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Adenoviral transduction of human 'clinical grade' immature dendritic cells enhances costimulatory molecule expression and T-cell stimulatory capacity

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Abstract

The therapeutic use of dendritic cells (DC) in antigen-specific anti-tumor vaccines, requires sufficient numbers of functional DC, the preparation of which should comply with the code of Good Manufacturing Practice. In addition, the expression of tumor specific antigen should be possible in these DC. As a preclinical step, the method reported here was developed in healthy volunteers. Monocytes (Mo) were isolated by leukapheresis from 12 donors, purified by elutriation and then cultured for 6 days in sealed bags in AIM-V serum free medium with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-13 (IL-13). Between 6×10^8 and 1×10^9 immature DC (iDC) could be differentiated from one leukapheresis. Cells displayed a characteristic iDC phenotype ($CD1a^+$, $CD14^-$, $CD80^+$, $CD86^+$, HLA DR^+, $CD83^-$), and had potent allogeneic and antigen dependent autologous T cell-stimulatory capacity. Moreover, iDC could be further differentiated into mature DC by CD40 ligation as assessed by CD83 expression and the upregulation of HLA-DR and costimulatory molecules. After infection with a recombinant adenovirus encoding for beta-galactosidase (βGal), 50% to 80% of iDC expressed βGal without toxicity. Adenovirus infection increased the expression of both costimulatory molecules and CD83, and also increased allogeneic stimulatory capacity. Thus, the method developed here allows us to use large numbers of functional iDC as will be required for therapeutic uses in man. These DC can express a transgenic protein.

Keywords: Human dendritic cell-Anti-tumor vaccination-Adenoviral transduction

Abbreviations: PBMC, peripheral blood mononuclear cell; PBL, peripheral blood lymphocyte; DC, dendritic cell; Mo, monocyte; CTL, cytotoxic T lymphocyte; S.D., standard deviation

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1. Introduction

The recent introduction of dendritic cells (DC) as antigen presenting cells in anti-tumor vaccination in man is based on data from fundamental research (Banchereau and Steinman, 1998) and murine experimental models (Schuler and Steinman, 1997). DC can present tumor antigens by fusing with tumor cells or by pulsing with crushed tumor, the peptides eluted from MHC class I molecules or tumor-derived RNA (Pardoll, 1998). To apply such strategies to human vaccination requires a sufficient quantity of fresh tumor, which in many diseases is not always possible. Alternatively, a gene encoding a tumor-specific antigen can be introduced into DC, so that the antigen will be expressed and presented as an endogenous protein by MHC class I molecules. Murine models have be used to demonstrate the ability of DC transduced by recombinant adenovirus to induce an anti-tumoral response in vivo (Gong et al., 1997; Ribas et al., 1997; Wan et al., 1997) and, more importantly, to induce regression of pre-established tumors (Song et al., 1997). When human DC are differentiated from peripheral blood monocytes (Mo), transduction with recombinant adenovirus of more than 50% of DC can be achieved (Arthur et al., 1997; Dietz and Vuk-Pavlovic, 1998; Diao et al., 1999; Diebold et al., 1999; Zhong et al., 1999). Human transduced DC are able to generate transgene-specific, class I-restricted CTL in PBL cultures from normal donors (Butterfield et al., 1998), but the ability of transduced DC to induce human tumor regression in vivo has not yet been reported.

The current use of DC in therapeutic protocols in man is limited by two restrictions: obtaining sufficient numbers of DC and the need to comply with the code of 'Good Manufacturing Practice' (GMP) which is often far removed from the protocols used for in vivo animal models or for in vitro research. DC can be generated in large numbers either from hemopoietic $CD34^+$ progenitors, or from peripheral blood monocytes (Mo) (Hart, 1997). DC from both sources can stimulate allogeneic mixed lymphocyte reactions (MLR) and present antigen to autologous T cells. We have developed the parameters for leukapheresis collection, Mo elutriation and in vitro differentiation of Mo-derived immature DC (iDC) from healthy volunteers in accordance with the GMP guidelines. We demonstrate in this report that iDC transduction by a recombinant beta-galactosidase (βGal) adenovirus not only preserves, but enhances, APC function. The protocol could be used in clinical trials of anti-tumor vaccination.

2. Methods

2.1. Leukocyte apheresis

In this study 12 healthy volunteer donors (25–43 years old) were subjected to leukapheresis at the Clinical Investigation Center of Saint-Antoine Hospital after informed consent was obtained. This study was approved by the institutional review board of the Henri Mondor Hospital. We used a Spectra cell separator (Cobe BCT, Lakewood, CO, USA) with the 'white blood cell set' and the mononuclear cell program according to the manufacturer's instructions. During the leukapheresis, acid-citrate-dextrose formula A (ACD formula A, Cat. No SD 1 008 0A, Macopharma, Tourcoing, France) was used at a ratio of 1:10–12. The collection flow-rate was 1 ml/min and the procedure was performed over 2 h.

2.2. Mononuclear cell separation

To minimize platelet contamination, peripheral blood mononuclear cells (PBMC) were washed once with BF 77 solution (Bruneau Laboratories, Boulogne-Billancourt, France) by centrifugation at 200 g for 10 min. The PBMC were then directly elutriated by counterflow centrifugation using a J-6M/E rotor (Beckman, Fullerton, CA, USA) in a 4 ml elutriation chamber as described previously (van Es and Bont, 1980). Briefly, BF 77 solution supplemented with 0.16% (w/v) human albumin (LFB, Courtaboeuf, France) was used as the flow medium during the elutriation process. Elutriation was performed at 15°C at 1300 g, at a constant rotor speed. The flow-rate was increased by steps of 1 ml/min from 13 to 19 ml/min. When the monocyte-rich fraction was detected in the Coulter Channelyzer-256 (Beckman Coulter, Inc, Fullerton, CA, USA) the rotor was turned off and the Mo were collected. The purity of the elutriated monocyte-rich fraction was determined using an EPICS® XL flow cytometer (Coulter). Mo

were gated in side scatter (SSC)/forward scatter (FSC) or identified by CD14 positivity in whole elutriated cell preparations using Mo2 (Coulter) anti-CD14 monoclonal antibody (mAb).

During the elutriation process, peripheral blood lymphocytes (PBL) were isolated and then frozen in 4% (w/v) human albumin with 10% DMSO (Sigma, St. Louis, MO, USA) for later use as autologous responders in functional assays.

When necessary, T cells were purified (purity >95%) from autologous elutriated PBL by negative selection using a cocktail of hapten-modified antibodies (CD11b, CD16, CD19, CD36 and CD56) followed by anti-hapten magnetic microbeads (pan T cell isolation kit; Miltenyi Biotec Inc, Auburn, CA, USA) according to the manufacturer's instructions.

PBL for use in allogeneic mixed leukocyte reactions (MLR) were collected by elutriation as described above from one unrelated healthy donor and cryopreserved in aliquots.

2.3. Culture medium and cytokines

DC were differentiated in serum free medium AIM-V glutamax (Life Technologies, Inc., Gaithersburg, MD, USA) supplemented with 100 UI/ml penicillin and 100 μg/ml streptomycin (Life Technologies Inc.). All functional assays were performed with RPMI 1640 supplemented with 10% heat-inactivated human AB-serum (Transfusion Sud-Est Francilien, Rungis, France), 1% non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (all obtained from Life Technologies Inc.).

Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) was purchased from Schering-Plough (Levallois Perret, France) and recombinant human IL-13 was kindly provided by Sanofi (Sanofi Recherche, Labège, France).

2.4. Immature dendritic cell generation.

Materials and practical facilities for in vitro DC generation were chosen according to GMP instructions. Mo were seeded in sealed gas-permeable bags (Cat. No.R2111 Baxter Healthcare corporation, Deerfield, IL) at 37°C in a humidified CO_2-containing atmosphere. Mo were cultured under non adherent conditions at a density of 2×10^6 cells per ml in total volumes of 70 to 100 ml. Cell differentiation was induced with 500 IU/ml GM-CSF and 50 ng/ml recombinant human IL-13. After 3 days, 50 ng/ml IL-13 were re-introduced into the culture. Cells obtained after 6 days in culture were defined as immature DC (iDC D6). iDC D6 viability was assessed by trypan blue exclusion. Cell yield was determined as the number of iDC D6 (DC morphology using light microscopy)/number of Mo on day 0 (FSC/SSC gate of the Mo population).

2.5. Dendritic cell maturation

Mature DC on day 8 (mDC D8) were obtained by culturing 1×10^6 iDC D6 with 2.5×10^5 irradiated (70 Gy) mouse fibroblastic L cells transfected with the human CD40Ligand (L-CD40L) (Fayette et al., 1997) (kindly provided by F.Brière, Schering Plough, Dardilly, France) in AIM-V glutamax serum-free medium in 6-well plates for 48 h. Adherent mDC D8 were removed with PBS 0.53 mM EDTA (Sigma) and washed in RPMI 1640 with 10% human AB-serum. We have established that L-CD40L, which could contaminate the mDC harvested on day 8, did not react with the antibodies used to characterize the mDC membrane phenotype.

2.6. Monoclonal antibodies

For membrane staining, the following mAbs conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were used: CD40 (MAB89, IgG1), CD80 (MAB104, IgG1), CD83 (HB15A, IgG2b) and CD64 (22, IgG1) (all obtained from Immunotech, Marseille, France); CD86 (IT2.2, IgG2b) (PharMingen, San Diego, CA, USA); CD1a (B-B5, IgG1) (Diaclone Besançon, France); HLA-DR (L243, IgG2a) (Becton Dickinson, San Jose, CA, USA); CD14 (Mo2, IgM), CD2 (T11, IgG1) (Beckman Coulter, Inc.). Corresponding isotype-matched control mAbs were used to establish background fluorescence.

2.7. Flow cytometry analysis

Cells were washed with staining medium (PBS, 2% fetal calf serum (FCS) (Techgen, Les Ulis,

France) and incubated on ice with conjugated-antibody protected from light. After 30 min, cells were washed with staining medium, then with PBS, and fixed with 1% para-formaldehyde (Sigma). At least 10 000 events were acquired for each test. Stained cells were analyzed using an EPICS® XL flow cytometer (Beckman Coulter, Inc.). Data analysis was performed with System II (2.1) software (Beckman Coulter, Inc.). Antigenic density was expressed as the mean fluorescence intensity (MFI).

Apoptosis was evaluated by the appearance of phosphatidylserine residues on the cell surface by labeling with an Annexin V-FITC kit (Bender MedSystems Diagnostics, Vienna, Austria) according to the manufacturer's instructions. Propidium iodide (PI) was added to exclude necrotic cells from the analysis.

2.8. Mixed lymphocyte reaction (MLR) assay

Stimulator iDC D6 were irradiated (30 Gy) and added in graded doses (312 to 8×10^4 cells) to allogeneic PBL (1×10^5/well). Cells were cultured in RPMI 1640 complete medium (see above) in 96-well round-bottomed plates. T cell proliferation was measured on day 5 of culture by a 16-h pulse with 1 μCi/well ^{3}H-thymidine (Amersham, Buckinghamshire, UK). ^{3}H-thymidine incorporation into DNA was measured using microplate scintillation counter filters (Top Count, Packard Instruments, Meriden, CT, USA). Proliferation assays were performed in six replicates, and results were expressed as mean counts per min (cpm)±S.D. (standard deviation).

2.9. Tetanus toxoid (TT) presentation assay

TT was purchased from Pasteur Mérieux Connaught (Marcy l'Etoile, France). The activity of TT was 4250 Lf/ml which was diluted in 0.9% (w/v) NaCl to achieve 425 Lf/ml and stored at 4°C before use. 1×10^4 iDC were cocultured with a constant number of 1×10^5 purified autologous T cells (see above). The cultures were set up in RPMI 1640 with 10% human AB-serum in the presence of increasing amounts of TT. The 96-well round bottom plates were incubated for 5 days at 37°C in an atmosphere of 5% CO_2. The cells were pulsed with 1 μCi of ^{3}H-thymidine, incubated for 16 h and harvested on microplate counter filters (Packard). Tests were performed in quadruplicate and the results expressed as mean cpm±S.D.

2.10. Recombinant βGal Adenovirus (Ad5βGal)

The replication-deficient Adenovirus vector is based on the adenovirus type 5 (Ad5) E1/E3 deletion mutant Ad5dl324 (Rosenfeld et al., 1991). The lacZ reporter gene was inserted in the E1 region under the control of the cytomegalovirus (CMV) promoter. Adenoviruses were amplified and purified as previously described (Eloit et al., 1990). Briefly, the viruses were grown on semiconfluent monolayers of 293 cells, an adenovirus type 5 (Ad5)-transformed human embryonic kidney cell line (ATCC CRL 1573). Adenoviruses were purified from cell lysates by banding twice in CsCl density gradients. Viral products were desalted using a PD-10 column (Amersham Pharmacia biotech, Freiburg, Germany) and stored at −80°C in PBS with 10% glycerol (v/v). The titer of viral stock of Ad5βGal was determined by the plaque-forming unit (pfu) assay using 293 cells. Three independent preparations were used for transduction assays.

2.11. DC transduction with Ad5βGal

Transduction of 2×10^5 DC was performed in suspension with adenovirus at 150 pfu/cell in 800 μl of AIM-V (Life Technologies, Inc) and seeded in 6-well plates. After incubation for 2 h at 37°C in 5% CO_2, the cells were adherent. From each well the supernatant was removed and 2 ml of AIM-V supplemented with 500 UI/ml GM-CSF and 50 ng/ml IL-13 were added. Forty-eight h after transduction the expression of the reporter lacZ gene was assayed by X-gal staining. Briefly, cells were washed once with PBS and fixed with 1% formaldehyde/0.2% glutaraldehyde (Sigma) in PBS for 10 min at room temperature. Fixed cells were rinsed twice with PBS and stained with X-gal substrate solution (PBS pH 7.4, 4 mmol/litre potassium ferrocyanide, 4 mmol/litre potassium ferricyanide, 4 mmol/litre $MgCl_2$ and 0.4 mg/ml X-gal)(Sigma) for 6 to 24 h. The percentage of blue cells was assessed using light

microscopy. The viability of the DC after transduction was determined by trypan blue exclusion.

3. Results

3.1. More than 6×10^8 Mo-derived iDC D6 can be generated in serum-free culture from one leukapheresis

A clinical protocol was developed in healthy donors to determine the optimal procedures for leukapheresis collection, mononuclear cell separation and DC differentiation. Twelve healthy donors were included. The low level of red cell contamination of the leukapheresis products (Table 1) permitted the direct isolation of mononuclear cells without an intermediate Ficoll step.

Twenty-five ml of each 120 ml leukapheresis product were processed by counterflow centrifugal elutriation (Table 1). Elutriation allowed a homogeneous population of Mo ($89\pm5\%$ $CD14^+$) to be obtained in terms of size (FSC) and granularity (SSC) (Fig. 1A). The majority of the $CD14^-$ cells in the elutriated fraction were $CD2^+$. Contamination by polymorphonuclear cells was consistently less than 8%.

For each donor, 2×10^8 elutriated cells were cultured in gas-permeable bags in AIM-V serum-free medium with GM-CSF and IL-13. After 6 days in culture, the cell viability was greater than 95% as estimated by trypan blue exclusion (Table 1). Apoptosis was studied in four donors: $8\pm2\%$ of the total cell population in the culture bags (Fig. 1B) were $Annexin^+$ and less than 4% were necrotic, PI^+. Of the viable iDC D6 population, based on forward (FSC) and side scatter (SSC) characteristics (Fig. 1B, gate R1), only $3\pm1\%$ of the cells were $Annexin^+$. The number of iDC on D6 (as determined by cell morphology) ranged from 1.4×10^8 to 2×10^8 (Table 1).

Thus, using a serum-free medium, the method permitted the differentiation of Mo into DC under non adherent conditions. When applied to the whole leukapheresis product, between 6×10^8 and 1×10^9 iDC D6 could be obtained.

Table 1
Efficiency differentiation of monocytes into immature dendritic cell (iDC)

Leukapheresis[a] (120 ml)	
PBMC	8×10^9 to 2×10^{10}
% monocytes	$20\pm10\%$
Hematocrit	4%
Elutriation process (on 25 ml)	
PBMC	$2.1\pm0.3\times10^9$
Collected monocytes	$3\pm0.85\times10^8$
Elutriation yield[b]	$64\pm12\%$
iDC differentiation in serum free medium (from 2×10^8 monocytes)	
Cell yield[c]	$85\pm15\%$
Viability	$\geq95\%$

[a] Healthy donors $n=12$.
[b] Number of recovered Mo/number of treated Mo in the PBMC fraction×100.
[c] Number of iDC D6 (DC morphology using light microscopy)/number of Mo on day 0 (FSC/SSC gate on Mo population)×100.

3.2. iDC are morphologically and phenotypically heterogeneous

Flow cytometric analysis of iDC D6 revealed an heterogeneous population which was larger in size and more granular than the initial Mo (Fig. 1B). Immunophenotypic analysis of the elutriated fraction before culture indicated a uniform population of Mo which were $CD14^+$, $CD64^+$, $HLA\text{-}DR^+$, $CD40^+$ and $CD86^+$ but which did not express CD1a, CD80 and CD83 (Fig. 1C). After 6 days in culture, an average of 54% of cells were $CD1a^+$ with marked variation between donors (18%–95%) (Fig. 1C). The monocyte/macrophage markers CD64 and CD14 were reduced, whereas CD80, absent in the initial Mo preparation, was found on $78\pm17\%$ of iDC D6. Only 53% of iDC D6 were $CD86^+$ but the intensity of expression was greater than that of the starting monocyte population (data not shown). The most striking phenotypic changes in the iDC D6 as compared to the starting Mo were the increased expression of CD40 (100-fold) and HLA-DR (10-fold) (data not shown). The mature DC marker, CD83 (Zhou and Tedder, 1995) was not detected on iDC D6.

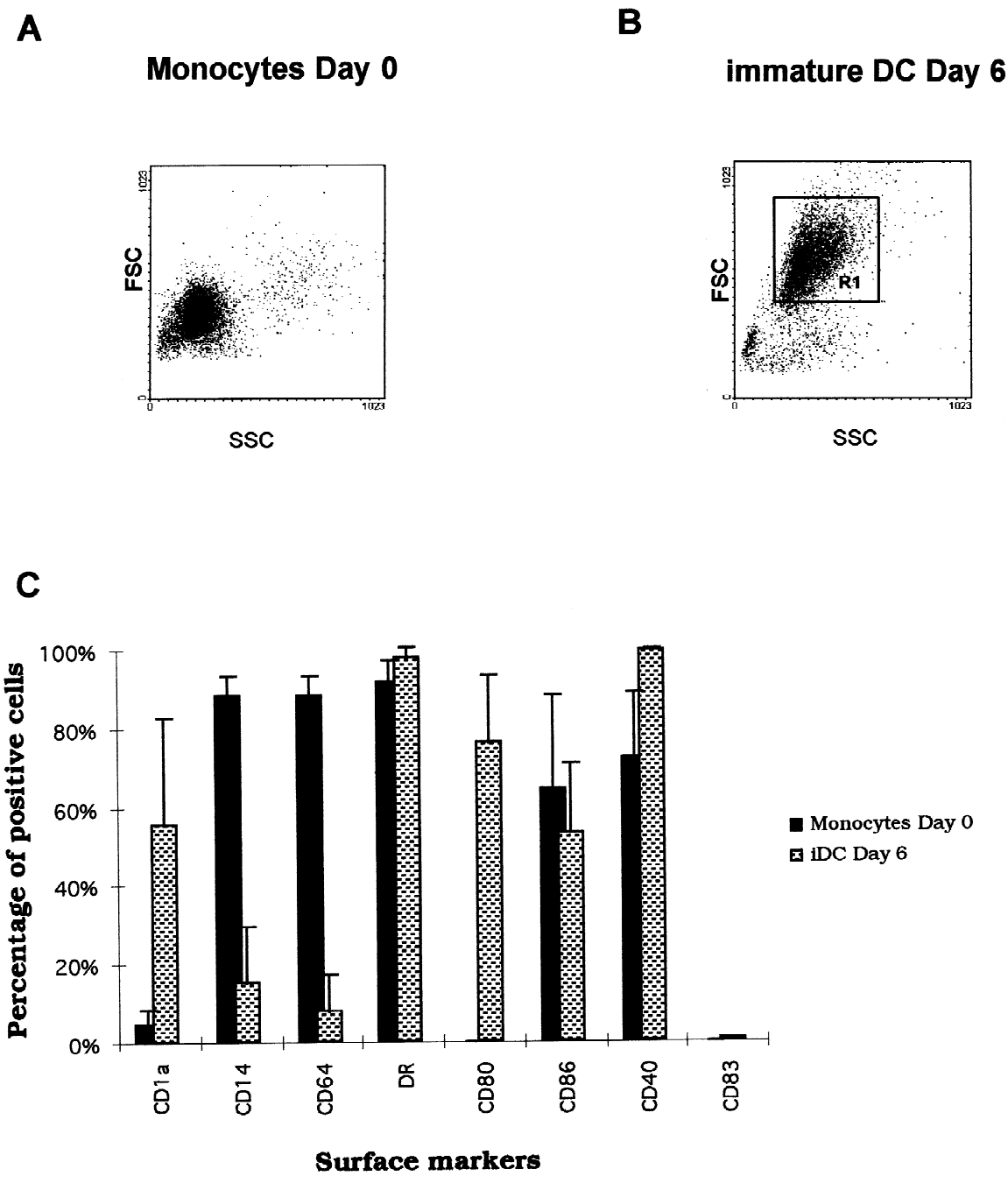

Fig. 1. Mo-derived DC display immature DC phenotype on day 6. Mo on day 0 were isolated by counterflow centrifugation and iDC were derived from Mo cultured with GM-CSF+IL-13 for 6 days using non adherent conditions in AIM-V serum free medium. (A) Mo and (B) iDC D6 differed in their forward (FSC) and side scatter (SSC) properties. (C) Mo and iDC D6 were analysed for the expression of surface markers by direct immunofluorescence. Results are expressed as the percentage of cells with a fluorescence intensity higher than that of appropriate isotype matched controls. Data are the mean±S.D. from 12 independent experiments.

3.3. iDC D6 have a potent allogeneic and autologous T cell-stimulatory capacity

iDC D6 were tested for their ability to induce allogeneic T cell proliferation and antigen specific autologous T cell proliferation. In allogeneic MLR using PBL as responders, a thymidine incorporation peak of 46 000±6000 cpm was obtained at a stimulator (iDC): responder (PBL) ratio of 1:5 (Fig. 2A). Significant thymidine incorporation, 5 times greater than the thymidine incorporation by PBL alone, was always found at a low ratio, i.e. one DC for 320 PBL.

In three donors we evaluated the ability of iDC D6

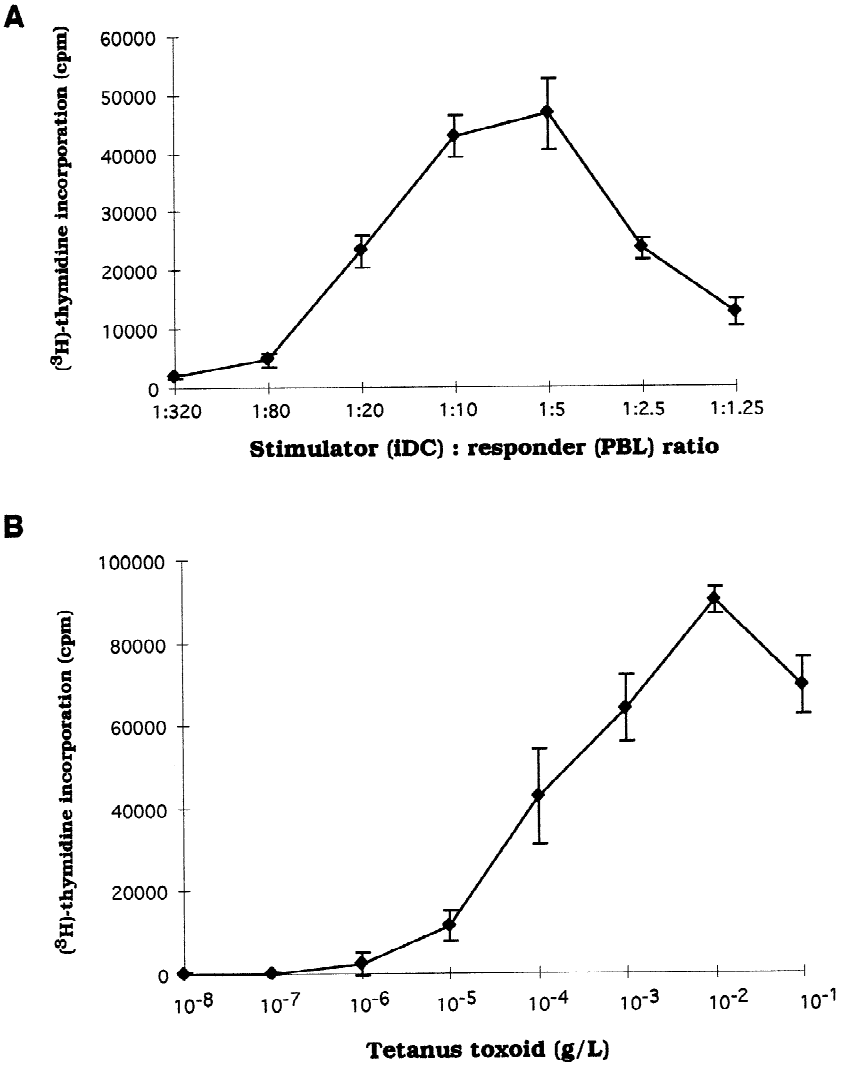

Fig. 2. iDC D6 display efficient functional capacity. (A) Allostimulatory capacity of iDC D6 in a MLR assay: 1×10^5 responder PBL were cultured with graded numbers of irradiated iDC D6 as indicated. T cell proliferation was measured on day 5 of culture by a 16-h pulse with ^{3}H-thymidine. Results are shown as the mean±S.D. of six replicates. Thymidine incorporation of responder cells alone was <250 cpm. The data are representative of eight independent experiments. (B) Presentation of TT to autologous purified T cells: 1×10^4 iDC and 1×10^5 purified T cells were co-cultured with graded doses of TT as indicated. Ag-specific proliferation of T cells was measured on day 5 by a 16-h pulse with ^{3}H-thymidine. Results are shown as the mean±S.D. of four replicates. ^{3}H-thymidine uptake by purified T cells cocultured with iDC D6 without TT (autologous MLR) was <3000 cpm in three independent experiments.

to stimulate purified autologous T cells in the presence of tetanus toxoid (TT). A TT concentration of 10^{-5} g/l was sufficient for pulsed DC to induce a proliferative response (Fig. 2B). In the absence of antigen, iDC did not induce T cell proliferation (autologous MLR). Thus, iDC D6 were effective as stimulator cells in allogeneic MLR and as soluble tetanus toxoid antigen presenting cells.

3.4. iDC D6 are able to differentiate into mature DC

We examined the capacity of iDC D6 to mature into mDC. Adherent iDC D6 were cultured from D6 to D8 in the presence of murine fibroblasts transfected with human CD40-ligand (L-CD40L). iDC rapidly formed aggregates around the L-CD40L and progressively lost their adhesiveness to plastic. mDC were morphologically more heterogeneous than iDC D6 in terms of SSC and FSC characteristics (Fig. 3A). Signaling via CD40 induced phenotypic changes on mDC D8 (Fig. 3B): the expression of the costimulatory molecules CD80 and CD86, and of CD40 was, in each case, increased by more than 1 log. The expression of HLA-DR showed a greater than 2-fold increase. CD1a was down-regulated; only 24% of mDC D8 were $CD1a^{+}$ as compared with 49% of iDC D6 (data not shown). 44% of mDC were positive for CD83 (Fig. 3B). By contrast, control adherent DC cultured from D6 to D8 on plastic in the absence of L-CD40L, exhibited an unchanged phenotype as compared with iDC D6.

3.5. Ad5βGal infection of iDC D6 enhances costimulatory molecule expression and T-cell stimulatory capacity

iDC D6 were infected in suspension with Ad5βGal and then left to adhere. 48 h after infection, 84±13% of the cells were viable as estimated by trypan blue exclusion, and 50–80% of the DC expressed βGal.

We evaluated the phenotypic and functional changes induced by transduction of DC with 150 pfu/cell of Ad5βGal in three donors. Two days after gene transfer, there was an increase in the membrane expression of CD40 and the costimulation molecules CD80 and CD86. Transduced DC expressed these molecules with a higher MFI than control DC cultured in parallel under adherent conditions but without adenovirus vector (Fig. 4A). Twelve per cent of transduced DC were positive for the CD83 maturation marker. The phenotypic changes in transduced DC were less than those induced by CD40 signaling (data not shown).

Untransduced and transduced DC were compared for their stimulatory capacity in the MLR assay (Fig. 4B). To obtain the maximal PBL proliferative response, 2 times less APC were required using transduced DC. Furthermore, when low stimulator (DC): responder (PBL) ratios were used (1:20 and 1:10), transduced DC were also more potent in PBL proliferation compared to untransduced DC.

These results show that iDC D6 infection with recombinant adenovirus enhanced the APC properties of iDC.

4. Discussion

The aim of this study was to define the in vivo conditions for leukapheresis collection and in vitro conditions for the preparation of iDC which could be used in a clinical trial of anti-tumor vaccination. The procedure had to fulfil the following requirements: (1) satisfy the code of Good Manufacturing Practices; (2) provide iDC capable of differentiating into mature DC when given appropriate stimuli; (3) provide iDC which could express a transgene and yet display the phenotypic and functional properties of APC after transduction.

Our protocol for DC differentiation is original in the combination of the following techniques:

(a) the use of peripheral blood Mo (Pickl et al., 1996) obtained by leukapheresis and purified by elutriation. This procedure provides a standardized protocol and the purity of the starting Mo population was always good regardless of the quality of the leukapheresis or the donor;

(b) culture under non adherent conditions, in a closed system, minimizing the risk of infection (Kowalkowski et al., 1998);

(c) the use of serum-free AIM-V medium which is approved for clinical use (Romani et al., 1996;

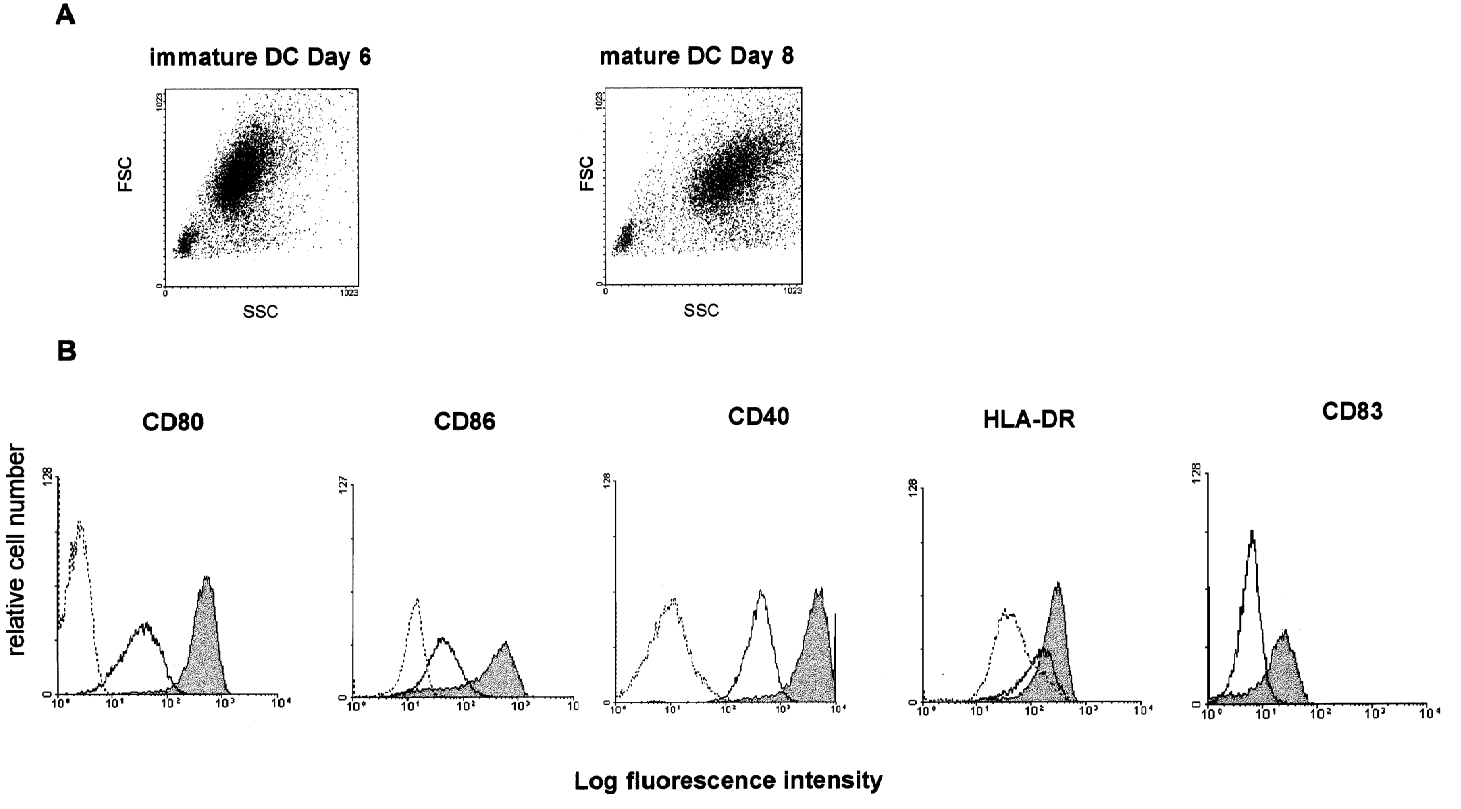

Fig. 3. CD40 ligation induces maturation of iDC. Mature DC Day8 were obtained after coculture for 48 h with L-CD40L. (A) mDC D8 display higher granularity (SSC) than immature DC Day 6. (B) Cell surface expression of activated molecules was compared in Mo day 0 (dotted lines), iDC D6 (solid lines) and mDC D8 (filled histogram). CD40 activated DC expressed CD80, CD86, CD40, HLA-DR and CD83 at high levels. One experiment representative of four is shown.

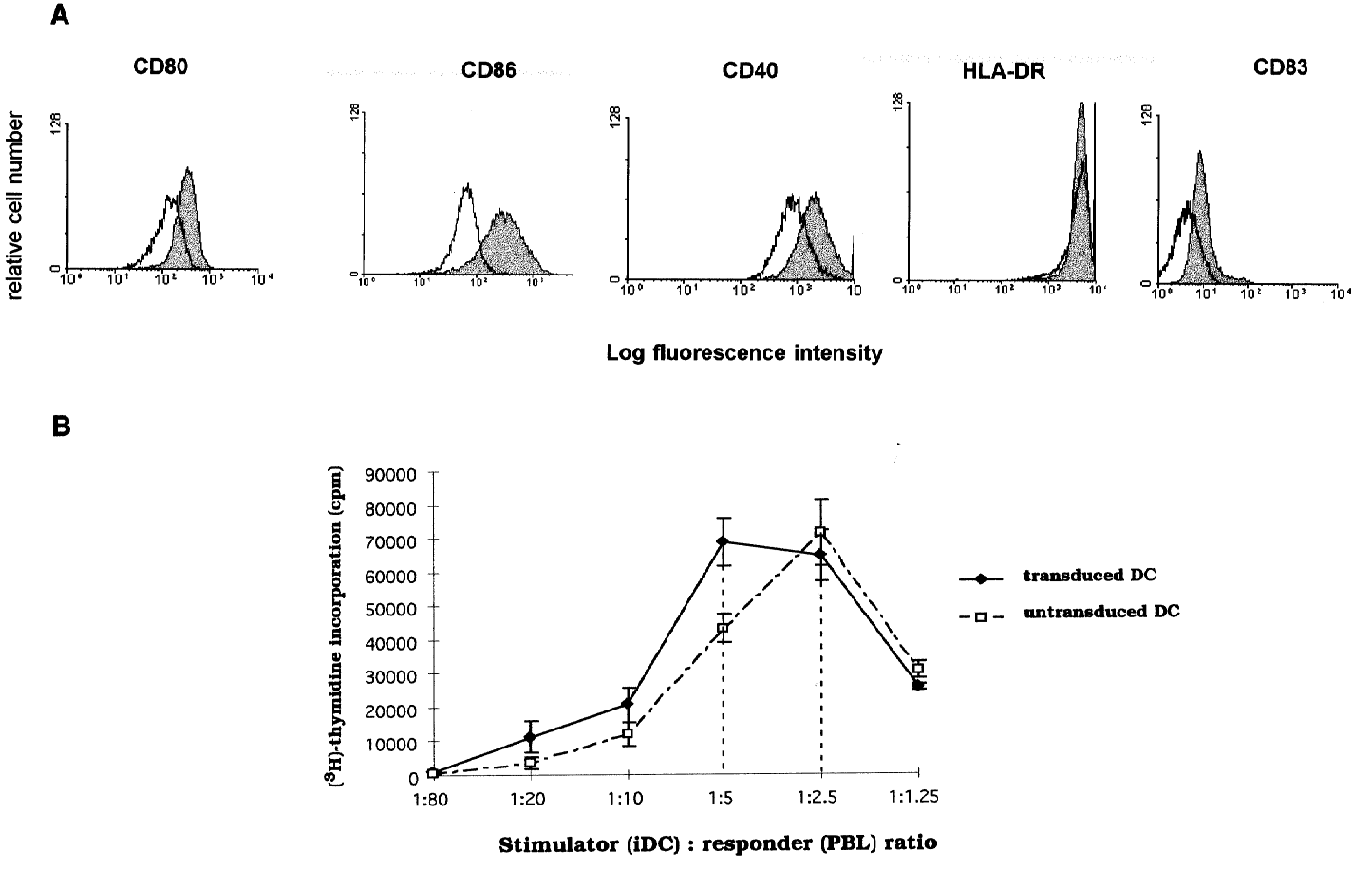

Fig. 4. Adenoviral transduction enhances costimulatory molecule expression and T-cell stimulatory capacity. iDC D6 were transduced with recombinant βgal adenovirus at 150 pfu/cells and cultured for 2 additional days. (A) Flow cytometry analysis of transduced DC (filled histograms) compared to untransduced DC (unfilled histograms). Adenoviral transduction induced an increase expression of costimulatory molecules and expression of CD83 on some cells. (B) Allostimulatory capacity of transduced DC (♦) and untransduced DC (□) in a MLR assay: 1×10^5 responder PBL were cultured with graded numbers of irradiated DC as indicated and T cell proliferation was measured on day 5 by a 16-h pulse with ^{3}H-thymidine. When transduced DC were used as stimulators, a lower number was required to induce an equal proliferative PBL response compared to untransduced DC. Results are shown as the mean±S.D. of six replicates.

Morse et al., 1997; Kowalkowski et al., 1998; Luft et al., 1998);

(d) the use of IL-13 and GM-CSF (Piemonti et al., 1995; Lopez et al., 1997; Boyer et al., 1999).

After 6 days in culture, up to 10^9 iDC can be generated. The clinical trials reported in man have used variable number of DC (from 5×10^6 to 2×10^7, depending on the differentiation protocol used). The clinical and/or immunological responses reported to-date in B cell lymphoma (Hsu et al., 1996), myeloma (Reichardt et al., 1999), melanoma (Nestle et al., 1998) and prostate cancer (Murphy et al., 1996) were partial. To test the influence of DC numbers on the response rate, it will be necessary to perform clinical trials with increased numbers of injected iDC. The large number of iDC that can be generated using our protocol makes such a trial possible and should permit standardization of the dose injected. Moreover, the iDC obtained from one leukapheresis can be frozen (data not shown) in aliquots containing the iDC number to be injected for each immunization. Serum-free AIM-V medium promotes high yield DC differentiation ($85\pm15\%$) which is better than that obtained with RPMI glutamax supplemented with 2% AB serum ($40\pm6\%$) (unpublished data). In addition, reduced numbers of apoptotic and necrotic DC ($<4\%$) should improve the efficiency of gene transfer.

Flow cytometric analysis of iDC D6 revealed a heterogeneous population with respect to size/structure characteristics and phenotypic expression, suggesting that several DC differentiation stages were present in the entire cell population in the bag. The variable expression ($54\pm27\%$) of CD1a and the absence of CD83 expression on iDC D6 was in contrast with the expression of CD1a by 100% of cells and of CD83 by 16 to 20% of cells following DC differentiation in RPMI glutamax supplemented with 2% AB serum (unpublished data). Other reports suggest that this difference might be due to the medium rather than the presence or absence of serum (Morse et al., 1997; Thurner et al., 1999).

The induction of an effective anti-tumor response is largely mediated by the generation of cytotoxic effectors. Endogenous antigen synthesis by DC will ensure the processing and presentation of tumor peptides by MHC class I molecules and the activation of a cytotoxic response. Mo-derived DC are difficult to transfect by classical methods (Arthur et al., 1997; Zhong et al., 1999) and therefore, we used adenovirus-mediated gene transfer in order to maximize the transfection rate. We have shown that adenoviral transduction of iDC D6 results in phenotypic changes which are associated with an increased capacity of iDC D6 to act as stimulator cells for T lymphocytes in allogeneic MLR. In fact, it has been reported in a model investigating the interaction between DC/helper T cells/CTL, that infection of iDC by influenza virus can replace the CD40 signal and mimic the help provided by $CD4^+$ T cells which is required for the priming of naive CTL (Ridge et al., 1998). Whereas Diao et al. (1999) have reported that the transduction of human PBMC-derived iDC by recombinant adenovirus leads to increased expression of CD86, others have found that adenoviral transfection has no effect either on the cell surface markers or on the function of iDC (Diebold et al., 1999; Zhong et al., 1999). A role for the differentiation medium in the alterations seen here is suggested by the fact that, like us, Diao et al. (1999) used AIM-V serum-free medium to generate DC.

The phenotypic changes following infection involve the whole population with a unimodal distribution for every marker studied. This indicates that although 20 to 50% of iDC do not express the transgene, they have all received a maturation signal. Two explanations can be put forward: either all the cells are infected but the transgene is expressed at low levels in some and is not detectable, or cytokine production by transduced DC promotes the maturation of the entire population via a paracrine loop (Kelleher and Knight, 1998). In both cases, DC maturation is probably induced by the vector alone and not by transgene expression, as beta-galactosidase is not expected to act as an immunomodulatory protein and has not been reported to induce DC maturation following non-viral-mediated transfection.

In conclusion, we have developed a DC differentiation method that provides large numbers of clinical grade iDCs that can be transfected by a recombinant adenovirus. This work was performed with the main objective of developing an anti-tumor vaccine for B cell lymphomas. However, it can be extended to anti-tumor vaccination for any cancer where a tumor antigen is available.

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