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Maturation of human iDCs by IL-18 plus PGE2, but not by each stimulus alone, induced migration toward CCL21 and the secretion of IL-12 and IFN-γ

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ABSTRACT

Dendritic cells (DCs) are potent antigen-presenting cells that initiate the primary immune response and whose functional properties *in vivo* depend on the maturation stimulus. We describe the functional properties of human monocyte-derived DCs after the maturation of immature DCs (iDCs) for 2 days with LPS (100 ng/ml), PGE2 (1 μg/ml), CD40L (1 μg/ml) or IL-18 (200 ng/ml) and with CD40L + PGE2 and IL-18 + PGE2 mixtures at the same concentrations as above. Neither IL-18 nor PGE2 alone stimulated IL-12 or IFN-γ secretion. When administered simultaneously to 1×10^6 iDCs/ml, IL-18 + PGE2 induced the secretion of 131.4 ± 6.7 pg IL-12/ml and 355 ± 87 pg IFN-γ/ml but there was no detectable IL-10 secretion. However, PGE2 alone stimulated the secretion of 208 ± 89 pg IL-10/ml whereas IL-18 alone did not stimulate the secretion of IL-10, IL-12, TNF-α or INF-γ. When the mixture of CD40L + PGE2 was used, only migration toward CCL19 and CCL21 was induced. CD40L did not stimulate the secretion of IL-10, IL-12, TNF-α or IFN-γ and did not stimulate migration toward CCL19 or CCL21. The extent of stimulation of T cell proliferation was essentially the same for all stimuli at the concentrations given above. New properties such as IL-12 and INF-γ secretion and migration toward CCL21 emerged when a mixture of IL-18 + PGE2 was employed. These data show that when the pairs of stimuli reported here were used simultaneously their effect was not additive. This system can be used to prepare mDCs with properties useful for cell therapy and also as a model to investigate the mechanisms of cytokine secretion and cell migration.

Introduction

Dendritic cells (DCs) initiate and modulate the immune response. In the peripheral circulation they capture and process antigens, express lymphocytic co-stimulator molecules, migrate to lymphoid organs, and secrete cytokines to initiate the immune response. The migration of DCs from the circulation to lymphoid tissue *in vivo* requires the acquisition of many specific properties such as secretion of and responses to cytokines, as well as the capture and processing of antigens *in vivo* (Steinman and Banchereau 2007). These functional properties and others are acquired during differentiation and the specific property acquired depends on the stimulus.

In vitro studies of DC differentiation and maturation have focused on the effect of the stimuli on the functional properties of DCs in order to be able to generate cells capable of migrating, secreting IL-12 and stimulating T cell proliferation, all of which are necessary for cell-targeted therapy (Dauer et al. 2008; Knippertz et al. 2009). DCs matured with a commonly used cocktail containing IL-1β + IL-6 + TNF-α + IFN-γ + PGE2; Lesterhuis et al. 2008; Song and Kim 2004) exhibited strong migratory capacity *in vitro* as well as *in vivo* and clinical trials have provided some evidence for effective immunological responses against tumors (Schuler et al. 2003; Salcedo et al. 2006;). However, clinical studies using a vaccine prepared with DCs matured with the mixture cited above have had

Abbreviations: CFSE, carboxyfluorescein-succinimidyl ester; DCs, dendritic cells; FCS, fetal calf serum; FITC, fluorescent isothiocyanate; GM-CSF, granulocye macrophage colony-stimulating factor; iDCs, immature dendritic cells; IFN-γ, interferon-gamma; Ig-γ, immunoglobulin-gamma; IL-4, interleukin-4; IL-12, interleukin-12; IL-18, interleukin-18; LPS, lipopolysaccharide; mDC, mature dendritic cells; MoDCs, human monocytes; NK, natural killer; PE, phycoerythrin; PGE2, prostaglandin-2; PHA, phytohemagglutinin; TGF-β, tumor growth factor-beta; TNF-α, tumor necrosis factor alpha.

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only limited clinical success (Lesterhuis et al. 2008; Song and Kim 2004). Kim et al. (2010) have reported that the use of the maturation cocktail described above followed by treatment with poly(I:C) enhanced the migratory and T cell stimulating capacity of DCs. In order to meet the requirements of the clinical applications of tumor immunotherapy, DCs should be strong IL-12 producers, have high migratory activity, and have T-cell stimulating ability as well as a stable phenotype amongst other properties.

The origin of the present study was the observation that DCs matured by various cytokines present different proteins that are differentially increased or decreased, as indicated by 2DE and mass spectrometry (Pereira et al. 2005). Our initial objective was to examine the multiple properties of mature DCs stimulated by cytokines used for maturation, IL-18 (Balkow et al. 2009), CD40L (Knippertz et al. 2009) and PGE2 (Lee et al. 2002). We observed that when pairs of stimuli were administered simultaneously the effect was not additive. We showed that DCs matured with IL-18 + PGE2 secreted significant amounts of IL-12 and IFN-γ, induced T cell proliferation and presented only modest amounts of migration to CCL19, whereas IL-18 or PGE2 alone did not produce any of these properties except for cell proliferation. Thus, compared to the result obtained of DCs can emerge or disappear when two stimuli are used simultaneously. DCs which secrete reasonable levels of IL-12 and IFN-γ should be useful for DC-targeted cell therapy. The emergence of new properties upon stimulation with cytokine mixtures is an interesting biological property, which may be useful in studies of the mechanism of control of cytokine secretion by mDCs and in the preparation of DC-targeted vaccines.

Materials and methods

Differentiation and maturation of DCs

DCs were prepared from human monocytes (MO-DCs) present in leukocyte concentrates obtained from healthy donors and provided by the Regional Hemotherapy Center of Ribeirão Preto. Monocytes were isolated with two Ficoll-Paque Plus gradients (GE Healthcare, Uppsala, Sweden) used successively as described by Lehner and Holter (2002) with the following two modifications: (1) whole blood was stored in a refrigerator at 4–6 °C for 1–3 h before and after leukocyte separation, and (2) after the second Ficoll gradient separation, cells were washed twice with PBS and centrifuged at 200 × *g* for 5 min rather than being submitted to one wash followed by centrifugation at 500 × *g* for 10 min. Monocytes were characterized by flow cytometry using monoclonal antibodies to CD45 and CD14 (Becton Dickinson, San Jose, CA, USA) in a FACSort cytometer (Becton Dickinson). Monocytes (1×10^6 cells/ml) were differentiated into iDCs in RPMI medium (Invitrogen, Carlsbad, CA, USA) supplemented with 200 ng/ml GM-CSF (Pepro Tech Inc., Rocky Hill, NJ, USA) and 50 ng/ml IL-4 (Pepro Tech Inc.), 10% fetal calf serum (FCS, Hyclone, Logan, UT, USA), 1% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA) in a moist chamber at 37 °C in the presence of 5% CO_2 for 5 days. After 2 days, 50% of the medium was changed. On day 5, iDCs (1×10^6 cells/ml) were stimulated for 48 h with LPS (100 ng/ml *E. coli* serotype 0128:B12, catalog no. L2887, Sigma), PGE2 (1 μg/ml, P5640, Sigma), CD40L (1 μg/ml, Pepro Tech Inc.) or IL-18 (200 ng/ml, MBL, Tokyo, Japan). When mixtures of stimuli were used the concentrations were the same as when stimuli were used separately. The concentrations of the stimuli and duration of the maturation period used were selected on the basis of the literature: PGE2 (Kim et al. 2008), CD40L (Baratelli et al. 2005), IL-18 (Yamaguchi et al. 2005), LPS (Alder et al. 2006) and a 48 h maturation period (Yamaguchi et al. 2005).

Immunophenotyping

After maturation, mDCs were characterized by immunophenotyping using a FACSort flow cytometer (Becton Dickinson) and the following monoclonal antibodies: CD14 phycoerythrin (PE) (BD Pharmingen, New York, NY, USA), CD1a-PE (BD Pharmigen), CD11c (BD Pharmingen), CD40-PE (BD Pharmingen), CD80-PE (BD Pharmingen), CD83-PE (HB15A, Coulter-Beckman, Marseille, France), CD86-PE (HA5.2B7, Immunotech, Marseille, France), CD209-PE (BD Pharmingen), CCR7-PE (BD Pharmingen), HLA-DR-PE (Becton Dickinson), and HLA-DR-FITC (fluorescein isothiocyanate, BD Pharmingen). Fc receptors were blocked with anti-CD16 and anti-CD32 antibodies and anti-mouse Ig-γ 1 and Ig-γ 2 antibodies were used as control. A total of 20,000 events/sample were acquired for each immunophenotypic analysis. Data are reported as mean ± SD for 4 independent experiments, each carried out in triplicate.

Phagocytosis

The phagocytic activity of iDCs and mDCs was determined as described by Chen et al. (1998) by measuring the incorporation of 70 kDa dextran-FITC particles (catalog no. FD705, Sigma) into the cells by flow cytometry. Approximately 2×10^5 cells in 500 μl RPMI were incubated at 4° or 37 °C with 50 μl of 10 mg/ml dextran-FITC particles. After 30 min or 1 or 2 h of incubation, the cells were washed four times by centrifugation in PBS containing 2% FCS in order to remove non-phagocytized particles. Finally, the cells were fixed in ice-cold PBS containing 5% paraformaldehyde and analyzed by flow cytometry. A total of 10,000 events/sample were acquired and five independent experiments, each in duplicate, were carried out. Data are reported as percent ± SD of the number of cells containing particles.

Migration

Migration was measured in a Boyden chamber (Neuro Probe, Cabin John, MD, USA) fitted with a 5 μm polycarbonate membrane (Millipore, Bedford, MA, USA) as described by Herman et al. (2007). Twenty-eight microliters RPMI medium containing 1% FCS plus chemokines was added to the lower compartment of a 48-well chamber. CCL19 or CCL21 (BD Pharmingen) were used separately, each at the concentrations of 250, 500 or 1000 ng/ml. After the addition of RPMI and of the chemokines the polycarbonate filter was placed on the lower part of the chamber containing RPMI and the chemokines. A rubber seal was then put in place and the upper part of the chamber was seated on top of the membrane. mDCs (5×10^4 cells/well) were added to the upper part of the chamber and incubated at 37 °C for 1.5 h. The filter was removed, washed and fixed in 70% methanol. It was then stained with Diff Quick dye (Baxter Diagnostics, Düdingen, Switzerland) and five fields were counted with a light microscope (Olympus, Tokyo, Japan) at 1000× magnification in triplicate for a total of 15 fields per treatment. Data are reported as mean ± SD for 3 independent experiments carried out in triplicate.

T cell proliferation

Two milliliters of mononuclear cells (1×10^7 cells/ml) in PBS containing 0.1% human albumin were labeled with 2.5 μM carboxyfluorescein-succinimidyl ester (CFSE, Molecular Probes, Carlsbad, CA, USA) at 37 °C for 10 min. Labeling was stopped by the addition of 20 ml RPMI containing 10% FCS at 4 °C and the mixture was incubated on ice in the dark for 5 min. The cells were washed twice and resuspended in RPMI supplemented with 10% FCS. 5×10^4 DCs and 5×10^5 CFSE-labeled mononuclear cells

were added to each well in 1 ml RPMI medium supplemented with 10% FCS. The experiments were carried out with iDCs or mDCs stimulated with LPS, IL-18, PGE2 + CD40L or PGE2 + IL-18. The positive control consisted of 5 μl 5 μg/ml phytohemagglutinin (PHA) added to 5×10^5 mononuclear cells labeled with CFSE in 1 ml RPMI medium containing 10% FCS. The cells were incubated at 37 °C for 5 days and then analyzed by flow cytometry using an R1 gate for lymphocytes. Data are reported as a fluorescence histogram (FL1-CFSE). After 18 h, 0.5×10^6 CFSE-labeled and non-labeled mononuclear cells were analyzed by flow cytometry to determine the labeling efficiency. A total of 10,000 events/sample were acquired. Data are reported as means ± SD for three independent experiments carried out in triplicate.

Cytokine secretion

Cytokines were determined by ELISA using 96-well plates after differentiation (5 days) and after maturation. The supernatant of iDC and mDC cultures containing 1×10^6 cells/ml was collected after differentiation or maturation to determine TNF-α, IL-10 and IFN-γ using a commercial kit (R&D Systems, Minneapolis, MN, USA) and IL-12p70 (eBioscience, San Diego, CA, USA) according to manufacturer's instructions. The range of the standard curve was 15–1000 pg/ml for IL-12p70 and TNF-α and 31–2000 pg/ml for IFN-γ and IL-10. Data are reported as means ± SD for three independent experiments carried out in triplicate.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) and by the Tukey–Kramer test to determine differences between groups, with the level of significance set at $P < 0.05$. GraphPad Prism software version 5.01 was used.

Results

Cell preparation

Monocytes

Human monocytes were prepared according to the protocol of Lehner and Holter (2002), who reported an average of 75% homogeneity for their preparations on the basis of double-label antibodies CD45/CD14 used for quantitation. We were able to reach this level of homogeneity only by storing the samples at 4–6 °C for 1–3 h before leukocyte separation and washing the cells after the second Ficoll-Paque Plus gradient by modifying the conditions from one centrifugation at 500 *g* × 10 min to two centrifugations at 200 *g* × 5 min, which reduced contamination by platelets. The average homogeneity of 10 preparations determined by cytometry was 68 ± 10% (mean ± SD) and the average total number of monocytes per preparation was $1.0 \pm 0.7 \times 10^8$. Flow cytometry analysis of a monocyte preparation indicated 75% homogeneity using double labeling with CD45/CD14 for quantitation.

Immature dendritic cells. Monocyte differentiation into iDCs was stimulated with GM-CSF and IL-4. The average total number of iDCs obtained in 10 preparations was $2.8 \pm 0.8 \times 10^7$. The percentage of the iDC markers CD1a and CD209 was 82 ± 12% and 71 ± 14%, respectively, for four independent preparations as shown in Table 1A. iDCs expressed high levels of CD11c (80.6 + 9.7%) and of CD80 (70.6 + 14.4%). The data in Table 1A show that the levels of expression of CD40, CD83 and CD86 were 15–25%, as has been reported for iDCs (Chen et al. 1998).

iDCs were also characterized in terms of phagocytic activity. The percent of cells that incorporated dextran particles labeled with FITC was 79 ± 7% at 37 °C, while the negative control at 4 °C presented 0–4% uptake (data not shown). The percent of mDC uptake of dextran at 37 °C was 5% when the cells were matured with LPS and 16% when they were matured with PGE2, compared to 79% uptake by iDCs, indicating that the maturation of the preparation had been successful.

Mature dendritic cells. iDCs were cultured in the presence of LPS, PGE2, CD40L, IL-18, CD40L + PGE2 or IL-18 + PGE2. Table 1B summarizes the results obtained with four mDC preparations after the addition of the 4 maturation stimuli alone or in combination. LPS, CD40L + PGE2 and IL-18 + PGE2 were the most effective inducers of maturation, as indicated by the high expression of the maturation marker proteins CD80, CD83 and CD86. The remaining stimuli, PGE2, CD40L or IL-18, induced less expression of CD83 than the mixtures. Data for LPS are included in Table 1B to facilitate comparison.

Morphology

iDCs were round with a central nucleus and short cytoplasmic prolongations. After maturation, the mDCs presented an irregular shape and an acentric nucleus. Long and thin cytoplasmic prolongations were demonstrable on the surface of mDCs, as described by Banchereau and Steinman (1998), but not on iDCs. No differences in the morphology of iDCs matured with different stimuli were observed (data not shown). All the data reported thus far demonstrate that the preparations of iDCs and mDCs have properties that are the same or similar to those described in the literature (Banchereau and Steinman 1998; Steinman and Banchereau 2007).

Functional properties of mDCs matured with different stimuli

mDCs stimulated with CD40L + PGE2 migrated toward both CCL19 and CCL21 (Fig. 1A), whereas mDCs stimulated with IL-18 + PGE2 migrated only toward CCL21. mDCs stimulated with LPS migrated toward CCL21 in a manner that was independent of CCL21 concentration, whereas migration toward CCL19 was concentration-dependent (data not shown). In contrast, IL-18 and PGE2 did not induce statistically significant migration when each stimulus was used separately. However, migratory activity toward CCL21 emerged when the stimuli were used in combination. The extent of migration obtained with LPS was almost 7-fold higher for CCL19 and 6-fold higher for CCL21 than for CD40L + PGE2 (Table 2).

T cell proliferation

When mDCs stimulated with LPS, IL-18, CD40L + PGE2 or IL-18 + PGE2 were incubated with mononuclear cells, they stimulated T cell proliferation to iDCs by 29–45% (Table 2 and Fig. 1B). The extent of T cell proliferation was the only property of mDCs that was independent of the specific stimulus used for maturation. The positive control, *i.e.*, mononuclear cells incubated with PHA, presented 80–90% stimulation of T cell proliferation under the same conditions (data not shown).

Cytokine secretion by mDCs after maturation with different stimuli

The data in Fig. 2 demonstrate that the stimuli used for iDC maturation to mDCs were specific with respect to secreted cytokines. For example, when the IL18 + PGE2 stimulus was used, mDCs secreted IL-12 and IFN-γ, but when IL-18 or PGE2 was used alone, only IL-10 was secreted after stimulation with PGE2, indicating the emergence of a new property when the mixture of stimuli was employed.

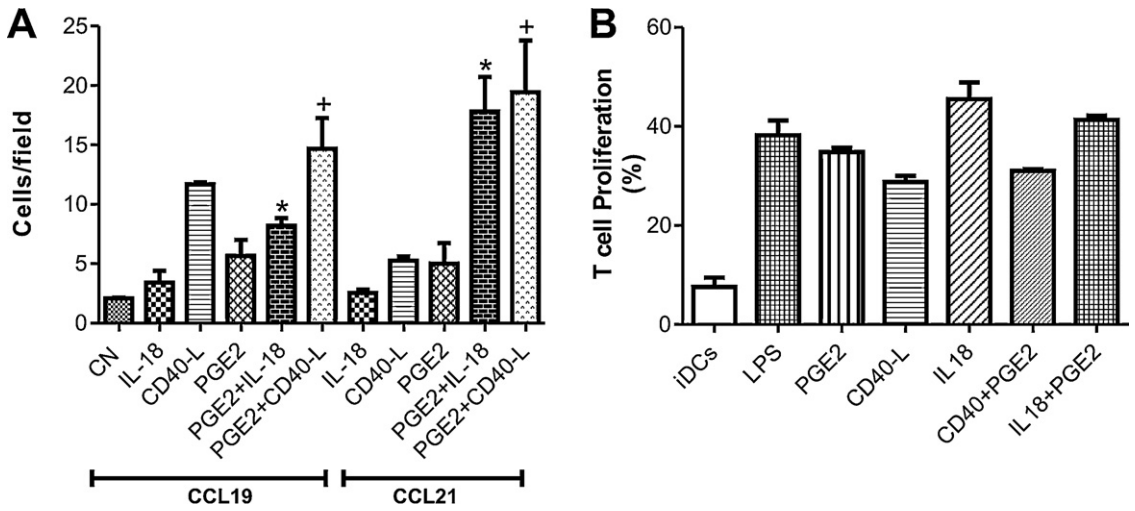

Fig. 1. Effect of maturation stimuli on human mDC migration toward CCL19 and CCL21 (A) and T lymphocyte proliferation (B). (A) Migration assays were carried out with mDCs matured with the stimuli indicated on the abscissa. The concentrations of the maturation stimuli and conditions are given in Table 2. The migration assay was carried out in a Boyden chamber for 1.5 h and the concentration of CCL19 and CCL21 was 1 μg/ml. Data are reported as mean ± SD for 3 experiments carried out in triplicate. The results of all assays reported in (A) were statistically different from controls, which consisted of RPMI medium without CCL19 or CCL21. $^{+}P < 0.05$ CD40L + PGE2 compared to CD40L. $^{*}P < 0.05$ IL-18 + PGE2 compared to PGE2. (B) T lymphocyte proliferation. iDCs and mDCs (1×10^4 cells/ml) were incubated separately with peripheral blood mononuclear cells (PBMCs) labeled with carboxyfluorescein-succinimidyl ester (CSFE) for 5 days. Cell proliferation was measured by the loss of fluorescence of cells initially labeled with CSFE. Data are reported as mean + SD for 3 experiments carried out in triplicate. The results of all assays reported in B were statistically different from controls, which consisted of RPMI medium without CCL19 or CCL21.

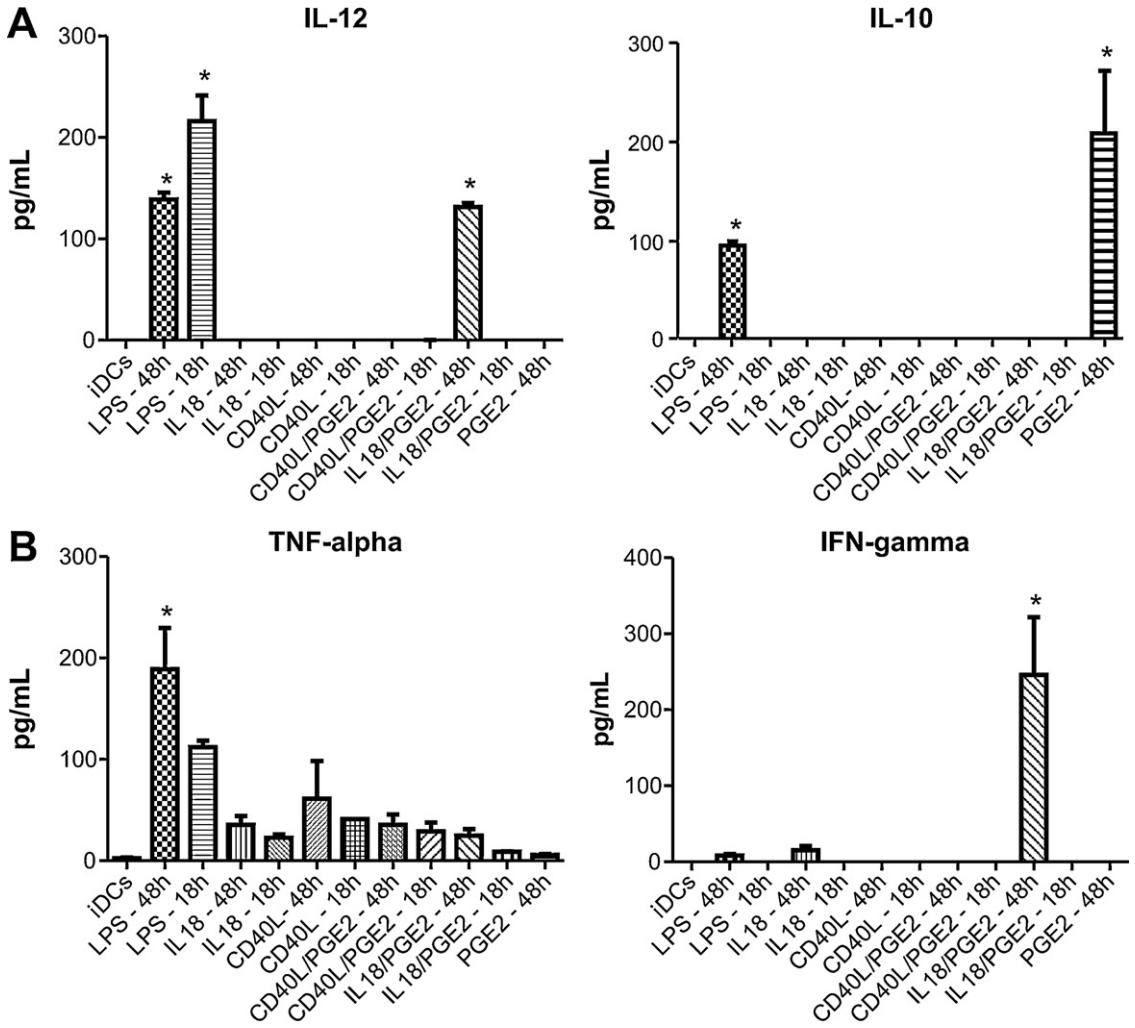

Fig. 2. (A and B) Effect of maturation stimuli on cytokine secretion by human mDCs. iDCs (1×10^6 cells/ml) were matured for 18 and 48 h with the stimuli, whose concentrations are given in Table 2. The culture supernatants were analyzed by ELISA. The bars indicate the mean ± SD of the cytokine concentration for 3 independent experiments carried out in triplicate.

Table 1
Effect of maturation stimuli on the expression of mDC surface antigen characteristic of dendritic cells during differentiation (iDCs) and maturation (mDCs). Data for 4 independent samples in triplicate from different donors are reported as percent mean ± SD. (A) iDCs were obtained after 5 days of human monocyte culture with GM-CSF/IL-4; (B) mDCs obtained after two additional days of culture with the stimuli listed below. Flow cytometry analysis was carried out using the specific markers, CD1a and CD209/DC-SIGN (for iDC), CD11C (for iDC and mDC) and CD40, CD80, CD83 and CD86 (for mDC).

Stimuli	CD1a	CD11c	CD40	CD80	CD83	CD86	CD209
(A)							
GM-CSF/IL-4	82 ± 11.8	80.6 ± 9.7	16.1 ± 19.4	70.6 ± 14.4	21.5 ± 14.5	24.7 ± 14.1	70.8 ± 14
Stimuli	CD1a	CD11c	CD40	CD80	CD83	CD86	CD209/DC-sign
(B)							
LPS	70.7 ± 17.8	93.5 ± 2.7	40.4 ± 18.7	94.6 ± 3.1	76.6 ± 15.7	92.5 ± 4.9	83.4 ± 7.0
PGE2	49.4 ± 18.3	82.4 ± 8.2	21.7 ± 23.8	64.2 ± 23.9	55.1 ± 31.2	49.8 ± 28.2	64.9 ± 9.8
CD40L	65.1 ± 5.4	81.8 ± 10	16.2 ± 9.9	59.7 ± 19.9	22.1 ± 8.4	57.3 ± 8.5	46.4 ± 18.3
IL-18	65.8 ± 19.7	77.3 ± 21.5	24.5 ± 20.3	69.7 ± 6.7	23.6 ± 27.6	49.6 ± 30.6	61.6 ± 16.6
CD40L + PGE2	94.7 ± 3.2	96.6 ± 2.7	43.1 ± 31.1	96.2 ± 2.3	83.6 ± 22	95.9 ± 2.9	86.7 ± 3.4
IL-18 + PGE2	75.3 ± 17.0	75.1 ± 16.4	49 ± 24.1	75 ± 13	63 ± 8.1	81.6 ± 12.1	70.2 ± 7.3

No CD14 in table.

Table 2 provides examples of a situation where mDCs matured by either PGE2 or IL-18 alone did not induce migration to CCL19 or IL-12 and IFN-γ secretion. However, when stimulated with a mixture of the two stimuli these functional properties emerged. Similarly, when either CD40L or PGE2 was used to mature mDCs, migration to both CCL19 and CCL21 was demonstrable.

Discussion

The *ex vivo* production of DCs capable of initiating, programming and regulating antigen-specific immune responses is necessary for successful cell-based therapy. Several laboratories have attempted to identify the stimulus or mixture of stimuli for iDC maturation that would increase the immunologic efficacy of these cells (Kim et al. 2010). We evaluated the effect of different stimuli as mixtures or alone on the maturation and functional properties of human MoDCs. An important result of the present research was the demonstration that when a mixture of two stimuli was used to mature iDCs the resulting functional properties of the mDCs were not the sum of the properties induced when each stimulus was administered alone. The data in Table 2 show two examples which demonstrate that new properties emerged when IL-18 + PGE2 were used simultaneously. These included mDC migration toward CCL21 as well as IL-12 and IFN-γ secretion. It is important to note that IL-18 and PGE2 alone did not induce the same functional properties when applied separately. The data are of special interest because the migration phenotype, and IL-12 and IFN-γ secretion, induced by IL-18 + PGE2 are essential properties for successful DC-based immunotherapeutic applications. IL-12 family members are an important link between innate and adaptive immunity and drive Th1 responses by increasing IFN-γ production, which is key for the clearance of intracellular pathogens (Henry et al. 2008).

Similarly, CD40L + PGE2 induced migration toward CCL19 and CCL21 when administered simultaneously for maturation, although when CD40L and PGE2 were used separately, no migration was observed. The CD40L + PGE2 combination improved the migration characteristics of mDCs more than those obtained with each stimulus alone. However, this combination of stimuli was unable to induce IL-12 or IFN-γ secretion (Table 2). PGE2, when used alone, stimulated IL-10 secretion but lost this property when administered together with IL-18 or CD40L. It has been shown that DCs cultured in the presence of PGE2 induced the differentiation of naïve T cells toward a helper T-cell type 1 (Th1) response, which was independent of IL-12 secretion in the basal state despite a slightly lower IFN-γ secretion compared with control cells. However, the function of cytotoxicity-stimulating autologous T cells was not increased by the addition of PGE2 (Lee et al. 2002). Immature DCs expressed the inflammatory chemokine receptors, CCR1 and CXCR4, but not CCR6, regardless of the presence or absence of PGE2. These data suggest ways to increase the efficacy of DC-based procedures by manipulating the maturation stimuli.

The present results are of interest because stimulation by IL-18 in combination with PGE2, in addition to inducing migration, induced IL-12 and IFN-γ secretion. Yamaguchi et al. (2005) and Boullart et al. (2008) also demonstrated that IL-18 alone does not induce IFN-γ secretion, which was obtained only from DCs stimulated with IL-12 + IL-18. IFN-γ is an important mediator of Th1 cell immunity required for effective immunity against intracellular pathogens (Fricke et al. 2006). It has been suggested that IFN-γ induces the secretion of IL-12 by myeloid DCs in the direction of a Th1 response (Nagai et al. 2006). IL-12 stimulates T cell proliferation in addition to inducing differentiation for the Th1 response, which is fundamental for an efficient antitumoral response (Sato et al. 2003). According to the classic model, IFN-γ is produced by natural killer (NK) cells and T lymphocytes, mainly Th1, and it has been suggested that IFN-γ production by DC could be the consequence of contamination with lymphoid cells (Frucht et al. 2001). However, as reported here, there was no significant secretion of IFN-γ by mDCs matured with LPS, IL-18, PGE2, CD40L or CD40L + PGE2 as stimuli. Only mDCs stimulated with the IL-18 + PGE2 mixture secreted significant amounts of IFN-γ. Fricke et al. (2006) reported that mycobacteria induced IFN-γ secretion by human DCs by triggering TLR2.

IL-18 + PGE2 induced a maturation profile similar to that obtained with LPS but with fewer new properties (see Table 2). LPS was included in the present study for comparison with other stimuli because it is a strong maturation stimulus, but it cannot be used in processes whose product is for human use. DCs stimulated with LPS and PGE2 were able to secrete IL-10, although these cells also secreted high levels of IL-12 and TNF-α. IL-10 produced by DCs promotes the development of regulatory T cells which control, *via* the production of IL-10 and TGF-β, the balance of the Th1/Th2 response (Litjens et al. 2004). It has also been reported that IL-18 induces migration of Langerhans cells to lymph nodes in response to an antigen in a murine model (Antonopoulos et al. 2008). Gutzmer et al. (2003) demonstrated that DC derived from monocytes and stimulated with IL-18 migrated in the direction of IL-18, but the migration of mDCs stimulated with IL-18 in the direction of chemokines CCL19 and CCL21 has not been reported previously. The IL-12 + IL-18 combination generated maximum IFN-γ secretion by macrophages and Mo-DC. This combination has a highly synergistic effect on macrophages, but a less pronounced effect on DC (Frucht et al. 2001).

DC activated by the toll-like receptor ligand R848 in the presence of IFN-γ and PGE2 secrete high levels of IL12-p70 and IL-23, in addition to inducing a migration phenotype (Lehner et al. 2008).

Table 2
Effect of maturation stimuli on the properties of human monocyte-derived dendritic cells. The surface antigens of the iDCs used in the experiments described here were CD1A (82 ± 12%), CD209 (71 ± 14%) and CD11C (80 ± 9.7%). The cells (10^6 cells/ml) were incubated in RPMI + 10% FCS for 48 h with the stimuli given in the second column. Three independent experiments were carried out in triplicate with each stimulus.

	Stimuli	Functional		Tests				
		Migration (cells/field)		T cell proliferation (%)	IL-12 secretion (pg/ml)	IL-10 secretion (pg/ml)	TNF-α secretion (pg/ml)	IFN-γ secretion (pg/ml)
		CCL19	CCL21					
1	LPS (100 ng/ml)	189.4 ± 26.1[a]	108.5 ± 7.6[a]	38.2 ± 7.2[b]	138.9 ± 20.0[c]	95.6 ± 6.0[c]	188.5 ± 78.8[c]	0
2	IL18 (200 ng/ml)	0	0	41.3 ± 2.1[b]	0	0	0	0
3	IL-18 (200 ng/ml) + PGE2 (1 μg/ml)	0	17.8 ± 6.6 [a,d,e]	45.5 ± 8.3[b]	131.4 ± 6.7[c,d,e]	0	0	355.0 ± 87.0[c,d,e]
4	PGE2 (1 μg/ml)	0	0	34.8 ± 2.1[b]	0	207.9 ± 63.2[c]	0	0
5	CD40L (1 μg/ml) + PGE2 (1 μg/ml)	27.4 ± 8.0 [a,d,f]	19.0 ± 4.7[a,d,f]	31.0 ± 0.9[b]	0	0	0	0
6	CD40L (1 μg/ml)	0	0	28.7 ± 3.1[b]	0	0	0	0

[a] $P < 0.05$ compared to control without CCL19 or CCL21.
[b] $P < 0.05$ compared with the control consisting of iDCs and mononuclear cells.
[c] $P < 0.05$ compared to iDC supernatant without maturation stimuli.
[d] $P < 0.05$ compared to PGE2 alone.
[e] $P < 0.05$ compared to IL-18 alone.
[f] $P < 0.05$ compared to CD40L alone.
0 = not significantly different from control. Statistical comparisons were analyzed statistically by analysis of variance (ANOVA) and by the Tukey–Kramer test.

It has also been reported that histamine and PGE2 increase the expression of CCL17 and CCL22 by monocyte-derived DCs in a dose-dependent manner (McIlroy et al. 2006). It is also known that PGE2 inhibits IL-12 secretion at sites of inflammation (Kaliński et al. 2001), but induces DC migration toward chemokines CCL19 and CCL21 (Scandella et al. 2002; Van Helden et al. 2006). In the present experiments there was no detectable migration by mDCs matured with PGE2 (Table 2). These cells also presented low levels of expression of the maturation markers CD80, CD83 and CD86.

The effects of different combinations of stimuli have been studied in order to identify an effective protocol for DC maturation. As cited above, Möller et al. (2008) demonstrated that the standard maturation cocktail containing poly(I:C) induced substantial IL-12 secretion compared to the cocktail containing PGE2. On the other hand, the cocktail containing PGE2 showed a superior migration-inducing effect but a low level of IL-12 secretion. Since secretion phenotypes are important properties of DC-based immunotherapeutic applications, the IL-18 + PGE2 combination provides a result which could be useful in dendritic cells based therapy applications. IL-12 and IFN-γ polarize the Th1 response, which induces antigen-specific T CD8 + cell proliferation (Sercan et al. 2006; Whitmire et al. 2005) and, importantly, the expression of molecules related to cytotoxicity (Kobayashi et al. 1989; Trinchieri, 2003).

Clinical trials in phase I/II have been carried out to show the possibilities of use of differentially matured DCs in human therapy. Lee et al. (2005) used vaccine produced using mDCs pulsed with autologous tumor lysates to treat advanced hepatocellular cancer which could provide better clinical survival. Slingluff et al. (2003) have used mDCs to treat patients with melanoma while Kikuchi et al. (2001) showed interesting results in patients with gliomas. Protocols of DC maturation using cytokines of clinical grade such as PGE2 and CD40L (Lee et al. 2002) and IL-18 or by combining a tumor lysate with IL18 (Yamanaka et al. 2005) have also been tested.

In view of the importance of obtaining DCs that will combine migratory capacity, IL-12 secretion and the ability to stimulate T cells, the present results provide relevant information that can be exploited in the future to produce tailor-made more efficient DCs for immunotherapeutic applications. Pilot clinical trials of antigen-pulsed DCs have been carried out for various types of cancer, including prostate cancer, colorectal cancer, multiple myeloma, and non-small cell lung cancer. These studies and others have shown that antigen-loaded DC cell therapy represents a safe and promising form of immunotherapy for a wide range of malignancies. Combination of certain stimuli, such as IL-18 and CD40L, with PGE-2 could be useful to prepare a cell-based vaccine that preferentially triggers a Th1 response. Identification of the precise mechanisms that underlie the emergence of desired properties of DCs will allow the understanding of DC plasticity and will be fundamental for the development of tailor-made cell-based vaccines.

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