Cytokine xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

IL-27 regulates HIF-1α-mediated VEGFA response in macrophages of diabetic retinopathy patients and healthy individuals

Q. Zhang[a,1], A.P. da Cunha[a,1], S. Li[b], Q. Hao[b], V. Kainz[a], Q. Huang[a], H.Y. Wu[a,*]

[a] Department of Ophthalmology, Novartis Institutes for Biomedical Research, Cambridge, MA 02139, USA
[b] Department of Analytical Sciences and Imaging, Novartis Institutes for Biomedical Research, Cambridge, MA 02139, USA

ARTICLE INFO

Keywords:
IL-27
The purinergic pathway
HIF1α
VEGFA
Human macrophages
Diabetic retinopathy

ABSTRACT

Human macrophages produce vascular endothelial growth factor A (VEGFA) for angiogenesis in diabetic retinopathy (DR). The regulatory function of IL-27 on human macrophages is not well understood. In particular, the effect of IL-27 on VEGFA response in human macrophages has not been investigated. We find that IL-27 suppresses *VEGFA* mRNA expression as well as protein secretion by human macrophages. The synergistic action of purinergic signaling and activation of hypoxia-inducible factor 1 alpha (HIF-1α) induces VEGFA production in a positive feedback loop. IL-27 signaling in human macrophages disrupts this positive feedback loop thus suppresses VEGFA production. Blockade of IL-27 signaling with a JAK2 antagonist reverses this downregulatory effect on HIF-1α and partially blocks the inhibitory effect on VEGFA production. Lastly, DR patient macrophages have a higher propensity to produce VEGFA and this is amplified by an *in vitro* challenge with the pro-inflammatory cytokine IL-1β. IL-27 suppresses VEGFA production by DR patient macrophages even in the presence of IL-1β challenge indicating a potential therapeutic use of IL-27 in the clinic.

1. Introduction

IL-27 was first described by S. Pflanz and colleagues in 2002 as specifically augmenting the production of interferon-γ (IFN-γ) from Th1 cells [1]. As Th1 cells contribute to the pathogenesis of a number of inflammatory diseases, IL-27 was initially viewed as a cytokine with pro-inflammatory activity. However, accumulating evidence has challenged this initial view and rallied an appreciation for its anti-inflammatory activities in mice and human diseases [2]. IL-27 is predominantly produced by myeloid cell populations including macrophages, inflammatory monocytes, microglia, and dendritic cells (DCs), although plasma cells, endothelial cells, and epithelial cells can also express IL-27 [3]. While the effect of IL-27 on effector T cell subsets and regulatory T cells (Tregs) is well documented, its autocrine function on macrophages is less well understood.

Recent evidence has implicated an important role of macrophages in regulating angiogenesis during steady state and in disorders of aging such as cancers, atheromatous heart disease and blinding diseases such as diabetic retinopathy (DR) [4–9]. The cardinal feature in the pathophysiology of DR is abnormal angiogenesis in the retinal vasculature [10]. Inflammatory macrophages can contribute to the process of neovascularization in DR by producing a variety of mediators including pro-angiogenic cytokines and growth factors and among these, VEGFA plays a critical role [11]. The mechanisms by which IL-27 affect human macrophage production of VEGFA is unknown. Purinergic signaling by adenosine triphosphate (ATP) and adenosine results in responses by macrophages that regulate VEGFA expression during angiogenesis in retinopathic diseases [12,13]. In the hypoxic retinal microenvironment in DR the transcription factor HIF-1α is upregulated in retinal endothelial cells for example and this in turn increases the expression of VEGFA to promote angiogenesis for oxygen supply. In addition, high glucose stimulation of rat retinal Müller cells induces HIF-1α and VEGFA expression via the activation of the Calmodulin-dependent kinase II (CaMKII)-CREB pathway [14]. Furthermore, inhibition of macrophages derived from monocytes isolated from synovial fluid of patients with rheumatoid arthritis (RA) by a $Ca2+/CaMKII$ pathway antagonist suppresses VEGFA production and may contribute to the therapeutic efficacy of this treatment in RA patients [15].

New treatments for DR are focused on neutralization of VEGFA activity in the eye [16,17]. Although these treatments can significantly reduce the risk of severe vision loss due to complications of angiogenesis they carry significant undesirable effects if used long term. VEGFA inhibitors are delivered directly into the eye and the frequency with which the treatment is carried out can lead to serious ocular adverse

* Corresponding author.
E-mail address: henry.wu@novartis.com (H.Y. Wu).
[1] These authors contributed equally to this paper.

https://doi.org/10.1016/j.cyto.2018.07.011
Received 18 April 2018; Received in revised form 3 July 2018; Accepted 7 July 2018

events such as infections, hemorrhage and retinal detachments [16,18]. Therefore, safer approaches that can target this critical angiogenic pathway involved in disease development and progression are needed to address this high unmet medical need. Here we demonstrate that IL-27 affects HIF-1α expression and purinergic signaling in human macrophages resulting in a significant inhibitory effect on VEGFA response in patients with DR. It has recently been demonstrated that IL-27 is significantly reduced in the aqueous humor [19] and serum [20] of DR patients compared to non-diabetic patients. Together our results point to a therapeutic potential of IL-27 in retinopathic diseases.

2. Material and methods

2.1. Human blood samples

Human blood was obtained from Research Blood Components LLC, US. using

protocol NEIRB #04-144 and patient informed consent approved by New England Independent Review Board (NEIRB). The protocol is in compliance with all federal, state and local laws pertaining to human research. We acquired DR patient and healthy individual blood samples in pairs from Bioreclamation IVT, US. under patient informed consent, protocol number 05035 in accordance with the Declaration of Helsinki ethical principles for medical research involving human subjects. Activities of Bioreclamation IVT and their collaborators were conducted in accordance with applicable laws, regulations, and ordinances. The study protocols are approved by the Novartis Research Center ethical committee.

2.2. DR patient information

Males and females Hispanic or Caucasian patients ranging from 56 to 77 years old who are diagnosed of type 1 or type II diabetes with diabetic retinopathy, diabetic nephropathy and hypertension. Patients are treated with Lantus, Novolog and/or Humolog as well as other medications depending on the individual condition. Sex matched controls are deemed healthy individuals (Hispanic) age between 32 and 54 years old. For our study on DR patient macrophages, patients were diagnosed with diabetes (type I or type II) for a least 10 years.

2.3. Media and reagents

Cells were cultured in RPMI 1640 medium with 10% FBS, L-Glutamine, Pen-Strep, Non-essential Amino Acids, Sodium Pyruvate, HEPES buffer, and 2-Mercaptoethanol, (all from Life Technologies, US). Recombinant human IL-27 and M-CSF were obtained from R&D Systems, US. Adenosine receptor antagonists 8-(3-Chlorostyryl) caffeine (CSC) and MRS 1754 hydrate as well as ATP and adenosine were obtained from Sigma, US. AZ960 was purchased from Selleckchem, US.

2.4. Cell preparation and treatment

Human blood was collected into EDTA-treated tubes. PBMCs were isolated using Ficoll-Paque Plus density centrifugation (500*g* for 25 min at room temperature without brake). Monocytes were purified using the Monocyte isolation kit II from Miltenyi Biotec, US. Non-monocytes were magnetically labeled using a cocktail of biotin-conjugated antibodies as well as anti-Biotin MicroBeads. Monocytes are enriched by depletion of the magnetically labeled cells through a MACS Separator (Miltenyi Biotec, US). All macrophage cultures were carried out in the presence of human M-CSF (100 ng/ml unless indicated otherwise) with or without recombinant human IL-27 (at 1, 10, 100 ng/ml unless indicated otherwise) for time indicated in the figures. Adenosine receptor antagonists and JAK2 inhibitors were added simultaneously with IL-27.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Culture supernatants were harvested and stored at −80 °C before used in ELISA. The VEGFA level was measured using VEGF DuoSet development kits (R&D Systems, US) according to the manufacturer's instructions.

2.6. RNA isolation and real time-PCR

Macrophages were lysed and mRNA prepared using TurboCapture 96 mRNA kit (Qiagen, US) according to the manufacturer's protocol. mRNA was used directly in cDNA synthesis by High-capacity cDNA reverse Transcription kit (Applied Biosystems, US) according to the manufacturer's protocol. All primer/probe mixtures were obtained from Applied Biosystems. Real-Time PCR reaction was carried out on ViiA 7 (Applied Biosystems, US) with TaqMan Gene Expression Assays using TaqMan Fast Advanced Master mix from Applied Biosystems. β-actin expression was used as internal control.

2.7. Adenosine detection by mass spectrometry

Culture supernatant was collected into methanol and stored at −80 °C until use. The methanol quenched samples were centrifuged. The supernatants were then transferred to a new Eppendorf tube and dried in vacuum (Savant SPD121P SpeedVac Concentrator, Thermo Scientific, US) at room temperature overnight. The vacuum dried supernatants were reconstituted in 50 μL water with 20 nM C-13 labeled adenosine (Cambridge Isotope Laboratories, US) added as the internal standard. The prepared samples were then analyzed using an LC/MS system of API-6500 QTrap (AB Sciex, US) coupled with a Shimadzu LC pump (LC-20AD) and a CTC auto sampler with DLW wash. For each sample, 4 μL was injected and separated using a SeQuant ZIC-pHILIC column (5 μm, 150 × 2.1 mm, EMD, US) maintained at 40 °C. A binary gradient was used for the elution, where mobile phase B is 100% acetonitrile with no additives and mobile phase A is 12 mM ammonium formate and 12 mM formic acid in 1:1 (v/v) mix of water and acetonitrile. ADP and adenosine were monitored from 0.5 to 4.5 min at ESI positive mode and mass transitions 428- > 136 and 268- > 136 respectively.

2.8. Western blot

Whole-cell lysates were extracted using RIPA buffer supplemented with protease and phosphatase inhibitors (both from ThermoFisher Scientific, US) according to the manufacturer's protocol. The cell lysate (30 μg of protein) was dissolved in sample buffer (Invitrogen, US) and boiled for 5 min at 100 °C. The sample was then separated by Criterion™ TGX™ precast gel electrophoresis and electrotransferred onto nitrocellulose membranes (Bio-Rad, US). The MagicMark™ XP Western Protein Standard (Invitrogen, US) was used as a molecular weight indicator. The membrane was blocked for 1 h using 5% milk in Tris-buffered saline and Tween 20 (TBST). Following three washes with TBST, the membrane was incubated overnight with rabbit anti-human HIF-1α (D2U3T) monoclonal antibody at 1:1000 dilution or anti-human β-actin antibody at 1:1000 dilution (both from Cell Signaling Technology, US). After three washes with TBST, the membrane was incubated with a secondary antibody (anti-rabbit IgG-HRP-linked antibody, at 1:1000 dilution; Cell Signaling Technology, US) for 1 h. The immunoreactive proteins were detected using SuperSignal™ West Femto Maximum sensitivity substrate (ThermoFisher Scientific, US) and imaged on FluorChem M (ProteinSimple, US). The densities of the bands were quantified with Image J software.

2.9. Flow cytometry staining and acquisition

Cells were washed (400 g, 5 min at 4 °C) with FACS buffer ($Mg2^+$

and $Ca2^{+}$ free HBSS with 2% FBS, 0.4% 0.5 M EDTA and 2.5% 1 M HEPES). Fcγ receptors were blocked by incubation with human Fc receptor binding inhibitor for 10 min at 4 °C. Cells were then stained with fluorescent anti-human cell surface molecule antibodies for 25 min at 4 °C in the dark. After staining, cells were washed again with FACS buffer before sample acquisition. Antibodies used for flow cytometry (all from Affymetrix eBioscience USA) were as follows: Human Fc receptor binding inhibitor purified (catalog number: 14-9161-73), anti-human CD73 APC (catalog number: 17-0739-42), anti-human CD11b APC-eFluor 780 (catalog number: 47-0118-42), anti-human CD14

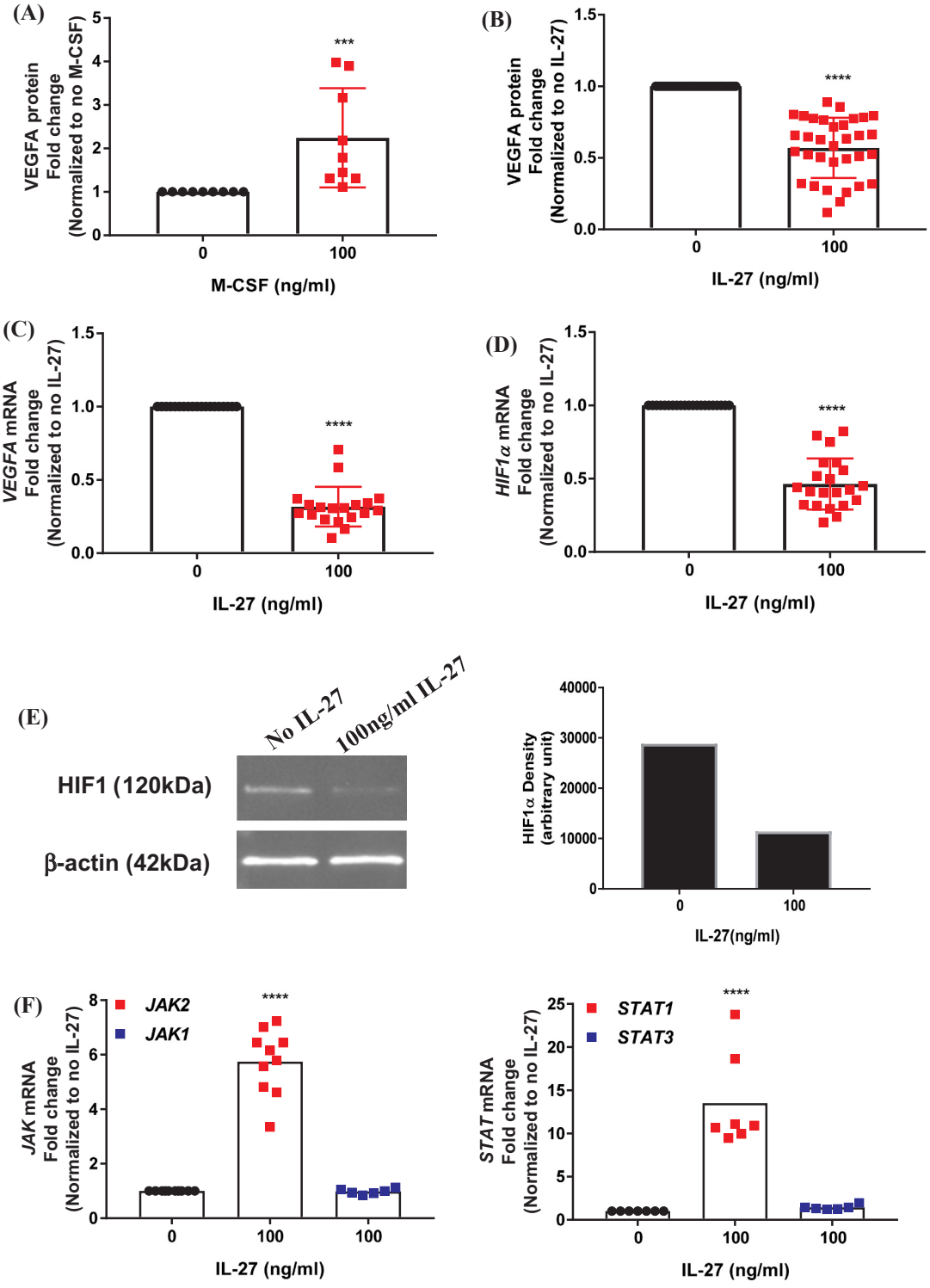

Fig. 1. The effect of IL-27 on VEGFA and HIF-1α in human macrophages. (A) Human macrophages were cultured in the absence or presence of M-CSF (100 ng/ml) alone or (B) M-CSF (100 ng/ml) with or without IL-27 for 3 days and VEGFA level was measured by ELISA. The level of VEGFA in culture supernatant following M-CSF stimulation was between 100 and 450 pg/ml. (C) *VEGFA* and (D) *HIF-1α* mRNA expression in human macrophages following 1 day culture with IL-27. (E) Quantification of HIF-1α protein in human macrophages by western blot following 3 day culture in the presence of M-CSF (100 ng/ml) with or without IL-27. (F) *JAK* and *STAT* mRNA expression in human macrophages following 1 day culture with or without IL-27. Each data point represents an individual. Data was normalized to the control as indicated in the y-axis.

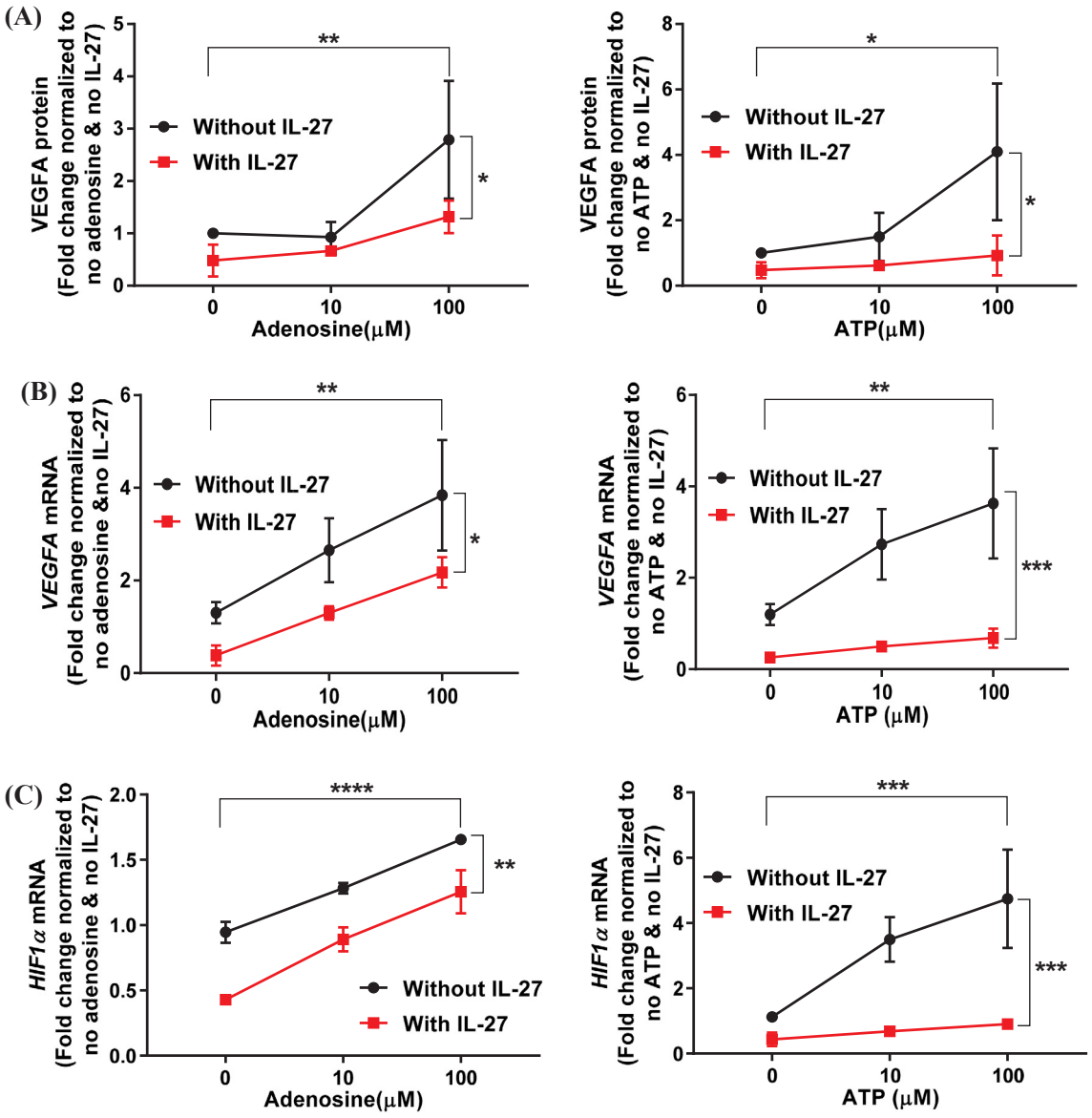

Fig. 2. Exogenous adenosine or ATP induces higher VEGFA response in human macrophages. (A) The production of VEGFA by human macrophages following 3 day culture with an ascending dose of adenosine or ATP in the absence or presence of IL-27 (100 ng/ml). (B) *VEGFA* and (C) *HIF-1*αmRNA expression following 1 day culture with an ascending dose of adenosine or ATP in the absence or presence of IL-27 (100 ng/ml). Data are pooled from 3 individuals. All data was normalized to the control as indicated in the y-axis.

eFluor® 450 (catalog number: 48-0149-42). The anti-human HIF-1α PE antibody was from Biolegend (catalog number: 359704). For phospho-flow analysis of JAK2 and STAT1 phosphorylation PBMCs were washed with phosphate-buffered saline (PBS) plated in 96 well plates at 10^5 cells per well in RPMI 1640 and stimulated with recombinant human M-CSF (100 ng/mL) alone or in the presence of human recombinant IL-27 (100 ng/ml) alone or JAK2 inhibitor AZ960 (1 μM) alone or both for 30 min. Cells were then labeled with anti-human CD11b APC-eFluor 780 and anti-human CD14 eFluor® 450 plus anti-human pSTAT1 pY701 Alexa Fluor 647 (Cell Signaling, catalog number: 8009S) or rabbit anti-human pJAK2 Y1008 purified (Cell Signaling, catalog number: 8082S) at room temperature for 30 min then washed. Samples labeled with pJAK2 antibody were subsequently incubated with anti-rabbit IgG PE (Cell Signaling, catalog number: 14705S) and washed before acquisition. Data were collected on a BD™ LSRFortessa X-20 flow cytometer (BD Biosciences, US). Analysis of data was carried out using FlowJo software (Treestar, US).

2.10. Statistical analysis

Statistical analysis was carried out using one-way or two-way analysis of variance (ANOVA) followed by Tukey's and/or Dunnett's multiple comparison tests. Results with p-values of < 0.05 were considered to be significant. All results are expressed as mean ± S.E.M. * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$

3. Results

3.1. IL-27 concomitantly suppresses VEGFA and HIF-1α expression in human macrophages

Macrophages are a major source of VEGFA driving angiogenesis in the eye [10]. To the best of our knowledge no previous study has investigated the regulatory role of IL-27 in VEGFA production by human macrophages. To examine this we derived human macrophages from multiple individuals by culturing enriched primary human monocytes

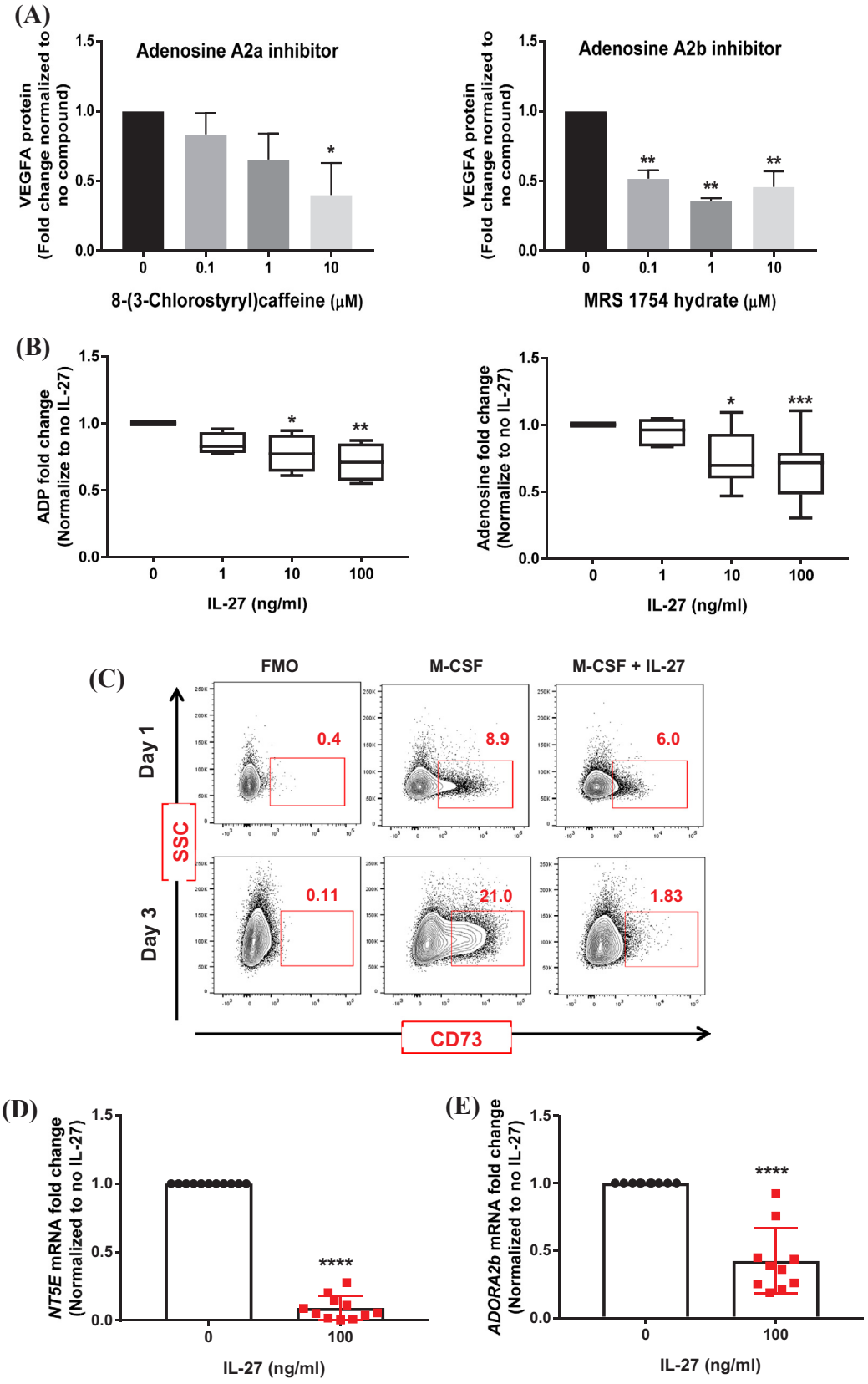

Fig. 3. IL-27 affects the purinergic pathway leading to downregulation of VEGFA in human macrophages. (A) Human macrophages were cultured in the presence of M-CSF (100 ng/ml) and an A2a (8-(3-Chlorostyryl)caffeine) or an A2b (MRS 1754 hydrate) antagonist for 3 days and VEGFA production was measured by ELISA. Data are pooled from 3 individuals. (B) The levels of ADP and adenosine in culture supernatant after 3 day culture were measured by mass spectrometry. Data are pooled from 4 individuals. (C) Cell surface CD73 expression on human macrophages after 1 or 3 day culture with M-CSF (110 ng/ml) alone or M-CSF and IL-27 (100 ng/ml) was measured by FACS. (D) The expression of *NT5E* mRNA in human macrophages after 1 day culture in the presence of M-CSF (100 ng/ml) with or without IL-27. (E) The expression of *ADORA2b* mRNA in human macrophages after 1 day culture in the presence of M-CSF (100 ng/ml) with or without IL-27. Each data point in 3d and 3e represents an individual. Data was normalized to the control as indicated in the y-axis.

as described in Methods. We assessed the level of human monocyte enrichment (by FACS using CD11b and CD14 as markers) and determined that a typical enrichment was from 40 to 50% before enrichment to above 90% after enrichment (Fig. S1). We found that human macrophages significantly increased VEGFA production upon stimulation with M-CSF (Fig. 1a). The addition of exogenous IL-27 significantly downregulated the production of VEGFA (Fig. 1b). Furthermore, IL-27 also suppressed *VEGFA* mRNA in human macrophages (Fig. 1c).

Supplementary data associated with this article can be found, in the

online version, at https://doi.org/10.1016/j.cyto.2018.07.011.

An important mechanism responsible for VEGFA production in human macrophages is the activation of HIF-1α [21]. We first measured the effect of M-CSF on the expression of *HIF-1α* mRNA in human macrophages. We did not observe an effect (Fig. S2). We went on to measure the effect of IL-27 on *HIF-1α* mRNA expression. We found that, similar to *VEGFA* mRNA, IL-27 significantly suppressed *HIF-1α*mRNA expression (Fig. 1d). To further confirm the downregulatory effect of IL-

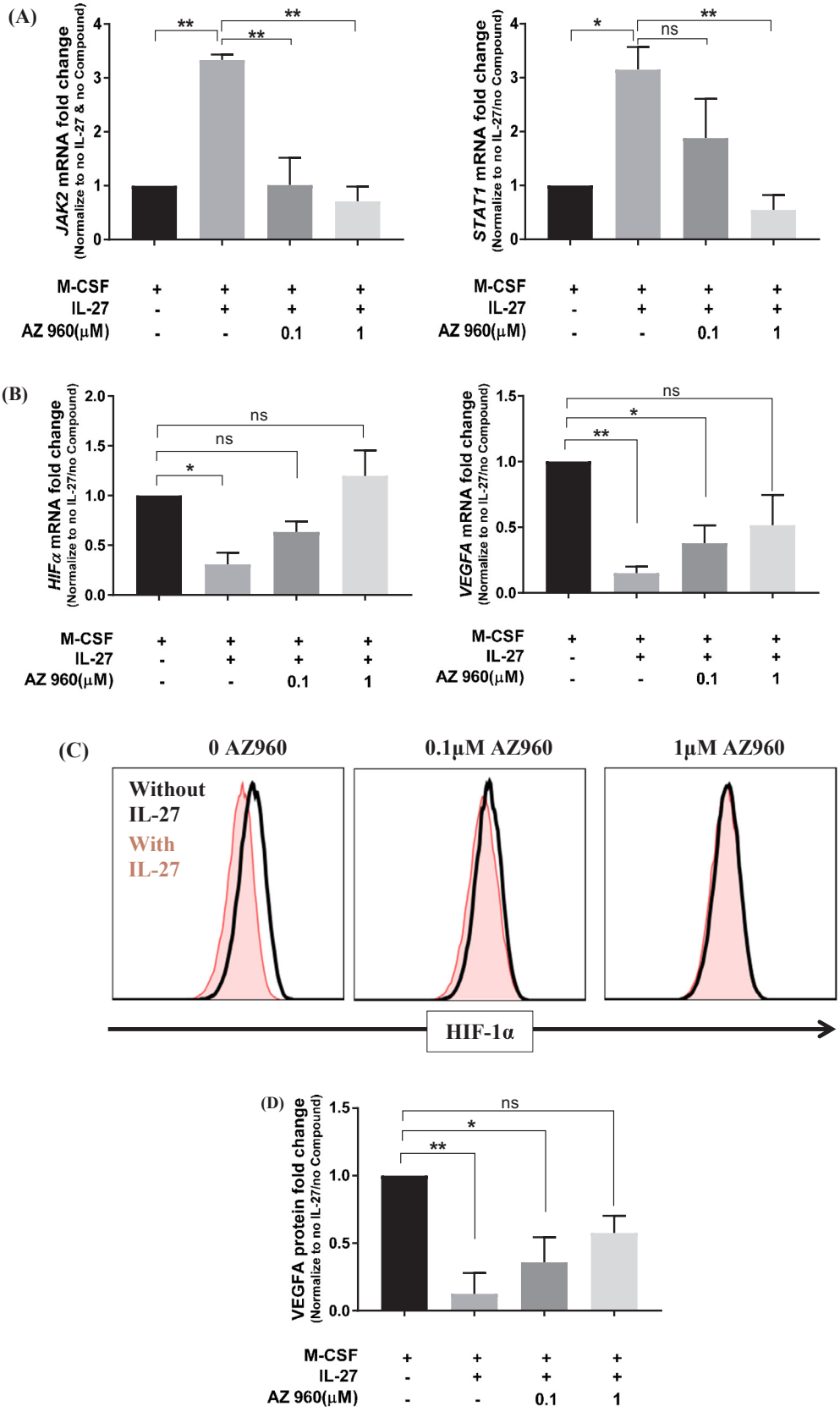

Fig. 4. Antagonizing JAK2 partially reverses the suppressive effect of IL-27 on VEGFA response in human macrophages. (A) Human macrophages were cultured for 3 days in the presence of M-CSF (100 ng/ml) with or without IL-27 (100 ng/ml) and with or without AZ960 as indicated. mRNA expression of *JAK2* and *STAT1* was measured after day 1 culture. (B) *HIF-1α* and *VEGFA* mRNA was measured after 1 day culture. (C) Representative (n = 3) FACS histograms showing HIF-1α expression in human macrophages after 3 day culture in the presence of M-CSF (100 ng/ml) with or without IL-27 (100 ng/ml) and with or without AZ960 as indicated. (D) VEGFA in culture supernatant following 3 day culture in the presence of M-CSF (100 ng/ml) with or without IL-27 (100 ng/ml) and with or without AZ960. All data are pooled from 3 individuals.

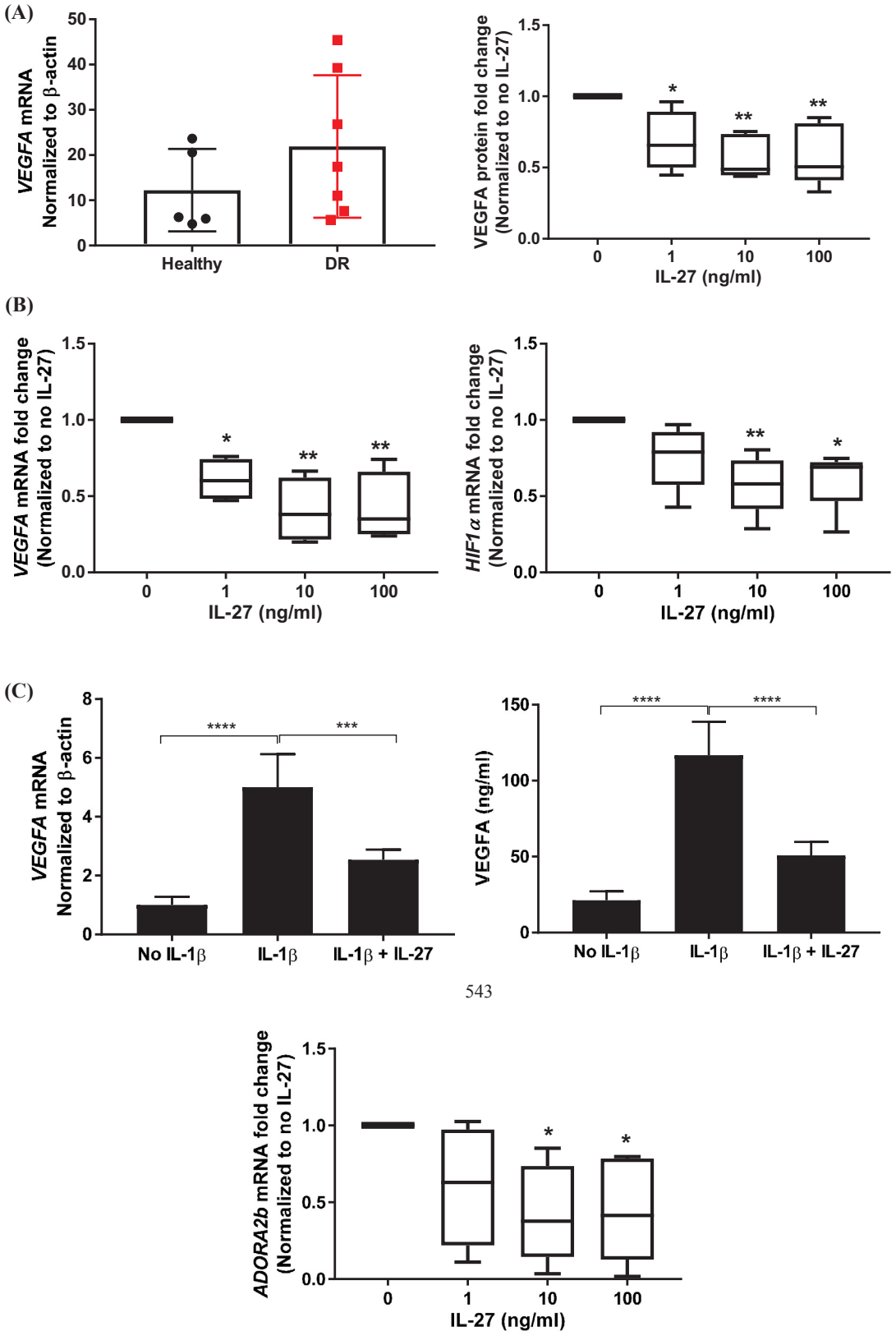

Fig. 5. IL-27 suppresses the heightened VEGFA response in DR patient macrophages. Macrophages from healthy individuals and DR patients were cultured in the presence of M-CSF (100 ng/ml) and (A) *VEGFA* mRNA expression was measured after 1 day culture. Each data point represents an individual. The effect of IL-27 on VEGFA production by DR patient macrophages was measured after 3 day culture. The data was pooled from 5 DR patients. (B) The effect of an ascending dose of IL-27 on *VEGFA* and *HIF-1α* mRNA expression in DR patient macrophages was measured after 1 day culture. The data was pooled from 5 DR patients. (C) Macrophages from DR patients (n = 3) were challenged with IL-1β (100 μg/ml) in the presence of M-CSF (100 ng/ml) with or without IL-27 (100 ng/ml) and *VEGFA* mRNA and VEGFA protein was measured after 1 day and 3 day culture respectively. (D) The effect of IL-27 on *ADORA2b* mRNA expression in DR patient macrophages was measured after 3 day culture. The data was pooled from 5 DR patients.

27 on HIF-1α we examined the level of HIF-1α protein in human macrophages cultured with IL-27. We found that, similar to mRNA expression, HIF-1α protein level in human macrophages was also downregulated by IL-27 (Fig. 1e). To confirm IL-27 signaling in human macrophages we examined mRNA expressions of IL-27 target genes JAK1, JAK2, STAT1, and STAT3. Interestingly, we found that only *JAK2* and *STAT1* mRNA were significantly increased in human macrophages (Fig. 1f). We are currently investigating whether this is a specific effect in human macrophages or it also occurs in other human immune cell types. Nonetheless, our results demonstrate that IL-27 signaling occurred and the effect on VEGFA and HIF-1α was not due to a continual M-CSF stimulation. Taken together, IL-27 suppresses VEGFA production by primary human macrophages and this suppression is associated with downregulation of HIF-1α expression.

3.2. Exogenous adenosine or ATP augments HIF-1α expression leading to an increase in VEGFA production and IL-27 suppresses HIF-1α expression thereby reduces VEGFA production

The expression of HIF-1α in cancer cells is regulated by the level of extracellular adenosine [22]. We hypothesize that purinergic signaling of human macrophages by adenosine or ATP augments VEGFA production via the upregulation of HIF-1α. To examine our hypothesis we first added exogenous adenosine or ATP to the culture. Fig. 2a shows that both adenosine and ATP stimulation of human macrophages increased VEGFA production (Fig. 2a). IL-27 significantly lowered both adenosine- and ATP-induced VEGFA production in human macrophages (Fig. 2a). To further examine the effect of adenosine and ATP we evaluated *VEGFA* mRNA expression in human macrophages. We found that in the presence of 100 μM adenosine or ATP there was a significant increase in *VEGFA* mRNA expression and stimulation of human macrophages with IL-27 significantly suppressed *VEGFA* mRNA expression (Fig. 2b). The suppressive effect of IL-27 on adenosine- and ATP-induced *VEGFA* mRNA expression corroborated well with its effect on *HIF-1α* mRNA expression in that IL-27 significantly downregulated adenosine- or ATP-induced increase in *HIF-1α*mRNA expression (Fig. 2c). Our findings shown here suggest that IL-27 affects the purinergic pathway in human macrophages and as a result IL-27 suppresses VEGFA production by human macrophages.

3.3. IL-27 suppresses VEGFA production by inhibiting autocrine purinergic signaling in human macrophages

Upon release ATP is immediately hydrolyzed to adenosine diphosphate (ADP) by membrane bound ectonucleoside triphosphate diphosphohydrolase-1 (CD39, *ENTPD1*) [23]. ADP undergoes further hydrolysis to form adenosine monophosphate (AMP) and AMP is then catalyzed by ecto-5′-nucleotidase (CD73, *NT5E*) to form adenosine for purinergic signaling [24]. It has been shown that HIF-1α activation enhances the expression of CD73 [25]. Thus, we hypothesize that upon activation with M-CSF human macrophages produce adenosine for autocrine purinergic signaling to initiate VEGFA production. To test this hypothesis we first added adenosine receptor A2a (8-(3-Chlorostyryl) caffeine) and A2b (MRS 1754 hydrate) antagonists [13,26–28] to the culture. We found that antagonizing A2a and A2b inhibited VEGFA production by human macrophages (Fig. 3a). To examine the effect of IL-27 on this pathway we used mass spectrometry to determine the level of ADP and adenosine in human macrophage cultures. We found that IL-27 significantly suppressed ADP and adenosine levels in human macrophage cultures (Fig. 3b). Using FACS we confirmed the downregulation of CD73 expression on human macrophages following 1 day and 3 days culture with IL-27 (Fig. 3c). These results suggest that IL-27 controls VEGFA response by regulating autocrine purinergic signaling in human macrophages. To further examine our hypothesis, we analyzed the effect of IL-27 on the expression of *NT5E* mRNA in human macrophages. Fig. 3d shows IL-27 suppressed the expression of *NT5E* mRNA in human macrophages. In addition, we also analyzed the effect of IL-27 on adenosine receptor A2b expression in human macrophages and show that IL-27 significantly affected A2b expression in human macrophages (Fig. 3e). Interestingly, it has been shown that adenosine binding to receptor A2b mediates VEGFA production and chemical inhibition of A2b signaling downregulates VEGFA production and ameliorates ocular inflammation in the rat model of streptozotocin-induced DR [27].

Collectively, our findings demonstrate that IL-27 can suppress the production of VEGFA from human macrophages by reducing HIF-1α expression. This in turn downregulates CD73 expression and lowers extracellular adenosine resulting in a disruption of the positive feedback loop for VEGFA production in human macrophages. Interestingly, in has been demonstrated that the level of CD39 expression by human monocyte derived macrophages was controlled by the *in vitro* autocrine effect of IL-27 [29].

3.4. Antagonizing IL-27 signaling molecule JAK2 in human macrophages reverses the suppressive effect on HIF-1α and VEGFA

To examine the dependency on IL-27 signaling in the regulation of VEGFA response in human macrophages we used a high affinity JAK2 antagonist AZ960 [30–32]. We also measured the expression of STAT1 which is a transcription factor downstream of JAK2 that is also activated by IL-27 signaling in human monocytes [33]. Fig. 4a shows that AZ960 suppressed IL-27 driven *JAK2* and *STAT1* mRNA expression in human macrophages. To further confirm the inhibitory effect of AZ960 on IL-27 signaling we used phospho-flow cytometry to demonstrate that IL-27-induced JAK2 and STAT1 phosphorylation in human macrophages was downregulated by AZ960 (Fig. S3). We next examined the effect of AZ960 on *HIF-1α* and *VEGFA* mRNA expression. Fig. 4b shows a reversal of the suppressive effect of IL-27 on *HIF-1α* mRNA and a partial reversal of the suppressive effect of IL-27 on *VEGFA* mRNA expression. This was recapitulated in the expression HIF-1α (Fig. 4c) and VEGFA proteins (Fig. 4d). These results further demonstrate that IL-27 downregulates VEGFA production by human macrophages via reducing HIF-1α expression. Interestingly, the suppressive effect of IL-27 on *NT5E* mRNA was also reversed by AZ960 (Fig. S4) suggesting that IL-27 may regulate purinergic signaling as well as HIF-1α expression in human macrophages.

3.5. IL-27 suppresses VEGFA response in DR patient macrophages

VEGFA stimulates the production of new blood vessels in the retina and it is an important factor for the development of DR in humans. Patients suffering from DR have high levels of VEGFA in the periphery and in the vitreous humor [34,35]. We hypothesize that macrophages from patients with DR have a higher VEGFA response. Fig. 5a shows that human macrophages from DR patients express a higher level of *VEGFA* mRNA compared to healthy individuals. IL-27 significantly suppressed VEGFA production by DR patient macrophages in day 3 culture. DR macrophages are not deficient in IL-27 expression compared to their healthy control counterparts (Fig. S5). IL-27 also suppressed *VEGFA* and *HIF-1α* mRNA expression in DR patient macrophages (Fig. 5b). A number of pro-inflammatory cytokines are consistently elevated in the vitreous humor of DR patients [36,37]. These pro-inflammatory cytokines propagate and maintain inflammation in the eye. The inflammatory cytokine IL-1β has been shown to activate macrophages as well as retinal pigment epithelial cells [38,39]. Thus, to simulate an inflammatory condition and to investigate the effect of IL-27 on VEGFA production in an inflammatory condition we added exogenous IL-1β to the culture with DR patient macrophages. Fig. 5c shows that activation of DR patient macrophages with IL-1β (100 μg/ml) increased the expression of *VEGFA* mRNA and the production of VEGFA after 3 day culture. IL-27 suppressed VEGFA production even under IL-1β stimulation which suggests that IL-27 has the

potential for clinical application. Finally, to examine the relationship between the effect of IL-27 on VEGFA production and adenosine signaling we measured adenosine receptor *ADORA2b* mRNA levels in DR patient macrophages after IL-27 stimulation. Fig. 5d shows that IL-27 suppressed the *ADORA2b* mRNA expression in DR patient macrophages. These findings suggest that IL-27 can control purinergic signaling and therefore regulate VEGFA response in DR patient macrophages.

4. Discussion

Here we demonstrate IL-27 controls VEGFA response by regulating HIF-1α and the purinergic pathway in human macrophages. IL-27 downregulates HIF-1α and reduces CD73 expression on human macrophages leading to a decrease in extracellular adenosine. IL-27 also downregulates *ADORA2b* mRNA expression further reducing the potential for purinergic signaling and subsequent HIF-1α activation in human macrophages. This effect is dependent on IL-27 as blockade of IL-27 signaling by JAK2 and STAT1 antagonism reduces the suppressive effect on VEGFA. In study of cancers, a number of reports have shown that STAT1 repressed HIF-1α activation which led to downregulation of HIF-1α target genes [40,41]. Using chromatin immunoprecipitation analyses one study demonstrated that STAT1 bound to HIF-1α and consequently dissociated HIF-1α complex from the hypoxia response element (HRE) promoter thereby rendering HIF-1α transcription inactive [41]. There could be a similar mechanism in which IL-27-induced STAT1 antagonizes HIF-1α activity and this negatively affects the purinergic pathway in human macrophages. This then results in a suppression of VEGFA expression in human macrophages. We are currently investigating these mechanisms. To the best of our knowledge, this is the first demonstration of the ability of IL-27 to control VEGFA expression in human macrophages. However, our studies are *in vitro* studies and the effect of IL-27 *in vivo* in healthy and in a disease setting is to be determined.

Diabetes is a lifelong progressive disease resulting from the body's inability to produce or utilize insulin to break down glucose in the circulation. The sustained hyperglycemia in diabetes induces inflammation in blood vessels throughout the body, including the eye. In the eye, chronic end organ damage is manifested as DR, the leading cause of blindness in adults of working age. The pathophysiology of DR is characterized by many features typical of inflammation. These include increased blood flow and vascular permeability, neovascularization [42], microglial cell activation [43,44] and increased expression of inflammatory mediators, including VEGFA [45,46]. VEGFA promotes retinal neovascularization [47], vascular permeability [48], and leukostasis [49]. It is thought that these soluble inflammatory mediators are secreted by immune cells like macrophages that invade the eye during the development of DR. Many studies have implicated immune cell infiltrates in promoting the pathogenesis of DR. Macrophages are found to be elevated in the vitreous humor of diabetic patients [50]. Also, macrophages are found in membranes surgically removed from diabetics [51]. In murine models of choroidal neovascularization (CNV) and oxygen-induced retinopathy (OIR), mice depleted of macrophages with intravitreous injections of clodronate liposomes had decreased ocular angiogenesis [52–54]. These findings support a pro-angiogenic role for macrophages in the eye. Also, mice lacking a key macrophage recruitment chemokine (CCL-2) develop spontaneous CNV [55]. Interestingly, treatment of CNV mice with IL-27 suppresses retinal CNV size [56]. To examine a potential therapeutic application of IL-27 in humans we first investigated the expression of *VEGFA* mRNA in macrophages from healthy individuals and patients with DR. We show that macrophages from DR patients spontaneously express a higher level of *VEGFA* mRNA. This increase in VEGFA response by DR patient macrophages is exacerbated when the macrophages are challenged with a pro-inflammatory stimulus IL-1β. The robust VEGFA response by DR patient macrophages maybe due to a higher expression of the adenosine receptor A2b which allows cells to respond to adenosine stimulation more readily compared to their healthy counterparts. IL-27 suppresses VEGFA production by DR patient macrophages via reducing HIF-1α and downregulating adenosine receptor A2b expression.

In summary, we demonstrate that IL-27 can regulate VEGFA production in human macrophages. We provide evidence to support a mechanism in which IL-27 signaling regulates the expression of HIF-1α and thereby affects purinergic signaling resulting in a disruption of the positive feedback loop that drives VEGFA production in human macrophages. Lastly, IL-27 is effective in suppressing VEGFA response in DR patient macrophages. Our novel findings shed new light on a previously unappreciated function of IL-27 and may potentially open a new avenue for IL-27 and other protein therapeutics to treat ophthalmic diseases such as diabetic retinopathy.

Acknowledgments and authorship

We thank Dr. Quintus G. Medley and Dr. Chris Wilson for their critical reading of the manuscript. Author contributions: Q. Zhang. and A.P. Cunha. designed, performed, and analyzed experiments and helped in writing the manuscript. S. Li., Q. Hao., and V. Kaniz performed, and analyzed experiments. Q. Huang. analyzed data and gave scientific input. H.Y. Wu conceived the project, led the study, designed and analyzed experiments, and wrote the manuscript, with contributions from all the other authors.

Conflict-of-interest disclosure

All authors are employees of Novartis Pharma AG, Cambridge, US.

References

[1] S. Pflanz, J.C. Timans, J. Cheung, et al., IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4+ T cells, Immunity 16 (6) (2002) 779–790.
[2] H. Yoshida, C.A. Hunter, The immunobiology of interleukin-27, Annu. Rev. Immunol. 33 (2015) 417–443.
[3] A.O. Hall, J.S. Silver, C.A. Hunter, The immunobiology of IL-27, Adv. Immunol. 115 (2012) 1–44.
[4] D.G. Espinosa-Heidmann, I. Suner, E.P. Hernandez, W.D. Frazier, K.G. Csaky, S.W. Cousins, Age as an independent risk factor for severity of experimental choroidal neovascularization, Invest. Ophthalmol. Vis. Sci. 43 (5) (2002) 1567–1573.
[5] G.K. Hansson, Inflammation, atherosclerosis, and coronary artery disease, N. Engl. J. Med. 352 (16) (2005) 1685–1695.
[6] J. Kelly, A. Ali Khan, J. Yin, T.A. Ferguson, R.S. Apte, Senescence regulates macrophage activation and angiogenic fate at sites of tissue injury in mice, J. Clin. Invest. 117 (11) (2007) 3421–3426.
[7] S. Nakao, T. Kuwano, C. Tsutsumi-Miyahara, et al., Infiltration of COX-2-expressing macrophages is a prerequisite for IL-1 beta-induced neovascularization and tumor growth, J. Clin. Invest. 115 (11) (2005) 2979–2991.
[8] P.R. Taylor, L. Martinez-Pomares, M. Stacey, H.H. Lin, G.D. Brown, S. Gordon, Macrophage receptors and immune recognition, Annu. Rev. Immunol. 23 (2005) 901–944.
[9] R.S. Apte, J. Richter, J. Herndon, T.A. Ferguson, Macrophages inhibit neovascularization in a murine model of age-related macular degeneration, PLoS Med. 3 (8) (2006) e310.
[10] R.S. Apte, Regulation of angiogenesis by macrophages, Adv. Exp. Med. Biol. 664 (2010) 15–19.
[11] N. Gupta, S. Mansoor, A. Sharma, et al., Diabetic retinopathy and VEGF, Open Ophthalmol. J. 7 (2013) 4–10.
[12] G. Cavallaro, L. Filippi, P. Bagnoli, et al., The pathophysiology of retinopathy of prematurity: an update of previous and recent knowledge, Acta Ophthalmol. 92 (1) (2014) 2–20.
[13] X. Du, X. Ou, T. Song, et al., Adenosine A2B receptor stimulates angiogenesis by inducing VEGF and eNOS in human microvascular endothelial cells, Exp. Biol. Med. (Maywood) 240 (11) (2015) 1472–1479.
[14] J. Li, S.Z. Zhao, P.P. Wang, S.P. Yu, Z. Zheng, X. Xu, Calcium mediates high glucose-induced HIF-1alpha and VEGF expression in cultured rat retinal Muller cells through CaMKII-CREB pathway, Acta Pharmacol. Sin. 33 (8) (2012) 1030–1036.
[15] J. Westra, E. Brouwer, I.A. van Roosmalen, et al., Expression and regulation of HIF-1alpha in macrophages under inflammatory conditions; significant reduction of VEGF by CaMKII inhibitor, BMC Musculoskelet Disord. 11 (2010) 61.
[16] D.M. Brown, P.K. Kaiser, M. Michels, et al., Ranibizumab versus verteporfin for neovascular age-related macular degeneration, N. Engl. J. Med. 355 (14) (2006) 1432–1444.
[17] R. Simo, J.M. Sundstrom, D.A. Antonetti, Ocular Anti-VEGF therapy for diabetic retinopathy: the role of VEGF in the pathogenesis of diabetic retinopathy, Diabetes

Care 37 (4) (2014) 893–899.
[18] P.J. Rosenfeld, D.M. Brown, J.S. Heier, et al., Ranibizumab for neovascular age-related macular degeneration, N. Engl. J. Med. 355 (14) (2006) 1419–1431.
[19] H. Chen, X. Zhang, N. Liao, F. Wen, Assessment of biomarkers using multiplex assays in aqueous humor of patients with diabetic retinopathy, BMC Ophthalmol. 17 (1) (2017) 176.
[20] A. Yan, H. You, X. Zhang, Levels of interleukin 27 and interleukin 35 in the serum and vitreous of patients with proliferative diabetic retinopathy, Ocul. Immunol. Inflamm. 26 (2) (2018) 273–279.
[21] T.D. Eubank, J.M. Roda, H. Liu, T. O'Neil, C.B. Marsh, Opposing roles for HIF-1alpha and HIF-2alpha in the regulation of angiogenesis by mononuclear phagocytes, Blood 117 (1) (2011) 323–332.
[22] S. Merighi, A. Benini, P. Mirandola, et al., A3 adenosine receptors modulate hypoxia-inducible factor-1alpha expression in human A375 melanoma cells, Neoplasia 7 (10) (2005) 894–903.
[23] E. Kaczmarek, K. Koziak, J. Sevigny, et al., Identification and characterization of CD39/vascular ATP diphosphohydrolase, J. Biol. Chem. 271 (51) (1996) 33116–33122.
[24] R. Resta, L.F. Thompson, T cell signalling through CD73, Cell Signal 9 (2) (1997) 131–139.
[25] K. Synnestvedt, G.T. Furuta, K.M. Comerford, et al., Ecto-5'-nucleotidase (CD73) regulation by hypoxia-inducible factor-1 mediates permeability changes in intestinal epithelia, J. Clin. Invest. 110 (7) (2002) 993–1002.
[26] L.M. Aguiar, D.S. Macedo, S.M. Vasconcelos, A.A. Oliveira, F.C. de Sousa, G.S. Viana, CSC, an adenosine A(2A) receptor antagonist and MAO B inhibitor, reverses behavior, monoamine neurotransmission, and amino acid alterations in the 6-OHDA-lesioned rats, Brain Res. 1191 (2008) 192–199.
[27] L. Patel, A. Thaker, The effects of adenosine A2B receptor inhibition on VEGF and nitric oxide axis-mediated renal function in diabetic nephropathy, Ren. Fail. 36 (6) (2014) 916–924.
[28] M.M. Van der Walt, G. Terre'Blanche, A. Petzer, A.C. Lourens, J.P. Petzer, The adenosine A(2A) antagonistic properties of selected C8-substituted xanthines, Bioorg. Chem. 49 (2013) 49–58.
[29] S.M. d'Almeida, G. Kauffenstein, C. Roy, et al., The ecto-ATPDase CD39 is involved in the acquisition of the immunoregulatory phenotype by M-CSF-macrophages and ovarian cancer tumor-associated macrophages: regulatory role of IL-27, Oncoimmunology 5 (7) (2016) e1178025.
[30] J.M. Gozgit, G. Bebernitz, P. Patil, et al., Effects of the JAK2 inhibitor, AZ960, on Pim/BAD/BCL-xL survival signaling in the human JAK2 V617F cell line SET-2, J. Biol. Chem. 283 (47) (2008) 32334–32343.
[31] T. Ikezoe, S. Kojima, M. Furihata, et al., Expression of p-JAK2 predicts clinical outcome and is a potential molecular target of acute myelogenous leukemia, Int. J. Cancer 129 (10) (2011) 2512–2521.
[32] J. Yang, T. Ikezoe, C. Nishioka, M. Furihata, A. Yokoyama, AZ960, a novel Jak2 inhibitor, induces growth arrest and apoptosis in adult T-cell leukemia cells, Mol. Cancer Ther. 9 (12) (2010) 3386–3395.
[33] G.D. Kalliolias, L.B. Ivashkiv, IL-27 activates human monocytes via STAT1 and suppresses IL-10 production but the inflammatory functions of IL-27 are abrogated by TLRs and p38, J. Immunol. 180 (9) (2008) 6325–6333.
[34] A.C. Cavusoglu, S. Bilgili, A. Alaluf, et al., Vascular endothelial growth factor level in the serum of diabetic patients with retinopathy, Ann. Ophthalmol. (Skokie) 39 (3) (2007) 205–208.
[35] K.M. Selim, D. Sahan, T. Muhittin, C. Osman, O. Mustafa, Increased levels of vascular endothelial growth factor in the aqueous humor of patients with diabetic retinopathy, Indian J. Ophthalmol. 58 (5) (2010) 375–379.
[36] N. Demircan, B.G. Safran, M. Soylu, A.A. Ozcan, S. Sizmaz, Determination of vitreous interleukin-1 (IL-1) and tumour necrosis factor (TNF) levels in proliferative diabetic retinopathy, Eye (Lond). 20 (12) (2006) 1366–1369.
[37] C. Mao, H. Yan, Roles of elevated intravitreal IL-1beta and IL-10 levels in proliferative diabetic retinopathy, Indian J. Ophthalmol. 62 (6) (2014) 699–701.
[38] P. Jayaraman, I. Sada-Ovalle, T. Nishimura, et al., IL-1beta promotes antimicrobial immunity in macrophages by regulating TNFR signaling and caspase-3 activation, J. Immunol. 190 (8) (2013) 4196–4204.
[39] Q. Shi, Q. Wang, J. Li, et al., A2E suppresses regulatory function of RPE cells in Th1 cell differentiation via production of IL-1beta and inhibition of PGE2, Invest. Ophthalmol. Vis. Sci. 56 (13) (2015) 7728–7738.
[40] M. Hiroi, K. Mori, Y. Sakaeda, J. Shimada, Y. Ohmori, STAT1 represses hypoxia-inducible factor-1-mediated transcription, Biochem. Biophys. Res. Commun. 387 (4) (2009) 806–810.
[41] A. Hosui, P. Klover, T. Tatsumi, et al., Suppression of signal transducers and activators of transcription 1 in hepatocellular carcinoma is associated with tumor progression, Int. J. Cancer 131 (12) (2012) 2774–2784.
[42] D.A. Antonetti, A.J. Barber, S.K. Bronson, et al., Diabetic retinopathy: seeing beyond glucose-induced microvascular disease, Diabetes 55 (9) (2006) 2401–2411.
[43] E. Rungger-Brandle, A.A. Dosso, P.M. Leuenberger, Glial reactivity, an early feature of diabetic retinopathy, Invest. Ophthalmol. Vis. Sci. 41 (7) (2000) 1971–1980.
[44] X.X. Zeng, Y.K. Ng, E.A. Ling, Neuronal and microglial response in the retina of streptozotocin-induced diabetic rats, Vis. Neurosci. 17 (3) (2000) 463–471.
[45] A.P. Adamis, J.W. Miller, M.T. Bernal, et al., Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy, Am. J. Ophthalmol. 118 (4) (1994) 445–450.
[46] L.P. Aiello, R.L. Avery, P.G. Arrigg, et al., Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders, N. Engl. J. Med. 331 (22) (1994) 1480–1487.
[47] E.W. Ng, A.P. Adamis, Targeting angiogenesis, the underlying disorder in neovascular age-related macular degeneration, Can. J. Ophthalmol. 40 (3) (2005) 352–368.
[48] D.R. Senger, D.T. Connolly, L. Van de Water, J. Feder, H.F. Dvorak, Purification and NH2-terminal amino acid sequence of guinea pig tumor-secreted vascular permeability factor, Cancer Res. 50 (6) (1990) 1774–1778.
[49] K. Miyamoto, S. Khosrof, S.E. Bursell, et al., Vascular endothelial growth factor (VEGF)-induced retinal vascular permeability is mediated by intercellular adhesion molecule-1 (ICAM-1), Am. J. Pathol. 156 (5) (2000) 1733–1739.
[50] H. Canataroglu, I. Varinli, A.A. Ozcan, A. Canataroglu, F. Doran, S. Varinli, Interleukin (IL)-6, interleukin (IL)-8 levels and cellular composition of the vitreous humor in proliferative diabetic retinopathy, proliferative vitreoretinopathy, and traumatic proliferative vitreoretinopathy, Ocul. Immunol. Inflamm. 13 (5) (2005) 375–381.
[51] P. Esser, K. Heimann, P. Wiedemann, Macrophages in proliferative vitreoretinopathy and proliferative diabetic retinopathy: differentiation of subpopulations, Br. J. Ophthalmol. 77 (11) (1993) 731–733.
[52] D.G. Espinosa-Heidmann, I.J. Suner, E.P. Hernandez, D. Monroy, K.G. Csaky, S.W. Cousins, Macrophage depletion diminishes lesion size and severity in experimental choroidal neovascularization, Invest. Ophthalmol. Vis. Sci. 44 (8) (2003) 3586–3592.
[53] S. Ishida, T. Usui, K. Yamashiro, et al., VEGF164-mediated inflammation is required for pathological, but not physiological, ischemia-induced retinal neovascularization, J. Exp. Med. 198 (3) (2003) 483–489.
[54] E. Sakurai, A. Anand, B.K. Ambati, N. van Rooijen, J. Ambati, Macrophage depletion inhibits experimental choroidal neovascularization, Invest. Ophthalmol. Vis. Sci. 44 (8) (2003) 3578–3585.
[55] J. Ambati, A. Anand, S. Fernandez, et al., An animal model of age-related macular degeneration in senescent Ccl-2- or Ccr-2-deficient mice, Nat. Med. 9 (11) (2003) 1390–1397.
[56] E. Hasegawa, Y. Oshima, A. Takeda, et al., IL-27 inhibits pathophysiological intraocular neovascularization due to laser burn, J. Leukoc. Biol. 91 (2) (2012) 267–273.