Generation of functional monocyte-derived fast dendritic cells suitable for clinical application in the absence of interleukin-6

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Abstract To develop dendritic cells (DCs)-based immunotherapy for cancer patients, it is necessary to have a standardized, reproducible, fast, and easy to use protocol for in vitro generation of fully functional DCs. Recently, a new strategy was described for differentiation and maturation of human monocyte (Mo)-derived fast-DCs with full T cell stimulatory capacity within only 48–72 h of in vitro culture. Interleukin (IL)-6 plus tumour necrosis factor (TNF)- α , IL-1 β , and prostaglandin (PG)-E₂ were used in this strategy to induce maturation of the generated DCs. The present study further modifies this strategy by excluding IL-6 from the cytokines cocktail used for DCs maturation. The results showed that maturation of fast-DCs without IL-6 did not significantly alter the morphology, phenotype and the yield of mature DCs ($P > 0.05$, compared with those generated with IL-6). Moreover, fast-DCs generated without IL-6 are functional antigen presenting cells, have the ability to induce tetanus toxoid-specific autologous T cell proliferation, and are suitable for gene delivery through adenoviral vector transduction as those generated with IL-6 ($P > 0.05$). In conclusion, the

present study proves that fully mature and functional Mo-derived fast-DCs can be generated in vitro without adding IL-6, which not only reduces the number of required recombinant cytokines, but may also resemble DCs development in vivo more closely.

Keywords Adenoviral expression · Interleukin-6 · Mo-derived fast-DCs · T cell proliferation · Tetanus toxoid

Introduction

Dendritic cells (DCs) are extraordinarily potent antigen presenting cells (APCs) that can not only initiate but also direct the nature of immune responses (Banchereau et al. 2000). The superior ability of DCs to present antigens to T-cells led to the development of DCs-based strategies for the purpose of enhancing the immune responses against tumours and infectious agents (Banchereau et al. 2000). However, circulating DCs in human blood are rare, <1% of peripheral blood mononuclear cells (PBMCs), and difficult to isolate and maintain in culture. Therefore, most experimental and clinical trials currently rely on the in vitro development of DCs (Fong et al. 2001). In traditional protocols, it is widely believed that the in vitro generation of mature DCs with full T cell stimulatory capacity from human monocytes (Mo) requires 5–7 days of differentiation with granulocyte-macrophage colony-stimulating

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factor (GM-CSF) and interleukin (IL)-4, followed by 2–3 days of maturation with proinflammatory cytokines and/or CD40 ligand (Bender et al. 1996; Bohnenkamp and Noll 2003; Feuerstein et al. 2000; Jonuleit et al. 1997; Thurner et al. 1999). However, there is increasing evidence that the time span required for DCs differentiation in vivo may be shorter. Indeed, it has been shown that a subpopulation of Mo differentiated into mature DCs upon reverse transmigration across an endothelial barrier within 48 h (Randolph et al. 1998).

Recently, a new strategy was described for differentiation and maturation of human Mo-derived fast-DCs with full T cell stimulatory capacity within only 48–72 h of in vitro culture (Dauer et al. 2003; Obermaier et al. 2003; Ramadan et al. 2004). In this strategy adherent blood Mo were cultured for 1 day with GM-CSF and IL-4 to generate CD14⁺CD1a⁺ immature DCs (1st step of fast-DCs generation: differentiation). Then, they were activated and converted to functional APCs (2nd step of fast-DCs generation: maturation) by culturing with the following proinflammatory mediators for either 1 or 2 days: tumour necrosis factor (TNF)- α , IL-1 β , IL-6 and prostaglandin (PG)-E₂ (Dauer et al. 2003; Obermaier et al. 2003; Ramadan et al. 2004). Mature DCs exhibit increased expression of the major histocompatibility complex (MHC) and costimulatory molecules (such as CD86) as well as secrete large amounts to immunostimulatory cytokines such as IL-12 thus rendering them highly effective inducers of antigen-specific T cell responses (Cella et al. 1997; Schnurr et al. 2000). Fast-DCs generated in only 2–3 days were capable of prime melanoma-, *A. fumigatus*- and cytomegalovirus-specific cytotoxic T cells as effectively as standard DCs generated in 7–10 days (Dauer et al. 2005; Ramadan et al. 2004, 2005a, b). Thus, the new strategy reduces labour, time and cost as well as renders cells less sensitive for external disruptive factors (Obermaier et al. 2003), and hence it may facilitate the use of DCs in clinical trials of cellular immunotherapy (Dauer et al. 2005; Ramadan et al. 2004). The present study further modifies the new strategy described for fast-DCs generation by excluding IL-6 from the cytokines cocktail used for their maturation, which not only reduces the number of recombinant cytokines required for DCs generation in vitro, but may also resemble DCs development in vivo more closely.

Materials and methods

Cell counting and viability

Cell counting was performed using a Neubauer counting chamber (Hawksely, UK) with at least 200 cells being counted per sample. Cell viability was assessed using trypan blue dye exclusion (Sigma–Aldrich Corp, St. Louis, MO, USA).

Generation of Mo-derived fast-DCs in the presence or absence of IL-6

Mo-derived fast-DCs were prepared from adherent blood Mo as previously described (Dauer et al. 2003; Ramadan et al. 2004), with some modifications. Briefly, PBMCs were obtained from healthy donors by Ficoll-Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation and were incubated (cell density 5×10^6 cells/mL) in complete RPMI medium (RPMI-1640 with 25 mM HEPES and 2 mM L-glutamine, BioWhittaker, Walkersville, MD, USA) containing 1% pooled human AB serum (C-Six Diagnostics, Mequon, WI, USA) at 37 °C and 5% CO₂ for 2 h. Informed consent was obtained from all donors before samples were collected. The non-adherent cells were removed by two gentle washes, and adherent blood Mos were incubated for 24 h in the above medium containing 800 U/mL GM-CSF (Immunex, Seattle, WA, USA) and 1,000 U/mL IL-4 (R&D Systems, Inc., Minneapolis, MN, USA) to generate immature DCs. Then, DCs were matured for 2 days with a mixture of 10 ng/mL IL-1 β , 10 ng/mL TNF- α (R&D Systems, Inc.) and 1 μ g/mL PG-E₂ (Sigma–Aldrich Corp) with or without 1,000 U/mL IL-6 (R&D Systems, Inc.).

Immunophenotyping of Mo-derived fast-DCs generated in the presence or absence of IL-6

Mature fast-DCs phenotype was determined by flow cytometric analysis as previously described (Ramadan et al. 2004). Sufficient numbers of cells ($\sim 2 \times 10^6$) were collected and immunophenotyped using a 3 colour-direct panel including fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- and peridinin chlorophyll-a protein (PerCP)-conjugated monoclonal antibodies to: CD1a, 3, 14, 40, 45, 83, 86 and HLA-DR along with the relevant isotype

controls. All antibodies were obtained from BD Biosciences (San Jose, CA, USA). To exclude dead cells, 7-amino-actinomycin D (7-AAD, Sigma–Aldrich Corp) was added to the samples. The cells were fixed with 2% paraformaldehyde before acquisition with a FACSCalibur™ flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA). List mode data were analysed using FloJo version 8 (Treestar, Ashland, OR, USA). CD83⁺DCs yield was calculated as the ratio of the output number of viable CD83⁺DCs relative to the input number of PBMCs using the following equation:

CD83⁺DCs yield

$$= \frac{\text{output number of viable CD83}^+\text{DCs} \times 100}{\text{input number of PBMCs}}$$

Antigen-loading of Mo-derived fast-DCs generated in the presence or absence of IL-6

The capacity to take up and process soluble antigen for presentation on MHC molecules is an important function of immature DCs, and is rapidly lost upon maturation (Sallusto and Lanzavecchia 1994). In the present study tetanus toxoid (TT) soluble antigen was used as a model antigen to induce autologous T cell proliferative response as previously described (Dauer et al. 2003). Immature fast-DCs were pulsed overnight at 37 °C and 5% CO₂ with 5 µg/mL TT soluble antigen (Calbiochem, La Jolla, CA) in complete RPMI medium containing 1% pooled human AB serum immediately prior to maturation. Then, they were used for autologous T cell proliferation assay after maturation in the presence or absence of IL-6.

Autologous T cell proliferation assay

Autologous T cell proliferation assay was performed as previously described (Dauer et al. 2003), with some modifications. Briefly, autologous T cells were purified from Mo-depleted PBMCs by negative selection using the Pan T Cell Isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). TT-pulsed mature fast-DCs generated in the presence or absence of IL-6 were harvested, irradiated (25 Gy) and cocultured in RPMI complete medium containing 10% pooled human AB serum at 37 °C and 5% CO₂ with autologous T cell at 1:20 ratio in 96-well round-bottomed plates for 5 days prior to the addition of

1 µCi ³H-thymidine (Na₂⁵¹CrO₄, ICN Pharmaceuticals, Cost Mesa, CA, USA). The proliferation was assessed by ³H-thymidine uptake after 18 h of incubation. Wells containing T cells alone (no stimulus) or with either TT antigen or irradiated nonpulsed fast-DCs were included as negative controls. The cells were harvested onto glass fibre paper using a 96-well cell harvester, and the radioactivity (counts per minute, CPM) was measured by a β-scintillation counter (Packard, Meriden, CT, USA).

Adenoviral transduction of Mo-derived fast-DCs generated in the presence or absence of IL-6

Introducing genes into DCs through adenoviral vector transduction is now an established method to potentiate a specific immune response against tumour and virus antigens (Cho et al. 2003; Lotem et al. 2006; Ramadan et al. 2004; Tian et al. 2007). The present study evaluated the effect of excluding IL-6 from the cytokines cocktail used for DCs maturation on adenoviral vector gene delivery and expression into fast-DCs and determined the suitable time for their adenoviral transduction. Fast-DCs preparation was transduced for 2 h with adenoviral vector containing green fluorescent protein gene (adeno-GFP, kindly provided by Dr. Gordon R. Sandford, Medical College of Wisconsin, Milwaukee, WI, USA) at multiplicity of infection (MOI) of 150 in complete RPMI medium containing 1% pooled human AB serum at 37 °C and 5% CO₂ as previously described (Keever-Taylor et al. 2001; Ramadan et al. 2004) either before or after fast-DCs maturation in the presence or absence of IL-6. Then, DCs were washed twice with medium to remove the virus. GFP gene expression was determined in adeno-GFP-transduced mature fast-DCs by fluorescence microscopy and flow cytometric analysis. Mock transduced (medium without adeno-GFP vectors) fast-DCs were used as negative control.

Statistics

Statistical analysis was performed with either Unpaired *t* test, for comparison between two groups, or one-way ANOVA (Bonferroni's multiple comparison test), for comparison between more than two groups, using GraphPad Prism version 4.03 for Windows (GraphPad Software Inc., San Diego, CA,

USA). P values of <0.05 , <0.01 and <0.001 were considered statistically significant, highly significant and very highly significant, respectively.

Results

Morphology, phenotype and $CD83^+$ yield of fast-DCs generated in the absence of IL-6

Forward and side scatter intensity (Fig. 1a) and light microscopy (Fig. 1b) indicated that fast-DCs generated in the absence of IL-6 had same size and granularity, were loosely adherent, showed cell aggregates, and developed the typical morphology in addition to the cytoplasmic projections (veils) characteristic for mature DCs as those generated in the presence of IL-6. In addition, phenotypic analysis

showed that fast-DCs generated in the absence of IL-6 were $CD3^-$ and $CD14^-$, down-regulated the immature DCs marker ($<20\%$ cells expressed $CD1a$), up-regulated the DCs lineage marker ($>70\%$ cells expressed $CD83$), and expressed mature DCs surface markers ($>98\%$ cells were $CD40^+$, $CD45^+$, $CD86^+$ and $HLA-DR^+$) to the same extent ($P > 0.05$, unpaired t test) as those generated in the presence of IL-6 (Fig. 2). Moreover, the percentage of viable $CD83^+$ DCs yield from the initial population of PBMCs did not significantly change ($P > 0.05$, unpaired t test) in fast-DCs generated in the absence of IL-6 compared with those generated in the presence of IL-6 ($4.44\% \pm 0.24\%$ vs. $4.72\% \pm 0.16\%$, respectively) as shown in Fig. 3. All of these results indicated that excluding IL-6 from the cytokines cocktail used for fast-DCs maturation did not significantly alter the morphology, phenotype and

Fig. 1 Morphological characterization of human mature Mo-derived fast-DCs generated in the presence or absence of IL-6. **a** Forward and side scatter intensity. **b** Light microscopy ($200\times$

magnification). *Dotted and solid arrows* indicate cell aggregates and hair-like cytoplasmic projections (veils), respectively

Fig. 2 Surface antigen phenotype characterizations of human mature Mo-derived fast-DCs generated in the presence or absence of IL-6. Data are presented as mean value $\pm$ standard deviation of five different experiments, $P > 0.05$, unpaired t test

Fig. 3 CD83⁺ yield of human mature Mo-derived fast-DCs generated in the presence or absence of IL-6. Individual values are shown, with means represented by horizontal bars, $P > 0.05$, unpaired t test. CD83⁺DCs yield was calculated using the following equation: CD83⁺DCs yield = output number of viable CD83⁺DCs $\times$ 100/input number of PBMCs

yield of mature DCs compared with those generated in the presence of IL-6.

Fast-DCs generated in the absence of IL-6 are functional APCs

Fast-DCs generated in the absence of IL-6 had the ability to take, process and present TT soluble antigen and were equally effective ($P > 0.05$, unpaired t test) in inducing TT-specific autologous T cell proliferative response as those generated in the presence of IL-6 ($44,830 \pm 3,023$ CPM vs. $42,860 \pm 2,338$ CPM, respectively) as shown in Fig. 4. No noticeable proliferation was obtained from wells containing

Fig. 4 Autologous T cell proliferative responses induced by irradiated human mature Mo-derived fast-DCs generated in the presence or absence of IL-6 and pulsed with TT antigen. Wells containing T cells alone (no stimulus) or with either TT antigen or irradiated nonpulsed fast-DCs were included as negative controls. Data are presented as mean CPM $\pm$ standard deviation of triplicate samples. There was no significant difference ($P > 0.05$, unpaired t test) between TT-specific proliferative response induced by TT-pulsed fast-DCs generated in the absence or presence of IL-6. § compared with T cells alone (no stimulus) or with either TT antigen or nonpulsed fast-DCs, one-way ANOVA (Bonferroni's multiple comparison test)

responder cells alone (<350 CPM) or with either TT antigen (<900 CPM) or irradiated nonpulsed fast-DCs ($<2,000$ CPM) indicating that the proliferative response was specific for TT antigen (Fig. 4).

Adeno-GFP-transduced fast-DCs generated in the absence of IL-6 had the ability to express GFP gene to the same extent ($P > 0.05$, ANOVA-Bonferroni's multiple comparison test) as those generated in the presence of IL-6 (Fig. 5). On the other hand, transduction of fast-DCs with adeno-GFP after maturation resulted in a significantly higher percentage ($P < 0.001$, ANOVA-Bonferroni's multiple comparison test) of fluorescent cells compared with transduction before maturation disregarded the presence or absence of IL-6 (Fig. 5). No GFP gene expression was detected in mock-transduced fast-DCs (data not shown). All of these results indicated that Mo-derived fast-DCs generated in the absence of IL-6 are functional APCs and suitable for gene delivery through adenoviral vector transduction.

Fig. 5 GFP gene expression of human mature Mo-derived fast-DCs generated in the presence or absence of IL-6 and transduced before or after maturation with adeno-GFP vectors. **a** Fluorescence microscopy (200× magnification). **b** Flow

cytometric analysis. Data are presented as mean value ± standard deviation of five different experiments. § compared with prematuration transduction, one-way ANOVA (Bonferroni's multiple comparison test)

Discussion

IL-6 is known to have a pleiotropic effect on cell growth, differentiation, survival and migration during immune responses, haematopoiesis and inflammation (Park et al. 2004). It regulates B cell differentiation and antibody production as well as T cell functions. In addition, IL-6 promotes CD4⁺Th₂ differentiation and inhibits CD4⁺Th₁ differentiation (Diehl et al. 2000). IL-6 is produced by a wide range of cell types such as fibroblasts (Chomarat et al. 2000) and various malignant tumours such as cervical carcinoma (Hess et al. 2000). Also, it is up-regulated during bacterial and viral infection as well as trauma (Heinrich et al. 2003). TNF- α , IL-1 β , IL-6 and PGE₂ were supposed to be essential cytokines for in vitro maturation of DCs and their production of interferon (IFN)- γ and IL-12 (Blanco et al. 2008; Bohnenkamp and Noll 2003; Feuerstein et al. 2000; Obermaier et al. 2003). In addition, PGE₂ was able to induce the final maturation of IL-12-deficient CD1a⁺CD83⁺DCs (Kalinski et al. 1998). Although the aforementioned cytokines were capable of inducing DCs maturation,

they inhibited DCs differentiation from Mo (Motta et al. 2010; Sallusto and Lanzavecchia 1994; Sombroek et al. 2002). Addition of TNF- α , IL-1 β or IL-6 to Mo cultures prevented the loss of CD14 as well as the appearance of CD1a molecules, and hence suppressed the in vitro generation of CD14⁺CD1a⁺ immature DCs (Bharadwaj et al. 2007; Motta et al. 2010; Sallusto and Lanzavecchia 1994). The inhibition of DCs development may be a way to ensure tumour cell survival, as the immune response becomes compromised (Motta et al. 2010). Indeed, it was found that IL-1 β and PGE₂ produced by tumour cells inhibited the Mo differentiation into immature DCs (Motta et al. 2010; Sombroek et al. 2002). Also, IL-6 secreted by human pancreatic cancer cells suppressed the generation of DCs from Mo via aberrant activation of signal transducer and activator of transcription 3, which may represent a novel target for the prevention of unwanted immune responses in autoimmune diseases through the control of DCs differentiation (Bharadwaj et al. 2007; Park et al. 2004). In addition, renal carcinoma cell lines inhibited the differentiation of haematopoietic

CD34⁺ progenitor cells into DCs through an IL-6-mediated mechanism (Menetrier-Caux et al. 1998). Moreover, IL-6 switched the differentiation of haematopoietic CD34⁺ progenitor cells and Mo from DCs to macrophages (Chomarat et al. 2000; Mitani et al. 2000; Ratta et al. 2002). An increase in the percentages of CD14⁺CD1a[−] macrophages was observed with the addition of IL-6 to Mo cultures containing GM-CSF and IL-4 (Mitani et al. 2000). Therefore, TNF- α , IL-1 β , IL-6 and PGE₂ cytokines cocktail was used in the protocol described here to induce maturation of Mo-derived CD14[−]CD1a⁺DCs (2nd step of fast-DCs generation), but not for Mo differentiation into immature DCs (1st step of fast-DCs generation).

Recently, it was also reported that several factors, such as IL-6, released by solid and liquid tumours affecting the maturation pathway of DCs (Liang et al. 2008; Orsini et al. 2003; Ratta et al. 2002). Pancreatic cancer patient sera containing high levels of IL-6 significantly blocked the maturation of Mo-derived CD14[−]CD1a⁺DCs by down-regulating their maturation markers (CD40, CD80, CD83 and MHC class II) and inhibiting their CD4⁺ and CD8⁺ T cells allostimulatory activity (Liang et al. 2008). In addition, a functional defect in DCs from peripheral blood of myeloma and chronic lymphocytic leukaemia patients was partially due to an overproduction of IL-6 (Orsini et al. 2003; Ratta et al. 2002). The maintenance of tolerogenic DCs, characterized by secreting lower IL-12 and higher IL-10 as well as inability to activate antigen-specific T cells, was increased in the presence of IL-6 (Bellone et al. 2006; Liang et al. 2008). IL-6 clearly decreased the lipopolysaccharide-induced expression of MHC-class II, CD40, 80, 86 and IL-12 (p40/p70) on DCs (Park et al. 2004). On the other hand, increased numbers of mature DCs were found in IL-6 knockout mice compared with their wild-type littermates (Park et al. 2004). All of these reports indicated that IL-6 blocked the transition of DCs from the resting/immature phenotype to the activated/mature phenotype.

The present study showed that excluding IL-6 from the cytokines cocktail used for fast-DCs maturation did not significantly alter the morphology, phenotype and yield of mature DCs compared with those generated in the presence of IL-6 (Figs. 1, 2 and 3). Moreover, Mo-derived fast-DCs generated in the absence of IL-6 are functional APCs, capable of

taking, processing and presenting antigen, have the ability to stimulate T cells, and suitable for gene delivery through adenoviral vector transduction (Figs. 4, 5). It was reported that the concentration of exogenous recombinant IL-6 required to block the generation of fully functional DCs supposed to equal 2,000 U/mL (Ratta et al. 2002), which is accurately double the concentration used in the present study. Moreover, it was found that TNF- α , IL-1 β , CD40 ligand and transforming growth factor β 1 blocked the inhibitory effects of IL-6 on DCs maturation (Mitani et al. 2000). Among these factors, TNF- α was most potent in interfering with the action of IL-6 (Mitani et al. 2000). All of these findings may explain the similarity shown in the present study between fast-DCs matured in the presence or absence of IL-6. In conclusion, the present study described a new strategy for in vitro generation of fully mature and functional fast-DCs from Mo without adding IL-6. This modification in the previously used protocol not only reduces the number of recombinant cytokines required for DCs generation in vitro, but may also resemble DCs development in vivo more closely.

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Conflict of interest The author has no potential financial conflict of interest.

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