5 expression in hMC. We believe it is likely an advantage that this therapeutic approach acts independently of VEGF regulation. Despite diabetic macular edema (DME) being the leading cause of vision loss in DR patients, a recent meta-analysis comparing the efficacies of aflibercept, ranibizumab, and bevacizumab predicted that only 3-4 out of every 10 DME patients will experience improvement after one year of anti-VEGF therapy.<sup>74</sup> Consequently, numerous combination therapies in which anti-VEGF therapy is combined with an additional target are currently in



development with the goal of achieving greater efficacy in a wider patient population than anti-VEGF alone.<sup>75,76</sup> Therefore, our data suggest that NFAT inhibition may be a potent and effective complement to anti-VEGF, though further study is certainly needed. Overall, these data suggest that NFAT inhibition can block the effects of both autocrine and paracrine sources of inflammation in hMC and represent the first report of the role of NFAT in regulating Müller cell expression of inflammatory genes.

Retinal microvascular endothelial cells demonstrate a wide range of molecular responses to the diabetic environment that contribute to the development of characteristic DR lesions.<sup>77</sup> Therefore, the identification of therapeutic targets whose inhibition could halt these endothelial responses would be highly beneficial in slowing DR pathogenesis. Although endothelial cell inflammatory responses to primary metabolic stimuli are lower in magnitude compared to those seen in Müller cells,<sup>57</sup> endothelial cells still become activated in response to cytokines and contribute to autocrine and paracrine inflammatory signaling.<sup>77</sup> Indeed, it is likely that the potent response of Müller cells to primary metabolic stimuli in the diabetic environment contributes heavily to the initiation and subsequent propagation of inflammation by endothelial cells. NFAT inhibition has been shown to mitigate vascular inflammation in a variety of disease contexts.<sup>47,49,51</sup> Importantly, previous findings demonstrated that NFAT inhibition attenuated TNF $\alpha$ -induced chemokine expression in hRMEC<sup>43,44</sup> and prevented diabetes-induced IL-10 reduction in STZ mice.<sup>50</sup> Therefore, we investigated whether INCA-6 could inhibit hRMEC expression of an array of inflammatory cytokines and chemokines, in a manner similar to the results we saw in hMC. When hRMEC were treated with IL-1 $\beta$  in the presence of INCA-6, NFAT inhibition caused a significant decrease in the expression of IL-1 $\beta$ , TNF $\alpha$ , IL-6, CCL2, and CCL5. Interestingly, NFAT showed differential regulation of inflammatory genes in hRMEC compared to hMC. Given the capacity of each of these cytokines to induce downstream pathological behaviors in endothelial cells, including leukostasis and vascular hyperpermeability,<sup>5,10,18,31,78</sup> we find it highly encouraging that INCA-6 attenuates cytokine expression across a range of genes in hRMEC. Particularly in the case of CCL2 and CCL5, whose primary function is chemotaxis of leukocytes, inhibition in hRMEC is significant owing to the immediate proximity of the endothelium to the circulation. Overall, these results demonstrate that NFAT regulates IL-1 $\beta$ -dependent inflammatory responses in both Müller cells and microvascular endothelial cells, suggesting that targeting NFAT might significantly inhibit retinal inflammation at multiple points along the pathogenic cascade of DR.

Increased leukocyte adhesion, or leukostasis, is considered a landmark event of NPDR and has been observed in both diabetic animals<sup>79-82</sup> and patients.<sup>83-86</sup> Adherent leukocytes contribute to DR pathogenesis in multiple ways, including eliciting and propagating local inflammation as well as occluding capillaries, leading to focal retinal ischemia.<sup>4,5</sup> The diabetic milieu induces the expression of several endothelial cell adhesion proteins leading to increased leukocyte adherence. Specifically, E-selectin is responsible for the initial tethering and rolling of leukocytes on the endothelium, while VCAM-1 and ICAM-1 are responsible for the arrest and firm anchoring of leukocytes.<sup>87,88</sup> Our previous studies demonstrated that targeting NFAT using INCA-6 or NFAT isoform-specific siRNA inhibited TNF $\alpha$ -induced expression and presentation of adhesion proteins in hRMEC and decreased leukocyte adherence to both hRMEC monolayers *in vitro* and blood vessel

walls *in vivo*.<sup>43</sup> Consistent with these data, NFAT inhibition has also proven efficacious in preventing inflammation-induced increases in adhesion molecule levels in a variety of disease contexts.<sup>46,47,51</sup> Therefore, we advanced the hypothesis that INCA-6 would mitigate IL-1 $\beta$ -induced leukocyte adherence owing to decreased NFAT-dependent leukocyte adhesion molecule expression; our results suggest that this is indeed the case. Furthermore, we confirmed that these changes had beneficial functional outcomes by demonstrating the efficacy of INCA-6 against leukocyte adhesion *in vitro* using the PPFC. Finally, we chose to utilize an acute inflammation model to confirm the therapeutic potential of our NFAT inhibitory strategy *in vivo*. Using intraocular injections of IL-1 $\beta$  and an intraocular dose of INCA-6 that had proven efficacious in our hands,<sup>43,68</sup> we modeled the increased leukostasis characteristic of early DR in a high throughput approach with a short experimental timeline and demonstrated that INCA-6 significantly attenuates this IL-1 $\beta$ -induced increase in retinal leukostasis. It is important to note that all our *in vitro* data are exclusively focused on endothelial cells' role in endothelial cell-leukocyte adhesion. However, NFAT was initially characterized in T-lymphocytes and extensive evidence exists supporting its role in leukocyte biology.<sup>89-91</sup> Therefore, it is possible that in the *in vivo* setting INCA-6 is acting not only on endothelial cells, as characterized by our PPFC experiments, but also on circulating immune cells. Further characterization of these effects will be sought in future studies.

The barrier function of the retinal endothelium depends on highly-organized junctional complexes that occlude the paracellular cleft between adjacent endothelial cells.<sup>92</sup> Metabolic dysfunction and increased retinal levels of inflammatory cytokines occurring in DR activate molecular signals that lead to junctional complex disorganization and the associated deficit of barrier function. The changes result in vascular hyperpermeability leading to the development of characteristic fundus lesions of NPDR and/or DME, the most common cause of vision loss in DR patients.<sup>4,5</sup> It is well established that IL-1 $\beta$  promotes retinal vascular hyperpermeability,<sup>5,28,93</sup> and evidence in other disease models suggests that NFAT is involved in control of vascular permeability. For instance, NFAT inhibition attenuated lung vascular permeability in an animal model of sepsis<sup>48</sup> and completely abrogated the retinal vascular hyperpermeability observed in Akita mice.<sup>50</sup> Therefore, we utilized ECIS to measure TEER across hRMEC monolayers and demonstrated that INCA-6 had significant efficacy in attenuating the decreased resistance seen in IL-1 $\beta$ -treated monolayers. We further supported these findings by using qFA to demonstrate that INCA-6 similarly rescued IL-1 $\beta$ -induced hyperpermeability in our model of acute retinal inflammation. Interestingly, in our ECIS experiments, treatment with INCA-6 alone caused an increase in resistance over vehicle, suggesting that INCA-6 can improve barrier function of even healthy hRMEC monolayers. Taken together, our findings demonstrate that NFAT inhibition mitigates multiple inflammatory responses in hRMEC.

A number of important questions remain before NFAT inhibition can be further developed as a therapeutic strategy. For instance, since INCA-6 prevents the binding of NFAT to the activating phosphatase CN, INCA-6 inhibits the action of all four calcium-dependent NFAT isoforms. However, it has been demonstrated that distinct NFAT isoforms can oppose each other in their control of inflammatory gene expression. Indeed, we have previously demonstrated that NFATc2 knockdown prevented TNF $\alpha$ -induced inflammatory responses in hRMEC while NFATc3 increased them.<sup>43</sup> Therefore, it is possible that greater efficacy

could be achieved by identifying the isoform(s) responsible for the behaviors observed here, which would then compel the development of isoform-specific therapies. Our intention in this study was not to investigate specific mechanisms of action of NFAT regulation of each of these events but rather to investigate if NFAT inhibition had broad efficacy across multiple pathogenic events characteristic of early DR. Therefore, isoform-specific studies were outside the scope of this investigation; however, future studies will involve investigation of the isoform-specific roles in controlling each pathogenic behavior with the hopes of developing an NFAT-directed therapeutic strategy with enhanced efficacy. Although NFAT inhibitors have only been tested in pre-clinical settings, CN inhibitors are approved for clinical use and are used in post-kidney transplant and chemotherapy regimens,<sup>39,94</sup> suggesting that therapeutic targeting of this signaling axis is tolerated in humans.

Our studies utilized an acute model of retinal inflammation to allow for the rapid assessment of efficacy of our NFAT inhibitory strategy, and it will be important to expand investigations of NFAT inhibition to diabetic animals with retinopathy. Furthermore, because a number of characteristic NPDR events, such as pericyte dropout or acellular capillaries, are not recapitulated by our acute inflammation model, we did not directly investigate the role of NFAT in their regulation. However, since chronic retinal inflammation is ultimately responsible for driving all types of NPDR lesions, we believe that by attenuating the propagation of early inflammation in DR, NFAT inhibition will likewise attenuate downstream events. Moreover, though our primary focus here was prevention of damage early in DR pathology, significant evidence from our lab and others suggests that NFAT inhibition could also be efficacious against the angiogenesis and neovascularization characteristic of late stage DR.<sup>47,68,95-100</sup> Of particular relevance, our previous work demonstrated that INCA-6 inhibits VEGF-induced hRMEC proliferation and tube formation and that both INCA-6 and the CN inhibitor, FK-506, significantly reduced pathologic neovascularization in a rat model of oxygen-induced retinopathy.<sup>68</sup> These findings further support the therapeutic potential of NFAT inhibition to slow or prevent DR progression at any stage.

There is an urgent need for therapies targeting early DR that prevent disease progression to the irreparable damage caused by PDR. Since early DR pathology involves a variety of altered behaviors in several retinal cells, identifying a therapy that can target multiple pathogenic events in multiple cell types is ideal. To this end, we have demonstrated that NFAT inhibition can attenuate multiple IL-1 $\beta$ -induced pathogenic cell behaviors in both hMC and hRMEC. Combined with our previous findings that NFAT inhibition can similarly prevent TNF $\alpha$ -induced retinal leukostasis,<sup>43</sup> we have significant evidence that NFAT is a target with substantial therapeutic potential for slowing retinal inflammation under DR-relevant conditions. Furthermore, previous studies have demonstrated that NFAT inhibition is similarly efficacious against hyperglycemia-induced retinal changes.<sup>50</sup> When considered in combination with our previous TNF $\alpha$  and current IL-1 $\beta$  findings, it appears that NFAT inhibition can block retinal pathogenic responses elicited by a range of diabetes-relevant stimuli, suggesting NFAT is an excellent target to globally slow the downstream inflammatory effects of the complex diabetic milieu. Overall, we have identified a multi-faceted therapeutic target to inhibit not only Müller cell amplification of diabetic

inflammation but also endothelial pathogenic responses, including increased inflammation, leukocyte adhesion, and vascular hyperpermeability.

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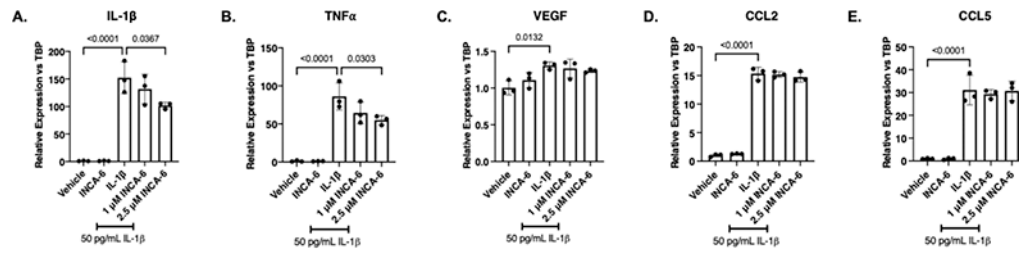
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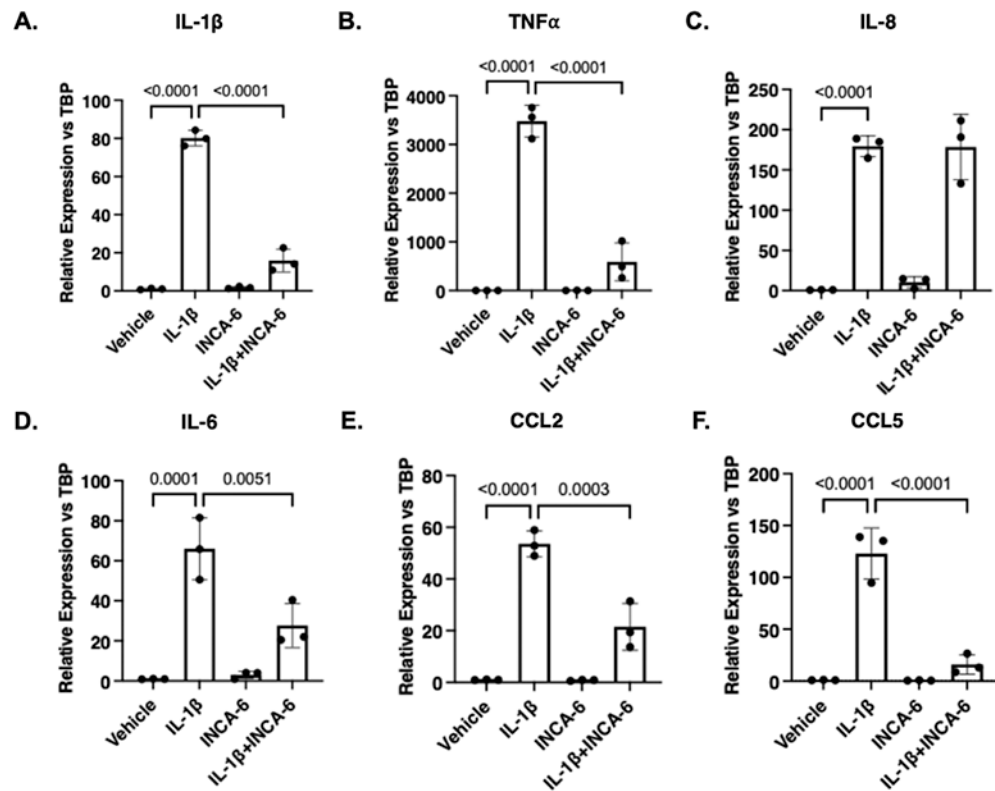
**Highlights**

- In DR, retinal inflammation initiates, propagates, and sustains disease progression
- Targeting NFAT attenuates IL-1 $\beta$ -induced Müller cell inflammation
- NFAT inhibition reduces IL-1 $\beta$ -induced microvascular endothelial cell inflammation
- NFAT inhibition rescues vascular hyperpermeability and leukostasis
- Targeting NFAT has therapeutic potential for attenuating retinal inflammation



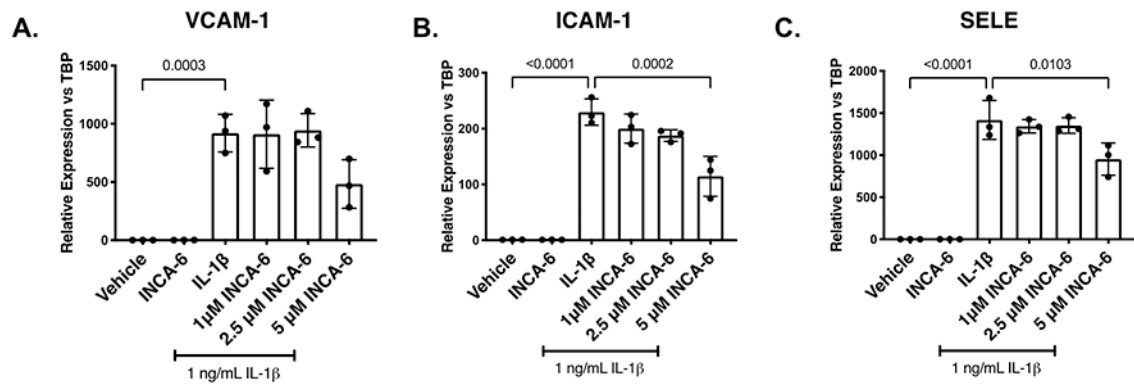
**Figure 1: NFAT inhibition attenuates cytokine expression in hMC.**

hMC were treated for 8hr with 50pg/mL IL-1 $\beta$  with or without INCA-6. Cells were collected and assayed for expression of (A) IL-1 $\beta$ , (B) TNF $\alpha$ , (C) VEGF, (D) CCL2, or (E) CCL5. Expression data are reported as fold induction over vehicle with bars representing mean  $\pm$  SD (n=3).

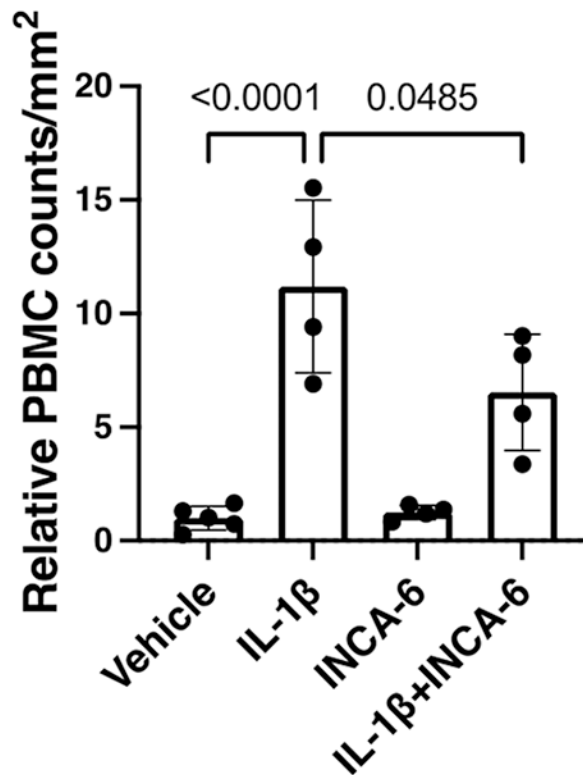


**Figure 2: INCA-6 inhibits IL-1 $\beta$ -induced cytokine expression in hRMEC.**

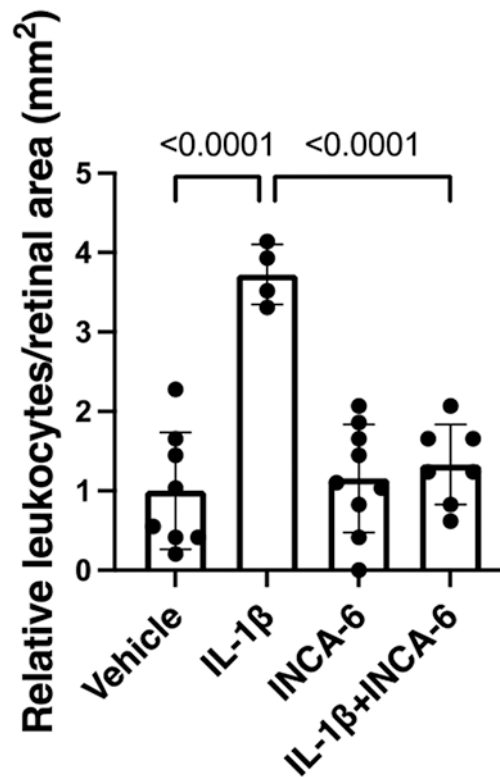
hRMEC were treated for 2hr with 1ng/mL IL-1 $\beta$  with or without 5 $\mu$ M INCA-6. Cells were collected and assayed for expression of (A) IL-1 $\beta$ , (B) TNF $\alpha$ , (C) IL-8, (D) IL-6, (E) CCL2, or (F) CCL5. Expression data are reported as fold induction over vehicle with bars representing mean  $\pm$  SD (n=3).



**Figure 3: INCA-6 inhibits IL-1 $\beta$ -induced expression of leukocyte adhesion molecules in hRMEC.** hRMEC were treated for 2hr with 1ng/mL IL-1 $\beta$  with or without INCA-6. Cells were collected and assayed for expression of (A) VCAM-1, (B) ICAM-1, or (C) E-selectin (gene name: SELE). Expression data are reported as fold induction over vehicle with bars representing mean  $\pm$  SD (n=3).

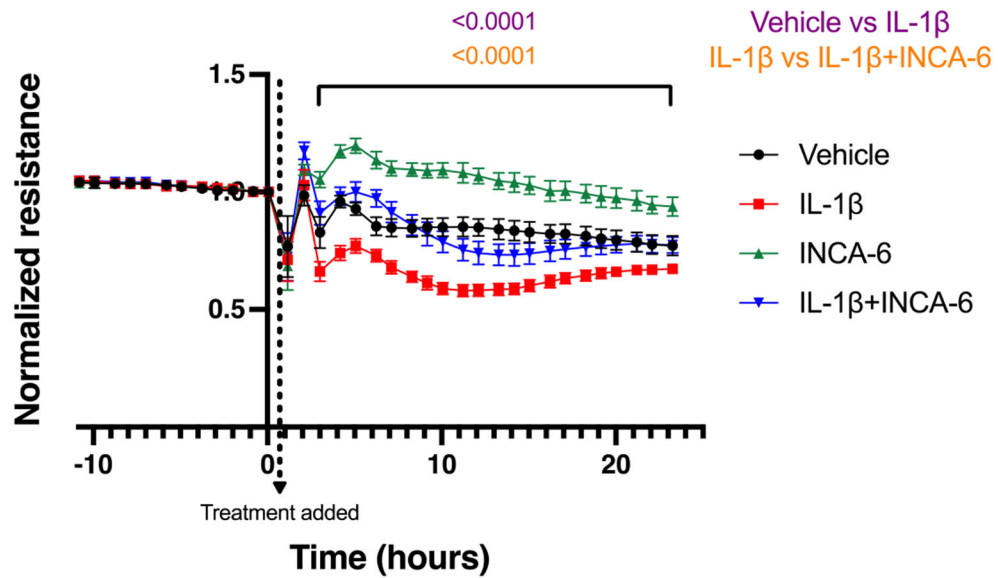


**Figure 4: INCA-6 prevents IL-1 $\beta$ -induced PBMC adhesion to hRMEC.** hRMEC were grown on slides until a confluent monolayer formed and then treated for 4hr with 1ng/mL IL-1 $\beta$  with or without 5 $\mu$ M INCA-6. Slides were placed in a PPFC, PBMC were flowed over the monolayers, and non-adherent cells were washed away. Adherent PBMC were counted in 8 fields per slide and averaged. Average counts were then divided by field area. Data are reported as fold induction over vehicle with bars representing mean  $\pm$  SD (vehicle: n=5; IL-1 $\beta$ : n=4; INCA-6: n=4; IL-1 $\beta$ INCA-6: n=4).



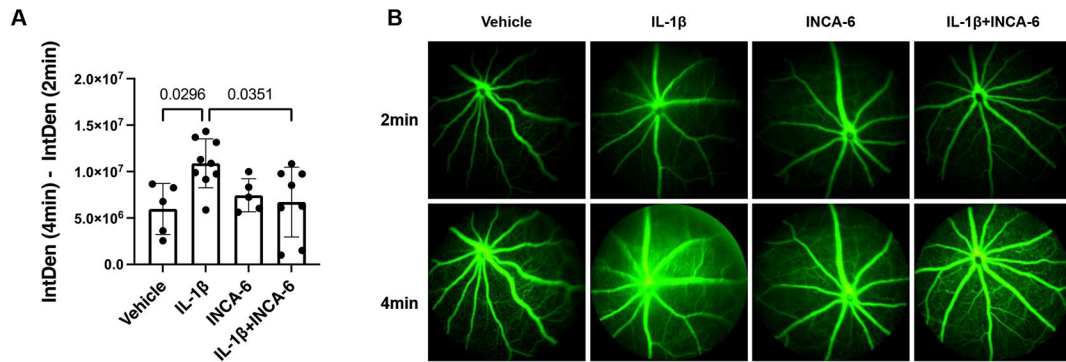
**Figure 5: INCA-6 prevents IL-1 $\beta$ -induced retinal leukostasis in an acute model of retinal inflammation.**

Mice received intravitreal injections of IL-1 $\beta$  +/- INCA-6. 12hr-post injection, leukostasis analysis was performed. Adherent leukocytes were counted in 4 fields per retina and averaged. Average counts were then divided by field area. Data are reported as fold induction over vehicle with bars representing mean +/- SD (vehicle: n=8; IL-1 $\beta$ : n=4; INCA-6: n=9; IL-1 $\beta$ +INCA-6: n=7)



**Figure 6: NFAT inhibition prevents IL-1 $\beta$ -induced hRMEC monolayer hyperpermeability.** hRMEC were allowed to settle and form a monolayer on 96-well ECIS plates. Once resistance plateaued, monolayers were treated with 1ng/mL IL-1 $\beta$  with or without 5 $\mu$ M INCA-6 and changes in resistance were monitored over 24hr. Resistance was normalized to plateaued resistance directly prior to treatment (timepoint 0). Data represent mean  $\pm$  SD (vehicle, IL-1 $\beta$ , and IL-1 $\beta$ +INCA-6: n=12; INCA-6: n=6).





**Figure 7: INCA-6 prevents IL-1 $\beta$ -induced vascular hyperpermeability in an acute model of retinal inflammation.**

Mice received intravitreal injections of IL-1 $\beta$  +/- INCA-6. 8hr post-injection, fluorescein was injected and fluorescent fundus images were captured at 2 and 4min post-fluorescein injection. (A) The difference in integrated density between images was calculated and recorded as a readout of vascular leakage. Bars represent mean +/- SD (vehicle: n=5; IL-1 $\beta$ : n=9; INCA-6: n=5; IL-1 $\beta$ +INCA-6: n=8). (B) Representative images of fluorescent fundus images obtained 2min (top row) and 4min (bottom row) after fluorescein injection.