Drug modification of LPS-stimulated human monocyte-derived dendritic cells

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ABSTRACT

Many drugs have been reported to convert dendritic cells (DCs) into a tolerogenic phenotype *in vitro*. However, there is evidence that an additional stimulus, such as lipopolysaccharide (LPS), may also be necessary for tolerogenic function *in vivo*. Little is known concerning the effects of drug modification on LPS-prestimulated DCs. In this study, monocyte-derived immature DCs were stimulated with LPS first and the influence investigated of six different agents on surface antigen expression, cytokine production and lymphocyte proliferation and cytotoxicity. Mycophenolic acid- and rapamycin-exposed DCs had little effect on surface antigen expression or functional activity towards lymphocytes. In contrast, treatment of immature dendritic cells with aspirin, dexamethasone, 1α,25-dihydroxyvitamin D3 (VD3) or butyric acid was associated with diminished expression of CD1a, CD1c, CD40, CD80 and CD83. Dendritic cell modification by aspirin, dexamethasone and VD3 were all associated with decreased production of tumour necrosis factor-α (TNFα). Furthermore, VD3 treatment was associated with a consistent and significant elevation of IL-6 production. Aspirin-, dexamethasone- VD3- and butyric acid-modified DCs suppressed interferon-γ production, proliferation and cytotoxicity in co-culture with allogeneic mononuclear cells, but inconsistent results were obtained with different allogeneic combinations. Different drugs show varying effects on DC phenotype. No single agent was consistently effective in suppressing the stimulation of allogeneic mononuclear cells and future work is needed to explore drug combinations.

KEY WORDS: Cytokines.
Dendritic cells.
Drug tolerance.
Transplantation.

Introduction

Dendritic cells (DCs) appear to act as critical modulators of the immune response, regulating both protective immunity and the induction of tolerance.[1,2] Dendritic cells may steer differentiating T cells towards a tolerogenic response directly, or act indirectly by promoting the expansion of regulatory T cells. Originally, it was thought that tolerance was mediated by immature DCs (i.e., naïve with respect to antigens and danger signals), but the current consensus is that tolerogenic DCs display a semi-mature or quasi-mature phenotype.[3,4] The therapeutic potential of tolerogenic DCs in transplantation and autoimmune disease is immense, but so far promising rodent models have not been translated into successful clinical trials.[5,6]

A growing literature suggests exposure to lipopolysaccharide (LPS) may be critical for induction of a tolerance function *in vivo*,[7–10] perhaps because LPS is essential for the adequate chemokine receptor 7 (CCR7) expression necessary for migratory activity.[9] Furthermore, it has been suggested that tryptophan metabolites produced by indoleamine-2,3-dioxygenase (IDO) could also be relevant to immune regulation,[11,12] and it seems LPS exposure is required to ensure IDO expression.[7]

Many agents, including several commonly used drugs, have been reported to switch DCs into a tolerogenic phenotype. These include dexamethasone,[13] 1α,25-dihydroxyvitamin D3 (VD3),[14] aspirin,[15] butyric acid,[16] mycophenolic acid[17] and rapamycin.[8] For each pharmacological agent, exploitation of this property therapeutically has been mooted in the context of autoimmune- and/or alloimmune-mediated disease. However, the conditions previously used to prepare these modified DC preparations differed with each drug, making direct comparisons difficult. Moreover, most of the previous data relating function to surface antigen expression were obtained with LPS-untreated cells. Previous *in vitro* studies indicating the advantage of LPS exposure used a modifying drug first, followed by LPS maturation,[9,10] although promising results *in vivo* have been obtained with unmodified LPS-matured cells.[10,11]

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Anderson *et al*.[9] claimed LPS activation was essential for CCR7-mediated tolerogenic function *in vivo*, but their dexamethasone/VD3-prepared DCs subsequently treated with LPS exhibited very modest CCR7 expression, much closer to that of immature DCs than to that of mature DCs.

The present authors hypothesise that there might be an advantage in exposing immature DCs to LPS long enough to initiate LPS-dependent signalling before exposure to potentially tolerogenic drugs. Therefore, the primary aim of this study is to investigate a new approach to generating DCs that could have tolerogenic potential. In addition, preparation of DCs under identical experimental conditions will allow direct comparisons to be made between individual drugs for the first time.

Materials and methods

Materials
Buffy coats (leucocyte-rich fraction) were obtained anonymously from blood donations given to the local Blood Transfusion Centre. Blood group AB serum was also obtained from voluntary blood donations. All fluorochrome-labelled antibodies were purchased from BD Pharmingen (Oxford, UK) except anti-CD1c-FITC (Miltenyi Biotech, Surrey, UK). Interleukin (IL)-4 and granulocyte-macrophage colony stimulating factor (GM-CSF) were purchased from Gentaur Molecular Products (Brussels, Belgium). All other reagents were obtained from Sigma-Aldrich, Poole, UK.

Dendritic cell preparation
Mononuclear leucocytes were isolated from buffy coats and allowed to adhere to plastic flasks. The adherent cells were then cultured in 1% human AB serum/RPMI-1640 for three days with IL-4 (15 ng/mL [195 u/mL]) and GM-CSF (50 ng/mL [613 u/mL]) to differentiate into immature DCs.[19]

Dendritic cell modification
The cells were washed, resuspended in fresh culture medium supplemented with fresh IL-4 and GM-CSF, and aliquoted into small flasks. Lipopolysaccharide (1 μg/mL) was added to each flask. Two hours later, apart from an LPS-only flask (positive control), one of the following was added to each flask: aspirin (4 mmol/L), dexamethasone (1 μmol/L), VD3 (10 nmol/L), butyric acid (0.5 mmol/L), mycophenolic acid (0.1 mmol/L) or rapamycin (10 ng/mL). The drugs were used at appropriate concentrations reported in the literature.[13–18] Three days later, the cells were analysed for surface antigen expression using flow cytometry, and the culture supernatant collected and stored at –70°C.

Flow cytometry
Flow cytometry was performed using fluorescein- and phycoerythrin-conjugated specific antibodies.[19] An anti-CD197-PerCP conjugate was used to detect CCR7. Data were collected on a BD FACSCalibur and analysed using Cell Quest Pro software. Isotype control values were deducted from those obtained with specific antibodies.

To detect the intracellular transcription factor, Foxp3 (FoxP3-PE staining kit, BD Pharmingen) was used.

Cytokine assays
Selected cytokines (i.e., IL-1β, IL-2, IL-5, IL-6, IL-8, IL-10, IL-12p70, interferon-γ [IFNγ], TNFα, TNFβ) were assayed by fluorescent bead immunoassay using the human Th1/Th2 11-plex kit (eBiosciences, Hatfield, UK), following the manufacturer's instructions, and results were analysed using FlowCytoMix Pro software. Low values (on the non-linear part of calibration curves, typically <100 pg/mL) were poorly reproducible and therefore generally not specified in the Results section.

Interferon-γ induction in mixed leucocyte culture
Mononuclear cells (10^6/mL) isolated from buffy coats, as described above, were cultured for five days with allogeneic dendritic cells (10^5/mL) in 10% human AB serum-RPMI. The cells were then harvested by centrifugation and the supernatants stored for IFNγ assay by fluorescent bead immunoassay. Secretion of IFNγ under such conditions correlates strongly (r=0.87) with tritiated thymidine incorporation.[20]

Cell proliferation assay
Cell proliferation was assayed with a dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA) cell proliferation kit (Perkin Elmer), using a modification to the manufacturer's instructions. In brief, DCs (5 x 10^4/mL) and allogeneic mononuclear cells (10^6/mL) were co-cultured in 10% human AB serum for five days at 37°C and 5% CO_2 before the addition of 5-bromo-2'-deoxyuridine (BrdU; final concentration: 10 μmol/L) and further incubation overnight. The cells were transferred to a MultiScreen HTS-BV plate (Millipore, Herts, UK) then washed, fixed and incubated with europium-labelled anti-BrdU antibody solution for 2 h. After further washing, the filter disks were cut out and placed into a 96-well low-fluorescence plate. DELFIA inducer reagent was then added and after 15 min the fluorescence associated with europium release was measured in a Victor X4 time-resolved fluorometer. Values obtained with autologous DC-mononuclear cell (MNC) combinations were deducted and the results expressed as corrected europium counts.

Cell-mediated lympholysis assay
Dendritic cells were co-cultured with allogeneic mononuclear cells (1 DC: 20 MNC) in 10% human AB serum

Table 1. Surface antigen expression of drug-modified dendritic cells.

	CD14	CD1a	CD1c	CD40	CD80	CD83	CD86	HLA-DR
LPS only	2(2)	44(18)	81(10)	39(27)	86(13)	77(23)	98(1)	99(1)
LPS-Asp	10(11)*	23(16)§	81(11)	19(14)†	64(25)‡	39(26)§	99(1)	99(1)
LPS-Dex	9(13)	28(14)‡	70(14)§	22(21)†	64(26)†	48(25)‡	95(7)	99(1)
LPS-VD3	13(11)*	32(19)†	66(17)‡	19(17)†	74(19)†	59(25)§	98(2)	99(1)
LPS-But	2(2)	23(14)§	77(10)*	11(11)†	70(26)*	46(29)‡	98(2)	99(1)
LPS-MPA	1(1)*	41(19)	81(13)	22(19)	87(13)	76(25)	97(6)	99(1)
LPS-Rapa	1(1)	44(18)	83(10)	24(23)	86(16)	67(23)	96(6)	99(1)

Results expressed as percentage positive cells and presented as mean (standard deviation) of 10 determinations.
Monocytes were allowed to differentiate into immature dendritic cells for six days and incubated with LPS after Day 3 with or without aspirin (Asp), dexamethasone (Dex), 1α, 25-dihydroxyvitamin D3 (VD3), butyric acid (But), mycophenolic acid (MPA) or rapamycin (Rapa).
Drug-modified dendritic cells were compared to the positive control (LPS only) and statistical significance assessed by paired *t*-test.
*$0.01<P<0.05$; †$0.001<P<0.01$; ‡$0.0001<P<0.001$; §$P<0.0001$.

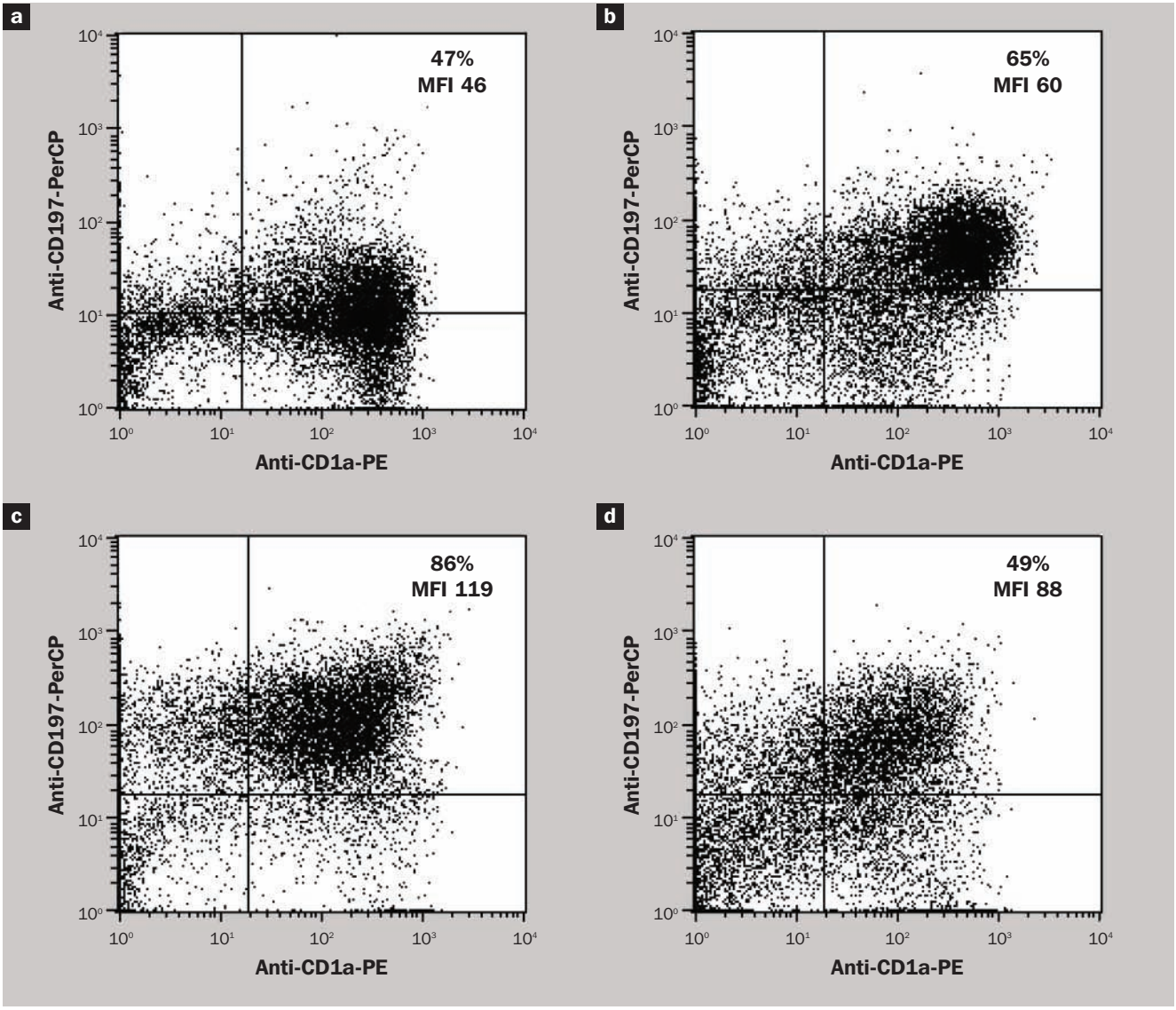

Fig. 1. Surface expression of CCR7 (CD197) on dendritic cells. Immature DCs after three days of culture **(a)** were allowed to continue in culture for a further three days **(b)**; were stimulated with LPS alone **(c)**; or with LPS followed by VD3 **(d)**. Results quantified both as percentage positive cells and as MFI. The data are representative of three determinations. VD3-modified DCs were chosen as an example of modified cells.

in RPMI medium for six days to generate cytotoxic T cells. The same mononuclear cells were cultured without allogeneic DC to provide CTL-free control cells. Lymphocytes from the DC donor were exposed to phytohaemagglutinin (L-PHA; 2 µg/mL) for two days to provide blasts for target cells. All cells were resuspended in 5% AB serum in RPMI for the cytotoxicity assay. Mononuclear cells (50 µL at 2 x 10^7/mL) were mixed with an equal volume of target cells (5 x 10^5/mL) in two sets of triplicates in a round-bottomed culture plate. The plate was gently centrifuged (100 x*g*; 3 min) before incubation for 4 h at 37°C in a CO_2 incubator. Forty-five minutes prior to the end of the incubation period, 12 µL either RPMI or Triton X-100 (lysis solution) was added to each set of triplicates. Finally, the plate was centrifuged at 200 x*g* for 5 min and the supernatants transferred to corresponding wells of an enzyme-linked immunosorbent assay (ELISA) plate. These supernatants were assayed for lactate dehydrogenase using a CytoTox 96 non-radioactive cytotoxicity kit (Promega). The mean CV for the triplicate determinations was 2.5%. For each set of values, the result obtained without lysis solution was calculated as the quotient of the result obtained with lysis solution (maximum lysis) and expressed as percentage cytotoxicity. The results were also expressed as a cytotoxicity ratio obtained by dividing the test percentage with the corresponding figure obtained with the unstimulated (no DC) mononuclear cell cultures.

Indolamine 2,3-dioxygenase assay

The IDO assay was based on previously published methodology.[21,22] Cells were washed (x2) and resuspended in 0.5 mL 50 mmol/L Tris-MOPS buffer (pH 7.4) containing 130 mmol/L NaCl, then disrupted by sonication for 30 sec on ice. The homogenate was centrifuged at 10,000 x*g* for 5 min at room temperature. Samples of the supernatant were taken for determination of protein concentration by the BCA assay using bovine serum albumin as a standard. Following this, 0.4 mL supernatant was added to an equal volume of 100 mmol/L potassium phosphate buffer (pH 6.5) containing 1 mmol/L L-tryptophan, 20 mmol/L methylene blue,

Table 2. Mean fluorescence intensity of surface antigen expression.

	CD14	CD1a	CD1c	CD40	CD80	CD83	CD86	HLA-DR
LPS only	4(1)	21(8)	29(13)	12(6)	93(64)	39(24)	391(197)	701(383)
LPS-Asp	5(2)	7(4)‡	21(7)*	8(5)†	41(33)†	13(8)†	348(120)	634(268)
LPS-Dex	5(2)	10(5)§	17(6)‡	8(2)*	41(27)‡	19(16)‡	204(148)‡	463(270)*
LPS-VD3	5(2)	13(6)‡	16(6)‡	7(3)†	55(38)†	24(14)†	271(140)*	479(263)*
LPS-But	4(1)	7(3)§	22(8)‡	6(2)†	49(32)†	16(12)‡	253(164)†	445(226)*
LPS-MPA	4(1)	20(20)	32(15)	9(2)	126(90)*	33(20)*	360(205)	523(257)
LPS-Rapa	4(1)	27(23)	29(11)	9(3)	94(58)	25(17)	363(210)	653(345)

The data summarised in Table 1 are presented here as mean fluorescence intensities.

*$0.01<P<0.05$; †$0.001<P<0.01$; ‡$0.0001<P<0.001$; §$P<0.0001$.

40 mmol/L ascorbic acid and catalase (200 u/mL; Sigma-Aldrich). Both the enzyme suspension and incubation buffer were preheated to 37°C before mixing. The mixture was incubated for a further 30 min at 37°C. The reaction was stopped by adding 0.4 mL 30% trichloroacetic acid and further incubated at 50°C for 30 min to hydrolyse *N*-formylkynurenine produced by IDO to kynurenine. The reaction mixture was then centrifuged at 10,000 *xg*, as above, to remove sediment. For spectrophotometric analysis, 75 mL of this mixture was added to an equal volume of 2% Ehrlich reagent (ρ-dimethylbenzaldehyde) in glacial acetic acid in a 96-well microtitre plate. Duplicate samples were run against a standard curve of defined kynurenine concentrations (0–100 µg/mL). Absorbance (*A*) was measured at 490 nm.

Statistical analysis

Statistical analysis was performed using Prism for Windows software from Graph Pad (San Diego, CA, USA). Significance values reported were always two-tailed values, with $P<0.05$ deemed statistically significant.

Results

CCR7 expression

Using flow cytometry, the study confirmed that LPS enhances CCR7 expression on dendritic cells (Fig. 1). Furthermore, after LPS exposure, none of the immunosuppressive drugs selected for study abolished CCR7 expression and generally had a modest effect (not shown). Immature DCs had readily detectable CCR7, and more than expected from the findings of Anderson and co-workers.[9]

IDO activity

Activity was detected in 10^6 LPS-stimulated DCs but was undetectable in six-day-old immature DCs, even when 10^7 cells were used. The specific activity in control (LPS-stimulated but drug-unexposed) cells was typically 15 nmol kyneurenine released/h/mg protein. Similar activities were still present after drug modification, except for VD3- and butyrate-modified DCs where the IDO activity was below the lower limit of detection (5 nmol kynurenine released/h/mg protein; data not given).

Surface antigen expression of drug-modified dendritic cells

The tolerogenic function of DCs is believed to be related to their degree of maturation and therefore the study compared the surface phenotypes of six different drug-modified DC preparations with unmodified LPS-matured DCs.

As expected,[23] exposure of immature DCs to LPS dramatically increased the expression of CD40, CD80 and CD83, while that of CD1a was decreased (data not shown). Little alteration in DC surface antigen expression was apparent after exposure to mycophenolic acid or rapamycin. However, treatment with aspirin, dexamethasone, VD3 and butyrate all resulted in significantly diminished expression of CD1a, CD40, CD80 and CD83. Analysis by quadrant statistics (percentage positive cells) is summarised in Table 1.

These same data analysed and expressed as mean fluorescence intensities yielded broadly similar results (Table 2). However, MFI analysis indicated that the expression of CD86 and HLA-DR was substantially reduced compared with control values after treatment with dexamethasone, VD3 or butyric acid. A significantly increased expression of CD80 in mycophenolic acid-treated dendritic cells was observed.

Although cells modified by aspirin, dexamethasone, VD3 and butyric acid all exhibited a quasi-mature phenotype, the patterns of surface antigen expression were not identical.

Table 3. Cytokine production of drug-modified dendritic cells.

	IL-6	IL-8	TNFα
LPS only	183(226)	9092(3653)	6689(6173)
LPS-Asp	627(1002)	9300(4477)	1468(2780)†
LPS-Dex	167(166)	10690(5462)	3823(2973)*
LPS-VD3	3086(3645)‡	10630(4898)	3997(4102)*
LPS-But	231(250)	10760(6119)	7621(5724)
LPS-MPA	171(193)	9697(5204)	1106(1290)†
LPS-Rapa	203(166)	10480(4280)	7960(5553)

Results (pg/mL) expressed as mean (standard deviation) of 10 determinations. Monocytes were allowed to differentiate into immature dendritic cells for three days then incubated with LPS after Day 3 with or without aspirin (Asp), dexamethasone (Dex), 1α, 25-dihydroxyvitamin D3 (VD3), butyric acid (But), mycophenolic acid (MPA) or rapamycin (Rapa) and the culture medium assayed for IL-6, IL-8 and TNFα at the end of the culture period. Cytokine production from drug-modified dendritic cells was compared to the positive control (LPS only) and statistical significance was assessed by paired *t*-test after logarithmic conversion.

*$0.01<P<0.05$; †$0.001<P<0.01$; ‡$P<0.0001$.

Most notably, aspirin-modified DCs did not consistently show a diminution in expression of CD86 or HLA-DR. Aspirin-modified DCs were also significantly smaller in size than the other DCs investigated (mean diameter: 11.9 μm *vs.* 14.6 μm; $P=0.0003$).

Cytokine production by drug-modified dendritic cells

Tolerance induction by DCs may depend on their cytokine milieux, and differential cytokine production might be an indication of differing function(s). Immature dendritic cells (after three or six days of culture) produced readily detectable levels of IL-8 in the culture medium. After stimulation with LPS, IL-8 was substantially increased and comparable amounts of IL-6 and TNFα were also produced. Other cytokines detected (i.e., IL-1β, IL-5, IL-10, IL-12) were present in trace amounts (<100 pg/mL). This confirmed observations previously reported in an earlier study.[23]

For the three cytokines that could be reliably detected, comparisons for the various drug-modified dendritic cell preparations were carried out. In preliminary determinations, the most striking relationship was a consistent and statistically significant elevation in IL-6 in cultures of VD3-treated cells. Less surprisingly, decreased production of TNFα was noted from aspirin-, dexamethasone- and VD3-treated cells.

These relationships were confirmed in a further series of 10 determinations (Table 3). Mycophenolic acid-treated cells were also associated with reduced TNFα production. No relationship was apparent with TNFα production in rapamycin-treated cells. All drug-modified DCs produced high amounts of IL-8.

Mixed leucocyte cultures

Drug-modified DCs were compared for their ability to stimulate the proliferation of allogeneic lymphocytes by measuring IFNγ secretion in co-cultures with allogeneic mononuclear cells. Eight allogeneic combinations were evaluated. Mean and median IFNγ concentrations were lower in association with aspirin-, dexamethasone- and VD3-modified DCs, but considerable variability was evident with different allogeneic combinations. The most noteable findings were marked decreases in IFNγ concentrations evident with aspirin (5/8) and VD3 (4/8). Dexamethasone- and butyric acid-modifications were sometimes associated with sharp decreases in IFNγ (3/8). Mycophenolic acid- and rapamycin-modified DCs yielded distribution of values similar to that obtained with the control co-cultures (with LPS-stimulated but drug-unmodified DCs) and the median IFNγ production was higher than that of their controls (Fig. 2).

On three occasions, the generation of $CD4^+CD25^+Foxp3^+$ cells was investigated. Only 4–7% of the allogeneic $CD4^+$ cells co-expressed CD25 and Foxp3 after exposure to unmodified (control) DCs. The proportion of these triple-positive lymphocytes was not significantly increased using any of the drug-modified DC preparations.

A further series of DC-MNC co-cultures was established to investigate the influence of drug-modified DCs on cellular proliferation and cytotoxicity (cell-mediated lympholysis). In all seven experiments, mycophenolic acid- and rapamycin-treated DCs behaved no differently from the positive (drug-free) controls. In five of the allogeneic combinations, however, aspirin-, dexamethasone-, VD3- and butyric acid-treated DCs all exerted some inhibition of proliferation and/or cytotoxicity. Collectively, this effect was not statistically significant. The relative degree of suppression varied according to allogeneic combination. There was never complete suppression for any modified DC in any allogeneic combination. An example is given in Figure 3. The proliferation and cytotoxicity values always correlated (r: 0.3–0.9), reaching statistical significance on three occasions.

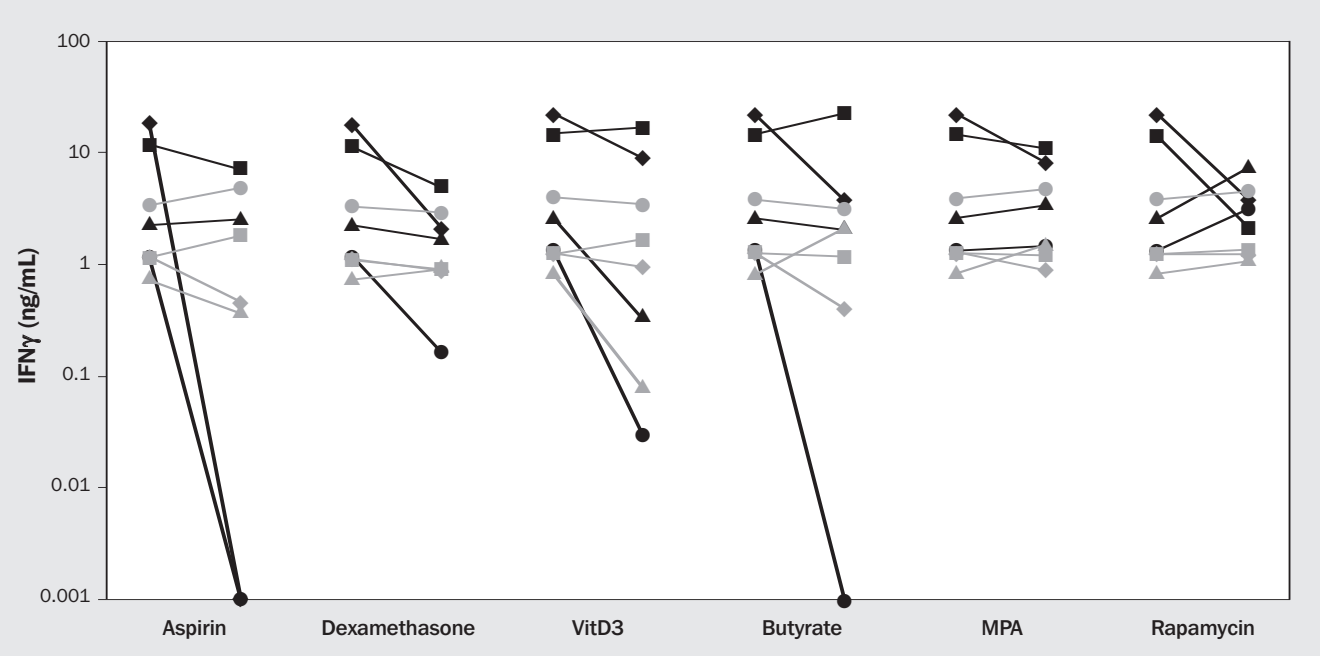

Fig. 2. Stimulation of allogeneic lymphocytes by drug-modified dendritic cells. Dendritic cells modified by the drugs indicated were co-cultured with allogeneic mononuclear cells, and interferon-γ secretion was measured after five days. Eight allogeneic pairs (separate symbols) were compared with the corresponding LPS-stimulated but drug-unmodified DC control cultures (left side).

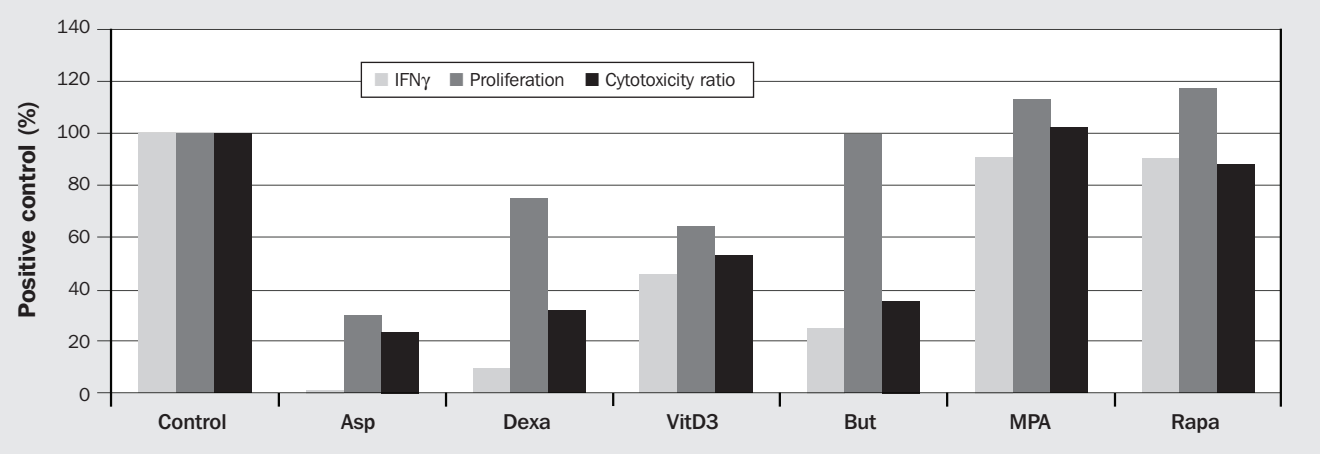

Fig. 3. Suppression of lymphocyte function by modified dendritic cells. LPS-stimulated but otherwise untreated DCs (control) and those modified with aspirin (Asp), dexamethasone (Dexa), 1α, 25-dihydroxyvitamin D3 (Vit D3), butyric acid (But), mycophenolic acid (MPA) or rapamycin (Rapa) were compared for their ability to stimulate allogeneic mononuclear cells. This is a single example from seven experiments featuring separate allogeneic combinations. The separate outputs of interferon-γ production, cellular proliferation and cytotoxicity have been normalised as a proportion of the positive control values for each.

The supernatants from these co-cultures were also assayed for IFNγ. The data obtained were broadly similar to those previously described (Fig. 3). The IFNγ values correlated strongly with the corresponding proliferation values, as expected (*r*: 0.63–0.9), and also correlated with the corresponding cytotoxicity values (*r*: 0.5–0.99).

Discussion

Dendritic cells have great potential as therapeutic agents. Tolerogenic DCs can be induced *in vitro*, but as yet no clinical trial has demonstrated efficacy of tolerance induction in humans *in vivo*.[5,6] Pharmacological modification of cells by a licensed drug has at least two strong potential advantages: i) the pharmacological agents used can be washed out before administration of the DCs, and ii) such tolerogenic cells are potentially incapable of promoting an unwanted pro-inflammatory response *in vivo*, unlike natural semi-mature DCs that could become activated and redifferentiate undesirably after administration.[24]

Typical or conventional DC preparation protocols require six days to obtain immature DCs, but the authors[19] and others[25,26] have shown that a three-day culture period provides cells that are phenotypically and functionally equivalent. Similarly, the conditions described in the literature for the preparation of drug-modified DCs have been very variable. This is the first time a comparison of six drugs under identical conditions has been made, and the authors have exposed the cells to LPS before drug application. As expected, those LPS-exposed cells expressed CCR7 and usually IDO, and therefore have the potential to induce tolerance *in vivo* as well as *in vitro*.

Under the conditions chosen, mycophenolic acid- and rapamycin-exposed DCs had little if any influence on surface antigen expression, cytokine production or functional activity towards lymphocytes. In contrast, aspirin-, dexamethasone-, VD3- and butyric acid-exposed DCs were to some extent similar in being associated with a decrease in key surface antigens and/or pro-inflammatory cytokines, despite substantial inter-individual variation. Some suppression of lymphocyte function was also associated with this group of modified DCs.

With the exception of mycophenolate and rapamycin, all the other drugs tested produced DCs with a quasi-mature phenotype when prepared under the conditions described. It is important to stress that a reduction in co-stimulatory molecules is not abolition, and a substantial decrease for abundant molecules such as CD86 and HLA-DR leaves a relatively high level of expression (Table 2). This may be important as CD80 and CD86 are essential to promote tolerance in murine bone marrow transplantation,[27] and mice exposed to LPS had increased levels of CD80 and CD86 on splenic DCs in association with tolerance induction.[28] Similarly, it may be pertinent that when activated B lymphocytes were compared with DCs for regulatory T-cell generation, the former were more potent inducers and also had higher levels of HLA-DR and CD80.[29] Indeed, generation of alloantigen-specific regulatory T cells depended partly on HLA-DR and CD80/86 expression.[29] There is some uncertainty in the literature about the surface phenotype of tolerogenic DCs, but a $CD1a^{low}$, $CD40^{low}$, $CD83^{low}$, $CD80^{high}$, $CD86^{high}$, $HLA\text{-}DR^{high}$ combination is entirely compatible with an anti-inflammatory function.[24]

Cytokine production is sometimes taken as a surrogate marker of DC maturation and function. The authors' LPS-stimulated DCs produced very little IL-12 or IL-10. This may be related to the use of a low concentration of human serum to supplement the culture medium, as both Pedersen *et al.*[30] and Burdek *el al.*[26] also reported barely detectable IL-12 production with similar concentrations of human serum or plasma. Lipids in human serum/plasma down-regulate both CD1a expression and IL-12 production,[31] possibly because CD1a-negative DCs do not respond to stimulation with IL-12 production.[32]

A new observation reported here for the first time was an increase in IL-6 production by an order of magnitude in DCs after VD3 exposure (Fig. 2, Table 3). Interestingly, there is now a growing body of literature implicating IL-6 as a potent anti-inflammatory and tolerogenic influence for both human and murine DCs.[33–38]

The authors' DCs have the same combination of surface antigen and cytokine secretion characteristics (CD80/CD86/HLA-DR^{high}; CD40^{low}; IL-6 secretion increased) as the functionally tolerogenic human DC preparations described by Torres-Aguilar *et al*.[39] However, the authors' VD3-negative DC preparations were functionally inhibitory in only about half the allogeneic combinations tested, and the effect was incomplete. This may be an indication that pharmacologically-modified DCs may be able to overcome some, but not all, histocompatibility barriers.

An insight provided by this study is that, when the influences on surface antigen expression and cytokine production are considered together, each drug investigated had a unique effect on DC maturation. This could be interpreted as an indication that different mechanisms or molecular pathways are being influenced by each drug. This raises the possibility that simultaneous treatment of immature DCs with two or more drugs might be more likely to yield a DC preparation that is more widely effective. Support for this concept is given by the recent report of synergy between mitomycin C and anti-CD154 monoclonal antibodies in preventing cardiac allograft rejection in mice.[40]

In conclusion, this study has shown that certain drugs can influence the properties of human DCs, even after prior stimulation with, and in the presence of, LPS. Most of the agents tested influenced DCs towards a less mature phenotype, but the combined pattern of surface antigen change and cytokine secretion was unique to each drug used. However, no single agent consistently generated DCs effective at suppressing the stimulation of allogeneic mononuclear cells, and is therefore unlikely to be effective alone *in vivo*. However, this work might be a helpful first step towards development of a new therapy, and the testing of drug combinations is suggested as the logical next step in this approach. □

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