

# TNF- $\alpha$ -induced ICAM-1 expression and monocyte adhesion in human RPE cells is mediated in part through autocrine VEGF stimulation

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**Purpose:** Local inflammation at the RPE cell layer is associated with inflammatory cell migration and secretion of proinflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$ . TNF- $\alpha$  upregulates intercellular adhesion molecule (ICAM)-1 expression on the RPE, which allows lymphocyte function-associated antigen-1 (LFA-1) to bind on leukocytes that contribute to leukocyte adhesion at sites of inflammation. Vascular endothelial growth factor (VEGF)-A<sub>165</sub>b is generated by alternative splicing of VEGF-A in the terminal exon, exon 8. VEGF-A<sub>165</sub>b is cytoprotective and antiangiogenic, but its effects on inflammation have not yet been elucidated. Therefore, we tested the hypothesis that VEGF-A<sub>165</sub>b regulates TNF- $\alpha$ -induced ICAM-1 expression and monocyte adhesion in RPE cells.

**Methods:** Primary RPE cells were pretreated with TNF- $\alpha$  alone, VEGF-A<sub>165</sub>b alone, VEGF-A<sub>165</sub>b with anti-VEGF-A<sub>165</sub>b, or the VEGFR-2 inhibitor ZM323881 before exposure to TNF- $\alpha$  for 24 h. Western blotting and monocyte adhesion assays were performed.

**Results:** VEGF-A<sub>165</sub>b and ZM323881 inhibited TNF- $\alpha$ -induced upregulation of ICAM-1 in RPE cells. The effect of VEGF-A<sub>165</sub>b was neutralized by an antibody to VEGF-A<sub>165</sub>b. VEGF-A<sub>165</sub>b ameliorated TNF- $\alpha$ -induced monocyte-RPE adhesion.

**Conclusions:** These findings indicate that VEGF-A<sub>165</sub>b inhibits TNF- $\alpha$ -mediated upregulation of ICAM-1 expression and increases monocyte-RPE cell adhesion, suggesting an anti-inflammatory property of VEGF-A<sub>165</sub>b in the eye.

The RPE is essential for visual function, including retinal chromophore regeneration, nutritional and metabolic support of photoreceptors, and phagocytosis and degradation of shed photoreceptor outer segments [1]. Functionally, RPE cell loss causes the progression of retinal degeneration. For instance, it has been reported that lymphocytes and macrophages migrate to the posterior compartment of the eye and secrete proinflammatory mediators, interleukin (IL)-1 $\beta$ , interferon (IFN)- $\gamma$ , and tumor necrosis factor (TNF)- $\alpha$  [2,3]. These inflammatory cytokines can target and impair RPE function, causing the pathogenesis of well-defined inflammatory diseases of the retina such as uveoretinitis and age-related macular degeneration [4,5].

Several studies have demonstrated that intercellular adhesion molecule-1 (ICAM-1), a transmembrane glycoprotein, binds to two integrins of the  $\beta$ 2 subfamily on leukocytes that mediate leukocyte adhesion and transmigration [6,7]. ICAM-1 is present at low levels on the cell surface of various

cell types but is upregulated in response to inflammatory mediators, including retinoic acid and the proinflammatory cytokine TNF- $\alpha$  [8,9]. Previous studies have shown that TNF- $\alpha$  induces the upregulation of ICAM-1 in many cell types, including smooth muscle cells [10], keratinocytes [11], intestinal epithelial cells [12], and endothelial cells [13].

Human vascular endothelial growth factor (VEGF)-A is produced by alternative splicing from eight exons within the VEGF gene to form different mRNAs encoding at least 14 different proteins in two families, the proangiogenic VEGF-A<sub>xxx</sub>a family and the antiangiogenic VEGF-A<sub>xxx</sub>b family, where xxx refers to the number of amino acids of the secreted isoform [14]. Exons 1–5 and the terminal exon, exon 8, are contained in all isoforms except exons 6 and 7, which encode heparin-binding domains, and can be included or excluded [15]. VEGF-A<sub>xxx</sub>b isoforms are formed by alternative distal splice site selection (DSS) in exon 8, forming an mRNA containing 19 bases coded by exon 8b whereas VEGF-A<sub>xxx</sub>a isoforms are generated by proximal splice site selection (PSS) resulting in encoding by 19 bases of exon 8a [15]. This alternative splicing generates proteins of the same length but with differing C-terminal amino acid sequences [16]. Exon 8a codes for CDKPRR and exon 8b codes for SLTRKD.

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Therefore, exon 8b lacks the cysteine (Cys) residue, which forms the disulfide bond [17], and the terminal two charged arginine (Arg) residues, which are involved with receptor signaling [18]. Exon 8b codes for serine (Ser) instead of Cys and a less basic C-terminal than exon 8a. The receptor binding domains are still present in VEGF-A<sub>165</sub>b, which acts as a competitive inhibitor of VEGF-A<sub>165</sub>a (i.e., it binds to the receptors but inhibits angiogenesis signaling) but also as a partial agonist of VEGFR-2 resulting in cell survival of RPE and endothelial cells [19] and neurons [20].

Angiogenic and antiangiogenic VEGF isoforms have been identified in the human retina, vitreous, and iris [16] and the rodent eye [21]. VEGF-A<sub>165</sub>b has also been shown to have an antiangiogenic effect in the rabbit cornea [22], mouse dorsal chamber, and mouse mammary gland [23]. Furthermore, VEGF-A<sub>165</sub>b is downregulated in diabetic retinopathy resulting in the switching to an angiogenic phenotype [16]. Therefore, distal splicing in the VEGF-A gene results in proteins that can act antagonistically on some effects (e.g., permeability, angiogenesis), but similarly on others (e.g., cytotoxicity, neuroprotection). VEGF-A<sub>165</sub>a has been shown to modulate inflammatory pathways, resulting in upregulation of ICAM-1 on retinal vascular endothelial cells [24], and VEGFR-2 has been shown to be expressed on RPE cells [25,26]. Furthermore, TNF- $\alpha$  has been shown to switch splicing of the VEGF gene from anti- to proangiogenic isoforms of VEGF-A in RPE cells [27] although the role of VEGF-A<sub>165</sub>b in inflammation in RPE, particularly in relation to regulating monocyte recruitment and adhesion processes, is not known. Therefore, the present study aimed to test the hypothesis that the effect of proinflammatory cytokine TNF- $\alpha$  on RPE cells to induce ICAM-1 expression and function is regulated by VEGF-A<sub>165</sub>b.

## METHODS

**Cell culture and treatment:** Human donor eyes were obtained within 10–30 h post-mortem from the Bristol Eye Bank (Bristol, UK) in Accordance with the Declaration of Helsinki, and the local ethics committee (United Bristol Healthcare Trust). Choroid-RPE sheets dissected from ocular globes were fragmented in a small amount of Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY):F12 (1:1) supplemented with GlutaMAX (Gibco), and RPE cells were digested for isolation with 0.3 mg/ml collagenase (Gibco) for 15 min at 37 °C. Cells and solution were collected and centrifuged at 450  $\times$ g for 10 min. Supernatant was discarded, and the pellet was resuspended in conditioned media with 25% (v/v) fetal bovine serum for 5 days to prevent fibroblast proliferation until the cell cultures became established.

After the first passage, the cell culture was maintained in DMEM:F12 (1:1) with 10% v/v fetal bovine serum (FBS) and antibiotic-anti-mycotic solution (Sigma Chemicals, St. Louis, MO). RPE cells were sub-cultured with trypsinization (trypsin/EDTA, Sigma). RPE cells at passage 3–4 were plated onto a T25 flask. Once the cells reached 80–100% confluence, experiments were performed. This resulted in cells that were RPE65 and keratin 18 positive, confirming that they are retinal pigmented epithelial cells. We called these cells primary RPE to distinguish them from immortalized cell lines.

**Enzyme-linked immunosorbent assay:** The RPE cells were pretreated with various doses (1, 2, 10, 20, and 100 ng/ml) of TNF- $\alpha$  for 24 h, and then the condition media were collected to perform enzyme-linked immunosorbent assay (ELISA). Total VEGF-A concentrations were measured using a DuoSet VEGF-A ELISA (R&D system Minneapolis, MN) following the kit's instructions. This kit has a lower affinity for VEGF-A<sub>165</sub>b than for VEGF-A<sub>165</sub>. Total VEGF-A (VEGF-A<sub>total</sub>) and VEGF-A<sub>xxx</sub>b were determined with sandwich ELISA, using a monoclonal biotinylated mouse anti-human VEGF-A or VEGF-A<sub>165</sub>b antibody, respectively (DY293 and DY3045, respectively; R&D Systems), and a standard curve for this assay was built with recombinant human VEGF-A<sub>165</sub>a and VEGF-A<sub>165</sub>b (R&D Systems) to correct for the affinity difference [28]. The total protein content in the conditioned media was measured using a Bradford assay from Bio-Rad Laboratories (Hercules, CA).

**Investigation of SRSF1 nuclear localization:** Primary RPE cells were treated with 20 ng/ml TNF- $\alpha$  for 12 h. After incubation, the cells were fixed in 4% paraformaldehyde for 5 min, and blocked with 1% normal goat serum in 0.05% Triton X-100 for 30 min. The cells were incubated overnight at 4 °C with a mouse monoclonal anti-Serine/arginine-rich splicing factor 1 (SRSF1) primary antibody (sc-33652, Santa Cruz, Santa Cruz, CA, diluted 1:100). A negative control was used where the primary antibodies were omitted. After washing in PBS-Tween (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>), the cells were incubated with Alexa555 (red)-conjugated secondary antibody (diluted 1:500; Invitrogen, Carlsbad, CA) for 1 h at 37 °C for 45 min. The nuclei of cells were counterstained with 1  $\mu$ g/ml Hoechst 33322 (Sigma) and then visualized under a Nikon E600 (Nikon, Tokyo, Japan) fluorescence microscope.

**Determination of ICAM-1 by western blot analysis:** RPE cells were treated with VEGF or TNF at the concentrations noted for 24 h. Inhibition experiments were performed by pretreating the cells for 1 h with the inhibitor or antibody before treatment with the growth factor. At the required

time, the cells were lysed in radioimmunoprecipitation assay (RIPA; Sigma) buffer for 5 min. The lysate was collected in a 1.5 ml microfuge tube and centrifuged at 12,000  $\times g$  for 15 min. The supernatant was kept at  $-80^{\circ}\text{C}$  until used. Protein concentration was determined using the Bradford protein assay kit (Bio-Rad, Hercules, CA). Twenty micrograms of protein was mixed with loading buffer and loaded onto a 15% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) electrophoresis. The proteins were transferred onto a nitrocellulose membrane and blocked with 5% nonfat dry milk in 1X Tris-buffered saline (50 mM, pH 8.0) containing 0.1% Tween-20 for 2 h. Membranes were incubated with primary and then secondary antibodies and developed using the Amersham Biosciences chemiluminescence ECL kit and Hyperfilm ECL (Piscataway, NJ). Primary antibodies used in this study were rabbit anti-ICAM-1 and mouse anti-alpha tubulin diluted 1:1,000. Horseradish peroxidase (HRP) conjugated anti-rabbit and HRP conjugated anti-mouse immunoglobulin G (IgG) were used as secondary antibodies at 1:2,000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoblots were quantitated using densitometry Scion image software package (a version of the NIH Image program developed at the US National Institutes of Health and available from the [Scion Corporation](#), Frederick, MD). Protein expression was reported as the expression relative to that of the respective control.

**Monocyte-RPE adhesion assay:** The human monocytic leukemia cell line (THP-1), a phagocytic cell that originated from the peripheral blood of a man with acute monocytic leukemia, was obtained from the American Type Culture Collection (ATCC, Mantissa, VA), and cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 IU/ml of penicillin, 100  $\mu\text{g}/\text{ml}$  of streptomycin, and 2 mM L-glutamine. THP-1 cells were grown at least 80–100% confluent and then trypsinized to dissociate the cells. Calcein AM (Sigma) was added to the cell suspension at a final concentration of 2.5  $\mu\text{M}$ . Cells were mixed gently by inversion and incubated in a  $\text{CO}_2$  incubator at  $37^{\circ}\text{C}$  for 1 h, and then centrifuged gently at 400  $\times g$  for 5–10 min. The supernatant was discarded, and the cell pellet was washed twice with PBS. After washing, cells were gently resuspended in RPMI-1640 with 10% FBS and 1% penicillin/streptomycin.

RPE cells were grown to confluence in six-well plates (confirmed visually with phase contrast microscopy) and then unstimulated as a control group or stimulated with 20 ng/ml TNF- $\alpha$  for 24 h. Cells were pretreated with 100 ng/ml VEGF- $\text{A}_{165}\text{b}$  for 1 h with or without 20 ng/ml TNF- $\alpha$  for 24 h. To confirm that monocyte adhesion to RPE cells was mediated through the binding of cell adhesion molecules ICAM-1, the

cells were treated with 100 ng/ml anti-ICAM-1 antibody after exposure with TNF- $\alpha$ . Calcein-labeled THP-1 ( $1 \times 10^6$ ) cells were overlaid onto the RPE cells and incubated at  $37^{\circ}\text{C}$  for 2 h. Cells were rinsed with PBS, and then monocyte-RPE adhesion was observed under a Nikon E600 fluorescent microscope. The number of fluorescence-labeled THP-1 was determined from five randomly selected lower power fields (10X) for each treatment from three independent experiments, and the fluorescence level was determined using a Wallac 1420 Victor plate reader (Wallac1420; PerkinElmer, Waltham, MA) with 485/530 nm excitation/emission filter sets.

**Statistical analysis:** Data were presented as means  $\pm$  standard error of the mean (SEM) from three or more independent experiments. Significance was assessed with one-way ANOVA (ANOVA) followed by Tukey's multiple comparison test in the [GraphPad Prism](#) program version 5 (GraphPad, San Diego, CA). Difference with p values less than 0.05 was considered statistically significant.

## RESULTS

**TNF- $\alpha$  induces ICAM-1 expression on RPE cells:** To confirm previous studies that identified TNF- $\alpha$ -induced upregulation of ICAM-1, we treated RPE cells with TNF- $\alpha$  for 24 h and measured ICAM-1 expression with western blot analysis. The level of ICAM-1 protein dose-dependently increased in cells treated with TNF- $\alpha$  (Figure 1A). The relative expression of ICAM-1 in cells treated with TNF- $\alpha$  at concentrations of 0.02, 0.2, 2, and 20 ng/ml were  $131.1 \pm 8.3\%$ ,  $160.7 \pm 13.6\%$ ,  $241.0 \pm 17.6\%$ , and  $422.5 \pm 19.2\%$  of the control, respectively (Figure 1B).

**TNF- $\alpha$  induced a proangiogenic VEGF isoform:** To confirm that TNF- $\alpha$  induced proangiogenic VEGF splicing, the amount of VEGF- $\text{A}_{xxx}\text{b}$  (antiangiogenic isoforms) and VEGF- $\text{A}_{\text{total}}$  in RPE cells treated with 20 ng/ml TNF- $\alpha$  was determined with ELISA. Cells treated with TNF- $\alpha$  (20 ng/ml) significantly increased VEGF- $\text{A}_{\text{total}}$  from the control cells (Figure 1C, control,  $179.4 \pm 1.1$  pg/mg protein; TNF- $\alpha$ -treated cells,  $256.6 \pm 5.9$  pg/mg protein). However, the amount of VEGF- $\text{A}_{xxx}\text{b}$  secreted from the TNF- $\alpha$ -treated cells was not different from the control (control,  $26.3 \pm 1.9$  pg/mg protein; TNF- $\alpha$ -treated cells  $26.5 \pm 1.3$  pg/mg protein). This was true for all doses investigated (Figure 1D). These results indicated that the increased amount of VEGF- $\text{A}_{\text{total}}$  by the TNF- $\alpha$  insult was restricted to the proangiogenic VEGF- $\text{A}_{xxx}\text{a}$  isoform. We have previously shown that VEGF- $\text{A}_{165}\text{a}$  expression in RPE cells is regulated by the splice factor SRSF1 in response to Insulin-like growth factor 1 (IGF). We stained cells for SRSF1 and found that TNF- $\alpha$  induced increased nuclear SRSF1 translocation (Figure 1E).

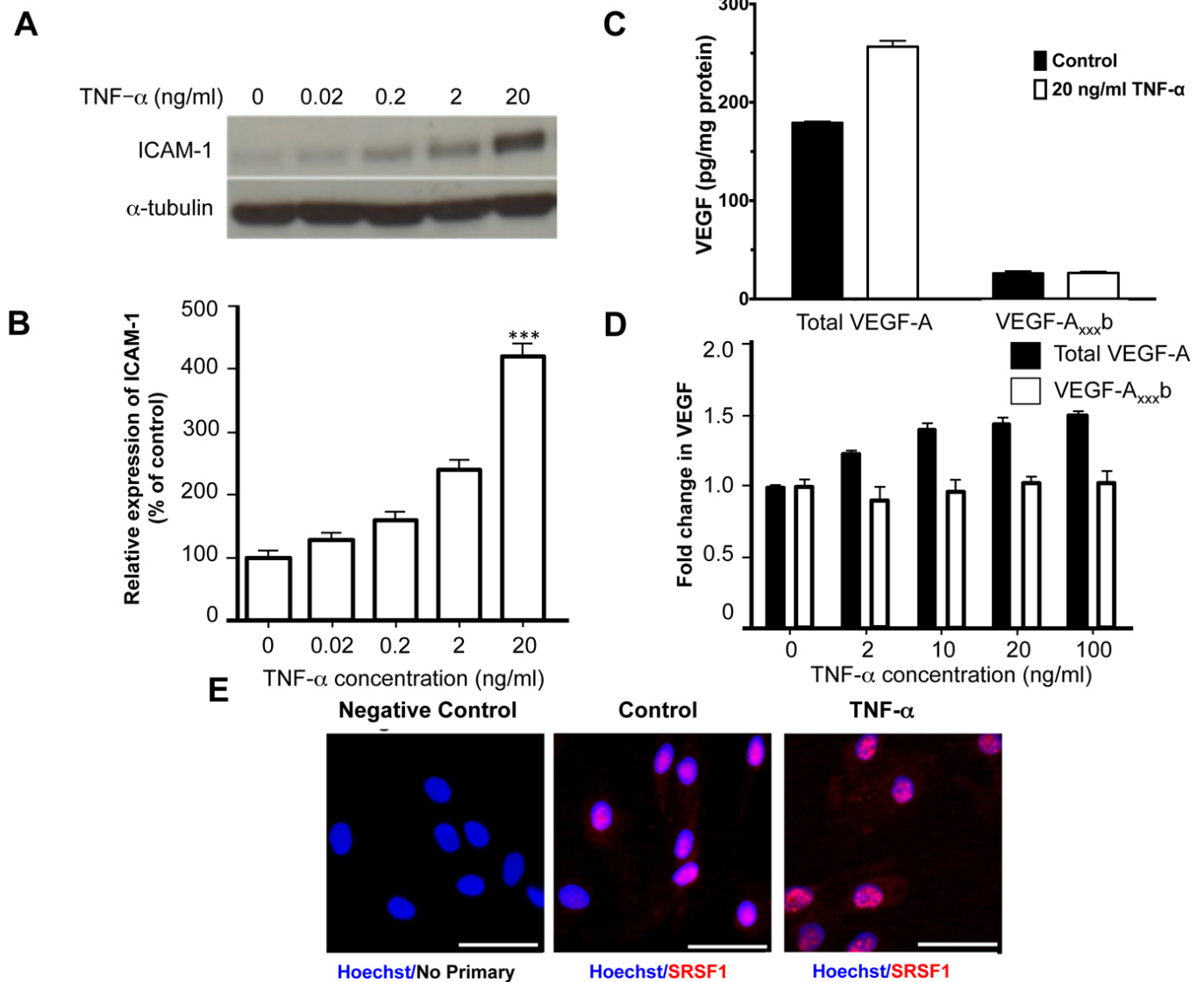


Figure 1. Primary RPE cells were treated with various concentrations of TNF- $\alpha$  (2, 10, 20, and 100 ng/ml) for 24 h or left untreated. **A:** Immunoblot showing tumor necrosis factor (TNF)- $\alpha$ -induced intercellular adhesion molecule-1 (ICAM-1) expression. **B:** Relative expression of the ICAM-1 protein. **C and D:** The amount of total vascular endothelial growth factor (VEGF) and VEGF-A<sub>165</sub>b proteins released from RPE cells into media determined with enzyme-linked immunosorbent assay (ELISA). **E:** Fluorescent micrographs showing nuclear localization of SRSF1 (red). Nuclei were counterstained with Hoechst (blue). Control RPE cells showed expression of SRSF1 in the nuclei and in the cytoplasm. TNF- $\alpha$  induced more nuclear localization of SRSF1. Scale bar = 20  $\mu$ m.

*The proangiogenic isoform VEGF-A<sub>165</sub>a-induced ICAM-1 expression:* VEGFR-2 has been shown to be present on RPE cells [25,27], and VEGF has been shown to induce ICAM-1 on retinal vascular endothelial cells [9]. Therefore, VEGF-A might also be able to act on RPE cells through VEGFR-2 to induce ICAM-1 on RPE cells. To test this, cells were treated with different concentrations of VEGF-A<sub>165</sub>a (1, 10, 50, and 100 ng/ml) for 24 h. The results showed that VEGF-A<sub>165</sub>a increased expression of ICAM-1 (Figure 2A) to 133.1 $\pm$ 8.2%, 203.0 $\pm$ 12.4%, and 397.3 $\pm$ 19.0% of the control, respectively

(Figure 2B). Additionally, the effect of VEGF-A<sub>165</sub>a was suppressed when the cells were pretreated for 1 h with 10 nM VEGFR-2 tyrosine kinase inhibitor ZM323881 (ZM323881, Calbiochem, Darmstadt, Germany) or an antibody to VEGF-A<sub>165</sub>a (Figure 2). The results suggested that the proangiogenic VEGF-A<sub>165</sub>a isoform itself mediated upregulation of ICAM-1 via the VEGFR-2 pathway. To determine whether the antiangiogenic isoform VEGF-A<sub>165</sub>b could counteract the effect of VEGF-A<sub>165</sub>a, cells were pretreated with 100 ng/ml VEGF-A<sub>165</sub>b for 1 h before exposure to VEGF-A<sub>165</sub>a

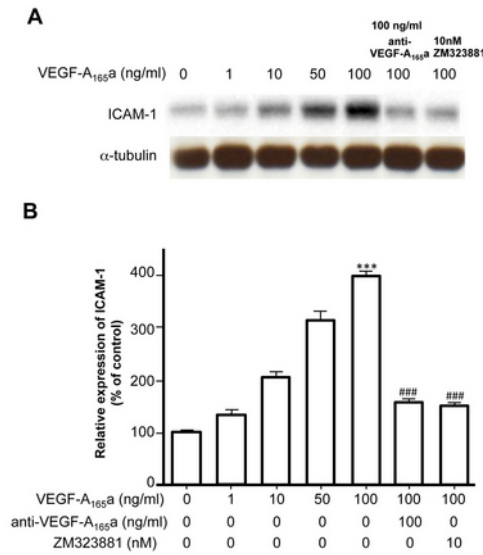


Figure 2. VEGF-A<sub>165a</sub>-induced ICAM-1 in primary RPE cells. **A**: Immunoblot for ICAM-1 expression. The effect was inhibited by anti-vascular endothelial growth factor (VEGF)-A<sub>165a</sub> and VEGFR-2 inhibitor, ZM323881. **B**: Relative expression of the intercellular adhesion molecule (ICAM-1) protein. ICAM-1 was normalized to  $\alpha$ -tubulin and expressed as a percentage of the control group. Data are expressed as the mean  $\pm$

standard error of the mean (SEM) of three independent experiments; \*\*\*p<0.001 compared with the control, ###p<0.001 compared with 100 ng/ml VEGF-A<sub>165a</sub>-treated group.

for 24 h. The results revealed that VEGF-A<sub>165b</sub> attenuated VEGF-A<sub>165a</sub>-induced ICAM-1 expression (Figure 3A), since ICAM-1 expression was decreased to 265.6 $\pm$ 6.6% of the control (Figure 3B).

*VEGF-A<sub>165b</sub> decreased TNF- $\alpha$ -induced ICAM-1 expression:* Since TNF- $\alpha$  induced VEGF-A<sub>165a</sub> expression and VEGF-A<sub>165a</sub> induced ICAM-1 expression, then it is possible that the TNF- $\alpha$  induced upregulation of ICAM-1 is, at least in part, due to its upregulation of VEGF-A<sub>165a</sub> and an autocrine effect on RPE cells. To test this, we determined whether TNF- $\alpha$

induced ICAM-1 expression could be blocked, at least in part, by inhibiting VEGF-A<sub>165a</sub>, either with VEGF-A<sub>165b</sub>, or by VEGFR-2 inhibition. To test this, cells were pretreated with 100 ng/ml VEGF-A<sub>165b</sub> or the VEGFR-2 inhibitor ZM323881 for 1 h before additional exposure to 20 ng/ml TNF- $\alpha$  for 24 h (Figure 4A). Both significantly decreased ICAM-1 expression to 272 $\pm$ 10.9% and 160 $\pm$ 11.3% of the control, respectively (compared with 422 $\pm$ 19.2% with TNF- $\alpha$  alone, Figure 4B). The inhibitory effect of VEGF-A<sub>165b</sub> was neutralized when the cells were simultaneously treated with an anti-VEGF-A<sub>165b</sub>

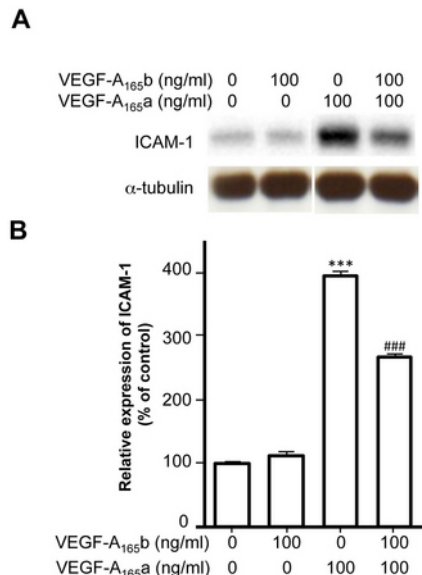


Figure 3. VEGF-A<sub>165b</sub> inhibited VEGF-A<sub>165a</sub>-ICAM-1 expression in primary RPE cells. **A**: Immunoblot for ICAM-1 expression in primary RPE cells. **B**: Relative expression of the intercellular adhesion molecule-1 (ICAM-1) protein. ICAM-1 was normalized with  $\alpha$ -tubulin (as the internal control) and expressed as a percentage of the control group. Data are expressed as the mean  $\pm$  standard error of the mean (SEM) of three independent experiments; \*\*\*p<0.001 compared with the control, ###p<0.001 compared with VEGF-A<sub>165a</sub> treated alone.

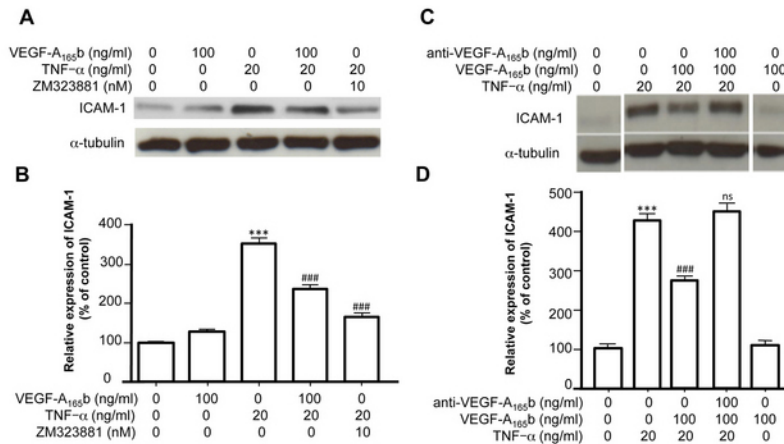


Figure 4. TNF induces ICAM-1 expression through VEGF induction. **A**: Immunoblot and **(B)** quantitation showing the effect of VEGF-A<sub>165</sub>b and ZM323881, a selective inhibitor of VEGFR-2 on TNF- $\alpha$ -induced ICAM-1 expression and **(C, D)** the inhibitory effect of VEGF-A<sub>165</sub>b was neutralized with an anti-VEGF-A<sub>165</sub>b antibody. Intercellular adhesion molecule-1 (ICAM-1) was normalized to  $\alpha$ -tubulin and expressed as a percentage of the control group. Data are expressed as the mean  $\pm$  standard error of the mean (SEM) of three independent experiments; \*\*\* $p$ <0.001 compared with the control, ### $p$ <0.001 compared with tumor necrosis factor (TNF)- $\alpha$  treated alone, *ns* was not significant when compared with TNF- $\alpha$  treated alone.

antibody (Figure 4C,D). These results indicated that the TNF- $\alpha$ -induced expression of ICAM-1 protein is mediated through endogenous VEGF-A<sub>165</sub>a upregulation.

*VEGF-A<sub>165</sub>b blocked TNF- $\alpha$ -induced monocyte-RPE adhesion:* To investigate whether inhibiting VEGF-A<sub>165</sub>a-mediated ICAM-1 expression with VEGF-A<sub>165</sub>b can functionally block the effects of TNF- $\alpha$ -induced monocyte-RPE adhesion, confluent RPE cells were treated with VEGF-A<sub>165</sub>b before TNF- $\alpha$  and monocyte-RPE adhesion was determined (Figure 5). The results showed that treatment of RPE cells for 24 h with TNF- $\alpha$  (20 ng/ml) caused a significant increase in monocyte adhesion and this effect was blocked by VEGF-A<sub>165</sub>b and neutralization with an anti-ICAM-1 antibody (Figure 5). However, the VEGF-A<sub>165</sub>b pretreatment alone had no effect on either monocyte adhesion or upregulation of ICAM-1 expression. These findings indicated that TNF- $\alpha$ -mediated ICAM-1 induced monocyte adhesion to RPE cells involving the VEGF pathway.

## DISCUSSION

In this study, primary RPE cells exposed to the proinflammatory cytokine, TNF- $\alpha$  upregulation of the inflammatory protein ICAM-1 and increased monocyte-RPE adhesion. TNF- $\alpha$  modulated the splicing of VEGF isoforms, increasing the nuclear translocation of the splicing factor SRSF1, which has been shown to result in production of the angiogenic isoform VEGF-A<sub>165</sub>a. Changing the VEGF-A<sub>165</sub>a:VEGF-A<sub>165</sub>b ratio appears to be a mechanism that could underlie the TNF- $\alpha$ -induced inflammatory responses in the RPE.

TNF- $\alpha$  has previously been shown to upregulate VEGF-A<sub>165</sub>a expression, and RPE cells have previously been shown to express VEGFR-2. We previously showed that 50 ng/ml TNF- $\alpha$  downregulated VEGF-A<sub>165</sub>b protein in RPE cells from 109 to 38 pg/mg protein, and upregulated total VEGF from 258 to 1,037 pg/mg. However, here we saw no downregulation of VEGF-A<sub>165</sub>b by 20 ng/ml TNF- $\alpha$  (the level of VEGF-A<sub>165</sub>b in these primary RPE cells was lower, 26 pg/mg, than in previous studies) and smaller, but consistent, upregulation of total VEGF. The total VEGF and VEGF-A<sub>165</sub>b levels vary from cell preparation to cell preparation (in this study and the previous study, eyes from at least three donors were used). However, the finding that TNF- $\alpha$  selectively upregulates the proangiogenic isoforms is consistent with previous findings.

The cells we used were freshly isolated RPE cells used at passages 3–4 at confluency or close to confluency. These cells are unlikely to have reached a fully differentiated state, as polarization, and full differentiation takes approximately 4 weeks of confluency [29]. However, in AMD and in uveoretinitis, RPE cells are likely to be in an injured state, so the findings here, although in a model that is more akin to an injury recovery, are perhaps more relevant than a fully differentiated cell culture model.

The key finding here is that TNF- $\alpha$  induces proinflammatory gene expression (e.g., ICAM-1) at least in part through increases in proangiogenic VEGF expression. Other pathways are clearly also involved, as neither VEGFR-2 inhibition nor VEGF-A<sub>165</sub>b completely abrogated the increase in expression, but the VEGF pathway appears to be a significant

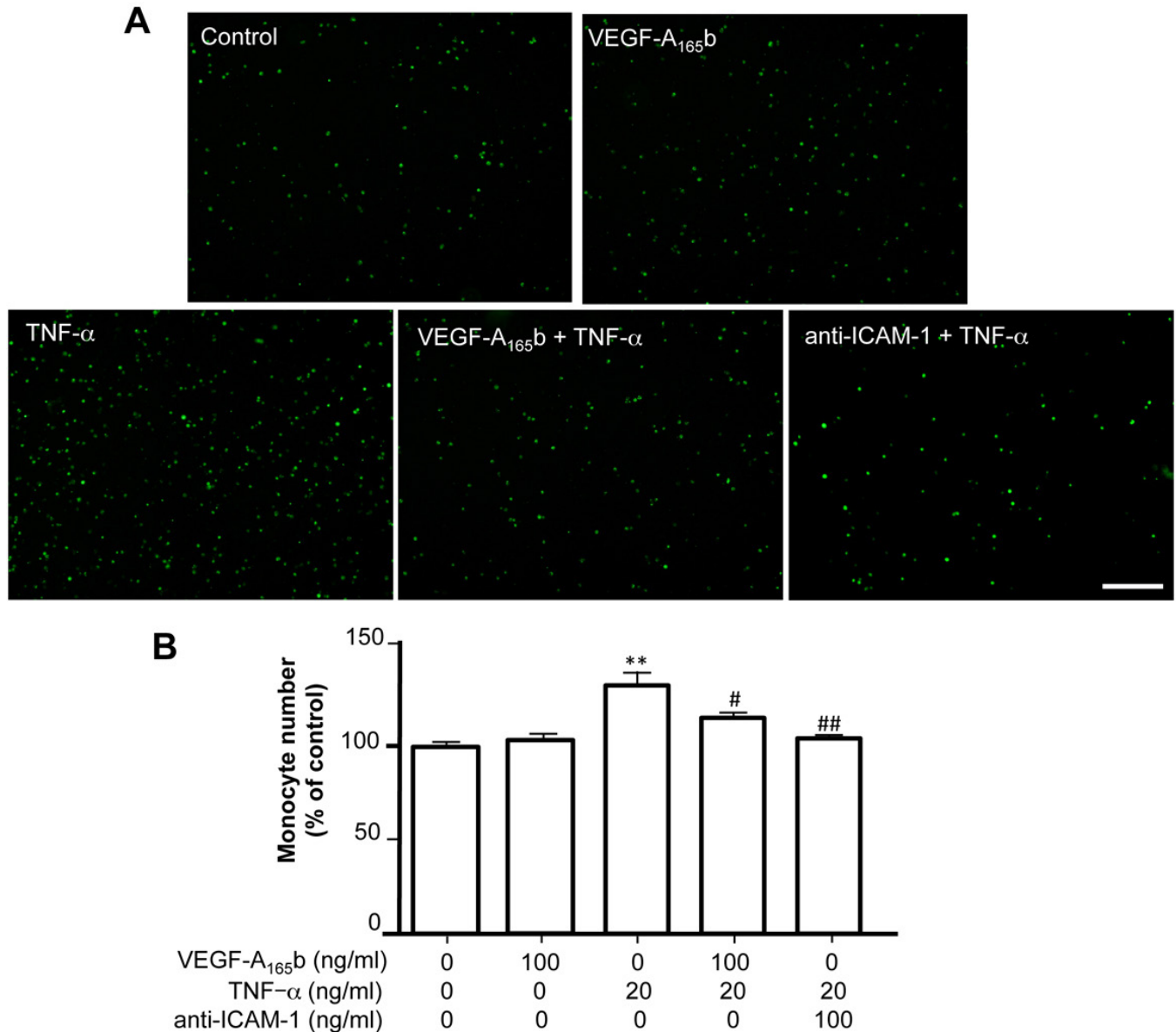


Figure 5. VEGF-A<sub>165</sub>b decreased the number of monocytes that adhered to RPE cells induced by TNF- $\alpha$ . **A:** Fluorescent micrographs from different treatments showing the adherence of calcein-labeled THP-1 (green) to RPE cells. **B:** The number of fluorescent-labeled THP-1 that adhered to the RPE cell monolayer. Data are expressed as mean  $\pm$  standard error of the mean (SEM); three independent experiments, each from six replicate wells; \*\*p<0.01 compared with control, ##p<0.01 and #p<0.05 compared with tumor necrosis factor (TNF)- $\alpha$ -treated alone. Scale bar = 200  $\mu$ m.

contributory factor. This has several implications for therapy. It would indicate that antiangiogenic drugs, such as ranibizumab, could be anti-inflammatory as well, and that part of their actions could be through an anti-inflammatory process. To do so, they would need to be able to prevent the VEGF induced by TNF- $\alpha$  from acting on the RPE outside the cell. We have not yet shown whether the TNF- $\alpha$ -VEGF-ICAM-1 pathway can mediate inflammatory processes in vivo, but these results suggest that inhibition of VEGF may act

through anti-inflammatory processes as well as antiangiogenic processes and that human recombinant antiangiogenic isoforms such as VEGF-A<sub>165</sub>b can be anti-inflammatory on RPE cells stimulated by TNF- $\alpha$ .

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