

ORIGINAL ARTICLE

Synovial fluid myeloid dendritic cells display important differences compared to monocyte-derived dendritic cells prepared *in vitro*

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The object of this study was to characterise synovial fluid dendritic cells (SFDCs) with regard to morphology, phenotype and responses to 1,25hydroxy-cholecalciferol (1,25D) and lipopolysaccharide (LPS), and to compare these characteristics with those of peripheral blood (PB) monocyte-derived DCs (MDDCs). SF was aspirated from knees with inflammatory effusions. PB samples were obtained contemporaneously. SFDCs were separated by flow cytometry. Morphology was determined on cytosmears. Expression of accessory molecules, cytokines and prostaglandin synthases mRNA was quantified by reverse transcription PCR. Analyses were performed on freshly prepared DCs and after incubation with 1,25D and LPS, separately and in combination. SFDCs and MDDCs displayed broadly similar morphology. Expression of accessory molecules, cytokines, cyclooxygenase-2 (COX-2) and prostaglandin E-synthase (PGES) was similar. SFDCs, but not MDDCs, expressed prostaglandin D-synthase (PGDS). PGDS was lost on incubation with SFDCs, but was induced by 1,25D in MDDCs. LPS in the presence or absence of 1,25D, induced interleukin 23 (IL23), IL1 β and tumour necrosis factor- α in SFDCs and MDDCs, with SFDC showing stronger expression of these cytokines. 1,25D in combination with LPS induced PGES and enhanced LPS induction of IL6 in SFDCs and MDDCs. LPS reduced 1,25D-induced expression of PGDS in MDDCs. SFDCs and MDDCs display similar basal characteristics but differ in PGDS expression and responsiveness to LPS and 1,25D. MDDCs have limitations as a model of SFDCs which have differentiated *in vivo*.

Clinical & Translational Immunology (2014) 3, e23; doi:10.1038/cti.2014.19; published online 8 August 2014

We have reported that myeloid dendritic cells (DCs) are a substantial minority population among mononuclear cells within synovial fluid (SF) from knee effusions in patients with rheumatoid arthritis and other inflammatory arthropathies.¹ We have further shown that DCs can be separated from other SF mononuclear cells of appropriate size and complexity by flow cytometry using a selection strategy, which utilises antibodies against CD11b (on myeloid cells), HLADR (antigen-presenting cells), CD11c (on human monocytes and DCs) and CD14 (present on human monocytes but not DCs). The resulting designation of CD11b⁺, HLADR⁺, CD11c⁺ cells as either DCs (CD14⁻) or monocytes (CD14⁺) has been confirmed by morphometric analysis of cell smears.¹

DCs can be generated *in vitro* through culture of peripheral blood (PB) monocytes for 1 week in the presence of granulocyte-macrophage colony-stimulating factor and interleukin 4 (IL4). The abundance of the starting population of cells from readily accessible PB samples and the reliability of the transformation, which utilises the readily available recombinant proteins, granulocyte-macrophage colony-stimulating factor and IL4, has led to the use of

monocyte-derived dendritic cells (MDDCs) generated *in vitro* as a core resource for the study of DCs.

While MDDCs have gained prominence in studies of human DCs because of their ready availability, questions remain regarding their authenticity compared with DCs differentiated *in vivo* at sites of inflammation, which provide multiple additional potential influences, including other cytokines, lipid mediators, adhesion molecules, stromal and structural elements and other cell types, which are not represented within *in vitro* cultures. The ability to isolate SFDCs from inflammatory knee effusions, coupled with convenience of venepuncture of the same subjects, provided the opportunity to test the authenticity of MDDCs as a model for DCs accumulating in inflamed joints of patients with arthritis.

In the studies described herein, we compare SFDCs and MDDCs with regard to their morphology, accessory molecule expression, inflammatory cytokine-expression profiles and the expression of prostaglandin synthases (PGSs) involved in inflammation. Utilising the same end points, we also examined the respective responses of SFDCs and MDDCs to two agents, lipopolysaccharide (LPS) and

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Received 21 May 2014; revised 7 July 2014; accepted 7 July 2014

1,25hydroxy-cholecalciferol (1,25D), which are known to influence, in different ways, the outcome of immune responses to immunological challenge through their effects on DCs. LPS is a complex bacterial cell wall glycolipid, which activates DCs, monocytes and other myelomonocytic cells through engagement of the pattern receptor TLR-4, thereby generating a 'danger signal', which increases immune responsiveness. By contrast, 1,25D, the biologically active form of vitamin D, has been shown to alter DC function in a way that favours lymphocyte tolerance to presented antigen and militates against autoimmunity as shown in animal models of multiple sclerosis and arthritis.^{2,3} These divergent effects made the outcome of combined stimulation with LPS and 1,25D of interest.

RESULTS

SFDCs and MDDCs display similar but distinct morphology

SFDCs freshly prepared using flow cytometry (Figure 1) and MDDCs both displayed the cytoplasmic and cell surface morphology of immature DCs, that is, medium size with smooth or ruffled borders (Figures 2a and b). However, the feature of eccentric location of the nucleus, which is regarded as a typical feature of immature DCs, was seen in MDDCs (Figure 2b), whereas the indented nucleus was often more centrally located in many SFDCs (Figure 2a). Culture of both SFDCs and MDDCs with the TLR-4 ligand LPS yielded the morphology characteristic of mature DCs (that is, variably located nuclei and veiled cytoplasm; Figures 2c and d).

SFDCs and MDDCs display similar expression of co-stimulatory molecules, inflammatory cytokines and the eicosanoid synthases PGES and COX-2, but differ in PGDS expression

The functional phenotypes of SFDCs and MDDCs were generally comparable with relatively similar expression of the co-stimulatory molecules CD80, CD83 and CD86 (Figure 3) and the proinflammatory cytokines IL23, IL1 β , tumour necrosis factor- α (TNF α) and IL6 (Figure 4) and the PGs cyclooxygenase-2 (COX-2) (Figures 5a and b) and prostaglandin E-synthase (PGES) (Figures 5c and d). A point of difference was the significant expression of the PGS prostaglandin D-synthase (PGDS) in SFDCs compared with basal expression by MDDCs ($P < 0.01$; Figures 5e and f).

Similar effect of LPS on expression of co-stimulatory molecules in SFDCs and MDDCs

As shown in Figure 3, in both SFDCs and MDDCs, LPS stimulation yielded significant upregulation of co-stimulatory molecules CD80 (Figures 3a and b) and CD83 (Figures 3c and d) ($P < 0.05$) but not CD86 (Figures 3e and f). CD14 was downregulated in response to LPS in both cell types (data not shown).

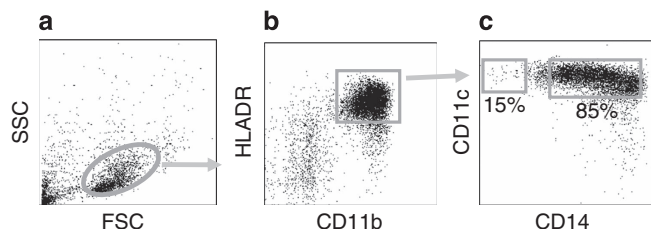


Figure 1 Four colour flow cytometric analysis of cells prepared from SF aspirates. (a) the selected gate based on forward and side scatter of light; (b) selection of HLADR⁺ and CD11b⁺ cells; (c) shows majority of HLADR⁺ CD11b⁺ CD11c⁺ cells are CD14⁺ monocytes and 15% are CD14⁻ myeloid DCs.

Differing effects of LPS on expression of cytokines in SFDCs and MDDCs

Although upon culture with LPS, both SFDCs and MDDCs upregulated IL23 (Figures 4a and b), IL1 β (Figures 4c and d) and TNF α (Figures 4e and f), the expression of these cytokines was substantially higher in SFDCs compared with MDDCs ($P < 0.05$, note differences in scales for SFDCs and MDDCs in Figures 4a–f). Upregulation of IL6 by LPS occurred to a similar extent in SFDCs and MDDCs (Figures 4g and h). LPS stimulation weakly upregulated IL12 in both SFDCs and MDDCs (data not shown).⁴

Effect of LPS on expression of PGs

Culture with LPS induced COX-2 in SFDCs and MDDCs (Figures 5a and b). LPS alone did not maintain nor induce PGDS (Figures 5e and f) or PGES (Figures 5c and d) expression in either cell type.

Effect of 1,25D on the morphology and phenotype of SFDCs and MDDCs

Culture with 1,25D for 48 h did not alter the morphology of SFDCs or MDDCs (not shown). Similarly, there was no significant effect of 1,25D on the expression of co-stimulatory molecules CD80, CD83 and CD86 (Figure 3) or of the inflammatory cytokines (Figure 4) in either SFDCs or MDDCs. Incubation with 1,25D upregulated PGDS significantly in MDDCs (Figure 5f), whereas the expression of PGDS was lost from fresh SFDCs when cultured for 2 days in medium with (Figure 5e) or without 1,25D (not shown in Figure 5, shown in Supplementary Figure 1). 1,25D alone did not significantly induce COX-2 (Figures 5a and b) or PGES (Figures 5c and d) in SFDCs and MDDCs.

Combinatorial effects of 1,25D and LPS on morphology and phenotype of SFDCs and MDDCs

The presence of 1,25D with LPS did not alter the morphological changes seen with maturation of SFDCs and MDDCs in response to LPS alone (not shown). Similarly, addition of 1,25D with LPS did not significantly alter mRNA expression of the co-stimulatory molecules CD80, CD83 and CD86 (Figure 3) or the cytokines, IL23, IL1 β and TNF α (Figures 4a–f), or PGS COX-2 (Figures 5a and b) in SFDCs or MDDCs compared with cells cultured with LPS alone. By contrast, in SFDCs and MDDCs, substantial further upregulation of IL6 (Figures 4g and h),^{5,6} and upregulation of PGES (Figures 5c and d), occurred in response to LPS in the presence of 1,25D ($P < 0.05$). The induction of PGDS in MDDCs by 1,25D was significantly diminished in the presence of LPS ($P < 0.0001$; Figure 5f).

Observations with MDDCs prepared from SF CD14⁺ monocytes

MDDCs prepared from SF CD14⁺ monocytes showed similar responses to LPS and 1,25D with regard to morphological changes and expression of surface molecules and genes of interest compared with PB MDDCs (data not shown).

DISCUSSION

Comparison between unstimulated SFDCs and MDDCs revealed many similarities. Cell surface morphology by microscopy and expression of accessory molecules, the inflammatory cytokines (IL1 β , TNF α , IL6, IL12, IL23) and the PGs (COX-2 and PGES) were similar. The main point of difference in unstimulated cells was the expression of PGDS in SFDCs but not MDDCs. There was also a morphological difference in the location of the nucleus (variable and more central in SFDCs, consistently eccentric in MDDCs).

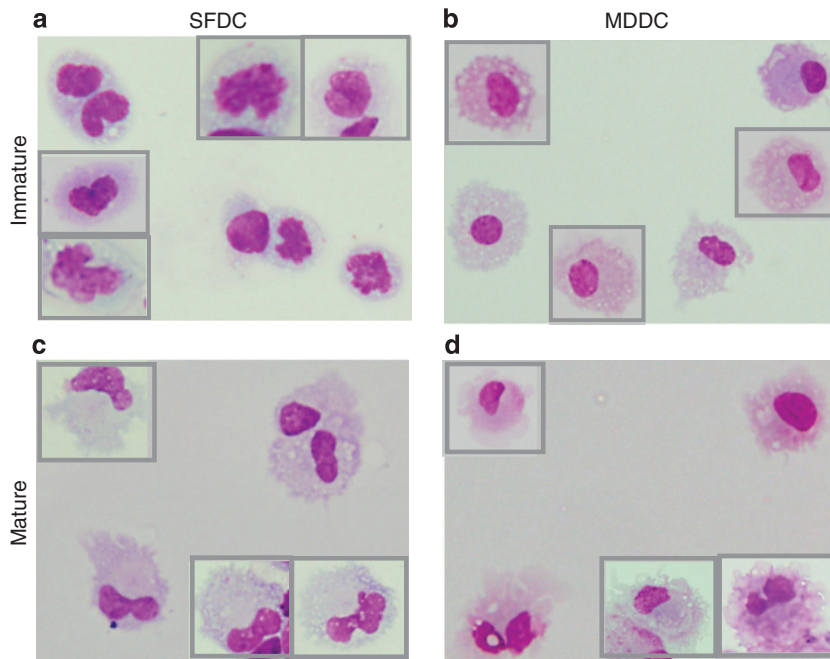


Figure 2 Comparison between morphology of SFDCs and MDDCs. Giemsa-stained cytospin preparations: (a) fresh SFDCs, (b) MDDCs from 7 days culture of peripheral blood monocytes in granulocyte–macrophage colony-stimulating factor/IL-4, (c) SFDCs after 2 days culture with LPS, and (d) MDDCs after 2 days culture with LPS. Because the density of cells would provide few examples at the magnification shown, the depicted fields have been supplemented by examples (cells surrounded by inset borders) from other fields.

The basis for the difference in nuclear location is uncertain, but could reflect the presence of additional factors that can influence maturation *in vivo* that are absent during maturation *in vitro*. The difference may also reflect variability in the stage of differentiation/maturation in SFDCs, which will not be synchronised to the extent that occurs with *in vitro* systems, such as generation of MDDCs, where the precursor monocytes will engage the stimuli for differentiation contemporaneously.

The expression of PGDS in fresh SFDCs may be an important observation. PGD₂ has been shown to play an active role in the resolution of experimentally induced inflammation *in vivo*.⁷ Furthermore, with DC isolated from other tissues, exogenous PGD₂ has been shown to retard DC migration and to induce regulatory T cells through engagement of the prostanoid receptor DP1.⁸ It is thus conceivable that endogenous PGD₂ generated by SFDCs may modulate immune responses within the synovium through autocrine and paracrine effects. We have shown in a rat model of polyarthritis that PGDS expression in DCs freshly isolated from synovium-rich tissues of arthritic hind-feet correlates inversely with clinical scores of disease severity.³ In addition, we have previously observed that the expression of PGDS by SFDCs and PGD₂ levels in SF in patients with inflammatory arthritis correlates inversely with the systemic disease activity markers, C-reactive protein and erythrocyte sedimentation rate.¹

Expression of PGDS by SFDCs was lost during 2 days of culture *in vitro* and this was not affected by 1,25D or LPS alone or in combination. By contrast, 1,25D upregulated PGDS in MDDCs, although upregulation was largely abrogated by co-culture with LPS. As the subjects under study were known either to have normal serum vitamin D levels or to have been taking a regular vitamin D supplement (data not shown), it can be inferred that *in vivo* differentiation of SFDCs occurred in the setting of vitamin D sufficiency. The extent to which 1,25D may influence PGDS

expression and other aspects of DC differentiation/maturation *in vivo* is uncertain. However, the present findings suggest that 1,25D has different effects on the expression of a number of inflammatory proteins, including PGDS, depending on the stage of maturation of DCs and the co-stimuli present.

1,25D can modulate the immune response leading to immunosuppression.^{9,10} These immunomodulatory effects appeared most pronounced when 1,25D is present during both differentiation and maturation of DCs.¹¹ However, such effects are not observed if the TLR-4 and the vitamin D receptor are stimulated with their agonist simultaneously.^{12,13} The immunomodulatory actions of 1,25D are thus complex and appear to change in the presence of danger signals such as TLR ligands.¹⁴ A protective effect of vitamin D has been shown in relation to respiratory infections in children.^{15,16} At least part of this effect may be attributed to effects of vitamin D on expression of proteins, which mediate mucosal barrier defence.¹⁷ In the present study, the cooperative interaction between 1,25D and the bacterial pattern receptor stimulus LPS, with regard to expression of IL6 and PGES by SFDCs and MDDCs, further supports the notion that vitamin D status may favourably condition innate-immune defence against pathogens.¹⁴ On the other hand, enhancement of PGES expression by 1,25D during LPS activation of DCs may be important in regulation of immune responses. For example, PGE₂ has been shown to redirect the differentiation of MDDCs towards myeloid-derived suppressor cells.¹⁸ These MDDC suppressor cells are involved in suppression of acute inflammatory mediators.¹⁹ IL6 is considered to be an inflammatory cytokine and has been implicated in autoimmune diseases and inflammation.²⁰ However, IL6 suppresses DC activation/maturation and may in some settings act as inflammation modulator.²¹ In the present study, due to limited numbers of SFDCs, co-stimulatory molecules, inflammatory cytokines and PGES were studied at the mRNA level. It remains to be determined whether levels of expression of message are matched by

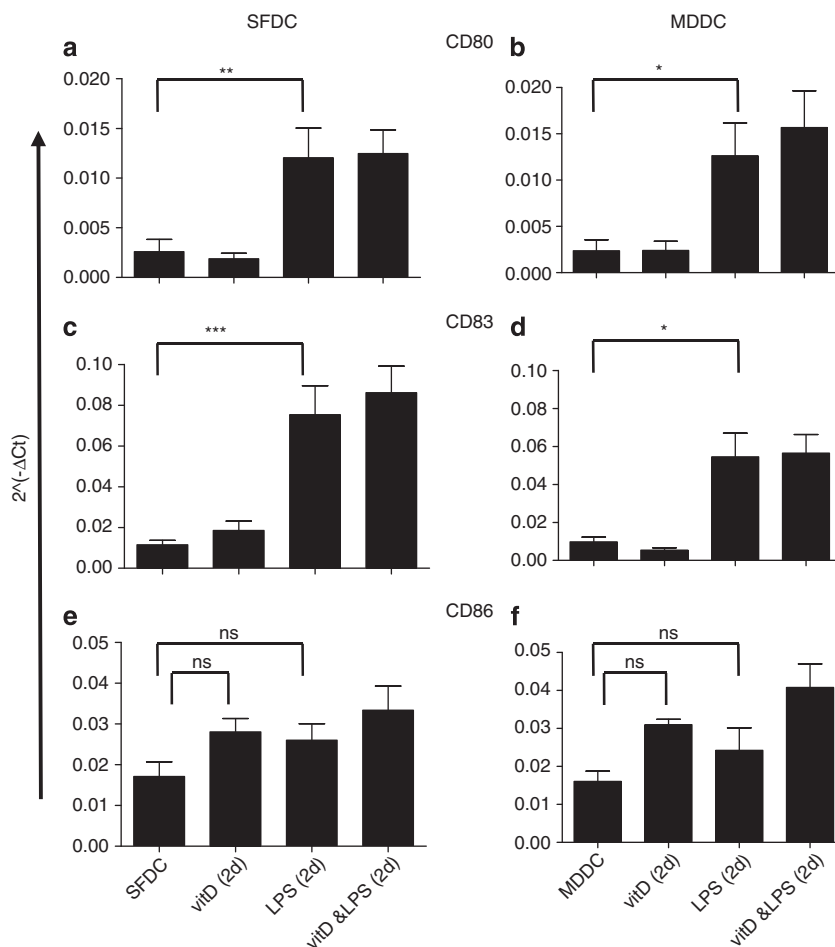


Figure 3 Expression of co-stimulatory molecules by SFDCs and MDDCs. The effect of 1,25D and LPS on the expression of mRNA for co-stimulatory molecules CD80 (a, b), CD83 (c, d) and CD86 (e, f) by SFDCs (a, c, e) and MDDCs (b, d, f). Mean + s.e.m. * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$, ns, not significant.

release of inflammatory mediators in SFDCs and MDDCs as measured at the protein level.

In a rat model of polyarthritis induced by intravenous injection of arthritogenic T cells from syngeneic donor rats in the prodrome for adjuvant-induced arthritis, we observed that vitamin D-replete recipients developed less-severe arthritis than their vitamin D-deficient counterparts. DC isolated from synovium-rich tissues from the vitamin D-replete rats expressed substantially more PGDS than DCs from synovium-rich tissues of vitamin D-deficient rats.³ While association does not establish causation, the known effects of PGD₂ in modulating DC and lymphocyte function and the inflammation-resolving actions of PGD₂ suggest a possible role for PGD₂ in disease control. In the above animal studies, the groups were markedly different in vitamin D status (severe deficiency vs upper normal range). By contrast, in human studies in rheumatoid arthritis to date, where the effects of vitamin D have been less clear cut, vitamin D deficiency has generally been less severe and serum 25 hydroxy-vitamin D levels in those regarded as replete, have often been towards the low end of the normal reference range.²² Thus, the comparisons have often been between moderate deficiency and borderline sufficiency, with the latter possibly being suboptimal for immunomodulatory and anti-inflammatory effects. In the present study, the combinatory effects of 1,25D and LPS on the expression by DCs of certain inflammatory proteins (IL6, PGES) suggest that

vitamin D could have proinflammatory effects through amplification of effector mechanisms in inflammatory diseases at least in the presence of bacterial pattern receptor ligands. It is conceivable that vitamin D may augment host defence through effects on pattern receptor responses while modulating autoimmunity through other mechanisms.^{5,6} Notwithstanding, the data are ambiguous with regard to the benefit or otherwise of vitamin D supplements in rheumatoid arthritis (and other autoimmune and inflammatory disease). Appropriately designed clinical trials in rheumatoid arthritis and other inflammatory diseases are therefore needed to determine the place of vitamin D supplements and to define targets for vitamin D status, as measured by serum assays of 25 hydroxy-vitamin D. Within this small sample of patients with different arthropathies, the responses seen by SFDCs were not obviously influenced by the type of arthropathy or treatment.

In conclusion, SFDC and MDDC share many similarities with regard to morphology, phenotype and basal expression of inflammatory cytokines and PGs. However, significant differences can occur in expression of PGDS, the synthase for the inflammation-resolving eicosanoid PGD₂, which is expressed basally by SFDCs but not by MDDCs. SFDCs and MDDCs also differ in responses to LPS. These responses can be influenced by the biologically active form of vitamin D, which can also induce PGDS in MDDCs. The findings highlight the need for studies that utilise DC isolated from inflammatory sites

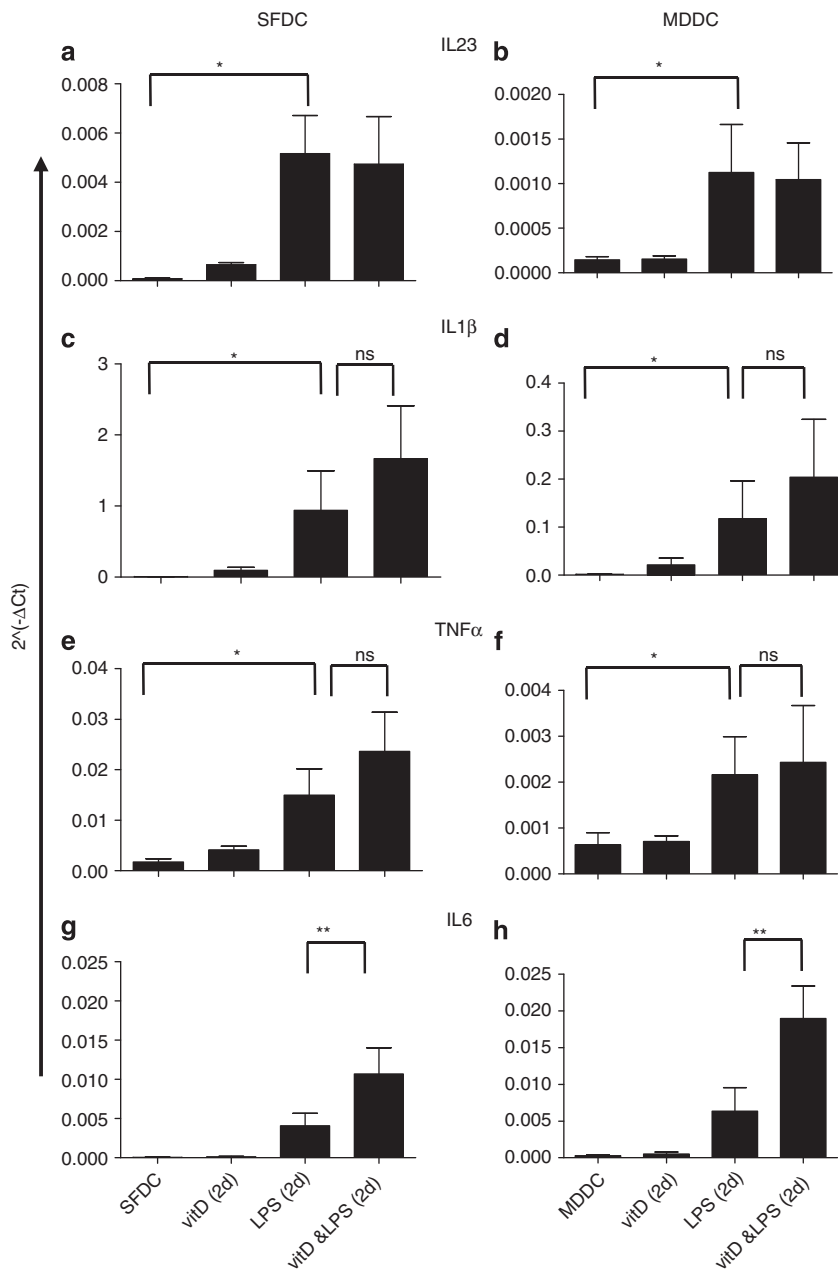


Figure 4 Expression of inflammatory cytokines by SFDCs and MDDCs. The effect of 1,25D and LPS on the expression of mRNA for the cytokines IL23 (a, b), IL1β (c, d), TNFα (e, f) and IL6 (g, h) by SFDCs (a, c, e, g) and MDDCs (b, d, f, h). Please note different scales have been used for SFDCs and MDDCs (a–f) to show the pattern of expression of IL23, IL1β and TNFα. Mean + s.e.m. **P*<0.01, ***P*<0.001, ns, not significant.

in order to complement studies of more conveniently obtained MDDCs. The studies also draw attention to the potentially complex effects of vitamin D on immune responses.

METHODS

Subjects

SF and paired PB samples were obtained from six patients with inflammatory knee effusions. Demographic and clinical details are shown in Table 1. All subjects gave informed consent, and the study protocol was approved by the Human Research Ethics Committee, Royal Adelaide Hospital.

Isolation of SF myeloid DCs

Mononuclear cells were isolated from SF by density gradient centrifugation over Lymphoprep, then incubated with a cocktail of (labelled) monoclonal

antibodies against CD11b (Alexa Fluor 488-lab), CD11c (phycoerythrin), HLADR (PE-cy5) and CD14 (APC) for 45 min at 4°C, as described previously.¹ Cells were gated by size (Figure 1a) for sorting into CD11b⁺ HLADR⁺ CD11c⁺ CD14⁻ (DCs) and CD11b⁺ HLADR⁺ CD11c⁺ CD14⁺ (monocytes) populations, using FACS Diva software (Becton Dickinson, San Jose, CA, USA), as described previously (Figures 1b and c).¹

Preparation of MDDCs

Lymphoprep was used to harvest PB mononuclear cells, from which, CD14⁺ monocytes were isolated by positive magnetic selection using anti-CD14 microbeads, according to the supplier's protocol (MiltenyiBiotec, Bergisch Gladbach, Germany). The purity of the CD14⁺ cell fraction, as assessed by flow cytometry using anti-CD14-FITC, was consistently more than 90%. To generate immature MDDCs, these cells were cultured at 0.5 × 10⁶ cells ml⁻¹ in

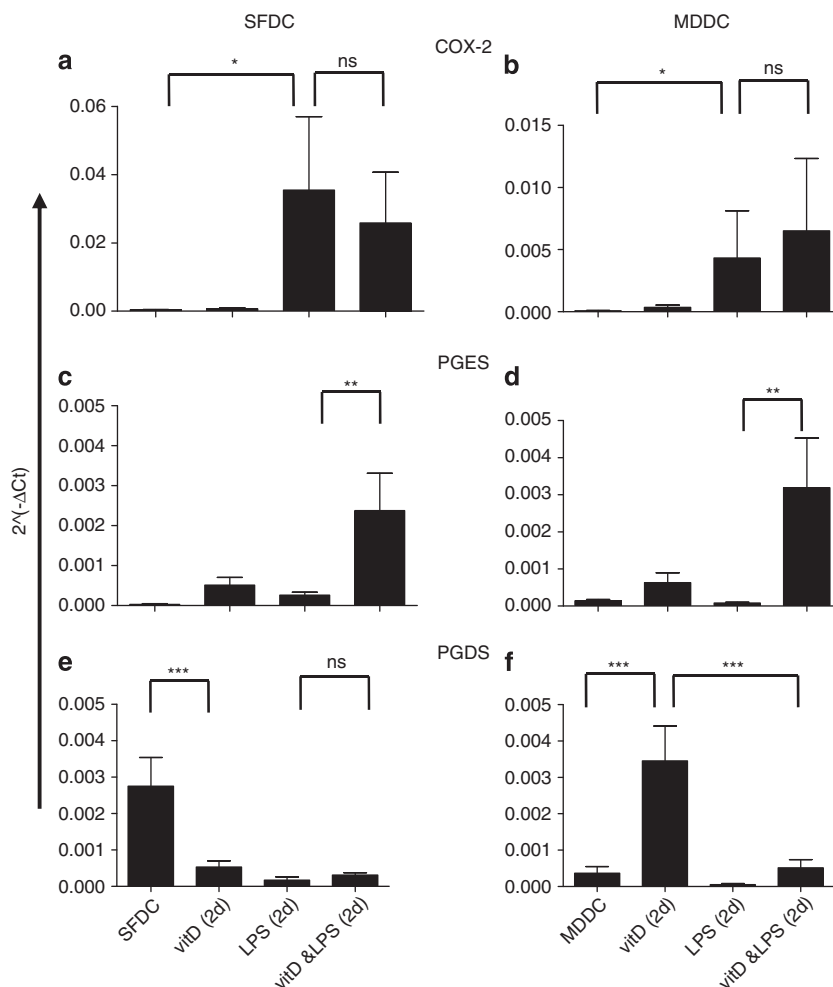


Figure 5 Expression of prostaglandin synthases by SFDCs and MDDCs. The effect of 1,25D and LPS on the expression of mRNA for prostaglandin synthases: cyclooxygenase (COX-2) (a, b), prostaglandin E-synthase (PGES) (c, d) and prostaglandin D-synthase (PGDS) (e, f) by SFDC (a, c, e) and MDDCs (b, d, f). Please note different scales have been used for SFDCs and MDDCs (a, b) to show the pattern of COX-2 expression. Mean + s.e.m. * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$, NS, not significant.

Table 1 Demographic and clinical characteristics of patients

Diagnosis	Disease duration	
	(years)	Treatment
Rheumatoid arthritis	22	DMARD + NSAID + fish oil
Rheumatoid arthritis	27	DMARD + adalimumab + fish oil
Psoriatic arthritis	6	DMARD + vitamin D + fish oil
Psoriatic arthritis	12	Fish oil
B27 + pauci-arthritis	18	DMARD + vitamin D + fish oil
RNP + polyarthritis	3	DMARD + NSAID + vitamin D + fish oil

Abbreviations: DMARD, disease-modifying anti-inflammatory drugs; NSAID, non-steroidal anti-inflammatory drugs. Adalimumab is a tumour necrosis factor inhibitor.

complete medium containing 10% fetal calf serum and 1 mM sodium pyruvate in the presence rhGM-CSF 100 ng ml⁻¹ (kindly provided by Dr T Hercus, Human Immunology, SA Pathology, Adelaide, Australia) and rhIL-4 50 ng ml⁻¹ (PeproTech, Rocky Hill, NJ, USA) for 7 days. Medium supplemented with cytokines was refreshed every 3rd day.

Culture of DCs

SFDCs and MDDCs, prepared as detailed above, were cultured for 48 h in complete medium supplemented with 10% fetal calf serum alone or

with LPS (from *Escherichia coli*, Sigma, St Louis, MO, USA), at a final concentration of 200 ng ml⁻¹, in the presence or absence of 1,25D (Sigma) 10 nM l⁻¹, which is physiologically appropriate.²³ Culture with medium alone was used as a control.

Cytology

Cytospin smears prepared from fresh and cultured cells were fixed and stained as described.²⁴

RNA isolation and quantitative reverse transcription PCR analysis of gene expression

Total RNA was extracted from fresh flow cytometrically sorted cell populations or from cultured cells, using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Total RNA was reverse transcribed to cDNA and amplified using the two-step reverse transcription PCR kit from Qiagen. RNA and cDNA quality was assessed using a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific, Wilmington, DE, USA) before samples were frozen at -70 °C until further use. Gene expression levels were investigated using commercially available specific primers for human genes obtained from Qiagen, including PGDS (QT-00022043), PGES (QT00208607), COX-2 (QT00040586), CD80 (QT00000497), CD83 (QT00069923), CD86 (QT00033915), IL1β (QT00021385), IL6 (QT00083720), IL12 (QT00000357), IL23 (QT00204078), TNFα (QT00029162) and a housekeeping gene *ACTB*

(β -actin, QT01680476). Real-time PCR was performed using the QuantiFast SYBR Green PCR kit (Qiagen) according to the supplier's protocol, in a Rotor-Gene 3000 real-time PCR machine (Corbett Research, New South Wales, Australia). A minimum of three replicate samples were processed in each experiment. Each PCR included a sample prepared without template and a sample prepared without primers as negative controls. The thermal conditions were 95°C for 5 min followed by 35 cycles of 95°C for 10 s and 60°C for 30 s. PCR product quality was monitored using post-PCR melt curve analysis. Fold changes were calculated using the formula $2^{(-\Delta\Delta Ct)}$, and $\Delta Ct = Ct(\text{target gene}) - Ct(\beta\text{-actin})$. Ct is the cycle at which the threshold line is crossed.²⁵

Statistical analysis

All data were analysed using GraphPad Prism V5.0 (GraphPad Software Inc., San Diego, CA, USA). Quantitative real-time reverse transcription PCR signals were normalised to β -actin. One-way analysis of variance with Neuman–Keuls test was used to determine significant differences between the groups. $P < 0.05$ was considered significant.

ACKNOWLEDGEMENTS

This work was funded in part by Research grant from Arthritis Foundation of Australia, Grant in aid, 2011. We are grateful to Mr Alan Bishop for his advice and assistance in flow cytometry and cell sorting, and Dr Melissa Gregory for valuable advice regarding reverse transcription PCR analysis.

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