Double-stranded RNA stimulation or CD40 ligation of monocyte-derived dendritic cells as models to study their activation and maturation process

Anna Maria Megiovanni, Françoise Sanchez, Jean Claude Gluckman, Michelle Rosenzwajg

Institut National de la Recherche Scientifique – Université Paris 7 EMI-0013 and laboratoire d'Immunologie Cellulaire et Immunopathologie de l'Ecole Pratique des Hautes Etudes, Institut Universitaire d'Hématologie, hôpital Saint-Louis, Paris, France

Michelle Rosenzwajg, M.D., Ph.D., INSERM EMI-0013, Institut Universitaire d'Hématologie, hôpital Saint-Louis, 1 avenue Claude Vellefaux, 75475 Paris Cedex 10, France. Phone: + 33 1 53 72 21 07 - Fax: + 33 1 53 72 22 37
E-mail: michelle.rosenzwajg@sat.ap-hop-paris.fr

Accepted for publication February 27, 2004

ABSTRACT. Monocyte-derived dendritic cells (DCs) were used as an *in vitro* model of myeloid DCs in order to determine a minimum marker pattern with which to characterize and distinguish different stages of DC activation and maturation. Phenotypic changes induced on immature DCs by two prototypic stimuli, poly I:C and CD40 ligation, were first examined. Both elicited HLA-DR, CD40, CD86 and CXCR4 upregulation, and CCR5 downregulation, but only CD40 ligand-stimulated DCs became $CD83^+/CCR7^+$, whereas poly I:C-stimulated DCs expressed lower CD83 levels and were mostly $CCR7^-$. CD40 ligation and poly I:C elicited increased production of inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor-α, of IL-10 and the CCL5 chemokine, but profiles differed as to higher IL-10, IL-12 and CCL22 (a CCR4 ligand important for T cell recruitment) levels for the former, and of CCL4 and CCL5 for the latter. Thus, a limited set of phenotypic markers, cytokine and chemokine production assays, may be used to distinguish the three stages in the life of DCs: immaturity, activation and full maturation. The ability of purified protein derivative-loaded DCs to stimulate autologous T cells to produce IL-2, IL-4 and interferon-γ indeed depended on their activation stage and endocytic activity, which decreased upon maturation. We then examined whether ligation of CD4, CCR5 and/or CXCR4, the receptor and coreceptors of human immunodeficiency virus envelope gp120, respectively, affected DC activation or maturation, neither a monoclonal antibody to the gp120-binding site on CD4 nor CCL5 nor CXCL12, the natural ligands of CCR5 and CXCR4, respectively, nor gp120 altered the DC activation and maturation processes.

Keywords: dendritic cells, cell activation, cell maturation, cytokines, Human immunodeficiency virus

INTRODUCTION

Dendritic cells (DCs) are professional antigen (Ag)-presenting cells that act as sentinels of the immune system [1]. In peripheral tissues, immature DCs (iDCs) capture Ags, upon which they acquire an intermediate stage of activation, which is a critical event in the initiation of adaptive immune responses. Following delivery of the early signals, DCs progressively lose the ability to capture Ags via phagocytosis, receptor-mediated endocytosis and macropinocytosis and produce inflammatory cytokines (tumor necrosis factor [TNF]-α, interleukin [IL]-6, IL-1β) and chemokines (CCL3, CCL4, CCL5) that promote macrophage and DC recruitment. Due to decreased expression of chemokine receptors that allow DC recruitment in peripheral tissues (CCR1, CCR2, CCR5, CXCR1), and upregulation of receptors (CXCR4, CCR7) for chemokines produced in lymphoid tissues (CCL19, CCL21), these alerted DCs are directed to migrate to draining lymphoid organs. This process is completed by terminal differentiation into fully mature DCs (mDCs), which occurs upon interaction with T cells [2], which they stimulate and polarize towards different cytokine secretion profiles [3-5].

The effect of danger or T cell-derived signals on iDCs has been extensively investigated [1, 6], but the transitional stages to early activated, referred to here as activated DC (aDCs), and then to mDCs are less well defined. Activation of iDCs is usually considered as representing the change from the resting state, and is assumed to lead to increased immunogenicity, whereas maturation is characterized by full Ag-presenting functions and takes place upon interaction with T cells. In order to determine phenotypic and functional characteristics that could be used to distinguish iDCs, aDCs and mDCs, monocyte-derived DCs were stimulated with two prototypic stimuli: double stranded RNA (dsRNA), which represents a virus-derived activation signal [7, 8] or CD40 ligand (CD40L), a T cell-derived irreversible maturation signal [9].

DCs are presumed to be among the first cells that encounter human immunodeficiency virus (HIV) and, upon binding or infection, it is assumed that they transport it to the draining lymph nodes to transfer it to T cells [10]. Indeed, DCs express several receptors of HIV envelope gp120 such as CD4, chemokine receptors CCR5 and CXCR4, the coreceptors of R5 and X4 strains, respec-

tively, as well as C-type lectins, the expression of which varies with DC activation/maturation [10-13]. Using monoclonal antibodies (mAbs) to CD4, CCR5 and CXCR4 natural ligands (CCL5 and CXCL12 respectively) and gp120, we took advantage of our *in vitro* model to examine whether ligation of CD4 and/or CCR5 or CXCR4 interfered with DC activation/maturation.

MATERIALS AND METHODS

Preparation and culture of cells

The culture medium was RPMI-1640, 2 mM L-glutamine, 50 µg/ml penicillin, 50 µg/ml streptomycin, 100 µg/ml neomycin, 10% heat-inactivated fetal calf serum (FCS) (Dutscher, Brumath, France), the endotoxin level of which was < 0.5 EU/ml (LAL kit, Biowhittaker, Walkersville, MA, USA).

Cytophereses from normal donors (Etablissement Français du Sang, Paris, France) were obtained according to institutional guidelines. Blood mononuclear cells were enriched by Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO, USA) centrifugation. T cells were depleted by sheep erythrocyte rosetting (Mérieux, Marcy l'Etoile, France) and B cells by M-450 Pan B/CD19 Dynabeads (Dynal, Oslo, Norway) immunomagnetic purification in phosphate-buffered saline (PBS), 2% FCS, resulting in 88 ± 7% pure $CD14^+$ cells. T cells (> 90% $CD3^+$ cells) obtained after rosetting were cryopreserved for use as responders in subsequent T cell reactivity assays.

DCs were generated in 6-well plates (Costar, Cambridge, MA, USA) by culturing 2×10^6 monocytes in 3 ml of medium supplemented with 10 ng/ml recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and 5 ng/ml IL-4 (both from R,D systems, Minneapolis, MN, USA). Cultures were fed every two days by removing one-third of the supernatant and adding fresh medium with full doses of cytokines [14]. Human soluble trimeric CD40 ligand (CD40LT, 500 ng/ml or 2 µg/ml, gift of Immunex, Seattle, WA, USA) or polyinosinic-polycytidylic acid sodium salt (poly I:C; 2 µg/ml; SIGMA) were added on culture day five.

Multiparameter flow cytometry analysis

Cells were washed in PBS, 2% FCS, incubated for 30 min at 4 °C with mAbs, washed and fixed in PBS, 1% paraformaldehyde, before analysis with a FACScalibur® cytometer (Becton Dickinson, San Jose, CA, USA). Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled mAbs were as follows: IgG1, IgG2a, CD83 (Beckman Coulter France, Villepinte, France), anti-HLA-DR (Becton Dickinson), IgG2b, CD1a, CD14, CD86, anti-CXCR4, anti-CCR5 (PharMingen, San Diego, CA, USA), anti-CCR7 (R,D Systems).

Endocytosis assay

To assess endocytosis, DCs were incubated in PBS, 2%FCS, with 1 mg/ml FITC-dextran (Sigma-Aldrich) at 37 °C to determine specific uptake, or at 4 °C to determine background binding. After one hr of incubation, cells were washed in cold PBS-FCS and analyzed by flow cytometry.

Cytokine determination in culture supernatants

DC (10^5/0.2 ml/well) were cultured for 6, 24 or 48 hrs under the conditions indicated. IL-6, IL-10, CCL4, CCL5, CCL17, CCL22 and TNF-α levels in supernatants were measured using kits from R,D Systems, IL-12p70 was assessed with a kit from Diaclone.

T-cell stimulation assay

T cells (1×10^6) from donors known to have been sensitized by *M. bovis* BCG vaccination, which is mandatory in France, were cultured in 1.5 ml of medium with 10% heat-inactivated normal human AB serum (Etablissement Français du Sang) in 24-well, cell culture plates (Costar) in the presence of 1×10^5 autologous DCs pulsed for 3 hrs with 20 µg/ml Protein Purified Derivative (PPD). Supernatants were collected after 24 hrs to evaluate IL-2, IL-4, IL-5, IL-10, interferon (IFN)-γ and TNF-α production with the Cytometric Bead Array (CBA) kit (Becton Dickinson).

CD4, CCR5 and CXCR4 ligation

CD4, CCR5 and CXCR4 ligands were incubated with culture day five DCs. Briefly, 3×10^6/ml DCs were incubated for one hr at 4 °C with 5 µg/ml CD4-specific mAbs SK3 (Becton Dickinson) or BL4 (Immunotech) as CD4 ligands, or with 2 µg/ml recombinant X4 or R5 gp120 (HIV-1IIIB and HIV-1BaL, respectively; NIH AIDS Research and Reference Reagent Program, Rockville, MD, USA). The mAb SK3 recognizes HIV gp120 binding site within CD4 first domain, whereas BL4 binds an epitope of the CD4 second domain not involved in gp120 binding [15]. The binding of gp120 to the cells was assessed using mAb 110H (2 µg/ml) (gift of Ali Amara, Institut Pasteur, Paris, France) and indirect immunofluorescence labeling with a goat anti-mouse PE-labeled mAb (ROCKLAND, WA, USA). Alternatively, soluble synthetic CCL5 (100 ng/ml) (gift of Claudio Vita, CEA, Gif sur Yvette, France) or CXCL12 (100 ng/ml) (gift of Françoise Baleux, Institut Pasteur) were added to day five iDCs for overnight incubation in 6-well plates (2×10^6 cells/3ml). DCs were then resuspended at 5×10^5 cells/ml, stimulated with CD40LT or poly I:C before plating in 96-well, round-bottomed plates for two days. Supernatants were collected after 24 hrs, and cell phenotype was analyzed after 48 hrs.

RESULTS

Changes in DC phenotype in response to poly I:C or CD40LT

In order to characterize DC activation and maturation patterns, we examined the phenotypic modifications elicited by prototypic stimuli, namely poly I:C and CD40LT [9, 16-18]. After five days, culture of monocytes with GM-CSF and IL-4 [14, 19], the resulting DCs were further cultured for two days with GM-CSF and IL-4 in the absence (control condition) or the presence of the stimuli, before FACS analysis (*Table 1* and *Figure 1A*). As expected [14, 19, 20], the control condition yielded $CD14^-$ iDCs (data not shown), 81 ± 4% of which were $CD1a^+$ All expressed HLA-DR, costimulatory molecules CD40 and low CD86, and low or no detectable membrane CD80, according to the donor. Maturation marker CD83 was ex-

Table 1

Analysis of DC phenotype in response to poly I:C or CD40LT stimuli. Culture day-5 DCs were cultured for a further 48 hrs with GM-CSF and IL-4 in the presence or not (control) of 500 ng/ml CD40LT (+ CD40LT) or 2 µg/ml poly I:C (+ poly I:C) before flow cytometry analysis. Data are means ± SEM. Δ MFI: differences between test and control mean fluorescence intensities. The paired Student's t-test was used for statistical analysis.

	Culture condition			Culture condition			
	marker	*n*	control	+ CD40LT	control *vs* *CD40LT*	+ Poly I: C	control *vs* *poly I: C* w
Cell percentages	CD1a (%)	14	81 ± 4	80 ± 3	*p > 0.05*	81 ± 4	*P > 0.05*
	CD83 (%)	15	4 ± 1	73 ± 5	*p < 0.001*	43 ± 8	*P < 0.001*
Δ MFI	CD40	5	100 ± 21	244 ± 31	*p = 0.03*	218 ± 28	*P = 0.02*
	CD80	5	6 ± 2	9 ± 3	*p > 0.05*	12 ± 4	*P > 0.05*
	CD86	14	44 ± 13	215 ± 27	*p < 0.001*	272 ± 50	*P < 0.001*
	HLA-DR	5	125 ± 60	516 ± 227	*p = 0.04*	294 ± 136	*P > 0.05*
	CCR5	12	22 ± 4	9 ± 1	*p = 0.003*	9 ± 2	*P = 0.003*
	CCR7	11	14 ± 4	101 ± 31	*p = 0.01*	20 ± 9	*P > 0.05*
	CXCR4	12	19 ± 5	37 ± 8	*p = 0.02*	31 ± 6	*P = 0.03*

pressed by only 4 ± 1% of the cells. As for chemokine receptors, the cells were $CCR5^+$ $CCR7^-$, CXCR4 expression being absent or very low.

Consistent with other reports [9, 16, 20, 21], culture with CD40LT induced a fully mature phenotype, with 73 ± 5% $CD83^+$ cells and increased HLA-DR, CD40 and CD86 but not of CD80 expression. CCR5 expression decreased whereas CXCR4 and CCR7 were up-regulated. DC stimulation with poly I:C led to only 43 ± 8% $CD83^+$ cells with lower expression of CD83 relative to their CD40LT-stimulated counterparts. HLA-DR, CD40 and CD86 expression was upregulated, that of CXCR4 increased moderately and that of CCR5 decreased, while CCR7 was low or undetectable. Thus, the phenotype elicited by poly I:C stimulation was intermediate between that of iDCs and mDCs. Differences in $CD83^+$ cell percentages and CCR7 expression levels reached statistical significance when comparing poly I:C- and CD40LT-treated DCs, allowing use of these markers to compare iDCs, aDCs and mDCs.

Because the differences noted here could be due to different kinetics of expression, the cell phenotype under each condition was also assessed six and 24 hrs *(Figure 1B)* after stimulation: CD86 upregulation occurred as early as six hrs after poly I:C and CD40LT stimulation, reaching a maximum by 24 hrs, whereas CD83, CXCR4 and CCR7 upregulation was only found after 24 hrs. Apart from CCR5 downregulation that became detectable later, at 48 hrs, all modifications were stable from 24 up to 48 hrs. Thus, under both conditions, the kinetics of these markers were comparable, and differences between aDCs and mDCs could not be accounted for by different kinetics.

Different cytokine and chemokine patterns are produced by iDCs, aDCs and mDCs

The patterns of cytokines and chemokines produced by DCs differ according to their activation/ maturation status [22-24]. Therefore, cytokine and chemokine production by DCs under the different conditions described above was assessed at different culture time points. First, supernatants were collected six, 24 and 48 hrs after stimulation to determine the maximum production level for each cytokine and chemokine assayed. TNF-α production peaked six hrs after stimulation, as compared to 24 hrs for IL-6, IL-10, IL-12 and CCL5 (*Figure 2* and data not shown).

Thereafter, only peak levels were considered *(Table 2)*. In control cultures, IL-6, IL-10, TNF-α as well as CCL4 and CCL5 levels were low, whereas consistent levels of CCL17 and CCL22 were produced. Relative to control cultures, CD40LT elicited strong IL-6, IL-10, CCL5 and CCL22 production without significantly modifying that of TNF-α, CCL4 and CCL17. Production of IL-6, IL-10, TNF-α, CCL4 and CCL5 increased after poly I:C activation. Thus, CD40LT and poly I:C elicited different cytokine and chemokine production profiles. Under all conditions tested, even with CD40LT (500 ng/ml), IL-12p70 production was rather low, but a higher CD40LT concentration (2 µg/ml) led to significant IL-12p70 production without globally modifying the cell phenotype or production of other chemokines and cytokines *(Table 2)*. IL-12p70 production by aDCs varied according to the donor, but it was lower than that of mDCs in most instances.

Endocytic activity of iDCs, aDCs and mDCs

Uptake of soluble Ags by iDCs via endocytosis and macropinocytosis is known to decrease upon maturation [1]. Therefore, we monitored FITC-dextran uptake by DCs under each condition used here. As expected, iDCs had a strong capacity to take up FITC-dextran. CD40LT-induced maturation reduced this ability whereas, in line with their activated phenotype, poly I:C-stimulated DCs had indeed a reduced capacity to take up FITC-dextran, but it was less important than for CD40LT-stimulated DCs *(Figure 3)*.

T cell activation by DC

Once established that, relative to iDCs or mDCs, aDCs have an intermediate capacity to capture Ags and an original cytokine and chemokine production pattern, we examined their capacity to stimulate autologous T cells when loaded with PPD as Ag. After 24-hr co-culture, cytokine production by the T cells was evaluated in supernatants *(Figure 4)*. In the absence of PPD, cytokine production by DCs alone, T cells alone, or DCs mixed with T cells was very low. When T cells were stimulated with PPD-loaded

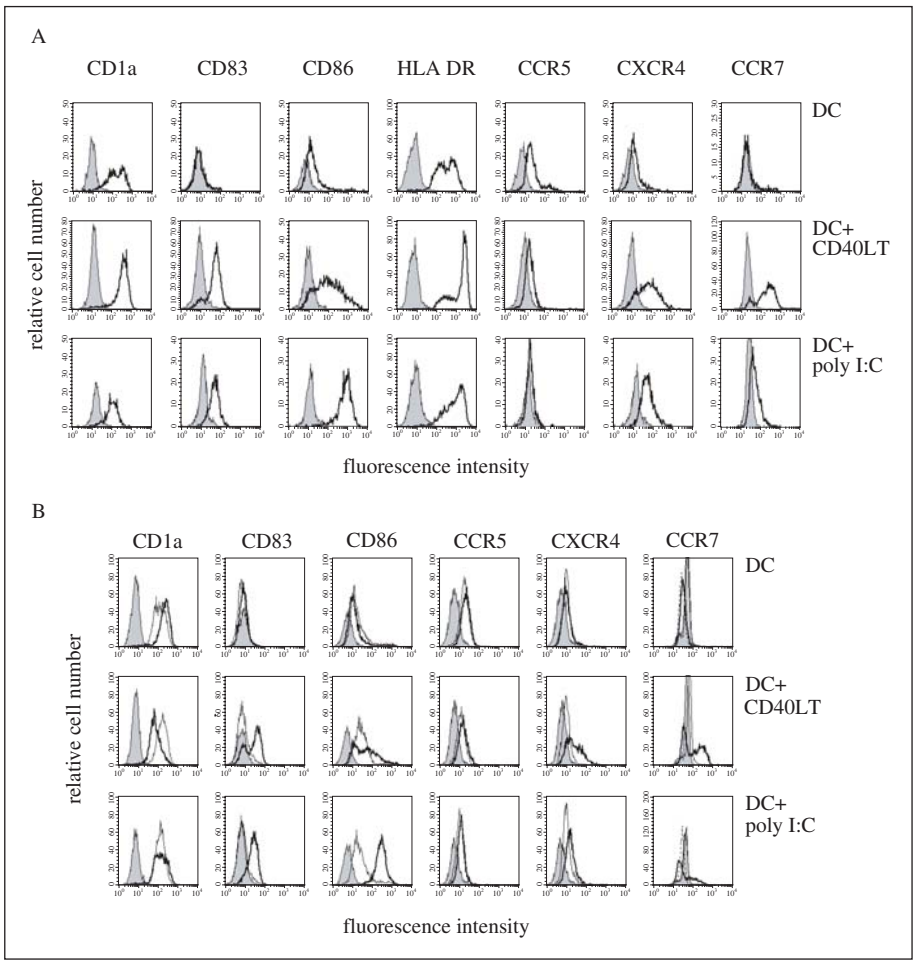

Figure 1

Analysis of surface molecule expression on DCs in response to poly I:C or CD40LT stimuli. Culture day-5 DCs were cultured for 48 hrs (A), or six or 24 hrs (B) with GM-CSF and IL-4 in the presence or not (DC) of 500 ng/ml CD40LT (DC + CD40LT) or 2 µg/ml poly I:C (DC + poly I:C). A. Shaded histograms indicate irrelevant isotype control mAb staining, and solid lines indicate relevant mAb staining. The results shown are a composite of data from two experiments representative of five to 15 (see *Table 1*). B. Shaded histograms indicate irrelevant isotype control mAb staining; thin lines and bold lines indicate relevant mAb staining after six or 24 hrs culture, respectively. The results shown are from one experiment representative of four.

iDCs, high IL-2, IFN-γ and IL-4 production was noted, which correlated with their high capacity to capture Ags and subsequently activate T cells. When T cells were stimulated with PPD-loaded aDCs or mDCs, lower IL-2, IFN-γ and IL-4 levels were found, which correlated with their lower capacity to capture Ags, but differences reached statistical significance ($P < 0.05$) only when PPD-loaded, stimulating mDCs were used. Production of IL-5, IL-10 and TNF-α did not significantly change under the different conditions (data not shown). No Th polarization was observed under the three conditions tested, which indicates a Th0 response.

Effect of gp120 receptor and co-receptor ligation on DC maturation

DCs express CD4, CCR5 and CXCR4, which act as HIV gp120 receptor and co-receptors, respectively [25-27] but their other functions on these cells are not known. Having defined discrete stages of DC activation/maturation, in which CCR5 and CXCR4 expression appears to be regulated, we examined whether such processes could be affected by ligation of these molecules which is known to play important roles in other cells such as lymphocytes and macrophages [12, 26, 27]. To this end, iDCs were incubated with CD4 mAbs that recognize (clone SK3) or not (clone BL4) the physiologically important first domain of CD4, especially the gp120 binding site. Alternatively, they were incubated with CCL5 or CXCL12 (CCR5 and CXCR4 natural ligands, respectively) before further culture for two days with GM-CSF and IL-4, with or without CD40LT or poly I:C. This did not lead to any significant modification of DC phenotype, chemokine and cytokine production relative to when these ligands were not used (*Figure 5A* and data not shown).

As a means to examine the effect of CD4 and CCR5 or CD4 and CXCR4 co-ligation, we used the capacity of gp120 to simultaneously bind to CD4 and CCR5 or CXCR4, according to whether it originates from R5 or X4 strains. Attachment of gp120 to DCs was controlled before inducing their activation or maturation and, as expected, it elicited some CD4 downregulation but did not alter DC-SIGN expression, or CXCR4 and CCR5 expression *(Figure 5B)*. However, gp120 binding did not affect the DC phenotype, cytokine and chemokine production patterns induced by subsequent poly I:C or CD40LT stimulation (*Table 3* and *Figure 5C*).

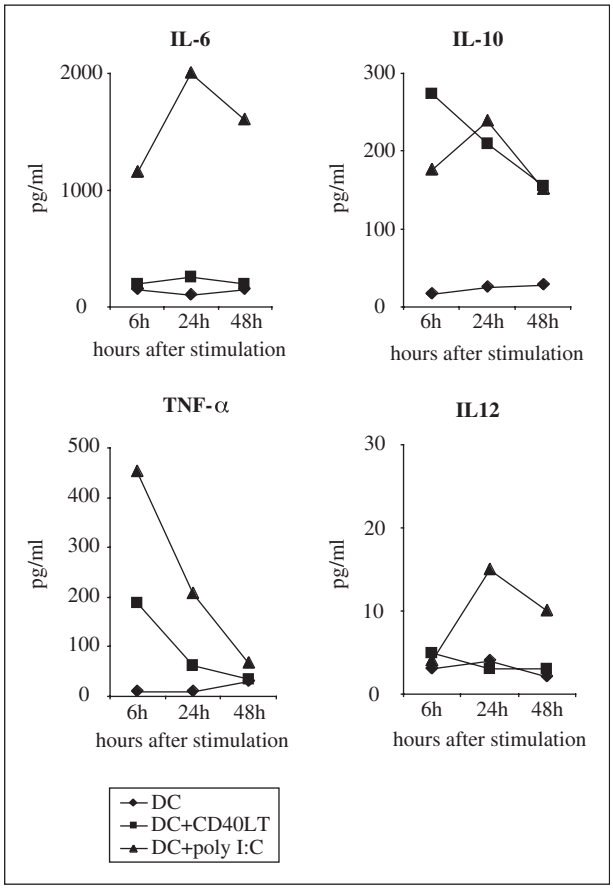

Figure 2
Cytokine production kinetics. Culture day-5 DCs (10^5/0.2 ml/well) were cultured with GM-CSF and IL-4 in the presence or not (DC) of 500 ng/ml CD40LT (DC + CD40LT) or 2 µg/ml poly I:C (DC + poly I:C). IL-6, IL-10, TNF-α and IL-12 levels were measured in culture supernatants at the indicated time points. Data from one experiment representative of four.

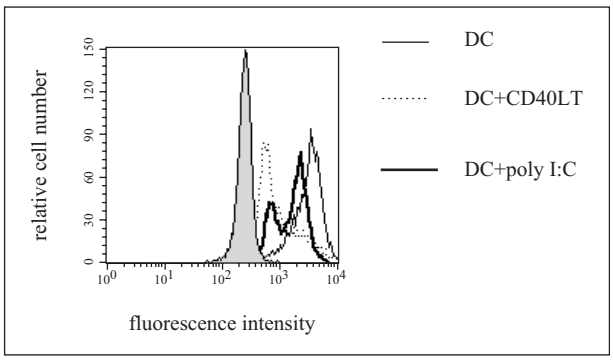

Figure 3
Endocytic activity of poly I:C- and CD40LT-stimulated DCs. Culture day-5 DCs were cultured for a further 48 hrs with GM-CSF and IL-4 in the presence or not (DC) of 500 ng/ml CD40LT (DC + CD40LT) or 2 µg/ml poly I:C (DC + poly I:C) before being incubated with FITC-dextran for 1 hr at 37 °C or at 4 °C in order to comparatively assess specific uptake and non-specific binding, respectively. The shaded histogram represents non-specific binding at 4 °C. Data are from one experiment out of 12.

DISCUSSION

During their life cycle, DCs undergo a maturation process that occurs through successive stages elicited by stimuli such as microbial products, inflammatory molecules and interactions with T cells. Here, we used monocyte-derived DCs [14, 19] as an *in vitro* model of *in vivo* myeloid DCs in order to characterize the successive stages of DC activation and maturation. We first examined the phenotypic changes induced on iDCs by two stimuli: poly I:C and CD40LT, both of which elicited HLA-DR and costimulatory molecule upregulation, as well as CCR5 downregulation and CXCR4 upregulation. The major differences between the two conditions concerned CD83 and CCR7 expression: only CD40 ligand-stimulated DCs became $CCR7^+$ whereas poly I:C-stimulated DCs expressed lower CD83 and were mostly $CCR7^-$. Thus, the phenotype of poly I:C-stimulated DCs was intermediate to that of iDCs and mDCs, and it was referred to as aDCs. It may be defined by the association of characteristics shared with mDCs – high HLA-DR and costimulatory molecule expression, CCR5 downregulation – with characteristics closer to iDCs – lower CD83 and lack of CCR7 expression.

Table 2
Cytokine and chemokine production by poly I:C or CD40LT stimulated DCs. Culture day-5 DCs (10^5/0.2 ml/well) were further cultured with GM-CSF and IL-4 in the presence or not (control) of CD40LT (+ CD40LT) or poly I:C (+ poly I:C) before supernatants were collected at time points corresponding to each cytokine or chemokine peak production as defined in preliminary experiments (see *Figure 2* for an example). Data are mean ± SEM cytokine levels. The paired Student's t test was used for statistical analysis. Significance of differences between experiments with 2 µg/ml *versus* 500 ng/ml CD40L: #$P = 0.03$; * $P = 0.04$.

	Cytokine (pg/ml)	*n*	Culture condition control	+ CD40LT	control *vs. CD40LT*	Culture condition + poly I: C	control *vs. poly I: C*
Experiments with 500 ng/ml CD40LT	IL-6	12	288 ± 39	529 ± 137	*p = 0.05*	938 ± 140	*p = 0.001*
	IL-10	12	20 ± 4	130 ± 33	*p = 0.006*	59 ± 18	*p = 0.06*
	IL-12	12	2 ± 0.3	7 ± 2	*p > 0.05*	61 ± 50	*p > 0.05*
	TNF-a	10	12 ± 1	151 ± 70	*p > 0.05*	149 ± 49	*p = 0.03*
	CCL4	10	331 ± 150	1506 ± 695	*p > 0.05*	6408 ± 1737	*p = 0.01*
	CCL5	12	43 ± 14	432 ± 179	*p = 0.045*	2314 ± 416	*p < 0.001*
	CCL17	10	3672 ± 421	4688 ± 121	*p > 0.05*	3952 ± 202	*p > 0.05*
	CCL22	10	6159 ± 1126	10669 ± 259	*p = 0.002*	7031 ± 1194	*p > 0.05*
Experiments with 2 mg/ml CD40LT	IL-6	8	339 ± 99	853 ± 163#	*p = 0.01*	982 ± 136	*p = 0.01*
	IL-10	8	22 ± 7	308 ± 90	*p = 0.02*	44 ± 10	*p = 0.002*
	IL-12	10	1 ± 0.1	31 ± 11	*p = 0.03*	21 ± 10	*p > 0.05*
	CCL5	7	29 ± 6	1168 ± 267*	*p = 0.007*	2826 ± 661	*p = 0.008*

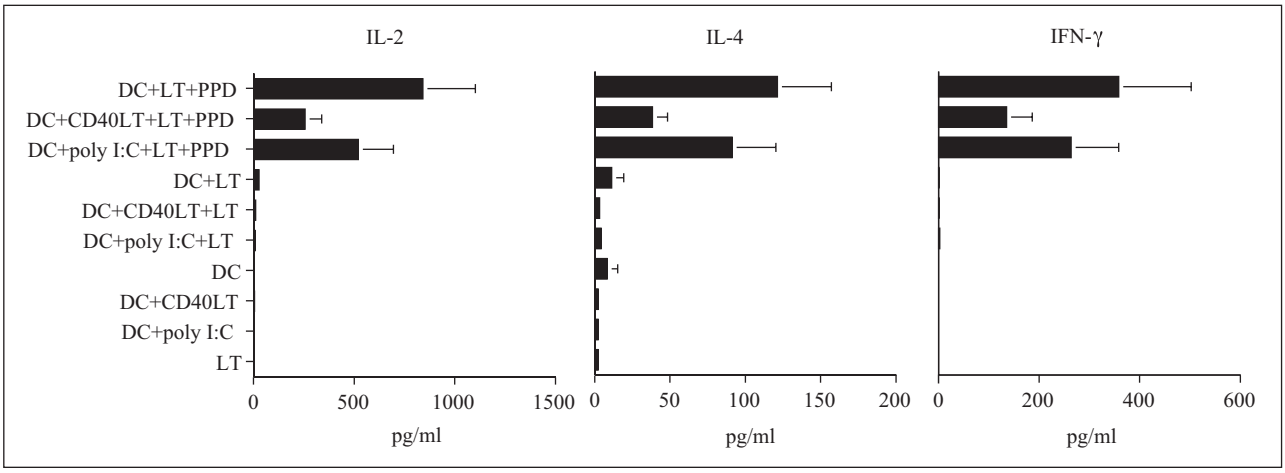

Figure 4

Analysis of poly I:C- or CD40LT-stimulated DC capacity to elicit PPD-specific reactivity of autologous T cells. Culture day-5 DCs were cultured for a further 48 hrs with GM-CSF and IL-4 in the presence or not (DC) of 500 ng/ml CD40LT (DC + CD40LT) or 2 µg/ml poly I:C (DC + poly I:C) before being pulsed with PPD and then cultured with autologous T cells. After 24-hr co-culture, supernatants were collected in order to assess IL-2, IL-4 and IFN-γ production. Data are means + SEM of five experiments.

Table 3

HIV gp120 does not affect DC cytokine and chemokine production. Culture day-5 DCs were incubated for 1 hr at 4 °C with gp120, binding of gp120 was confirmed by flow cytometry (see *Figure 5*), and cells were further cultured with GM-CSF and IL-4 in the presence or not (control) of 2 µg/ml CD40LT (+ CD40LT) or poly I:C (+ poly I:C) before supernatants were collected and processed as indicated in the legend of Table 2. Data are means ± SEM. For IL-6, IL-10 and CCL5, $n = 5$; for IL-12, $n = 7$. Under the three conditions tested, no statistically significant differences in cytokine production were noted depending on whether cells had been preincubated with gp120.

Culture conditions	Cytokines (pg/ml)	gp120 added None	gp120 X4	gp120 R5
control	IL-6	290 ± 70	340 ± 93	360 ± 145
	IL-10	17 ± 2	17 ± 2	22 ± 5
	IL-12	1 ± 0	1 ± 0	2 ± 0
	CCL5	34 ± 8	35 ± 10	35 ± 9
+ CD40LT	IL-6	710 ± 124	830 ± 243	810 ± 208
	IL-10	360 ± 123	371 ± 148	357 ± 145
	IL-12	34 ± 15	26 ± 15	29 ± 15
	CCL5	1190 ± 373	1012 ± 358	1032 ± 318
+ Poly I:C	IL-6	820 ± 147	760 ± 174	820 ± 218
	IL-10	38 ± 4	40 ± 5	32 ± 6
	IL-12	15 ± 10	19 ± 14	17 ± 12
	CCL5	2351 ± 741	2327 ± 734	2037 ± 720

We then examined whether aDCs and mDCs could be distinguished by functional characteristics as assessed by cytokine and chemokine production patterns. Both conditions corresponded to increased production of inflammatory cytokines IL-6 and TNF-α, of IL-10, and of CCL5, but profiles differed as to higher IL-12, IL-10 and CCL22 levels for mDCs, and of CCL5 and CCL4 for aDCs. IL-12 is a key cytokine for Th1 cell stimulation [4, 28-30] but IL-10, which is simultaneously produced, may serve as a counter-regulatory signal [31-33]. Time-dependent chemokine production by DCs is important for regulating their migratory capacity to lymph nodes and subsequent interaction with T cells [34]. CCL4 and CCL5, both being CCR5 ligands, are produced by aDCs in response to viral infection [35], and they direct DCs to lymph nodes where their subsequent interaction with T cells elicits CCL22 production, a ligand of CCR4 expressed by T cells that is important for T cell recruitment [23].

The capacity to stimulate autologous T cells after loading DCs with PPD was then evaluated and was found to correlate with their capacity to capture and process Ags. The iDCs, which had the highest capacity to endocytose FITC-dextran, had the highest capacity to stimulate T cells under these conditions, whereas mDCs had the strongest decreased capacity, which correlated with their decreased Ag capture capacity. This autologous T cell reactivity to PPD-loaded DCs, showing a Th0 rather a Th1 response profile, correlated with the DC cytokine production pattern noted with iDCs, aDCs and mDCs, respectively.

We finally examined whether ligation of CD4 and/or CCR5 or CXCR4, HIV gp120 receptor and co-receptors,

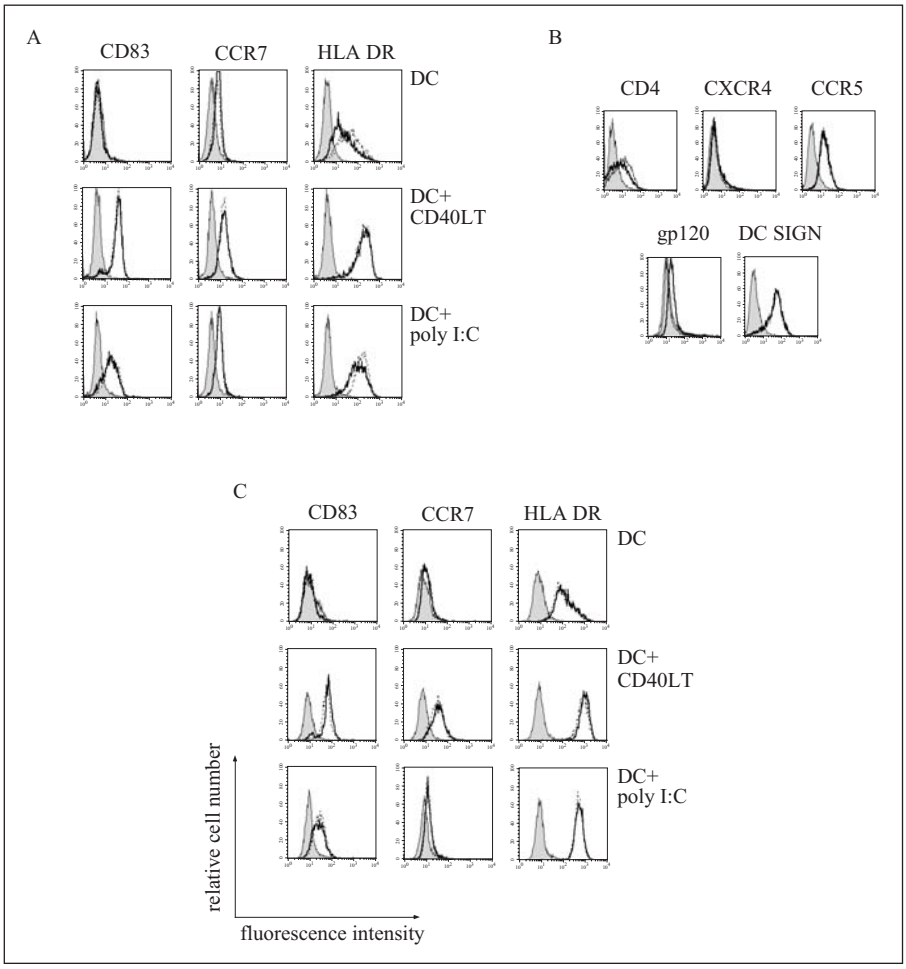

Figure 5

Effect of CD4 ligation or gp120 binding on DC activation and maturation. A. Culture day-5 DCs were incubated or not (control DCs) with SK3 anti-CD4 mAb (SK3-DC), and cultured for a further 2 days with GM-CSF and IL-4 in the presence or not (DC) of 2 µg/ml CD40LT (DC + CD40LT) or poly I:C (DC + poly I:C): histograms represent specific staining of control DCs (solid line) or SK3-DCs (dotted line); shaded histograms represent staining with an irrelevant isotype control mAb; data are from one experiment out of three. B. Culture day-5 DCs were incubated or not for 1 hr at 4 °C with 2 µg/ml gp120, after which they were collected and labeled for CD4, CXCR4, CCR5, DC-SIGN and gp120 cell surface expression using specific mAbs; shaded histograms represent control staining with an irrelevant isotype control mAb, thin and bold histograms represent the indicated staining in the absence or in the presence of gp120, respectively; data are from one experiment out of seven. C. Culture day-5 DCs were incubated or not (control DCs) with 2 µg/ml gp120 (gp120 DCs) for 1 hr at 4 °C and cultured for a further 48 hrs with GM-CSF and IL-4 in the presence or not (DC) of 2 µg/ml CD40LT (DC + CD40LT) or poly I:C (DC + poly I:C); histograms represent specific staining of control DCs (solid line) or gp120 DCs (dotted line); shaded histograms represent staining with an irrelevant isotype control mAb; data are from one experiment out of seven.

respectively, could modify the phenotypic and functional patterns displayed by DCs exposed to poly I:C or to CD40LT signals. This showed that neither the individual ligation of these receptors by CD4 mAbs or by their natural ligands nor gp120 binding to DCs affected their activation and maturation processes. Moreover, gp120 binding to the cells did not result in significant rise of intra-cytoplasmic Ca^{2+} (data not shown). Our data are consistent with those of Kawamura *et al.* that show that DC pretreated with gp120 do not exhibit impaired MHC class II and co-stimulatory molecule expression, or altered allogeneic stimulatory capacity. They also show that HIV gp120-treated DCs retain the ability to respond to further maturational stimuli. Although the consequences of HIV infection of DCs are controversial [36-41], our data are in line with findings that *in vitro* or *in vivo* HIV infection does not significantly affect DC differentiation, phenotype or Ag-presenting function [11, 12, 39].

In conclusion, our data indicate that a limited set of phenotypic markers (CD83, CD86, HLA-DR, CCR5, CCR7, CXCR4), and cytokine (IL-6 and IL-10 more than IL-12) and chemokine (CCL4, CCL5, CCL22) production assays may be used to distinguish the three stages in the life of DC, namely immaturity, activation and full maturation. Using such markers, it was found that CD4, CCR5 and CXCR4 ligation, either separately by their specific ligands or in association, by HIV gp120, did not affect the monocyte-derived DC activation/maturation process.

Acknowledgements. We thank Immunex (Seattle, WA, USA) for the gift of CD40LT, and the NIH AIDS Research and Reference Reagent Program (Rockville, MD, USA) for providing recombinant gp120 samples. We are grateful to Dr. Claudio Vita (CEA, Gif sur Yvette, France) and to Dr. Françoise Baleux, (Institut Pasteur, Paris, France) for sharing with us CCL5 and CXCL12, respectively, and to

Dr. Ali Amara (Institut Pasteur) for the gift of anti-gp120 mAb 110H. We finally thank Dr. Sarah Boudaly for helpful advice in discussing and revising the manuscript.
This work was supported by the Agence Nationale de Recherche contre le SIDA, the Association de Recherche contre le Cancer, the Comité de Paris de la Ligue Nationale contre le Cancer, and the Association pour la Recherche sur les Déficits Immunitaires Viro-Induits (Paris, France).

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