IFN-γ Arms Human Dendritic Cells to Perform Multiple Effector Functions

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IFN-γ Arms Human Dendritic Cells to Perform Multiple Effector Functions

Loredana Frasca,2* Maria Nasso,* Fabiana Spensieri,* Giorgio Fedele,* Raffaella Palazzo,* Fabio Malavasi,† and Clara Maria Ausiello*;

Dendritic cells (DCs) are central players in immunity and are used in immune-adoptive vaccine protocols in humans. IFN-γ, mandatory in Th-1 polarization and endowed with regulatory properties, is currently used to condition monocyte-derived DCs (MDDC) in cancer therapy and in clinical trials to treat chronic infectious diseases. We therefore performed a wide analysis of IFN-γ signaling consequences on MDDC multiple effector functions. IFN-γ itself induced IL-27p28 expression and survival but did not promote relevant CCR7-driven migration or activated Th-1 cell recruitment capacity in MDDC. Administered in association with classical maturation stimuli such as CD40 or TLR-4 stimulation, IFN-γ up-regulated IL-27 and IL-12 production, CCR7-driven migration, and activated Th-1 cell recruitment, whereas it decreased IL-10 production and STAT3 phosphorylation. CD38 signaling, which orchestrates migration, survival, and Th-1 polarizing ability of mature MDDC, was involved in IFN-γ-mediated effects. Thus, IFN-γ is a modulator of multiple DC effector functions that can be helpful in MDDC-based vaccination protocols. These data also help understand the dual role exerted by this cytokine as both an inducer and a regulator of inflammation and immune response. The Journal of Immunology, 2008, 180: 1471–1481.

Dendritic cells (DCs)3 are central players in immunity, bridging the innate to the adaptive arms of the immune system. They act as sentinels of infection and inflammation in the tissue, subsequently undergo the maturation process, and then migrate to the site of T and B cell priming, namely lymph nodes (LNs).

Monocyte-derived DCs (MDDC) are currently used in immune-adoptive vaccine protocols to treat cancer patients (1). Recently, IFN-γ has been included as a stimulus to condition MDDC ex vivo in these protocols, by virtue of its ability to induce potent Th-1 skew and proinflammatory activity (2, 3).

Monocyte-derived DCs (MDDC) in cancer therapy and in clinical trials to treat chronic infectious diseases. We therefore performed a wide analysis of IFN-γ signaling consequences on MDDC multiple effector functions. IFN-γ itself induced IL-27p28 expression and survival but did not promote relevant CCR7-driven migration or activated Th-1 cell recruitment capacity in MDDC. Administered in association with classical maturation stimuli such as CD40 or TLR-4 stimulation, IFN-γ up-regulated IL-27 and IL-12 production, CCR7-driven migration, and activated Th-1 cell recruitment, whereas it decreased IL-10 production and STAT3 phosphorylation. CD38 signaling, which orchestrates migration, survival, and Th-1 polarizing ability of mature MDDC, was involved in IFN-γ-mediated effects. Thus, IFN-γ is a modulator of multiple DC effector functions that can be helpful in MDDC-based vaccination protocols. These data also help understand the dual role exerted by this cytokine as both an inducer and a regulator of inflammation and immune response. The Journal of Immunology, 2008, 180: 1471–1481.

Studies based on the use of IFN-γ and IFN-γR knockout mice have confirmed the critical role of IFN-γ in Th-1 immunity, by using intracellular pathogens as infectious models (reviewed in Ref. 3, 5). However, emerging evidence indicates IFN-γ also is a master regulator of immune response and inflammation, a concept witnessed by evident beneficial effects in experimental systems of autoimmunity (reviewed in Ref. 6). Possible anti-inflammatory/ regulatory effects have been studied in immune cells other than DCs. IFN-γ has been shown to regulate B cell motility (7) and T cell survival (8), regulatory T cell activation (9), and more recently, suppression of Th-17 (10, 11).

Given the apparent paradoxical roles of IFN-γ in immunity, it is mandatory to understand the effects of IFN-γ signaling on MDDC immune players, especially in the light of future immune therapeutic applications either as a pharmacological agent (5, 12) or in ex vivo MDDC-based vaccination (1).

In this respect, we considered it profitable to undertake a wider analysis of IFN-γ administration consequences on MDDC effector functions. Our previous studies on human MDDC suggested IFN-γ would act as a maturation stimulus leading to up-regulation of various markers of mature (m)MDDC and decrease of dextran uptake (13). Among the markers up-regulated by IFN-γ, we found the CD38, recently shown by our group to be involved in several effector functions of mMDDC (14). In particular, we demonstrated that CD38 receptorial activity regulates IL-12p70 secretion (13) and in turn, Th-1 commitment, migratory ability guided by CCR7, and resistance to spontaneous apoptosis (14).

We decided to use IFN-γ either as a unique stimulus or in combination with classical maturation stimuli, such as LPS, a CD28 (TLR-4) binder, or CD40 ligation (CD40lig). In particular, the effect of the latter stimulus, in combination with IFN-γ, appeared of greater interest, considering that targeting of CD40-mediated pathways has undergone phase 1 clinical evaluation in advanced-stage cancer patients (15).

A question we concomitantly addressed concerned the outcome of CD38 signaling stimulation or inhibition on the IFN-γ-induced functional activities of MDDC (13, 14).
**Materials and Methods**

Reagents and mAbs

Ultra-pure *Escherichia Coli* LPS, PD98059, and SB203580 were from Cayla-InvivoGen Europe; PMA, ionomycin, brefeldin A, and PHA were from Sigma-Aldrich. Human recombinant GM-CSF was from R&D Systems. Culture supernatants of IL-4–62 cell line were the source of IL-4. CCL-21 was from PepTecBio. IL-2 was from Roche. As CD38 blocking reagents we used human recombinant soluble CD38 and blocking anti-CD38 mAb ABT135 (13, 14). As CD38 agonistic reagent we used IB4 mAb (13, 14). Anti-CD40 mAb (G28–5) was from ATCC. Affinity-purified goat anti-p-p44/42 MAPK (Thr 202/Tyr204 and ERK1/2), anti-p-p38 MAPK (Thr180/Tyr182), anti-p-IκBα, anti-p-IκBβ, anti-HLA-DR, FITC-conjugated anti-CD83, FITC-conjugated anti-CD1a, FITC-conjugated anti-CD86, and anti-TLR-4 mAbs were from ICN Cappel. Anti-CCR7 mAb was from R&D Systems. FITC-conjugated anti-CD40 cross-linker was from Cayla-InvivoGen Europe; PMA, ionomycin, brefeldin A, and PHA were from Sigma-Aldrich. Human recombinant GM-CSF was from R&D Systems.

**Cell lines**

CD40L-, control empty vector-, and transfected-J558L cells (16) were cultured in RPMI 1640 (Invitrogen Life Technologies), supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin (HyClone), and 0.05% phenol red-free, autolipidated, sodium pyruvate (1 mM), 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin (HyClone), and 0.05% phenol red-free. Mouse anti-β tubulin was from Invitrogen Life Technologies. Mouse anti-STAT1 was from Transduction Laboratories.

**Purification and culture of MDDC**

Monocytes were purified as described (13, 14) and cultured at 5 × 10^6/ml in complete medium in the presence of culture supernatants of IL-4–62 cell line, corresponding to 500 U/ml IL-4 and quantified by ELISA, and 50 ng/ml rGM-CSF. After 6 days, immature (i)MDDC were stimulated (1 × 10^7/ml) by LPS (1–100 ng/ml), human recombinant IFN-γ (5–500 U/ml), by CD40ig, or combined stimuli. Optimal CD40ig was obtained by culture with CD40L-expressing J558L cells at multiple cell ratios (10:1) or by cross-linked anti-CD40 mAb (13) with comparable effects. In some experiments, suboptimal stimulation was obtained by a 10-fold dilution of stimulatory CD40L-J558 cell number or CD40 mAb. Phenotype was analyzed after 48 h by flow cytometry in a FACSscan (BD Biosciences) using CellQuest software (13, 14). In signaling experiments, when indicated, MDDC were treated with ERK1/2 inhibitor PD98059 (100 μM) or p38 inhibitor SB203580 (20 μM) for 1 h and further stimulated with LPS or CD40ig. In IFN-γ priming experiments, MDDC were stimulated with 500 U/ml IFN-γ for 48 h, washed, and further stimulated with LPS or CD40ig. In experiments where CD38 involvement was evaluated, MDDC were treated with 500 U/ml IFN-γ in the absence or presence of either CD38 blocking or agonistic reagents.

**Cytokine measurement by enzymatic assays and TaqMan real-time RT-PCR analysis**

IL-12p70, IL-10, and IL-23 production by MDDC were assessed by ELISA after 48 h of stimulation as described (13, 14); IL-23 (eBioscience) detection limit was 15 pg/ml. IFN-γ, IL-5, and IL-17 in cocultured T cells were measured as described (13, 14); IL-17 (Quantikine; R&D Systems) detection limit was 15 pg/ml.

**MDDC chemotaxis and activated T cell recruitment assays**

For measurement of chemotaxis, we used 5 μm pore size polycarbonate filters (Corning Costar) (14). For chemotaxis of activated T cells (PHA blasts), a dilution of 1/3 of the MDDC culture supernatants in the final volume of 600 μl was added to the transwell bottom chamber while 4 × 10^5 PHA blasts (cell input) were added to the upper chamber. Recruited T cells were counted after 3 h by FACs (14). Scores for chemotaxis experiments are calculated as percentage of migrated cells vs cell input.

**PHA blasts activation**

PHA blasts were obtained by treating PBMC (1–5 × 10^6/ml) with PHA (1.5 μg/ml in complete medium). Cells cultured for 48 or 72 h were used as “activated” T cells (60–80% CXCR3 positive cells, measured by FACs) in recruitment assays and targets in cytotoxic assays, respectively.

**Apoptosis detection**

Apoptosis of MDDC was detected by APOTEST-FITC (DakoCytomation) after 48 h of stimulation (14).

**Isolation of T lymphocytes for MDDC-T cell cocultures**

T cells were purified from PBMC as described (13). Anti-CD4 conjugated microbeads (Miltenyi Biotec) were used to obtain CD8^+ T cells by negative selection. Purity of cell preparations was assessed by FACs.

**MDDC-T cell cocultures for induction of CTLs**

A total of 2 × 10^6 allogeneic CD14-depleted PBMCs, or purified CD8^+ T cells, were cultured with either n or IFN-γ-treated MDDC as APCs.
performed with different MDDC preparations.

The capacity of T cells to kill allogeneic PHA blasts was assessed by 51Cr-release assay. A total of 5 × 10^3 51Cr-labeled targets were plated in 96-well round-bottom plates with different effector numbers. Percentage of specific lysis was calculated as described (17).

Cytotoxic assay

The capacity of T cells to kill allogeneic PHA blasts was assessed by 51Cr-release assay. A total of 5 × 10^3 51Cr-labeled targets were plated in 96-well round-bottom plates with different effector numbers. Percentage of specific lysis was calculated as described (17).

MDDC-allogeneic T cell MLR and Th1/Th2 polarization

MDDC-induced allogeneic T cell proliferation was assessed as described (14). For polarization, we used cord blood T cells (14). For IL-17 determination, CD14^- cells (10^5) were cultured with allogenic MDDC (10^5) in 96-well plates (Corning Costar) in a final volume of 0.2 ml and supernatants harvested on day 12.

### Table II. Comparative analysis of CCR7 and TLR surface expression

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>CCR7</th>
<th>TLR-4</th>
<th>TLR-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>nt</td>
<td>6.4 ± 1.6</td>
<td>7.4 ± 1.5</td>
<td>33.5 ± 16</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>52.7 ± 6.3^b</td>
<td>40.1 ± 5.9^b</td>
<td>58 ± 15^b</td>
</tr>
<tr>
<td>IFN-γ LPS</td>
<td>61.8 ± 7.5</td>
<td>13.3 ± 5.6</td>
<td>ND</td>
</tr>
<tr>
<td>IFN-γ CD40lig</td>
<td>55.7 ± 10.0</td>
<td>25.7 ± 5.7</td>
<td>ND</td>
</tr>
<tr>
<td>LPS</td>
<td>50.1 ± 9.1^b</td>
<td>3.9 ± 0.9</td>
<td>22.4 ± 14</td>
</tr>
<tr>
<td>CD40lig</td>
<td>39.8 ± 1.1^b</td>
<td>6.7 ± 5.3</td>
<td>ND</td>
</tr>
</tbody>
</table>

^a MDDCs were either left nt or treated for 48 h as in Table I. Mean of percentage of expression (±SE) of each marker expression are reported.

^b Increased expression with respect to nt of each single stimulation is statistically significant (p < 0.05; Student’s t test (n = 5)).

### FIGURE 1. Dose response of maturation marker’s expression following IFN-γ stimulation. MDDC were either left nt or treated for 48 h in the presence of different doses of IFN-γ (A) and (B) and in combination with TLR4 (LPS) or CD40 (CD40lig) stimulation. Results are expressed as the mean ± SE of fluorescence intensity (MFI) (A) and (C) or percentage of positive cells (B). Results are from one of three independent experiments performed with different MDDC preparations.

### FIGURE 2. Analysis of cytokine expression by ELISA (A) or quantitative RT-PCR (B). MDDC were either left nt or treated for 48 h with IFN-γ, LPS, CD40lig, or combined stimuli as indicated. Data are reported as amount (pg/ml, mean values ± SE) of cytokines secretion measured by ELISA in 26 (IL-12p70), 10 (IL-10), and 4 (IL-23) experiments performed with different MDDC preparations. *, Differences in increased (IL-12p70) or decreased (IL-10) cytokine secretion induced by double with respect to single stimulated MDDC are significant (p < 0.05; Student’s t test). B, MDDC were either left nt or treated using 100-fold lower IFN-γ dose in combination with 10-fold lower level of LPS or CD40 stimulation. Data are reported as amount (pg/ml, mean values ± SE) of IL-12p70 and IL-10 secretion measured by ELISA in MDDC cultures derived from three different donors. +, Differences in increased (IL-12p70) or decreased (IL-10) cytokine expression induced by double with respect to each single stimulus are significant (p < 0.05; Student’s t test). C, MDDC were either left nt or treated as in Fig. 2A for either 5 or 24 h. Quantitative RT-PCR for IL-12/IL-23p40 and IL-12p35, IL-27p28 and EBI3, and IL-23p19 gene expression by MDDC was performed. mRNA transcript levels are expressed as fold increase over those measured in ntMDDC at 5 h. Results from one representative of three independent experiments, performed with different MDDC preparation, are shown.
Intracellular staining
Cytofix-Cytoperm-Perm/Wash protocol (BD Bioscience) was used for IL-4 and IFN-γ detection in T cells from polarization experiments and for Bcl-2 and Bax detection in MDDC (14).

Western blot analysis
Stimulated MDDC were lysed in RIPA buffer, composed of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, and 1 mM EDTA, with protease inhibitors (Complete Mini, EDTA free, 1 tablet for 10 ml of RIPA buffer, Roche Diagnostics), on ice for 20 min at 1 × 10^6 cells/ml, centrifuged 10 min at 12000 xg, and protein concentration measured by Bradford assay (Bio-Rad). Cell lysate quots (20 μg) were mixed with equal amounts of 2 × sample buffer (100 mM Tris-HCl, (pH 6.8) 25% glycerol, 2% SDS, 0.01% bromphenol blue, and 1 mM DTT), boiled (5 min), and proteins separated by 12% SDS-PAGE and transferred onto a nitrocellulose (0.22 μm) membrane (GE Healthcare). Nonspecific binding was blocked by TBS 5% nonfat milk, 0.1% Tween 20 for 1 h at room temperature. Immunoreactive protein was detected by incubating blots with anti-phosphorylated (or unphosphorylated or β-tubulin as control)-proteins overnight at 4°C. Blots were washed in TBS 0.1% Tween 20, incubated with HRP-conjugated goat anti-rabbit IgG (Bio-Rad) (to reveal p-proteins) or HRP-conjugated goat anti-mouse IgG (GE Healthcare) (to reveal control proteins) developed with the ECL reagents from Pierce. Specific phosphorylation levels were expressed as fold increase of basal conditions (iMDDC).

Statistical analysis
Statistical analyses were conducted using the SPSS statistical software package. Statistical significance of Student’s t test was set at p < 0.05.

Results
IFN-γ effects on maturation markers and TLR up-regulation by MDDC
As a first step, we performed a wide phenotypical analysis of MDDC from a high number of donors matured with IFN-γ alone or in combination with TLR-4 or CD40 activation, confirming that IFN-γ itself did not represent a “null event” (13) but determined a phenotype resembling that of mMDDC (Tables I and II). We also found up-regulation of other molecules important in DC biology, such as CCR7, TLR-4, and TLR-2 (Table II). Fig. 1, A and B, reports typical dose-response curve of selected maturation marker expression following IFN-γ treatment, revealing that effects could be observed also at low concentration. Fig. 1C shows that IFN-γ at 100-fold lower dose than that used in Table I and II still significantly increased expression of selected maturation markers in combination with 10-fold lower levels of LPS or CD40lig.

IFN-γ effects on IL-12 family cytokines and IL-10 expression by MDDC
IFN-γ did not induce significant levels of IL-12p70 in MDDC (Fig. 2A). Only in MDDC prepared from 7 of 26 individuals could we detect low levels of IL-12p70 secretion. When we considered this group of seven donors, the low IL-12p70 production obtained after IFN-γ treatment was significantly increased with respect to stained with Annexin V to assess apoptosis and PI to assess cell death. Cumulative apoptosis values (means ± SE of 3–6 independent experiments, depending on the condition analyzed), are presented as percentage of Annexin V^+ MDDC, determined as percentage of Annexin V^+ cells to total cells. Decreased apoptosis of MDDC treated with double stimuli with respect to single stimulated MDDC is statistically significant (p < 0.05; Student’s t test) in three experiments performed. The value of decreased apoptosis of IFN-γ-treated MDDC with respect to nt is indicated (p < 0.05; Student’s t test in three experiments performed).

E, MDDC were either nt or treated with IFN-γ, LPS, or CD40lig for 24 h, and Bcl-2 and Bax were measured by intracellular staining. Cumulative Bcl-2:Bax ratio values are expressed as mean of six independent experiments, performed with different MDDC preparations.

FIGURE 3. CCL21-driven chemotaxis (A), T cell recruitment capacity (B and C), survival (D), and anti- and proapoptotic factors expression (E). A, A total of 1.25 × 10^6 MDDC, either nt or treated as Fig. 2A, were added to the transwell upper chamber. In the lower chamber, CCL21 was added at the concentration of 10 ng/ml. Results are expressed as percentage of migrated cells with respect to the cell input (1.25 × 10^6 MDDC). Reported are data representing the mean values ± SE of three independent experiments performed with different MDDC preparations. *, Increased migration of MDDC treated with double stimuli with respect to migration of each single stimulated MDDC is statistically significant (p < 0.05; Student’s t test). B, A total of 4 × 10^5 PHA blasts were added to the transwell upper chamber. In the lower chamber, a 1:5 dilution of supernatants derived from MDDC, either nt or stimulated for 48 h as in Fig. 2A, was added. Data (mean values ± SE) are expressed as percentage of migrated PHA blasts with respect to the cell input (4 × 10^5 PHA blasts). The mean values of three independent experiments, performed with different MDDC preparations, are reported. *, Increased recruitment capacity of MDDC treated with double stimuli with respect to single stimulated MDDC are statistically significant (p < 0.05; Student’s t test). C, Recruitment capacity was assessed as in Fig. 3B using stimuli at the indicated doses. Reported is one representative of two experiments performed with different MDDC preparations. D, MDDC were either nt or treated as in Fig. 2A. Cells were counted and the percentage of early apoptotic (Annexin V^+

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An analysis of APC capacity of IFN-γ-treated MDDC. A, CD14+ cells (i) and (iii) or CD8+ T cells (ii) and (iv), as indicated, were cultured with MDDC either left nt or treated for 48 h with IFN-γ. After 6 days, recovered cells were used as effectors in cytotoxic assays using 51Cr-labeled PHA blasts, derived from the same individual source of MDDC used in primary cultures, as targets. In the assays in (iii) and (iv), cold NK-target K562 cells were plated at the indicated K562:labelled PHA blast ratio. Results are reported as percent of specific lysis (% of killing) of targets PHA blasts and are from one representative experiment of four performed. B, Cord blood-derived T cells were cocultured with MDDC either left nt or treated as in Fig. 2A. After 12 days, recovered T cells were re-stimulated with PMA/ionomycin for IFN-γ and IL-4 intracellular staining. Results are shown as the mean values ±SE of percent of positive cells (measured by FACS), derived from three independent experiments. *Increase of IFN-γ expression in cultures treated with double stimuli with respect to single stimulus is statistically significant (p < 0.05; Student’s t test). The value of increased IFN-γ expression of IFN-γ-treated with respect to ntMDDC is also indicated and is statistically significant (p < 0.05; Student’s t test). C, CD14+ cells were cocultured with MDDC either left nt or treated as in Fig. 2A. After 12 days, IFN-γ, IL-5, and IL-17 secretion were measured in the culture supernatants by ELISA. Results are from one representative experiment of four performed with different MDDC prepreations. D, MDDC were either left nt or treated as in Fig. 2A for 48 h, no treatment [45.4 ± 16.4 pg/ml IFN-γ-treated MDDC vs 5.7 ± 4 pg/ml ntMDDC, p < 0.05 (n = 7)]. When MDDC were treated with IFN-γ plus LPS or CD40lig, synergic effects on IL-12p70 were found in accordance with previous reports (18–20). In contrast, IL-10 release significantly decreased in MDDC when stimulated by double stimuli, compared with MDDC stimulated by LPS or CD40lig alone. IL-23 release did not increment significantly upon double stimulation (Fig. 2A). Fig. 2B shows IL-12p70 and IL-10 counter-regulation, using 100-fold lower IFN-γ dose, in combination with 10-fold lower level of TLR-4 or CD40 stimulation. We measured the transcription of IL-12 family subunits by quantitative RT-PCR (Fig. 2C). IFN-γ alone induced significant IL-27p28 transcription (270.3 ± 75-fold increase of IFN-γ-treated vs 0.8 ± 0.2-fold increase of ntMDDC, p = 0.037 and n = 3, at 5 h). Together with stimulation through TLR-4 or CD40, IFN-γ significantly increased transcription of IL-27 and IL-12p70 subunits. IL-23p19 transcription increase was not statistically significant.

The effects of IFN-γ on MDDC CCR7-driven migration

IFN-γ consistently up-regulated CCR7 and CD38, driving homing to LNs (13, 14, 21) (Table II); however, CCR7 expression does not always guarantee acquisition of CCL19/CCL21 responsiveness (21, 22). We assessed CCL21-driven chemotaxis of IFN-γ-treated MDDC and found that they possessed poor capacity to sense CCL21 gradients, although IFN-γ positively influenced CCR7-guided migration induced by TLR-4 or CD40 activation (Fig. 3A).

IFN-γ effects on MDDC T cell recruitment ability

MDDC produce different types of chemokines depending on the factors used for maturation (23, 24). Among inflammatory chemokine genes, those encoding for IP10/CXCL10, Mig/CXCL9, and I-TAC/CXCL11 (23, 24) contain IFN-γ-responsive elements. They all bind CXCR3 (25), expressed on activated T cells mainly of Th-1 subset. IFN-γ-treated MDDC recruited activated T cells with low efficiency (Fig. 3B). However, concomitant IFN-γ plus either CD40lig or LPS administration increased this activity in a significant manner (Fig. 3B). In both cases, the effects were evident even when a dose of 5 U/ml IFN-γ was used to treat MDDC as shown in Fig. 3C.

IFN-γ effects on MDDC survival

mMDDC are more resistant to growth factor withdrawal apoptosis than ϕMDDC (14). Fig. 3D shows a significantly decreased ability of IFN-γ-treated MDDC to undergo spontaneous apoptosis (measured by Annexin V/iodide propidium (PI) staining) as compared with mMDDC. Moreover, IFN-γ treatment conferred significant additive effect to survival of MDDC stimulated by either LPS or CD40lig. Expression of Bcl-2 and Bax, anti- and proapoptotic factors, respectively, was analyzed by intracellular staining (14). As resumed in Fig. 3E, IFN-γ increased Bcl-2:Bax ratio to levels comparable to those achievable after LPS or CD40lig stimulation, both treatments increasing MDDC half-life (14, 26).

IFN-γ effects on APC capacity of MDDC

Whether or not IFN-γ is truly a maturation stimulus or a cofactor in human DC biology needs clarification (13). To test APC capacity of IFN-γ-treated MDDC, we first analyzed ability to induce washed, and cultured in different numbers with allogeneic T cells for 6 days. Proliferation was assessed in triplicate by [3H]thymidine incorporation. Results are reported as mean cpm, minus background proliferation (8 cpm) and are from one representative of six independent experiments.

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FIGURE 5. Signaling in response to IFN-γ, TLR-4, and CD40 stimulation in MDDC. A, MDDC were treated with the indicated stimuli for either 30 min or 2 h. Amounts of pSTAT1, pSTAT3, pERK1/2, pp38, and SAPK/JNK p46 and p56 were determined by Western blotting. Data are reported as both blots and fold increase of phosphorylated protein level as measured by densitometric analysis (for SAPK/JNK the sum of p46 plus p56 is reported) in treated with respect to ntMDDC. Data are from one representative of four independent experiments, performed with different MDDC preparations. B, MDDC were treated as in Fig. 2A, either in the absence or presence of ERK1/2 inhibitor PD98059 or p38 inhibitor SB203580 for 48 h. Expression of maturation markers and TLRs was measured as in Table I and II. Results of three independent experiments are expressed as the percent of expression of each marker with respect to expression of the same marker by MDDC cultured with the correspondent stimulus in the absence of inhibitor, considered as 100% of expression (mean ± SE of expression of each markers in MDDC not treated with inhibitor was for IFN-γ; CD38 (MFI) = 101 ± 9, CD80 (MFI) = 55 ± 2, CD83 (%) = 10 ± 2, TLR-2 (%) 45.8 ± 3, and TLR-4 (%) 69.3 ± 7; for LPS: CD38 (MFI) = 140 ± 10, CD80 (MFI) = 110 ± 9, and CD83 (%) = 50 ± 12; for CD40lig: CD38 (MFI) = 30 ± 5, CD80 (MFI) = 55 ± 12, and CD83 (%) = 60 ± 9; for IFN-γ plus LPS: CD38 (MFI) = 160 ± 9, CD80 (MFI) = 170 ± 12, and CD83 (%) = 60 ± 10; and for IFN-γ plus CD40lig: CD38 (MFI) = 80 ± 10, CD80 (MFI) = 65 ± 4, and CD83 (%) = 80 ± 5). Results relative to IL-12p70 and IL-10 secretion measured by ELISA (pg/ml) are reported and calculated in the same way (mean of cytokine release by MDDC not treated with inhibitor was for IFN-γ: IL-12p70 = 101 ± 9 and IL-10 = 12 ± 5; for LPS: IL-12p70 = 600 ± 55 and IL-10 = 4100 ± 20; for CD40lig: IL-12p70 = 400 ± 9 and IL-10 = 990 ± 30; for IFN-γ plus LPS: IL-12p70 = 1400 ± 130 and IL-10 = 3500 ± 10; and for IFN-γ plus CD40lig: IL-12p70 = 1050 ± 90 and IL-10 = 200 ± 5). Results are the mean values of three independent experiments performed with different MDDC preparations. *, Inhibition of expression of either surface markers or cytokine release with respect to MDDC stimulated in the absence of inhibitor is statistically significant (p < 0.05; Student’s t test).
FIGURE 6. Priming effects of IFN-γ treatment on MDDC effector functions. A, MDDC were either pre-treated with IFN-γ or left nt. After 48 h, IFN-γ or nMDDC were either left nt or treated as indicated. Amounts (pg/ml) of IL-12p70 or IL-10 are measured by ELISA and data reported as mean (±SE) of three experiments. *, Increased (IL-12p70) or decreased (IL-10) cytokine production by MDDC, primed with IFN-γ with respect to unprimed MDDC and relative to the correspondent type of re-stimulation, are statistically significant (p < 0.05; Student’s t test). B, MDDC were pretreated as in A. After 24 h, IFN-γ or nMDDC were either left nt or re-stimulated for the indicated time points. Quantitative RT-PCR for IL-12 family genes expression by MDDC was performed. Results expressed as percentage of Annexin V positive cells. C, MDDC were pretreated as in A. After a further 48 h of culture, MDDC (1.25 × 10^5 MDDC) were added to the transwell upper chamber. In the lower chamber, CCL21 was added at the concentration of 100 ng/ml. Data (mean values ± SE) are expressed as percentage of recruited cells with respect to the cell input (4 × 10^5 PHA blasts) and are from three independent experiments performed with different MDDC preparations. *, Increased migration capacity of MDDC, primed with IFN-γ as compared with unprimed MDDC relative to the correspondent type of re-stimulation, are statistically significant (p < 0.05; Student’s t test). D, MDDC were pretreated as in A. A 1/3 dilution of culture supernatant collected after a further 48 h of culture was added to the transwell lower chamber. A total of 4 × 10^5 PHA blasts were added to the transwell upper chamber. Data (mean values ± SE) are expressed as percentage of recruited cells with respect to the cell input (4 × 10^5 PHA blasts) and are from three independent experiments performed with different MDDC preparations. *, Increased T cell recruitment capacity of MDDC, primed with IFN-γ as compared with unprimed MDDC relative to the correspondent type of re-stimulation, are statistically significant (p < 0.05; Student’s t test). E, MDDC were pretreated as in A. After a further 48 h of culture, cells were stained with Annexin V to assess apoptosis and PI to assess cell death. Cumulative apoptosis values (means ± SE of three independent experiments) are presented as percentage of Annexin V^+ cells. *, Reduced percent of apoptosis of MDDC, primed with IFN-γ with respect to the unprimed and relative to the correspondent type of restimulation, are statistically significant (p < 0.05; Student’s t test).

activation of CTLs. Therefore, allogeneic CD14^−, or 95% pure CD8^+ T cells, were cultured for 6 days with nt or IFN-γ-treated MDDC. Cytotoxicity of recovered T cells was assessed against PHA blasts from the same individual source of MDDC of primary cultures. IFN-γ-treated MDDC possessed a certain ability to expand CTLs, compared with nMDDC, when CD14^− cells were used as responders in primary cultures. In contrast, they were less efficient than nMDDC when enriched CD8^+ T cell populations were used as responder (Fig. 4A). Killing was not due to mere activation of NK cells during primary stimulation, as demonstrated by cold competition assays using unlabelled K562 cells, at different ratios as NK targets.

We then assessed polarization capacity by using allogeneic cord blood-derived T cells as responder (13). IFN-γ-treated MDDC could elicit a certain level of IFN-γ production by allogeneic naive T cells as demonstrated by intracellular staining (data not shown),
or treated with IFN-γ. Similar results for IFN-γ production were obtained by using a mixed naive and memory T cell population (Fig. 4C). By using this culture system, small amounts of IL-17 were produced by responder T cells when using MDDC matured by CD40lig and significantly reduced when using MDDC stimulated by CD40lig plus IFN-γ (p = 0.043 and n = 4). IFN-γ-treated MDDC turned out to be unable to elicit proliferation of purified T cells (Fig. 4D); nevertheless, double stimulation induced a stronger T cell proliferation compared with coculture with TLR-4 or CD40 stimulated MDDC.

**IFN-γ signaling in MDDC**

Signaling in response to IFN-γ administration alone or in combination with classical stimuli is presently unknown in human DCs. MDDC maturation is mainly driven by p38 activation (27), whereas, in other cell systems, IFN-γ signaling has been shown to involve ERK1/2 and JNK in addition to STATs activation (2, 28). Our previous data (13) also suggested activation of NF-κB pathway by IFN-γ. We therefore decided to analyze activation of STAT1 and STAT3, all MAPKs, and IκBα, the NF-κB inhibitor. Western blot analysis showed STAT1 phosphorylation induced very early by IFN-γ and only at a later time by CD40lig or LPS stimulation (Fig. 5A and data not shown, respectively). STAT1 activation tended to be enhanced upon double stimulation, whereas STAT3 phosphorylation decreased. IκBα, p38, ERK1/2, and SAPK/JNK were induced by all stimuli but with different intensities. A general intensification of phosphorylation, upon double stimulation, was observed for all proteins analyzed, except ERK1/2.

Then we used inhibitors of either ERK1/2 (PD98059) or p38 (SB203580) and assessed phenotype and cytokine secretion. IFN-γ-induced phenotypic changes and TLR-2 and TLR-4 up-regulation were preferentially abolished by ERK1/2 inhibitor, whereas LPS-mediated effects mainly depended on p38 activation (27) (Fig. 5B). Concerning maturation induced by CD40lig, both inhibitors exerted down-regulatory effects. Upon double stimulation, a predominant role of the p38 pathway was apparent.

Low IL-12p70 levels occasionally produced by IFN-γ-treated MDDC were completely inhibited by blocking ERK1/2 and not p38, whereas IL-10 was unaffected in the presence of either inhibitor. In MDDC stimulated through TLR-4 or CD40, IL-12p70 secretion was preferentially inhibited by p38, less by ERK1/2 blocking (Fig. 5B). However, p38 seemed to be the major player in

whereas MDDC stimulated through either LPS or CD40lig, in the presence of IFN-γ, significantly increased responder T cell-produced IFN-γ as compared with single stimulated MDDC (Fig. 4B).
IL-12p70 secretion upon double stimulation. IL-10 was inhibited by both inhibitors but with different intensities.

**Outcome of IFN-γ priming on MDDC multiple effector functions**

We next addressed the priming effect of IFN-γ by treatment of MDDC with the sole IFN-γ and exposure, in the next 48 h, to LPS or CD40lig. IFN-γ pretreatment resulted in a higher amount of IL-12p70 secretion and a partially impaired capacity to release IL-10 (Fig. 6A). This effect was seen also after pretreatment with 10- and 100-fold lower IFN-γ doses (data not shown). IFN-γ primed for increased transcription of both IL-12/IL-23p40 and IL-12p35 subunits, IL-23p19 and IL-27 (p28 and EB13), the latter increment more evident at 24 h (Fig. 6B). Fig. 6, C and D, show that MDDC stimulated by IFN-γ from the first, possessed both a CCR7-driven migratory and a T cell recruitment ability statistically more efficient than that of previously ntMDDC (p < 0.05) upon TLR-4 or CD40 stimulation. Fig. 6E reports the priming effect on induction of resistance to spontaneous apoptosis, showing IFN-γ priming significantly (p < 0.05) prolonging MDDC survival.

**CD38 signaling involvement in IFN-γ-induced functions of MDDC**

We recently discovered that CD38 receptorial activity regulates, in nMDDC, IL-12p70 secretion, Th-1 polarization, CCR7-driven migration, and survival (14). Since IFN-γ highly up-regulates CD38, we wanted to establish whether CD38 signaling was involved in IFN-γ-mediated effects. We treated MDDC with IFN-γ in the presence of reagents acting as CD38 blocking or agonist. Blocking reagents interfering with cross-talk between CD38 and its counter-receptor CD31 (both expressed by MDDC (13, 14)) prevented CD83 up-regulation induced by IFN-γ (13). In contrast, concomitant stimulation by CD38 agonistic reagents increased IFN-γ-induced CD83 up-regulation (Fig. 7A). MDDC recovered from 7 of 26 individuals analyzed (26.9%) responded to IFN-γ itself by producing small amounts of IL-12p70. IFN-γ treatment, in the presence of CD38 blocking reagents, totally suppressed IL-12p70 (Fig. 7B) without changing IL-10 secretion (data not shown (13)). In contrast, simultaneous IFN-γ and CD38 agonist reagents stimulation resulted in release of significantly (p < 0.05) increased levels of IL-12p70 (Fig. 7B (13)), being IL-10 secretion unaffected (data not shown). Concerning CCR7-driven migration and capacity to recruit activated T cells, MDDC simultaneously stimulated by IFN-γ and CD38 agonist reagents significantly increased the ability to sense CCL21 (Fig. 7C), whereas supernatants recovered in this culture condition possessed a significantly (p < 0.05) enhanced T cell recruitment activity (Fig. 7D). Fig. 7E shows inhibition of the protective effect of IFN-γ on growth factor withdrawal apoptosis in the presence of CD38 blocking reagents. Restoration of signaling by concomitant provision of agonist CD38 reagent during IFN-γ treatment restored protection. Th-1 polarizing ability was impaired in MDDC stimulated by IFN-γ in the presence of CD38-blocking reagents (Fig. 7F) because, in this condition, responder T cells released reduced amounts of IFN-γ.

**Discussion**

In this article, novel data support the view that encounter with IFN-γ changes the fate of human DCs through modulation of a wider array of effector functions than previously shown. Our findings may have important implications in clinical settings, being that MDDC are currently used in cancer adoptive immune therapy (1). They can also provide a tool for future studies aimed at a deeper dissection of the complex network in which IFN-γ exerts both suppressive and immune stimulatory activities (6).

Our data indicate that IFN-γ induces IL-27p28 expression in human DCs and strongly synergizes with classical maturation stimuli in sustaining high levels of IL-27 transcription, an effect observed only in murine macrophages so far (29). Since IL-27 arms naive T cells to respond to IL-12p70 through up-regulation of the specific receptor (10, 30), we may have highlighted an additional mechanism through which IFN-γ can promote Th-1 commitment. Recently, it has become clear that the predominant in vivo role of IL-27 is negative immune regulation: IL-27 down-regulates T cell activation in general but also selectively suppresses Th-17 and Th-2 cells (10, 30, 31). An intriguing speculation is that by enhancing or sustaining over time IL-27 production in the presence of microbial products (TLR-4 ligands) or T cell help (CD40lig) (Figs. 2 and 6), IFN-γ ensures homeostatic down-regulation of immunity once infection vanishes. Moreover, IL-27 production could be instrumental to decrease Th-17-driven chronic inflammatory responses (10). In this respect, we detected IL-17 production by a mixed population of memory and naive T cells cocultured with CD40lig-stimulated MDDC not evident in cultures containing MDDC stimulated through CD40lig in the presence of IFN-γ (Fig. 4). Worthy of closer examination is the possibility that IFN-γ allows sufficient IL-27 production by DCs to counteract the action of IL-23, which is not significantly increased in the system we explored (Fig. 2), a possible explanation for IFN-γ beneficial effects in autoimmunity (3, 6–9). Comparison of amounts of release of both IL-27 and IL-23 will better explain the reciprocal immune regulatory role of these two cytokines (30). These data also reinforce the idea of using IFN-γ in *Mycobacterium Tuberculosis* infection (5), characterized by production of IL-17, a cytokine exacerbating chronic infection (10). A recent related discovery is the potent antitumor activity of IL-27 (10). In this respect, we suggest that IFN-γ treatment of autologous MDDC used in cancer therapy may be beneficial, especially if provision for stimulation through CD40lig is taken into account (15, 32). To avoid the recently observed unwelcome long-term effect in animal models treated with Abs to CD40 (33), CD40lig could be provided on MDDC themselves ex vivo instead of systemically.

Very few studies have analyzed IFN-γ effect on DC trafficking capacity (36–38). We observed additive effects between IFN-γ and TLR-4 or CD40 stimulation on this function (Figs. 3 and 6). The increased migration probably did not only rely on the limited (and nonstatistically significant, Tables I and II) increase of CD38 and CCR7 expression. Thus, integration of multiple signaling pathways may be envisaged. The increased efficiency of CCR7-driven migration implies a further level of “enhancer/regulatory effect” of IFN-γ on human DC functions. Since this phenomenon occurs as a priming effect too, a fashionable interpretation is that IFN-γ can prepare the DCs in the tissue for a subsequent migration step, later induced either by stimulation with TLR-binding factors (LPS) or cross-talk with effector CD4+ T cells homing to inflamed tissues. Noteworthy, after IFN-γ priming, we observed an increase of both IL-12p70 secretion and CCR7-driven migration. This observation, on one hand, supports the “signal response modules” theory (39), re-analyzing the concept that LN-directed migratory and strong IL-12p70 release ability are mutually exclusive phenomena. In contrast, acquisition of both characteristics at the same time, especially when IFN-γ is used in combination with CD40lig.
(Fig. 2, A and B, and 3), can be important in MDDC-based vaccination. Also worth noting, we did not find inhibitory effects of IFN-γ on MDDC migration, a phenomenon observed by using the classical maturation mixture for adoptive immune therapy (36). This likely reflects different ways of maturing MDDC, implying integration of different activation pathways (36, 39).

Why CCR7 and CD38 up-regulation is independent of migratory ability acquisition is an issue still under investigation. However, CCR7 signaling, like CD38 signaling (13, 14), also stimulates proinflammatory differentiation programs and protection from apoptosis (21). Consequently, the advantage to gain CCR7 confers proinflammatory differentiation programs and protection ever, CCR7 signaling, like CD38 signaling (13, 14), also stimulates proinflammatory differentiation programs and protection from apoptosis (21). Consequently, the advantage to gain CCR7-mediated functions in DCs.

Combined IFN-γ and TLR-4 or CD40 stimulation also conferred to MDDC a greater capacity to attract activated Th-1 cells (25) (Figs. 3, B and C). Not having measured chemokines release, this is an issue we are currently addressing; we are aware that these present preliminary observations. IFN-γ can increase IP10/CXCL10 secretion by CD40L stimulated pDCs (40) while acting as a cofactor inducing CXCR3-binding chemokines in conventional DCs (24) in humans. Although IFN-γ itself induced sufficient CXCR3 binding chemokines in mouse DCs (41), in our hands, supernatants of MDDC treated with the sole IFN-γ did not show a significantly enhanced chemo attractant potential compared with iMDDC culture medium. Accordingly, preliminary intracellular staining experiments revealed a slight increase of IP10/CXCL10 expression in IFN-γ-treated (as opposed to nt) MDDC but lower than that obtained in TLR-4 stimulated MDDC (unpublished observations). Although incomplete, the evidence so far obtained suggests IFN-γ effects an amplification of DC recruitment ability, likely instrumental in driving effector T cells into inflamed tissues, a phenomenon possibly also operative in LNs to ensure retention of Th-1 cells at the cell priming sites (42). Further, this function can be considered important in MDDC-based vaccine protocols: once MDDC stimulated through IFN-γ and CD40lig have migrated to the LNs, they may be able to attract and retain the proper helper T cells. The APC capacity of IFN-γ-treated DCs (13) remains a challenging issue, although to some extent we have confirmed previous results, such as the ability to stimulate cytolytic activity and weak Th-1 polarization of allogeneic T cells (19). Our results suggest a T cell help dependency of CD8+T cell killing activity induced by IFN-γ-treated DCs, revealing a way to regulate cellular adaptive immune responses. At the moment, we are unable to explain the inability of IFN-γ-treated DCs to induce responder T cell’s proliferation. “IFN-γ modified” mouse DCs have been shown to ameliorate the status of autoimmune diseases through expression of IDO (43). However, use of 1-methyl-tryptophan, a typical IDO inhibitor (44), allowed us to exclude IDO-mediated effects (unpublished observations).

Finally, the positive IFN-γ effect on MDDC survival (Figs. 3 and 6) can be viewed as a further way to confer stronger immune stimulatory potential to tissue resident or recruited DCs already at the time of innate immunity activation, eventually prolonging their longevity during, or even before, the encounter with pathogen- or T cell-derived stimuli.

Signaling induced by IFN-γ has not been addressed in human DCs so far. Our data reveals that IFN-γ-mediated phenotypic changes particularly rely on ERK1/2 and less on p38 activation (Fig. 5). Western blot data confirm, in the human DC setting, capacity to induce typical phosphorylation of STAT1 and STAT3 (3, 38) and show NF-κB pathway activation, (see IkBa, in Fig. 5) confirming previous findings using N-acetyl cysteine to inhibit IFN-γ effects (13). We show reproducible additive affects on STAT1 and MAPKs activation, similar data by Zhao et al. (45) on macrophages, not so evident only for ERK1/2 activation (Fig. 5 and data not shown for TLR4 stimulation). The most interesting result concerns STAT3 phosphorylation, which is always reduced in case of IFN-γ administered together with either CD40 (Fig. 5) or TLR-4 (data not shown) stimulation. Previous studies have demonstrated that binding of activated STAT3 to IL-10 promoter is required for efficient IL-10 gene expression (46). Thus, reduced STAT3 activation would explain the significant suppression of IL-10 production by IFN-γ. Conversely, it has been shown that IL-10 is responsible for STAT3 activation in mouse cells, suggesting that blocking of STAT3 activation, upon provision of double stimuli, may be linked to inhibition of IL-10 production in these conditions (47, 48). However, the blocking represents an early event (30 min, Fig. 5) in our system and may be independent from IL-10. It is known that STAT3 inhibits full up-regulation of DC maturation markers and IL-12p70 secretion (48–50). Thus, concomitant administration of IFN-γ and CD40 (or TLR-4) stimulation may relax the STAT3-mediated inhibitory activity on APC functions, favoring a sort of “hyperactivation” of DCs. Other authors have nicely shown that disruption of STAT3 signaling, in either macrophages or bone marrow-derived DCs, renders them capable of restoring responsiveness of tolerant T cells from tumor-bearing mice (49, 50). Thus, we have found another effect, promoted by CD40 plus IFN-γ stimulation, that can be beneficial in cancer therapy where immune regulatory mechanisms should be relaxed.

The final piece of data corroborates previous observations on CD38 activity in human DCs (14). It should be underlined that two novel observations have been made in this study. First, concomitant induction of IFN-γ stimulation and CD38 signaling consistently increased CCL21 driven migration (Fig. 3). Second, in the same type of stimulation, MDDC’s capacity to recruit activated Th-1 cells is augmented, suggesting a synergy between IFN-γ and CD38 signaling in inducing CCR7-regulated signals and release of CXCR3-binding inflammatory chemokines. Previous (13, 14) and novel findings suggest important implications of CD38 signaling in inflammation, being CD31, the CD38 counter-receptor, expressed not only on DCs themselves but by various parenchymal, endothelial, B, and all cell types residing in, or homing to, inflamed tissues. It is also intriguing that CD31 is peculiarly expressed by naive T cells (51) encountering DCs in LNs. Thus, it is conceivable to regard CD38 signaling as an additional “licensing stimulus” that inflamed tissues or LN environments confer to mDCs or DCs conditioned by IFN-γ, expressing high CD38 levels.

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