



Penha, Rafael and Higgins, John and Mutamba, Shilla and Barrow, Paul and Mahida, Yashwant R. and Foster, Neil (2016) IL-36 receptor is expressed by human blood and intestinal T lymphocytes and is dose-dependently activated via IL-36 β and induces CD4+ lymphocyte proliferation. *Cytokine*, 85 . pp. 18-25. ISSN 1096-0023

Access from the University of Nottingham repository:

<http://eprints.nottingham.ac.uk/41582/1/Penha%20et%20al%20Cytokine.pdf>

Copyright and reuse:

The Nottingham ePrints service makes this work by researchers of the University of Nottingham available open access under the following conditions.

This article is made available under the Creative Commons Attribution Non-commercial No Derivatives licence and may be reused according to the conditions of the licence. For more details see: <http://creativecommons.org/licenses/by-nc-nd/2.5/>

A note on versions:

The version presented here may differ from the published version or from the version of record. If you wish to cite this item you are advised to consult the publisher's version. Please see the repository url above for details on accessing the published version and note that access may require a subscription.

For more information, please contact eprints@nottingham.ac.uk

IL-36 receptor is expressed by human blood and intestinal T lymphocytes and is dose-dependently activated via IL-36 β and induces CD4+ lymphocyte proliferation

Rafael Penha¹ John Higgins¹, Shilla Mutamba¹, Paul Barrow¹, Yashwant Mahida² and Neil Foster^{1*}

School of Veterinary Medicine and Science¹ and the Institute of Infection, Immunity and Inflammation². University of Nottingham, UK

*Corresponding author:

Neil Foster

School of Veterinary Medicine and Science

Sutton Bonington campus

University of Nottingham

Nottingham

NG7 2NR

Tel: 0115 9516433

Email: n.foster@nottingham.ac.uk

Abstract

We show that IL-36R is expressed by T (CD4+ and CD8+) and B (CD19+) lymphocytes in human blood and also by CD4+ T lymphocytes in the intestinal lamina propria. IL-36R protein was mostly stored in the cytoplasm of CD4 lymphocytes and B cells, during steady and the greatest expression of IL-36R mRNA was measured in CD4+ (T helper) lymphocytes. IL-36 β , which functions via IL-36R induced rapid and significant ($P < 0.05$) proliferation of CD4+ lymphocytes, within 48h. IL-36R expression was also maintained on the surface of circulating CD4+ lymphocytes which enter the intestinal lamina propria.

In conclusion our study is the first to show that (1) all human blood lymphocytes express IL-36R; (2) IL-36R expression is maintained by circulating CD4+ lymphocytes which enter the intestinal lamina propria and (3) IL-36R/IL-36 β induces rapid CD4 lymphocyte proliferation. The possible significance of these results in the context of human disease is discussed.

1. Introduction

There are relatively few published studies regarding the biology of IL-36 receptor (IL-36R) and its cytokine ligands IL-36 α , IL-36 β , IL-36 γ and IL-36Ra, since this novel arm of the IL-1 family was discovered during the human genome project. Fewer studies still have reported on the biological expression and function of these molecules in human immune cells. There is now mounting evidence, from murine studies, that the IL-36R/IL-36 α axis may have an important role in skin disorders [1-3]. This could also be the case in humans since missense mutations within *IL-36RN* genes, which encode for IL-36RA, an IL-36R antagonist, have been shown to be associated with pustular psoriasis [4]. In another study in which this association was made, peripheral blood mononuclear cells, isolated from these patients produced high concentrations of proinflammatory cytokines when cultured with IL-36 α [5]. The expression of IL-36R by immune cells and the effect of IL-36 α - γ on these cells may also be important in the aetiology of many other inflammatory pathologies [reviewed 6]. Murine studies have shown that IL-36R is expressed on naive CD4⁺ Th cells and that low concentration of IL-36 α - γ (100 ng/ml) directly induces proliferation of these cells [2]. In patients with psoriatic arthritis, both IL-36R and IL-36 α expression is increased in the synovial lining and also in infiltrating CD 138⁺ plasma cells [7]. Although studies by Lamacchia *et al.*, [8] have shown that the levels of expression of IL-36R and IL-36 α , in the synovium of collagen-induced arthritic mice, is not correlated with disease progression. Nevertheless, some of these studies do suggest that IL-36R-expressing lymphocytes may proliferate in response to novel IL-36 cytokines and this may have some affect on inflammatory arthritis. If this is so then IL-36R must also be expressed by cells entering the peripheral tissues from the blood.

The aim of the study we report was to determine whether human lymphocytes express IL-36R and if so to investigate the cellular distribution and biological significance of this receptor.

2. Materials and methods

2.1. Reagents

Unless otherwise stated all laboratory reagents were purchased from Sigma, Poole, UK and all antibodies were purchased from Serotec, Oxford, UK. Recombinant human IL-36 β proteins and murine anti-human IL-36R were supplied by the Amgen, Corporation, Seattle, Washington, USA. All reagents were measured for LPS contamination by Amgen and University of Nottingham prior to use.

2.2. Bioethics

All studies were conducted following approval by local ethics committee following written patient/participant consent.

2.3. Endotoxin assays

A chromogenic Limulus Amebocyte Lysate (LAL) assay (Kinetic-QCL, TMLonza, USA) was performed on IL-1 reagents at Amgen and at the University of Nottingham by standard methods. Assay sensitivity was between 0.005 and 50.0 EU/ml. All reagents tested (IL-36R, IL-36 β) contained \ll 0.05 Pg/ml LPS. As an additional test for LPS contamination, THP1 monocytes (which are IL-36R negative but react to LPS) were cultured with up to 500 ng/ml of IL-36R or IL-36 β prior to measurement of TNF- α over a 24h period. All tests were shown to be negative for TNF α and, therefore, LPS at the concentrations used in the work described here.

2.4. Cell isolation and culture

2.4.1. Isolation of peripheral blood lymphocytes and cell sorting

Blood products from healthy donors were obtained from the National blood transfusion service (Sheffield, UK). Isolation of peripheral blood mononuclear cells (PBMC) was performed using differential centrifugation in histopaque 1077 (Sigma-Aldrich, UK) by standard methods. The isolated PBMC were incubated with magnetic microbeads conjugated with mouse anti-human CD19⁺, anti-human CD4⁺, CD3⁺ or anti-human CD8⁺ antibodies (Miltenyi Biotec, Bisley, UK) for 20 min at 6°C. The labeled cells were separated by passing the cell suspension over a magnetic-activated cell sorter (AutoMACS, Miltenyi Biotec, Germany). Each population was carefully removed from the columns and were separately kept in culture in RPMI 1640 media supplemented with 10% (v/v) Fetal Calf Serum, 200mM L-glutamine and 5 mL of antibiotic solution containing 100 IU of penicillin and 1mg of streptomycin per mL (Sigma-Aldrich, UK).

2.4.2. Isolation of human ileal lamina propria cells

Fresh, histologically normal mucosal samples, surplus to clinical requirements, were obtained from human terminal ileum resected (as part of right hemicolectomy specimens) for tumour. Ethical committee approval was provided by the Nottingham Research Ethics Committee. Mucosal samples were obtained at least 5 cm from the tumour. The mucosal samples were washed thoroughly with calcium- and magnesium-free Hanks balanced salt solution (HBSS; Gibco BRL, Gaithersburg, MD, USA), to remove adherent luminal material. Lamina propria cells were isolated from mucosal samples using a previously described technique [9].

Briefly, dissected strips of mucosa were weighed and incubated in 1 mmol/l dithiothreitol solution (DTT) (Sigma, Poole, Dorset, UK) for 20 minutes at room temperature. To remove the epithelial cells, mucosal strips were treated three times with 1 mmol/l ethylenediamine tetra-acetic acid (BDH, Poole, Dorset, UK) at 37°C for 30 min. Between each incubation step the strips were washed with calcium and magnesium-free HBSS.

The mucosal samples, denuded of epithelial cells, were subsequently cultured at 37 °C in RPMI containing 10% fetal calf serum (Gibco) in 60 mm tissue culture dishes (Costar Corp, Cambridge, MA, USA). During culture, cells appeared both in suspension and attached to the tissue culture dishes. After a 24h culture period, these cells were collected by transferring the pieces of mucosal tissue to culture dishes containing fresh media and incubating the original dishes (containing cells only) at 4°C for 60 min. Following pipetting to detach adherent cell, the cells were counted and viability tested using Trypan blue (4 µg/ml) and were found to be between 90-100% viable.

Depending on down-stream applications, the isolated lamina propria cells were suspended in appropriate medium at a density of 1×10^6 cells/ml. To stabilize RNA prior to extraction, samples for qRT-PCR analysis were immediately pelleted and suspended in *RNAlater* RNA Stabilization Reagent according to the Manufacturer's recommendations (QIAGEN, Crawley UK). Total RNA was extracted from the cells, reverse-transcribed into cDNA and amplified in a typical real time PCR reaction. A relative quantification analysis using GAPDH as the reference gene and IL-36R as the target gene was performed on a LightCycler® 480 analyser. HT 29 cells and THP-1 cells were used as positive and negative controls respectively. FACS analyses were also performed on migrated cells to ascertain overall CD3⁺ population, CD4⁺

population within the CD3⁺ populations and expression of IL-36R protein by CD4⁺ cells. Each analysis was performed in triplicate on 5 separate occasions.

2.4.3. HT 29 cell cultures

The human colonic epithelial carcinoma cell line, HT-29 (was a kind gift from Professor Susan Watson, University of Nottingham) was used as a positive control for IL-36R expression, as previously reported [10] Cells were cultured in Dulbecco's modified Eagle's medium supplemented with glucose (25mM), Sodium Bicarbonate (44mM), Transferrin (10mg/l), sodium pyruvate (1mM), L-glutamine (2mM), 0.1 mM non-essential amino acids (NEAA) and 10% FCS. Cell cultures were maintained in humidified incubators at 37°C, 5% CO₂. The medium was changed every day to prevent glucose exhaustion.

2.5. Flow Cytometry

Following cell sorting, FACS analyses were performed by standard methods. Briefly, 1×10⁶ test cells of each purified cell population (CD19⁺, CD4⁺ and CD8⁺) were resuspended in FACS buffer (BSA 1% w/v); EDTA (2 mM). The cells were blocked in FACS buffer containing human serum (10% v/v) for 15 min and some cell preparations were permeabilized with 10% (w/v) Saponin (Sigma-Aldrich, UK). Permeabilized and non-permeabilized cells were incubated for 45min with mouse anti-human IL-36R antibody (Amgen, USA) or with IgG2a isotype control conjugated with FITC for negative controls (Abcam, UK). Cells were then analysed using an FACSCanto II analyser (Becton Dickinson, USA). Samples were acquired using the CellQuest pro software (Becton Dickinson) and analysed using the WinMDI 2.8

software. Cell viability was assessed by propidium iodide uptake (20 µg/mL for 10 min) via FACS analysis and was found to be >90% in all cases.

2.6. Analysis of IL-36R expression

2.6.1 Analysis of IL-36R mRNA expression by quantitative real time RT-PCR (qRT-PCR in real time)

Prior to qRT-PCR analysis, RNA was first reverse-transcribed into cDNA in a reverse transcription (RT) reaction using the Transcriptor First Strand cDNA Synthesis Kit (Roche, West Sussex, UK) as per supplier's instructions. A random hexamer primer was used as detailed in the manufacturer manual. The total reaction volume was 20 µl made up of Transcriptor Reverse Transcriptase reaction buffer (4 µl), Deoxynucleotide (dNTP) mix (2 µl), random hexamer primer (2 µl), protector RNase inhibitor (0.5 µl), Transcriptor Reverse transcriptase (0.5 µl) and a maximum of 11 µl RNA (up to 1µg of total RNA was used). The following program was used on the thermal cycler: 10 minutes at 25°C, 30 minutes at 55°C, 5 minutes at 85°C then hold at 4°C. All samples were run in duplicate.

For qRT-PCR, a reaction volume of 25 µl was used on the LightCycler® 480 analyser (Roche, West Sussex, UK). This consisted of 12.5 µl LightCycler® 480 Probes Master (2x concentration), 2 µl cDNA, 1.5 µl forward primer, 4.5 µl reverse primer (Table1), 0.88 µl hydrolysis probe (Universal Probe Library, Roche, West Sussex, UK) and 3.63 µl PCR-grade water. The working concentration for all primers and probes was 0.5 µM. The expression of the IL-36R gene was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) through relative quantification using the $2^{-\Delta\Delta CT}$ method [11]. THP-1 cells were used as a negative control and HT29 cells were used as positive controls for IL-36R expression. Primer

sequences are shown in table 1 and as previously reported [12-13] and PCR product obtained was also shown to be of correct size as that previously reported [12].

2.6.2. Analysis of IL-36R protein expression by immunocytochemistry

An aliquot of approximately 10^3 purified CD4⁺, CD8⁺ and CD19⁺ cells was separately collected from cell culture, washed in 1mL of PBS and centrifuged at 250 g for 5 min. Cells were suspended in 500 μ L of PBS and centrifuged at 800rpm in the CytoSpin 4 (ThermoScientific, UK) to lay the cells in the slides. Initially the cells were fixed on ice cold solution of acetone and methanol 1:1 (v/v) for 2 min and the slides were washed for 10 min in PBS. Cells were incubated for 20 min at RT with 10% (v/v) human serum (Sigma Aldrich, UK) to block unspecific binding. Slides were then incubated for 1h at RT with mouse anti-human IL-36R (25 μ g/mL, Amgen, Seattle, WA, USA) and incubated for 1h at RT with goat anti-mouse IgG1 conjugated with fluorescein isothiocyanate (FITC). Slides were rinsed with PBS and incubated for 20min with Phalloidin-Rhodamine (200ng/mL, P1951, Sigma-Aldrich, UK). The cells were washed and mounted with Prolong Gold anti-fade reagent containing 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, Paisley, UK). The detection of IL-36R was compared with negative control cells, incubated without the primary antibody.

2.7. Lymphocyte proliferation assays

A standard WST-1 cell proliferation assay (Roche Diagnostics Ltd., West Sussex, UK) was used (as per manufacturer's instructions) to measure proliferation of lymphocytes (10^4 cells/100 μ l RPMI medium) after 48h culture in IL-36 β (100 ng/ml). We deliberately used a very short culture period to ascertain true proliferation rather than increased survival which was shown to be problematic in other studies. Results from the colourimetric assay were compared by measuring absorbance at 450 nm on a Labtech LT-

4000 ELISA Plate Reader and to assess mitochondrial enzyme activity, these were compared to a standard curve obtained from control lymphocytes. Lymphocytes cultured with Concanavalin A (conA) (75µg/ml) over the same time period were used as a positive control while unstimulated lymphocytes were used as a negative control.

2.8. Statistical Analysis

An ANOVA test with a one way classification was used to determine significant differences, at the 95% confident interval, in the numbers of lymphocytes which had divided after 24h. A post-hoc test (Tukey's) was then used to identify means which were significant. Statistical data was analysed using SPSS software, licenced to the University of Nottingham.

3. Results

Initially we investigated IL-36R expression in non-adherent cells in blood buffy coats and found this to be positive (above that expressed by the positive control, HT-29 cells). We then found that most of the IL-36R signal was coming from the CD3+ (pan T cell) fraction (Fig 1). When we examined IL-36R expression in the granulocyte fraction (below the buffy coat) we found that these cells did not express the receptor and was comparable to the negative control cell line (THP1) (Fig 1). The next set of experiments investigated IL-36R mRNA expression in blood lymphocyte subsets compared with HT29 cells (positive control). Quantitative PCR analysis showed that in all lymphocyte subsets, constitutive IL-36R mRNA expression was greater than that measured in HT29 cells but this increase was significant ($P < 0.05$) only in CD4+

T cells (Fig 2A). We then incubated CD4⁺ lymphocytes with varying concentrations of IL-36 β , to ascertain whether this would alter IL-36R expression relative to constitutive levels. Our results showed that all concentrations of cytokine (10-500 ng/ml) significantly increased IL-36R expression above constitutive levels but this was greatest at 100-200 ng, at which there was a plateau in IL-36R mRNA expression (Fig 2B).

We then used fluorescent microscopy to investigate the cellular distribution of IL-36R in lymphocytes. IL-36R immunoreactivity was detected on the cell membranes of CD4⁺ and CD8⁺ T lymphocytes as well CD19⁺ B lymphocytes (Fig 3). In all cases the immunoreactivity was comparable to that observed in HT29 cells (Fig 3 panel B) which have previously been used as an IL-36R positive control. In contrast, a negative control panel (without IL-36R antibody and secondary antibody only) showed no non-specific binding to these cells (control overlay D). However, this study also showed what appeared to be cytoplasmic localisation of IL-36R in both CD4⁺ and CD19⁺ lymphocytes, as well as HT29 cells but this was much less clear in CD8⁺ lymphocytes (Fig 3 panel B). To further investigate both expression and localisation of IL-36R protein, we used FACS analysis on non-permeabilised (cell membrane only) and permeabilised (cell membrane and cytoplasm) cells. Our results show that most of the measured IL-36R protein in CD4⁺ cells was within the cell cytoplasm with an associated increased shift in IL-36R compared with non-permeabilised cells (Fig 4A). In contrast to CD4⁺ lymphocytes, the levels of IL-36R protein detected in CD8⁺ lymphocytes was comparable in both permeabilised and non-permeabilised cells, implying that most expression was membrane associated (Fig 4B). In both CD19⁺ B lymphocytes (Fig 4C) and in HT29 cells (Fig 4D) IL-36R protein expression was increased in permeabilised cells but the associated shifts between IL-36R protein

expression in permeabilised and non-permeabilised cells was not as large as that measured in CD4+ lymphocytes. To investigate whether ligation of IL-36R had any physiological effect on CD4+ lymphocytes, we measured lymphocyte proliferation after culturing the cells with IL-36 β for 48h. Our studies showed that CD8+ T lymphocytes and CD19+ B lymphocytes did increase in number in cultures exposed to IL-36 β but this change was not statistically significant. However, IL-36 β did induce CD4+ T lymphocytes to proliferate at significantly higher ($P < 0.05$) numbers than unstimulated controls (Fig 5). In all cases, proliferation was low, probably due to the short culture period used.

The dynamic circulation of immune cells from blood into tissues can have profound effects on the expression of numerous proteins. With this in mind, we then investigated whether expression of IL-36R was maintained, lost or reduced when CD4+ lymphocytes enter peripheral tissues. We chose surgically resected (normal) human intestinal mucosal tissues for this analysis. Initially (following cellular isolation from the lamina propria; LP) we measured the population of CD3+ (pan T cells) lymphocytes and within this population the number of CD4+ cells, to ascertain whether CD4+ cells were a good representative population to study (i.e in sufficient numbers within the tissues). Our results show that 75% of cells which migrated from the LP tissues were CD3+ (Fig 6A & B) and of these, 55% were CD4+ (Fig 6C & D). We then measured IL-36R mRNA expression in isolated intestinal LP cells by qPCR and compared this to expression by HT29 cells (positive control) and THP1 monocytes (negative control). This showed that LP cells expressed IL-36R at higher levels than HT29 cells (Fig 7A) and when we further analysed the CD4+ sub-population within the total isolated LP population (by FACS analysis) we found that they expressed IL-36R on their cell membranes (Fig 7B).

4. Discussion

The study we report is the first to show that human blood lymphocytes express the novel IL-1 family cytokine receptor IL-36R. The study therefore shows differences in the expression of IL-36R between humans and mice. In mice IL-36R has been reported to be expressed by splenic CD4⁺ T lymphocytes but not by CD8⁺ T lymphocytes or B lymphocytes [2]. Our study also differs from that of Foster *et al.*, [14] which reports a lack of expression of IL-36R in human lymphocytes. It is possible that that some anomalies have arisen due to differences in this study and ours. We have used three different methods to show expression of IL-36R mRNA and protein in human Th cells, CTLs and B lymphocytes. In our studies all reagents were LPS tested and the IL-36R antibody used was monoclonal, unlike the polyclonal antibody used in the aforementioned study. We also used the same PCR probe we successfully reported on before [12-13] and visualised the PCR product, using an agarose gel, which was found to be the correct size for IL-36R (data not shown). Our data shows differences in the expression pattern of IL-36R in different lymphocytes, with CD4⁺ lymphocytes (and to a lesser extent B lymphocytes) containing large cytoplasmic stores of the receptor, whereas in CD8⁺ lymphocytes most of the IL-36R protein is found on the cell membrane. We then studied whether, or not, IL-36 β altered expression of IL-36R in CD4⁺ lymphocytes and found that IL-36R expression plateaued when the cells were incubated with 100-200 ng/ml IL-36 β . Human IL-36 α - γ have been shown before to be effective at relatively low doses, when compared to murine homologues [12-13]. The current study is, therefore, consistent with the latter since culture of CD4⁺ cells with IL-1 β at 500 ng/ml decreased IL-36R expression,

when compared to expression at 100-200 ng/ml, which also indicates a negative feedback loop shown in a previous paper [12].

Classic IL-1 cytokines are known to indirectly induce T cell proliferation by enhancing APC maturation and survival [15-16] and we have recently shown that this is also the case for IL-36 β and IL-36 γ -stimulated human monocyte derived dendritic cells (MDDCs) [12]. Few studies have reported the direct effect of classic IL-1 cytokines on lymphocyte proliferation. However, Ben-Sasson *et al.*, [17] have shown that antigen-primed murine CD4⁺, Th cells proliferate in response to IL-1 β . In this study, CFSE analysis showed that IL-1 β stimulation was responsible for only about a 2 fold increase in CD4⁺ Th cells and that the overall 7-8 fold increase measured at the end of the experiments was mostly due to enhanced survival of IL-1 β -stimulated Th cells. Similarly, IL-1 β has also been shown to induce the proliferation of human IL-17 producing, CD4⁺ Th cells [18] and IL-17/Th memory cell populations [19]. Due to measuring proliferation after only 48h, our studies show that there is a rapid effect of IL-36 β which cannot be due merely to increased survival as has been shown in some other studies mentioned above. However, the down-side to using a short incubation period was that quite low numbers of cells had proliferated and also we cannot say if CTL or B cell proliferation may have surpassed that of CD4⁺ lymphocytes after a longer period of time. Nevertheless the study did show some functionality of this receptor in CD4⁺ lymphocytes.

If expression of IL-36R by lymphocytes has some functional significance in disease within peripheral tissues, then expression of IL-36R must be maintained on the cell membrane of lymphocytes as they enter the tissues from circulation. We found that IL-36R mRNA was expressed in the lamina propria at higher levels than was

expressed by HT-29 (positive) cells. Intestinal samples were then denuded of epithelium and cultured to allow migration of cells from the LP. Consistent with a previous study [9], 75% of the cells which migrated from the LP, after 36h, were CD3⁺ T cells and of these 55% expressed CD4. However, we show for the first time that the CD4⁺ population within the LP also expressed IL-36R. Usually $\gamma\delta$ T cells, which reside in the intestine, do not express CD4 [20] but rare $\gamma\delta$ /CD4⁺ T phenotypes have been shown to be expanded in patients with rare genetic disorders such as CD3 δ deficiency, a form of severe combined immunodeficiency [21]. Intra-epithelial lymphocyte populations are known to increase in patients with various intestinal disorders [22] but the model we have used denudes the intestinal epithelium which would also preclude intra-epithelial lymphocytes from the CD4/IL-36-R population we have measured. Our results, therefore, most likely indicate that CD4⁺ lymphocytes maintain IL-36R expression as they enter peripheral tissue from the blood. Thus, expression of IL-36R by CD4⁺ lymphocytes, and their activation via IL-36R cytokine ligands, could have a role in human intestinal and blood disorders.

5. Disclosures

The authors have none to declare.

Acknowledgements

The study was funded by a Nottingham University studentship awarded to NF and YM.

References

1. Blumberg, H., Dinh, ES., Trueblood, J., Pretorius, D., Kugler, N., Weng, ST., Kanaly, JE., Towne, JE., Willis, CR., Kuechle, MK., Sims, JE. & Peschon, JJ. (2007). Opposing activities of two novel members of the IL-1 ligand family regulate skin inflammation. *J. Exp. Med*, 204, 2603–2614.

2. Vigne. S., Palmer, G., Martin, P., Lamacchia, C., Strebel, D., Rodriguez, E., Olleros, ML., Vesin, D., Garcia, I., Ronchi, F., Sallusto, F., Sims, JE & Gabay C. (2012). IL-36 signaling amplifies Th1 responses by enhancing proliferation and Th1 polarization of naive CD4+ T cells. *Blood*, **120**, 3478-3487.

3. Tortola, L., Rosenwald, E., Abel, B., Blumberg, H., Schafer, M., Coyle, AJ., Renaud JC., Werner, S., Kisielow, J. & Kopf, M. (2012). Psoriasisiform dermatitis is driven by IL-36-mediated DC-keratinocyte crosstalk. *J. Clin. Invest*, **122**, 3965-3976.
4. Marrakchi, S., Guigue, P., Renshaw, BR., Puel, A., Pei, XY., Fraitag, S., Zribi, J., Bal, E., Cluzeau, C., Chrabieh, M., Towne, JE., Douangpanya, J., Pons, C., Mansour, S., Serre, V., Makni, H., Mahfoudh, N., Fakhfakh, F., Bodemer, C., Feingold, J., Hadj-Rabia, S., Favre, M., Genin, E., Sahbatou, M., Munnich, A., Casanova, JL., Sims, JE., Turki, H., Bachelez, H. & Smahi, A. (2011). Interleukin-36-receptor antagonist deficiency and generalized pustular psoriasis. *N. Engl. J. Med*, **365**, 620–628.

5. Onoufriadis, A. , Simpson, MA., Pink, AE., Di Meglio, P., Smith, CH., Pullabhatla, V., Knight, J., Spain, SL., Nestle, FO., Burden, AD., Capon, F., Trembath, RC. & Barker JN. (2011). Mutations in IL36RN/IL1F5 are associated with the severe

episodic inflammatory skin disease known as generalized pustular psoriasis. *Am. J. Hum. Genet.* **89**, 432–437.

6. Van de Veerdonk & Netea MG. (2013). New insights in the immunobiology of IL-1 family members. *Front Immunol*, **4**, 167.

7. Frey, S., Derer, A., Messbacher, ME., Baeten, DL., Bugatti, S., Montecucco, C., Schett, G. & Hueber AJ. (2013). The novel cytokine interleukin-36 α is expressed in psoriatic and rheumatoid arthritis synovium. *Ann Rheum Dis*, **72**, 1569-1574

8. Lamacchia, C., Palmer, G., Rodriguez, E., Martin, P., Vigne, S., Seemayer, CA., Talabot-Ayer, D., Towne, JE. & Gabay C. (2013). The severity of experimental arthritis is independent of IL-36 receptor signalling. *Arthritis Res Ther*, **15**, R38.

9. Mahida, YR., Galvin, AM., Gray, T., Makh, S., McAlindon ME., Sewell, HF. & Podolsky, DK. (1997). Migration of human intestinal lamina propria lymphocytes, macrophages and eosinophils following the loss of surface epithelial cells. *Clin. Exp. Immunol.* **109**, 377-386.

10. Towne, JE., Garka, KE., Renshaw, BR., Virca, GD. & Sims JE. (2004). Interleukin (IL)-F6, IL-F8 and IL-F9 signal through IL-1Rrp2 and ILRAcP to activate the pathway leading to NF-Kb and MAPKs. *J Biol Chem.* 279: 13677-13728.

11. Livak, KJ. & Schmittgen, TD. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, 25: 402-408.
12. Mutamba, S., Allison, A., Mahida, Y., Barrow, P. & Foster N. (2012). Expression of IL-1Rrp2 by human myelomonocytic cells is unique to DCs and facilitates DC maturation by IL-1F8 and IL-1F9. *Eur J Immunol*, **42**, 607-617.
13. Higgins J, Mutamba S, Mahida Y, Barrow P. & Foster N.(2015). IL-36 α induces maturation of Th1-inducing human MDSC and synergises with IFN- γ to induce high surface expression of CD14 and CD11c. *Hum Immunol.* **76**: 245-253.
14. Foster, AM., Baliwag, J., Chen, CS., Guzman. AM., Stoll SW., Gudjonsson, JE., Ward, NL. & Johnston A. (2014). IL-36 promotes myeloid cell infiltration, activation, and inflammatory activity in skin. *J Immunol*, **192**, 6053-6061.
15. Wesa. A. & Galy, A. (2002). Increased production of pro-inflammatory cytokines and enhanced T cell responses after activation of human dendritic cells with IL-1 and CD40 ligand. *BMC Immunol*, **3**, 14.
16. Guo, Z., Zhang, M., An, H., Chen, W., Liu, S., Guo, J., Yu, Y. & Cao X. (2003). Fas ligation induces IL-1beta-dependent maturation and IL-1beta-independent

survival of dendritic cells: different roles of ERK and NF-kappaB signaling pathways.

Blood, **102**, 4441-4447.

17. Ben-Sasson, SZ., Hu-Li, J., Quiel, J., Cauchetaux, S., Ratner, M., Shapira, I., Dinarello, CA. & Paul WE. (2009). IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. *Proc Natl Acad Sci U S A*, **106**, 7119-7124.

18. Acosta-Rodriguez, EV., Napolitani, G., Lanzavecchia, A. & Sallusto F. (2007). Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol*, **8**, 942-949.

19. Rao, DA. Tracey, KJ. & Pober JS. (2007). IL-1alpha and IL-1beta are endogenous mediators linking cell injury to the adaptive alloimmune response. *J Immunol*, **179**, 6536-6546.

20. Hayday, AC. (2000). [gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annu. Rev. Immunol*, **18**, 975-1026.

21. Garcillán, B., Mazariegos, MS., Fisch, P., Res, PC., Muñoz-Ruiz, M., Gil, J., López-Granados, E., Fernández-Malavé, E. & Regueiro JR. (2005). Enrichment of the rare CD4 $\gamma\delta$ T-cell subset in patients with atypical CD3 δ deficiency. *J Allergy Clin Immunol*. **133**, 1205-1208.

22. Chang, F., Mahadeva, U. & Deere H. (2005). Pathological and clinical significance of increased intraepithelial lymphocytes (IELs) in small bowel mucosa. *APMIS*, **113**, 385-399.

Figure Legends

Table 1. Primer sequences and probe numbers for the quantification of the gene expression.

Primer sequences and probe numbers are shown for IL-36R gene and the reference gene GADPH. Acc= GenBank accession number.

Figure 1. IL-36R mRNA is expressed by non-adherent and CD3+ cells within human blood buffy coats.

Quantitative PCR analysis was used to show IL-36R expression in blood cells, relative to HT-29 (positive) cells which were given the arbitrary value of 1. IL-36R was detected in the non-adherent cells of the buffy coat and within this population most expression was detected in CD3+ lymphocytes (T cells). Expression of IL-36R was not detected in blood granulocytes, which may have contaminated the buffy coat, or in THP1 cells used as a negative control. Asterisk denotes significant increase ($p = 0.05$) in mRNA expression above that measured in HT-29 cells. Error bars show standard deviation from the mean. Each experiment was performed in triplicate on 5 separate occasions.

Figure 2. IL-36R mRNA is expressed by human blood lymphocytes and is dose-dependently increased by IL-36 β in CD4 $^+$ cells

(A) Quantitative PCR analysis showing IL-36R expression in T and B lymphocytes relative to HT-29 (positive) cells (not shown). (B) Expression of IL-36R mRNA is dose-dependently and significantly increased in CD4 $^+$ lymphocytes by IL-36 β but plateaus between 100-200 ng/ml. Asterisk denotes significant increase ($p = 0.05$) in mRNA expression above that measured in HT-29 cells (A) or unstimulated CD4 $^+$ lymphocytes (B). Error bars show standard deviation from the mean. Each experiment was replicated 3 times on 5 separate occasions.

Figure 3. Localisation of IL-36R by immuno-fluorescence in the cytoplasm and cell membrane of CD4 $^+$ and CD8 $^+$ T lymphocytes and CD19 $^+$ B lymphocytes in human blood.

Each panel shows immunofluorescent images of (A) cell nuclei (stained with DAPI); (B) IL-36R isolated by mouse anti-human IL-36R and secondary anti-mouse/FITC on the cell membrane (open arrows) and cytoplasm (closed arrows); (C) β -actin (stained with Phalloidin) and (D) shows overlay images taken from control cells (secondary anti-mouse FITC antibody only) with no FITC signal detected. Each panel is representative of 3 replicates isolated on 5 separate occasions. Scale bar (bottom left) = 10 μ M.

Figure 4. IL-36R is expressed in the cytoplasm and cell membranes of T and B lymphocytes isolated from human blood

FACS histograms showing the expression of IL-36R on the cell membranes (non-permeabilised cells) and cytoplasm (permeabilised cells) relative to isotype controls.

(A) CD4⁺ T helper cells; (B) CD8⁺ cytotoxic T lymphocytes; (C) CD19⁺ B lymphocytes and (E) HT-29 (positive) cells. All FACS plots are representative of 3 replicate plots per group performed on at least 3 separate occasions.

Figure 5. IL-36 β induces early proliferation of IL-36R expressing CD4⁺ T helper cells.

Lymphocyte subsets were aliquoted into 96 well plates at a concentration of 10⁴ cells per 100 μ l RPMI, containing 100 ng/ml IL-36 β . For each subset additional well contained lymphocytes in RPMI only (negative control) and lymphocytes in media containing conA (75 μ g/ml). After 48 h culture the numbers of lymphocytes were enumerated using a WST-1 cell proliferation assay, read at 450 nm. The optical density measured in each sample was then compared to the optical density obtained from standard curves of each lymphocyte subset ranging from 10³-10⁶ cells. Asterisk denotes a significant increase ($p = 0.05$) in lymphocyte numbers compared with the number of cells in the unstimulated population. The data shows a mean from 3 replicates performed on 3 separate occasions.

Figure 6. Percentage of CD3+ and CD4+ lymphocytes migrating into human LP.

FACS analyses were performed on cells, which had migrated from the LP, to ascertain; (A) Expression of CD3 (compared with isotype control) within the LP cell population; (B) Percentage of CD3+ cell population as a total of migrated LP cells; (C) Expression of CD4 (compared with isotype control) within the LP cell population; (D) Percentage of cells expressing CD4 within the CD3+ population. Each FACS histogram is representative of 3 replicates performed on 5 separate occasions.

Figure 7. IL-36R is expressed by CD4+ lymphocytes migrating into human LP.

(A) Quantitative PCR analysis was used to show IL-36R mRNA expression in migrated LP cells, relative to HT-29 (positive) cells which were given the arbitrary value of 1. IL-36R was detected in LP cells at a higher level than in HT-29 cells. In comparison, no expression of IL-36R was detected in THP1 cells. (B) CD4+ lymphocytes within the LP population express IL-36R protein, shown relative to the isotype control. Asterisk denotes significant increase ($p = 0.05$) in mRNA expression above that measured in HT-29 cells. Error bars show standard deviation from the mean. Each experiment was replicated 3 times on 5 separate occasions.

Fig 1. Penha
et al.,

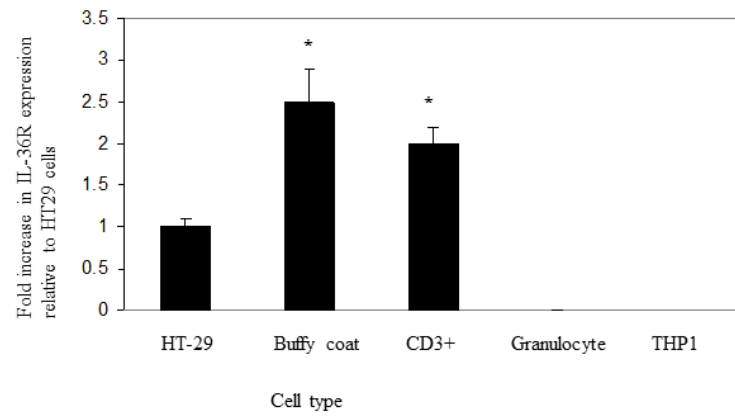


Fig 2. Penha et al.,

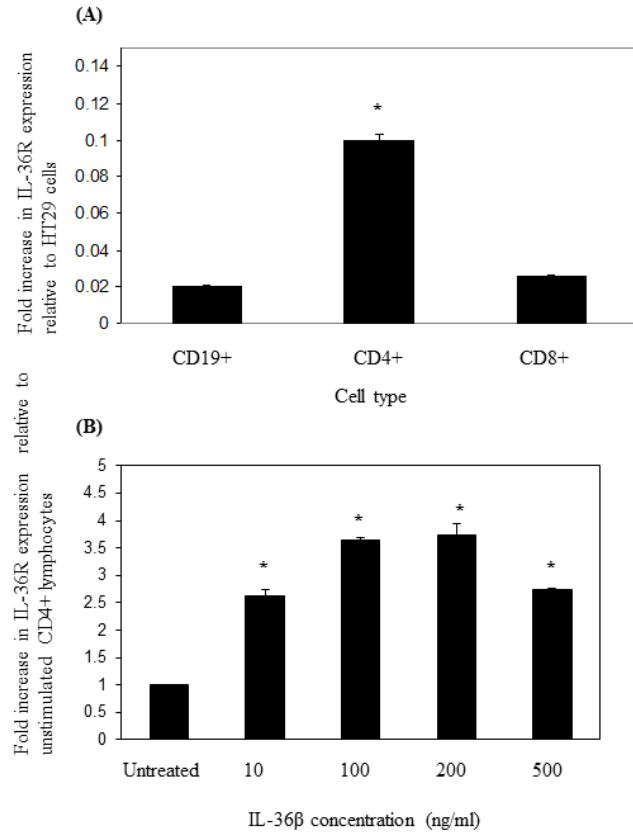


Fig 3. Penha
et al.,

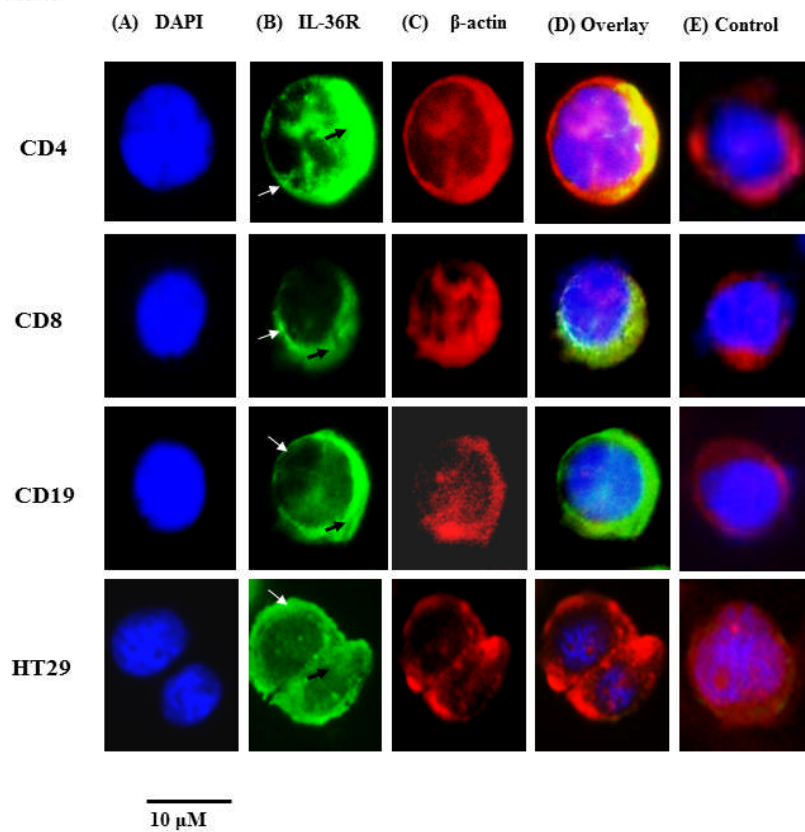


Fig 4. Penhha et al.,

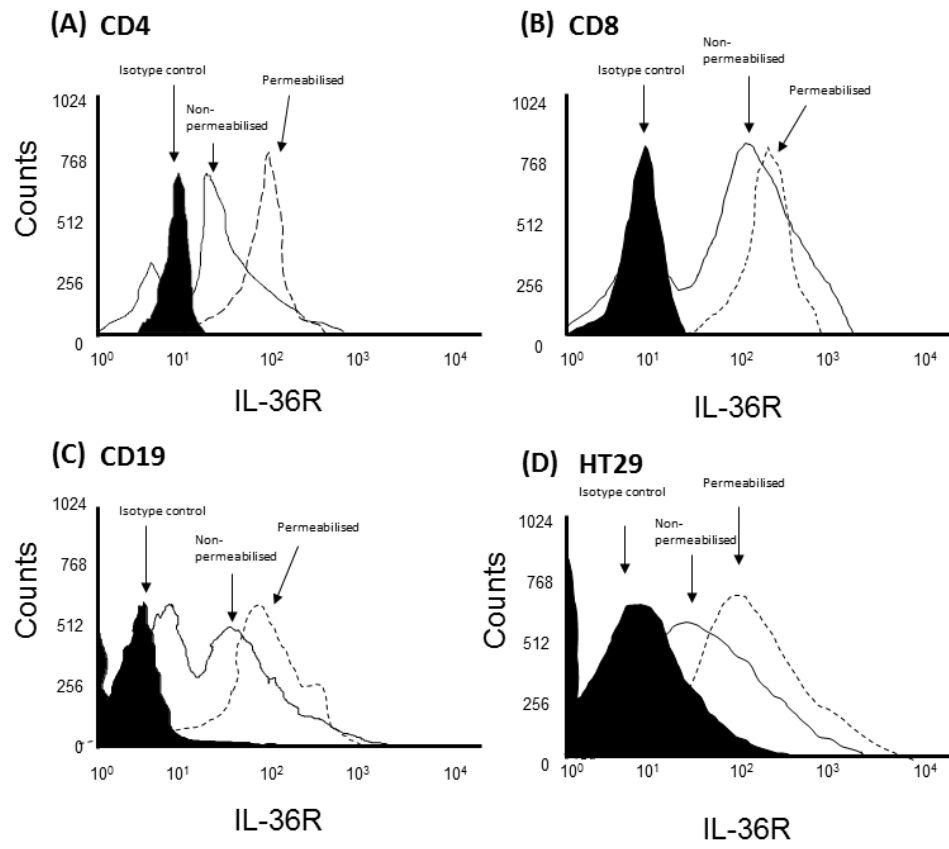


Fig 5. Penha et al.,

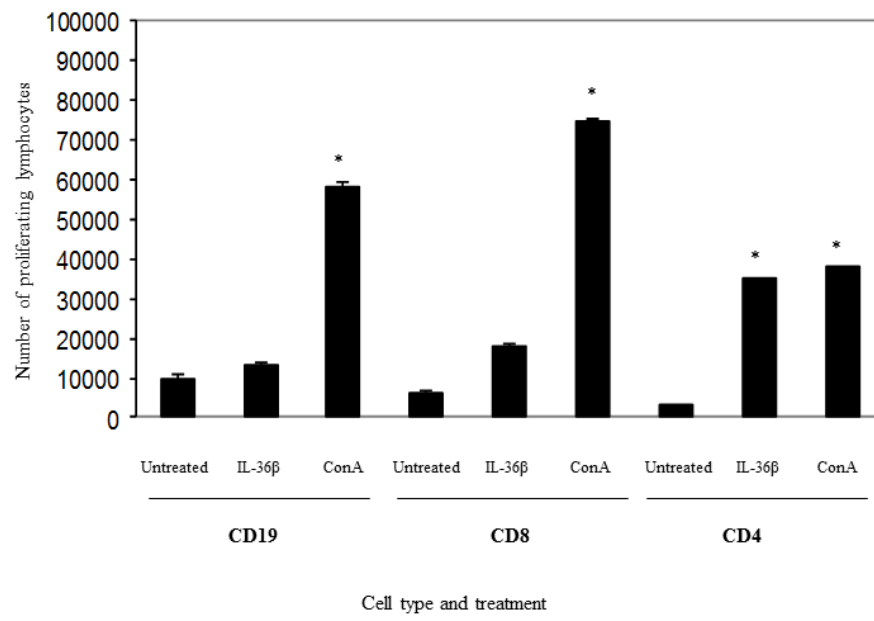


Fig 6. Penha
et al., (A)

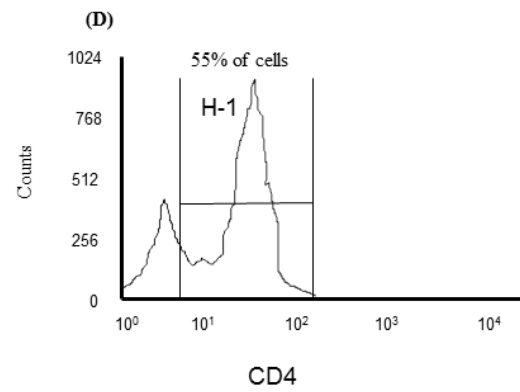
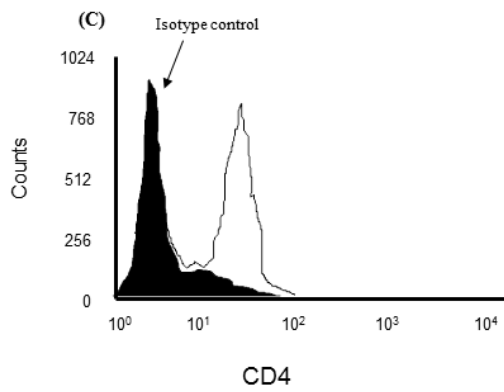
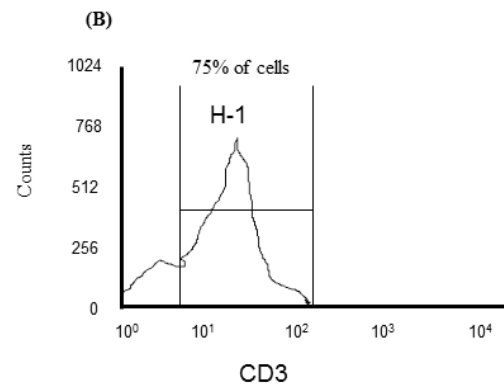
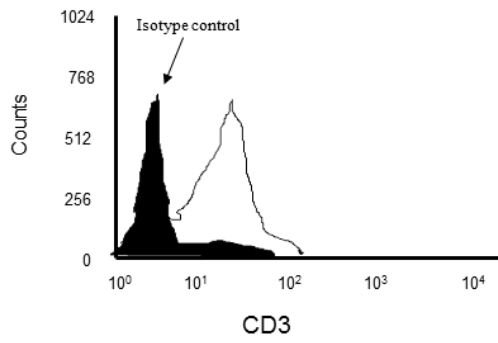


Fig 7. Penha et al.,

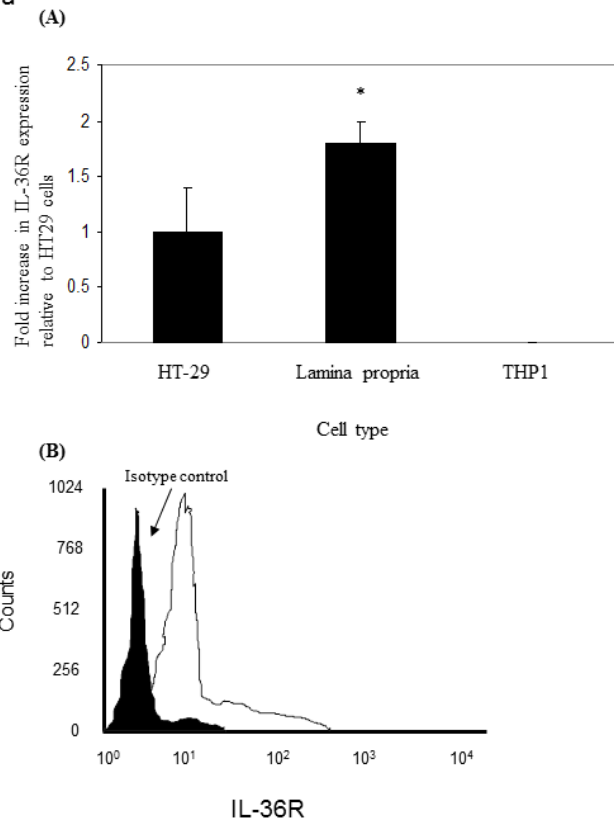


Table 1. Penha et al.,

GENE NAME	Acc. No.	Forward primer Sequence	Reverse Primer Sequence	Probe No.
GAPDH	NM002046	5'-CTCTGCTCCTCCTGTTGAC-3'	5'-ACGACCAAATCCGTTGACTC-3'	60
IL-36R	AF284434	5'-GCTGGAGTGCCACAGCATA-3'	5'-GCGATAAGCCCTCCTATCAA-3'	24