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Extensive characterization of dendritic cells generated in serum-free conditions: regulation of soluble antigen uptake, apoptotic tumor cell phagocytosis, chemotaxis and T cell activation during maturation *in vitro*

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Dendritic cells (DC) play a key role in the initiation of primary immune response, and pilot clinical studies have demonstrated their ability to induce efficient antitumor immunity. However, the DC used in these clinical trials were generated with various serum sources and were poorly characterized. Obtaining fully characterized DC in controlled and reproducible culture conditions is thus of major interest. We demonstrate that X-VIVO 15 medium supplemented with 2% human albumin can be used to obtain DC. The phenotypic and functional characteristics of these clinical-grade DC were analyzed according to their differentiation stages. CD83⁻ immature DC, obtained in the presence of granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4, were able to endocyte soluble antigens and internalize apoptotic tumor cells, and also expressed receptors for inflammatory chemokines. Tumor necrosis factor-α (TNF-α) induced irreversible DC maturation in association with a decreased ability to uptake antigens and an increased allostimulatory capacity. CD83⁺ mature DC became responsive to EBI1 ligand chemokine (ELC), a chemokine specifically expressed in secondary lymphoid organs. In addition, mature DC obtained with TNF-α produced IL-12 and some IL-10 in response to CD40 stimulation. In conclusion, we present well-defined culture conditions allowing the control of DC maturation for clinical or fundamental studies. *Leukemia* (2000) **14,** 2182–2192.

Keywords: dendritic cells; cellular differentiation; immunotherapy; chemotaxis

Introduction

Very promising clinical trials have been reported recently using dendritic cells (DC) pulsed with tumor peptides or tumor cell lysates.[1–8] However, these DC were obtained with either fetal calf serum (FCS), allogenic or autologous human plasma, or serum, and in some cases, with monocyte conditioned medium. Moreover, several points remain unclear, especially concerning the traffic and homing of reinfused DC. Given the complex biology of DC, there is an urgent need to define methodologies making it possible to generate DC in well-defined and reproducible culture conditions. In addition, it seems necessary to ascertain that these clinical-grade DC will have all the recently identified biological properties that are assumed to be important to their function *in vivo*.

DC undergo a differentiation pathway in two major stages, an immature and a mature stage, according to a set of phenotypic and functional characteristics.[9,10] Immature DC obtained *in vitro* from monocytes by culture with granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 or GM-CSF and IL-13 are analogous to peripheral tissue DC, ie Langerhans cells and interstitial DC. These immature DC are able to pick up antigen (Ag) with high efficiency using specialized receptors, such as Fc receptors (FcR),[11,12] mannose receptor (MR)[13] and phagocytic receptors, especially CD36 and the $\alpha v\beta 5$ integrin.[14] They can thus internalize proteins, whole cell lysates, RNA, and apoptotic cells. In contrast, they only express low levels of costimulatory molecules necessary for T cell activation. Following exposure to maturating signals, mainly given by Ag, inflammatory cytokines or bacterial products, DC lose their phagocytic and endocytic capacities[13,14] but increase MHC class I, MHC class II, CD80, and CD86 expression and become highly potent Ag presenting cells (APC). The transition from immature to mature DC is associated with a switch in chemokine receptor expression. Maturing DC down-regulate CCR1 and CCR5, the receptors for inflammatory chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β and RANTES, and concomitantly they up-regulate CCR7, the receptor for EBl1 ligand chemokine (ELC)/MIP-3β and secondary lymphoid-tissue chemokine (SLC) which are expressed constitutively in secondary lymphoid organs.[15–18] These changes in chemokine receptor expression are important for the *in vivo* traffic of DC. Immature DC are recruited by inflammatory chemokines into Ag entry sites. Upon activation by Ag and inflammatory stimuli, they lose CCR1 and CCR5 and acquire CCR7 expression. Mature DC could then enter lymphatic vessels and migrate to the T cell area of draining lymph nodes where they present Ag-derived epitopes to naive and memory T cells.

These various characteristics of DC have been reported individually by different investigators using different culture conditions. In particular, all studies concerning chemokine receptor modulation and phagocytosis of apoptotic cells have been performed only with DC obtained in the presence of FCS or human serum. Xenogenic Ag may be immunodominant and hamper the development of specific antitumor immunity.

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Several groups have thus focused on the generation of monocyte-derived DC in FCS-free conditions using medium supplemented with 1–10% of autologous plasma,[3,4,19–23] autologous serum,[24] or pooled human AB serum.[6,20,25–27] However, even autologous serum could be a problem since it contains numerous proteins, especially antibodies[28] which could modify Ag capture and processing. In addition, some tumor Ag such as MUC-1 in several cancers or monoclonal immunoglobulin in multiple myeloma are present in serum at variable levels.

We and others have previously reported obtaining DC that are able to activate T lymphocytes in serum-free media alone.[29–34] However, these DC were not fully characterized according to the recent biological properties reviewed above. In this report, we detail the different stages of DC maturation, ie Ag uptake, DC migration and T cell activation, in fully defined culture conditions. We demonstrate that immature DC could be reproducibly generated from cancer patients in serum-free culture medium supplemented only with human albumin (HA). These immature DC could phagocyte apoptotic tumor cells and endocyte soluble Ag via MR. Their maturation into highly efficient APC could be induced by the association of tumor necrosis factor-α (TNF-α) and prostaglandin E2 (PGE2) instead of monocyte-conditioned medium and render them responsive to ELC/MIP3-β. In particular, PGE2 greatly enhance DC migration induced by this chemokine, as previously reported for spontaneous DC locomotion.[21] This may guide DC trapping into lymphoid organs after *in vivo* reinjection.

Materials and methods

Generation of DC

Apheresis cells (AC) were collected from patients with MM during mobilization of hematopoietic precursors with cyclophosphamide and recombinant human granulocyte colony-stimulating factor (G-CSF, filgrastim; Neupogen, Amgen-Roche, Neuilly-sur-Seine, France) and they were frozen in liquid nitrogen. AC were thawed, washed twice and allowed to adhere to plastic for 2 h in RPMI 1640 supplemented with 2% of clinical-grade HA (LFB, Courtaboeuf, France). Non-adherent cells were discarded and adherent cells were cultured in the presence of 100 ng/ml of GM-CSF (Leucomax; Sandoz, Basel, Switzerland) and 25 ng/ml of IL-4 (R&D Systems, Minneapolis, MN, USA) for 7 days either in RPMI 1640 supplemented with 10% FCS (reference medium) or in X-VIVO 15 (BioWittaker, Walkersville, MD, USA) supplemented with 5% AB serum, 5% autologous serum, 5% autologous plasma or 2% HA. HA batches were tested before use for their ability to support DC growth from three different donors. Four different HA batches were screened for the present study and one of them was excluded on the basis of a poor cell viability after 7 days of DC culture. We chose X-VIVO 15 because it is a serum-free culture medium already used for clinical-grade generation of cells. It contains purified albumin, recombinant transferrin and recombinant insulin. Fresh medium containing GM-CSF and IL-4 was added on days 2 and 4. After 5 days of culture, GM-CSF and IL-4-containing medium was added with (mature DC) or without (immature DC) inflammatory mediators: TNF-α (R&D Systems) at 20 ng/ml, PGE2 (Sigma Chemical, St Louis, MO, USA) at 1 μg/ml, IL-1 (R&D Systems) at 10 ng/ml, IL-6 (provided by Sanofi, Montpellier, France) at 3 ng/ml. After 48 h, cells were collected and analyzed for surface Ag expression by flow cytometry or used for functional studies. The cell yield was calculated as follows (cell number at day 7 of culture/cell number put into culture) × 100(%). To study the stability of DC phenotype, immature and mature DC cultures were washed out of cytokines and replated for 3 days in culture medium without cytokine. Cell yield, cell morphology and cell phenotype were then analyzed.

Phenotypic analysis by flow cytometry

To characterize the DC phenotype, the following monoclonal antibodies (MoAbs) were used: CD1a-PE, CD14-PE, CD36-FITC, CD80-PE, CD83-PE, HLA-DR-FITC (Immunotech, Marseille, France); CCR5-PE, CD51/CD61-FITC, CD86-FITC, MR-PE (Pharmingen, San Diego, CA, USA) and the isotype-matched murine IgG (Immunotech). For $\alpha v\beta 5$ staining, cells were first incubated with an $\alpha v\beta 5$ primary MoAb (Chemicon, Temecula, CA, USA) followed by FITC-conjugated goat anti-mouse Ig (Immunotech). Analyses were performed with a FACScan apparatus (Becton Dickinson).

Endocytosis through MR

Endocytosis at the cellular level was studied using lysine-fixable FITC-dextran, MW = 40 000 (Molecular Probes, Eugene, OR, USA) as previously described.[35] Immature and mature DC were collected at day 7 and incubated at 37°C for 7, 15 and 30 min or at 4°C for 30 min (background uptake) with 1 mg/ml of FITC-dextran. DC were then washed with cold PBS supplemented with 1% FCS and 0.02% NaN_3, and fluorescence was analyzed with a FACScan apparatus.

Induction of apoptosis in malignant plasma cells

XG-1 is a multiple myeloma cell line previously established in our laboratory whose characteristics have been described in detail elsewhere.[36] XG-1 cells (2.5 × 10^5/ml) were incubated with 4 μg/ml of cycloheximide (CHX) in RPMI 1640–10% FCS supplemented with 3 ng/ml of IL-6 at 37°C. The kinetic of cell apoptosis was monitored using double staining with Annexin-V FITC (Boehringer Mannheim, Meylan, France) and propidium iodide (PI) (Sigma) as previously described.[14] Early apoptotic cells were stained only by Annexin-V (Annexin-V^+/PI), whereas secondary necrotic cells also incorporated PI due to a loss of membrane integrity (Annexin-V^+/PI^+). After CHX treatment, tumor cells were washed three times in X-VIVO 15–2% HA before coculture with DC.

Phagocytosis of tumor cells

Immature and mature DC were dyed green using PKH67-GL (Sigma) and cultured for 2 h to allow release of unbound dye. XG-1 cells were dyed red using PKH26-GL (Sigma) according to the manufacturer's instructions before being induced to undergo apoptosis by CHX for 6–8 h. Then red-dyed XG-1 cells were cocultured with immature or mature green-dyed DC at a ratio of 1:1 in X-VIVO 15–2% HA.[14] After 90 min at 37°C, green and red fluorescences were analyzed with a FACScan. In blocking experiments, XG-1 cells and DC were coincubated at 4°C.

Table 1 Phenotypic analysis of DC obtained in different culture conditions from patients with MM

Medium	Cytokines	Cell yield (%)	Viability (%)	Mean % of positive cells (MFI)[a]				
				CD14	HLA-DR	CD83	CD80	CD86
RPMI-FCS	GM/IL-4	9	93	10 (32)	100 (180)	3	80 (72)	82 (53)
	GM/IL-4/TNF	10	94	4	100 (465)	86 (47)	98 (120)	90 (126)
	GM/IL-4/TNF/PGE2	10	95	3	100 (417)	94 (65)	98 (190)	100 (188)
XV-AB serum	GM/IL-4	12	92	80 (52)	100 (115)	0	77 (32)	90 (22)
	GM/IL-4/TNF	14	90	25 (69)	100 (224)	25 (40)	95 (60)	85 (50)
	GM/IL-4/TNF/PGE2	13	86	40 (50)	100 (173)	52 (80)	90 (60)	100 (95)
XV-autologous plasma	GM/IL-4	18	90	40 (35)	100 (90)	0	25 (30)	90 (65)
	GM/IL-4/TNF	19	95	20 (29)	100 (125)	20 (55)	35 (42)	90 (110)
	GM/IL-4/TNF/PGE2	23	92	12 (60)	100 (145)	80 (65)	80 (58)	110 (160)
XV-autologous serum	GM/IL-4	22	89	50 (35)	100 (95)	9 (12)	28 (26)	85 (68)
	GM/IL-4/TNF	25	90	26 (66)	100 (120)	17 (25)	39 (33)	90 (92)
	GM/IL-4/TNF/PGE2	23	93	40 (70)	100 (115)	59 (66)	72 (68)	95 (150)
XV-HA	GM/IL-4	16	89	21 (13)	100 (172)	0	82 (46)	85 (80)
	GM/IL-4/TNF	16	94	5	100 (270)	55 (43)	100 (110)	95 (97)
	GM/IL-4/TNF/PGE2	20	90	3	100 (251)	85 (66)	100 (136)	100 (133)

Adherent AC from four MM patients were cultured concurrently with GM-CSF and IL-4 in RPMI 1640-10% or in X-VIVO 15 medium supplemented with 5% AB serum, 5% autologous plasma, 5% autologous serum or 2% HA. At day 5, fresh medium was added with GM-CSF and IL-4, GM-CSF, IL-4, and TNF-α or GM-CSF, IL-4, TNF-α, and PGE2. At day 7, cells were harvested, counted, and the percentages of cells expressing CD14, HLA-DR, CD83, CD80, and CD86 were determined by FACS analysis. Data are means obtained with the four patients.
[a]The mean of fluorescence intensity (MFI) obtained with each MoAb is indicated in parentheses. The MFI obtained with isotype-matched control antibodies was always set between 4 and 6.

Chemotaxis assay

Immature and mature DC (2×10^5 cells) were added in 200 μl of RPMI-1% HA in the upper chamber of a 5-μm pore Transwell (Costar, Cambridge, MA, USA) with 600 μl of ELC/MIP-3β diluted at 100 ng/ml in the same medium in the lower chamber. After a 4-h incubation at 37°C, cells which had migrated into the lower chamber were collected and counted microscopically. Results are expressed as the percentage of input cells that migrated into the lower chamber (percentage of migrating cells).

Cytokine analysis

Immature and mature DC obtained after 7 days of culture in X-VIVO 15–2% HA were harvested, washed, and plated at 4×10^5/ml in RPMI 1640–5% FCS with or without CD40L-transfected L cells (10^5/ml, a gift from Sem Saeland, Schering-Plough, Dardilly, France). When indicated, recombinant human IFN-γ (1000 U/ml, R&D Systems) was added. Supernatants were collected 30 h after stimulation and stored at –70°C. Amounts of IL-10 and p70 IL-12 were measured by ELISA according to the manufacturer's protocols (R&D Systems).

Allogenic mixed lymphocyte reaction (MLR)

Resting T lymphocytes were purified from healthy volunteers' peripheral blood by two cycles of negative selection using CD14- and CD19-coated microbeads (Dynal, Oslo, Norway) followed by a cocktail of CD16, CD56, and HLA-DR MoAbs (Immunotech) and goat anti-mouse Ig microbeads (Dynal). The purity of $CD3^+$ T cells was >97%. Graded numbers of mitomycin-treated DC (50 μg/ml) were added to 1.5×10^5 allogenic T cells in 200 μl of RPMI-5% AB serum. After 5 days of culture, T cell proliferation was measured by tritiated thymidine incorporation (1 μCi/well) during the last 12 h. The results are expressed as mean counts per minute (c.p.m.) ± s.d. determined in sextuplet culture wells.

Statistical analysis

For each culture condition (GM-CSF + IL-4, GM-CSF + IL-4 + TNF, and GM-CSF + IL-4 + TNF + PGE2), experiments were repeated with cells from at least six different patients and statistical significance was assessed by the nonparametric Mann–Whitney test or the nonparametric Wilcoxon test for pairs.

Results

Optimization of culture conditions for reproducible generation of serum-free DC

In our earlier experiments, X-VIVO 15 medium alone was shown to substain DC generation.[31] The cell yield was 9% of AC put into culture. However, we observed increased adherence of cells to culture plates, resulting in decreased cell recovery on day 7. In addition, in some cases, significant DC death occurred, whereas DC generated in the presence of FCS were always fully viable. To improve DC generation in FCS-free medium, we tested several culture conditions, including various concentrations of AB serum, autologous plasma, autologous serum, and HA. We found that 5% of AB serum, autologous plasma and autologous serum or 2% of HA were optimal concentrations for cell yield. The percentage of viable cells obtained after 7 days of culture in the presence of GM-

Table 2 Phenotypic analysis of DC obtained in XV-HA supplemented with GM-CSF, IL-4, and TNF-α with or without PGE2

Cytokines	*CD14*	*HLA-DR*	*CD83*	*CD80*	*CD86*
GM/IL-4TNF					
Mean percentage ± s.d.	2.6 ± 8.2	100	64 ± 19	100	100
Mean MFI + s.d.	—	299 ± 183	44 ± 7	140 ± 49	251 ± 211
GM/IL-4/TNF/PGE2					
Mean percentage ± s.d.	1.5 ± 4	100	83 ± 12[b]	100	100
Mean MFI ± s.d.	—	265 ± 124	61 ± 25[a]	190 ± 85	345 ± 271

Adherent AC from 15 patients were cultured in X-VIVO 15 medium supplemented with 2% HA. At day 5, fresh medium was added with GM-CSF, IL-4, and TNF-α with or without PGE2. Cells were harvested on day 7 and the percentage of cells expressing CD14, HLA-DR, CD83, CD80, and CD86 was determined by FACS analysis.
[a]Indicated $P < 0.01$ when compared to GM/IL-4/TNF group.
[b]Indicates $P < 0.05$ when compared to GM/IL-4/TNF group.

Table 3 Receptor profile of serum-free DC

Culture conditions	*Mean % of positive cells ± s.d. (MFI)*			
	MR	*CD36*	*$\alpha v\beta 3$*	*$\alpha v\beta 5$*
GM/IL-4	98 ± 2 (233)	88 ±5 (89)	0	87 ± 10 (43)
GM/IL-4/TNF	80 ± 15 (95)*	37 ± 13 (47)**	0	68 ± 8 (32)*
GM/IL-4/TNF/PGE2	74 ± 15 (91)*	25 ± 10 (33)**	0	58 ± 20 (36)*

Adherent AC from six patients were cultured for 5 days in X-VIVO 15–2% HA supplemented with GM-CSF and IL-4. At day 5, fresh medium was added with either GM-CSF and IL-4 alone, with GM-CSF, IL-4, and TNF-α or with GM-CSF, IL-4, TNF-α and PGE2. On day 7, the expression of MR, CD36, $\alpha v\beta 3$, and $\alpha v\beta 5$ was studied by FACS analysis. Data are expressed as mean values with DC from the six patients.
[a]Indicates $P < 0.01$ when compared to GM/IL-4 group.
[b]Indicates $P < 0.05$ when compared to GM/IL-4 group.

CSF and IL-4 was 93% with FCS, 92% with AB serum, 90% with autologous plasma, 89% with autologous serum and 89% with HA (Table 1). As cell viability was only 72% in X-VIVO 15 alone, X-VIVO 15 alone was not further used. The primary read-out system was cell yield and membrane phenotype (CD14, HLA-DR, CD80, CD83, CD86) to discriminate between DC and monocyte/macrophage. These experiments were repeated with AC from four different MM patients. As detailed in Table 1, the cell yield obtained in the presence of GM-CSF and IL-4 reached 12% with AB serum, 18% with autologous plasma, 22% with autologous serum, and 16% with HA. X-VIVO 15–2% HA supplemented with GM-CSF and IL-4 was the most efficient culture condition for obtaining clinical-grade $CD14^{-/low}$ $CD83^{-}$ $HLA\text{-}DR^{++}$ immature DC expressing high amounts of CD80 and CD86. The overall phenotype of these DC was very similar to that of immature DC generated in the presence of FCS (Table 1). Using X-VIVO 15 medium supplemented with AB serum, autologous plasma, or autologous serum, the percentage of $CD14^{+}$ cells was much higher (up to 80% in X-VIVO15-AB serum), and the resulting cells expressed a lower density of HLA class II and costimulatory molecules. Thus, starting from adherent-AC, X-VIVO 15–2% HA was able to support the generation of a high number of DC ($CD14^{-}$, $HLA\text{-}DR^{++}$, $CD80^{++}$, $CD86^{++}$) without the addition of unidentified xenogenic proteins, allogenic proteins, human antibodies or autologous tumor antigens. We obtained similar results with AC from 15 different patients with MM (data not shown).

Having established an optimal serum-free culture medium for obtaining DC, we next attempted to identify a combination of adjuvants allowing full maturation of these DC as evaluated by up-regulation of CD80 and HLA-DR molecules and induction of CD83 expression. TNF-α, IL-1, IL-6, and PGE2 have recently been shown to improve the maturation of DC generated in FCS-free conditions. We tested these stimuli in each culture medium by adding them on day 5 of culture for 2 additional days. CD83, a specific marker of mature DC was detected after 24 h and reached maximum expression within 48 h (data not shown). Again, X-VIVO 15–2% HA was proven to be the best culture medium for obtaining clinical-grade mature DC (Table 1). A combination of PGE2 and TNF-α induced CD83 expression in up to 83% of cells, compared to 64% with TNF-α alone ($P = 0.007$) (Table 2). PGE2 also cooperated with TNF-α for CD80 and CD86 upregulation on DC (Tables 1 and 2). When PGE2 was added on day 5 without TNF-α, DC maturation was completely inhibited, as shown by the absence of CD83 expression on day 7 (results not shown). In addition, the percentage of $CD14^{+}$ cells was higher in the presence of GM-CSF + IL-4 + PGE2 than with GM-CSF and IL-4 alone, suggesting that PGE2, when used without TNF-α, induced at least some immature DC to revert to macrophage-like cells, although GM-CSF and IL-4 were continuously present in the culture (data not shown). In the same way, when immature DC, obtained in X-VIVO15–2% HA supplemented with GM-CSF and IL-4, were harvested on day 7, extensively washed and replated in culture medium without any cytokine for 3 additional days, they again adhered to plastic culture flasks and expressed CD14 (data not shown). This is consistent with reversion of these immature DC to macrophage-like cells, as previously described.[19] In contrast, the cell morphology and cell phenotype of mature DC generated by the addition of TNF-α alone or TNF-α + PGE2 for 2 days were not markedly affected after withdrawal of cytokines, indicating an irreversible mature stage. It should be noted that in our

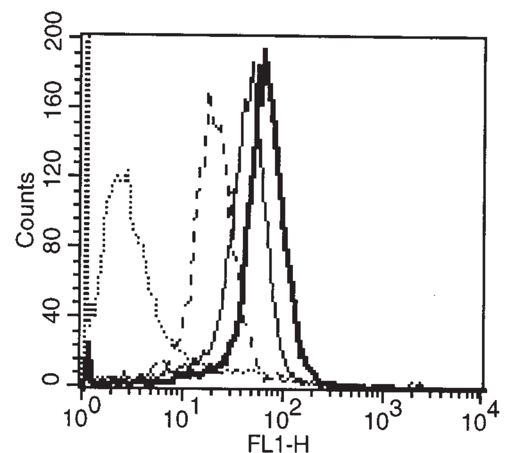
culture conditions IL-1 and IL-6 had no additional effect on DC maturation. Given the superiority of X-VIVO 15–2% HA as a DC culture medium, it was subsequently used for functional DC characterization.

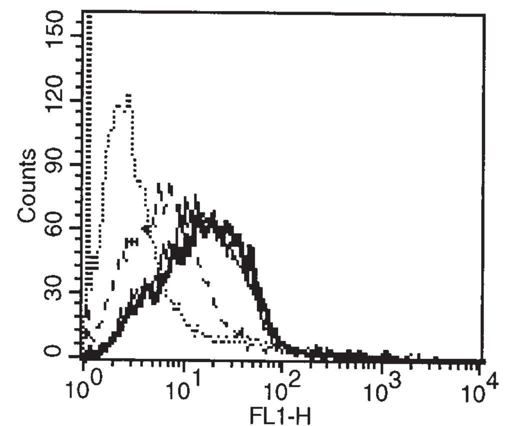
Maturation of serum-free DC decreases Ag uptake

Several observations have demonstrated that immature DC efficiently capture Ag using several mechanisms. One of them is soluble Ag endocytosis through MR. Immature DC obtained in X-VIVO 15–2% HA by a 7-day culture with GM-CSF and IL-4 highly expressed MR, and this expression was significantly decreased by more than 50% with DC maturation induced by TNF-α alone ($P = 0.03$) or by TNF-α + PGE2 ($P = 0.03$) (Table 3). Similarly, endocytosis of Dextran-FITC by mature DC obtained with TNF-α or TNF-α + PGE2 was substantially decreased compared to immature DC (Figure 1). These results confirm those previously reported for DC generated in the presence of FCS. Phagocytosis of apoptotic cells is another way of Ag entry and plays a major role in the cross-priming phenomenon. Recently, several phagocytic receptors were identified on DC obtained in the presence of human serum, and it was shown that monocyte-conditioned medium, which leads to irreversible DC maturation, down-regulates their expression.[14] In X-VIVO 15–2% HA medium, immature DC expressed high levels of CD36 and $\alpha v\beta 5$ integrin. In these culture conditions, $\alpha v\beta 3$ integrin was not detected (Table 3). In the presence of TNF-α, CD36 and $\alpha v\beta 5$ expression was significantly decreased by more than half ($P = 0.002$) and by 20–35% ($P = 0.03$), respectively. PGE2 had no additive effect with TNF-α on the decrease of phagocytic receptor expression. We used apoptotic myeloma cells to test the phagocytic potential of DC generated in X-VIVO 15–2% HA. As shown in Figure 2, after 6 h of culture with 4 μg/ml of CHX, 60% of the XG-1 myeloma cells displayed features of early apoptotic cell death, ie binding of Annexin-V but no incorporation of PI. XG-1 cells were dyed red, induced to apoptosis and then cocultured at 37°C with green dyed immature or mature DC. Data from one representative experiment out of three are shown in Figure 3. More than a third of immature DC engulfed apoptotic XG-1 after 90 min of coculture. Only 10–12% of immature DC were double stained after coculture with non-apoptotic XG-1 cells (data not shown). The uptake of myeloma cells by immature DC was visually confirmed on cytospins of dyed cocultures (data not shown). Phagocytosis was completely blocked by low temperature (Figure 3). Induction of DC maturation yielded a decrease in phagocytic activity. Indeed, only 12% of mature DC obtained after the addition of TNF-α internalized apoptotic XG-1 after 90 min of coculture (Figure 3). The same decrease in phagocytosis occurred with mature DC obtained with TNF-α + PGE2 (Figure 3).

Maturation of serum-free DC modifies their response to chemokines

CCR5, a receptor for inflammatory CC chemokines, was detectable on immature DC generated in X-VIVO 15–2% HA, but its expression was significantly reduced by a 48-h incubation with TNF-α ($P = 0.03$). PGE2 did not induce a further significant decrease (Figure 4) ($P = 0.25$). As no MoAb was available to measure CCR7 expression, we tested the response of our clinical-grade DC to MIP-3β in a chemotaxis assay. The





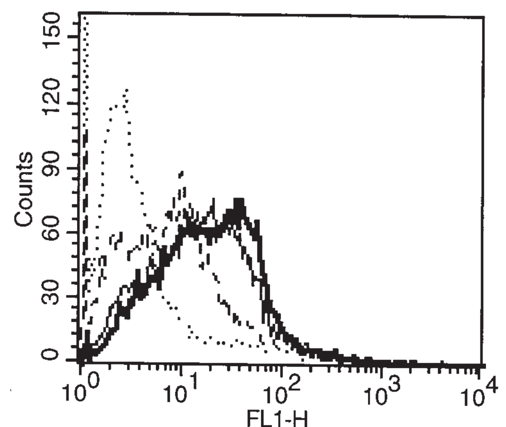


Figure 1 Effect of DC maturation on FITC-Dextran endocytosis. Immature and mature clinical-grade DC were incubated with FITC-Dextran for 7 min (broken lines), 15 min (solid lines), and 30 min (bold lines) at 37°C. Dotted lines represent the uptake background at 4°C. Immature DC (a) were obtained by 7 days of culture in X-VIVO 15–2% HA supplemented with GM-CSF and IL-4 and mature DC were generated by the addition of TNF-α alone (b) or TNF-α and PGE2 (c) for the last 48 h of culture.

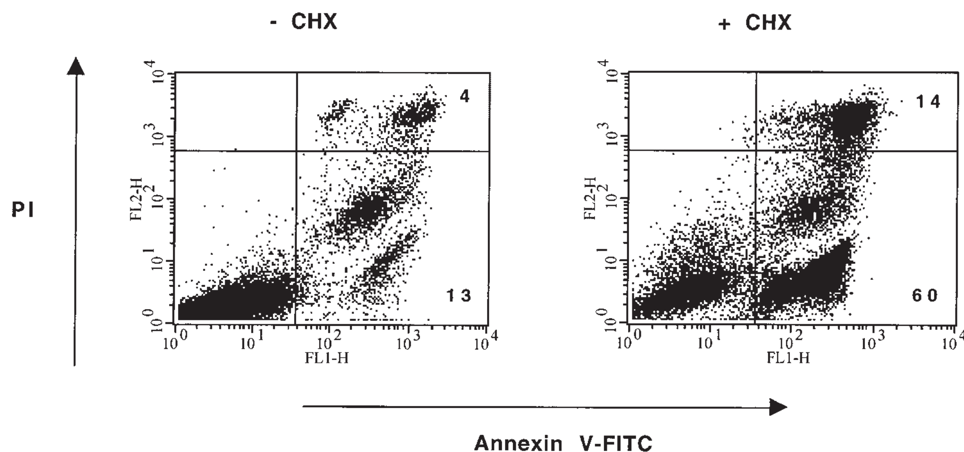


Figure 2 Cycloheximide induces apoptosis of the XG-1 myeloma cell line. XG-1 cells were incubated with or without 4 μg/ml of cycloheximide (CHX) in RPMI 1640–10% FCS supplemented with IL-6. After 6 h, myeloma cells were labeled with Annexin V-FITC and PI. Early apoptosis is defined by Annexin^{+}/PI^{-} staining, whereas late apoptotic and necrotic cells were Annexin^{+}/PI^{+}.

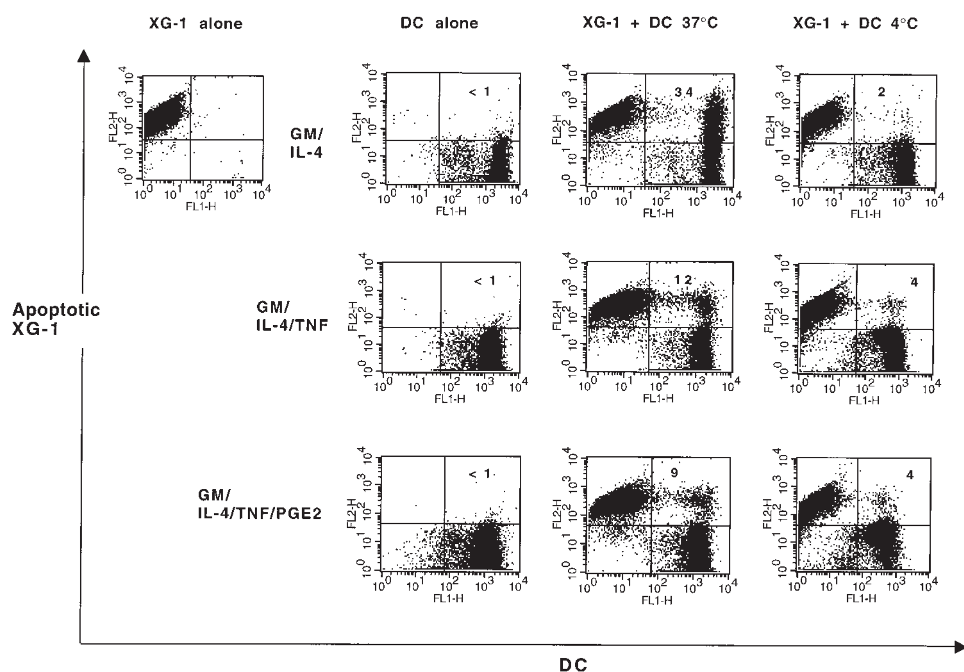
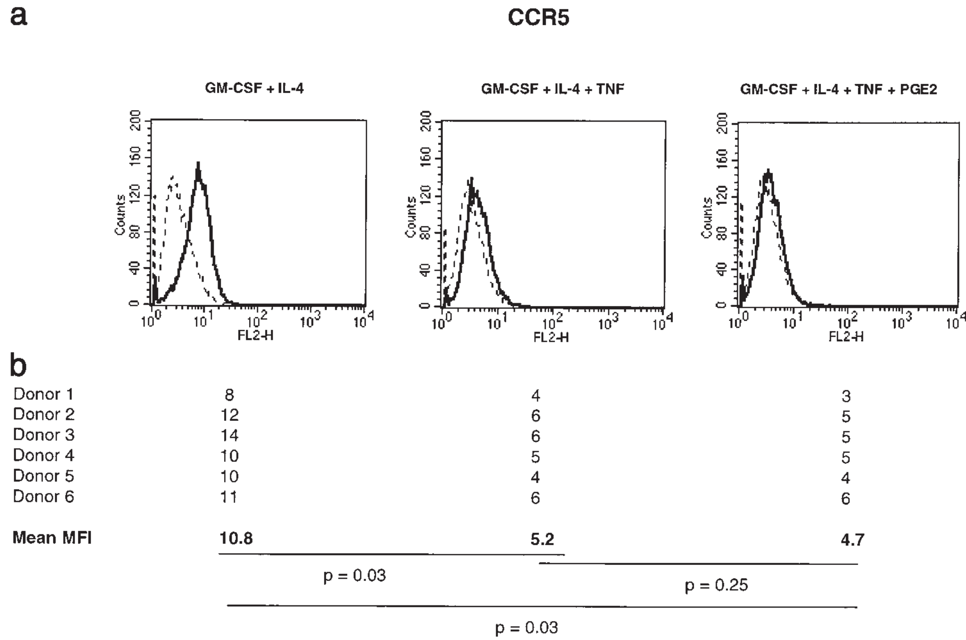


Figure 3 Immature but not mature DC phagocytose apoptotic tumor cells. XG-1 cells were labeled with the PKH26-GL red fluorescent cell-linker compound and induced to undergo apoptosis by cycloheximide for 6 to 8 h. Immature and mature DC were dyed green with PKH67-GL and cocultured with apoptotic XG-1 at 37°C or 4°C at a ratio of 1:1. After 90 min, cells were analyzed by FACScan. Uptake of apoptotic XG-1 cells by DC was evaluated by the percentage of double positive cells. The results are representative of one experiment out of three.

migration of DC from one patient in the absence and presence of MIP-3β is shown in Figure 5a, and the data obtained for six patients with MIP-3β are outlined in Figure 5b. Immature DC cultured with GM-CSF and IL-4 did not respond to MIP-3β (mean percentage of migrated cells: 0.7%, n = 6). The addition of TNF-α on day 5 significantly enhanced the DC response (P = 0.002), with a mean of 14.2% migrating cells (n = 6). Interestingly, PGE2 acted synergistically with TNF-α since 31% to 67% (mean: 48.8%, n = 6) of TNF-α + PGE2-maturated DC migrated to the lower chamber of the transwell during a 4-h incubation at 37°C (P = 0.002 when compared to TNF-α-matured DC). This was associated with a slight increase in spontaneous DC migration (a mean of 7% of input DC were found in the lower chamber in the absence of MIP-3β).

Maturation of serum-free DC modifies their cytokine production

We assessed the production of IL-10 and IL-12 by immature and mature DC in response to CD40 ligation. In some experiments, IFN-γ was also added. Immature DC generated with GM-CSF/IL-4 failed to produce p70 IL-12 but produced very high amounts of IL-10 after CD40 triggering (Table 4). The addition of IFN-γ combined with CD40 stimulation resulted in a 30-fold decrease in IL-10 production by CD40-activated immature DC. Induction of DC maturation with TNF-α resulted in a dramatic decrease in CD40-induced IL-10 production (mean reduction of 10-fold) in association with the induction of IL-12 expression. The addition of IFN-γ further inhibited IL-10 production by mature DC. Note that for one



	GM-CSF + IL-4	GM-CSF + IL-4 + TNF	GM-CSF + IL-4 + TNF + PGE2
Donor 1	8	4	3
Donor 2	12	6	5
Donor 3	14	6	5
Donor 4	10	5	5
Donor 5	10	4	4
Donor 6	11	6	6
Mean MFI	**10.8**	**5.2**	**4.7**

p = 0.03 (GM-CSF + IL-4 vs GM-CSF + IL-4 + TNF); p = 0.25 (GM-CSF + IL-4 + TNF vs GM-CSF + IL-4 + TNF + PGE2); p = 0.03 (GM-CSF + IL-4 vs GM-CSF + IL-4 + TNF + PGE2)

Figure 4 Effect of DC maturation on CCR5 expression. CCR5 expression was analyzed on immature DC obtained by adherent AC-culture in X-VIVO 15–2% HA medium supplemented with GM-CSF and IL-4 for 7 days and on mature DC obtained by the addition of TNF-α or TNF-α + PGE2 from day 5 to 7. The results obtained from one patient are illustrated in the upper panels (a) and the mean fluorescence intensity (MFI) obtained with DC from six different patients are indicated in the table (b). The MFI obtained with isotype-matched control antibody was set between 3 and 5.

patient the addition of IFN-γ combined with CD40 stimulation induced IL-12 production by immature DC and enhanced IL-12 production by mature DC. This is in accordance with previous reports showing that IFN-γ could be a cofactor for CD40-induced IL-12 production.[37,38] However, for the other three patients' tested samples, IFN-γ reduced IL-12 production by DC obtained in the presence of GM-CSF/IL-4 and TNF-α. Finally, the induction of a fully mature DC with TNF-α and PGE2 resulted in a reduced IL-10 and IL-12 production after CD40 stimulation as compared to TNF-α alone.

Maturation of serum-free DC enhances their ability to stimulate allogenic T cells

As shown in Figure 6 for one representative experiment out of five, maturation of DC obtained in X-VIVO 15–2% HA made them as potent allogenic T cell stimulators as mature DC generated in RPMI-10% FCS. TNF-α alone gave the same results as the association of TNF-α and PGE2.

Discussion

The aim of our study was to obtain DC with fully identified biological properties in order to improve the vaccination protocols in patients with cancers. First, the use of fully defined culture conditions will allow better reproducibility in obtaining DC, reduce the internalization and presentation of unidentified xenogenic, allogenic, or autologous proteins, and thus limit immune responses that are not specific to tumor antigens. Second, these clinical-grade DC should be able to capture *in vitro* tumor Ag by endocytosis of proteins or by phagocytosis of apoptotic cells. Third, after *in vivo* injection, these DC should be able to migrate to lymph nodes in order to present antigen-derived peptides to T lymphocytes. Fourth, these DC should produce cytokines favoring differentiation of naive $CD8^+$ T cells into cytotoxic T cells. Fifth, since it is not possible to control the DC environment after *in vivo* infusion, the injected DC should have a stable phenotype after withdrawal of cytokines used during *ex vivo* culture.

We have shown here that X-VIVO 15 supplemented with 2% HA was an efficient medium to get clinical-grade DC that have all of these characteristics. This culture medium allows a fully viable DC with a cell yield of 16% to be obtained, starting from fresh or frozen AC. A 5 h-apheresis makes it possible to harvest approximatively 40 to 50×10^9 cells. Thus, 6 to 8×10^9 DC can be reproducibly generated, allowing at least six vaccinations with 10^9 DC. Since the use of a closed culture system should be more safe than culture plate of flask, we are currently testing clinical-grade culture bags recently shown to support DC generation from adherent PBMC.[39] Some investigators have shown that monocyte-conditioned medium could help in obtaining mature DC in FCS-free culture medium.[19,20] As this maturating effect seemed to be mainly due to the presence of TNF, IL-1 and IL-6,[21] we have looked for the best cytokine combinations making it possible to control DC maturation *in vitro*. We found that IL-1 and IL-6 did not improve maturation of DC compared with TNF-α alone. On the contrary, addition of PGE2 can further increase this maturation.

Having defined the best culture condition to obtain immature and mature DC, we studied their biological properties in view of a therapeutic use. Immature $CD83^-$ $CD14^{low}$ DC obtained in the presence of GM-CSF and IL-4 expressed HLA-DR, CD80, and CD86 as well as endocytic and phagocytic receptors, namely MR, CD36, and $\alpha v\beta 5$. Several studies demonstrate that tumor Ag requiring internalization and processing must be added to immature DC.[40] Thus, these immature DC could be pulsed with proteins, tumor cell lys-

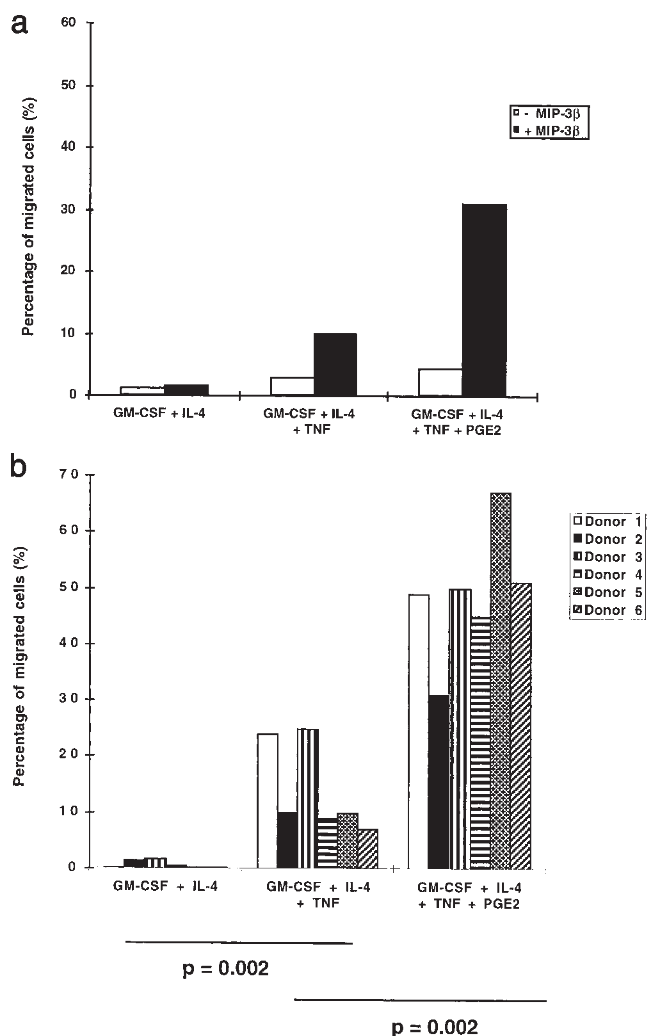


Figure 5 Mature but not immature DC migrate in response to ELC/MIP-3β. Migration of immature and mature DC is expressed as the percentage of input cells that migrate into the lower chamber of a 5-μm pore Transwell during 4-h incubation at 37°C. This assay was performed on DC obtained from six different patients. The difference of cell migration in the absence and presence of MIP-3β is shown for DC from one patient (a) and the percentage of cells induced to migrate in the presence of MIP-3β is shown for DC obtained from six different patients (b).

ates, or RNA. Endocytosis of mannose receptor-targeted tumor Ag, in particular mannan-MUC-1, by immature DC could also be an interesting way to generate antitumor immunity in cancers that express underglycosylated MUC-1. Obtaining DC in a serum-free medium, in particular in X-VIVO 15 medium supplemented with HA, would be a prerequisite to get such an anti-MUC-1 CTL response. In fact, human serum contains natural anti-Galα(1,3)Gal antibodies that crossreact with MUC-1 and switch the anti-MUC-1 immune response from cellular to humoral after immunization with MUC-1 conjugated with mannan.[28] In addition, immature clinical-grade DC obtained in X-VIVO 15–2% HA were able to phagocyte apoptotic tumor cells, as previously reported for the phagocytosis of apoptotic monocytes. A recent report has shown that immature phagocytic DC derived from CD34+ hematopoietic precursors can capture Ag from irradiated apoptotic acute myeloid leukemia cells and induce autologous CTL against leukemic cells.[41] Stimulation of immature DC obtained in X-VIVO 15–2% HA with TNF-α combined with GM-CSF and IL-4 for 2 additional days led to obtaining cells corresponding phenotypically and functionally to mature DC. These cells expressed CD83 and higher levels of HLA-DR, CD80, and CD86 compared to GM/IL-4 immature DC. They were able to activate allogenic T cells with the same efficiency as mature DC obtained in the presence of FCS. In addition, these mature DC only weakly expressed endocytosis receptors such as the mannose receptor or phagocytosis receptor, eg $\alpha v\beta 5$ and CD36. Similarly, these mature DC had a reduced ability to endocyte dextran or to phagocyte apoptotic tumor cells. Depending on the HLA phenotype of cancer patients, such mature DC could be pulsed *in vitro* with identified antigenic tumor peptides, especially derived from MAGE-type genes which bind directly to MHC molecules and could be used to obtain antitumor CTL. The different steps of DC maturation *in vitro* are summurized in Figure 7.

The response to chemokines was similarly modulated for DC obtained with defined culture medium and DC obtained in FCS-containing medium. In fact, immature DC obtained in X-VIVO 15–2% HA expressed CCR5 and did not respond to MIP-3β. Thus, after *in vivo* injection, these cells would probably be preferentially trapped into inflammatory sites where MIP-1α, MIP-1β or RANTES are produced. This could explain why In^{111}-labeled immature DC generated in AIM V serum-free medium and injected in cancer patients are not found in lymph nodes.[30] In contrast, after TNF-α treatment, DC obtained in X-VIVO 15–2% HA lost CCR5 expression and became responsive to MIP-3β in a Transwell assay. It could be hypothesized that a higher proportion of these mature DC would be trapped in T cell areas of lymph nodes where MIP-3β is produced and would initiate an efficient antitumor immune response.

We have shown that PGE2, which was previously shown to enhance DC maturation in FCS-free media,[21,27] can play a role in DC migration. In our culture conditions, PGE2 did not substantially modify the phenotype of DC generated with GM/IL-4 and TNF, except for an increase in CD83 expression, and had no additive effect with TNF-α for T cell activation. However, PGE2 enhanced DC migration in response to MIP-3β by increasing CCR7 expression and/or through the stimulation of DC motility, as previously described.[21] It could be very profitable to direct DC migration into lymphoid organs.

In order to induce the generation of antitumor cytotoxic T cells, DC should drive the differentiation of naive T cells toward the type 1 subset expressing IFN-γ and IL-2. IL-12 is the main cytokine involved in the polarization of CD4+ T cells toward Th1 cells. In the model of anti-EBV T cell response, it has been demonstrated that IL-10 expression by lymphoblastoid cell lines is associated with the emergence of type 2 CD8+ T cells[42] that produce IL-4 and IL-10 and are non-cytotoxic.[43] On the contrary, type 1 CD8+ T cells produce IFN-γ and IL-2 and are cytotoxic. Thus, DC generated *in vitro* for antitumor vaccination purposes should ideally produce IL-12 and no IL-10. Also, IL-10 has an additional deleterious effect on DC maturation. In fact, DC treated with IL-10 during the maturation phase induce antigen-specific anergy of CD4+ and CD8+ T cells.[30,44] DC obtained in X-VIVO 15–2% HA, unlike those obtained in the presence of FCS,[45,46] produced only slight amounts of IL-12 but high amounts of IL-10 in response to CD40 ligation, in agreement with studies showing that myeloid DC were able to produce IL-10, particularly following CD40 stimulation.[47,48] However, we show that TNF-α-induced DC maturation resulted in the induction of IL-12 production and dramatic inhibition of IL-10 synthesis upon

Table 4 Cytokine production by serum-free DC

Culture conditions	Cytokine production (pg/ml)					
	No stimulation		CD40 stimulation		CD40 + IFN γ stimulation	
	IL-10	IL-12	IL-10	IL-12	IL-10	IL-12
XV-HA GM/IL-4	25 ± 9 (16–35)	0	1619 ± 529 (1105–2360)	5.5 ± 6.3 (0–11)	50 ± 57 (14–115)	158 ± 274 (0–476)
XV-HA GM/IL-4/TNF	0	0	137 ± 104 (62–285)	84 ±23 (55–105)	7 ± 7 (0–21)	299 ± 518 (0–898)
XV-HA GM/IL-4/TNF/PGE2	0	0	64 ± 61 (0–145)	7.2 ± 8.8 (0–18)	0	16 ± 29 (0–50)

Adherent AC from four patients were cultured in X-VIVO 15 medium supplemented with 2% HA. At day 5, fresh medium was added with GM-CSF, IL-4, and TNF-α with or without PGE2. Cells were harvested on day 7 and cultured at 4×10^5/ml in medium alone, in the presence of CD40 L-transfected L cells or with CD40 L-transfected L cells and IFN-γ. After 24 h, supernatants were harvested and cytokine concentrations were determined by ELISA. The results are expressed as the mean ± s.d. of four experiments and the ranges are indicated in parentheses.

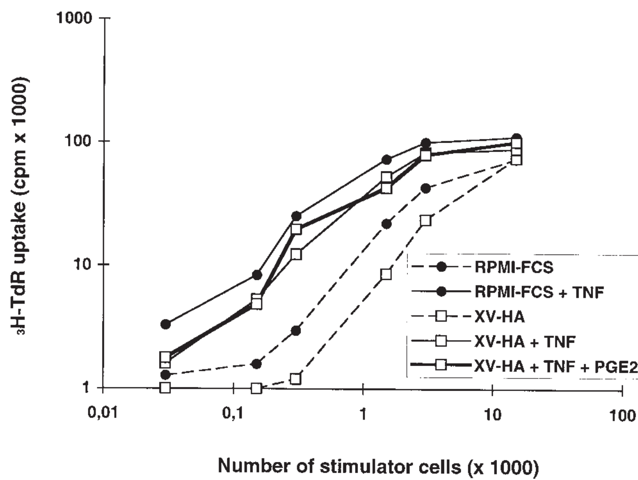


Figure 6 Mature DC efficiently activate allogenic T cells. Adherent AC were cultured with GM-CSF and IL-4 in X-VIVO 15–2% HA or in RPMI 1640–10% FCS. At day 5, fresh medium was added with GM-CSF and IL-4, GM-CSF + IL-4 + TNF-α or GM-CSF + IL-4 + TNF-α + PGE2. At day 7, DC were harvested, mitomycin-treated and used in graded numbers as stimulator cells for 1.5×10^5 purified allogenic T cells. Cell proliferation was evaluated by a 12-h pulse with ^{3}H-TdR after 5 days of coculture. Data are the mean ^{3}H-TdR incorporation determined in sextuplet culture wells. The ^{3}H-TdR incorporation rate of purified T cells was less than 400 c.p.m. The results are representative of one experiment out of five.

CD40 activation. Thus, these mature DC should be able to trigger the differentiation of naive T cells into type 1 T cells. The addition of PGE2 further inhibited IL-10 production but also IL-12 production, in agreement with a recent study.[45] However, mature DC obtained in X-VIVO 15–1% autologous plasma in the presence of TNF-α, IL-1, IL-6 and PGE2 were recently shown to be potent inducers of anti-melanoma cytotoxic CD8$^+$ T cells.[44] Additional studies should be performed to conclusively determine the benefit of adding PGE2 to obtain DC for immunotherapy strategies.

Finally, we have shown that mature DC generated with X-VIVO 15 medium, 2% HA, GM-CSF, IL-4 and TNF with or without PGE2 had a stable phenotype after cytokine withdrawal. These results are in accordance with those of Jonuleit and coworkers[21] who used X-VIVO 15 supplemented with 1% autologous plasma to generate DC, although Romani and colleagues[19] reported that TNF-α, contrary to monocyte conditioned medium, is not sufficient to obtain irreversible DC maturation in RPMI 1640-FCS.

Recently, several DC-based therapeutical trials have been performed in a wide range of cancers and have led to very interesting biological and sometimes clinical results. Surprisingly, these studies have not used DC obtained in fully defined culture conditions. In follicular lymphoma[5] and multiple myeloma,[8] DC were purified from peripheral blood by a series of density gradient centrifugations. Aside from the low frequency of DC precursors in blood, circulating DC constitute a heterogeneous compartment that comprises a variable proportion of immunostimulating and tolerogenic DC.[47] In melanoma, Nestle *et al*[1] used immature monocyte-derived DC generated in the presence of FCS. Immature tumor-Ag pulsed DC, generated without FCS, were also reinfused to patients with prostate cancer.[3,4] The ability of these cells to migrate to lymph nodes in order to activate tumor-specific CTL was not discussed in these works. To circumvent this problem, Thurner *et al*[2] have recently used in patients with advanced melanoma, a homogeneous population of mature monocyte-derived DC obtained without xenogenic protein but in the presence of autologous uncontrolled monocyte-conditioned medium and human plasma. Theoretically, even allogenic or autologous serum should be avoided since they contain proteins such as antibodies or tumor antigens that could interfere with further DC use. Such a protocol was also used in renal cell carcinoma[6,7] but, in that case, the maturation step was performed in the presence of TNF-α and PGE-2 without any characterization of cytokine production.

In conclusion, generating mature DC by controlling the critical steps of DC generation and homing *in vivo* remains an important challenge for the development of immunotherapy strategies.

Acknowledgements

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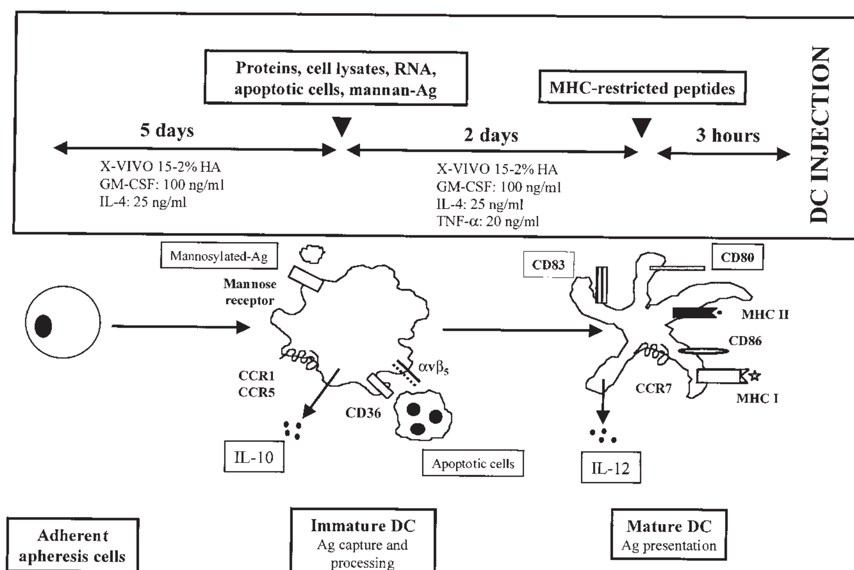

Figure 7 DC maturation in serum-free medium. Immature DC obtained in X-VIVO 15-HA in the presence of GM-CSF and IL-4 could be used to capture tumor antigens that required internalization and processing such as proteins, whole cell lysates, RNA, apoptotic cells or mannose-receptor targeted peptides. After *in vitro* maturation in the presence of TNF-α, DC could be pulsed with HLA-restricted peptides such as the MAGE antigens and used to generate antitumor CTL *in vitro* or after *in vivo* reinfusion to the patient.

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