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IL-36 γ has proinflammatory effects on human endothelial cells

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Abstract

Interleukin-36 cytokines are predominantly expressed by epithelial cells. Significant upregulation of epidermal IL-36 is now a recognised characteristic of psoriatic skin inflammation. IL-36 is known to induce inflammatory responses in dendritic cells, fibroblasts and epithelial cells. Although vascular alterations are a hallmark of psoriatic lesions and dermal endothelial cells are well known to play a critical role in skin inflammation, the effects of IL-36 on endothelial cells are unexplored.

We here show that endothelial cells including dermal microvascular cells express a functionally active IL-36 receptor. Adhesion molecules VCAM-1 and ICAM-1 are upregulated by IL-36 γ stimulation and this is reversed by the presence of the endogenous IL-36 receptor antagonist. IL-36 γ stimulated endothelial cells secrete the proinflammatory chemokines IL-8, CCL2 and CCL20. Chemotaxis assays showed increased migration of T cells following IL-36 γ stimulation of endothelial cells. These results suggest a role for IL-36 γ in the dermal vascular compartment and it is likely to enhance psoriatic skin inflammation by activating endothelial cells and promoting leukocyte recruitment.

Introduction

Interleukin-36 (IL-36) cytokines are part of the wider IL-1 family and include IL-36 α , IL-36 β , IL-36 γ and their inhibitor, the IL-36 receptor antagonist (IL-36Ra)(1). IL-36 binds to the IL-36 receptor (IL-36R) and this is followed by recruitment of an accessory protein (IL-36RAcP), which is shared by other IL-1 members(2). Downstream intracellular signalling results in NF- κ B and AP-1 activation and the expression of proinflammatory mediators in susceptible cells. Whilst the receptor antagonist is able to bind to the receptor, it does not recruit the AcP receptor, so signaling does not occur and thus the Ra exerts antagonist effects(3, 4). IL-36 γ is thought to be mainly produced by keratinocytes and other epithelial cells in response to stimuli such as fungi, inflammatory mediators such as TNF- α and IL-1, bacteria, rhinovirus infection and smoke(5-9). IL-36 has stimulatory effects on a range of cell types including epithelial cells, fibroblasts and immune cells(6, 10-12). IL-36 expressed by epithelial cells has been documented in several tissues including the lung; however, the majority of research has focused on the skin and specifically psoriasis(6).

Psoriasis is an immune mediated inflammatory disease affecting 2% of the world population(13). Psoriasis pathology is associated with hyperkeratotic plaques and epidermal inflammatory cell infiltrates. This leads to visible raised erythematous scaly lesions. T cells and the adaptive immune system are key players in psoriasis. However, innate inflammatory mediators such as IL-1 family members, IL-23, IL-10 family members, CCL20, IL-8, TNF- α and antimicrobial peptides are now well recognised for their role in both the initiation and maintenance of psoriatic inflammation(14, 15).

IL-36 γ is highly up-regulated in psoriasis lesions on both the mRNA and protein level(16). Expression levels of IL-36 γ also correlate with levels of other cytokines such as IL-17, IL-23 and TNF α in psoriasis lesions(5). Transgenic mice in which keratinocytes overexpress IL-36 α are susceptible to a psoriasis like inflammation following 12-O-Tetradecanoylphorbol-13-acetate treatment(17). Using the same model, mice which were deficient in the IL-36Ra showed chronic skin abnormalities and enhanced plaque development. Mice deficient in the IL-36R were protected from plaque development(18). Also of note, generalized pustular psoriasis (GPP), a severe form of psoriasis, has been linked to loss-of-function mutations in the IL-36Ra. These mutations result in a less stable protein with resultant reduced control over IL-36 mediated responses(19).

The pathophysiologic contribution of endothelial cells (ECs) in psoriatic inflammation is well recognised. Psoriasis lesions show a developed vascular network, most notably in the papillary dermis. Studies have confirmed these capillaries are wide, dilated, tortuous and leaky(20-22). The combination of endothelial activation followed by angiogenesis leads to enhanced and sustained leukocyte recruitment/migration to the lesion and thus tissue inflammation(23, 24). Upregulation of adhesion molecules such as VCAM-1 (Vascular Cell Adhesion Molecule 1) and ICAM-1 (Intercellular Adhesion Molecule 1) has been detected, which increases adhesion of leukocytes and leads to extravasation(25).

Several currently used medications for psoriasis may also work by affecting ECs along with immune cells. Methotrexate is thought to inhibit adhesion molecule expression(26). Moreover, Efalizumab, which was efficient for psoriasis treatment when available, works by blocking LFA-1(Lymphocyte function-associated antigen 1) on leukocytes so they are unable to bind to the adhesion molecule ICAM-1 on ECs(27). A number of proinflammatory cytokines upregulated in psoriatic lesions including TNF- α and IL-17 are capable of activating ECs (23, 28).

The purpose of this study was to determine the effect of IL-36 γ on ECs.

Methods

Generation of IL-36 proteins

As IL-36 and IL-36Ra both require N-terminal processing to become fully active(3), recombinant active forms of IL-36 γ (IL-36 γ S18) and IL-36Ra (IL-36 RaV2) were used throughout. Constructs of IL-36 γ S18 and IL-36 RaV2 containing N-terminal His-SUMO fusion partners were generated and expressed in BL21 (DE3) Codon + *E. coli* as described before(29). Proteins were subsequently purified using Ni²⁺ -affinity and size exclusion chromatography. Once purified, the SUMO tag was removed using the Ulp1 protease, and the final active recombinant cytokines were purified by Ni²⁺ -affinity, ion exchange and size exclusion chromatography.

Cell culture and stimulations

Umbilical cords were supplied by Bradford Royal Infirmary under the approval and processing of Ethical Tissue Bradford. HUVEC (Human Umbilical Vein Endothelial Cells) from 5 donors were isolated from umbilical cords in a previously described method(30). HDLEC (Human Dermal Lymphatic Endothelial Cells) from pooled donors were supplied from PromoCell (Heidelberg, Germany). Cells were incubated in an atmosphere of 95% humidity and 5% CO₂ at 37°C. All experiments were performed between the passages of 2-5 for both cell types. Endothelial cell media (PromoCell) was used containing penicillin/streptomycin (100U/100mg/ml) and fungizone (2.5 μ g/ml) (both Life Technologies, Carlsbad, USA). Cells were plated out on 6 well plates (Greiner Bio-One, Stonehouse, UK) pre coated with 10% v/v gelatine solution overnight. Relevant cells were incubated with NF- κ B inhibitor (IMD-0354) (Merck Millipore, Billerica,USA) 1 μ M for 1 hour prior to stimulation. The specificity of IMD-0354 at the doses used as previously been documented (31).

IL-36 Receptor confirmation by quantitative PCR (qPCR)

Cells were washed with PBS and RNA was isolated using Quick-RNA MiniPrep (Zymo Research, Irvine, USA) according to the manufacturer's instructions. RNA was converted to cDNA using RevertAid first strand cDNA synthesis kit (Thermo Scientific, Waltham, USA), according to the manufacturer's instructions. cDNA from previously isolated and cultured primary human primary keratinocytes and fibroblasts were used as positive controls(32). Quantitative PCR (q-PCR) was conducted using the CFX Connect™ Real-Time PCR Detection System. Reaction was performed using the QuantiFast SYBR Green PCR Kit (Qiagen). Primers for the IL-36R (IL1RL2) were purchased from Qiagen. Data was analysed using BIO-RAD CFX manager software version 3.0. Results were normalised against the housekeeping gene U6snRNA. The ratio between Ct value for the target gene and the Ct value for the housekeeping gene was then calculated. The average values for the different cell types were then expressed as a ratio of the keratinocyte value (1.00).

IL-36 Receptor confirmation by Immunocytochemistry

HUVEC and HDLEC were seeded onto gelatin coated coverslips overnight. Cells were washed in TBS and fixed in 4% formaldehyde for 20 mins. Cells were then blocked for 1 hour in 5% BSA in TBS. Cells were incubated overnight with mouse anti-human CD31 (1:1000) (Dako, Glostrup, Denmark) and rabbit anti-human IL-36R (1:500) (Novus Biologicals, Littleton, USA) or Rabbit IgG isotype control (1:500, Abcam, Cambridge, UK). Cells were washed with TBST and secondary donkey anti-rabbit Alexa 594 conjugated, and donkey anti-mouse Alexa 488 conjugated were added (both 1:1000, both Invitrogen, Carlsbad, USA).

NF- κ B and c-JUN analysis by Western Blot

Cells were stimulated as before, for 1 hour. Cells were lysed with CelLytic M lysis buffer (Sigma-Aldrich), containing protease inhibitor cocktail (Roche Applied Bioscience, Rotkreuz, Switzerland) and phosphatase inhibitor (Thermo Scientific, Loughborough, UK). Protein concentration was determined by Bradford Assay and 20ug of total protein was separated on any kDa mini protean gel (Bio-Rad, Hemel Hempstead, UK). Proteins were

blotted onto 0.2 μ m PVDF trans-blot pack (Bio-Rad). Membranes were probed with either rabbit anti-human phospho-NF- κ B (1:1000), rabbit anti-human Phospho-c-Jun (both Cell signalling Technology, Leiden, Netherlands), or mouse anti-human GAPDH (Santa Cruz Biotechnology, Dallas, USA), in Tris-buffered saline 0.1% Tween-20 (TBST) containing 5% BSA overnight at 4 °C. Mouse anti-rabbit or Donkey anti mouse, HRP-conjugated secondary antibodies were used at 1:5000 for 1 hour at room temperature.

NF- κ B and c-JUN activity by Immunocytochemistry

Gelatin coated coverslips were placed into 6 well plates, and HUVEC cultured and stimulated as before. Following 1 hour of treatment, cells were washed in TBS and fixed in 4% formaldehyde for 20 mins. Cells were then blocked for 1 hour in 5% BSA in TBS. Cells were then incubated overnight at 4 °C with either rabbit anti-human phospho-NF- κ B(1:1000) or rabbit anti-human phospho-c-JUN (1:1000) (both Cell signalling Technology, Leiden, Netherlands). Cells were washed with TBST and a secondary donkey anti-rabbit Alexa 594 (1:1000) conjugated antibody was added. Sheep anti-human Von Willebrand factor (FITC conjugated) (Abcam, Cambridge, UK) was used as a background stain.

Chemokine analysis by ELISA

Following stimulation, CCL2 and IL-8 concentrations were measured using ELISA kits from eBioscience (San Diego, USA). CCL20 was measured using R&D systems kit (Minneapolis, USA). ELISAs were carried out according to the manufacturer's protocols. Reproducibility of supernatants were confirmed by triplicate testing, with <10% error.

Endothelial Cell adhesion molecule analysis by flow cytometry

Following stimulation, cells were scraped in PBS. 1×10^5 cells per treatment were analysed. Cell surface Fc receptor block was performed with 5% BSA in PBS for 30 minutes, cells were then centrifuged and the supernatant discarded and the pellet resuspended in PBS. The following antibodies and relevant isotype controls (BioLegend, San Diego, USA) were then added, all at a 1 in 300 dilution: Alexa Fluor 488 anti-human CD54 (ICAM-1) and PE anti-

human CD106 (VCAM-1). After 1 hr incubation, cells were centrifuged, and pellets resuspended in PBS. Cells were then analysed by the Beckman Coulter CyAn™ ADP Analyser, using the Summit software version 4.1.

Chemotaxis assay

Chemotaxis plates (101-216, 8µm pore size) were purchased from Neuro Probe, USA. 30µl of supernatant was placed in the bottom chamber. The porous insert was then placed on top. Blood was obtained from 7 healthy volunteers. Mononuclear cells were isolated from blood by Lymphoprep density gradient centrifugation and resuspended in RPMI containing 10% FCS. The suspension was then purified using a MACS magnetic separation column (Miltenyi Biotec) using microbeads for CD14 negative selection followed by CD4 positive selection. T cell purity (<90%) was confirmed by FACS CD4/CD3 selection. Isolated T cells were resuspended in RPMI and 5×10^4 cells in 20 µl were added to the top chamber. Cells were incubated in an atmosphere of 95 % humidity and 5% CO₂ at 37°C for 2 hours. Following incubation, the suspension in the bottom chamber containing migrated cells was removed and placed in counting slides (BIO RAD, USA, 1450011). The cell count was performed using the BIO RAD TC20 automated cell counter. The percentage of cells that had migrated was calculated for each treatment. The percentage of cells that migrated for media only was set to 0 and other treatment values were expressed as a migration index.

Statistical analysis

Statistical significance was calculated using a one-way ANOVA with a Bonferroni's multiple comparisons test, unless stated. Analysis was performed using GraphPad Prism software (GraphPad Software Inc, La Jolla, CA, USA). Error bars represent the standard error of the mean (SEM). *: p<0.05 from indicated controls.

Results

The IL-36R is present on endothelial cells

Whilst no stimulatory effects of IL-36 on ECs have previously been documented, expression of the IL-36 receptor (IL-36R) first needed to be confirmed. For analysis of receptor expression, two different EC types were used, HUVEC (Human Umbilical Vein Endothelial Cells) and HDLEC (Human Dermal Lymphatic Endothelial Cells). A range of cell types are known to express the IL-36R including fibroblasts and keratinocytes. Keratinocytes are known to highly express the receptor when compared to other cell types and human T cells show no expression(10). qPCR analysis confirmed expression of the IL-36R by both HUVEC and HDLEC (Figure 1A). ICC expression confirmed the expression at protein level on both HUVEC and HDLEC (Figure 1B).

IL-36 γ activates NF- κ B and c-JUN

IL-36 γ stimulation resulted in increased NF- κ B and c-JUN activation (Figure 2). Concentrations of 10 and 50ng/ml of IL-36 γ resulted in increased detection of the phosphorylated active versions of NF- κ B and c-JUN (AP-1) in the nucleus of HUVEC and also in whole protein lysate. The activation of both NF- κ B and c-JUN was reduced by the addition of the RA. NF- κ B inhibitor only partially inhibited IL-8 secretion suggesting AP-1 may also play a role (Figure 4A).

Adhesion molecules ICAM-1 and VCAM-1 are upregulated following IL-36 γ stimulation

Proinflammatory cytokines are known to upregulate adhesion molecules on ECs thereby facilitating leukocyte recruitment. With regard to IL-36 γ FACS analysis confirmed both ICAM-1 and VCAM-1 were upregulated after stimulating ECs with IL-36 γ for 48 hr (Figure 3). HUVEC and HDLEC both showed adhesion molecule upregulation in a dose dependent manner. Stimulation with 10 ng/ml showed minimal upregulation but 50 ng/ml of IL-36 γ resulted in a significant upregulation of surface expression of both ICAM-1 and VCAM-1. Median fluorescence intensity (MFI) of independent experiments was determined. Statistical

analysis performed showed the MFI for untreated controls was statistically significant when compared to IL-36 50 ng/ml MFI, for both cell types and adhesion molecules.

Endothelial cells secrete chemokines following IL-36 stimulation

To assess the functional significance of IL-36R receptor expression on ECs, chemokines known to be secreted by ECs that are of relevance for psoriatic inflammation were measured. Following 48 hr IL-36 γ stimulation, ECs supernatants were analysed for chemokines by ELISA. ECs secreted IL-8, CCL2 (MCP-1) and CCL20 in a dose dependent manner (Figure 4 A-C). ECs are known to react very sensitively to endotoxin contamination and for this reason the IL-36 preparation was boiled prior to stimulation to confirm no contamination was present. Boiled controls subsequently produced similar secretion levels to untreated cells thus excluding endotoxin contamination in the recombinant protein. Both 10 and 50 ng/ml of IL-36 γ resulted in significant chemokine production when compared to untreated control. In the presence of the IL-36Ra secretion levels were reduced in a dose dependent manner and concentrations of 10, 50 and 100 ng/ml of the Ra all lowered secretions in comparison to IL-36 γ stimulation alone. The effect of IL-36Ra also confirms the presence of the IL-36R on both EC types.

Supernatant from IL-36 γ stimulated ECs is a chemoattractant for T cells

Having observed chemokine secretion by ECs following IL-36 γ stimulation, the chemotactic potency of the endothelial cell conditioned media was assessed. CCL20, which we found to be secreted by ECs following IL-36 γ stimulation is known to be a chemoattractant for lymphocytes and has a documented role in psoriasis(33). Untreated cell supernatant, media alone and IL-36 γ + media were used as negative controls and rhCCL20 as a positive control. Supernatant from IL-36 γ stimulated ECs proved to be a chemoattractant for T cells when compared to negative controls (Figure 4D). A dose dependency for the IL-36 γ effect on endothelial cells' capacity to increase T cell migration was seen. Chemotaxis assays could confirm that IL-36Ra at 100 ng/ml was sufficient to inhibit the stimulatory effects of IL-36 γ 10 ng/ml. When stimulating with the Ra at 100 ng/ml, the migration of T cells was reduced and comparable to untreated supernatant. This suggests IL-36 γ induced chemokine secretion was indeed responsible for T cell migration.

Discussion

Psoriasis is a common chronic inflammatory skin disorder affecting around 2% of the population. The IL-1 family member, IL-36 has been linked to psoriasis pathogenesis and disease severity. In psoriasis and other inflammatory diseases, ECs are activated by proinflammatory cytokines, resulting in enhanced leukocyte recruitment. However, so far the effect of IL-36 on ECs has been unexplored.

Here, we describe that the IL-36 receptor is expressed by both HUVEC and HDLEC. We assessed the functional activity of the receptor by assessing the biologic response of ECs to IL-36 γ stimulation, including adhesion molecule upregulation and chemokine production. Both adhesion molecules ICAM-1 and VCAM-1 were upregulated following IL-36 γ stimulation. Adhesion molecules are involved in leukocyte extravasation, which allows the movement of leukocytes to the site of inflammatory responses. Selectins are responsible for the rolling and capture of leukocytes whereas ICAM-1 and VCAM-1 are involved in the adhesion and transmigration. The integrins expressed on leukocytes, LFA-1 and VLA-4 (Very Late Antigen-4) bind to ICAM-1 and VCAM-1 respectively(34). In the context of psoriasis pathogenesis, both these adhesion molecules are required for T cell adhesion and migration into the skin(17, 35).

IL-36 stimulation and subsequent chemokine secretion has been shown to be dependent on the activation of transcription factors AP-1 and NF- κ B (36). We report activation of both these transcription factors in ECs (Figure 2). ECs responded with chemokine production to IL-36 γ stimulation. CCL2 (MCP-1) has a documented importance in psoriasis and other inflammatory diseases by recruiting monocytes(37). IL-8, which promotes neutrophil recruitment, EC survival and angiogenesis, was also secreted following IL-36 γ stimulation(38). The importance of angiogenesis in psoriasis is well recognised(39), and local EC secretion of IL-8 induced by IL-36 γ could be a contributing factor. CCL20 was secreted, which binds to CCR6, which is highly expressed on IL-17 and IL-22 producing lymphocytes and the majority of T cells that infiltrate the skin in psoriasis are CCR6 positive(33). Chemotaxis assays confirmed increased T cell migration following IL-36 γ EC stimulation, which could be due to CCL20 secretion. Although keratinocytes are a main source of CCL20 (33, 40), our findings indicate that ECs can contribute to the cutaneous CCL20 as well as CCL2 production. For all chemokines analysed, the IL-36Ra was able to dampen the effect in a dose dependent manner. This finding was also mirrored in the

chemotaxis assay where T cell migration was reduced when cells had been co-stimulated with the Ra.

It is hypothesized environmental triggers can cause keratinocytes to secrete IL-36 γ independently of proinflammatory cytokines such as TNF α (17, 35). It is thus possible that IL-36 γ could be important in the initiation of EC activation.

Our here presented findings could also have implications for other diseases. Lung bronchial epithelial cells secrete IL-36 γ in response to cytokines such as TNF α (6). Cigarette smoke, the causative agent of Chronic Obstructive Pulmonary Disease (COPD) is also known to cause IL-36 γ secretion in bronchial epithelial cells(7). However, the exact role of IL-36 in respiratory disease is still unknown. IL-36 α has been shown to be expressed in the synovium of both psoriatic and rheumatoid arthritis patients (41), while both α and γ have been implicated in Crohn's disease in mouse models (42). In COPD, Crohn's disease and rheumatoid arthritis, like many chronic inflammatory diseases, angiogenesis and EC activation occurs(43-45). Therefore IL-36 may also have a role in EC activation and leukocyte recruitment in these diseases. Murine models of liver damage/liver inflammation have also identified enhanced expression of IL-36 γ , and elevated CCL20 levels(46). EC induced secretion of CCL20 by IL-36 γ could be one of the potential sources of this.

IL-36 γ serum levels are enhanced in psoriasis patients(16). In recent years psoriasis has been suggested as an independent risk factor for atherosclerosis(47, 48). In atherosclerosis, endothelium activation and initial inflammatory signalling represent an important stage of disease development(49). Increased serum levels of IL-36 γ and therefore possible increased endothelium activation of the arteries could accelerate atherosclerosis disease progression. Of note, generalised pustular psoriasis (GPP) can be associated with loss of function mutations in IL-36Ra and results in reduced control over IL-36. However the risk factor of GPP on cardiovascular disease is largely unexplored.

In summary, our results suggest that IL-36 γ has a role in activating the endothelium further enhancing lymphocyte recruitment and thus enhancing psoriatic inflammation. These findings add to the growing importance of IL-36 γ in both psoriasis initiation and maintenance. Further studies will help decipher the significance of IL-36 γ and its interactions with other EC stimulating mediators such as TNF- α and IL-17 in human psoriatic tissue.

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Author Contributions Statement

CB performed EC culture and experiments

AA contributed to chemotaxis and PCR experiments and analysis of data obtained

MW contributed to setup and planning of the IL-36 experiments, writing and reading of the manuscript

MS contributed to IL-36 experiment planning and critical discussion of results obtained as well as manuscript correction

DL contributed to experiments with HDMEC, experimental planning and critical discussion

AG contributed to EC cell experimental setup, experimental planning and discussion

Additional Information

Competing financial interests

None declared

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Figure 1. Expression of the IL36R on human endothelial cells. A) qPCR was performed and relative mRNA expression determined. Keratinocyte expression was set to 1.00 and HUVEC, HDLEC and fibroblast expression of the receptor was quantified relative to keratinocyte expression. Samples: HUVEC n = 5, HDLEC n=3 (pooled donors) 3 biological replicates, keratinocytes, fibroblasts n=2. Standard error of mean depicted on graph. B) ICC confirmation of receptor on HUVEC and HDLEC. Magnification x 40.

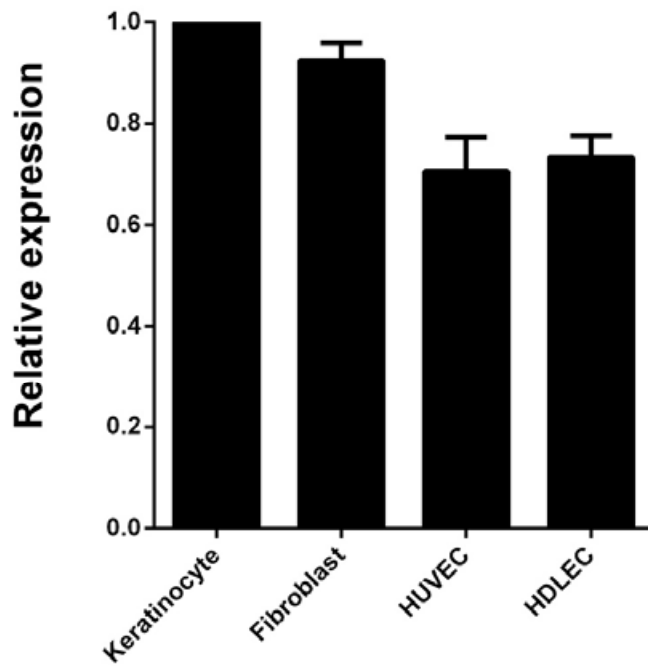
Figure 2. IL-36 γ induces NF- κ B and c-JUN in HUVEC. Western blot (A) and ICC analysis (B) of pNF- κ B-p65 and pc-JUN. Phosphospecific antibodies detect active form of NF- κ B or c-JUN in the nucleus of HUVEC. Increased detection of active form of both detected in whole cell lysate via western blot. Magnification x 40.

Figure 3. Endothelial cell adhesion molecule upregulation following IL-36 γ stimulation. Following 48 hr stimulation with IL-36 γ , ECs (HUVEC and HDLEC) were stained with labelled antibodies specific for ICAM-1 or VCAM-1 and analysed by flow cytometry for surface expression. Relevant isotype controls were used as negative control. Fluorescence beyond the “isotype” line in the depicted histograms thus represents specific binding of antibody demonstrating detectable expression. A representative experiment is shown (sample sizes: HUVEC n=3, HDLEC n=3).

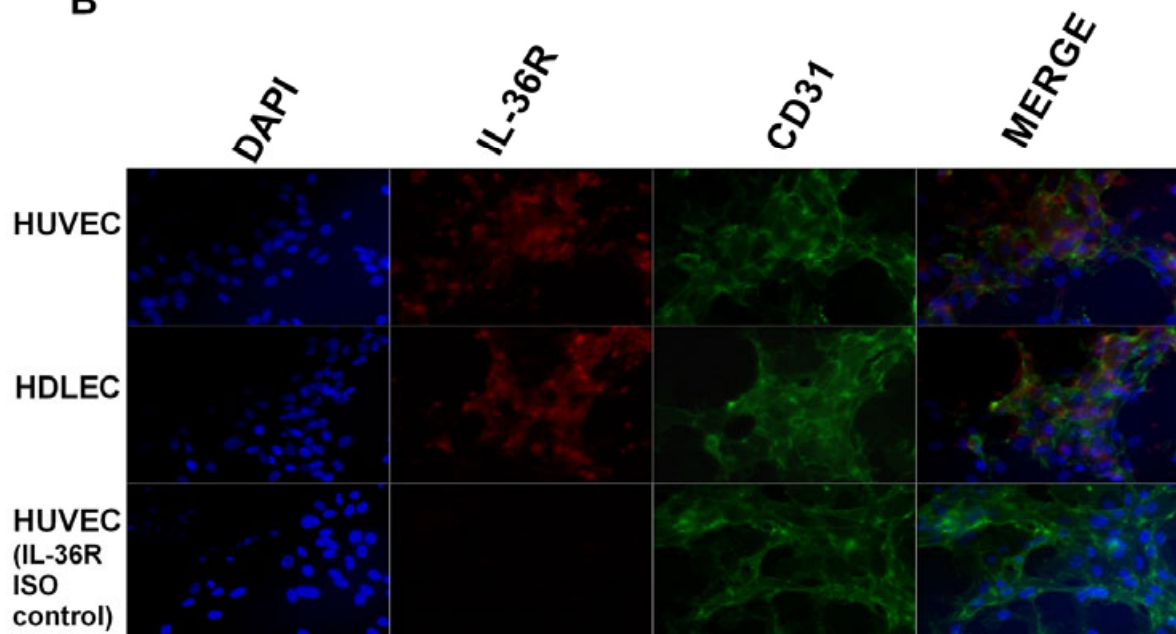
Figure 4. ECs secrete chemokines which are a chemoattractant for T cells. Following 48hr stimulation, the cell supernatant was tested for CCL20, CCL2 and IL-8 by ELISA (A-C). Sample sizes: HUVEC n=5, HDLEC n=3 (3 biological replicates of pooled donors). The supernatant from treated and untreated ECs was removed and tested for its chemoattractant ability using a chemotaxis assay. The supernatant was placed in the bottom chamber and T

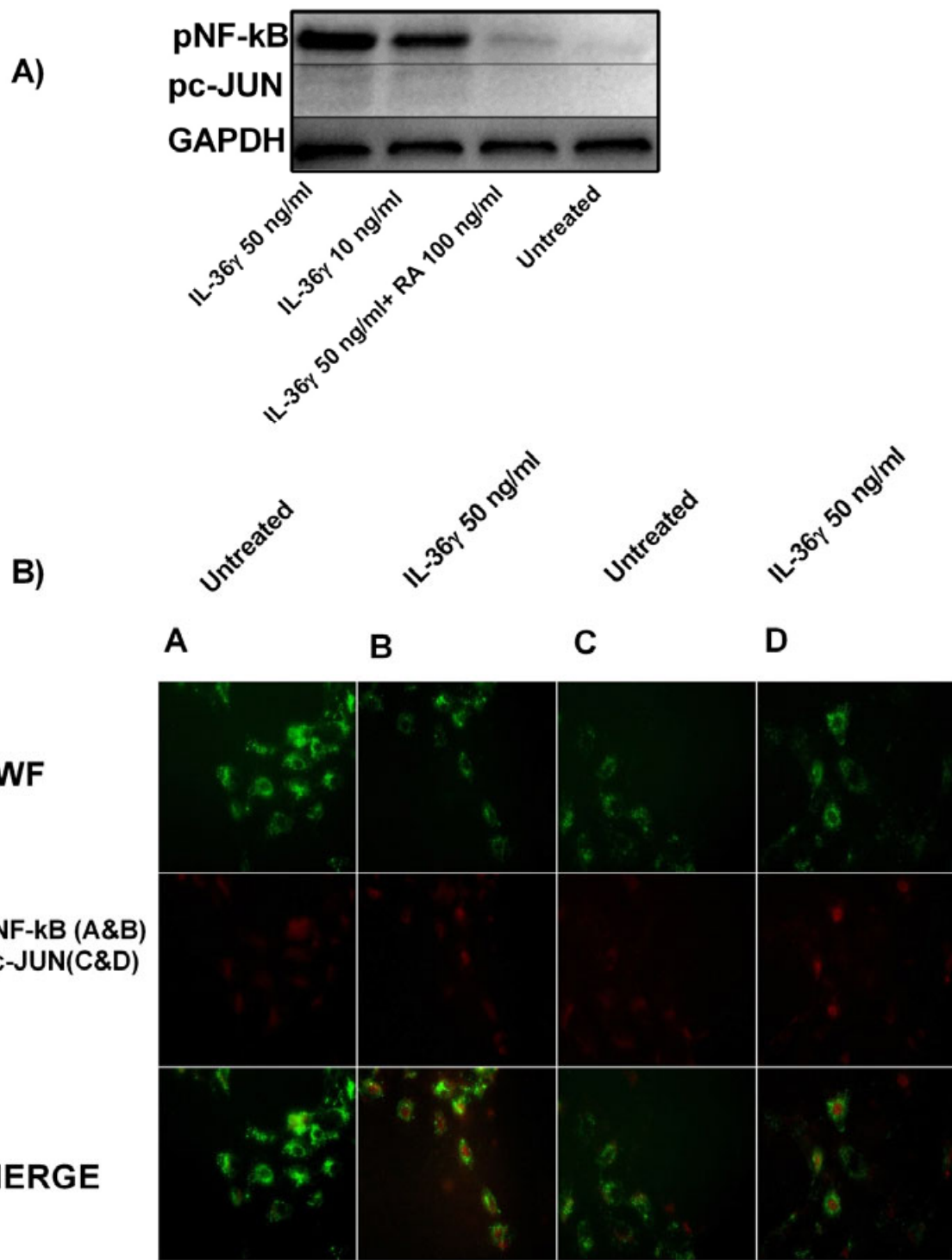
cells above. The number of migrated cells in the bottom chamber was then measured. Sample size: n=7. Standard error of mean depicted. ANOVA *: p<0.05 from relevant controls.

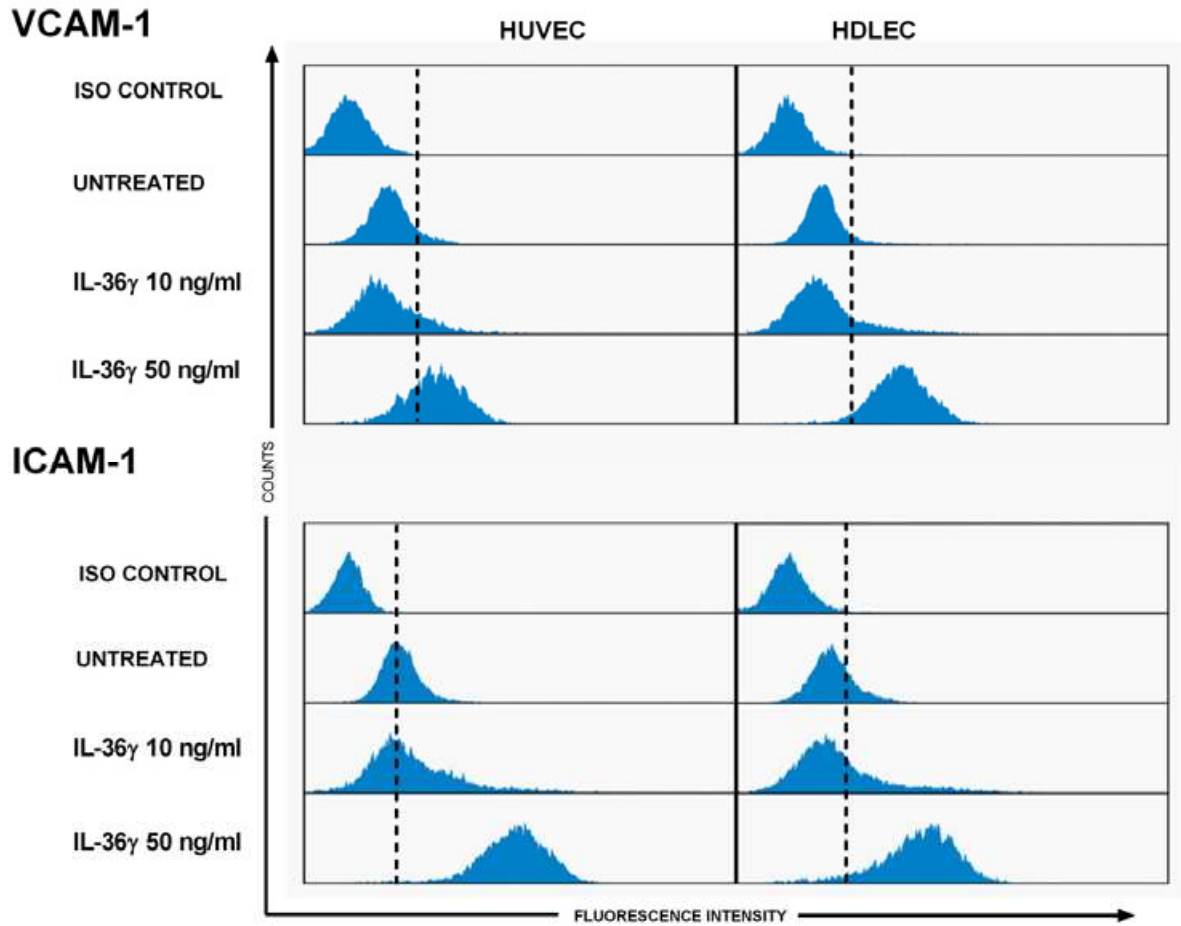
A)

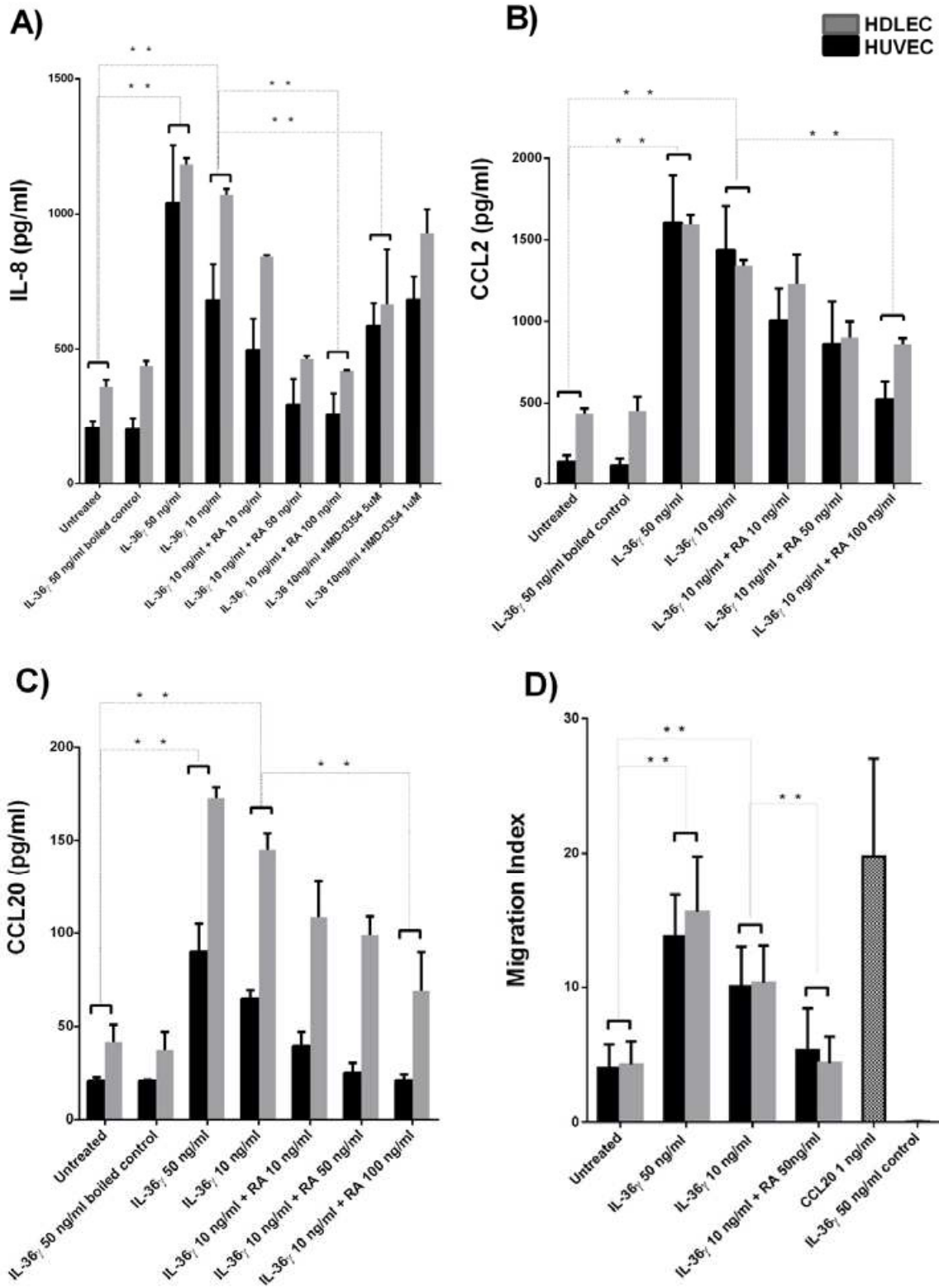


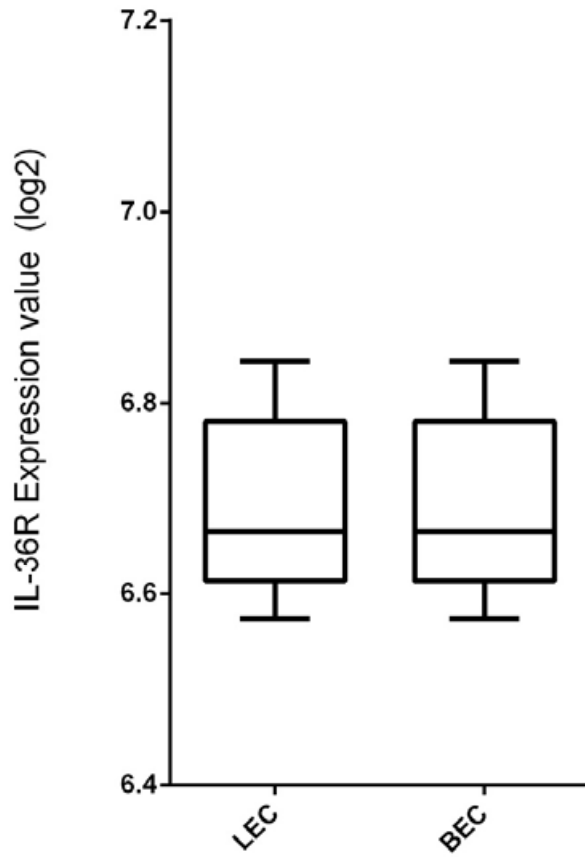
B











Additional figure 1-

IL-36R expression on both HDLEC (Human dermal lymphatic endothelial cells) and HDBEC (Human Dermal Blood Endothelial Cells). Data obtained from the "Normalised Log2 transformed signal", from Affymetrix microarray profiling.