

IFN- α 2a induces IP-10/CXCL10 and MIG/CXCL9 production in monocyte-derived dendritic cells and enhances their capacity to attract and stimulate CD8⁺ effector T cells

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Abstract: Type I IFNs are immunomodulatory factors that possibly influence the properties of tissue-resident dendritic cells. Here, we have investigated the capacity of IFN- α 2a to enhance DC chemoattractive and stimulatory capacity toward CD8⁺ T lymphocytes. Phenotypically, IFN- α 2a-treated DC (IFN-DC) showed an increased expression of costimulatory and antigen-presenting molecules, maintained even after withdrawal of the cytokine. IFN- α 2a enhanced DC stimulatory capacity toward CD8⁺ T cells, as assessed by increased MLR responses and induction of MART-1_{26–35}-specific CTLs in vitro. No functional CCR7 chemokine receptor could be induced. Instead, high amounts of IP-10/CXCL10 and MIG/CXCL9 chemokines were produced. Freshly isolated CD8⁺RO⁺ cells and PHA-activated CD8⁺ T cells migrated efficiently in response to IFN-DC-conditioned medium, and the migration could be inhibited by neutralizing the CXCR3 receptor on responder cells. These results suggest that type I IFNs could enhance the elicitation of class I-restricted effector functions in vivo in the periphery by modulating DC chemoattractive properties. *J. Leukoc. Biol.* 71: 669–676; 2002.

Key Words: type I IFNs · chemokines · CTL · tumor immunity

INTRODUCTION

The fate of immune reactions depends on the precise recruitment of immunocompetent cells [1]. Dendritic cells (DC) initiate and control adaptive immune responses by sensing inflammatory stimuli in the periphery. Tissue-resident DC are activated not only by antigenic materials provided by pathogens (virus, ds-RNA, bacteria, and their soluble products) but also by the cytokines induced upon inflammation at the site of antigen deposition. Once activated, DC acquire an increased stimulatory capacity toward T lymphocytes and peculiar chemotactic properties [2]. Priming of naive antigen-specific T cells can take place once mature DC have reached the secondary lymphoid organs (SLO), and elicitation of effector functions requires an efficient recruitment and activation of memory T cells in the periphery. The traffic of immunocompe-

tent cells between SLO and periphery is directed by chemokines [3].

Since their discovery as antiviral [4] and antiproliferative agents, type I interferons (IFNs) have been used largely in human immunotherapy and until now, still represent an effective possibility of immune intervention for a number of malignancies and chronic viral infections [5]. Recently, renewed attention has been focused on their role as immunomodulatory factors promoting immune cell differentiation, activation, and survival [6–10]. The cellular source of type I IFNs in humans has been identified recently as the plasmacytoid monocyte [11, 12], thus strengthening the role of DC and type I IFNs as a link between innate and adaptive immunity.

Patterns and kinetics of chemokine production by DC following lipopolysaccharide (LPS), tumor necrosis factor α (TNF- α), or CD40L have been described in detail [13]. Conversely, no data have been shown so far regarding chemokine production by DC upon IFN type I stimulation. In this respect, IFN-inducible protein 10 (IP-10)/CXCL10, monokine induced by IFN- γ (MIG)/CXCL9, and IFN-inducible T-cell α chemoattractant (I-TAC)/CXCL11 chemokines, specifically attracting CXCR3⁺ T cells, are induced by IFNs on different cell types [3].

DC generated from peripheral blood monocytes in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 [14] provide a convenient experimental system to study the biology of bona fide tissue-resident DC in vitro. Monocyte-derived DC can produce low levels of type I IFNs in the absence of viral infection [15]. Up to 10,000 IU/ml can be detected upon stimulation in vitro with viral and nonviral agents [16], thus suggesting that DC themselves could be the target of an autocrine production of type I IFNs.

Recently, these cytokines have been shown to induce differentiation and phenotypic maturation in bone marrow or monocyte-derived DC, functionally resulting in enhanced allostimulation and antigen presentation [7–9]. In spite of these major advances, however, less is known about the effects of type I IFNs on terminally differentiated DC [16], and no data

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are available concerning the possible induction of mechanisms promoting T-cell recruitment.

In this work, we addressed the production of chemokines potentially attracting T lymphocytes by monocyte-derived DC stimulated with IFN- α 2a. We show that under these in vitro conditions, DC produce substantial amounts of IP-10/CXCL10 and MIG/CXCL9 chemokines and efficiently attract CD8⁺ T cells expressing the CXCR3 cognate receptor. Concomitantly, the elicitation of class I-restricted effector functions is enhanced strongly.

MATERIALS AND METHODS

Media and reagents for cell culture

Cells were cultured in RPMI 1640 supplemented with 1% kanamycin, 2 mM L-glutamine, 1% nonessential amino acids, 1% Na-pyruvate, 5×10^{-5} M 2-mercaptoethanol, and 10% fetal calf serum (FCS; Gibco, Paisley, UK), thereafter referred to as complete medium. LPS content in serum was tested by LIMULUS LAL assay, and only LPS-free batches were used. Human recombinant IL-4 was produced in our laboratory. GM-CSF was kindly provided by Novartis (Basel, Switzerland).

Generation of DC, CD8⁺RA⁺, and CD8⁺RO⁺ T cells from peripheral blood lymphocytes

Immature DC (iDC) were generated from human peripheral blood mononuclear cells according to published methods [14]. Briefly, monocytes were purified by positive sorting using anti-CD14-conjugated microbeads (Miltenyi, Bergisch Gladbach, Germany). Sorted cells were cultured for 5–6 days in complete medium supplemented with 50 ng/ml GM-CSF and 1000 U/ml IL-4, thereafter referred to as IL4/GM-CSF medium.

T cells were purified by negative sorting of CD8⁺ lymphocytes followed by positive sorting with anti-CD45RA- and anti-CD45RO-conjugated microbeads (Miltenyi). Isolated cells were assessed immediately for phenotype and chemotaxis.

Induction of DC maturation

iDC were cultured in IL4/GM-CSF medium with 1 μ g/ml LPS from *Salmonella abortus equi* (Sigma Chemical Co., St. Louis, MO) or 10^4 – 30 IU/ml IFN- α 2a (Hoffmann-La Roche, Basel, Switzerland) in serial dilutions 48 h prior to use.

Fluorescein-activated cell sorter (FACS) analysis

IFN- α receptors were detected by goat anti-human IFNR-I antibodies followed by secondary rabbit anti-goat fluorescein isothiocyanate (FITC)-conjugated antibodies and by rabbit anti-human IFNR-II followed by goat anti-rabbit FITC-conjugated antibodies (R&D Systems, Abingdon, UK, and SBA, Birmingham, AL).

DC-phenotypic modulation was evaluated by cell-surface staining using FITC-conjugated mouse antibodies to human CD80 (clone BB1), CD86 (clone IT2.2), human leukocyte antigen (HLA)-ABC (clone G46-2.6), HLA-DR (clone TU36), and CD83 (clone HB15e). Anti-CCR7 antibody (clone 2H4) was used in combination with biotinylated anti-mouse immunoglobulin (Ig)M plus streptavidin-phycoerythrin (PE). The phenotype of cytotoxic T lymphocytes was assessed in three-color staining using anti-CD45RO-CyChrome, anti-CD45RA-FITC, and anti-CXCR3-PE. All antibodies were purchased from PharMingen (San Diego, CA).

Samples were analyzed on a FACSCalibur (Becton Dickinson, Mountain View, CA) using propidium iodide (PI) to exclude dead cells. PI⁺ cells never exceeded 14% of the total population. FACS staining data are shown as histograms or expressed as median fluorescence intensity (MFI) values, calculated as the ratio between the experimental MFI and that observed upon staining with an isotype-matched, control antibody.

Mixed lymphocyte reaction assay

iDC, LPS- or IFN-treated, were washed five times, diluted in fresh culture medium, and used as allogeneic stimulators. Cells were seeded in 96-well round-bottom culture plates (Nunc, Roskilde, Denmark) to have serial antigen-presenting cell (APC) dilutions ranging from 2×10^4 to 500 DC/well together with freshly purified CD8⁺ cells (10^5 /well). After 6 days of incubation, cells were pulsed with 1 μ Ci ³H-thymidine (TdR) per well (specific activity, 5 mCi/mmol; Amersham Pharmacia Biotech, Zürich, Switzerland) for 16–18 h and were finally harvested on filter paper. Proliferative responses were measured as TdR incorporation by an automatic β -counter. Tests were performed in triplicates, and results were expressed as the mean count per minute (cpm). SD values never exceeded 10%.

In vitro priming

Autologous DC from an HLA-A2⁺ healthy donor were left immature or treated with 1000 IU/ml IFN- α 2a and used as APC (4×10^5) to stimulate 10^6 -purified CD8⁺ lymphocytes in the presence of 10 μ g/ml MART-1_{26–35} peptide. Cultures were then expanded with low doses of IL-2, rested, and restimulated in the same conditions. Specificity was then assessed by ⁵¹Cr release assay, using Na8 HLA-A2⁺/MART-1[–] melanoma cells as target (kindly provided by F. Jotereau, Nantes, France), pulsed with MART-1_{26–35} peptide or p21ras_{55–63} as control. Data are expressed as (% Δ -specific lysis = % MART-1_{26–35}-specific lysis – % p21ras_{55–63}-specific lysis). Finally, antigen-specific cytolytic T lymphocyte (CTL) clones were derived from positive cultures by cloning the bulk populations in limiting dilution, according to standard techniques.

Chemokine detection

IP-10/CXCL10 and MIG/CXCL9 production was determined by quantitative enzyme-linked immunosorbent assays (ELISAs) using cell-culture supernatants at 48 h stimulation. Antibody pairs and standards were provided by PharMingen. I-TAC/CXCL11 gene expression was assessed by reverse transcriptase-polymerase chain reaction (RT-PCR).

RT-PCR

Single-strand cDNA was synthesized from 2 μ g total RNA using Moloney leukemia virus RT (BRL, Gaithersburg, MD) in the presence of deoxynucleotide triphosphate and dithiothreitol, according to the manufacturer's instructions. The following primers were used: CCR7, 5'-GATTACATCGGAGACAA-CACC-3', 5'-TAGTCCAGGCAGAAGAGTCG-3', amplifying a product of 1067 bp [17]; I-TAC/CXCL11, 5'-GCTATAGCCTTGGCTGTGATATTG-3', 5'-GATTTGGGATTTAGGCATCGTTGT-3', amplifying a product of 219 bp [18]; and β -actin, 5'-CACCACACTGTGCCCCATC-3', 5'-CTAGAAGCATT-GCGGTGGAC-3', amplifying a 650 bp product. cDNA were amplified by PCR using the following conditions: 35 cycles, each including 30 s at 94°C and 20 s at 58°C, and 20 s at 72°C, followed by a 10 min final extension at 72°C. PCR products were resolved on 1.5% agarose gels containing ethidium bromide.

Migration assay

Cell migration was measured in 48-well Transwell plates (5 μ m pores; Corning Costar Europe, Badhoeve, The Netherlands). iDC, LPS- or IFN-treated DC, or T cells were diluted at 2×10^6 cells/ml, and 50 μ l cell suspension was added to each Transwell insert (10^5 total cells/well), prewetted with 50 μ l plain RPMI medium; 500 μ l recombinant chemokines (rSLC and rIP-10; PharMingen), control medium containing IFN- α 2a at 1000 IU/ml, or DC-conditioned medium were distributed in the lower chambers. In some experiments, purified anti-CXCR3-neutralizing monoclonal antibody (mAb; clone 1C6; kindly provided by S. Qin, Millennium Pharmaceuticals, Cambridge, MA) or normal mouse IgG1 was added to the cell suspension at a final concentration of 50 μ g/ml. After 3 h incubation at 37°C, the Transwell inserts were lifted, and cells from the lower chamber were collected in a separate tube; each well was rinsed twice with 500 μ l of a buffer containing 2 mM ethylenediaminetetraacetate and 0.5% FCS in phosphate-buffered saline without Ca⁺⁺ and Mg⁺⁺. Migrated cells were identified by FACS staining for the expression of CD11c or CD8. Each sample was acquired on a FACSCalibur for 60 s. Data on transmigrated cells from duplicate wells are expressed as % of total cells \pm SD.

RESULTS

DC phenotypic modulation induced by IFN- α 2a

Biological effects of type I IFNs are initiated upon binding to a common heterodimeric receptor that is distributed on most cell types [19]. This heterodimeric structure contains two subunits, IFNR-I and IFNR-II, determining selectivity and affinity to different ligands [20]. By using specific antibodies, we could detect significant expression of IFNR-I and IFNR-II on monocyte-derived iDC after a 5 day differentiation in IL4/GM-CSF medium (Fig. 1A), thus suggesting that tissue-resident DC might respond to type I IFNs.

iDC were then cultured in the presence of 1000 IU/ml IFN- α 2a (IFN-DC) and were analyzed 48 h later for the expression of CD80, CD86, CD83, HLA-DR, and HLA-ABC markers. We observed an up-regulation of CD80 and CD86 upon IFN- α 2a treatment and of HLA-DR and HLA-ABC, as expected (Fig. 1B). Similar increases were induced by LPS stimulation, considered as a positive control of in vitro terminal maturation. DC activation induced by IFN did not result in CD83 expression, in contrast to LPS-DC (Fig. 1B). Comparable effects were observed upon treatment with IFN- α 2a doses ranging from 30 to 3000 IU/ml. Remarkably, doses as low as 30 IU/ml were still able to induce up to 2.6-fold increases of CD86

expression compared with iDC (Fig. 1C). Finally, IFN-DC were washed extensively and recultured in IL4/GM-CSF medium without IFN- α 2a. Phenotypical analysis of IFN-DC rested for 2 days revealed a sustained up-regulation in the expression of CD86 and HLA-ABC (Fig. 1D). Such an increased expression of costimulatory and HLA molecules on DC upon IFN- α stimulation maintained even after withdrawal of the cytokine could have important consequences on their function as professional APC.

Increased stimulatory capacity of IFN-DC

The importance of cytotoxic T lymphocytes in the regression of human malignancies and chronic viral infections treated eventually with IFN- α has been documented [21, 22]. The stimulatory capacity of IFN-DC in our experimental system was analyzed with two types of assays, both targeting class I-restricted immune responses.

First, allogeneic CD8⁺ peripheral blood lymphocytes were cocultured in vitro with serial dilutions of iDC, IFN-DC, or LPS-DC, and proliferative responses were measured after 6 days of culture. Data shown in Figure 2A indicate that IFN-DC are more effective stimulators as compared with iDC; positive-control LPS-DC induced the highest level of proliferation.

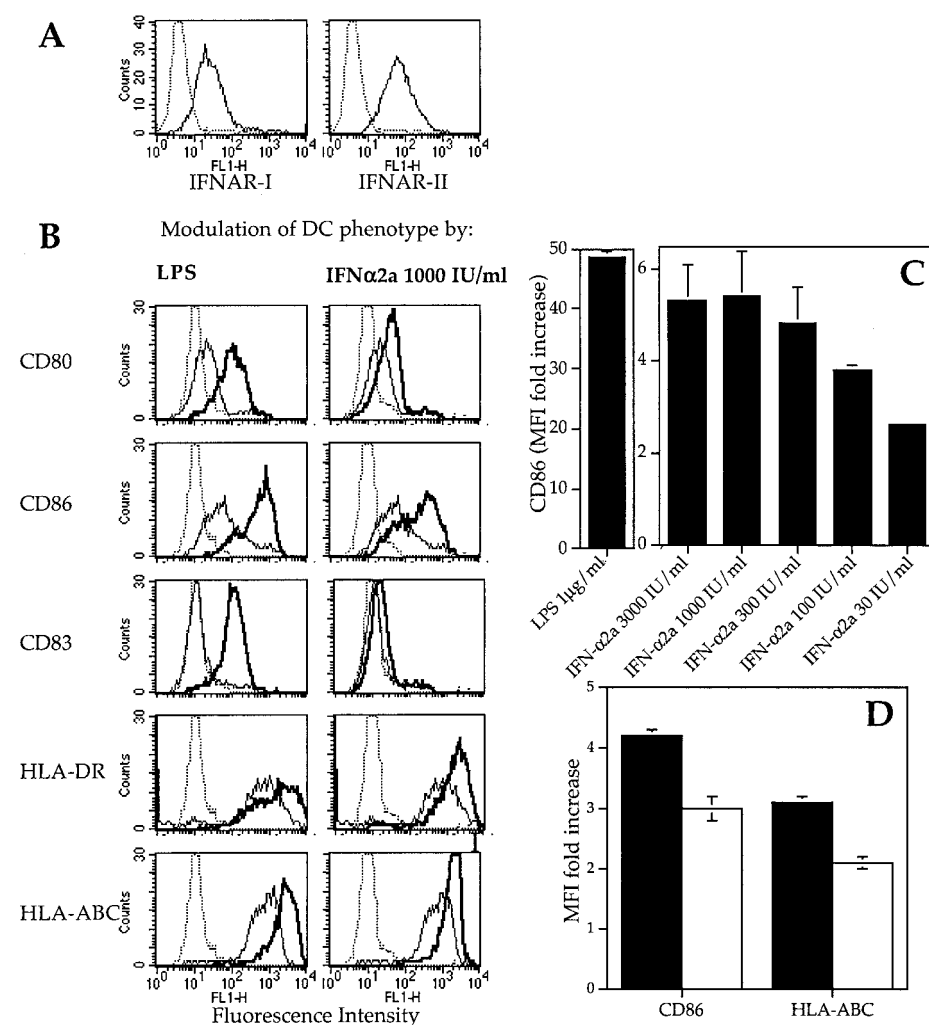


Fig. 1. (A) IFN- α receptor expression on monocyte-derived iDC. IFNR-I- and -II-specific staining are shown on the left and right panels, respectively. Dotted lines represent the background staining with isotype-matched control antibodies followed by the respective secondary antibody. (B) Modulation of CD80, CD86, CD83, HLA-DR, and HLA-ABC expression on iDC upon maturation induced by 1 μ g/ml LPS (left panels) or 1000 IU/ml IFN- α 2a (right panels). In each plot, dotted lines represent the background staining with isotype-matched control antibodies, thin lines show the staining profile of iDC, and overlaid black lines indicate the phenotype of mature DC. (C) Dose-response of IFN-specific modulation of CD86. (D) Stability of IFN-induced phenotype. IFN-DC treated as described above (solid bars) or washed and recultured for 48 h in the absence of IFN- α 2a (open bars) were assessed for CD86 and HLA-ABC expression. (C and D) Data are given as MFI-fold of increase compared with iDC. Results represent mean values \pm SD of four different experiments performed independently.

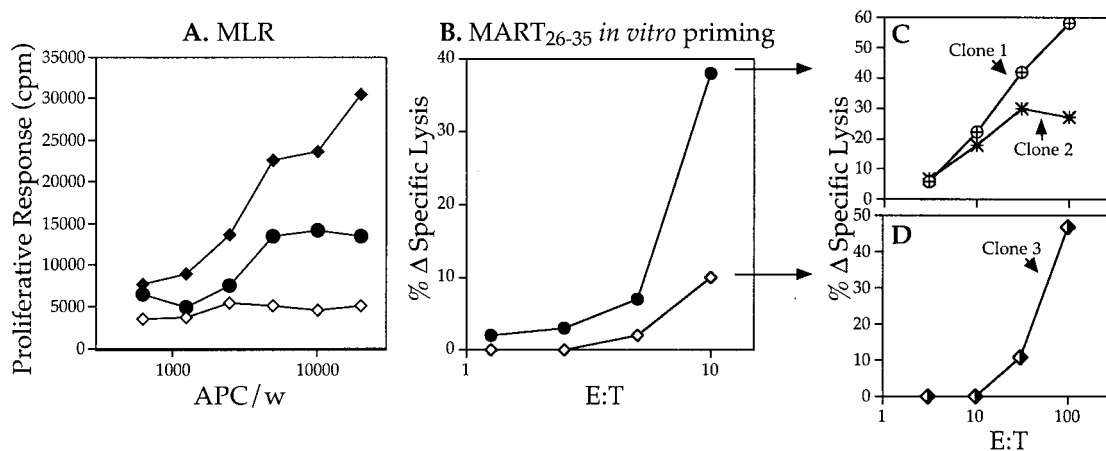


Fig. 2. Increased stimulatory capacity of IFN-DC. (A) Monocyte-derived DC, kept iDC (◇), matured by LPS (LPS-DC; ◆), or treated with IFN α 2a (IFN-DC; ●) were used as stimulators in a mixed leukocyte reaction (MLR) assay. Proliferative responses of allogeneic-purified CD8⁺ T cells were determined after 6 days of culture by thymidine incorporation. Representative data of one out of three experiments performed are shown. (B) *In vitro* priming of CD8⁺ T cells specific for MART-1₂₆₋₃₅. Autologous iDC (◇) or IFN-DC (●) were used as APC in two rounds of stimulation. Specificity of each bulk culture was assessed as capacity to lyse HLA-A2-expressing melanoma cells pulsed with the MART-1₂₆₋₃₅ peptide. Data are given as mean of triplicates and expressed as % Δ -specific lysis. Cytotoxic activity of specific CD3⁺CD8⁺ CTL clones derived from each bulk coculture is shown (C, clones 1 and 2; D, clone 3). CTL reactivities are representative of three different experiments.

Second, CD8⁺ T cells purified from peripheral blood lymphocytes of an HLA-A2⁺ healthy donor were stimulated *in vitro* in the presence of MART-1₂₆₋₃₅ peptide using autologous iDC or IFN-DC as APC. After two rounds of stimulation in the same conditions, the respective T-cell lines were assessed for antigen specificity in cytotoxicity assays using Na8 melanoma cells pulsed with MART-1₂₆₋₃₅ or control peptide as targets. As shown in Figure 2B, the effector function of the CTLs expanded in the presence of IFN-DC was significantly higher than that of the T-cell line obtained using iDC as APC: 38% versus 10% Δ -specific lysis at 10:1 = E:T ratio. Specific CD8⁺CD3⁺ T-cell clones prepared from each bulk culture confirmed the functional results. Clones 1 and 2, derived from the line restimulated with IFN-DC, and clone 3, raised with iDC as APC, showed 42%, 30%, and 11% Δ -specific lysis at 30:1 = E:T ratio, respectively (Fig. 2, C and D). Thus, the DC phenotypic modulation induced by IFN- α 2a is consistent with an increased stimulatory capacity of class I-restricted immune responses.

As professional APC, mature DC could prime naive T cells resident in secondary lymphoid organs (SLO). In the context of inflammation or therapeutic IFN- α administration, however, events leading to effector T-cell chemoattraction at the inflammatory site might determine the fate of the immune response. To get a first insight into the possible mechanisms underlying leukocyte migration in our experimental system, the migratory/chemoattractive properties acquired by DC upon IFN- α treatment *in vitro* were analyzed in detail.

Stimulation with IFN- α 2a does not result in functional CCR7 expression required for homing to SLO

Maturing DC are known to up-regulate the CCR7 receptor [23] and to migrate to secondary lymphoid organs in response to SLC/CCL21 and ELC/CCL19 chemokines, produced constitu-

tively therein [24, 25]. Therefore, we addressed the migratory behavior of IFN-DC as compared with iDC and LPS-DC.

Expression of the CCR7-specific gene could be induced upon treatment with 1000 and 300 IU/ml IFN- α 2a, although at an apparently lower magnitude as compared with LPS-DC (Fig. 3A). However, IFN-DC failed to express the specific protein on the cell surface (Fig. 3B) and did not acquire the capacity to migrate in response to rSLC/CCL21 chemokine used in a range from 100 to 1000 ng/ml (Fig. 3C). As expected, the profiles obtained with iDC were negative in all three experimental settings (Fig. 3, A–C). In contrast, the CCR7 gene and protein expression were induced in LPS-DC (Fig. 3B), and migration to rSLC/CCL21 could also be measured (Fig. 3C).

Thus, based on these types of reactivities, it is unlikely that exposure to IFN- α could result in DC migration to SLO *in vivo*. However, DC might acquire the capacity to chemoattract T cells, thus enhancing specific immune responses at the site of activation. Although unable to respond to SLO-specific chemokines, the possibility that IFN-DC could effectively produce chemotactic factors attracting T lymphocytes *in vitro* was explored further.

Chemokines specific for T-cell chemoattraction are induced by IFN- α 2a stimulation

Chemokines IP-10/CXCL10, MIG/CXCL9, and I-TAC/CXCL11 are induced in response to IFNs [3]. They bind a unique receptor, the CXCR3 [26], and are involved in the recruitment of activated lymphocytes [27, 28]. Therefore, we examined the production of these chemoattractants in our DC cultures. As shown in Figure 4, iDC were unable to produce any of the chemokines. Administration of IFN- α 2a at different doses induced a consistent production of IP-10/CXCL10 and MIG/CXCL9, as well as I-TAC/CXCL11 gene expression. Remarkably, over 700 pg/ml/10⁶ cells of IP-10/CXCL10 were detected upon DC stimulation with doses of IFN- α 2a as low as

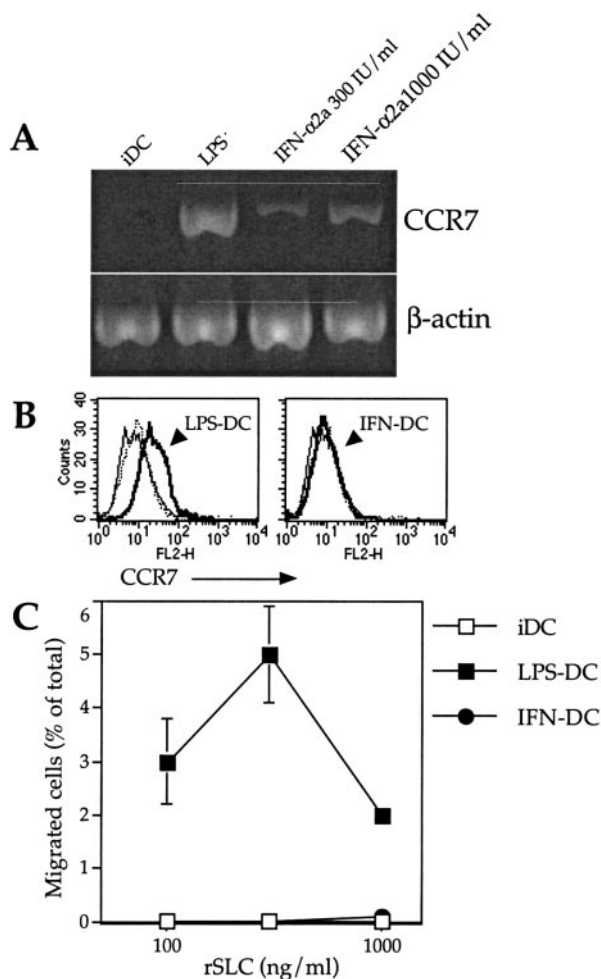


Fig. 3. Induction of CCR7 gene expression in the absence of CCR7 protein detection and responsiveness to SLC chemokine in IFN-DC. (A) RT-PCR analysis of CCR7 mRNA expression in iDC, LPS-DC, or DC treated with 300 and 1000 IU/ml IFN- α 2a; samples were collected and analyzed after 48 h culture. (B) Cell-surface expression of CCR7 protein as assessed by FACS staining using specific mAb. In each plot, dotted lines represent the background staining with isotype-matched control antibodies, thin lines show the staining profile of iDC, and overlaid black lines indicate the phenotype of DC upon stimulation with 1 μ g/ml LPS or 1000 IU/ml IFN- α 2a (left and right panels, respectively). (C) Migratory responses to different concentrations of rSLC chemokine of iDC (\square), LPS-DC (\blacksquare), and IFN-DC (\bullet). Migrated cells are expressed as % of total. Data, given as mean of duplicates \pm SD, are representative of three experiments performed independently.

10 IU/ml (Fig. 4A). Furthermore, up to 7645 pg/ml/ 10^6 cells of IP-10/CXCL10 and 1698 pg/ml/ 10^6 cells of MIG/CXCL9 were measured upon stimulation with 3000 IU/ml IFN- α 2a (Fig. 4, A and B, respectively). I-TAC/CXCL11 gene expression was clearly detected at 1000 IU/ml IFN- α 2a concentration (Fig. 4C). Positive control of chemokine production was given by stimulation with LPS [13], itself a potent inducer of type I IFN production by monocyte-derived DC [16]. In those conditions, 7862 pg/ml/ 10^6 cells of IP-10/CXCL10 and 7772 pg/ml/ 10^6 cells of MIG/CXCL9 (Fig. 4, A and B), as well as I-TAC/CXCL11-positive gene expression (Fig. 4C), were measured.

Thus, IFN- α 2a signaling triggers very efficiently IP-10/CXCL10 and MIG/CXCL9 production by monocyte-derived DC in vitro. It is interesting that IP-10/CXCL10 induction is sustained

even at very low doses of IFN- α 2a. These results might have important consequences on T-cell chemoattraction.

Chemotaxis of freshly isolated CD45RA⁺ and CD45RO⁺ CD8⁺ T cells

To gain insight into the consequences of such a high induction of IP-10/CXCL10 release upon IFN- α stimulation, freshly isolated CD45RA⁺ and CD45RO⁺ CD8⁺ T cells were assessed for chemotaxis.

Cytotoxic T-cell subsets were purified from peripheral blood lymphocytes as homogeneous populations (Fig. 5, A and C). Both were positive for CXCR3 chemokine receptor expression (Fig. 5, B and D) and represented possible targets of IP-10/CXCL10-mediated chemotaxis. However, when both populations were assessed for migration toward conditioned medium derived from iDC, IFN-DC, or LPS-DC, as well as rIP-10/CXCL10 and control medium, different patterns of response were observed (Fig. 5E). CD8⁺RO⁺ T cells migrated very efficiently, not only in response to rIP-10/CXCL10 (4.6% of total cells) but also to IFN-DC-conditioned medium (2.8% of total cells), and chemoattraction toward iDC supernatants or control medium was comparably low (1.7% and 1.4%, respectively). In contrast, CD8⁺RA⁺ cells migrated poorly toward rIP-10/CXCL10 (2% of total cells), and furthermore, none of the conditioned medium induced its chemoattraction (0.6%, 0.2%, 0.4%, 0.5% of total cells migrated in response to control, iDC, LPS-DC, and IFN-DC medium, respectively).

The differences in the migration patterns observed could be related to a difference in CXCR3 expression between freshly isolated CD45RA⁺ and CD45RO⁺ CD8⁺ T cells. Indeed, the corresponding CXCR3 MFI values measured by FACS in Figure 5, B and D, were 102.7 versus 228.7 for CD45RA and CD45RO, respectively.

Chemotaxis of activated T cells

Several studies document that activation of T lymphocytes leads to an increased expression of CXCR3 and enhanced migration in response to the cognate chemokines [26–29]. Therefore, the chemotactic properties of the supernatants derived from our DC cultures were analyzed further on activated T cells. Polyclonal T-cell lines were used as responders for chemotaxis, assessed as described above. In this case, values of migrated cells were expected to be of a higher magnitude than those obtained with freshly isolated T cells, as a consequence of phytohemagglutinin (PHA) activation [27].

In our settings, IFN-DC- and iDC-conditioned media were equally efficient in inducing CD4⁺ T-cell chemoattraction: 24.1% and 24.7% of cells migrated, respectively, versus 13.1% in the presence of control medium (Fig. 6A). Conversely, IFN-DC-conditioned medium attracted CD8⁺ T cells more efficiently than iDC supernatant: 18.6% and 8% of cells migrated, respectively, as compared with 5.4% for control medium (Fig. 6A). Chemoattraction of CD4⁺ and CD8⁺ subsets toward positive-control, LPS-DC-conditioned medium was induced strongly (39.7% and 33.0%, respectively; Fig. 6A).

The CD8⁺ T-cell migration was analyzed by using DC stimulated with different doses of IFN- α 2a (Fig. 6B). Strikingly, supernatants of DC stimulated with doses as low as 30

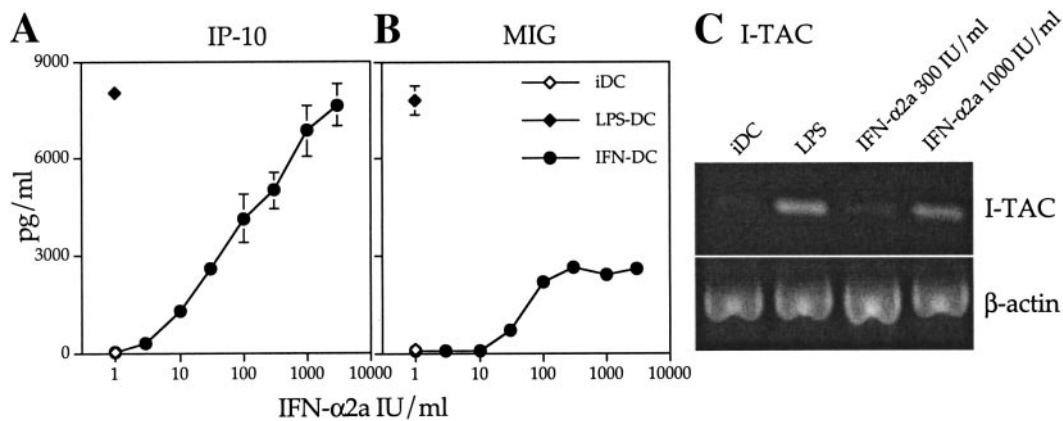


Fig. 4. Production of IFN-inducible chemokines. IP-10 (A) and MIG (B) content in DC culture supernatants was measured after 48 h incubation by specific ELISAs. DC were left immature (\diamond) or stimulated with 1 μ g/ml LPS (\blacklozenge) or with different concentrations of IFN- α 2a (\bullet). Data, given as mean of triplicates \pm SD and expressed as pg/ml, are representative of three experiments performed independently. (C) RT-PCR analysis of I-TAC gene expression using specific primers was performed on DC, treated as indicated, and collected after 48 h stimulation.

IU/ml IFN- α 2a were still able to attract up to 18.4% of the total CD8 $^{+}$ cells, as compared with 51% in the presence of rIP-10/CXCL10 and 4.6% for control medium (Fig. 6B). Finally, the requirement for the cognate receptor in these responses was assessed in the presence of anti-CXCR3 antibodies. Indeed, CXCR3 neutralization resulted in 65% inhibition of CD8 $^{+}$ T-cell chemoattraction toward rIP-10/CXCL10 and up to 93% inhibition toward IFN-DC medium (Fig. 6B).

Altogether, upon IFN- α stimulation *in vitro*, DC not only stimulated antigen (Ag)-specific class I-restricted immune responses more efficiently but also acquired a strong chemoattractive capacity toward memory and activated CD8 $^{+}$ T cells. That migration was sustained by engagement of the CXCR3 receptor on responder cells.

DISCUSSION

Type I IFNs are among those cytokines that coordinate immune responses. They are produced by DC upon viral infection but

can also be induced by a variety of nonviral agents such as intracellular and extracellular bacteria, parasites, and fungi [11, 12, 16]. Thus, stimulation of tissue-resident DC by type I IFNs, as well as recruitment and activation of immunocompetent cells at the site of infection *in vivo*, might play an essential role for the elicitation of effective immune responses.

As pharmaceuticals, type I IFNs are used in the clinical treatment of chronic viral infection and human malignancies [5]. In both instances, CD8 $^{+}$ T-cell expansion and stimulation appear to be required for the implementation of therapeutic effects [21, 22]. More recently, a role as vaccine adjuvants has also been proposed [30]. In this respect, the use of immunologically defined adjuvants eventually promoting CTL recruitment and elicitation of effector functions in the peripheral tissues could represent an important feature of immune intervention.

Previous studies have shown that exposure of CD34 $^{+}$ or CD14 $^{+}$ precursor cells to type I IFNs promotes differentiation and maturation toward a DC type endowed with professional

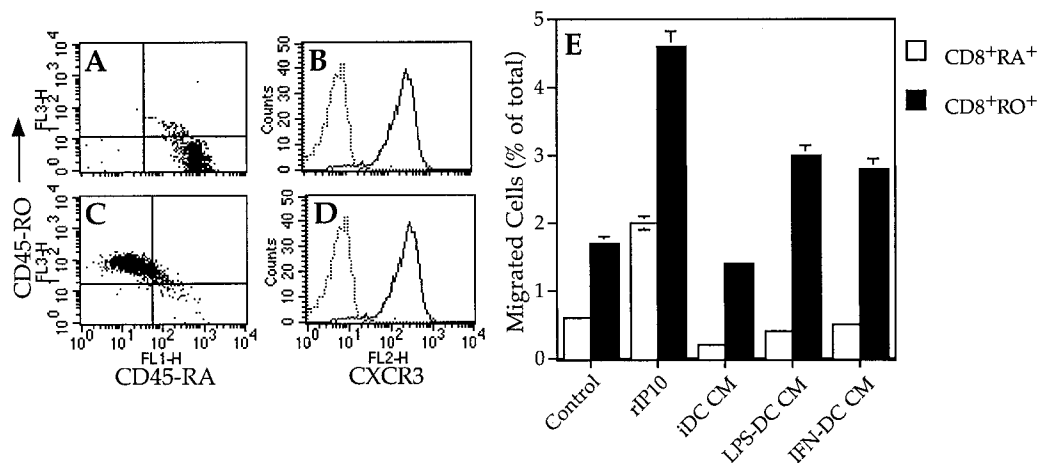


Fig. 5. Migratory response of freshly isolated CD8 $^{+}$ RA $^{+}$ - and CD8 $^{+}$ RO $^{+}$ -rested T cells. (A–D) Phenotypic analysis of sorted cells using anti-CD45RA-FITC, anti-CD45RO-CyChrome, anti-CXCR3-PE mAb, or isotype-matched controls. (E) Migratory response of CD8 $^{+}$ RA $^{+}$ (open bars) and CD8 $^{+}$ RO $^{+}$ (solid bars) T-cell populations to conditioned medium (CM) derived from DC left iDC or stimulated with 1 μ g/ml LPS (LPS-DC) or 1000 IU/ml IFN- α 2a (IFN-DC). Medium containing 1000 IU/ml IFN- α 2a was used as control of background migration; positive control for migration was obtained in response to 40 ng/ml rIP-10. Migrated cells are expressed as % of total and are given as mean of duplicate wells \pm SD. Results are representative of three experiments performed independently.

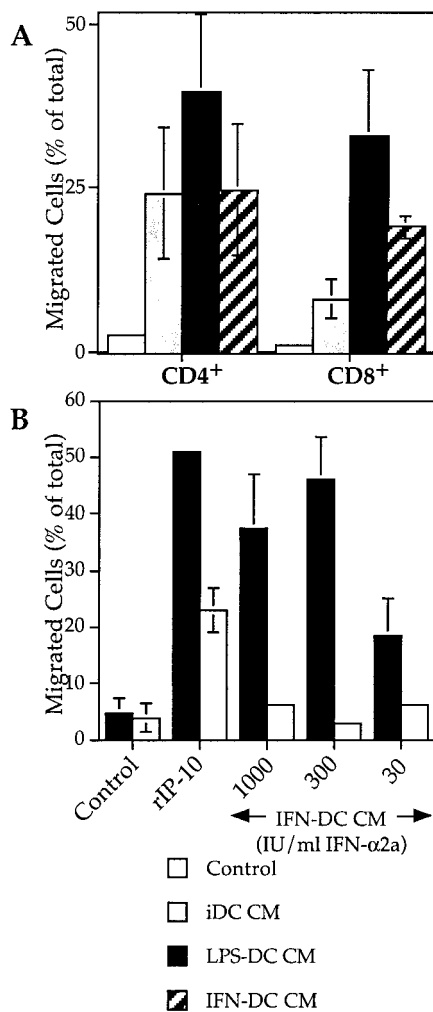


Fig. 6. Chemotaxis of activated T cells and effect of anti-CXCR3 neutralizing antibodies. (A) A polyclonal, PHA-activated T-cell line containing 44% CD4⁺ and 38% CD8⁺ T cells was assessed for migratory capacity in response to control medium (open bars) or CM-derived from iDC (gray bars), LPS-DC (solid bars), or IFN-DC (hatched bars). (B) The effect of anti-CXCR3 neutralizing mAb (open bars) or matching isotype controls (solid bars) on the chemotaxis of activated CD8⁺ T cells. Chemotaxis was assessed toward control medium, rIP-10 at 60 ng/ml, or CM of IFN-DC treated with 1000, 300, and 30 IU/ml IFN-α2a in the presence of 50 μg/ml 1C6 or mouse IgG₁ antibodies, as indicated. In both diagrams, migrated cells are expressed as % of total. Data, given as mean of duplicate wells ± SD, are representative of six experiments performed independently.

APC capacities [7–9]. Administration of type I IFN in vivo resulted in the enhancement of antigen-specific immune responses [10]. Furthermore, experimental models targeting monocyte-derived DC have demonstrated that IFN type I treatment promotes the expression of costimulatory and HLA molecules but fails to induce terminal maturation [16]. Consistent with these data, treatment of iDC with IFN-α2a in our experimental settings enhanced expression of CD80/CD86 and HLA molecules indeed in the absence of CD83. Most importantly, DC activated with IFN-α2a were potent stimulators of Ag-specific, class I-restricted effector functions as compared with untreated DC.

The knowledge of the biological effects induced by type I IFN on DC was extended further by showing that upon IFN-α2a

stimulation, monocyte-derived DC did not express the CCR7 receptor on cell surfaces and did not respond to recombinant SLC-driven chemoattraction. Although we cannot exclude that other inflammatory stimuli present at the site of antigen challenge might induce terminal maturation of tissue-infiltrating DC in vivo, type I IFN per se might not be sufficient to promote DC homing to SLO. Instead, IFN-α2a stimulation in vitro at doses as low as 100 IU/ml induced the production of high amounts of IP-10/CXCL10 and MIG/CXCL9 chemokines. Because no concomitant expression of the cognate CXCR3 receptor is detectable on iDC [23] or IFN-DC (unpublished results), other cell types are the likely functional targets of these chemoattractants. Indeed, we have observed efficient chemotaxis of memory and activated CD8⁺ T cells toward IFN-DC-conditioned medium. In particular, CD45RO⁺CD8⁺ T cells appear to be significantly more efficiently attracted than their CD45RA counterpart. Furthermore, conditioned media from IFN-DC displayed a significantly higher chemoattraction capacity toward activated CD8⁺ T cells as compared with iDC-conditioned media. Engagement of CXCR3 receptor on responder cells was found to be crucial in determining those migratory events.

Considering that monocyte-derived DC represent a model of tissue-resident DC in vivo, our data have a number of important implications. IFN-treated DC could attract in the periphery antigen-specific effector cells and also display an effective presenting capacity for relatively weak antigens such as TAA. In this respect, IFN-DC were indeed powerful stimulators of CTL specific for the MART-1 antigen. Thus, rather than migrating to SLO, DC exposed to type I IFNs could display their APC function at the site of the antigenic challenge. In this respect, it is of interest that upon vesicular stomatitis virus or recombinant *Listeria monocytogenes* infection, effector memory cells were found to be localized preferentially in nonlymphoid tissues [31].

Implications potentially related to anti-tumor-immune responses are particularly intriguing. Impaired DC mobility and sequestration of immunocompetent cells into malignant tissues have been described as potential mechanisms of tumor-immune escape not affecting antigen-presenting functions [32, 33]. In this scenario, enhancement of T-cell recruitment and activation into the malignant tissue, where professional APC might be present already, could offer the possibility of inducing specific immune responses. Conversely, IP-10/CXCL10 is known to inhibit neovascularization of tumor masses and to provide an anti-tumor effect in mouse models [34–36]. Thus, local administration of type I IFNs in vivo could not only enhance specific anti-tumor rejection but also nonspecific mechanisms of tumor necrosis.

Taken together, our data provide novel insights into the molecular mechanisms underlying the immunomodulatory effects of type I IFN and strongly support their use as biological adjuvants in immunotherapy, eventually used in TAA-targeted vaccines.

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