

PHENOTYPIC AND FUNCTIONAL ANALYSES OF STANDARD AND FAST DENDRITIC CELLS (DC) FOR DC BASED IMMUNOTHERAPY

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Abstract: DC based vaccines have been shown to effectively induce high numbers of circulating tumor specific T cells, but their production for clinical use is time-consuming and laborious. Standard DC are frequently derived from peripheral blood monocytes after culture them in GM-CSF and IL-4 for 5 days in presence of a maturation cocktail of pro-inflammatory cytokines: IL-1 β , IL-6, TNF- α and PGE2, for additional 48 hours. Recently it has been published that monocytes cultured in GM-CSF and IL-4 for 24 hours and matured with the cocktail mentioned above for additional 24 hours, lead to obtain fast DC (herein named Fast-DCs). Although Fast-DCs may be suitable to be used as antigen vehicle for cancer immunotherapy, their use for this purpose is still to be demonstrated. In this work a comparison of the surface expression of DCs differentiation markers and co-stimulatory molecules (CD83, CD80, CD209, CD38 and HLA-DR); the expression of the receptor for the CCL19/CCL21 chemokines (CCR7); and the functional analyses such as priming of the naive T cells, cytokine production by CD4+ and CD8+ T cells and

generation of cytotoxic CD8+ T cells were assessed side by side using Standard DC and Fast-DC as APCs. Despite it has been described that the incubation of Standard DC with recombinant human IFN- γ induces expression of CD38 and CD83 this was not observed in Fast-DCs, however, the expression of all surface markers analyzed was very similar between both cellular types and furthermore, upon maturation the induction of CCR7 was higher in Fast-DCs than in Standard DC. On the other hand, the cellular yield was remarkably reduced in Standard DC. Fast-DCs were suitable for expansion of CD8 Cytotoxic T cells precursors specific for HLA-A2 restricted peptide from melanoma from naive individuals and efficiently primed CD4 T cells specific for defined antigens from naive individuals. Altogether our results suggest that Fast-DCs may be suitable for priming T cells and for efficient antigen delivering of DC based cancer vaccines.

INTRODUCTION

DCs are highly specialized APCs important to delivery the antigen to the immune system and to elicit potent T cell mediated immune responses of T cells. DCs pulsed with antigen *in vitro* that activate T cells able to recognize and destroy tumor cells in an antigen specific manner, *in vivo*, have been proved in a wide variety of animal models and in numerous human clinical trials (1-4). In most of these studies, Standard DCs (DCs derived from peripheral blood monocytes in the presence of GM-CSF and IL-4 during 6-7 days (5)) has been commonly used. Recently, Dauer *et al* (6) reported a novel strategy for the development of mature DCs from monocytes within 48 h of *in vitro* culture (herein named Fast-DCs) that seem to be functionally similar to Standard DCs. In this work, we compared side by side on Standard DC and Fast-DCs their sensitivity to express DC activation markers, sustained expression of a lymph node-homing receptor and their proficiency as APCs to stimulate T cells after their stimulation with a cocktail of pro-inflammatory cytokines. Altogether, the results presented here suggest that Fast-DCs are potent APC suitable for priming T cell responses required for DC based cancer vaccines.

OBJECTIVES

To characterize and to compare Fast-DCs (2 days) vs. Standard DCs (7 days) derived from peripheral blood monocytes using pro-inflammatory stimuli.

Specific:

- To obtain Fast-DCs and Standard DCs derived from peripheral blood monocytes using IL-4 and GM-CSF and the maturation cocktail IL-1 β , IL-6, TNF- α and PGE2.
- To compare expression of maturation surface markers and co-stimulatory molecules in both cell types.
- To analyze the functional capacity of DCs as professional APC to induce antigen specific CD4+ and CD8+ T cells.

METHODS

Isolation and cell culture: Monocytes from peripheral blood of healthy donors ($n=9$ Fast-DCs, $n=7$ Standard DCs) were isolated using RosetteSep (StemCell Technologies) and frozen in RPMI, FBS and DMSO. Cells were cultured $1-2 \times 10^6$ cells/ml in AIM-V supplemented with IL-4 (750U/ml) and GM-CSF (1000U/ml) (CellGenix). Standard DCs were cultured during 5 days (37°C/5% CO₂) then cultured with proinflammatory cytokines (CellGenix) during 48 hours (TNF- α 1000U/ml, IL-1 β 10ng/ml, IL-6 10ng/ml and PGE2 1 μ g/ml). For Fast-DCs, monocytes were cultured during 24 hours with IL4, GM-CSF, after that cocktail was added during 24 hours more. To measure the effect of LPS/IFN- γ on the CD38 and CD83 expression on Fast-DCs, the cells were stimulated with LPS (1 μ g/ml) and RhlFN- γ (500U/ml) in a protocol similar to that described in (7). CD4+ and CD8+ T cell were purified by positive selection from PBMCs by MACS column (Miltenyi Biotec GmbH).

DCs phenotype characterization: Fast-DCs and Standard DCs were collected in PBS+10%FBS and stained with mAbs: CD209 (DC-SIGN), HLA-DR, CD14, CD80, CD86, CD83, CD38 (Becton Dickinson) and CCR7 (R&D Systems) with standard cell staining protocol. Cells were analyzed in CyAnTM ADP (Beckman Coulter) and FACScan (BD Biosciences, San José). Data were analyzed in Summit 4.3 (Beckman Coulter), FlowJo 7.2.5 (Tristar) and FCS express 3.0 (DeNovo Software).

Melan-A peptide specific CD8 T cell line generation: Mature Fast-DCs, pulsed with Melan-A peptide (ELAGIGILTV, 10M), were co-cultured with CD8+ T cells from HLA-A0201 donor (1:5 ratio Fast-DCs:CD8 T Cells). After 48 hours IL-2 (300U/ml) and IL-7 (5ng/ml) were added. Cells were collected after 10 days of culture (adding cytokines every 72 hours) counted and re-stimulated with peptide pulsed Fast-DCs (1:10 ratio). Cells were stained with mAbs: CD8 (Caltag) and Melan-A Tetramer (Dako Cytomation) and analyzed in CyAn Flow Cytometer (Dako-Cytomation).

Cytotoxicity assays: Peptide pulsed Fast-DCs were co-cultured with peptide specific CD8+ T Cells generated as described above (1:2 ratio) in a final volume of 200 μ l of AIM-V and 1 μ l of Monensin (Becton Dickinson GolgiStopTM) and 1 μ l of anti-CD107a (Becton Dickinson) were added. As a negative control an irrelevant peptide was used (*Leishmania spp.*, DSLTNLRAL). After five hours of incubation, the cells were collected with PBS + 0.02% Na₃ + 0.5mM EDTA. Cells were stained with CD8 and analyzed by FC.

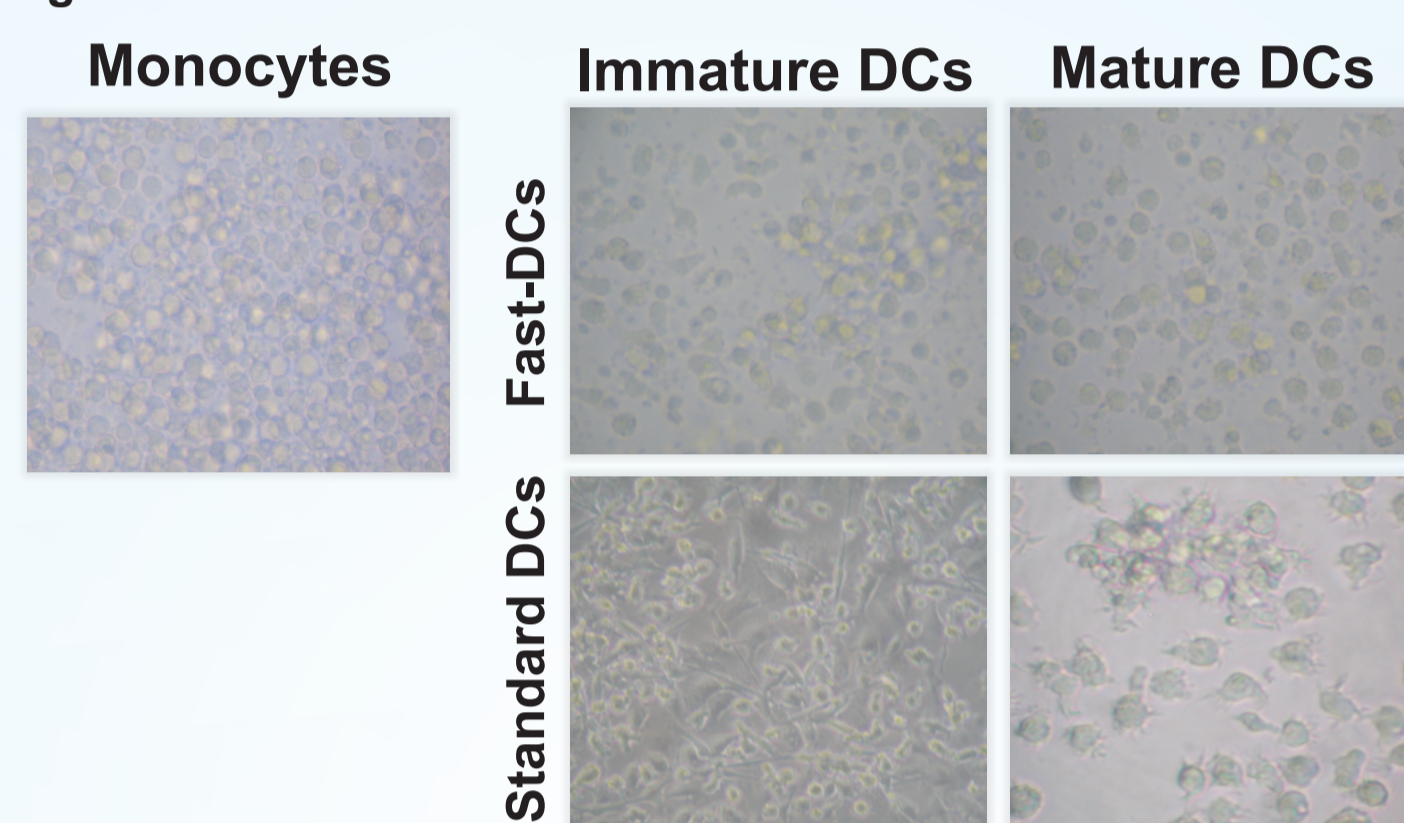
Ex vivo IFN- γ measurement in CD4 and CD8 T cells: Purified CD4 and CD8 T cells from two healthy donors were co-cultured with Standard and Fast-DCs matured with cocktail and pulsed with antigen (*M. tuberculosis*, H37Rv sonicate (300 μ g/ml). Brefeldin A was added in the last 12 hours of incubation. 48 hours later, cells were collected and stained with CD3, CD8, CD45RO, CD62L and CD27. Fixation and permeabilization of cells was done with Cytofix/Cytoperm (Becton Dickinson), for intra-cellular staining of IFN- γ detected by FC.

Statistical analysis: Statistical analysis (Mann-Whitney Test) and graphs were made in Prism 5.0 (GraphPad). p value <0.05 was considered significant.

RESULTS AND DISCUSSION

In order to compare the functional proficiency of Standard and Fast-DCs, morphological, phenotypical and functional analyses of both types of cells were performed. To produce both types of DCs thawed stocks of purified monocytes were used. After culturing monocytes *in vitro* with IL-4 and GM-CSF and incubating with maturation cocktail the yields of Fast-DCs were three fold higher than that achieved with Standard DCs. Morphologically, Fast-DCs were very similar to freshly isolated monocytes; instead Standard DCs showed morphologic changes characterized by size increase and changes in cell shape (Fig. 1).

Figure 1.



The phenotypic analysis of DCs in response to pro-inflammatory cytokines cocktail showed typical maturation markers with a significant increase of CD80 and CD83 (Fig. 2) in both DCs types ($p=0.0001$ Fast-DCs Fig. 2A and $p=0.0006$ Standard DCs Fig. 2B). Although there was an initial expression of CD86 marker (Fig 3), its expression on both Fast-DCs and Standard DCs was significantly increased upon maturation ($p=0.0002$ and $p=0.0006$ respectively). Whereas all Fast-DCs samples up-regulated the expression of CCR7 after maturation ($p=0.0022$), this was not observed in Standard DCs ($p=0.5350$) (Fig. 4A). There was a down-regulation of the monocyte marker CD14 in Fast-DCs and Standard DCs compared with its expression on monocytes (Fig. 4B).

Figure 2.

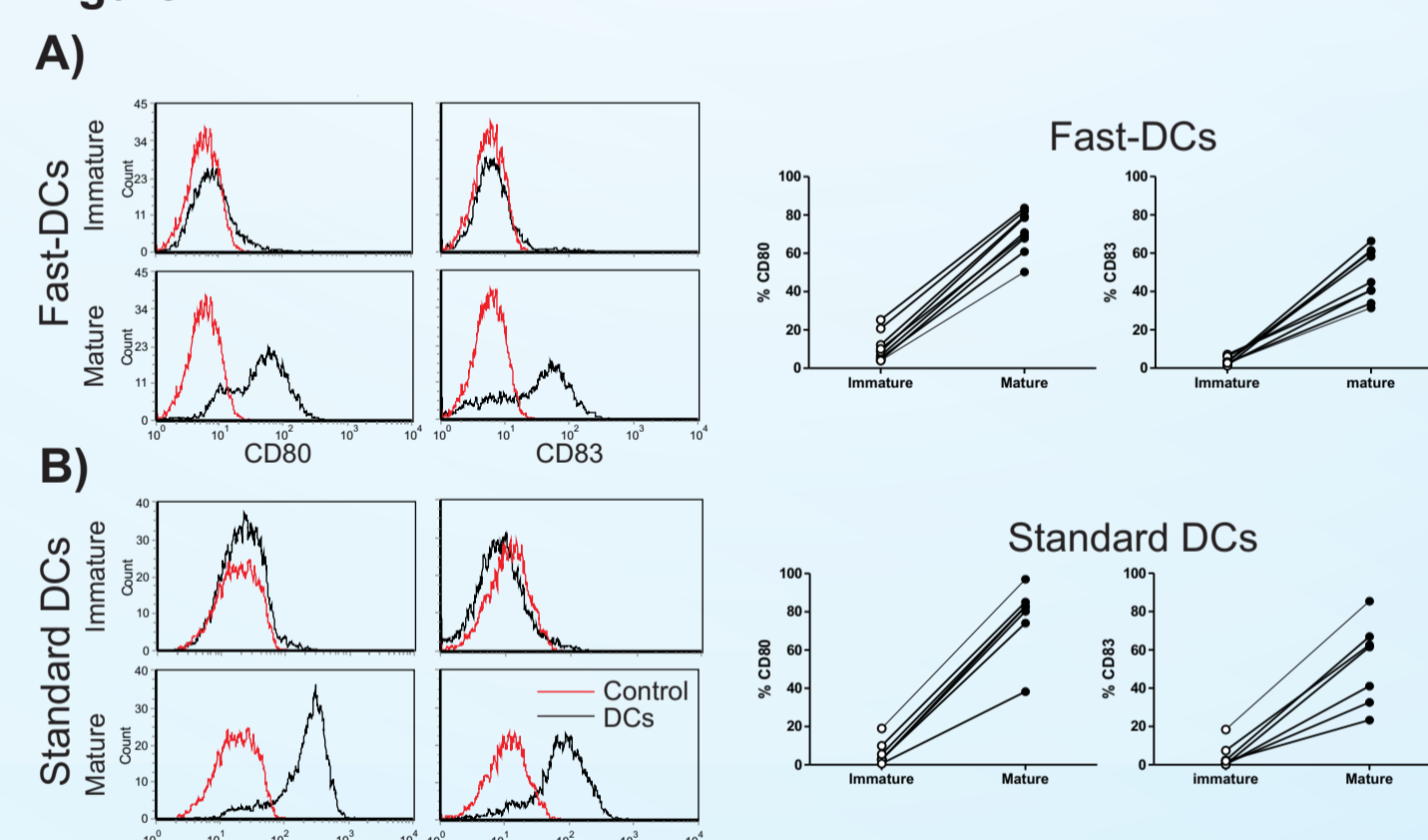


Figure 3.

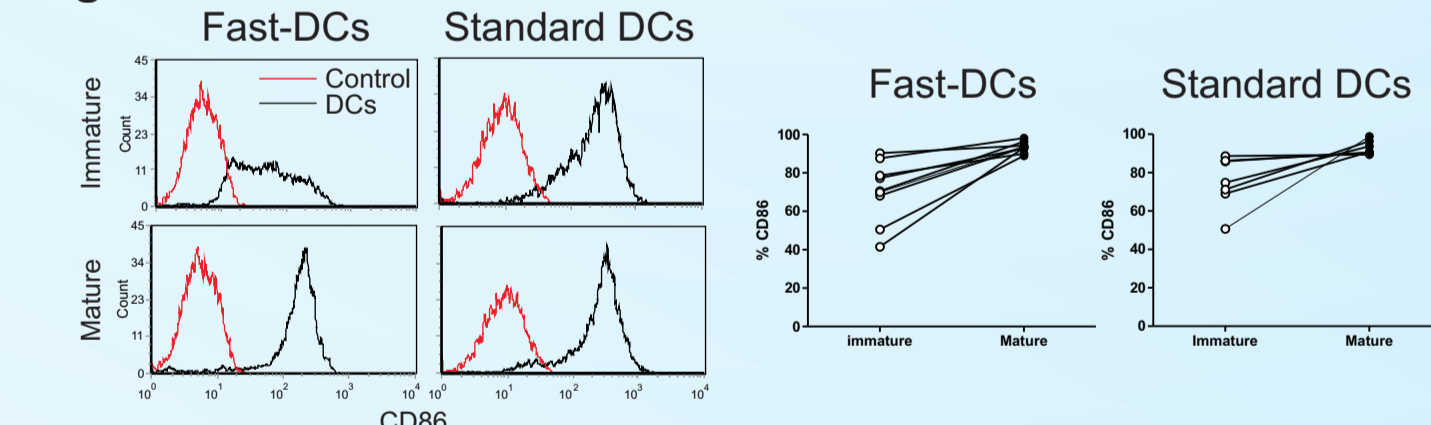
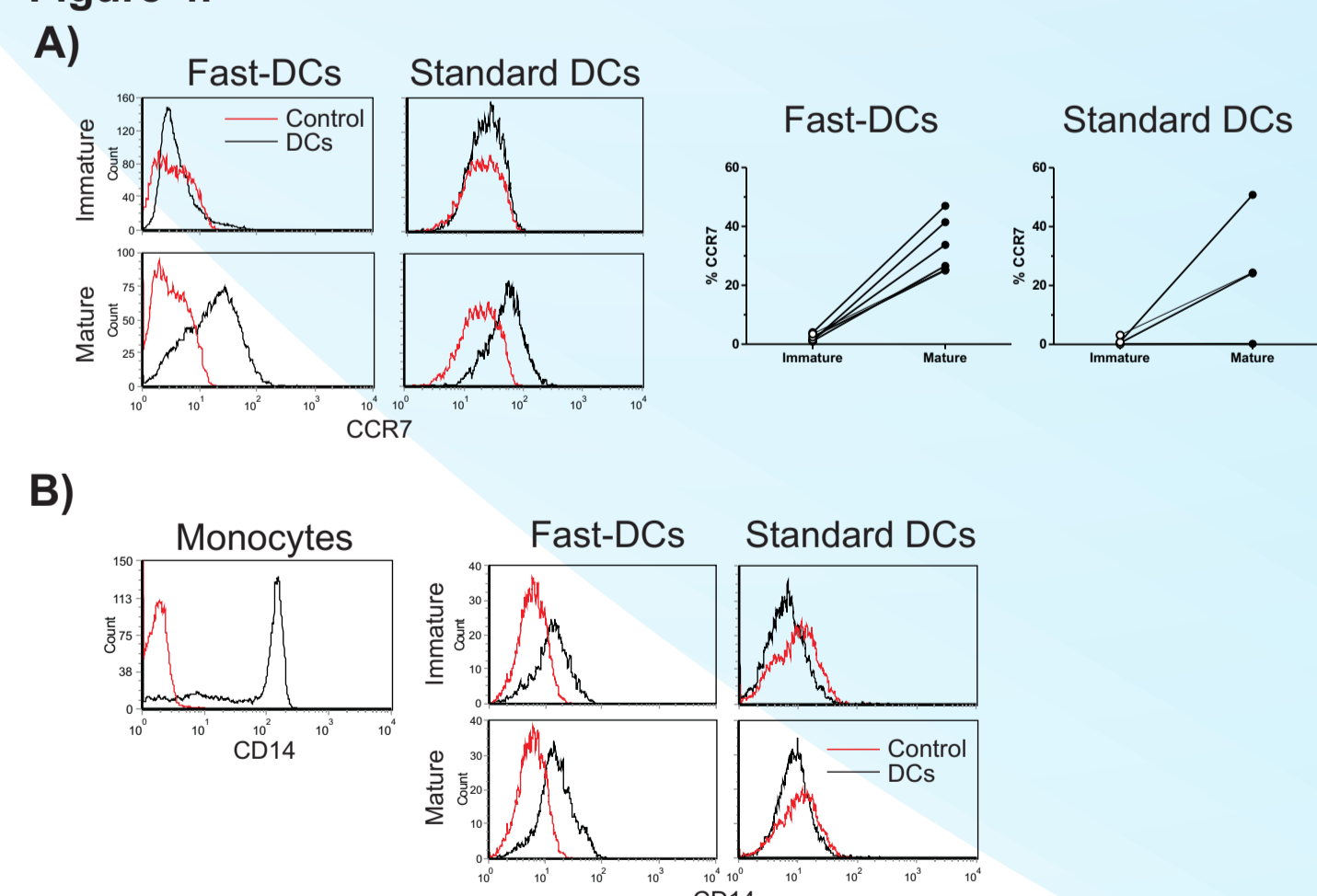


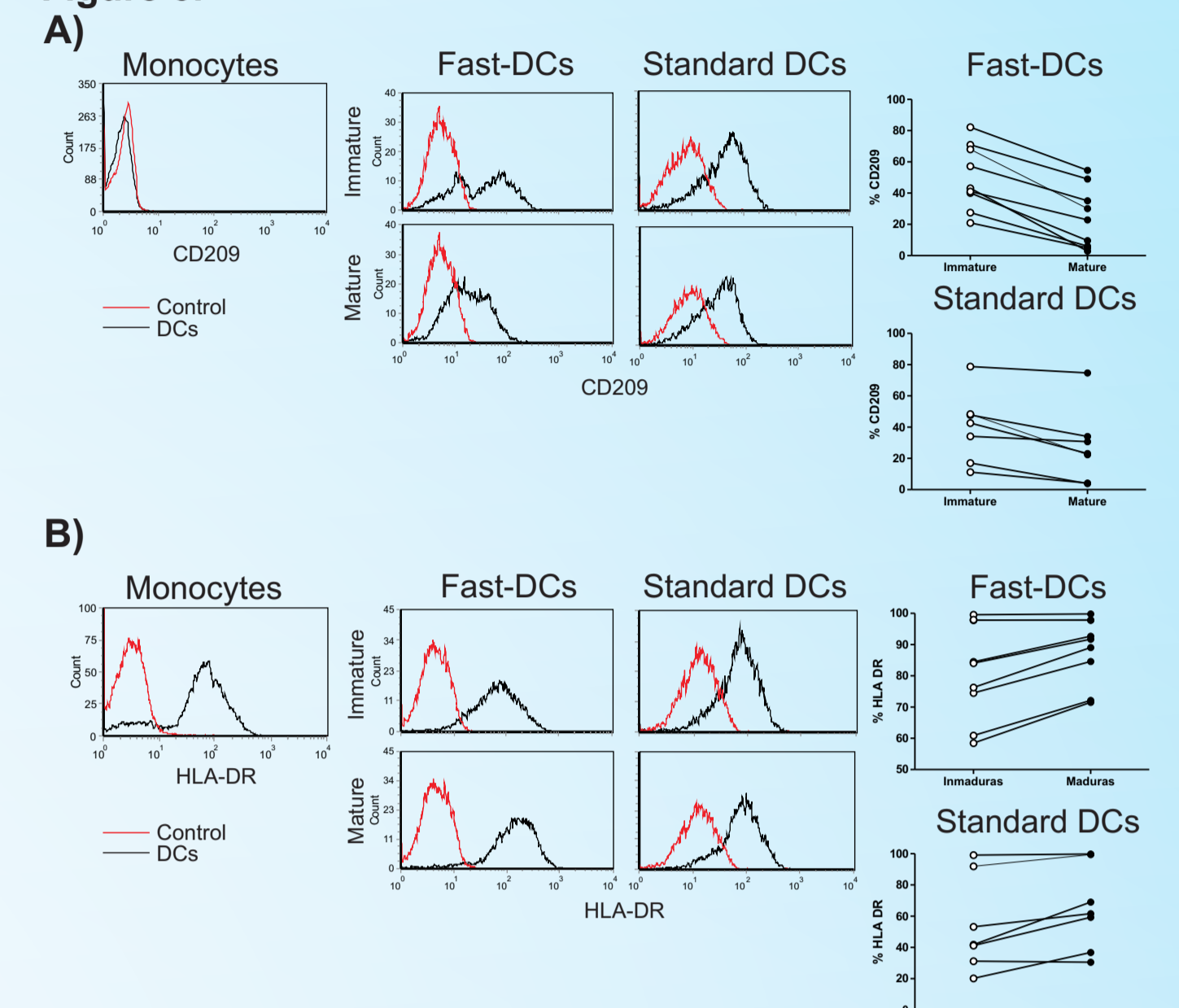
Figure 4.



Meanwhile in Fast-DCs the expression of CD209 (DC-SIGN) decreased in the presence of the cocktail ($p=0.0244$), in Standard DCs there was no significant difference ($p=0.2086$) between immature and mature DCs upon maturation (Fig. 5A). Finally, there was no significant difference in the over-expression of MHC class II in both types of mature DCs in most of the analyzed donors (Fig. 5B). Of note, there was a remarkable variance in the expression of CD209 and HLA-DR markers in both Standard and Fast-DCs throughout the individuals analyzed (Fig. 5 panels A and B).

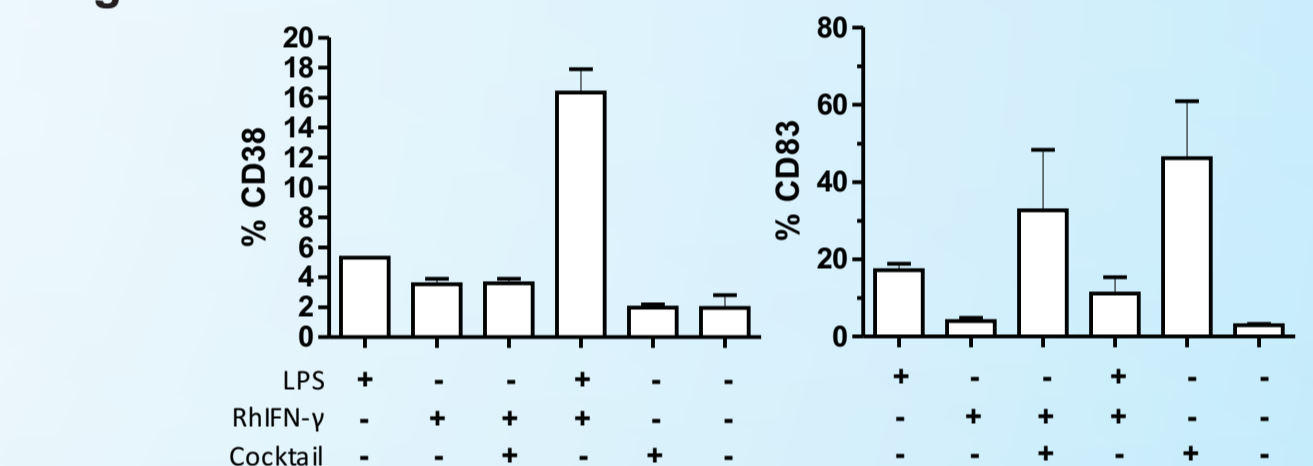
Recently, an effect of IFN- γ on Standard DCs in promoting Th-1 development of T cells has been described (7). This effect that seems to be mediated by TLR4 stimulation is p38 activation pathway dependent and is accompanied by CD38 and CD83 up regulation. The effect of IFN- γ in up-regulating CD38 and CD83 expression in LPS/IFN- γ stimulated Fast-DCs was explored. The results shown in Figure 6 suggest that although the double stimulation of Fast-DCs with LPS and IFN- γ remarkably up-regulate CD38 expression, this effect was not

Figure 5.



accompanied by CD83 over-expression. Furthermore, by analyzing the up-regulation of CD83 on these cells, it was found that Fast-DCs efficiently up-regulate CD83 expression just in response to cytokine cocktail. Of note, this over-expression of CD83 induced on Fast-DCs by the cocktail was remarkably higher compared to that achieved by stimulating the cells with LPS plus IFN- γ (Fig. 6).

Figure 6.



Functional assays were performed with T cells using Standard and Fast-DCs as APCs matured with cocktail and pulsed with antigen (*M. tuberculosis* sonicate). Using FC, the intracellular production of IFN- γ in purified CD4+ and CD8+ T cells from 2 healthy donors was measured. Table-1 shows that upon stimulation with both types of DCs there was a significant production of IFN- γ production in both CD4+ and CD8+ T cells in response to the antigen. The capacity of both types of DCs to elicit terminal differentiation of effectors T cells was assessed by measuring the down modulation of CD27 on CD4 and CD8 T-cells responding to the antigen, the results in Table 1 suggest that while both types of DCs induced a remarkably down-modulation of DC27 on CD8 this effect was not observed in CD4 T-cells. In these experiments, the stimulation of both CD4 and CD8 T-cells with antigen pulsed Standard or Fast-DCs did not produce significant differences in the expansion of four memory T cell subpopulations examined (Naïve (CD62L+/CD45RO-), TCM (CD62L+/CD45RO+), TEM (CD62L-/CD45RO+), TEF (CD62L-/CD45RO-) (data not shown).

Table 1.

	IFN- γ +				CD27+			
	CD4+	CD8+	CD4+	CD8+	D1	D2	D1	D2
Control	0,2	0,1	0,3	0,2	10	14	7,1	7,4
Fast-DCs + Sonicate	0,3	0,4	1	0,5	8,1	8,4	1,1	2,4
Standard DCs + Sonicate	0,2	0,3	0,5	0,3	7,1	8,7	0,9	2,6

Once the high similarity of the phenotype of mature Fast-DCs and Standard DC was proved, we evaluated the functional capacity of Fast DC to prime naive CD8 T cells specific for a tumor antigen. To do so, a specific Melan-A CD8+ T cell line specific for the Melan-A epitope ELAGIGILTV was generated and re-stimulated with Fast-DCs pulsed with this epitope. As shown in Figure 7, a T cell population specific to the peptide (16.62% positive tetramer in CD8+ T cell gate) was evidenced in the culture upon re-stimulation. Finally CD8+ cytotoxic T Lymphocytes were analyzed by the expression of CD107a by FC, after stimulation of the peptide specific T cell line with peptide pulsed Fast-DCs a population of 62.67% CD8+/CD107a+ double positive T cells were evidenced in these cultures (Fig. 8).

Figure 7.

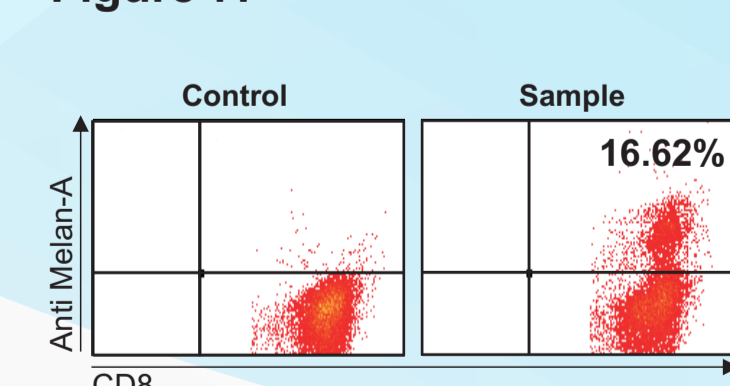
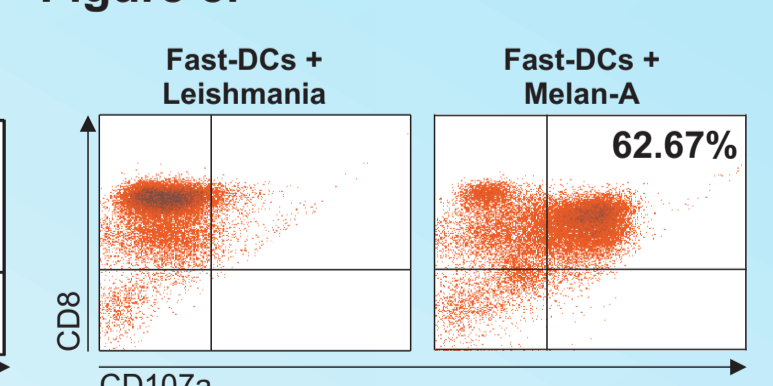


Figure 8.



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