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Pimecrolimus does not affect the differentiation, maturation and function of human monocyte-derived dendritic cells, in contrast to corticosteroids

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SUMMARY

Clinically, corticosteroids (CS) are among the first line drugs in the therapy of autoimmune and allergic diseases and potently inhibit the activation of immune cells. However, due to their pleiotropic mode of action, the prolonged use of CS is generally associated with a range of undesirable side-effects. In this study, we compared the activity of pimecrolimus, a novel immunomodulatory drug for the treatment of inflammatory skin disorders, and the CS dexamethasone (Dex) and beta-methasone-valerate (β-MSV) in different *in vitro* assays addressing the cytokine-induced differentiation and maturation of monocyte-derived dendritic cells (M-DC), the susceptibility of M-DC to drug-induced apoptosis and the potency of differentiated M-DC to induce primary T cell activation. In contrast to pimecrolimus, Dex and β-MSV strongly induced apoptosis of M-DC precursors if added at the start of the DC differentiation culture. Flow cytometric analysis of surviving cells on day 6 of culture showed that the expression of several DC-specific antigens such as CD1a, CD40 and CD80 was inhibited by 50% to 80% at concentrations between 1 nM and 10 nM of either Dex or β-MSV. Furthermore, the presence of CS during the final maturation of M-DC inhibited the synthesis of IL-12p70, the expression of critical DC costimulatory molecules, such as CD83 and CD86 and impaired their ability to activate primary $CD4^+$ T cell proliferation. In contrast, pimecrolimus did not inhibit the LPS-induced secretion of IL-12, surface expression of costimulatory molecules or the maturation of M-DC into potent stimulators of T cells. Taken together, these data indicate that pimecrolimus does not interfere with the differentiation and viability of dendritic cells and their precursors or with the function of mature M-DC to prime naïve T lymphocytes, and thus may have a lower potential than CS to interfere with DC-mediated immunosurveillance.

Keywords T cells dendritic cells apoptosis cytokines allogeneic MLC

INTRODUCTION

For more than four decades, corticosteroids (CS) have been the mainstay in the treatment of allergic rhinitis and asthma, as well as for inflammatory skin diseases, such as atopic dermatitis [1–3]. It is well known, however, that CS treatment can be associated with a range of undesirable side-effects, which limit their use in milder forms of the disease or in the therapy of infants and adolescents. The medical need to improve the therapeutic profile of CS has fostered intense research into the mechanism of their action. Intracellularly, CS bind to the cytoplasmic glucocorticoid receptor (GR), a member of the steroid hormone receptor superfamily, which translocates to the nucleus to function as a ligand-activated transcription factor [4]. Previous data suggested that the inhibition of a variety of immunoregulatory genes by CS may be caused by direct transcriptional induction and cytoplasmic expression of IκBα [5,6] which would prevent nuclear translocation of NF-κB dimers and immune gene activation. However, CS were also shown to repress NF-κB-driven genes without IκBα involvement by directly interfering with the interaction of NF-κB subunits with the basal transcription machinery [7]. In line with this mechanism, various publications demonstrated a direct negative interference of nuclear GR-CS complex monomers with components of other transcription factors, particularly AP-1 (reviewed in 8). Given that the induction of gene expression is known to involve AP-1 and NF-κB in many different immune and nonimmune cell types, a pleiotropic mode of action of CS appears almost inevitable.

Previously, the therapeutic effects of CS as immunosuppressive drugs have been thought to be primarily due to their action on T cells and on monocytes/macrophages. It was reported that CS inhibited the signal transduction by the IL-2 receptor [9] and the synthesis of inflammation-associated cytokines such as IL-2 and IFNγ in T cells [10] and IL-1, IL-6, IL-12 and TNFα in monocytes and macrophages [11–14].

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The crucial role of dendritic cells (DC) to initiate specific immune responses by priming of naïve T cells made them the focus of recent work that addressed the effects of CS on their differentiation, maturation and function. Whereas generally, these studies showed inhibition of DC at different levels, there is still no coherent picture about which of the diverse aspects of DC biology are primarily affected by CS in a concentration-dependent fashion. Whereas several reports showed inhibition of DC surface antigens, such as CD1a, CD40, CD80 and HLA-DR [15,16], that was associated with a decreased functional capacity to stimulate primary or recall T cell responses [17–19], however, other investigators failed to confirm these findings [20,21]. Furthermore, it was demonstrated recently that CS induced apoptosis of human monocytes *in vitro*, but the relevance of this finding in the context of DC differentiation was not addressed [22]. With respect to Langerhans' cells (LC) as a *bona fide* dendritic cell type, topical application of CS to guinea pig skin was shown to cause a rapid and marked decrease in the number of LC in the epidermis [23]. Recently, these results were corroborated and extended to show that CS, including the low-potency steroid hydrocortisone, mediated the inhibition of MHC class II expression and caused a long-lasting depletion of LC after epicutaneous application to murine skin *in vivo* [24]. Whether this CS-mediated reduction of epidermal LC was due to migration or cell death *in situ* is still an unresolved issue. Finally, tacrolimus, a calcineurin inhibitor used clinically to prevent transplant rejection and, more recently, used as topical immunomodulator for the treatment of allergic eczema, was shown to inhibit the maturation of epidermal LC into T cell stimulatory cells even more potently than CS [25].

Pimecrolimus, the youngest member of the triad of calcineurin inhibitors, was specifically developed for the treatment of inflammatory skin diseases [26]. Extensive clinical studies with pimecrolimus 1% crème (Elidel®) demonstrated high efficacy and safety in adults, children and infants [27–32]. Furthermore, oral pimecrolimus was shown be highly affective in psoriasis patients, without evidence of side-effects including kidney functions, local and systemic immune responses [33]. Pimecrolimus was shown to inhibit the cytokine synthesis and proliferation of T cells and the secretion of pro-inflammatory mediators from human mast cells and basophils after specific activation via the T cell receptor or the high affinity receptor for IgE, respectively [34,35]. A selective mode of action of pimecrolimus was suggested by the absence of inhibitory activity on the proliferation or cytokine production by cell lines of endothelial, keratinocyte, fibroblast, and melanocyte origin [27] and by its lack of epidermal LC-depleting activity when administered to murine epidermis [24], in sharp contrast to CS. However, its potential to modulate the differentiation or activation of dendritic cells has not been investigated so far. The present study was carried out to compare the activity profile of pimecrolimus with that of CS regarding the inhibition of human monocyte-derived DC differentiation, terminal maturation and the modulation of their functions.

MATERIALS AND METHODS

Cell preparation and culture

Mononuclear cells (MNC) were isolated from leukapheresis samples of healthy human volunteers by density gradient centrifugation over Lymphoprep® (Nycomed Pharma, Oslo, Norway). Monocytes or primary $CD4^+$ T cells or Pan T cells were purified from MNC aliquots by a negative immunomagnetic separation technique using the appropriate kits (Miltenyi Biotec, Bergisch Gladbach, Germany) allowing unmodified isolation of the target cells. Magnetically labelled and unlabelled cells were separated on the Auto-MACS machine (Miltenyi Biotec) using the DEPLETE program. The purity of the eluted monocytes and T cells was greater than 95% as verfied by flow cytometric analysis using fluorochrome-conjugated monoclonal antibodies specific for the relevant lineage markers. The culture medium (CM) was RPMI1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 50 μg/ml each of penicillin and streptomycin (all purchased from Gibco/Invitrogen Life Technologies, Paisley, UK).

Differentiation of dendritic cells

Purified monocytes were seeded into Costar® 6-well plates at a cell density of 7×10^5 cells/ml and their differentiation into dendritic cells was induced by adding recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF, 300 U/ml) and recombinant human interleukin-4 (IL-4, 200 U/ml) both from Novartis (CTA, Biomolecule Production, Novartis Pharma AG, Basel, Switzerland). The test compounds pimecrolimus (SDZ ASM 981, Novartis Pharma AG), dexamethasone or beta-methasone-valerate (Dex or β-MSV, both from Sigma-Aldrich, St. Louis, MO, USA) were added to the M-DC cultures at final concentrations ranging from 10^{-9}M and 10^{-6}M as indicated in the description of the each experiment. The cultures were fed on day 3 by exchanging half of the medium per well for fresh CM supplemented with GM-CSF and IL-4 at the concentrations used when initiating the cultures. Where appropriate, the CM was also supplemented with the two-fold final concentration of compound to maintain consistent culture conditions throughout the experiment.

Activation of dendritic cells

Following a six-day culture, aliquots of 1×10^6 DC were stimulated by 100 ng/ml bacterial lipopolysaccharide (LPS, Sigma-Aldrich) in Costar® 12-well plates in the absence or presence of test compounds. Each of the compounds (Dex, β-MSV or pimecrolimus) were added to a final concentration of 1 nM, 10 nM and 100 nM. After 2 days, the activated DC were analysed for expression of CD83 and CD86 by flow cytometry as described below. IL-12p70 synthesis was determined after activation of M-DC with LPS (100 ng/ml) used in combination with IFNγ (100 U/ml, Roche Diagnostics, Indianapolis, IN, USA) and culture supernatants were harvested and analysed after 8 h by ELISA specific for the heterodimeric form of IL-12 (R & D Systems Minneapolis, MN, USA or Bender Medsystems, Vienna, Austria).

Primary allogeneic MLC

As responders in one-way MLC, 1×10^5 $CD3^+$ or purified $CD4^+$ T cells were added to $2{\cdot}5 \times 10^3$ allogeneic M-DC in 100 μl medium per well of 96-well round-bottom microtiter plates (Costar®, Corning Inc. NY, USA). Subsequently, 100 μl of culture medium containing appropriate dilutions of compounds (pimecrolimus, Dex or β-MSV) covering a range from 10^{-10} M to 10^{-6} M were added to each well to determine the inhibition of M-DC stimulated T cell proliferation. When using mature M-DC that were treated with LPS in the presence of compounds (see above), a potential carry-over into the MLC was minimized by transfer of washed, LPS-activated M-DC into fresh CM and subsequent incubation for at least 16 h at 37°C in polypropylene tubes before they were used as stimulator cells. LPS-activated, com-

pound-treated DC aliquots were seeded into 96-well round-bottom microtiter plates starting with the amount of $1{\cdot}25 \times 10^3$ cells/100 μl CM to give a DC/T cell ratio of 1/80, followed by serial two-fold dilution of the DC suspension to give ratios of 1/160, 1/320, and 1/640. All samples were assayed in quadruplicate. T cell proliferation was measured by adding 1 μCi/well of tritiated thymidine (specific activity 3000 mCi/mMol, Amersham, UK) during the last 16 h of a 5-day culture period. The results are expressed as the mean cpm ± SD of quadruplicate wells. $CD4^+$ T cells and M-DC were also seeded separately in order to control for background proliferation.

Flow cytometry

Cells were stained on ice with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated murine monoclonal antibodies for 30 min in phosphate-buffered saline supplemented with 2% FCS and 0·1% sodium azide. After extensive washing, 7-amino-actinomycin D (7-AAD, Sigma-Aldrich) was added to each sample at a final concentration of 4 μg/ml prior to FACScan™ measurements thus enabling the exclusion of dead cells during analysis of specific fluorescence. Appropriate evaluation of specific cell staining was achieved by compensation of nonspecific fluorescence in each channel. The following monoclonal antibodies (BD Biosciences/BD PharMingen, San José, CA) were used for surface marker analysis of monocytes or immature and mature DC: anti-CD40-FITC (5C3); anti-CD80-PE (L307·1); anti-CD83-PE (HB15e); anti-CD86-FITC or -PE (FUN-1); anti-CD14-FITC (FMC-32, Chemicon, Temecula, CA, USA) or anti-CD1a-PE (T6-RD1, Coulter Corp., Miami, FL, USA). Surface antigen expression profiles of DC were measured by flow cytometry in a FACScan (Becton Dickinson, Mountian View, CA, USA) and the data analysed using Windows® Multiple Document Interface (WinMDI) flow cytometry applications software.

Apoptosis detection

Cell death by apoptosis was determined by staining with Annexin V-PE and the vital dye 7-amino-actinomycin D (7-AAD) as outlined in the instructions of the Annexin V-PE apoptosis detection kit (BD PharMingen). Briefly, cells were washed in cold PBS and resuspended in binding buffer (10 mM Hepes/NaOH, pH 7·4, 140 mM NaCl, 2·5 mM $CaCl_2$). 1×10^5 cells in 100 μl were mixed with 5 μl of Annexin V-PE or 5 μl 7-AAD or both reagents and incubated for 15 min at 25°C in the dark. 400 μl binding buffer was then added to each sample and cells were immediately analysed by flow cytometry. In addition, apoptosis was analysed by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) method using the APO-BRDU™ Kit (BD PharMingen) as described by the manufacturer. This assay system employs propidium-iodide to stain the whole genomic DNA content of the permeabilized cells together with a FITC-labelled antibromo-deoxyuridine (BrdU)-specific monoclonal antibody reacting with the BrdU-modified 3′-OH DNA ends generated by enzymatic activity of terminal deoxynucleotidyl transferase (TdT).

RESULTS

CS and Pimecrolimus show similar potency to inhibit the primary MLC

Although CS are generally known as immunosuppressive agents, the actual potency with which CS inhibit T cell stimulation via dendritic cells has not been determined so far. Therefore, the CS Dex and β-MSV were compared to each other and to pimecrolimus in the primary MLC using purified $CD4^+$ T cells and M-DC as responders and stimulators, respectively. As shown in Fig. 1, the both Dex and β-MSV inhibited the MLC with IC_{50} values in the low nanomolar range (4·7 nM and 6·4 nM, respectively). Pimecrolimus showed an IC_{50} value of 1·6 nM, indicating that all three

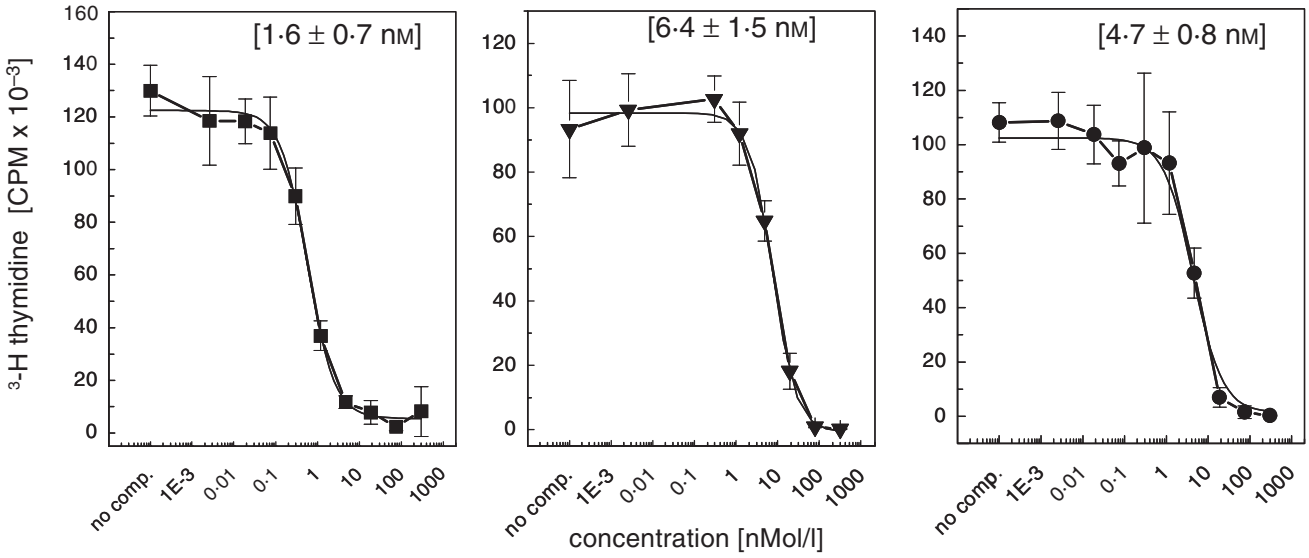

Fig. 1. Potencies of pimecrolimus, β-MSV and Dex to inhibit the human allogeneic MLC. M-DC were generated by GM-CSF and IL-4 in the absence of compounds and used on day 6 at a cell ratio of 1/40 as stimulators of allogeneic T cells. Inhibition of T cell proliferation by pimecrolimus (■), β-MSV (▼), or Dex (●) was determined by addition of [^{3}H]-thymidine during the last 16 h of the 5-day MLC. IC_{50} values were determined with the Origin® software by sigmoidal curve fitting of the plotted cpm (mean of quadruplicate wells) and are indicated within each graph as the average IC_{50} (± s.d.) obtained in five independently performed experiments using different donors.

compounds inhibited DC-mediated T cell growth stimulation with similar potency.

CS mediate apoptosis of early monocytic DC precursors in vitro

It was reported previously that CS induced profound apoptosis of human monocytes in a dose-dependent fashion, which was prevented by the addition of an antagonist of GR or by IL-1β [22]. The activity of CS or of calcineurin inhibitors to induce apoptosis of monocytes in cultures supplemented with GM-CSF plus IL-4 and the kinetics of cell death during DC differentiation *in vitro* has not been studied so far. Immediately after initiating the differentiation of monocytes to DC in the presence of GM-CSF and IL-4, pimecrolimus, β-MSV or Dex were added at different concentrations (3, 10, 30 and 100 nM) to the cultures. Cell samples were taken before and 24 h, 48 h, 72 h and 6 days after the start of DC differentiation. Apoptosis was analysed by cell staining with annexin-V in combination with the DNA intercalating dye 7-AAD followed by flow cytometry. This method is useful to quantify the number of apoptotic cells that have already proceeded to intermediate and late stages of apoptosis and thus bear 7-AAD permeable nuclear membranes (Annexin V and 7-AAD-positive cells). Figure 2a reveals that the proportion of apoptotic/dead cells increased from 25% to 35% on day 1 to about 50% to 55% on days 2 and 3 if the cells were cultured in the presence of 10 nM or higher concentrations of β-MSV. Because the bulk of apoptosis occurred during the first 3 days of culture, the yield of viable cells on day 6 decreased to about 10–15% of the number that was seeded at the start of the DC differentiation. Comparable results for cell death kinetics were obtained using the TUNEL assay that identifies cells bearing fragmented chromosomal DNA (Fig. 2b). In the case of Dex or β-MSV, the percentage of TUNEL-positive cells increased from 8% at the start of the culture to about 25%, 35%, and 27% on days 1, 2, and 3, respectively. Both assays revealed that the level of apoptosis obtained in the presence of pimecrolimus was practically indistinguishable from that found in control cultures. The observation that CS-induced apoptosis peaked on days 2 and 3 of culture and decreased to reach control levels on day 6 indicated that monocytes and early monocytic DC precursors are the main targets of the pro-apoptotic action of CS.

DEX and β*MSV inhibit the differentiation of DC from monocytes* in vitro

M-DC that were cultured for 6 days of in the presence of GM-CSF and IL-4 showed an immature phenotype characterized by the expression of CD1a, CD40 and CD80 and the absence of surface CD14 (Fig. 3, control panel). CS dose-dependently inhibited the acquisition of these DC antigens with high potency (Table 1) because the emergence of the characteristic $CD1a^{+}/CD14^{neg}$ and $CD40^{+}/CD80^{+}$ expression profile was almost completely prevented if M-DC were differentiated in the presence of 10 nM of either β-MSV or Dex (Fig. 3). In clear contrast to CS, pimecrolimus did not inhibit CD1a, CD40 or CD80 expression and the $CD1a^{+}/CD14^{neg}$ profile was only marginally affected by pimecrolimus at a high concentration, such as 1 μM which was used in the experiment shown in Fig. 3. The data shown in Table 1 indicate that > 50% reduction of CD1a and CD80 expression is obtained at 1 nM of β-MSV or Dex, and greater than 80% inhibition of all markers is seen at 10 nM.

Inhibition of CD83 and CD86 induction and IL-12 secretion by CS but not pimecrolimus

Activation of immature DC by bacterial antigens, such as LPS, is known to induce the final maturation of DC, which is associated with a strong induction of CD83 and CD86. Both molecules play a role as important ligands for costimulation of T cells [36,37]. Figure 4a shows that the presence of Dex or β-MSV during LPS-mediated DC stimulation dose-dependently inhibited the induction of CD83 and CD86 whereas the same concentrations of pimecrolimus were without effect. The data presented in Table 2 indicate that about 50% inhibition of CD83 and CD86 up-regulation was obtained when using about 10 nM of each CS, and that the surface levels of CD83 or CD86 were decreased by 80% or 70%, respectively, at a concentration of 100 nM. Because pimecrolimus did not interfere with final DC maturation on the basis of surface marker expression, we asked whether it could block cytokine production in activated M-DC. We particularly focused on IL-12p70 as a DC-specific cytokine, because it is the relevant heterodimeric molecule for the differentiation of Th1 cells. As shown in Fig. 4b, pimecrolimus did not reduce IL-12 synthesis whereas β-MSV dose-dependently inhibited the secretion of IL-12p70.

Final maturation of DC in the presence of β*-MSV impaired function of DC to stimulate T cells in primary MLC*

DC were induced to mature by LPS activation for two days in the absence or presence of β-MSV or pimecrolimus. Subsequently, DC were washed and incubated overnight in the absence of compounds, in order to minimize any carry-over artifacts as much as possible. Drug-treated and control DC were then used as stimulators of primary allogeneic T cells in the MLC. Figure 5 shows that DC activation in the presence of β-MSV impaired the function of M-DC to stimulate the proliferation of T cells. This is consistent with the view that the inhibition of CD83 and CD86 expression decreases the stimulatory potential of DC. In contrast,

Table 1. Dose-dependent inhibition of surface marker expression on compound-treated M-DC

	Control	Pimecrolimus [nM]				β-MSV [nM]				Dex [nM]			
		300	100	30	10	30	10	3	1	30	10	3	1
CD1a	98·0§	98·4	98·8	98·8	98·6	4·1	4·1	9·0	35·0	0·8	2·9	4·9	46·6
CD40	94·9	97·6	96·6	99·5	95·4	8·1	18·0	27·3	69·0	13·2	12·5	18·7	62·9
CD80	46·8	41·6	41·7	38·2	32·7	1·4	1·7	4·6	11·3	0·9	1·7	1·5	18·9

§Percent of surface maker positive cells on day 6 of M-DC culture. Data obtained in one out of three independently performed experiments with similar results.

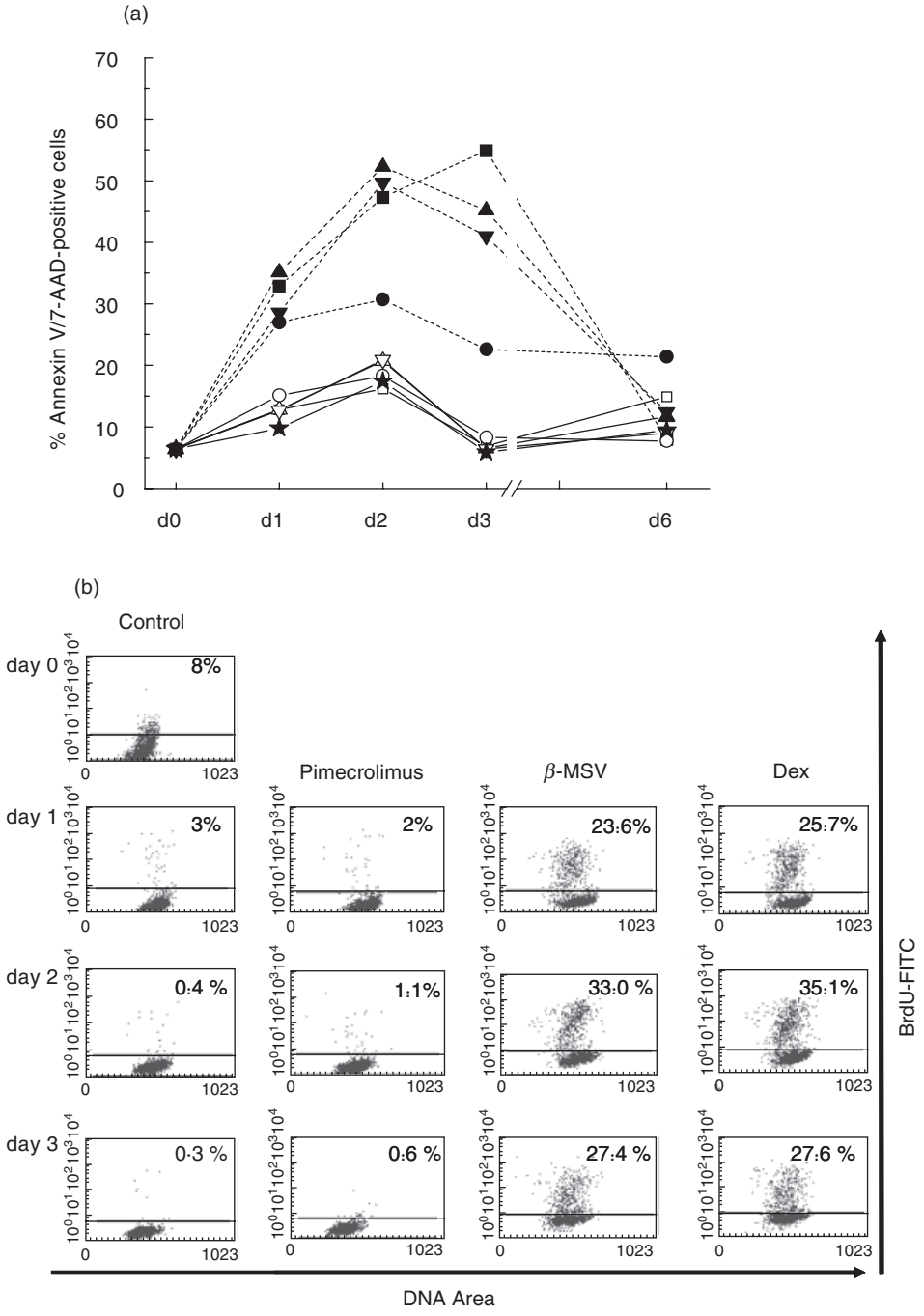

Fig. 2. CS but not pimecrolimus mediate apoptosis of monocytic DC precursor cells. Differentiation of DC from purified monocytes was initiated on day 0 by adding GM-CSF and IL-4. Cell aliquots were harvested at the indicated time points and subjected to flow cytometry following (a) staining with annexin-V plus 7-AAD or (b) following labelling by the TUNEL method. (a) The percentage of annexin-V/7-AAD-stained cells in absence of compounds (★) and in the presence of different concentrations of pimecrolimus (3 nM ○; 10 nM ▽; 30 nM △; 100 nM □) and β-MSV (3 nM ●; 10 nM ▼; 30 nM ▲; 100 nM ■) was determined at the start of each culture (day 0) on the consecutive days as indicated. (b) Apoptosis of cells cultured in the absence or presence of compounds (100 nM each) as determined by the TUNEL method.

pimecrolimus did not impair T cell stimulation by activated M-DC, in line with its failure to inhibit costimulatory antigens.

DISCUSSION

In this study, we have shown that Dex and β-MSV profoundly inhibited cytokine-induced differentiation of human monocytes to dendritic cells (DC) *in vitro*. Blockade of DC differentiation was caused by the CS-mediated induction of apoptosis of monocytic DC precursors and the prevention of surface expression of CD1a, CD40 and CD80 on the remainder of nonapoptotic cells. Furthermore, when administered during LPS-induced maturation of immature DC, CS inhibited the up-regulation of critical costimulatory molecules, the synthesis of IL-12p70 and impaired the

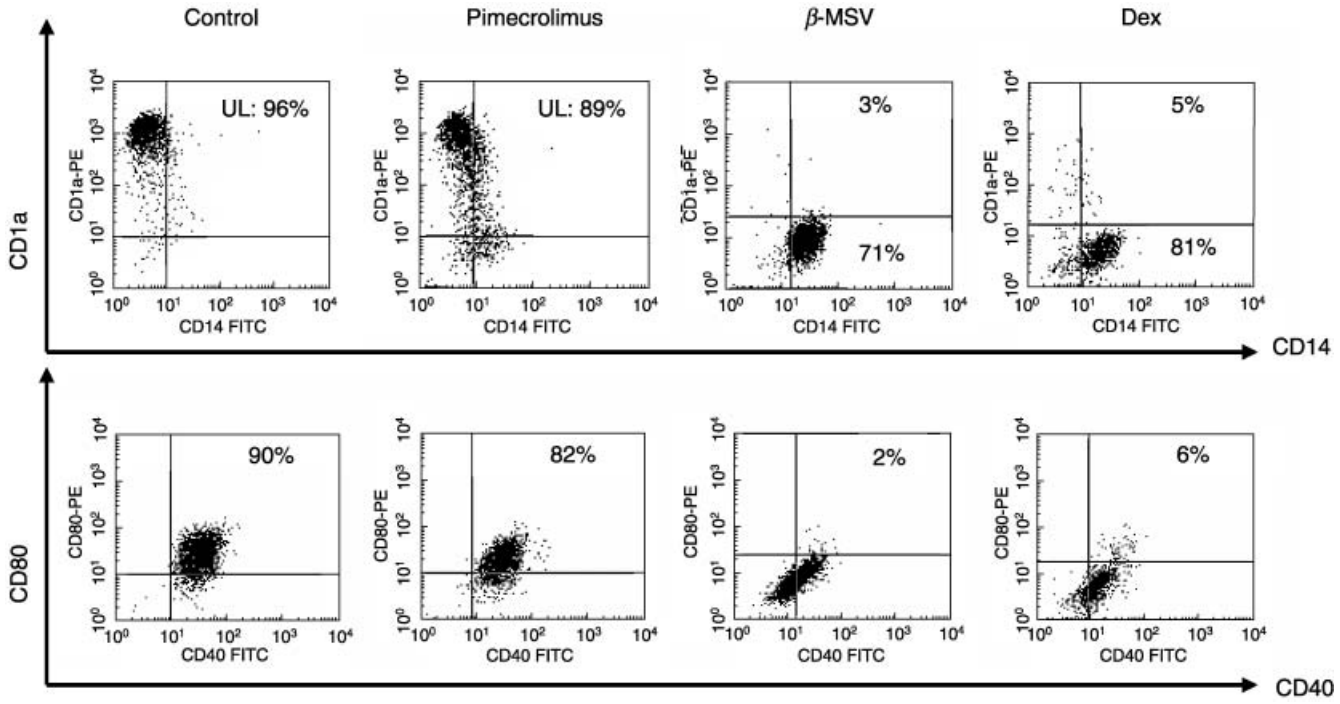

Fig. 3. CS but not pimecrolimus inhibited the differentiation of DC *in vitro*. Differentiation of DC from monocytes was induced by GM-CSF and IL-4 in the absence (control) or the presence of pimecrolimus (1 μM), β-MSV and Dex (10 nM each). After 6 days, cells were harvested, stained by fluorochrome-conjugated mAb and analysed for coexpression of CD1a/CD14 and CD40/CD80 by flow cytometry. UL: upper left.

Table 2. Inhibition of induction of costimulatory molecules on LPS-activated M-DC

	Pimecrolimus [nM]			β-MSV [nM]			Dex [nM]		
	100	10	1	100	10	1	100	10	1
CD83	12·0 (± 13·4)	8·8 (± 8·8)	9·3 (± 7·5)	79·3 (± 14·2)	60·5 (± 19·1)	16·5 (± 12·2)	85·3 (± 10·4)	65·0 (± 14·9)	26·7 (± 9·5)
CD86	1·8 (± 2·9)	3·8 (± 5·7)	2·3 (± 4·5)	71·3 (± 7·9)	56·3 (± 7·2)	20·5 (± 16·3)	68·7 (± 9·9)	44·3 (± 12·8)	6·3 (± 8·0)

Values denote percentage inhibition (mean ± SEM) obtained in four independent experiments and were calculated by using the formula (MFI, mean fluorescence intensity):

$$\frac{\text{MFI stimulated M-DC} - \text{MFI compound-treated M-DC}}{\text{MFI stimulated M-DC} - \text{MFI unstimulated M-DC}} \times 100\%$$

function DC to stimulate primary T cell proliferation in the allogeneic MLC. In clear contrast to CS, pimecrolimus did not induce apoptosis of monocytic DC precursor cells and it did not interfere with DC differentiation, maturation, IL-12 secretion or their T cell-stimulatory activity in allogeneic MLC.

Although several investigators reported that CS or and its synthetic analogs inhibited DC differentiation and/or terminal maturation [15–21], there is still disagreement about the main immunological effects of CS-mediated inhibition of DC differentiation and function. For instance, although some studies demonstrated that CS blocked the expression of typical DC differentiation antigens such as CD1a, CD40, CD80 [16,17], other investigators failed to confirm an inhibitory effect of CS on the expression of these surface antigens [21] or even observed higher levels of expression of CD40, CD80 or HLA class II antigens on CS-treated DC as compared to control cells [20]. Similarly, contradictory results were obtained with respect to the inhibition of DC activation and functional maturation. Whereas some authors showed an impaired ability for T cell growth stimulation in allogeneic MLC if DC were activated in the presence of CS [17,19], other groups did not observe a decreased T cell stimulatory capacity of M-DC that were activated to mature in the presence of Dex [21] or when exposed to a synthetic analogue of vitamin D3 [38]. The results of the current study indicate that CS indeed inhibit DC differentiation and terminal maturation resulting in an impaired ability to stimulate naïve allogeneic T cells.

DC are known to influence the differentiation of naïve T cells into effector T cells expressing a Th1 of Th2 cytokine profile. In this respect it should be considered that CS profoundly suppress the production of the biologically active IL-12p70 heterodimer as demonstrated in most studies addressing this issue [15,16,18–21]. The failure of DC to secrete IL-12 after activation

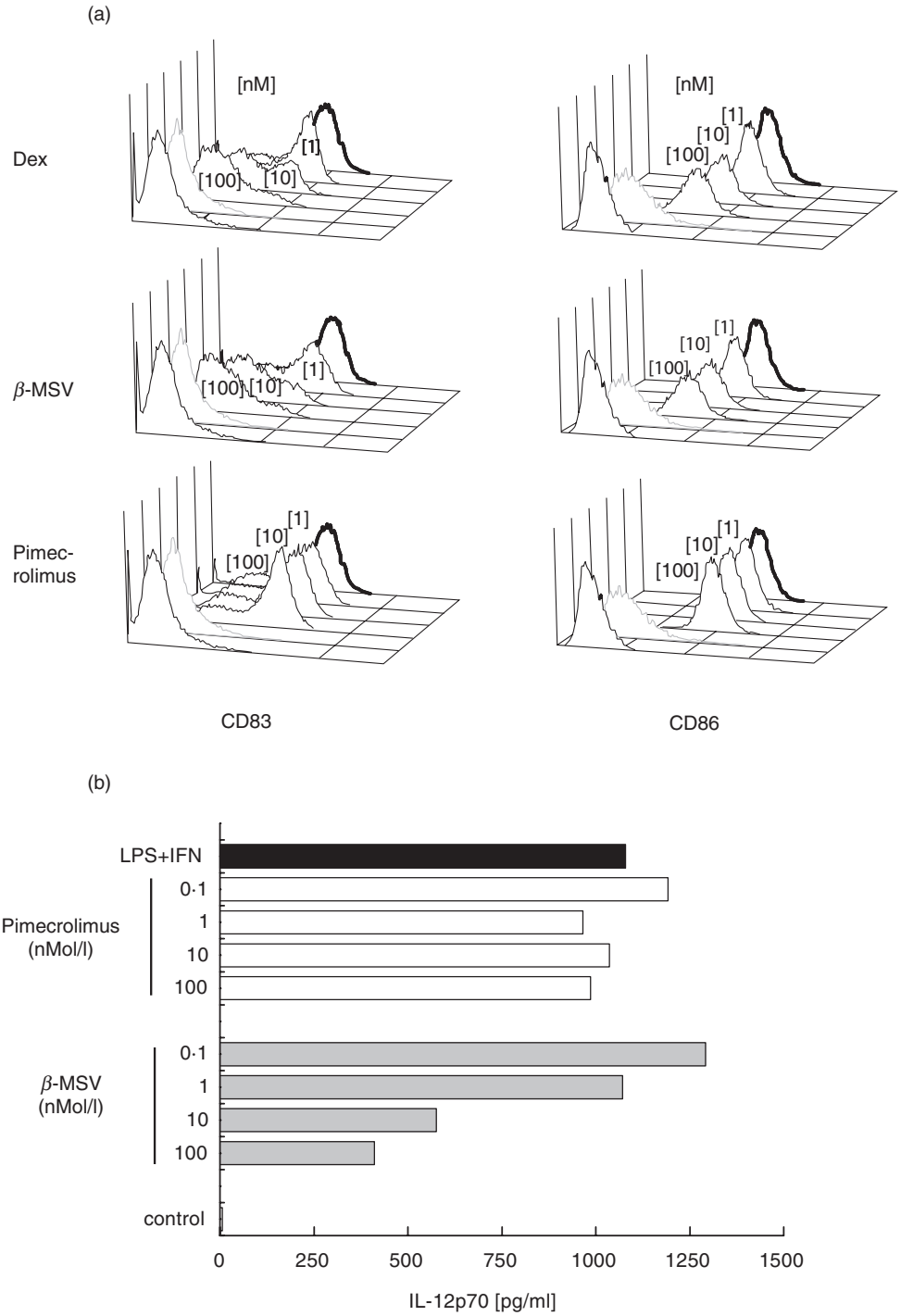

Fig. 4. Inhibition of terminal DC maturation by Dex and β-MSV, but not pimecrolimus. DC were differentiated from monocytes in the absence of compounds. (a) Immature DC obtained on day 6 were activated by LPS (100 ng/ml) in the presence of β-MSV, Dex or pimecrolimus for 48 h at the concentrations indicated next to the overlayed histograms. The relevant isotype staining control (thin line at the front) and the levels of CD83 and CD86 expression on unstimulated DC (grey lines) and DC stimulated in the absence of compounds (bold lines) is shown for comparsion. (b) Immature DC were activated by LPS (100 ng/ml) and IFNγ (100 U/ml) and culture supernatants were harvested after 16 h for the determination of IL-12 p70 levels by ELISA. Data are representative of four (a) and two (b) independently conducted experiments.

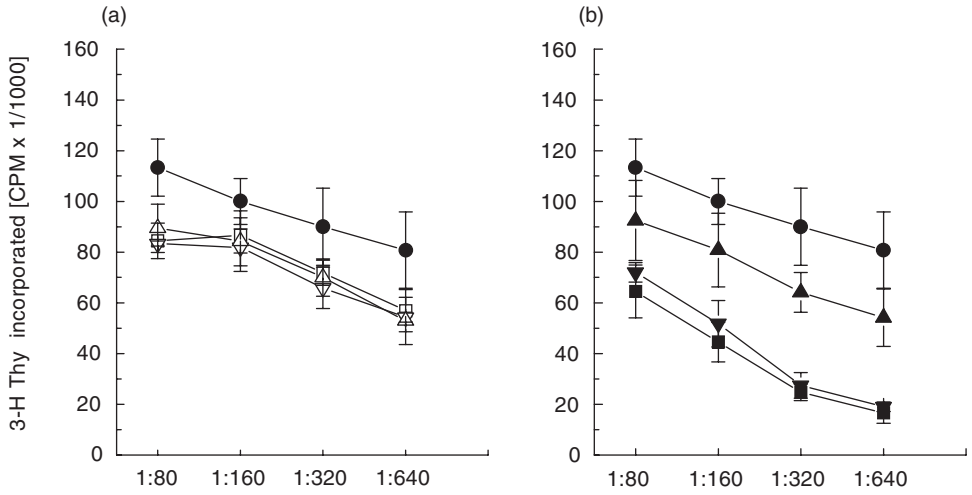

Fig. 5. β-MSV-treated DC were less potent to stimulate allogeneic T cell proliferation, as compared to pimecrolimus-treated DC. Final DC maturation was induced by LPS for 2 days in the absence (●) or (b) presence of β-MSV (▲ 1 nM; ▼ 10 nM; ■ 100 nM) or (a) pimecrolimus (△ 1 nM; ▽ 10 nM; □ 100 nM). DC were extensively washed, incubated for another 16 h in fresh culture medium before addition to the T cells at the ratios indicated on the *x*-axis. The proliferation of T cells was determined in the absence of compounds by [^{3}H]-thymidine incorporation during the final 16 h of the 5-day MLC. The data shown were obtained in one out of two independent experiments with similar results.

is known to favour the differentiation of Th2 effector cells, which produce higher levels of IL-4 and low levels of IFNγ [18,21]. In the case of autoimmune disorders, the promotion of Th2 development via inhibition of IL-12 synthesis and the impaired differentiation of IFNγ-producing Th1 cells may be benefical for disease management [38]. However, in the context of allergic diseases, a shift of the cytokine balance towards a Th2 profile caused by prolonged treatment with CS may eventually exacerbate the allergic disposition, as recently suggested [13,39]. Primary activation in the presence of Dex was shown to polarize T cells for preferential secretion of IL-4 and IL-13, even if secondary stimulation occurs in the absence of CS [40], and this may lead to enhanced production of total and allergen-specific IgE antibodies after cessation of therapy [41,42]. Apart from blocking IL-12 synthesis in DC and monocytes, CS also interferes with IL-12 receptor signalling in T cells via inhibition of Stat4 phosphorylation and reduced Stat4-dependent IFN-regulatory factor-1 promoter activity thereby hampering Th1 differentiation and IFNγ synthesis [43]. Owing to the failure to inhibit IL-12 synthesis in DC, pimecrolimus is not expected to have Th2 promoting activities.

An influence on DC differentiation and function was, however, observed for tacrolimus (FK506) which is a potent calcineurin inhibitor used in transplantation and more recently for the topical treatment of atopic eczema [44]. Tacrolimus was shown to decrease the expression of the high affinity receptor for IgE (FcεRI) on both Langerhans'cells (LC) and another DC-type, termed inflammatory dendritic epidermal cells, IDEC [25,45]. Moreover, skin-derived LC exposed to tacrolimus *ex vivo* had severely reduced T cell stimulatory ability together with a reduced expression of MHC class I and II antigens as well as costimulatory molecules, such as CD40 and CD80. Surprisingly, β-MSV was found to be less potent than tacrolimus to inhibit the LC-mediated stimulation of allogeneic T cells [25]. More recently, rapamycin, which, like pimecrolimus and FK506 also binds to macrophilin-12 (also know as FKBP-12) as its intracellular receptor, was shown to interfere with the differentiation and maturation of human M-DC. In that study, tacrolimus did not inhibit the differentiation of immature CD1a-positive M-DC, although it caused some decrease of CD40 and CD83 expression on mature M-DC [46].

Pimecrolimus and tacrolimus are similar structurally and share a common intracellular binding receptor, FKBP-12, which, in its ligand-activated form, inhibits the activity of the phosphatase calcineurin. Consequently, both compounds inhibit the nuclear translocation of NFAT, a critical transcription factor in T cells and mast cells. Whereas steroids and tacrolimus were shown to affect the function of human DC and LC, our results clearly demonstrate that pimecrolimus lacks DC-modulating activity and thus appears to be distinct from both steroids and tacrolimus in this regard. The higher selectivity of pimecrolimus appears advantageous because it would preserve the critical ability of DC and skin LC to act as antigen-presenting cells for efficient priming of naïve T cells to *de novo* antigens. In fact, evidence for this notion has recently been obtained in a murine contact hypersensitivity model by showing that systemic administration of pimecrolimus (but not tacrolimus) did not inhibit the primary sensitization of T cells for an epicutaneously applied contact allergen, whereas about equipotent suppression of the recall response was obtained for pimecrolimus and tacrolimus when given during the time frame of re-challenge with the contact allergen [47].

Taken together, our data indicate that pimecrolimus is not expected to interfere with the function of DC as critical sentinels of immune surveillance whereas it inhibits the activation of effector T cells, a key event in inflammatory skin diseases such as atopic dermatitis.

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