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## Cellular Immunology

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## Fast generation of dendritic cells

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## ARTICLE INFO

## Article history:

Received 21 May 2009

Accepted 4 September 2009

Available online xxxxx

## Keywords:

Dendritic cells

Fast generation

IL-12

IL-10

## ABSTRACT

Dendritic cells (DC) are potent antigen presenting cells capable of inducing immune responses. DC are widely used as vaccine adjuvant in experimental clinical settings. DC-based vaccines are normally generated using a standard 8 day DC protocol (SDDC). In attempts to shorten the vaccine production we have developed fast DC protocol by comparing two different fast DC protocols with SDDC. DC were evaluated by FACS analysis, and the optimal profile was considered: CD14<sup>low</sup>, CD80<sup>high</sup>, CD83<sup>high</sup>, CD86<sup>high</sup>, CCR7<sup>high</sup>, HLA class I and II<sup>high</sup>. FACS profiles were used as the selection criteria together with yield and morphology. Two fast DC protocols fulfilled these criteria and were selected for functional analysis. Our results demonstrate that DC generated within 5 days or 48 h are comparable with SDDC both phenotypically and functionally. However, we found that 48 h DC were more susceptible than SDDC to the IL-10 inducing stimulus of TLR ligands (R848 and LPS). Thus to determine the clinical relevance of fast DC protocols in cancer settings, small phase I trials should be conducted monitoring regulatory T cells carefully.

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## 1. Introduction

Dendritic Cells (DC) are potent antigen presenting cells (APC) with the unique capability to prime and control T cell mediated immune responses. DC are found throughout the body where they capture and process antigen for presentation. Upon encounter with appropriate stimulation DC differentiate into mature DC which are characterized by decreased endocytic activity, up-regulation of major histocompatibility complex (MHC) class I and II molecules and co-stimulatory molecules (CD86, CD80), and responsiveness to inflammatory chemokines [1]. DC's unique capacity to prime antigen specific immune responses has lead to development of DC-based vaccine therapies which are currently being tested against various forms of cancer in clinical settings [2,3].

Circulating blood DC only account for <1% of PBMC and are difficult to maintain in culture. This low number can be increased by treatment with flt-3 ligand and GM-CSF [4]. However, most DC-based vaccines currently use DC generated *in vitro* from CD34<sup>+</sup> progenitor cells or blood monocytes [5,6]. Using blood monocytes, conventional *in vitro* protocols require 8–10 days to generate mature DC. Monocytes are cultured for 5–7 days with GM-CSF and IL4 to generate immature DC and subsequently another 1–3 days with maturation stimuli to generate a population of immunogenic mature DC [2,7]. Several groups have shown that it is possible to generate DC in 2–3 days cell culture [8–10]. These fast protocols

may more closely resemble the development of DC from monocytes *in vivo* [11].

When generating DC-based vaccines for clinical use it is mandatory that the production occurs under compliance with Good Manufacturing Procedures (GMP). This includes validated clean rooms, trained staff and GMP materials. These requirements make the generation of vaccines laborious. The generation of fast DC will shorten the time from the venesection of patient to DC-based vaccine and therefore be beneficial in the clinical setting.

In the present study, we aimed at developing a fast DC method fulfilling the criteria of DC displaying a highly matured and immunogenic profile including high expression of functional CCR7 for homing to the draining regional lymph nodes. Here, we report on results from a comparison of a standard (8 days), an intermediate (5 days) and a short (48 h) protocol. To our knowledge this is the first report directly comparing protocols for three time points.

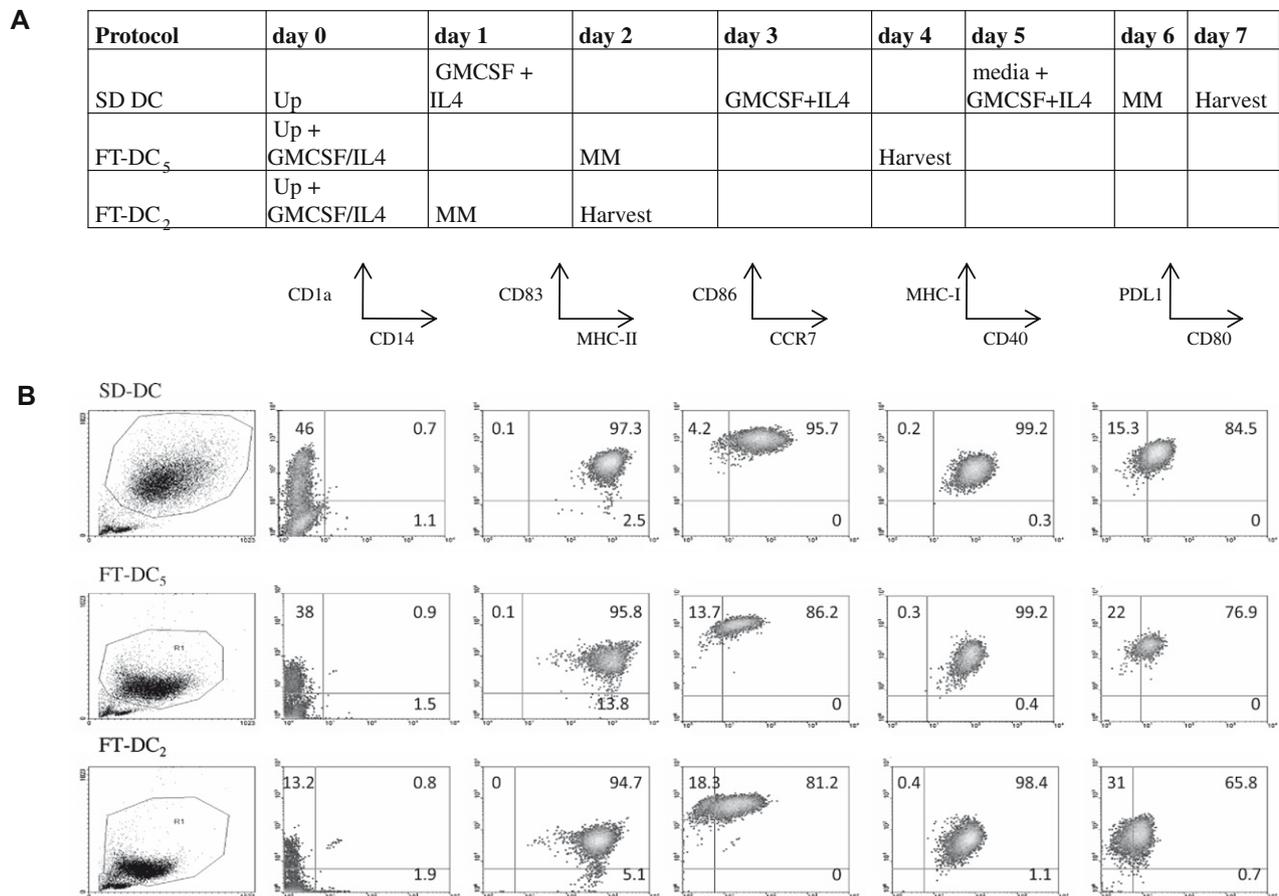
## 2. Materials and methods

## 2.1. Preparation of DC from PBMC of healthy donors

Monocyte-derived DC were generated as previously described by Romani et al. [6] with some modifications. Briefly, peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of healthy donors by Ficoll gradient centrifugation (Nycomed, Oslo, Norway). Monocytes were isolated by plastic adherence and subsequently cultured for 5 days with GM-CSF and IL-4 (1400 U/ml and 700 U/ml, respectively, Gentaur, Brussels, Belgium) in AIM-V

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**Fig. 1.** Protocol and comparative phenotypic analysis of SDDC and fast DC by flow cytometry. (A) A schematic overview of the two selected fast protocols compared with the standard protocol. The addition of cytokines, loading of antigen and time of harvest are given. Loading: Addition of antigen, MM: maturation stimuli. One of three performed experiments is presented. (B) Expression of selected surface markers on SDDC and fast DC at the time of cell harvest is assessed by labeling cells with mAb and subsequent FACS analysis and results are depicted as density plots.

(Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1% autologous plasma. At day 5 the immature DC were ready for loading of antigen. At day 6 IL-1 $\beta$  (10 ng/ml) (Gentaur), TNF $\alpha$  (10 ng/ml)(Gentaur), IL-6 (10 ng/ml)(Gentaur) and PGE<sub>2</sub> (1 mM) (Sigma–Aldrich) were added as maturation stimuli. After one additional day the DC were harvest and cryopreserved for later use.

The preparation of the fast DC was based on methods described previously [12,13] and the method currently used for our vaccine product MelCancerVac<sup>®</sup> with some modifications. For the FT-DC<sub>5</sub> protocol monocytes were cultured for 2 days with GM-CSF and IL-4, and for 2 days with maturation stimuli. For the FT-DC<sub>2</sub> protocol monocytes were cultured with GM-CSF and IL-4 for 24 h and exposed to maturation stimuli for another 24 h (see Results, Fig. 1A). Adherent cells were gently scraped off the plastic prior to harvest.

To induce IL-12 production 2.5  $\mu$ g/ml Resiquimod (R848, 3 M, St. Paul, MN, USA), 0.1  $\mu$ g/ml LPS (Sigma–Aldrich, St. Louis, MO, USA) and 1  $\mu$ g/ml CD40L (R&D systems, Abingdon, UK) were added to the cell cultures at day 6, 2 or 1 for SDDC, FT-DC<sub>5</sub> and FT-DC<sub>2</sub>, respectively, when indicated. Supernatants were collected at the time of harvest and kept for cytokine analysis.

## 2.2. Flow cytometry

Cells to be analyzed were incubated for 15 min with 5% human AB serum (Lonza, Basel, Switzerland) prior to labeling with fluorescein-conjugated antibodies. For analysis of DCs, the following mouse monoclonal antibodies were used: anti-CD14 (M5E2, Becton

Dickinson [BD], San Jose, CA), anti-HLA-D (T $\ddot{u}$ 39, BD), anti-CD40 (5C3, eBioscience, San Diego, CA), anti-CD80 (2D10.4, eBioscience), anti-CD86 (FUN-1, BD), anti-CCR7 (150503, R&D systems, Abingdon, UK), anti-CD83 (HB15e, BD), anti-PD-L1 (MIH1, eBioscience and appropriate isotype controls (eBioscience and BD). For analysis of DC-activated T cells, the cells were labeled with a CMV peptide (NLVPMVATV) loaded MHC class I pentamer (proimmune USA) for 20 min and subsequently labeled with the following mouse monoclonal antibodies: anti-CD8 (RPA-T8, eBioscience), anti-CCR7 (150503, R&D systems), anti-CD28 (CD28.2, eBioscience), anti-CD62L (DREG-56, R&D systems), and anti-CD45RA (HI100, R&D systems). All labeled cells were analyzed on a FACS Calibur (BD) or for the pentamer analysis FACS aria (BD). Data analyses were conducted using WinMDI or DIVA software.

## 2.3. Cytokine measurements

Supernatants were collected from all cell culture conditions at the day of harvest and after 48 h of washout culture for IL-10, IL-12(p70) and IL-23 measurement using enzyme linked immunosorbent assay (ELISA) kits purchased from eBioscience and manufacture's descriptions were followed.

## 2.4. Endocytic activity

Endocytic activity was assessed by incubating immature DC for 4 h with FITC labeled dextran ( $M_w$  40,000 Da, Sigma–Aldrich)

(3 mg/ml) at 37 °C. Cells were washed extensively with PBS and analyzed by flow cytometry. Extracellular FITC signal was assessed by incubating cells on ice during incubation with FITC dextran.

### 2.5. Co-culture of T cells and DC

Autologous DC were incubated with or without 5 μM CMV pp65 peptide, NLVPMVATV (Schafer-N, Copenhagen, Denmark) for 2 h before they were co-cultured with T cells from CMV positive donors. The DC: T cell ratio was 1:20 for *in vitro* stimulation of 8 days prior to FACS analysis. The culture conditions were 37 °C, 5% CO<sub>2</sub>. On day 2 20 U/ml of IL-2 (Gentaur) was added.

### 2.6. IFN $\gamma$ Enzyme-linked immunospot (EliSpot) assay

EliSpot analysis was performed directly without *in vitro* stimulation as previously described [14]. Briefly, 96 well plates with nitrocellulose membrane bottoms (Multiscreen HTS system, Millipore, Roskilde, Denmark) were coated with 7.5 μg/ml mouse anti-IFN $\gamma$  mAb (1-D1k, Mabtech, Nacka Strand, Sweden) in 7 μl DPBS and incubated at room temperature over night. Subsequently plate were washed and blocked with 200 μl AIM-V per well. Lymphocytes were added in three decreasing concentration (5 × 10<sup>5</sup>, 10<sup>5</sup>, 5 × 10<sup>4</sup>) together with 10<sup>4</sup> DC either unloaded or preloaded with 5 μM CMV pp65 peptide, NLVPMVATV (Schafer-N, Denmark) for 2 h before use. Plates were incubated for 18–24 h. Subsequently plates were washed and 0.75 μg/ml biotinylated anti-IFN $\gamma$  mAb was added (7-B6-biotin, Mabtech) in 75 μl DPBS. Next step was addition of streptavidin (Mabtech) and spots were developed by BCIP/NBT enzyme substrate (Mabtech). The spots were numerated by digitalized EliSpot counter (ImmunoSpot, CTL Inc., USA).

### 2.7. Allogeneic mixed lymphocyte reaction (MLR)

DCs prepared as described above were treated with 20 μg/ml mitomycin-c (Sigma-Aldrich) at 37 °C for 1 h and washed extensively before they were added to allogeneic monocyte depleted PBMC (10<sup>5</sup> cells) in 96-well plates (Nunc, Denmark). After 4 days, BrdU incorporation was measured by pulsing them with BrdU for 8 h, and incorporated BrdU was analyzed by ELISA (Cell Proliferation ELISA kit, Roche). Each experimental point was set up in quadruplicate.

### 2.8. Migration assay

To test the functional capacity of the expressed CCR7 on the generated DC a migration assay against the ligand for CCR7 CCL19/MIP-3 $\beta$  was performed as described previously [15] with some modifications. Briefly, a chemotaxis chamber designed for 96 well plates (Chemo TX system MBA96 (Neuro probe, Gaithersburg, MD, USA) was used. Media containing CCL19 (MIP-3 $\beta$ , PeproTech, London, UK) in decreasing concentrations (200 ng/ml, 100 ng/ml, 50 ng/ml) was plated. A polycarbonate filter (5 μm pore size, Neuro Probe) was placed on top of the wells and cells to be tested (8 × 10<sup>4</sup>) were placed carefully on the filter above each well. The plate was incubated at 37 °C/5% CO<sub>2</sub> for 90 min. Cells that migrated to the lower chamber were counted using FACS Calibur (BD).

## 3. Results

### 3.1. Screening of fast protocols by comparison to standard DC (SDDC) method

Several surface markers are present on mature immunogenic DC including maturation markers and co-stimulatory molecules

for T cell activation. Phenotypic characterization was made by FACS including expression of CD1a, CD14, HLA-ABC, HLA-DR, CD40, CD80, CD83, CD86, PD-L1 and CCR7 in order to find the optimal time point and length of exposure for differentiation cytokines and maturation stimuli. The aim was to obtain a FACS profile comparable to that of SDDC which is CD1a<sup>+</sup>, CD14<sup>-</sup>, HLA-ABC<sup>+</sup>, HLA-DR<sup>high</sup>, CD40<sup>+</sup>, CD80<sup>+</sup>, CD83<sup>high</sup>, CD86<sup>high</sup>, CCR7<sup>high</sup>. DC Morphology and DC yield were also included in the evaluation of the various fast generated DC.

Beside the standard differentiation cytokines GM-CSF and IL-4 we tested IFN $\alpha$  and IL-15 as differentiation cytokines. However, addition of IFN $\alpha$  and IL-15 resulted in less optimal DC regarding surface markers, DC yields or morphology compared to GM-CSF and IL-4 (data not included).

The screening of various methods resulted in the selection of one fast (48 h) and one intermediate (5 days) protocol. According to the 5 day protocol monocytes were cultured with GM-CSF and IL-4 for 2 days and maturation stimuli for 2 days. In the 48 h protocol the monocytes were cultured for 24 h with GM-CSF and IL-4 and the with maturation stimulus for subsequent 24 h (Fig. 1A). These protocols will in the following be referred to as FT-DC<sub>5</sub> and FT-DC<sub>2</sub>, respectively.

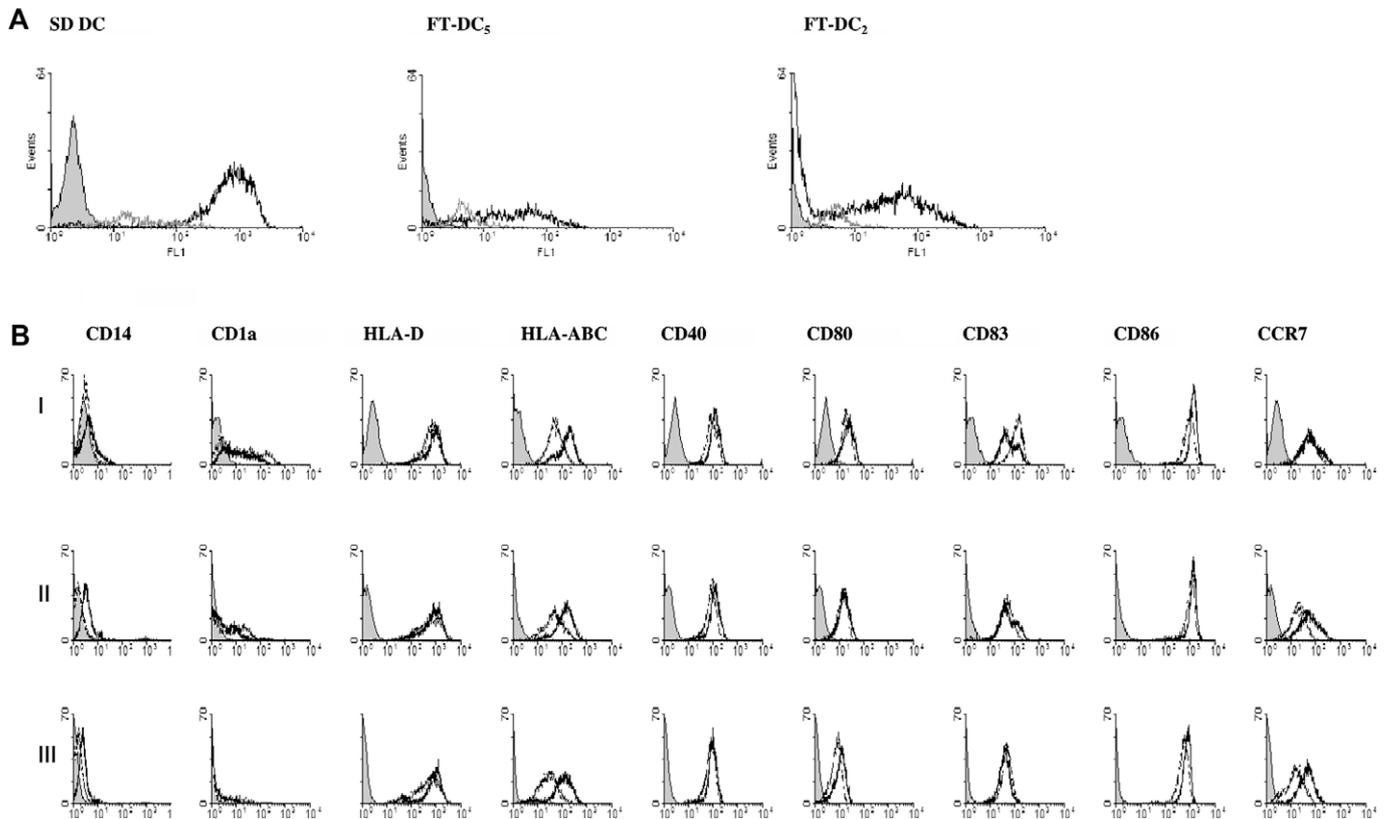
Both methods fulfilled the criteria set up for phenotypic characteristics (Fig. 1B). FT-DC<sub>5</sub> and FT-DC<sub>2</sub> are smaller in size and less granulated when compared to SDDC (forward and side scatter plot, Fig. 1B). The expression of CD1a is lower with decreased culture time with 45%, 38% and 13.2% positive cells for SDDC, FT-DC<sub>5</sub> and FT-DC<sub>2</sub>, respectively. The expression of MHC class II and CD83 are equally high, however, with a more heterogeneously expression for FT-DC<sub>5</sub> and FT-DC<sub>2</sub> (Fig. 1B). The expression of both CCR7 and CD80 is decreased by approximately 10% for FT-DC<sub>5</sub> and 15% for FT-DC<sub>2</sub> compared with SDDC (Fig. 1B). However, both FT-DC<sub>5</sub> and FT-DC<sub>2</sub> express high levels of CCR7 (86% and 81%, respectively). CD86, CD40 and MHC class I are expressed equally for FT-DC<sub>5</sub>, FT-DC<sub>2</sub> and SDDC (Fig. 1B). All tested DC express high levels of PD-L1, however fewer molecules per cell were expressed by FT-DC<sub>5</sub> and FT-DC<sub>2</sub> shown by decrease in MFI (see Fig. 2b). In general the MFI values decreased with decreased culture time for the DC. In addition, both FT-DC<sub>5</sub> and FT-DC<sub>2</sub> resulted in between 20–50% higher cell yield with the characteristic DC protrusions for the fast DC compared with the yield from the SDDC (data not shown).

In summary we selected FT-DC<sub>5</sub> and FT-DC<sub>2</sub> for further functional analyses because (1) both methods generated DC's displaying high quality FACS profiles and (2) high DC yields and DC morphology. Furthermore, we were interested in investigating the intermediate time point between 2 and 8 day protocols i.e. FT-DC<sub>5</sub> to examine if important functional events occur at this stage. In addition, no previous work on fast generation of DC has compared both the fast and intermediate protocol to the standard protocol.

### 3.2. Functional analyses of the fast DC compared to the standard DC

#### 3.2.1. Comparison of endocytic activity

The ability to take up and process antigen is characteristic for immature DC and this capacity is down regulated upon maturation where the presentation of antigen becomes optimal. MelCancerVac<sup>®</sup> consists of autologous DC loaded with a tumor cell lysate, thus, the vaccine is dependent on the ability to take up antigen. To evaluate the capacity of the FT-DC<sub>5</sub> and FT-DC<sub>2</sub> to take up soluble antigen, FITC conjugated dextran was added to the monocytes cultured with GM-CSF and IL-4 for 5, 2 or 1 day for the SDDC, FT-DC<sub>5</sub> and FT-DC<sub>2</sub> protocols, respectively. Both FT-DC<sub>5</sub> and FT-DC<sub>2</sub> take up dextran, however, less efficiently compared to SDDC reflected by lower MFI as shown in Fig. 2A.



**Fig. 2.** (A) Uptake of FITC labeled dextran by immature SDDC and fast DC. Each histogram shows the measurement of the cell co-cultured with FITC labeled dextran at 37 °C (black line) and non specific labeling of the cells (grey line). The filled histograms represent appropriate isotype controls. One of three performed experiments is presented. (B) Phenotype of mature DC remains stable for 48 h in cytokine-free medium for both SDDC and FT DC. I SDDC, II FT-DC<sub>5</sub> and III FT-DC<sub>2</sub> at the time of harvest (dotted line) and after 48 h (solid lines). Isotype control is depicted as the solid line. One of three performed experiments is presented.

### 3.2.2. Comparison of phenotypic stability

When DC used for immune therapy are injected intradermally it takes up to 48 h (or more) for the DC to migrate to the draining lymph node. Therefore the stability of the generated DC is important. To investigate if FT-DC<sub>5</sub> and FT-DC<sub>2</sub> obtain a stable phenotype during the short differentiation time, the cells were re-cultured without exogenously added cytokine for additional 48 h after harvest. The results in Fig. 2B show that the stability of the chosen phenotypic markers over a period of 48 h is comparable for FT-DC<sub>5</sub> and FT-DC<sub>2</sub> and SDDC.

### 3.2.3. Migration assay

Migration assay against CCL19/MIP-3 $\beta$  was conducted using a transwell system to test the functionality of the cell surface expressed CCR7. All three types of DC were capable of specifically migrating under the chemokine gradient indicating that CCR7 expressed by DC are functional. However, the percentage of cells migrating increased with decreased culture time of the DC (see Table 1).

### 3.2.4. Comparison of cytokine secretion

Production of immune regulatory cytokines by DC is a critical factor for T cell activation. To assess Th 1/Th2 cytokine secretion by FT-DC<sub>5</sub> and FT-DC<sub>2</sub> IL-10 and IL-12 levels were measured. Furthermore we analyzed the secretion of IL-23 which is believed to be important for proliferation of Th<sub>17</sub> CD4<sup>+</sup> T cells [16].

The secretion was tested using supernatants from the day of harvest and supernatants from 48 h after the time of harvest during which DC had been cultured in media without any added protein or cytokines. The concentration of IL-12(p70) measured

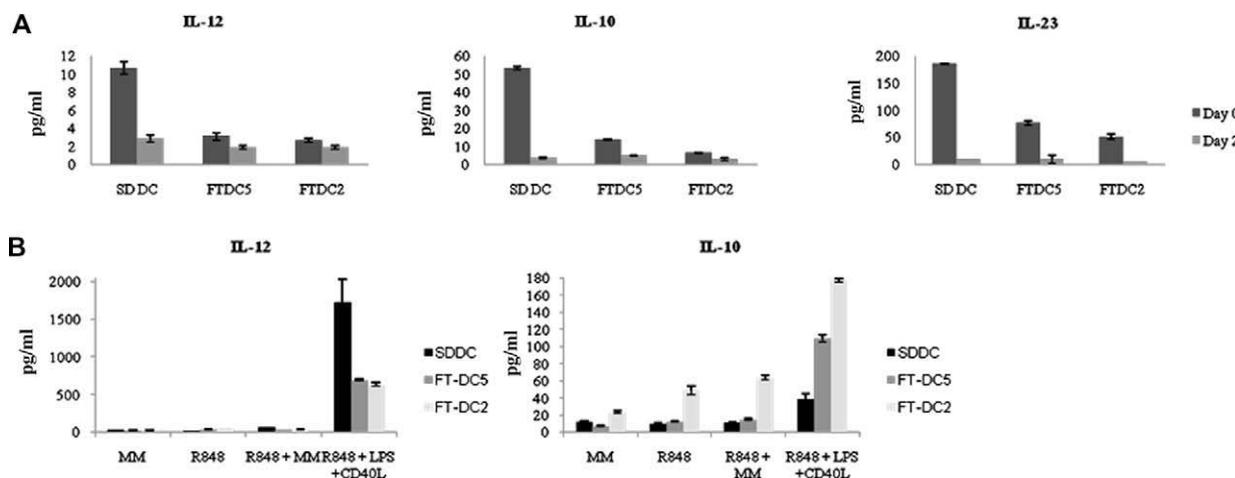
**Table 1**

Results from migration assay. Numbers listed are the percentage of cells migrating against two different concentrations of the chemokine CCL19/MIP-3 $\beta$ .

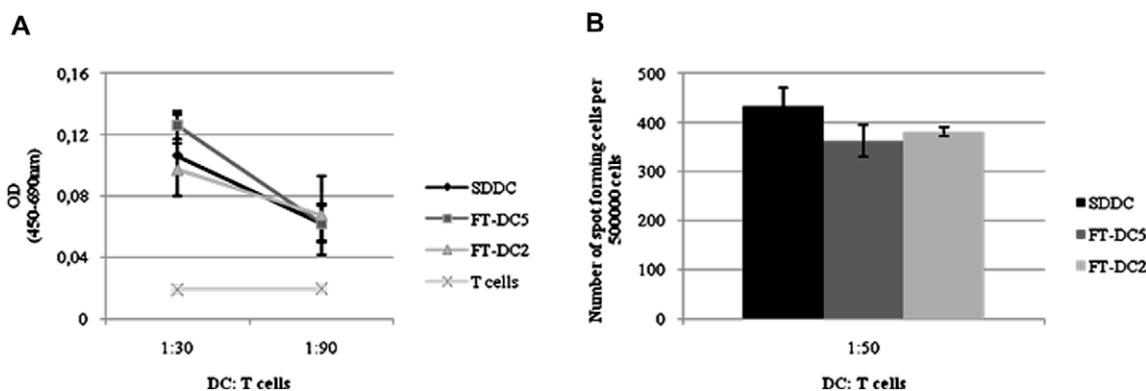
CCL19	SDDC	FT-DC <sub>5</sub>	FT-DC <sub>2</sub>
200 ng	6.2	44.5	59.4
100 ng	3.7	14.7	33.0

was as expected low due to the presence of PGE<sub>2</sub> in the maturation cocktail [17]. SDDC secrete higher concentrations of all three cytokines, i.e. IL-10, IL-12 and IL-23 (Fig. 3B) than the fast generated DC. Also in the washout SDDC cultures, the levels of secreted cytokines were higher although the general cytokine levels are very low in these culture supernatants.

By testing the TLR7/8 agonist Resiquimod (R848) alone or in combination with the maturation cocktail we did not detect a further induction of IL-12p70 production, however, IL-10 secretion was elevated in FT-DC<sub>2</sub> when stimulated with the combination of maturation cocktail and R848 (Fig. 3B). To test our three DC preparations for optimal cytokine secretion capacity R848 was used in combination with LPS and CD40L. This resulted in a significant increase in secretion of IL-12p70 compared to maturation cocktail alone or the combination with R848. However, the IL-10 secretion was also increased significantly, in particular in FT-DC<sub>2</sub> (Fig. 3B). Thus it seems that SDDC are more resistant than FT-DC to the IL-10-inducing effect of the R848, LPS, CD40L cocktail, probably reflecting the less fully differentiated status of the FT-DC. In titration experiments of R848 we did not detect notable changes in either IL-12p70 or IL-10 secretion when added in the range of 1–10  $\mu$ g/ml (data not shown).



**Fig. 3.** Cytokine secretion of the DCs measured on culture supernatants. (A) Supernatants were harvest at time for cell harvest and after additional 48 h DC culture without added cytokines. The concentrations of IL-10, IL-12(p70) and IL-23 were measured by ELISA. Presented is one of three performed experiments using different donors. (B) Cytokine secretion upon stimulation with R848 alone or in combination with maturation cocktail or LPS and CD40L was measured in culture supernatants by ELISA.



**Fig. 4.** T cell stimulatory capacity. (A) Comparison between the allogeneic T cell proliferative response induced by mature SDDC, FT-DC<sub>5</sub> and FT-DC<sub>2</sub>. The incorporation of BrdU was used as a measurement of proliferation. Presented is one of three performed experiments. (B) IFN $\gamma$  secreting T cells were enumerated by EliSpot assay. The number of IFN $\gamma$  secreting T cells was determined in triplicates. One of three performed experiments is presented.

### 3.2.5. T cell stimulatory capacity

The capacity to promote T cell proliferation was assessed in an allogeneic MLR, and we tested the T cell stimulatory capacity of FT-DC<sub>5</sub> and FT-DC<sub>2</sub> compared to SDDC. T cells were co-cultured with any of the 3 types of DC (SDDC, FT-DC<sub>5</sub> or FT-DC<sub>2</sub>) for 4 days and proliferation was measured by incorporation of BrdU. The results in Fig. 4A show similar T cell stimulatory capacity between the 3 types of DC measured by T cell proliferation.

Antigen-specific T cell stimulation was analyzed using material from HLA-A2 and CMV positive donors. The 3 types of DC were pulsed with a HLA-A2 restricted immunogenic CMV-derived peptide. The number of CMV peptide reactive T cells was quantified as IFN $\gamma$  releasing T cells in EliSpot assays when co-cultured with DC presenting the CMV peptide. The results obtained from these experiments show that all 3 types of DC can present and activate antigen-specific T cells to the same degree (Fig. 4B).

### 3.2.6. Phenotypic characterization of T cell populations after stimulation with either of the 3 types of DC

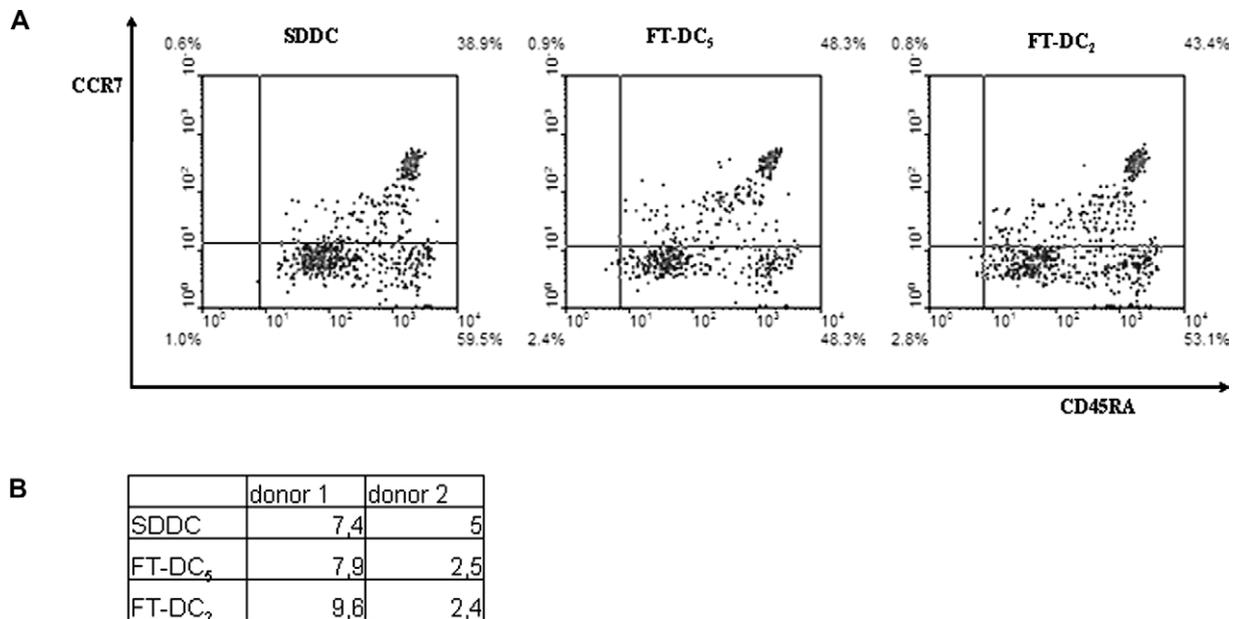
To assess if FT-DC<sub>5</sub> and FT-DC<sub>2</sub> have the capacity to expand immunogenic effector T cell populations in an antigen specific manner comparable with that of SDDC, the phenotypes of the DC/antigen exposed T cell populations were examined. T cells were co-cultured with DC loaded with a CMV-derived HLA-A2 restricted

peptide for 8 days and T cells were subsequently characterized for surface marker expression by flow cytometry including HLA-A2 peptide loaded pentamer, CD8, CCR7, CD62L, CD45RA and CD28. The results in Fig. 5A show comparable expression of CCR7 and CD45RA within the CD8 positive population stimulated with any of the three types of DC. In addition to the phenotypes, the number of antigen-specific T cells (pentamer positive) was assessed. We observed that for some donors the number of Ag specific T cells decreased from SDDC to FT-DC whereas for other donors the numbers were equal (Fig. 5B).

## 4. Discussion

Production of DC for therapeutic purposes has to fulfill several aspects regarding DC function such as up-regulation of maturation markers, expression of co-stimulatory molecules, capacity to prime Th1 responses, and ability to home to lymph nodes. In the majority of clinical trials based on immune therapeutic DC vaccines, DC are generated using the standard protocol of 8–10 days [2].

This report compares for the first time a SDDC protocol with two fast protocols i.e. an intermediate 5 day protocol and a short 48 h protocol. Much work has been carried out by Dauer and colleagues regarding fast generation of DC [8] and several groups have elaborated on this pioneering work [9,10,13,18,19]. Several factors



**Fig. 5.** (A) Phenotypic characterization of T cell populations after 8 days of stimulation with one of the three types of DC. Gates: The lymphocyte population was gated on using forward/side scatter and subsequently CD8<sup>+</sup> cells were gated. The expression of CCR7 and CD45RA within the CD8<sup>+</sup> population is shown in density plots for each T cell population. One of four performed experiments is presented. 10,000 cells within the lymphocyte population were acquired. (B) The percentage of pentamer specific cells obtained after 8 days of stimulation with either of the DC types preloaded with peptide are listed.

have been tested previously in other studies including differentiation cytokines (GM-CSF/IL4, IFN $\alpha$ ) [8,10] and maturation stimuli (standard cocktail, IFN $\gamma$ , CD40L, LPS and R848) [8,10], and isolation of monocytes (CD14<sup>+</sup> MACS isolation, plastic adherence and Elutra systems) [8–10,18]. The results presented here demonstrate that it is possible to shorten the DC protocol to 5 days or even 48 h and generate DC of comparable quality to SDDC regarding both phenotype and functionality. The immature fast DC efficiently endocytosed dextran, underwent DC maturation, then up-regulated the lymph node directing chemokine receptor CCR7, and acquired T cell stimulatory capacity comparable to standard 8 days DC, however, a decrease in the number of Ag specific T cells after 8 days *in vitro* stimulation in some donors was observed. This is probably partly due to the difference in size of the DC (Fig. 1B) and we speculate that this could be circumvented by increasing the number of DC given per vaccine. Furthermore, regarding cytokine secretion we observed differences in susceptibility to IL-10 inducing effects of TLRs (see below).

We have in this study analyzed relevant cell surface markers for immunogenic DC and most importantly we found that functional CCR7 expression remained high in FT-DC<sub>5</sub> and FT-DC<sub>2</sub> (86% and 81%, respectively). Stability analysis revealed that CCR7 expression increased in both FT-DC to the same level of SDDC in the washout culture. We speculate that this increase in CCR7 expression could occur *in vivo* subsequent to injection of vaccine, as it is independent of cytokine stimulation. The functionality of CCR7 was assessed by migration assay and we observed that DC generated using either protocol had the capacity to migrate, however, the percentage of migrating cells increased with decreased time in culture. The assay utilized for this experiment is based on a transwell system and the cells migrate through a membrane with a fixed pore size. This might give smaller cells an advantage over larger cells which is our explanation to the correlation between increasing number of cells migrating and the cell size.

The ligands for CCR7, the chemokines CCL19 and CCL21 are primarily produced in T cell rich paracortical areas of lymph nodes. Hence the expression of CCR7 on monocyte-derived DC for vaccine

purposes is a prerequisite for the induction of an effective immune response. The addition of PGE<sub>2</sub> in the maturation cocktail is very important for both expression and functionality of CCR7 [17]. However, DC activated with PGE<sub>2</sub> often fail to secrete IL-12(p70) when used in the combination commonly referred to as the 'golden standard' maturation cocktail (IL-1, IL-6, TNF $\alpha$  and PGE<sub>2</sub>). It has furthermore repeatedly been demonstrated that PGE<sub>2</sub> inhibits the secretion of IL-12(p70) even in combination with powerful IL-12(p70) stimuli such as CD40L [8]. Despite this IL-12 inhibitory effect it has been demonstrated that PGE<sub>2</sub> *per se* is important for DC's ability to stimulate T cells. Alldawi and co-workers showed that T cell proliferative responses to DC were enhanced by the addition of PGE<sub>2</sub> to maturation cocktail [18]. Accordingly several vaccination studies have obtained induction of T cell responses despite the use of PGE<sub>2</sub> on injected DC [3,7,20]. This correlates well with our results from both allogeneic and CMV peptide specific T cell stimulation experiments where we observed that both fast and standard DC induced T cell proliferation. Furthermore, fast DC are equally capable of stimulating a Th1 directed immune response similar to SDDC as shown by IFN $\gamma$  production of autologous T cells co-cultured with DC.

Dauer and co-workers have shown that despite the presence of PGE<sub>2</sub> in the maturation cocktail, IL-12(p70) production can be activated by additional stimuli through TLR4 and TLR7/8, and thus, IL-12(p70) secretion is not irreversibly inhibited by PGE<sub>2</sub> [17]. Thus, after injection of DC and during their migration to lymph nodes and in the T cell zone of lymph nodes, DC could encounter adequate stimuli to induce IL-12(p70) secretion. In line with this to ensure that IL-12(p70) production could indeed be induced in DC generated using our protocol and that fast and standard DC were equally susceptible to stimulation, we stimulated DC with R848, maturation cocktail, LPS and CD40L in different combinations (Fig. 3B). We observed that IL-12(p70) secretion could be induced in all three types of DC, however, strongest in the SDDC, and that IL-10 production was concomitantly increased. Results showed that SDDC were more resistant than FT-DC to the IL-10-inducing effect of R848, LPS and CD40L cocktail. This difference may reflect the stage of DC differentiation in the fast versus the standard DC at

the time of cell harvest, the standard DC already being terminally differentiated. Thus the plasticity of FT-DC and in particular of 48 h DC can perhaps be an advantage as they might fully differentiate during migration to lymph nodes and be potent producers of cytokines. Results from the EliSpot assay (Fig. 4B) demonstrate that fast DC are equally capable of stimulating Ag specific Th1 responses (IFN $\gamma$ ). However, they might also be more susceptible to immune suppressive tumor-derived factors. Hence, these observations raise concerns about using fast generated DC in clinical settings. However, it is not known if the level of IL-10 potentially produced by DC generated in 48 h will have harmful effects *in vivo*.

Furthermore it has been shown that subsequent to stimulation with LPS DC only secrete IL-12 transiently and become exhausted and are no longer capable of inducing Th1 directed immune responses [21], whereas DC generated using our protocol matured with the maturation cocktail (IL-1 $\beta$ , IL-6, TNF $\alpha$  and PGE $_2$ ) can be induced to secrete IL-12 48 h subsequent to harvest, and hence do not become exhausted (unpublished data Pedersen AW).

Our studies show that both FT DC and SDDC secrete IL-23 suggesting that CD8 T cells can receive help from activated Th $_{17}$  cells. In line with this it has been suggested that IL-23 has additional effects on conventional CD4 Th1 cells, thus, bypassing the crucial need for IL-12(p70) to induce Th1 directed response [22].

Although the standard method (8–10 days) can be used for the generation of DC that have been proven to efficiently induce antitumoral immune responses *in vivo* [3,7,20] this time span may not reflect the kinetics for DC differentiation from monocytes under physiological conditions. Monocytes represent a pool of circulating precursor cells capable of rapid differentiation into mature DC after transit into inflamed or infected tissue [11]. All the reported results of functional fast DC including the present results support these findings.

The reason for the inclusion of both the 5 days and the 48 h protocol in the present study was to assess if crucial DC development occurred in this intermediate stage which would affect the quality of the resultant DC. The intermediate protocol yielded DC less susceptible to IL-10 induction by R848. In addition, we found a decrease however not significant in CCR7 expression between FT-DC $_5$  and FT-DC $_2$ . In general, we observed a trend towards decreased but yet comparable values in all experimental set up in FT-DC $_2$  compared to FT-DC $_5$  and we speculate that this trend is due to the difference in size of the cell (Fig. 1) and can be dealt with by increasing the number fast DC used. Implementing a fast protocol in the production of DC-based cancer vaccines will be less laborious, more cost efficient and potentially more physiological correct. However, to determine the clinical relevance of fast DC protocols small phase I trials including limited number of patients should be conducted and regulatory T cells (induced by IL-10) should be monitored carefully.

## Acknowledgments

This work is part of the PhD. thesis of P. Kvistborg at the university of Copenhagen and DanDrit biotech, Denmark. P. Kvistborg was partly supported by ministry of science technology and innovation. We thank Per thor Straten and Rikke Bæk Sørensen at Center for Cancer Immune Therapy, Herlev, Denmark for kind help conducting part of the T cell FACS analysis. We thank 3 M who kindly provided the Resiquimod.

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