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IL-36α induces maturation of Th1-inducing human MDDC and synergises with IFN-γ to induce high surface expression of CD14 and CD11c

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Short Title: IL-36α induces expression of key MDDC molecules

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Abstract

We show that IL-36α induced maturation of human MDDCs and stimulated differentiation of IFN-γ producing (Type 1) CD3+ lymphocytes but was not as effective as IL-36β in doing so. For the first time, we also show that IL-36α induced expression of CD14 by MDDCs and this was highly potentiated by co-cultured with IFN-γ. In contrast, lipopolysaccharide (LPS) did not increase CD14 expression by MDDCs, suggesting that if MDDCs represent a physiologically relevant population *in vivo*, they need to be stimulated by relevant inflammatory cytokines prior to CD14 expression and detection of LPS, expressed by Gram negative bacteria. IFN-γ synergised with IL-36α to restore the high levels of CD11c expression by MDDCs, which was reduced by culture with these cytokines in isolation. IL-36α / IFN-γ synergy also correlated with increased binding of the opsonic complement protein (iC3b) to MDDCs. However although IL-36α increased the phagocytic capacity of MDDCs for Salmonella Typhimurium 4/74 this was not synergistically increased by IFN-γ ($p > 0.05$).

In conclusion we report the hitherto unknown effects of IL-36α on the innate cell function of human MDDCs.

Introduction

In the past decade, new members of the IL-1 family of cytokines have been described but there is still very little data reported on their biological effects and virtually nothing is known about these cytokines in the context of the human immune system. Recently it has been shown that human monocyte derived dendritic cells (MDDCs) mature when stimulated with novel IL-1 cytokines (IL-36β or IL-36γ) and that IL-36β-matured MDDCs induce differentiation of T helper (type 1) (Th1) lymphocytes [1]. However, it is not known whether IL-36α, which also signals via the same receptor (IL-36R), has the same effect on maturation. Furthermore, nothing has been reported regarding the effect of novel IL-1 cytokines in conjunction with other DC maturation signals such as IFN-γ. IFN-γ is known to increase the expression of maturation markers, such as CD80, CD86, CD83 and HLA-DR, on the surface of MDDCs [2, 3], while other studies have shown that MDDC maturation is also associated with increased expression of HLA-DR [4] and CD83 [5] and decreased expression of CD1a [6]. IFN-γ is also known to potentiate the maturation of MDDCs by IL-1β [2] but it is not known whether IFN-γ potentiates maturation of MDDCs by novel IL-1 cytokines.

Another important function of IFN-γ is in clearing pathogenic bacteria, such as salmonella, and in this case the highest concentration of IFN-γ is produced by Th1 lymphocytes which enter intestinal tissue from the blood [7]. IFN-γ produced by these cells activates innate immune cells by increasing phagocytosis and upregulating cellular killing pathways [8-11]. Therefore, the induction of Th1 cells by novel IL-1 cytokines may have an effect on bacterial immunity, while it has also been reported that culture of TLR4 expressing MDDCs with IFN-γ also induces production of IL-12 and IL-27 which makes lymphocytes more receptive to IL-12 [3]. Thus, IFN-γ may

have a bi-directional effect on the activation of DCs and Th1 lymphocytes and it is possible that this may be affected by novel IL-1 cytokines, which induce Th1 proliferation, via DC maturation.

There is some evidence to suggest that MDDCs do represent a physiological population which replenish resident tissue DCs following infection [12-13]. However, differentiation of monocytes to MDDCs is associated with loss of CD14 expression and an increase in CD11c is increased. These are contradictory effects since both are important in the recognition of Gram negative bacteria. The important role of CD14 in LPS recognition by innate immune cells has been known for a number of years [reviewed 14], while CD11c increases phagocytosis of iC3b-opsonized particles [15] and has been shown to induce LPS responsiveness [16]. However, nothing has been reported on the effect of IFN-γ on CD14 and CD11c expression by MDDCs matured with novel IL-1 cytokines and it is possible that these MDDCs remain responsive to Gram negative bacteria (by maintaining CD14 expression) when stimulated with IFN-γ, or other cytokines which are produced during infection.

The aims of the study we report were; (1) to determine whether IL-36α increased maturation of MDDCS. (2) To determine whether IFN-γ synergised with IL-36α to induce maturation of MDDCs (as is the case with IL-1β). (3) Determine how these events may influence expression of CD14 and CD11c, which are important in detecting gram negative bacteria.

Materials and Methods

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-36α and IL-36β proteins, and murine anti-human IL-36R were supplied by the Amgen, Corporation, Seattle, Washington, USA.

Bioethics

All studies were conducted following approval by local ethics committees.

Endotoxin assays

A kinetic chromogenic LAL assay (Lonza, USA) was performed by a verified technician prior to shipment of IL-1 reagents by Amgen. Assay sensitivity is between 0.005-50.0 EU/ml. Results showed LPS concentrations of < 0.209, which are equivalent to << 0.01 pg/ml in 100 ng IL-1 protein, were used in cell culture. Similar results were obtained at Nottingham prior to use. As an additional test for LPS contamination we used a THP 1 sensitivity assay which showed that the cells did not produce TNFα in response to IL-1 reagents but did produce TNFα when stimulated with bacterial lipopolysaccharide (data not shown).

FACS analyses

FACS analyses were performed by standard methods. Briefly, 1 x 10^6 test cells per group 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 prior to incubation for 45 min with relevant antibodies (see table 1). Cells were then analysed using a FACSCanto II analyser (Becton Dickinson, USA). Samples were acquired using CellQuest pro software (Becton Dickinson, USA) and analysed using 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.

Differentiation and culture of monocyte derived dendritic cells (MDDCs)

Blood products from healthy donors were obtained from the National blood transfusion service (Sheffield, UK). Isolation of peripheral blood monocytes (PBMs) was performed using differential centrifugation in histopaque 1077 by standard methods. Briefly, 10^6 cells/ml were cultured in 6 well plates containing supplemented RPMI media (see above) with additional GM-CSF (50 ng/ml) and IL-4 (10 ng/ml) for 5 days.

Analysis of MDDC phenotype and maturation status.

Expression of CD1a, CD11c, CD14 and HLA-DR were measured by FACS analyses (as previously stated). The phenotype of these cultured cells was $CD11c^{high}$, $CD1a^{high}$, $HLA\text{-}DR^{high}$, and $CD14^{low}$, consistent with their differentiation to immature MDDCs.

FACS analysis of maturation status following culture of MDDC with IL-36α, IL-36β and IFN-γ.

To assess IL-36α-dependant maturation of MDDCs, changes in HLA-DR, CD1.a, CD40, CD80, CD83 and CD86 expression were assessed by FACS analysis on the surface of day 5 (Immature) MDDCs which had been washed 3 times in PBS prior to incubation with IL-36α (500 ng/ml) for 48h. Expression of these maturation markers was then compared with expression of these markers on the surface of unstimulated day 5 MDDCs which were washed and cultured in media only for a further 48 or, as an additional control, MDDCs incubated with relevant isotype controls.

In a separate series of experiments, maturation of MDDCs, following culture with IL-36α or IL-36β (500 ng/ml), was compared with maturation following culture with

IFN-γ (100 ng/ml) or following co-culture with IL-1 cytokine and IFN-γ. In other experiments, MDDCs were cultured with *S*. Typhimurium LPS (100 ng/ml).

T lymphocyte proliferation assays

Following maturation of MDDCs with IL-36α, the MDDCs were co-cultured with allogeneic CD3+ lymphocytes at a ratio of 1:50 for 96h.

T cell proliferation was measured after 96h using CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, USA) according to manufacturers instructions. Values were compared to those obtained from a standard curve with a maximum value of 1 x 10^6 CD3+ lymphocytes. As a positive control CD3+ lymphocytes were cultured for 96h in media containing Concanavalin A (10 μg/ml). Further comparison was made between IL-36α-matured MDDCs and MDDCs matured with IL-36β (500 ng/ml) and IL-1β (100 ng/ml)) prior to proliferation analysis and as a negative control CD3+ lymphocytes were cultured for 96h in media containing no additional supplements. All samples were cultured in triplicate and repeated on 3 separate occasions.

Determination of the cytokine profile of IL-36α-matured MDDCs and T lymphocytes stimulated with IL-36α -matured MDDCs

Immature MDDCs were stimulated with IL-36α (500 ng/ml) or IL-1F2 (10 ng/ml or 100 ng/ml) for 48h. After 48h, a standard ELISA analysis was used to measure IL-12 p70 and IL-18 in the MDDC supernatants. As a negative control, cell supernatants were obtained from immature (day 5) MDDCs which had been washed and cultured for 48h in media containing no supplements. As a positive control (and to provide comparative data) the affect of IL-36β (500 ng/ml) or IL-1 β (100 ng/ml) on IL-12 and IL-18 production was also measured. To determine T cell phenotypes differentiated by IL-1-matured MDDCs, analysis of IL-10 and IFN-γ concentrations

were performed using standard ELISA analyses in supernatants obtained after 96h co-culture of MDDCs with CD3+ lymphocytes (as stated above).

Measurement of the phagocytic capacity of MDDC cultured with or without cytokine.

S. Typhimurium strain 4/74 was cultured to mid-log phase prior to counting colony forming units (CFU) and assessment of growth. When required for use, *Salmonella* were diluted to give a multiplicity of infection (MOI) of 10 prior to co-culturing with day 6 MDDC, day 5 MDDC cultured with IL-36α (500 ng/ml) for 48h prior to the addition of Salmonella or day 5 MDDC cultured with IL-36α (500 ng/ml) and IFN-γ (100 ng/ml) for 24h prior to the addition of Salmonella. After 2h culture, the MDDCs were washed 3 times by centrifugation in PBS and then lysed using Triton x-100 (0.5% v/v) (Sigma). Salmonella CFU were then counted in lysed cells. Each experiment was performed in quadruplicate on 3 separate occasions.

Measurement of iC3b binding to IL-36α stimulated MDDCs

Day 5 MDDCs, which had been incubated with IL-36α (500 ng/ml), IFN-γ (100 ng/ml) or co-cultured with IL-36α and IFN-γ, for 48h were washed 3 times in PBS. The cells were incubated for 30 min with 2.5 UG human iC3b protein (Calbiochem, Nottingham UK), washed 3 times in PBS and then incubated with mouse anti-human ic3b (neoantigen) (1 µg) (monoclonal antibody IgG2b anti human iC3b (neoantigen) conjugated to APC, for 30 min at 4 ^{0}C. The cells were then washed 3 times in PBS prior to FACS analyses.

RNA extraction

RNA extraction (using RNeasy mini kits) and DNase digestions were performed using standard procedures recommended by the kit manufacturer (Qiagen, Crawley, UK). The concentration of RNA was determined using a Nanodrop (ND1000)

spectrophotometer (NanoDrop technologies, Wilmington, DE, USA) according to the manufacturer's instructions. A260/A280 ratio greater than 1.8 was considered to be pure. Reverse transcription was performed using a Transcriptor First Strand cDNA Synthesis Kit (Roche, West Sussex, UK) according to manufacturers instructions.

Measurement of IL-36R mRNA expression by quantitative PCR

RNA was extracted from blood monocytes, MDDCs and HT29 (IL-36R positive control) cells prior to measurement of IL-36R expression. Gene-specific primers and probes were designed using the Roche Universal Probe Library (UPL) Assay Design Centre software. To allow differentiation of the amplified cDNA from contaminating genomic DNA, intron-spanning primers (primers that annealed to exon sequences on both sides of an intron or on exon/exon boundaries) were designed (Roche Diagnostics Corporation, 2007). The LightCycler® 480 Probes Master Kit (Roche, Burgess Hill, West Sussex UK) was used for qRT-PCR and the manufacturer's guidelines for use with LightCycler® 480 Multiwell Plate 96 were followed. Gene-specific primers were obtained from Invitrogen, (Paisley, UK) via Fisher Scientific (Loughborough, UK) and 5-carboxyfluorescein (FAM)-labelled probes were obtained from Roche Diagnostics.

The following forward and reverse IL-36R primer sequences were used:

Forward: GCTGGAGTGTCCACAGCATA

Reverse: GCGATAAGCCCTCCTATCAA

Genbank accession number AF284454, UPL number 24.

Expression of a house keeping gene (Glyceraldehyde-3-phosphate dehydrogenase) (GAPDH) was used as a reference comparison.

Forward: CTCTGCTCCTCCTGTTCGAC

Reverse: ACGACCAAATCCGTTGACTC

The PCR cycling program used was as follows: Pre-incubation (1 cycle; 95°C for 10 minutes), Amplification (45 cycles; 95°C for 10 seconds, 60°C for 40 seconds and 72°C for 1 second) and Cooling (1 cycle; 40°C for 10 seconds). All experiments were performed in triplicate and PCR product was assessed using standard acrylamide gels. A relative quantification method based on the relative changes in mRNA expression of the target gene versus changes in mRNA expression of GAPDH was used to quantify IL-36R expression. Relative expression of the target gene to the reference gene (calibrator given the arbitrary unit of 1) was calculated using the LC480 software program (Roche). The Advanced Relative quantification efficiency corrected calculation model was used according to LC 480 user manual (Roche). PCR product was also eluted by gel electrophoresis and was shown to be of the expected amplicon size of 80 base pairs (data not shown). The human colonic carcinoma cell line HT29 (a kind gift from Professor Susan Watson, University of Nottingham) was used as a positive control of IL-36R expression and human monocytic THP-1 cells (purchased from the European collection of cell cultures (Salisbury, Wilts UK), were used as a calibrator (negative control) as previously published (1).

Statistical Analysis

An analysis of variance (ANOVA) test with a one-way classification was used to calculate significant differences ($P < 0.05$), between test and control samples following cytokine ELISA analyses and the difference between the number of cells, within total cell populations, expressing CD1a or HLA-DR following stimulation with IL-1 cytokines and IFN-γ. Tukey's test was used post hoc to determine significance between individual means. Minitab software was used for all statistical analyses. To statistically analyse the effect of cytokine on the phagocytosis of Salmonella, a students t-test was performed to compare Salmonella numbers in IL-36α cultured

MDDCs with untreated MDDCs or MDDCs cultured with both IL-36α and IFN-γ with untreated MDDCs. Statistical analysis was performed using Mintab software licensed to Nottingham University.

Results

IL-36α induces maturation of human MDDCs

We investigated whether, or not, IL-36α induced MDDC maturation, as previously shown with IL-36β and IL-36γ. In comparison with isotype controls (Fig 1A), expression of both HLA-DR and CD1a was increased on the surface of immature MDDCs (monocytes cultured for 5 days with GM CSF/IL-4) and cultured for 48h in culture media without cytokines (Fig 1B). A distinct population of CD1a ^{high}/HLA-DR^{med} MDDCs (which represented > 70% of the total population in all replicates) was gated as (R1) for comparison with the same population following cytokine treatment. When immature MDDCs were cultured with IL-36α for 48h CD1a expression decreased and HLA-DR expression was increased, with the R1 population representing < 40 % of the total MDDC population in all replicate experiments (Fig 1C). As an additional marker of MDDC maturation, CD83 was measured on the surface of all control and test samples. Our data shows that CD83 expression was increased on the surface of MDDCs cultured with IL-36α for 48h compared to MDDCs which had been cultured without cytokine for the same time period (Fig 1D).

IL-36 α induces expression of CD40 but not CD80 or CD86 on the surface of MDDCs.

We investigated the expression of essential co-stimulatory molecules, required for full activation of cognate lymphocytes, by MDDCs cultured with IL-36α. Following incubation of immature MDDCs with IL-36α (500 ng/ml) for 48h. Our data showed

that IL-36α increased expression of CD40, compared to either unstimulated MDDCs or isotype controls (Fig 2A). However, IL-36α had no effect on either CD80/CD86 expression (Fig 2A) or CD86, which remained at levels expressed by unstimulated MDDCs.

IL-36α stimulates production of pro-inflammatory cytokines (IL-12p70 and IL-18 from human MDDCs

We next investigated whether production of IL-12p70 and/or IL-18 from immature (day 5) MDDCs was stimulated following culture with IL-36α for 48h. We compared our results to MDDCs cultured with IL-36 β or IL-1β for the same time period, both of which are known to stimulate IL-12p70 and IL-18. Our results show that IL-36α stimulated a significant increase ($P < 0.05$) in IL-12p70 compared to IL-12p70 concentrations measured in the supernatants isolated from unstimulated MDDCs (Fig 3A). However, IL-12p70 concentrations induced by IL-36α were lower than those induced by either IL-36 β or IL-1β but this difference was not significant ($P > 0.05$) (Fig 3A). IL-36α (and IL-36 β or IL-1β) also stimulated a significant increase in IL-18 production when cultured with immature MDDCs for 48h (Fig 3B). The concentration of IL-18 induced by IL-36 β was, again, lower than IL-18 production stimulated by either IL-36 β or IL-1β and in the latter case the difference was significant ($P < 0.05$) (Fig 3B).

IL-36α -matured MDDCs induce proliferation of Th1 lymphocytes in allogeneic MDDC/CD3+ lymphocyte cultures

MDDCs, which had been matured by culture with IL-36α for 48h, were cultured for 5 days with allogeneic CD3+ lymphocytes to determine whether they increased lymphocyte proliferation and skewed the phenotypic characteristic towards Th1 (pro-inflammatory), as is the case with IL-1β and IL-36β, or Th2 (anti-inflammatory). Our

results showed that IL-36α induced a > 2 fold increase in CD3+ numbers compared to the number of CD3+ lymphocytes cultured for 5 days without MDDCs (Fig 6A). When the effect of IL-36α-matured MDDCs on lymphocyte proliferation was compared to positive controls, IL-36α maturation induced a comparative response to con A (> 2 fold increase) but a lesser effect when compared to either IL-36β or IL-1β (> 3 fold) (Fig 4A). IL-36α -matured MDDCs also stimulated a significant increase ($P < 0.05$) in IFN-γ concentration in the supernatants isolated from allogeneic MDDC/CD3+ lymphocyte cultures but this was also significantly lower ($P < 0.05$) than IFN-γ concentrations induced by either IL-36β or IL-1β-matured MDDCs (Fig 4B). IL-10 concentration in supernatants isolated from allogenic cultures was slightly raised (~ pg/ml) when MDDCs were matured by IL-36α, IL-36β or IL-1β but this was not significant ($P < 0.05$) (Fig 4C).

IL-36α and IL-36β induces CD14 expression by human MDDCs and synergise with IFN-γ to produce a CD14^{high}/CD11c^{high} MDDCs phenotype

FACS analyses were used to measure expression of CD14 and CD11c on the surface of immature MDDCs cultured with IL-1 cytokines and/or IFN-γ for 48h and these were compared to relevant isotype controls (Fig 5A). Although this study was primarily about IL-36α, nothing has been published regarding the affect of IL-36β on these important innate immune molecules. Our data showed that monocytes expressed a characteristic CD14^{high}/CD11c^{low} phenotype (Fig 5B) and when these were differentiated into MDDC following 5 days of culture with GM CSF and IL-4 the phenotype shifted to the characteristic phenotype of MDDCs, CD14^{low}/CD11c^{high} (Fig 5C). We then stimulated MDDCs with LPS for 48h and this had no effect on relative CD14 or CD11c expression (Fig 5D). However, when MDDC were cultured with either IFN-γ (Fig 5D); IL-36α (Fig 5E) or IL-36β (Fig 5F)

the phenotype shifted towards increased CD14 expression and decreased expression of CD11c. Our next series of experiments investigated whether IL-36α or IL-36β synergised with IFN-γ to affect expression of CD14 and CD11c. We show that there was a clear increase in CD14 expression and a return to high CD11c when MDDCs were co-cultured for 48h with IL-36α and IFN-γ (Fig 5G) or IL-36β and IFN-γ (Fig 5I).

IL-36α and IFN-γ synergistically increases binding of iC3b on the surface of MDDCs but not phagocytosis of Gram negative bacteria (Salmonella Typhimurium)

Since CD 11c binds iC3b to the surface of cells, and CD11c expression was synergistically increased on the surface of MDDCs cultured with both IL-36α and IFN-γ, we investigated whether, or not, this also lead to synergistic increases in binding of iC3b on the surfaced of MDDCs. The synergistic effect of IL-36α and IFN-γ was measured by increased interaction of iC3b (an opsonin which binds to CD11c) on the surface of MDDCs when compared to either cytokines alone, although both cytokines increased iC3b interaction in comparison to unstimulated MDDCs (Fig 6A). However, our data shows that although IL-36α stimulated MDDCs phagocytosed more bacteria than unstimulated (age matched) MDDCs the increase was not significant ($P > 0.05$) but was significant ($P < 0.05$) if MDDCs were cultured with both IL-36α and IFN-γ (Fig 8B). When MDDCs were stimulated with IFN-γ only, the number of bacteria phagocytosed increased still further (compared with co-culture of MDDCs with IL-36α and IFN-γ) but this increase was not significant (Fig 6B)

IL-36α and IFN-γ stimulate similar levels of CD1a and CD83 expression by MDDCs but IL-36α is less potent in inducing HLA-DR

To test whether any synergistic effects attributed to stimulation of MDDCs with both IL-36α and IFN-γ were simply due to increased MDDC maturation, we measured expression of CD1a, CD83 and HLA-DR on the surface of immature MDDCs which had been subsequently cultured with IL-36α or IFN-γ or co-cultured with IL-36α and IFN-γ for 48h. When MDDCs were cultured with IL-36α or IFN-γ or co-cultured with IL-36α and IFN-γ for 48h, expression of CD1a (Fig 7A) and CD83 (Fig 7B) was comparable and all were above isotype control levels. However, when HLA-DR expression by MDDCs was measured following these treatments, IL-36α induced less expression than was measured for IFN-γ, which itself induced a similar level of HLA-DR expression measured when MDDCs were co-cultured with IL-36α and IFN-γ (Fig 7C).

Discussion

Our results show that IL-36α induces maturation of human MDDCs, as measured by increased expression of CD83 and HLA-DR and decreased expression of CD1a. IL-36α also stimulated IL-12p70 and IL-18 production from MDDCs and the differentiation of (IFN-γ-producing) Th1 lymphocytes in allogeneic MDDC/CD3+ lymphocyte cultures. This effect has previously been shown for IL-36β and IL-36γ [1], although our results indicate that IL-36α is less potent than IL-36β in this regard. These results are also in accordance with a recent murine study which reported that IL-36β induced the expression of maturation markers on the surface of bone marrow derived dendritic cells (BMDDC) and promotes Th1 proliferation in vitro and in vivo [17]. However, our study also shows that IL-36α has a differential effect on the expression of co-stimulatory molecules, having no effect on CD80 or CD86 expression but increasing CD40 expression. This is different to the previously

reported effect of IL-36 β which induces both CD40 and CD80 expression on the surface of human MDDCs [1] and probably also reflect the reduced effect of IL-36α compared to IL-36β. It could also be the case that IL-36α and IL-36β differentially effect the production of IL-36RA, which although not tested here is an inhibitor of both cytokines via IL-36R. However, IL-36α did not increase the effect of IFN-γ on the expression of standard maturation markers such as HLA-DR, CD83 or CD1a and generally IL-36α only weakly induced HLA-DR expression, when compared to IFN-γ. However, these results may also show a different effect of the novel IL-1 cytokines when compared to IL-1β since a previous study has shown that both IL-1β and IFN-γ induce maturation of human MDDC and that these 2 cytokines synergise to increase this effect [2].

The immune response to Gram negative bacteria requires not only the induction of Th1 responses by APCs but also, initially, that the APCS can detect the presence of the bacteria. We therefore investigated the effect of IL-36α on CD14 expression and whether any additional affect on expression was provided by IFN-γ, which is an important cytokine in response to Gram negative bacteria. CD14 is an essential component in the detection of bacterial LPS [18] and CD14 gene knock-out mice are resistant to endotoxaemia induced by LPS injection [19]. Furthermore, LPS binding of CD14 decreases its spatial proximity to Toll-like receptor 4 (TLR4) and it is via TLR4 that the intracellular signalling cascade, which results in the production of pro-inflammatory cytokines, is initiated [20]. More recently it has been reported that CD14 also augments murine TLR4 endocytosis by dendritic cells, induced by LPS [21]. Our results showed that both IL-36α and IFN-γ upregulated expression of CD14, when compared with unstimulated MDDCs, whereas LPS was unable to do so. Previous studies have shown that LPS upregulates CD14 expression by human

monocytes, which were also stimulated for 48h [22]. However, when MDDCs were cultured with IL-36α and IFN-γ, CD14 expression on the surface of MDDCs increased even further. Thus, IFN-γ synergises with IL-36α to induce high levels of CD14 expression by human MDDCs. Therefore CD14 expression, which is lost when monocytes differentiate into MDDCs, is replenished when MDDCs are cultured with IL-36α and this effect of IL-36α is synergistically enhanced by IFN-γ.

We also investigated whether, or not, these cytokines affected expression of CD11c. CD11c is a receptor for iC3b, which opsonizes micro-organisms to increase phagocytosis [15], while transfection of CD11c genes into CHO cells has been shown to induce LPS responsiveness [16]. Thus, expression of both CD11c and CD14 are required for the efficient detection and phagocytosis of Gram negative bacteria such as Salmonella. Furthermore, when CD11c is blocked on the surface of Langerhans cells, proliferation of allogeneic T lymphocytes is prevented [23], which suggests that CD11c is a vital molecule in the induction of adaptive immunity (atleast allogeneic response) by DCs. As monocytes differentiate into (immature) MDDC, CD11c is expressed on their cell membrane but our results showed that when immature MDDCs were cultured with IL-36α, IL-36β or IFN-γ, expression of CD11c was supressed, while culture of MDDCs with LPS had no effect on CD11c. This latter result is in accordance with a recent study which has reported that LPS down-regulates expression of CD11c by murine splenic DCs and BMDCs but fails to do so in human MDDCs [24]. However, when we cultured MDDCs with either IL-36α and IFN-γ or IL-36β and IFN-γ, the expression of CD11c was restored to the high levels expressed by immature (unstimulated) MDDCs. In accordance with these results, we also found that IL-36α and IFN-γ synergised to increase binding of iC3b. The role of CD11c in bacterial uptake by human MDDCs is, however, complex since one study has shown

that blockade of CD11c has little effect on uptake of opsonized yeast particles and in this study CD11b appeared to be the most dominant complement receptor [25]. In contrast, Ben Nasr et al., (2006) [26] have previously reported that uptake of the Gram negative bacteria *Francisella tularensis* is dependent on both CD11c and CD11b. In our study we show that although increased CD11c expression due to IL-36α/IFN-γ synergy was also associated with increased iC3b binding to MDDCs this was not fully correlated with *Salmonella* uptake. Our results showed that although IL-36 α increased *S*. Typhimurium uptake by MDDCs and that this was increased to significant levels when MDDCs were cultured with IL-36 α and IFN-γ, neither of these treatments increased uptake above that measured when MDDCs were incubated with IFN-γ alone. We did not study the effect of IL-36α on CD11b expression (as investigated in previous studies mentioned above) and so it is possible that although IL-36α increased CD11c expression and subsequent iC3b binding to MDDCs, CD11b may have a more dominant role in phagocytosis of *S*. Typhimurium. Some studies have indicated that MDDCs are a physiological DC population generated *in vivo* during inflammation (12-13), possibly serving to replenish spent peripheral DC populations. If this is the case, then our study also suggests that they require additional signals, not only provided by IL-36α alone but also other cytokines such as IFN-γ.

Authorship

John Higgins and Shilla Mutamba performed most of the experimental work. Paul Barrow and Yashwant Mahida were involved in the concept and development of the study. Neil Foster performed some of the experimental work and was involved in the initial concept, development and writing of the study.

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Conflict of interest

The authors declare no financial or commercial conflict of interest

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Figure Legends

Figure 1. IL-36α induces maturation of human MDDCs

Immature MDDCs (day 5) were cultured in media alone or in media containing IL-36α (500 ng/ml) for 48h. After 48h FACS analyses were performed to measure surface expression of HLDR, CD83 and CD1a. FACS density plots were used to analyse HLA-DR and CD1a expression in treated and untreated MDDCs. (A) Shows isotype control IgG3.PE/IgG2a.FITC; (B) Gated population (R1) set according to the distinct CD1a ^{high}/HLA-DR^{med} popluation observed in day 5 MDDCs washed and cultured for 48h without further cytokine stimulation (>70% of all cells). (C) Shows a shift in gated R1 population from CD1a ^{high}/HLA-DR^{med} (< 40% of cells) to CD1a$^{med/low}$/HLA-DR^{high} when day 5 MDDCs are cultured with IL-36α for 48h. (D) shows that IL-36α increases CD83 expression on the surface of MDDCs above that measured in unstimulated MDDCs, cultured over the same time period (7 days). FACS data shown are representative of data obtained on 5 separate occasions.

Figure 2. IL-36α differentially effects expression of co-stimulatory molecules on the surface of MDDCs

Immature MDDCs (day 5) were cultured in media alone or in media containing IL-36α (500 ng/ml) for 48h. After 48h FACS analyses were performed to measure surface expression of CD80, CD86 and CD40.

IL-36α did not increase expression of CD80 (A) or CD86 (B) above that measured in unstimulated MDDCs or isotype controls. IL-36α did increase expression of CD40 above that measured in unstimulated MDDCs or isotype controls (C). FACS histograms shown are representative of data obtained on 3-5 separate occasions.

Figure 3. IL-36α stimulates IL-12p70 and IL-18 production from MDDCs

IL-12p70 and IL-18 production was compared by ELISA analysis in culture supernatants from Immature MDDCs (day 5) cultured in media alone or when cultured in media containing IL-36α (500 ng/ml) for 48h. As an additional control, induction of IL-12p70 and IL-18 by IL-36**α** was also compared to day 5 MDDCs cultured with IL-36β and IL-1β for 48h.

(A) Shows a significant increase ($P < 0.05$) in IL-12p70 production by MDDCs cultured with IL-36α, IL-36β or IL-1β for 48h compared to unstimulated controls. IL-36α induced lower IL-12p70 production compared to IL-36β or IL-1β but this was not significant. (B) Shows a significant increase ($P < 0.05$) in IL-18 production by MDDCs cultured with IL-36α, IL-36β or IL-1β for 48h compared to unstimulated controls. IL-36α induced lower IL-18 production compared to IL-36β and significantly lower ($P < 0.05$) IL-18 production when compared to IL-1β. Each data point is a mean of 3 replicates performed on 3 separate occasions. Bars show standard deviation from the mean.

Figure 4. IL-36α-matured MDDCs induce proliferation of (IFN-γ-producing) Type1 lymphocytes in allogeneic MDDC/CD3+ lymphocyte cultures

(A) Allogeneic CD3+ lymphocytes were cultured for 5 days with MDDCs matured with IL-36α, IL-36β or IL-1β for 48h. Proliferation of these lymphocytes was then compared to CD3+ lymphocytes cultured without cytokine stimulation or CD3+ lymphocytes stimulated with con A for the same time period. IL-36α-matured MDDCs induced > 2 fold increase in CD3+ lymphocyte population and was comparable to con A but lower than either IL-36β or IL-β (> 3 fold increase).

(B) A significant increase ($P < 0.05$) in IFN-γ concentration was measured in culture supernatants removed from IL-36α-matured MDDC/CD3+ lymphocytes compared with unstimulated CD3+ lymphocytes cultured over the same time period. However, IL-36α-matured MDDCs stimulated a significantly lower ($P < 0.05$) IFN-γ concentration when compared to either IL-36β or IL-1β-matured MDDCs cultured with CD3+ lymphocytes over the same time period. (C) IL-36α, IL-36β or IL-1β-matured MDDCs stimulated increased concentration of IL-10 in MDDC/CD3+ lymphocyte cultures but this was not significant ($P > 0.05$). Each data point is a mean of 3 replicates performed on 3 separate occasions. Bars show standard deviation from the mean.

Figure 5. IL-36α and IL-36β induces CD14 expression by MDDCs and synergise with IFN-γ to produce a $CD14^{high}/CD11c^{high}$ MDDCs phenotype.

FACS analyses were used to measure expression of CD14 and CD11c on the surface of MDDCs cultured with IL-1 cytokines (500 ng/ml) and IFN-γ (100 ng/ml) for 48h. (A) Isotype controls, IgG2a.PE and IgG1.FITC. (B) = Human monocytes expressing a characteristic $CD14^{high}/CD11c^{low}$ phenotype. (C) When monocytes were converted to MDDCs by GMCSF and IL-4 the phenotype shifts to a characteristic $CD14^{low}/CD11c^{high}$. (D) When MDDCs were stimulated with LPS (100 ng/ml) for 48h the phenotype remained $CD14^{low}/CD11c^{high}$. However when MDDCs were stimulated with either (E) IFN-γ, (F) IL-36α or (G) IL-36β for 48h, CD14 expression increased and CD11c expression decreased and when MDDCs were co-cultured with (H) IL-36α and IFN-γ or (I) IL-36β and IFN-γ, CD14 expression increased further and CD11c expression returned to the higher levels measured in unstimulated cells. FACS density plots shown are representative of plots obtained on at least 5 separate occasions.

Figure 6. IL-36α and IFN-γ synergise to increase the phagocytic capacity and iC3b expression in human MDDCs

(A) Showing increased phagocytosis of *S*. Typhimurium 4/74 was measured after 2h in 5 day old MDDCs matured with IL-36α (500 ng/ml) for 48h compared with Salmonella CFU isolated from unstimulated 7 day old (age matched) MDDCs. A significant increase ($P < 0.05$) in phagocytosis of Salmonella was measured after 2h in MDDCs cultured with both IL-36α (500 ng/ml) and IFN-γ (100 ng/ml) for 48h prior to exposure to bacteria. Each experiment was performed in quadruplicate on 3 separate occasions, error bars show standard deviation and the large bar shows a significant difference ($P < 0.05$) between the CFU isolated from MDDCs cultured with IL-36α and IFN-γ compared to unstimulated, age matched MDDCs. (B) IL-36α and IFN-γ synergistically increase iC3b interaction with the MDDC membrane above that measured when MDDCs are stimulated with either cytokine alone. FACS histograms show representative plots obtained on 3 separate occasions.

Figure 7. IL-36α- stimulated MDDCs express similar levels of CD1a and CD83 but lower HLA-DR expression than IFN-γ-stimulated MDDCs

FACS analyses were also used to measure expression of CD1a, CD83 and HLA-DR on the surface of human MDDCs cultured with IL-36α (500 ng/ml), IFN-γ (100 ng/ml) or following co-culture with IL-36α and IFN-γ for 48h. FACS histograms (A) show equivalent expression of CD1a following cytokine treatment; (B) shows identical expression of CD83 following cytokine treatment and (C) shows reduced expression of HLA-DR following incubation with IL-36α compared to IFN-γ or IL-36α/IFN-γ co-culture. All plots are representative of data obtained in triplicate on 5 separate occasions.

Fig 1 Higgins et al

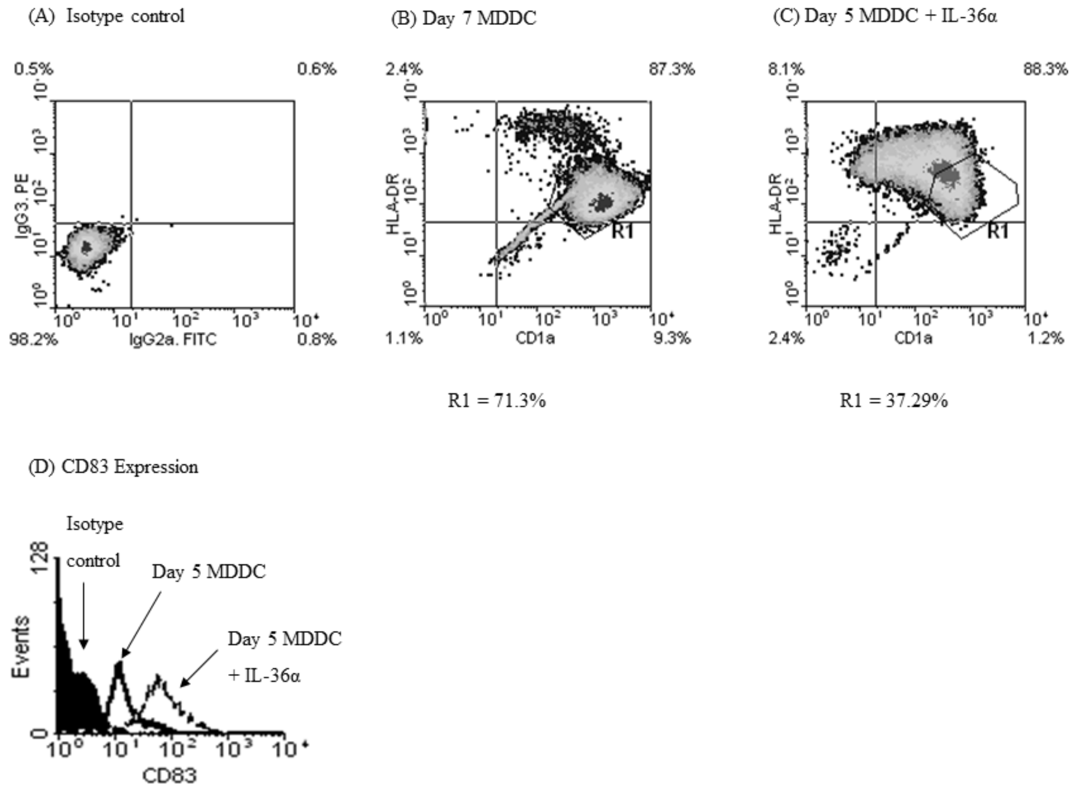
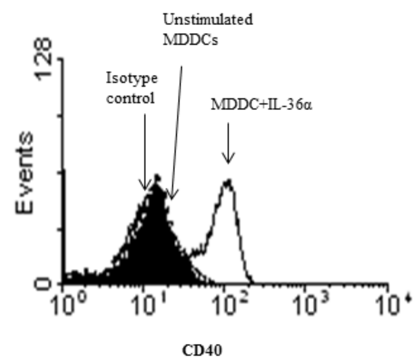


Fig 2 Higgins et al

(A)



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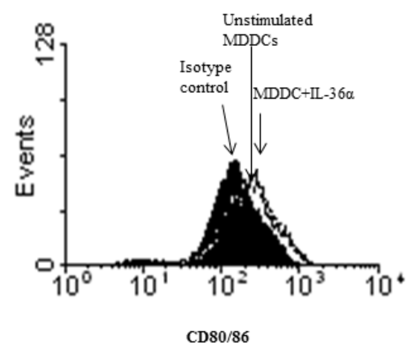
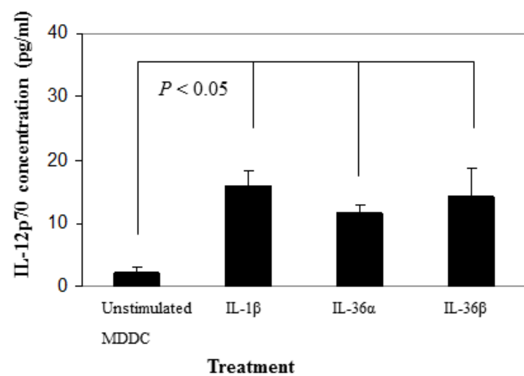


Fig 3 Higgins et al

(A)



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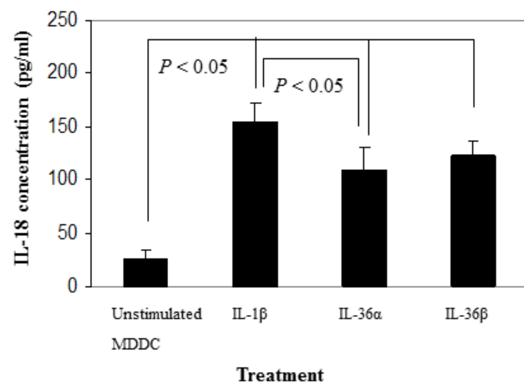


Fig 4 Higgins et al

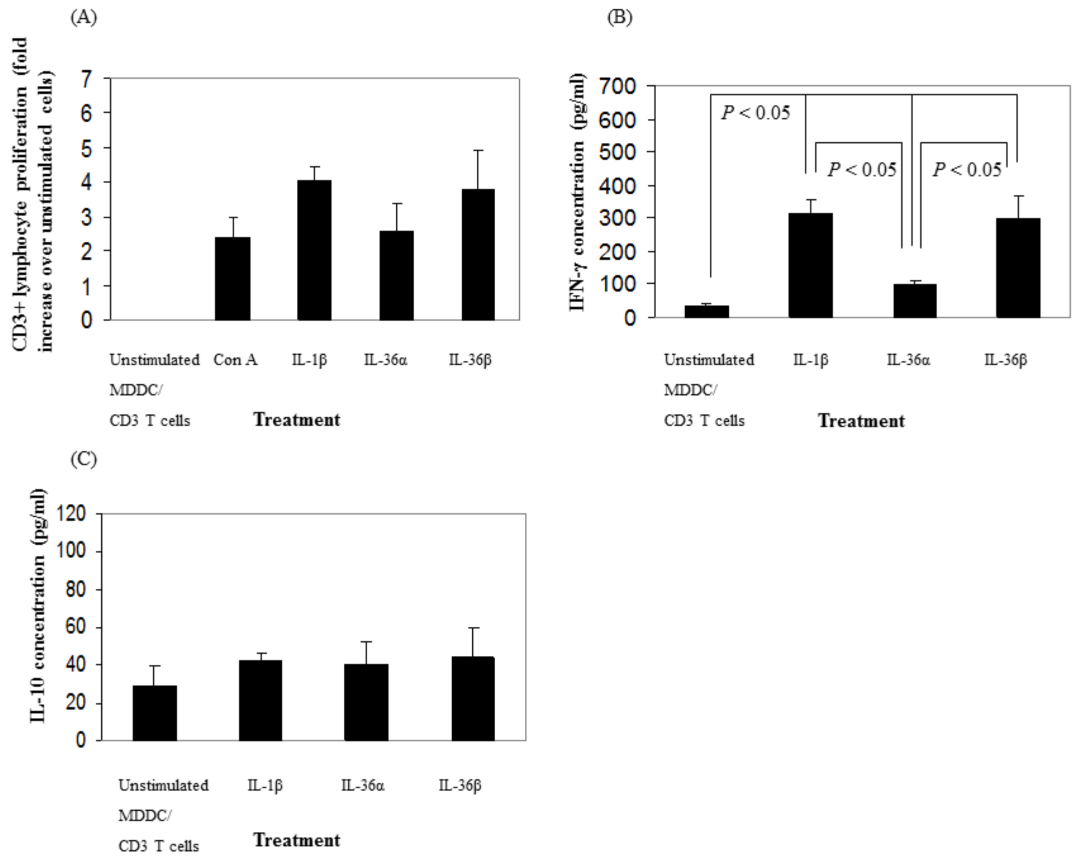


Fig 5 Higgins et al

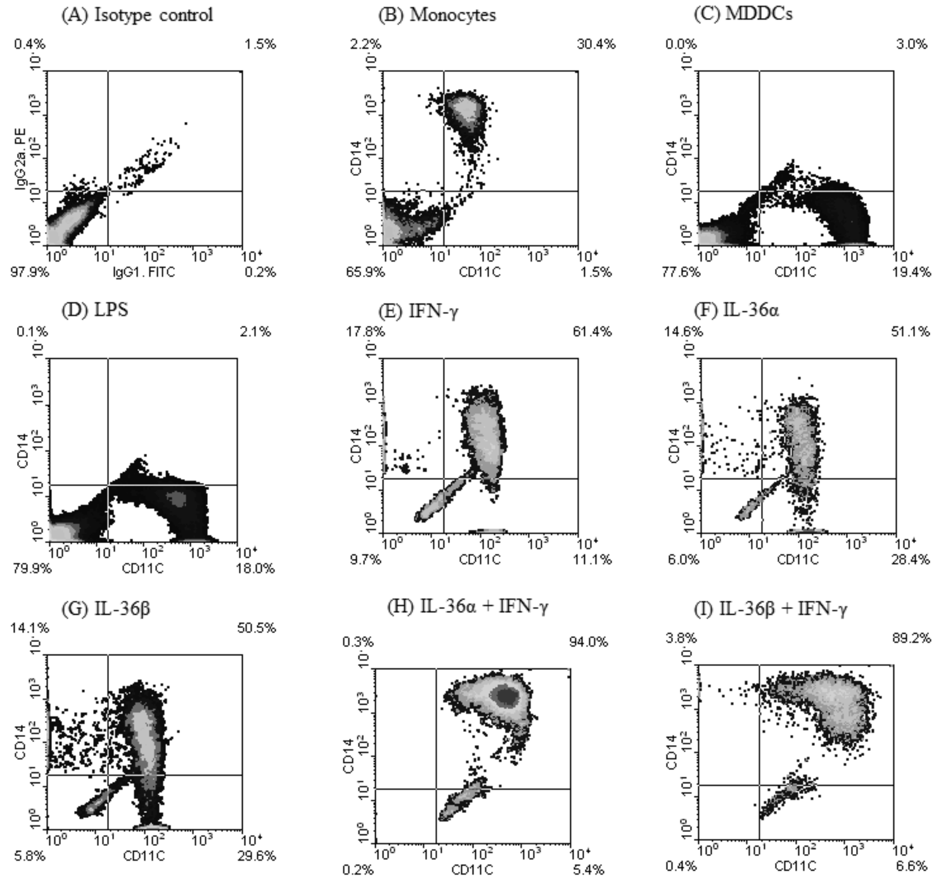


Fig 6 Higgins et al

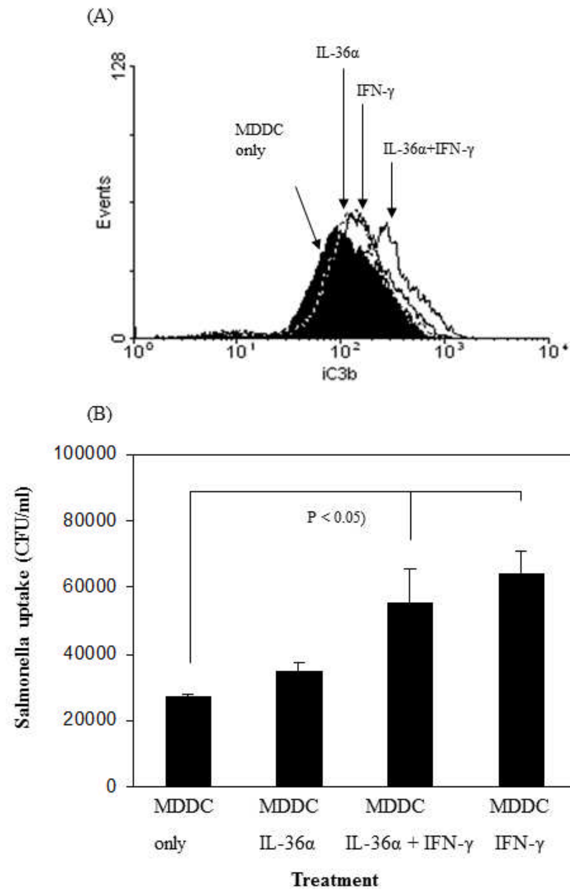
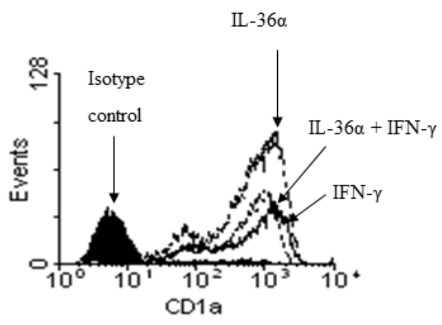


Fig 7 Higgins et al

(A)



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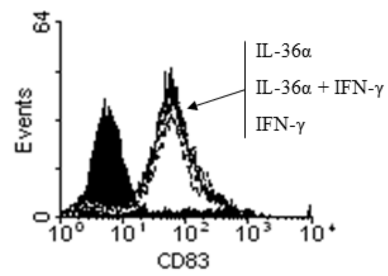

(C)