Dendritic cells generated in the presence of interferon-α stimulate allogeneic CD4+ T-cell proliferation: modulation by autocrine IL-10, enhanced T-cell apoptosis and T regulatory type 1 cells

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Keywords: CD4 T cell subsets, cytokines, T lymphocytes

Abstract

Dendritic cells (DCs) generated in the presence of IFN-α (IFN-DCs) exhibit high expression of major histocompatibility and co-stimulatory molecules and a potent ability to stimulate CD8+ T-cell responses. Here, we found that IFN-DCs were more potent stimulators of bulk and purified CD8+ T-cell proliferation, as compared with IL-4-DCs. In contrast, IFN-DCs were less efficient than IL-4-DCs in stimulating allogeneic CD4+ T-cell proliferation, due to a weak induction of naive CD4+CD45RO− T-cell proliferation by these DCs. However, both DC populations induced similar levels of proliferation of memory CD4+CD45RO+ T cells. IFN-DCs and IL-4-DCs exhibited a similar phenotype and production of IL-10 following maturation induced by CD40 ligation. In contrast, IFN-DCs produced higher levels of IL-10 during the first days of differentiation. In addition, neutralization of IL-10 during the differentiation of DCs increased the expression of DC-LAMP and MHC class II by IFN-DCs, and the ability of IFN-DCs to stimulate allogeneic CD4+ T-cell proliferation at similar levels, than IL-4-DCs. Independently of IL-10 production, IFN-DCs were found to induce higher levels of CD4+T-cell apoptosis, this effect being more sticking on naive T cells. Finally, we demonstrated that IFN-DCs induced a differentiation bias of naive CD4+ T cells towards Th1 and Tr1 cells, compared to IL-4-DCs. Taken together, these results indicate that, despite the induction of Tr1 cells and enhanced apoptosis of naive CD4+ T cells, IFN-DCs are potent stimulators of CD8+ and memory CD4+ T cells, and induce a strong polarization of naive CD4+ T cells towards Th1 cells, further supporting their use in immune-based therapy.

Introduction

Dendritic cells (DCs) are professional APCs able to induce primary immune responses against pathogens (1–3). Immature DCs reside in peripheral tissues, as sentinel. After contact with danger signals, DCs undergo maturation and migrate to secondary lymphoid organs, where they first stimulate CD4+ T cells (1–3). Once activated, CD4+ T cells ‘licence’ DCs to stimulate CD8+ T lymphocytes via cognate interactions as CD40/CD40 ligand (CD40L) or soluble mediators (4–6).

DCs can be derived from blood circulating monocytes cultured in the presence of different combinations of cytokines including GM-CSF plus IFN-α, in vitro (7,8). IFN-α is a potent non-specific anti-viral cytokine produced by many cell types including DCs (9). DCs generated from peripheral blood monocytes in the presence of GM-CSF and IFN-α (IFN-DCs) exhibit a DC morphology, express low levels of CD1a, maintain a weak expression of CD14 and CD16 and highly express MHC class I and II and co-stimulatory molecules (7,8,10). IFN-α-treated DCs are potent inducers of CD8+ T cell-mediated immunity (8–11). McRae et al. (12) reported, however, that IFN-α may inhibit monocyte differentiation towards DCs. In addition, it has been suggested that IFN-α-
treated blood DCs produced high levels of IL-10 and induced regulatory T cells (Tr1) (13). IL-10 is a potent immunoregulatory cytokine reported to modulate the immunobiology of DCs. Thus, IL-10 alters the differentiation and the maturation of DCs, blocks IL-12 secretion and impairs CD4+ T-lymphocyte responses (14,15). IL-10-treated DCs have also been described as tolerogenic DCs (16,17).

We have previously reported that IFN-DCs were able to prime IFN-γ production and cytotoxic activity by HIV-specific CD8+ T cells (10). It has recently been suggested that IFN-DCs induced a Th1 immune response (18). The aim of the present study was to further characterize the ability of DCs generated in the presence of GM-CSF and IFN-α to induce CD4+ T-cell responses and a CD4+ T-cell polarization, as compared with DCs generated in the presence of GM-CSF and IL-4 (IL-4-DCs).

Methods

In vitro differentiation of DCs

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats [Etablissement Français du Sang (EFS), Rungis, France] by Ficoll density gradient centrifugation on MSL (Medium for Separation of Lymphocytes, Eurobio, Les Ulis, France). The percentage of monocytes among PBMC was determined by flow cytometry using forward scatter and side scatter properties (FSC/SSC). Monocellular cells were resuspended in RPMI 1640 with ultra-glutamine (BioWhittaker Europe, Verviers, Belgium) penicillin±streptomycin (100 IU/ml) and 10% AB serum (EFS). Cells were then seeded into 12-well plates (Costar, Cambridge, MA) at the concentration 1 × 10^6 adherent cells/ml. Plates were incubated at 37°C for 1 h. Non-adherent cells were kept for subsequent T-lymphocyte isolation. The adherent cell population was rinsed gently and resuspended in RPMI 1640 supplemented with 10% FCS (Dutscher, Brumath, France) and penicillin±streptomycin for 24 h of stimulation. After stimulation, cells were saturated with PBS±azide (0.01%) and fixed with PBS± formaldehyde (4%) (Sigma-Aldrich). Cells were subsequently incubated with anti-IL-10±PE mAb (Diaclone S.A., Besançon, France) in PBS±azide (0.01%) and washed twice with PBS containing azide (0.01%) and BSA (0.2%) and then fixed using PBS formaldehyde (1%) (Sigma-Aldrich, St Louis, MO). Intracellular staining of DC-LAMP was performed as described below for the detection of cytokine, using a buffer containing saponin (0.5%) (Sigma-Aldrich) and an anti-DC-LAMP±PE mAb provided by Coulter-Immunotech SA. Analyses were performed using FACScalibur and CellQuest software (Becton Dickinson, Mountain View, CA) on at least 1000 events. Gating was performed according to light scattering properties.

Maturation of DCs

At day 7, DCs were stimulated for 48 h by human CD40L transfected mouse L-cells (L40L cells), kindly provided by Dr J. Blanchereau, at a ratio of 5 × 10^4 L40L cells to 10^6 DCs in the presence or absence of rhIFN-γ (1000 IU/ml/10^6 cells, Roussel-Uclaf, France). Phenotype and cytokine production were then assessed at single cell level by flow cytometry.

Intracellular detection of IL-10 production by DCs by flow cytometry at single cell level

In order to prevent cytokine secretion, Brefeldin A (10 µg/ml/10^6 cells) (Sigma-Aldrich) was added to cell cultures in the last 24 h of stimulation. After stimulation, cells were saturated with RPMI 1640, 10% FCS, incubated with anti-CD83±PECy5 mAbs (Coulter-Immunotech) for 30 min at 4°C and fixed with PBS formaldehyde (4%) (Sigma-Aldrich). Cells were subsequently incubated with anti-IL-10±PE mAb (Diaclone S.A., Besançon, France) in PBS±azide (0.01%) BSA (0.2%) saponin (0.5%) (Sigma-Aldrich) for 25 min at room temperature. Cells were washed twice before flow cytometric analysis.

Measurements of IL-10 secretion

Cell-free culture supernatants were collected after 2, 4 and 6 days of DCs cultures. IL-10 was quantified using Quantikine® sandwich immunoassay kits from R&D Systems according to the manufacturer’s protocol. Assays were performed in triplicate. The detection limit was 10 pg/ml.

Purification of T cell subsets

Non-adherent PBMC were re-incubated in medium overnight at 37°C. The recovered fraction, referred to as ‘bulk CD3+ T cells’ contained >90% CD3+ T lymphocytes and <1% monocytes (as assessed by flow cytometry). T lymphocytes were frozen until use.

CD4+ T lymphocytes were purified from bulk T lymphocytes using the CD4+ isolation kit twice (Mylenyi Biotec, Bergisch-Gladbach, Germany). The recovered positively selected cell fraction, referred to as ‘CD8+ T lymphocytes’ contained >90% CD3±CD8+ T lymphocytes.

In some experiments, the recovered negatively selected CD4+ cell fraction was further washed and incubated with CD45RO+ magnetic beads (Mylenyi Biotec) for 30 min at 4°C.
The CD4+CD45RO+ cells were subsequently separated using magnetic columns (Myltenyi Biotec) according to the manufacturer’s instructions. CD45RO+ and CD45RO- fractions were collected separately. This generally resulted in obtaining >90% purity of both CD4+ T cell subsets, as assessed by flow cytometry (data not shown).

**Apoptosis detection**

Apoptosis of DC-stimulated allogeneic CD4+, CD4+CD45RO+ and CD4+CD45RO- T cells was assayed by staining with annexin V–FITC and propidium iodide (Apoptosis detection kit, Coulter Immunotech) according to the manufacturer’s instructions. Cells were then analyzed by flow cytometry.

**Analysis of cytokine production by DC-stimulated allogeneic CD4+ T cell subsets**

CD4+, CD4+CD45RO+ or CD4+CD45RO- T cells were co-cultured for 6 days with extensively washed IFN-DCs or IL-4-DCs, generated in the presence of either anti-IL-10 mAbs or control IgG mAb. Cells were then harvested and restimulated with 25 ng/ml PMA (Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich) for 8 h. In addition, naive CD4+CD45RO- T cells were restimulated with plate-bound anti-CD3 mAbs (1 μg/ml, Coulter Immunotech) and soluble anti-CD28 mAbs (1 μg/ml, Coulter Immunotech) for 8 h. Brefeldin A (10 μg/ml, Sigma-Aldrich) was added during the last 4 h of culture. Cells were stained, using anti-CD3–PerCP or anti-CD3–APC and anti-CD8–PerCP or anti-CD8–APC (BD Pharmingen), for 30 min at 4°C and fixed with PBS formaldehyde (4%) (Sigma-Aldrich). Cells were subsequently incubated with anti-IFN-γ–FITC and anti-IL-10–PE mAbs (Diaclone) in PBS-azide (0.01%), BSA (0.2%), saponin (0.5%) (Sigma-Aldrich) for 25 min at room temperature. Cells were washed twice before flow cytometric analysis. Analyses were performed using FACScalibur and CellQuest software (Becton Dickinson) on at least 5000 events.

**Statistical analysis**

Data were expressed as mean ± SD. Statistical comparisons were performed using Student’s t-test. Significance was considered for P < 0.05.

**Results**

**Immature IFN-DCs are more potent to induce allogeneic CD8+ T lymphocyte proliferation but stimulate less efficiently allogeneic CD4+ T lymphocytes as compared with immature IL-4-DCs**

We first assessed the ability of immature IFN-DCs to stimulate allogeneic CD3+ T-cell proliferation. DCs were co-cultured with purified allogeneic CD3+ T cells at DC/T ratios ranging from 1:80 to 1:10; [3H]thymidine incorporation was measured after 4 days. As illustrated in Fig. 1(A), we found that IFN-DCs induced higher levels of allogeneic CD3+ T-cell proliferation than IL-4-DCs from the same donor, as previously reported by Santini et al. (8). Interestingly, unlike IL-4-DCs, IFN-DCs induced the proliferation of purified CD8+ T lymphocytes (Fig. 1B). In contrast, the level of allogeneic CD4 T-cell proliferation was significantly lower when IFN-DCs were used as presenting cells as compared with IL-4-DCs (Fig. 1C). Taken together, these data indicate that the proliferation of allogeneic CD3+ T cells induced by immature IL-4-DCs was mainly related to the proliferation of CD4+ T cells, whereas the proliferation of allogeneic CD3+ T cells induced by immature IFN-DCs was dependent on the proliferation of both CD4+ and CD8+ T cells.

**Immature IFN-DCs induce similar levels of memory CD4+CD45RO+ T-cell proliferation but stimulate less efficiently naive CD4+CD45RO- T-cell proliferation as compared with immature IL-4-DCs**

We further assessed whether immature IFN-DCs and immature IL-4-DCs differ in their ability to induce the proliferation of naive and memory CD4+ T cell subsets. As shown in Fig. 2, IFN-DCs were less potent than IL-4-DCs to stimulate the proliferation of naive CD4+CD45RO- T cells (4.1-fold less). In contrast, the levels of memory CD4+CD45RO+ T-cell proliferation induced by IFN-DCs and IL-4-DCs did not differ. These data suggest that the proliferation of allogeneic CD3+ T cells induced by immature IL-4-DCs was mainly related to the proliferation of CD4+ T cells, whereas the proliferation of allogeneic CD3+ T cells induced by immature IFN-DCs was dependent on the proliferation of both CD4+ and CD8+ T cells.

**Mature IFN-DCs stimulate less efficiently allogeneic unfractionated CD4+ and naive CD45RO T cells as compared with mature IL-4-DCs**

Considering that maturation of DCs spontaneously occurs during an allogeneic DC/T co-culture, we further asked the question whether the low levels of CD4+ T-cell proliferation induced by IFN-DCs were dependent on a defective maturation of this DC population following CD40 ligation. We thus assessed: (i) the expression of the co-stimulatory molecules, CD80 and CD86, of HLA-DR and of the DC markers CD83 and intracellular DC-LAMP following maturation of both DC populations induced by CD40 ligation (40L cells) in the presence or absence of IFN-γ for 48 h; (ii) the ability of both mature DC populations to induce the proliferation of CD4+ T cell subsets.

As illustrated in Fig. 3(A), despite a trend towards a lower expression of CD83 on IFN-DCs, the similar expression of intracellular DC-LAMP, of HLA-DR, CD80 and CD86 following maturation of both DC populations suggests that IFN-DCs

...
CD4⁺ T-cell responses induced by IFN-α-treated DCs

Donor A

Donor B
mature as efficiently as IL-4-DCs following T-cell derived signals.

To characterize the stimulatory properties of mature IFN-DCs, both DC populations that had been stimulated in the presence of L40L cells, (mature DCs) were subsequently co-cultured with purified CD4+, CD4*CD45RO+, CD4*CD45RO- or CD8+ T cells. As shown in Fig. 3(B), CD40 ligation increased the stimulatory abilities of both DC populations, for all T cell subsets. However, as observed for immature DCs, mature IFN-DCs were less potent than mature IL-4-DCs to stimulate the proliferation of unfractionated allogeneic CD4+ and naive CD4*CD45RO+ T cells (mean: 1.7- and 2.5-fold less, respectively, for the three donors tested). In contrast, the levels of memory CD4*CD45RO+ T-cell proliferation induced by IFN-DCs and IL-4-DCs were similar. As expected, maturation of IL-4-DCs significantly increased their ability to stimulate the proliferation of allogeneic CD8+ T cells. Interestingly, even following maturation, IFN-DCs were more potent than mature IL-4-DCs to induce allogeneic CD8+ T-cell proliferation.

**IFN-DCs and IL-4-DCs exhibit a similar ability to produce IL-10 following T-cell derived signals**

CD4+ T-cell responses have been reported to be influenced by the IL-12/IL-10 balance (19,20). We previously reported that mature IFN-DCs and IL-4-DCs exhibited similar proportions of IL-12-producing cells following CD40 ligation. We further quantified IL-10-producing DCs at the single cell level following maturation. Stimulated DCs were stained for membrane expression of CD83 and intracellular IL-10 production. We found that the proportion of IL-10-producing IFN-DCs was similar to that of IL-10-producing IL-4-DCs, following stimulation with L40L cells alone (data not shown) or in combination with IFN-γ (Fig. 4A). As IL-10 was also reported to be produced by CD4+ T cells (21), we neutralized IL-10 secreted throughout the DC/T co-culture in order to assess the impact of IL-10 production on the levels of CD4+ T-cell proliferation. As shown in Fig. 4(B), the levels of CD4+ T-cell proliferation were not affected by the neutralization of IL-10 produced throughout the co-culture of CD4+ T cells and either IFN-DCs or IL-4-DCs.

Taken together, these data indicate that lower levels of allogeneic CD4+ T-cell proliferation induced by IFN-DCs are not dependent on defective maturation of this DC population or enhanced IL-10 production during the proliferation assay.

**IFN-DCs spontaneously produce higher amounts of IL-10 than IL-4-DCs during the first steps of differentiation**

As autocrine IL-10 production by DCs during their differentiation was described to also influence the ability of DCs to induce allogeneic CD4+ T-cell proliferation (22), we measured the concentrations of IL-10 in culture supernatants of differentiating IFN-DCs and IL-4-DCs, at days 2, 4 and 6. As shown in Table 1, when monocytes were differentiated in the presence of IL-4, IL-10 production was detected from day 2 and decreased throughout the culture. At days 2 and 4, cells cultured in the presence of IFN-α produced significantly higher amounts of IL-10 than cells cultured in the presence of IL-4. Conversely, at day 6, IFN-DCs and IL-4-DCs were found to secrete similar levels of IL-10.

**Neutralization of IL-10 produced during the differentiation significantly increases the expression of DC-LAMP and MHC class II molecules and the ability of immature IFN-DCs to stimulate allogeneic CD4+ T-lymphocyte proliferation**

To gain an insight into the role of IL-10 in the modulation of IFN-DC differentiation, secreted IL-10 was neutralized during the whole differentiation using anti-IL-10 mAbs. As illustrated in Fig. 5(A and B), the intracellular expression of DC-LAMP strongly increased in both IL-4-DCs and IFN-DCs, when neutralizing anti-IL-10 mAbs were added to the culture. Levels of MHC class II molecules were also significantly increased on IFN-DCs, generated in the presence of anti-IL-10 mAbs. In contrast, the neutralization of IL-10 had little effect on the expression of the co-stimulatory molecules CD80 and CD86 and other markers (CD14, CD16, CD1a, CD83) on both DC populations (data not shown). These results suggest that the neutralization of IL-10 weakly affects the stage of differentiation of IFN-DCs but dramatically increases the expression of molecules related to the pathway of MHC class II antigen presentation.

Moreover, in order to characterize the impact of IL-10 produced throughout the differentiation of IFN-DCs on their ability to stimulate allogeneic CD4+ T cells, DCs generated in the presence of neutralizing anti-IL-10 mAbs were co-cultured with unfractionated, memory and naive allogeneic CD4+ T cells, CD4+ T cells induced by IFN-4, IL-10 production was detected from day 2 and decreased throughout the culture. At days 2 and 4, cells cultured in the presence of IFN-α produced significantly higher amounts of IL-10 than cells cultured in the presence of IL-4. Conversely, at day 6, IFN-DCs and IL-4-DCs were found to secrete similar levels of IL-10.

**Fig. 1.** Proliferation of unfractionated, memory and naive allogeneic CD4+ T cells induced by immature IFN-DCs and IL-4-DCs. Immature IFN-DCs (black symbols) and IL-4-DCs (white symbols) generated from monocytes, as previously described, were co-cultured with allogeneic unfractionated CD4+ T cells, allogeneic purified naive CD4*CD45RO- or memory CD4*CD45RO+ T cells at a DC/T ratio of 1:10 for 4 days. Thymidine incorporation was measured after an 18 h pulse with 1 μCi of [3H]thymidine. Results, representative of two different donors, are shown as mean ± SD of triplicate values.

**Fig. 2.** Proliferation of unfractionated, memory and naive allogeneic CD4+ T cells induced by immature IFN-DCs and IL-4-DCs. Immature IFN-DCs (black) and IL-4-DCs (white) generated from monocytes, as previously described, were co-cultured with allogeneic unfractionated CD4+ T cells (C) at different DC/T ratios for 4 days. Thymidine incorporation was measured after an 18 h pulse with 1 μCi of [3H]thymidine. Results from two different donors (left and right panels) are shown as mean ± SD of triplicate values.*P < 0.05 between IFN-DCs and IL-4-DCs.
with allogeneic CD4+ T cells. Addition of anti-IL-10 mAbs during the differentiation dramatically increased the ability of IFN-DCs to stimulate the proliferation of allogeneic CD4+ T cells (Fig. 5C). Allogeneic CD4+ T-cell proliferation induced by IL-4-DCs generated in the presence of either control mAbs or anti-IL-10 mAbs did not differ (Fig. 5D).

Thus, the neutralization of IL-10 during the differentiation increases the ability of IFN-DCs to induce allogeneic CD4+ T-lymphocyte proliferation, at similar levels to IL-4-DCs.

Immature IFN-DCs, whether differentiated in the presence of anti-IL-10 or control mAbs, induce higher levels of apoptosis of total, memory and naive CD4+ T cells compared to IL-4-DCs.

Several studies have previously reported that type I IFNs could promote activation-induced cell death (AICD) of CD4+ T cells (23). To investigate the hypothesis that decreased levels of CD4+ T-cell proliferation induced by IFN-DCs may also result from enhanced programmed cell death, we assessed the binding of annexin V by CD4+ T cells following 4 days of culture in the presence of either IFN-DCs or IL-4-DCs. As illustrated in Fig. 6, CD4+ T-cell apoptosis was significantly higher (1.3-fold) following stimulation with IFN-DCs than with IL-4-DCs. Interestingly, the neutralization of IL-10 produced by differentiating DCs did not affect the levels of CD4+ T-cell apoptosis induced by either immature IFN-DCs or IL-4-DCs. We further analyzed DC-induced apoptosis of allogeneic memory CD4+CD45RO+ and naive CD4+CD45RO− T cells. A significantly higher apoptosis of both CD4+ T-cell populations was observed when immature IFN-DCs were used as APC as compared with immature IL-4-DCs. This phenomenon was more pronounced for naive as compared to memory CD4+ T cells (2.2-fold and 1.2-fold, respectively). Altogether, these data suggest that the lower levels of unfractonated and naive allogeneic CD4+ T-cell proliferation induced by IFN-DCs may be, at least in part, related to higher levels of IFN-DC-induced apoptosis of CD4+ T lymphocytes, included memory and above all naive CD4+ T cells, as compared with IL-4-DCs.

Fig. 3. Phenotypic and functional characterization of mature IFN-DCs. Mature DCs were generated from monocyte-derived DCs following stimulation with L40L cells and rhIFN-γ for 48 h. (A) Phenotypic characterization of mature IFN-DCs. Cells were stained with the relevant mAb or isotype control and analyzed by flow cytometry for expression of CD83, DC-LAMP, HLA-DR, CD80 and CD86. Histograms represent the mean ± SD (n = 3 donors) of percentages of positive cells (left panels) or of mean fluorescence intensity (right panels) of mature IFN-DCs (black) or mature IL-4-DCs (white). (B) Proliferation of unfractionated, memory and naive allogeneic CD4+ T cells and allogeneic CD8+ T cells induced by immature and mature IFN-DCs and IL-4-DCs. Immature and mature IFN-DCs and IL-4-DCs generated from monocytes, as previously described, were co-cultured with allogeneic unfractionated CD4+ T cells, allogeneic purified naive CD4+CD45RO− or memory CD4+CD45RO+ T cells or allogeneic CD8+ T cells at a DC/T ratio of 1:10 for 4 days. Thymidine incorporation was measured after an 18 h pulse with 1 μCi of [3H] thymidine. Results, representative of three different donors, are shown as mean ± SD of triplicate values.
IFN-DCs stimulate IFN-γ production by both unfractionated CD4+ and memory CD4+CD45RO+ T cells similarly to IL-4-DCs

In order to characterize the ability of IFN-DCs to induce a Th1 response, we first co-cultured unfractionated CD4+ and memory CD4+CD45RO+ T cells with either allogeneic IFN-DCs or IL-4-DCs for 6 days. We then restimulated T cells with PMA and ionomycin for 8 h and assessed by flow cytometry at the single cell level, intracellular IFN-γ production by T cells. As illustrated in Fig. 7, IFN-DCs stimulated the production of IFN-γ by both CD4+ and CD4+CD45RO+ T cells, as efficiently as IL-4-DCs. We did not detect IFN-γ+IL-10+ T cells in the presence of either IFN-DCs or IL-4-DCs (data not shown).

Enhanced induction of Th1 and Tr1 cells by IFN-DCs

Finally, to assess the polarization of naive CD4+CD45RO+ T cells induced by DCs, we co-cultured naive T cells with either immature allogeneic IFN-DCs or IL-4-DCs for 6 days. We then restimulated T cells with PMA and ionomycin or a combination of plate-bound anti-CD3 mAb and soluble anti-CD28 mAb for 8 h and assessed by flow cytometry at the single cell level, the intracellular production of IFN-γ and IL-10 by T cells. As compared with IL-4-DCs, IFN-DCs induced a significantly higher proportion of IFN-γ+IL-10+ Th1 cells following either PMA/ionomycin or anti-CD3/anti-CD28 mAb stimulation. IFN-DCs also induced a significantly higher proportion of

Table 1. Production of IL-10 throughout the differentiation of DCs

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<th>Day 2</th>
<th>Day 4</th>
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<tr>
<td>IFN-DCs</td>
<td>190.4 ± 46.2*</td>
<td>103.1 ± 16.9*</td>
<td>54.7 ± 5.8</td>
</tr>
<tr>
<td>IL-4-DCs</td>
<td>88.4 ± 25.7</td>
<td>55.2 ± 9.8</td>
<td>43.7 ± 5.8</td>
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IFN-DCs and IL-4-DCs were generated as described in Methods. IL-10 was measured by ELISA in cell-free culture supernatants collected after 2, 4 and 6 days of culture. Results are shown as mean ± SD obtained from three different donors. The limit of detection of IL-10 was 10 pg/ml.

*P < 0.05 between IFN-DCs and IL-4-DCs.
IFN-γ+IL-10+ Tr1 cells whatever the stimulation used, compared to IL-4-DCs (Figs 8C and 9C). Interestingly, the proportion of Tr1 cells induced by either IFN-DCs or IL-4-DCs was not affected by the neutralization of IL-10 during the differentiation of DCs (Figs 8B and 9B). Taken together, these data demonstrate that IFN-DCs induce a strong polarization of allogeneic CD4+CD45RO− naive T cells towards Th1 and Tr1 cells, and that, in these experimental conditions, Th1 and Tr1 generation does not involve IL-10 production by differentiating DCs.

Fig. 5. Neutralization of IL-10 production throughout the differentiation modulates the phenotypic characteristics of immature IFN-DCs and IL-4-DCs and increases the ability of IFN-DCs to stimulate allogeneic CD4+ T-cell proliferation. Monocytes were cultured in the presence of rhGM-CSF and either rhIFN-α or rhIL-4 in the presence of 2 µg/ml of either anti-IL-10 mAbs or control isotypic mAb, for 7 days. (A and B) Expression of HLA-DR, DC-LAMP, CD80 and CD86 was analyzed following staining with the relevant mAb or isotype control. Histograms represent the mean ± SD (n = 3 donors) of percentages of positive cells (A), or of mean fluorescence intensity (B). (C and D) Immature IFN-DCs (C) or IL-4-DCs (D) differentiated in the presence of either neutralizing anti-IL-10 mAb (triangles) or control mAb (squares) were cocultured with allogeneic purified CD4+ T cells at different DC/T ratios for 4 days. Thymidine incorporation was measured after an 18 h pulse with 1 µCi of [3H]thymidine. Results, representative of two different donors, are shown as mean ± SD of triplicate values. *P < 0.05 between DCs generated in the presence of anti-IL-10 mAb and control IgG.
Discussion

Results from the present study indicate that immature DCs generated in the presence of IFN-α are more potent stimulators of bulk and purified CD8⁺ T-cell proliferation, as compared with immature IL-4-DCs. In contrast, immature IFN-DCs are less efficient than their counterparts generated in the presence of IL-4 to stimulate allogeneic CD4⁺ T-cell proliferation, due to a weak induction of naive CD4⁺CD45RO⁻ T-cell proliferation by IFN-DCs. However, memory CD4⁺CD45RO⁺ T cells equally proliferate in the presence of either IFN-DCs or IL-4-DCs. Following maturation induced by CD40 ligation, IFN-DCs exhibit a phenotype and levels of IL-10 production similar to IL-4 DCs but remain less potent in inducing CD4⁺ T-cell proliferation.

Compared to IL-4 DCs, IFN-DCs produce higher levels of IL-10 during the first days of differentiation. We further demonstrate that neutralization of IL-10 during the
differentiation of DCs increases the ability of immature IFN-DCs to stimulate allogeneic CD4+ T-cell proliferation at similar levels than IL-4-DCs. Immature IFN-DCs were found to induce higher levels of apoptosis of CD4+ T cells, a feature more striking for naive T cells, and to induce a differentiation bias of naive CD4+ T cells towards Th1 and Tr1 cells; these later findings were however not found to be related to IL-10 secretion.
We first assessed the ability of IFN-DCs to induce the proliferation of T cell subsets. We found that immature IFN-DCs were potent to stimulate the proliferation of bulk CD3+ T cells, as previously reported (7,8). Immature IFN-DCs were found to induce a strong proliferation of purified allogeneic CD8+ T cells. IFN-α-treated DCs have been reported to be specialized in inducing CD8+ T-cell responses, based on the high expression of TNF-α and IL-15 and their ability to produce...
CD8+ T-cell attractive chemokines (8,11,24). In addition, we have previously reported that IFN-DCs were potent inducers of HIV-specific CD8+ T cells producing IFN-γ and exerting specific cytotoxicity (10). IFN-DCs differentiated from monocytes isolated from CML patients were also reported to stimulate anti-CML specific CD8+ T cells (25,26). The

Fig. 9. Enhanced induction of Th1 and Tr1 cells by immature IFN-DCs following anti-CD3/anti-CD28 mAbs restimulation. Purified naive CD4+CD45RO− T cells were co-cultured with allogeneic immature IFN-DCs and IL-4-DCs differentiated in the presence of either control IgG (A) or anti-IL-10 mAbs (B) at a DC/T ratio 1:10 for 6 days. Naive T cells were then restimulated with plate-bound anti-CD3/soluble anti-CD28 mAbs for 8 h. Brefeldin A was added in the last 4 h of culture and IFN-γ and IL-10 production was then assessed by flow cytometry at the single cell level. Cells were first gated on lymphocytes, according to light scattering properties and subsequently on CD8+ expression, because of down-modulation of CD3 molecules following anti-CD3 mAb treatment. The numbers indicate the percentage of positive cells in each quadrant, defined according to the relevant isotypic control. (A and B) Results from the same donor are shown, representative of three different experiments performed with the cells of three donors. (C) Histograms represent the mean ± SD of percentages of IFNγ+IL-10− Th1 cells and IFNγ+IL-10+ Tr1 cells following stimulation with anti-CD3/anti-CD28 mAbs (n = 6 donors).
induction of purified CD8+ T-cell proliferation strongly suggests that immature IFN-DCs can stimulate CD8+ T lymphocytes independently of CD4+ T-cell help or CD40 ligation, as recently reported in a mouse model in vivo (27). The potent CD8 T-cell activation by IFN-DCs could be dependent on IL-15 secretion. Thus, IL-15 was demonstrated to control the development and the differentiation of CD8+ T cells (28). Interestingly, Pulendran et al. (29) have recently demonstrated that immature murine DCs, differentiated in the presence of GM-CSF and IL-15, were able to stimulate the proliferation of CD8+ T cells, conversely to murine IL-4-DCs. We further aimed at better characterizing CD4+ T-cell responses induced by IFN-DCs. We found that IFN-DCs were consistently less potent than IL-4-DCs in stimulating the proliferation of purified allogeneic CD4+ T lymphocytes. Therefore, we may consider that the proliferation of allogeneic CD3+ T cells induced by IL-4-DCs was mainly related to the proliferation of CD4+ T cells, whereas the proliferation of allogeneic CD3+ T cells induced by IFN-DCs was dependent on the proliferation of both CD4+ and CD8+ T cells. Considering that allogeneic stimulation activates both naive and memory CD4+ T lymphocytes to the same extent (30), we then analyzed the proliferation of CD4+ T cell subsets and showed that IFN-DCs stimulated memory CD4+ T-cell proliferation as IL-4-DCs. These results are in agreement with previous results demonstrating that tuberculin-pulsed immature IFN-DCs induced similar levels of proliferation of autologous CD4+ T lymphocytes to IL-4-DCs (31). In contrast, IFN-DCs stimulated less efficiently naive CD4+ T-cell proliferation, compared to IL-4-DCs. These results differ from those of another study reporting that immature IFN-DCs stimulated naive CD4+ T cells similarly to IL-4-DCs (18). This discrepancy might be explained by the use, in the latter study, of DCs differentiated from monocytes for 5 days rather than 7 days, and the assessment of primary mixed lymphocyte reaction after 6 days. Thus, we performed kinetic experiments indicating that the optimal proliferation and IL-2 production in MLR was detected after 4 and not 6 days of co-culture (data not shown).

The weak levels of naive CD4+ T-cell proliferation induced by immature IFN-DCs could be related to an impaired DC maturation, and especially a low expression of co-stimulatory and MHC class II molecules. However, despite a trend to a lower expression of CD83 by IFN-DCs, the similar expression of intracellular DC-LAMP, of HLA-DR, CD80 and CD86 following maturation of both DC populations suggests, as previously reported (18), that IFN-DCs mature as efficiently as IL-4-DCs following T-cell derived signals. In addition, although the proliferation of naive and memory CD4+ T cells increased when mature DCs rather than immature DCs were used as APC, mature IFN-DCs were consistently less potent than IL-4-DCs to induce the proliferation of unfractionated and naive CD4+ T lymphocytes.

To gain an insight into the mechanisms leading to the low level of CD4+ T-cell proliferation induced by IFN-DCs, we further analyzed the role of the IL-12/IL-10 balance. Indeed, the cross-regulatory roles of IL-12 and IL-10 produced by APCs were demonstrated to affect CD4+ T-cell functions (19,32). Here, we demonstrate that IL-10 production by IFN-DCs and IL-4-DCs following maturation was similar. This is in agreement with our previous study demonstrating that the proportion of IFN-DCs and IL-4-DCs producing IL-12 did not differ following maturation (10). In addition, neutralization of IL-10 during the co-culture did not alter the levels of CD4+ T-cell proliferation induced by either IFN-DCs or IL-4-DCs, suggesting that, in our experimental conditions, IL-10 production by maturing DC or CD4+ T cells did not result in decreased proliferation of CD4+ T lymphocytes.

Other studies have reported that autocrine production of IL-10 throughout their differentiation may alter DCs, leading to an impairment of the phenotype and T-cell stimulating properties (14,33). We thus measured the production of IL-10 by differentiating DCs and showed that IFN-DCs secreted higher amounts of IL-10 than IL-4 DCs, during the first steps of differentiation, with a peak at day 2. We cannot exclude that IL-10 was also produced by contaminating T cells but it has been reported that T cells stimulated by IFN-α alone did not produce IL-10 (34).

We further addressed the effect of neutralization of IL-10 produced throughout the differentiation of DCs on the phenotypic characteristics of IFN-DCs and IL-4-DCs. Expression of CD1a, CD14, CD16, CD83 (data not shown), CD80 and CD86 remained unchanged on IFN-DCs despite addition of anti-IL-10 mAbs. Conversely, neutralization of IL-10 throughout the differentiation induced a strong up-regulation of the expression of DC-LAMP and HLA class II molecules on IFN-DCs. This is in agreement with recent studies showing that IL-10 induced an accumulation of invariant chain/class II molecule complexes within the DC rather than at the cell surface (35). DC-LAMP was originally described as a marker of the initiation of the maturation process for all subsets of DCs (36). In addition, DC-LAMP could be involved in antigen presentation, due to its expression in MHC class II compartments (36). Taken together, these data strongly suggest that autocrine secretion of IL-10 weakly affects the phenotype of IFN-DCs, but decreases IFN-α-induced up-regulation of molecules involved in MHC class II antigen presentation. Moreover, we characterized the effects of the neutralization of IL-10 during the differentiation of DCs on their ability to stimulate CD4+ T cells. Our results demonstrated that, as opposed to IL-4 DCs, the neutralization of IL-10 during the differentiation increased the ability of IFN-DCs to induce allogeneic CD4+ T-lymphocyte proliferation, similar to that of IL-4-DCs. The increased allogeneic CD4+ T-cell proliferation in the presence of anti-IL-10-treated IFN-DCs may result from a significant increase in HLA-DR expression and MHC class II pathway antigen presentation.

Expansion of activated CD4+ T cells is the net result of CD4+ T-cell proliferation and activation-induced cell death. Low expansion of CD4+ T cells may thus result from increased apoptosis. We investigated the levels of CD4+ T-cell apoptosis induced by both DC populations. Our results indicated that stimulation with IFN-DCs led to higher levels of CD4+ T-cell apoptosis, as compared with IL-4-DCs. Of note, the neutralization of IL-10 during the differentiation of IFN-DCs did not influence the levels of CD4 T-cell apoptosis, suggesting the lack of direct relationship between increased apoptosis by IFN-DC and production of IL-10 by differentiating IFN-DCs. IFN-DCs were found to induce the AICD of both naive and memory CD4+ T cells at a higher level compared to IL-4-DCs, with a more striking increase of apoptosis of naive CD4 T cells.
as compared with memory T cells. Our results are in agreement with previous reports demonstrating that: (i) naive CD4+ T cells are more sensitive to IFN-α than activated CD4+ T cells (37), (ii) IFN-α increases AICD of CD4+ T cells by up-regulation of the expression of Fas/Fas-L (23), and (iii) DC-activated memory CD4+ T cells are more resistant to Fas-mediated AICD than naive CD4+ T cells (38,39). The enhanced apoptosis could be mediated by the secretion of IFN-α by IFN-DCs (18) rather than Fas/Fas-L interactions between T cells and DCs, since IFN-DCs were not found to express Fas-L (8).

Low expansion of CD4+ T cells may also result from the suppression of CD4+ T-cell proliferation by regulatory T cells (40). It has been suggested that IFN-α-treated DCs could induce regulatory T lymphocytes (Tr1) among naive T cells (13). To address this question, we analyzed the polarization of naive CD4+ T cells induced by either IFN-DCs or IL-4-DCs. Our results indicate that a higher proportion of IFN-γ+ IL-10+ Tr1 cells was generated by immature IFN-DCs, as compared with IL-4 DCs. Furthermore, this induction of Tr1 cells was not found to depend on IL-10 secretion, as previously described for IFN-α-treated blood circulating DCs (13). We also performed kinetic studies indicating that Tr1 cells were not detected before 8 h of stimulation (data not shown); results in agreement with a study reporting on the lack of induction by IFN-DCs of Tr1 cells following 5 h of restimulation with PMA and ionomycin (18). Tr1 cells were described to be generated in the presence of IL-10 and IFN-α (41) which was also reported to be secreted by immature IFN-DCs (18). In addition, a recent report suggests that IFN-α could induce the up-regulation of ILT3 and ILT4 molecules on DCs, triggering the generation of regulatory T cells (42).

We found that immature IFN-DCs strongly polarized naive CD4+ T cells towards IFN-γ+ IL-10- Th1 cells, as recently reported (18). The induction of Th1 cells by IFN-DCs was reported to be independent of IL-12 and IL-18 but to depend on IFN-α secretion by IFN-DCs (18). The present study further demonstrates that, like IL-4-DCs, immature IFN-DCs stimulate the production of IFN-γ by allogeneic memory CD4+CD45RO+ T cells. Thus, our results strongly support that IFN-α induces the generation of a strong Th1 response by acting at the DC level, as previously suggested in in vivo studies (8,24,43).

Therefore, IFN-DCs affect T cells by different pathways (Fig. 10). Stimulation of CD8+ T cells may involve IL-15 secretion by IFN-DCs (8,24,26,28,29,44). Apoptosis and Th1/Tr1 polarization of naive CD4+ T cells was not found to be related to IL-10 secretion but may be dependent on IFN-α secretion by IFN-DCs (13,18). Finally, autocrine production of IL-10 by differentiating IFN-DCs may mostly limit the proliferation of memory CD4+ T cells. Thus, neutralization of IL-10 increased the expression of MHC class II molecules without affecting the expression of co-stimulatory molecules that may result in an increase in CD4+ T-cell proliferation limited to the memory population. This hypothesis is also supported by another study by our group demonstrating that the neutralization of IL-10 production by differentiating IFN-DCs derived from HIV-infected patients dramatically increased the proliferation of autologous CD4+ T cells induced by IFN-DCs pulsed with recall antigens (involving mostly memory CD4+ T cells) (31).

In conclusion, despite the induction of Tr1 cells and enhanced apoptosis of CD4+ T cells, immature IFN-DCs are potent stimulators of CD8+ and memory CD4+ T cells, and induce a strong polarization of naive CD4+ T cells towards Th1 cells. Therefore, our results further support the use of IFN-DCs in immune-based therapy aimed at inducing strong antigen-specific immune responses in the treatment of cancers and chronic viral infections.
Acknowledgements

We thank Dr. K. Jay for helpful discussion on DCs. We also gratefully acknowledge Drs. E. Tartour and S. Kaveri for helpful discussions and rereading the manuscript. We thank Dr. S. Lebecque for providing us anti-DC-LAMP mAb for preliminary experiments. This work was supported by a grant from Ensemble Contre le SIDA (ECS), France. C.C. is a recipient of a fellowship from the Ministère de l’Education Nationale (France). The authors declare that they have no competing financial interests.

Abbreviations

AICD  activation-induced cell death
APCs  antigen-presenting cells
CD40L  CD40 ligand
DCs  dendritic cells
DC-LAMP  dendritic cell lysosome associated membrane protein
FAS-L  Fas ligand
GM-CSF  granulocyte macrophage colony stimulating factor
IFN-DCs  DCs generated in the presence of GM-CSF and IFN-α
IL-4-DCs  DCs generated in the presence of GM-CSF and IL-4
ILT  immunoglobulin-like transcript
MLR  mixed lymphocyte reaction
PECy5  phycoerythrin-cyanin-5

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