Abstract

Human monocytes differentiate into dendritic cells (DC) or macrophages according to the nature of environmental signals. Monocytes stimulated with granulocyte-macrophage colony-stimulating factor (GM-CSF) plus interleukin-4 (IL-4) yield DCs. We tested here whether interferon-gamma (IFNγ), a potent activator of macrophages, may modulate monocyte differentiation. Addition of IFNγ to IL-4 plus GM-CSF-stimulated monocytes switches their differentiation from DC to CD14-CD64+ macrophages. IFNγ increases M-CSF and IL-6 production by IL-4 plus GM-CSF-stimulated monocytes by acting at the transcriptional level, and acts together with IL-4 to upregulate M-CSF but not IL-6 production. IFNγ also increases M-CSF receptor internalization. Results from neutralizing experiments show that both M-CSF and IL-6 are involved in the ability of IFNγ to skew monocyte differentiation from DC to macrophages. Finally, this effect of IFNγ is limited to early stages of differentiation. When added to immature DC, IFNγ upregulates IL-6 but not M-CSF production and does not convert them to macrophages, even in the presence of exogenous M-CSF. In conclusion, IFNγ shifts monocyte differentiation to macrophages rather than DCs through autocrine M-CSF and IL-6 production. These data show that IFNγ controls the differentiation of antigen-presenting cells and thereby evidence a new mechanism by which IFNγ orchestrates the outcome of specific immune responses.

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Introduction

Peripheral blood monocytes can differentiate into dendritic cells (DC) or macrophages depending on environmental factors encountered during their migration from blood to peripheral tissues (1-4). Transendothelial trafficking (5) and culture in the presence of serum from systemic lupus erythematosus (through the presence of IFNα) induce monocyte differentiation into immature DC (6). Upon contact with IL-4 plus GM-CSF (cytokines that could be produced by tissue mast cells), monocytes also differentiate into immature DC (2-4). Addition of TGF-β or exposition of monocytes to GM-CSF plus IL-15 lead to DC with features of Langerhans cells (7, 8). In contrast, M-CSF is a potent macrophage differentiation factor (1). IL-6 (9, 10) and IL-10 (11) also shift monocyte differentiation from DC to macrophages. Tumor cells produce IL-6 and M-CSF that shift the differentiation of CD34+ progenitors from DC to macrophages (9). Fibroblasts, via IL-6 production, up-regulate functional M-CSF receptor (CD115) expression and autocrine M-CSF consummation by monocytes, thereby switching their differentiation from DC to macrophages (10).

Dendritic cells are the most potent antigen-presenting cells (12). In the periphery, immature DC capture antigens and, upon contact with stress factors (such as microbial components), migrate to the lymphoid organs and undergo a maturation process. They express high levels of co-stimulatory and accessory molecules, upregulate MHC-I and -II molecules and neoexpress CD83. In the lymph nodes, mature DC prime naive antigen-specific T cells (12). In contrast to DC, macrophages are effector cells that produce various mediators and have evolved to ingest as many pathogens as possible. Although they present antigens, macrophages are less efficient than DC and unable to prime naive T cells (13). Interferon-gamma (IFNγ), released during early and late stages of the immune response by natural killer cells and activated T cells, respectively, regulates several aspects of the immune response (14). In addition to direct antiviral activity, IFNγ orchestrates leukocyte-endothelium interaction (14) and plays a crucial role in vivo in “cancer immunosurveillance” (15). IFNγ is a potent activator of macrophages. Upon contact with IFNγ, monocyte-macrophages undergo biochemical and morphological modifications that allow them to perform their functional activities (16). INFγ stimulates macrophage antimicrobial and tumoricidal activities and accessory cell functions, and modulates proteasome gene expression (16). IFNγ also acts on uncommitted myeloid immature DC to polarize them into Th1 cell-promoting effector cells that produce high levels of IL-12 upon stimulation (17). We tested here whether IFNγ could be involved in
monocyte differentiation, and report that IFNγ switches monocyte differentiation from DC to macrophages, at least partly via an autocrine production of M-CSF and IL-6.

Methods

Cytokines. All human and murine recombinant cytokines were from R&D Systems (Abingdon, UK).

Human monocyte differentiation. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque (Life technologies, Cergy Pontoise, France) density gradient centrifugation. Monocytes were purified from PBMC by positive selection using a magnetic cell separator (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). Purity, assessed by FACS analysis using a FITC-labeled anti-CD13 mAb, was >98%. Monocytes were differentiated into DC by 5 days culture in complete medium (CM) consisting in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 10 mM Hepes and 0.1 mM non essential amino acids (all from Life technologies) at 5x10⁶ cells/5 ml/well in 6-well tissue culture plates (Costar, Cambridge, MA) with 20 ng/ml IL-4 and 20 ng/ml GM-CSF. Macrophages were obtained by culturing monocytes for 5 days in CM with 2 ng/ml GM-CSF and 20 ng/ml M-CSF (18). In some experiments, monocytes were cultured for 5 days in CM with 20 ng/ml GM-CSF. All these cultures were also performed in the presence of different concentrations of IFNγ (2 to 50 ng/ml) added at different time points, from day 0 to day 5. In neutralization experiments, monocytes in GM-CSF + IL-4 containing or not 25 ng/ml IFNγ were treated at day 0 and day 2 with 30 µg/ml neutralizing anti-human M-CSF mAb (R&D Systems) plus 30 µg/ml neutralizing anti-human IL-6 mAb (Diaclone, Besancon, France) or with 30 µg/ml mouse isotype control mAbs (BD Pharmingen, San Diego, CA). In other experiments, day 5 immature DC were recultured in GM-CSF + IL-4 without or with 25 ng/ml IFNγ alone, 25 ng/ml IFNγ plus 100 ng/ml M-CSF, or 100 ng/ml M-CSF plus 100 ng/ml IL-6 or were recultured in CM without cytokine. Finally, in some experiments cells were stimulated with 20 ng/ml LPS (Sigma, Saint-Louis, MO).

Mixte lymphocyte reaction (MLR) with human cells. Dendritic cells and macrophages, obtained as described above, were washed, irradiated (3000 rads) and cultured in quintuplicate at 4x10², 2x10³ or 10⁴ cells/200 µl/well in 96-well flat-bottomed plates with 10⁵ allogenic T cells purified from PBMC by rosetting with sheep red blood cells (the purity, assessed by FACS analysis using a FITC-labeled anti-CD3 mAb, was >95%). In some experiments, day 5 immature DC were treated or not with 25 ng/ml IFNγ. After 48 hours, cells were or not stimulated with 2 ng/ml LPS for 24 hours and
used in MLR assays at 2×10^4 cells/ml with 10^6 allogenic purified T cells. After 5 days, cells were pulsed during the last 16 hours with 0.25 µCi/well [3H]-thymidine (Amersham, Uppsala, Sweeden). Thymidine incorporation was measured by standard liquid scintillation counting. Results are expressed in cpm (mean of quintuplicate values).

**Murine cells.** C57BL/6 (IA^b^) and Balb/c (IA^d^) mice were from Harlan (Gannat, France). Murine DC were generated as described (19) by culturing bone marrow cells from C57BL/6 mice in CM supplemented with 50 µM β-mercaptoethanol (β-ME) and containing 3 ng/ml GM-CSF. In some experiments, 25 ng/ml murine IFNγ was added at the beginning of the culture. After five days, the phenotype of the cells was analyzed by FACS and MLR were performed as follows. Briefly, allogenic CD4^+ T cells from Balb/c mice were purified by incubation with a FITC-labeled anti-murine CD4 mAb (BD Pharmingen) followed by positive selection using anti-FITC mAb coated-microbeads (Myltenyi Biotec). After 5 days culture in GM-CSF, myeloid APCs were depleted in Gr1-positive cells by incubation with an anti-Gr1 mAb (Caltag, Burlingame, CA) followed by anti-mouse Ig mAb-coated beads (Dynal, Oslo, Norway). In 96 well flat-bottomed culture plates (Costar), 4×10^5 CD4^+ T cells plus 5×10^4 APCs were cultured in triplicate for 72 hours. During the last 16 hours, [3H]-thymidine was added. Results are expressed in cpm x 10^-3 as mean ± s.d., n=3.

**FACS analysis.** The phenotype of cells was analyzed by cytofluorometry using a FACSVantage cytofluorometer (BD Biosciences, Erembodegem, Belgium). For human cells, the following mAbs were used: FITC-labeled anti-CD1a (Immunology products, Groningen, The Netherlands), -CD14 (Dako, Glostrup, Denmark), -CD64 (Caltag), -CD86, and -HLA-DR (both from BD Pharmingen) mAbs, unlabeled anti-mannose receptor (MR) (Research Diagnostic, Flanders, NJ), -MHC-I, -CD83 (both from Beckman Coulter, Villepinte, France) revealed by FITC-labeled anti-mouse IgG Ab (Silenus, Melbourne, Australia) and goat anti-CD115 Ab (R&D Systems) revealed by FITC-labeled anti-goat IgG Ab (Silenus). To analyze intracellular expression of CD115 and RFD7, cells were fixed and permeabilized using the Intrastain kit (Dako) before staining with anti-CD115 or -RFD7 mAbs (Serotec, Oxford, UK) revealed by FITC-labeled anti-goat IgG Ab or anti-mouse IgG Ab, respectively. Murine cells were phenotyped using FITC-labeled anti-CD11b, -CD11c, -CD86, -IA^b^ (all from BD Pharmingen), biotin-labeled anti-F4/80 mAb (Caltag) revealed by FITC-labeled streptavidin (Molecular Probes, Eugene, OR) and PE-labeled anti-CD11c (BD Pharmingen) and –Gr1 mAbs. Isotype control mAbs were from BD Pharmingen. Results are expressed as a percentage of positive cells or in MFI values after subtraction of the MFI obtained with the control mAb.
Analysis of mRNA expression by RT-PCR. In freshly purified human monocytes and in monocytes cultured in GM-CSF + IL-4 in the absence or presence of 25 ng/ml IFNγ for 2, 4, 8 or 16 hours, the expression of the mRNA encoding IL-6, IL-10, M-CSF and CD115 was determined by RT-PCR. Briefly, RNA was extracted using Trizol reagent (Life technologies) and the single-strand cDNA was synthesized using 2 µg of total RNA by reverse transcription using an oligo-dT primer (Amersham). PCR reactions were performed with cDNA corresponding to 50 ng of total RNA and primers designed to amplify the coding sequence of the cytokines and cytokine receptor. PCR reaction was as follows: 94°C for 5 min, 30 cycles 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min followed by a final extension at 72°C for 5 min. RNA integrity and cDNA synthesis was verified by amplifying GAPDH cDNA. The amplified fragments were size-separated on a 1% agarose gel and visualized by ethidium bromide.

Quantification of human cytokines and soluble IL-6 receptor. IL-6, M-CSF and gp80 (soluble IL-6 receptor) were quantified in the cell-free culture supernatants by ELISA (R&D Systems) (sensitivity of 0.7, 9 and 6.5 pg/ml, respectively). Results are expressed in ng/ml or in µg/ml (as mean ± s.d., n=4).

Phagocytosis and cytochemistry. In order to analyze endocytic properties, cells were incubated for 20 min at 37°C with FITC-labeled dextran (40,000 MW), Staphylococcus aureus or latex beads (2 µm) (all from Molecular Probes). After extensive washings, cells were analyzed by FACS. Results are expressed in MFI or as a percentage of fluorescent cells. Nonspecific esterase activity was analyzed using the alpha naphthyl acetate esterase kit (Sigma) following the manufacturer instructions. Cell staining was analyzed by light microscopy.
Results

**IFNγ shifts monocyte differentiation from DCs to activated macrophages.**

CD1a−CD14+ monocytes cultured with GM-CSF + IL-4 differentiate after 5 days into CD1a+CD14− immature DC (2,3) (Table I). When cultured in GM-CSF + IL-4 containing IFNγ, monocytes differentiate into CD1alowCD14+ cells (Table I). Compared to monocyte-derived immature DC, IFNγ-treated cells express CD64 and CD86, express higher levels of MHC-I and -II molecules, lower levels of mannose receptor (MR) (Table I) and present reduced T cell costimulatory properties (Fig. 1A, left panel). Microscopic observation shows that, while immature DC are mostly non adherent round cells, IFNγ-treated cells form a network of adherent elongated cells (Table I & Fig. 1B). In contrast to macrophages, LPS-stimulated immature DC undergo a maturation process. They present morphological changes associated with veils (Table I & Fig. 1B), neoxpress CD83 (Table I), upregulate MHC-II, CD40, CD80 and CD86 expression (data not shown) and acquire potent costimulatory properties (Fig. 1A, right panel). Upon LPS stimulation, IFNγ-treated cells are adherent, do not present veils (Table I & Fig. 1B), do not acquire CD83 expression (Table I) and the expression of MHC-II and costimulatory molecules (CD40, CD80 and CD86) is not modulated (data not shown). Moreover, these cells have limited costimulatory properties compared to mature DC (Fig. 1A, right panel). Lastly, in response to 2 ng/ml LPS, IFNγ-treated cells produce undetectable levels of bioactive IL-12, in contrast to immature DC (835 ± 160 pg/ml) (data not shown). IFNγ-treated cells do not express Langerhans cell-associated markers (langerhin and E-cadherin) (data not shown). Together, these data suggest that GM-CSF + IL-4 + IFNγ-treated monocytes present a macrophage phenotype.

Some macrophage subsets, such as monocytes differentiated into macrophages in the presence of M-CSF, are characterized by CD14, CD64, RFD7 and MR expression and by potent phagocytic and nonspecific esterase activities (Table I). IFNγ modulates macrophage phenotype and functions: it upregulates MHC-I, HLA-DR and CD64 expression (20) and downregulates CD14 (21), RFD7 (22) and MR (23) expression as well as phagocytic properties (14, 24) and nonspecific esterase activity (Table I). In agreement with these data, monocytes cultured in GM-CSF + IL-4 + IFNγ express CD86 and CD64 but not RFD7 and CD14, and present reduced endocytic and nonspecific esterase activities (Table I). In parallel, IFNγ poorly modulates macrophage accessory cell function (25; Fig. 1A, right panel). Together, these data show that IFNγ skews monocyte differentiation from immature DC to IFNγ-stimulated macrophages.
By comparing the respective properties of IL-4 and IFNγ on GM-CSF-treated monocytes (Table I, left and middle panels), it appears that IL-4 and IFNγ have an additive effect on the upregulation of MHC-I and MHC-II molecule expression (26). This additive effect was observed at any time point analyzed during the differentiation process (from day 1 to day 5; data not shown). Moreover, IL-4 and IFNγ have also an additive effect in upregulating CD64 and CD86 expression on day 5-differentiated macrophages. Together, these observations suggest a more complex dialog between IL-4 and IFNγ than just a decrease in monocyte-differentiating cell sensitivity to IL-4 mediated by IFNγ (27).

**IFNγ prevents murine bone marrow progenitor differentiation into DC.**

We therefore tested whether the ability of IFNγ to skew myeloid cell differentiation from DC to macrophages could be extended to an IL-4-independent model of DC generation. In the presence of GM-CSF, murine bone marrow-derived progenitors differentiate into DC (19). Bone-marrow progenitors were incubated for 5 days with GM-CSF in the absence or presence of IFNγ. Under the aegis of GM-CSF, MHC-II-negative progenitors give rise to a major population of DC (75 to 90% of the cells according to the experiments; n=5) characterized by MHC-II, CD11c, CD86 and F4/80 expression and potent T cell stimulatory properties (Table II). In addition to DC, macrophages (adherent, CD11c-negative, Gr1-negative, CD11b-positive and low or no MHC-II) and granulocytes (non adherent, Gr1-positive and MHC-II-negative) are also present (19; data not shown).

When progenitors are cultured in the presence of GM-CSF plus IFNγ for 5 days, no CD11c-positive cells are generated (Table II). In addition to a minor proportion of Gr1-positive granulocytes (15 to 32%), the Gr1-negative and CD11c-negative cells express low or undetectable levels of MHC-II, but express CD86, F4/80 and CD11b (Table II). They do not express B, T or NK cell markers (data not shown). Finally, in contrast to DC, these CD11c-negative CD11b-positive cells present poor MLR-stimulating activity (Table II). These data show that IFNγ skews murine bone-marrow progenitor differentiation from DCs to macrophage-like cells. They also support a direct effect of IFNγ on DC precursors.

**IFNγ upregulates M-CSF production by monocytes.**

M-CSF is the most potent macrophage differentiation factor described (1). We then tested whether IFNγ may affect M-CSF production by monocytes. Human monocytes were cultured in GM-CSF + IL-4 containing or not IFNγ and M-CSF was quantified in the supernatants at different time-points during the differentiation process. Monocytes cultured in GM-CSF + IL-4 produce M-CSF (Ref. 28 & Fig. 2A). M-CSF production is maximal at day 1 and declines during the 5-day
period culture (Fig. 2A). Surprisingly, the presence of IFNγ together with GM-CSF + IL-4 results in a sustained (Fig. 2A) and dose-dependent upregulation of M-CSF production (maximal increase of 300% ± 42 using 25 ng/ml IFNγ; mean ± sd, n=4) (Fig. 2B). M-CSF mRNA expression is induced by culturing monocytes in GM-CSF + IL-4 (28) and is upregulated by IFNγ as early as 2 hours after stimulation (Fig. 3).

We next evaluated the respective role of IL-4 and IFNγ on M-CSF production (Fig. 2B). Monocytes were exposed to GM-CSF in the absence or presence of IL-4 and/or different concentrations of IFNγ. M-CSF was quantified in the 24 h supernatants. GM-CSF induces M-CSF production (Ref. 28 & Fig. 2B) in a dose-dependent manner (data not shown). IL-4 and IFNγ have antagonistic effects on GM-CSF-induced M-CSF production as they downregulate (28) and upregulate M-CSF production, respectively (Fig. 2B). Surprisingly, when added together, IFNγ and IL-4 have an additive effect on the upregulation of M-CSF production (Fig. 2B). Monocytes in GM-CSF produce higher levels of M-CSF when stimulated with IFNγ plus IL-4 than with IFNγ alone (increase of 300% ± 42 and 80% ± 19 using 25 ng/ml IFNγ, respectively; mean ± sd, n=4) (Fig. 2B). Finally, IFNγ also upregulates murine M-CSF production by bone marrow progenitors (data not shown). Thus, we report for the first time that IFNγ is a potent inducer of M-CSF production by human and murine myeloid cells.

**IFNγ upregulates IL-6 production by monocytes.**

IL-6 shifts monocyte differentiation from DC to macrophages by enhancing M-CSF consummation (9, 10). We then evaluated whether IFNγ may control the production of IL-6 by monocyte-differentiating DC. Human monocytes were maintained in GM-CSF + IL-4 containing or not IFNγ and IL-6 was quantified in the supernatants during the differentiation process. Monocytes in GM-CSF + IL-4 produce IL-6 (Fig. 2C). Maximal IL-6 levels are obtained at day 1 and then decline time-dependently (Fig. 2C). Addition of IFNγ upregulates IL-6 production in a time- (Fig. 2C) and dose-dependent manner (with a maximum obtained using 50 ng/ml) (Fig. 2D). IL-6 mRNA expression is induced by culturing monocytes in GM-CSF + IL-4 and is upregulated by IFNγ (with an effect detectable 2 hours after stimulation) (Fig. 3). Analysis of the respective roles of IL-4 and IFNγ on IL-6 production shows that GM-CSF induces IL-6 production by monocytes and that IL-4 and IFNγ down and upregulates GM-CSF-induced IL-6 production, respectively (Fig. 2D). In contrast to M-CSF, no additive effect of IL-4 + IFNγ on IL-6 production is observed as IL-4 partly prevents IFNγ-induced IL-6 production (Fig. 2D). A previous study reported that soluble IL-6 receptor (gp80) cooperates with IL-6 and M-CSF in switching monocyte differentiation to macrophages (10). We observed that IFNγ
only slightly upregulated gp80 production with a maximum occurring at day 3 (0.26 ± 0.05 and 0.35 ± 0.06 ng/ml, in the absence or presence of IFNγ respectively; mean ± sd, n=4). Lastly, IL-10 has also been shown to shift monocyte differentiation into macrophages (11). We failed in detecting IL-10 production (data not shown) nor an upregulation of IL-10 mRNA expression upon treatment with IFNγ (Fig. 3). Together, these data show that IFNγ upregulates IL-6 production by monocytes in GM-CSF + IL-4 by acting, at least partly, at the transcriptional level.

**IFNγ downregulates CD115 cell surface expression on monocytes cultured in GM-CSF + IL-4.**

M-CSF consummation is associated with CD115 internalization (29). We then evaluated whether the addition of IFNγ to monocytes cultured in GM-CSF + IL-4 results in a modulation of CD115 expression. Monocytes in GM-CSF + IL-4 express CD115 and this expression remains stable during the differentiation process (Ref. 10, 28 & Fig. 2E). The presence of IFNγ in GM-CSF + IL-4 results in a decrease in membrane CD115 expression, observed at day 1 and maximal at day 3 (Fig. 2E). CD115 expression returns to basal level at day 5 (Fig. 2E). Analysis of CD115 expression in permeabilized cells shows similar levels of expression on monocytes cultured in GM-CSF + IL-4 with or without IFNγ (Fig. 2E), thereby suggesting an internalization of CD115 by IFNγ-treated cells. In accordance with these data, a decrease in M-CSF concentrations in the day 3 supernatants of monocytes in GM-CSF + IL-4 + IFNγ is observed (Fig. 2A). Moreover, IFNγ slightly enhances CD115 mRNA expression on monocytes in GM-CSF + IL-4 (Fig. 3). Together, these data suggest that the upregulation of M-CSF and IL-6 production by monocytes induced by IFNγ is associated to an autocrine enhancement of M-CSF consummation.

**Both M-CSF and IL-6 are involved in the effect of IFNγ on monocyte differentiation into DC.**

In neutralizing experiments, we evaluated whether autocrine IL-6 and M-CSF production induced by IFNγ was involved in its ability to skew monocyte differentiation from DC to macrophages. Monocytes in GM-CSF + IL-4 were treated or not with IFNγ in the absence or presence of neutralizing anti-M-CSF and/or anti-IL-6 mAbs. At day 5, cells were stimulated with LPS for 24 hours before to analyze CD83 expression. Results show that IFNγ-treated cells partly recover the ability to acquire CD83 expression in the presence of neutralizing mAbs (61% ± 10; mean ± sd, n=4) (Fig. 2F). When anti-M-CSF mAb or anti-IL-6 mAb were used alone, CD83 expression was also slightly recovered (35% ± 8 and 29 % 5, respectively) (Fig. 2F), thereby suggesting that both cytokines participate to the effect of IFNγ on monocyte differentiation. No significant effect was observed with control mAbs (Fig. 2F). As control, the neutralizing mAbs do
not affect IL-4 + GM-CSF-induced monocyte differentiation into DC (Fig. 2F). Together, these data show that IFNγ shifts differentiating DC towards macrophages at least partly by upregulating autocrine of M-CSF and IL-6.

**Immature DC do not produce M-CSF nor differentiate into macrophages in response to IFNγ.**

We then evaluated whether IFNγ may reconvert immature DC towards macrophages. IFNγ has been reported to polarize immature DC into DC1 that produce high levels of IL-12 and present potent costimulatory properties upon stimulation (17). In accordance with these data, we show that immature DC exposed to IFNγ retain a phenotype of CD1a⁺ CD14⁻ DC (Table III). Moreover, upon LPS stimulation, immature DC exposed to IFNγ acquire veils (data not shown), neoexpress CD83 and present potent T cell costimulatory properties (Table III).

We tested whether IFNγ may control M-CSF and IL-6 production by immature DC. IFNγ does not modulate M-CSF production (Fig. 4A) nor mRNA expression (data not shown) by immature DC. Kinetic experiments show that the ability of monocytes cultured in GM-CSF + IL-4 to produce M-CSF in response to IFNγ is maximal at day 1 and decreases time-dependently during the differentiation process (Fig. 4A). In contrast, immature DC retain the ability to produce IL-6 in response to IFNγ (Fig. 4B). These data show that the regulation of M-CSF production in response to IFNγ is tightly regulated during the differentiation process from monocytes to DC.

We evaluated whether IFNγ may reconvert immature DC to macrophages when exogenous M-CSF is added. In the presence of a high concentration of M-CSF (100 ng/ml), IFNγ-treated immature DC in GM-CSF + IL-4 retain a DC phenotype: they express CD1a but not CD14 (Table III), present a morphology of immature DC (data not shown) and, upon LPS stimulation, acquire CD83 expression (Table III) and veils (data not shown). In agreement with other (10), they also present a decrease in cell surface CD115 expression (Table III), thereby suggesting a consumption of M-CSF by immature DC. Finally, even in the presence of exogenous M-CSF and IL-6, immature DC in GM-CSF + IL-4 retain a DC phenotype (Table III). As a positive control (30), upon GM-CSF and IL-4 removal, immature DC acquire CD14 expression and convert towards adherent macrophages (Table III). Thus, these data suggest that, in the presence of GM-CSF + IL-4, immature DC may express functional CD115 but are resistant to IFNγ-, IL-6- and M-CSF-induced macrophage differentiation.
Discussion

IFNγ produced by NK or activated T cells participates to the control of the innate and adaptive phases of the immune response (14). IFNγ exerts most of its effects on APCs: it activates macrophages and polarizes immature DC into Th1-cell promoting effector DC (14, 16, 17). We show here for the first time that IFNγ also participates to the control of APC differentiation: IFNγ switches monocyte differentiation to activated macrophages at least in part via autocrine activation by IL-6 and M-CSF.

M-CSF is a major macrophage differentiation factor (1) that skews CD34+ progenitor and monocyte differentiation from DC to macrophages (9, 10, 30). By enhancing functional M-CSF receptor expression and M-CSF consummation (10), IL-6 cooperates with M-CSF in this process (9, 10). In agreement with these data, we report that IFNγ switches monocyte differentiation into macrophages via an upregulation of autocrine IL-6 and M-CSF production.

GM-CSF induces M-CSF production by monocytes (10, 28) in a dose-dependent manner with a maximum at 100 ng/ml (data not shown). In our experimental conditions, the concentrations of M-CSF present in the supernatants of monocytes in GM-CSF + IL-4 were lower than those reported by others (10, 28). The use of 20 ng/ml instead of 100 ng/ml GM-CSF in our study may explain this difference.

We also show that monocytes treated with IL-4 plus GM-CSF produce IL-6. Levels of IL-6 induced by IL-4 plus GM-CSF were lower than those induced by GM-CSF alone. Thus, it appears that IL-4 inhibits GM-CSF-induced M-CSF (28) and IL-6 production. It is therefore tempting to speculate that the inhibitory effect of IL-4 on M-CSF and IL-6 production, may contribute to explain its DC-differentiation factor property.

Although IFNγ antagonizes many IL-4-mediated responses (27), it has been also previously observed that IL-4 and IFNγ act synergistically to enhance MR-dependent phagocytosis (31) and CD23 expression on macrophages (32). We report that whereas IFNγ upregulates IL-6 and M-CSF production, IL-4 downregulates their production. However, when used together, they mutually inhibit their respective effects on IL-6 production and have an additive effect on the upregulation of M-CSF production. Together, these observations suggest a complex dialog between IL-4 and IFNγ not limited to antagonistic effects.

IFNγ antagonizes many physiological responses mediated by IL-4, by acting at posttranscriptional (27) or transcriptional levels. IFNγ and IL-4 activate Stat1 and Stat6, respectively (33, 34). IFNγ inhibits IL-4-mediated Stat6 activation by
inducing the expression of SOCS1 (35). The antagonist effect of IFNγ on IL-4-induced inhibition of IL-6 production could be related to the SOCS1-mediated Stat6 inhibition. Moreover, expression of IL-6 gene is under the control of transcription factors, including AP-1, NF-κB and NF-IL-6 (36). Whereas NF-IL6 is strongly inhibited by IL-4 (37), it is poorly affected by IFNγ (38). Consequently, the ability of IL-4 to down-regulate IFNγ-induced IL-6 production could be related to the strong inhibition of NF-IL6 which is mediated by IL-4 and not counteracted by IFNγ.

In accordance with data showing a differential regulation of M-CSF and IL-6 gene expression in monocytes (39), we report that IFNγ + IL-4 have an additive effect of M-CSF production. The role of members of the Stat family in the regulation of M-CSF expression is poorly documented. Nevertheless, we could hypothesize that the down-regulation of IL-4-induced activation of some members of the Stat family mediated by IFNγ may contribute to explain this effect. In addition, the increase of M-CSF production induced by IL-4 + IFNγ could be also indirect. M-CSF increases M-CSF mRNA expression by GM-CSF-activated monocytes (40). M-CSF receptor gene expression is controlled by transcription factors including NF-κB, AP-1 and PU-1. PU-1 is activated by IFNγ (41) and interacts with the interferon regulatory factor 4 (IRF4) which expression is increased by IL-4 (42). Thus, the additive effect of IFNγ and IL-4 on M-CSF production could be consecutive to an increase of M-CSF receptor transcription. Additional experiments are required to determine the effects of IL-4 + IFNγ on the expression of the transcription factors involved in IL-6 and M-CSF production.

DC and macrophages have different roles in the immune response. While DC initiate specific immune responses, macrophages and especially IFNγ-activated macrophages exhibit potent bactericidal and antitumoral activities (14). When added to differentiated uncommitted immature DC, IFNγ polarizes them into Th1 cell-promoting effector DC (17). Th1 cells produce IFNγ, IL-2 and TNFβ and evoke cell-mediated immunity and phagocyte-dependent inflammation.

In addition to acting on mature APC functions, IFNγ may also act on DC/macrophage precursors to favor their differentiation into macrophages. Interestingly, IFNγ acts mainly at the initial stages of monocyte differentiation. The ability of cells to produce M-CSF in response to IFNγ varies with the status of differentiation. In contrast to monocytes, immature DC express CD115 but do not revert to macrophages upon contact with IL-6, M-CSF or IFNγ. The mechanisms underlying this resistance remain to be identified. These data also suggest that the effects of IFNγ are tightly
controlled according to the status of cell differentiation. Lastly, in agreement with data showing that environmental cytokines tightly control monocyte differentiation into immature DC and macrophages and the inter-conversion into one another (4, 30), we observed that the removal of IFNγ from the culture at the early stages of differentiation partly prevents its blocking effect on DC differentiation. IL-10 also shifts monocyte differentiation from DC to macrophages (11). IL-10 and IFNγ give rise to macrophage-like cells with divergent antigen presentation and endocytosis properties (43). These observations suggest that monocyte differentiation and the function of the cells generated are also tightly controlled by the nature of cytokines encountered.

DC are the only APCs that prime naive T cells and initiate specific immune responses (12). Consequently, they have a central role in vaccine strategies and especially in anti-tumor immunotherapies (44, 45). Tumor-specific vaccination using antigen-loaded autologous DC has reached the stage of human clinical trials. These studies have been made possible by the development of methods for obtaining large number of DC. Most of these studies have been carried out with \textit{ex vivo} generated monocyte-derived DC (44, 45). Thus, to identify factors that control monocyte differentiation is crucial in order to optimize DC generation. Our results show that IFNγ skews monocyte differentiation from DC to macrophages, suggesting that the presence of IFNγ-producing cells together with monocytes could interfere with the differentiation process into DC. Finally, in agreement with others, we report that IFNγ-stimulated macrophages present undetectable or low levels of markers that are usually considered as macrophage markers (RFD7, CD14 and MR). This observation points out that the expression of CD1a and/or CD14 is sometimes not sufficient to clearly distinguish between monocyte-derived macrophages or DC and that the study of additive morphologic, phenotypic and functional parameters is required.

In conclusion, our data show that, in addition to a direct effect on APC functions, IFNγ skews monocyte differentiation from DC to macrophages and thereby evidences a new mechanisms by which IFNγ may control the outcome of the immune response.

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### Table I: Characteristics of monocytes cultured for 5 days with GM-CSF plus IL-4, GM-CSF or M-CSF in the absence or presence of IFNγ.

<table>
<thead>
<tr>
<th>IFNγ</th>
<th>GM-CSF + IL-4</th>
<th>GM-CSF</th>
<th>M-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD1a</td>
<td>320</td>
<td>70</td>
<td>36</td>
</tr>
<tr>
<td>CD14</td>
<td>5</td>
<td>8</td>
<td>590</td>
</tr>
<tr>
<td>CD64</td>
<td>4</td>
<td>112</td>
<td>58</td>
</tr>
<tr>
<td>CD86</td>
<td>8</td>
<td>76</td>
<td>18</td>
</tr>
<tr>
<td>CD83</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>CD83*</td>
<td>130</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>MHC-I</td>
<td>335</td>
<td>1112</td>
<td>744</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>539</td>
<td>1115</td>
<td>319</td>
</tr>
<tr>
<td>RFD7</td>
<td>3</td>
<td>3</td>
<td>290</td>
</tr>
<tr>
<td>MR</td>
<td>271</td>
<td>175</td>
<td>160</td>
</tr>
<tr>
<td><strong>Endocytic Activity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dextran</td>
<td>395</td>
<td>142</td>
<td>131</td>
</tr>
<tr>
<td>S. aureus</td>
<td>34%</td>
<td>7%</td>
<td>nd</td>
</tr>
<tr>
<td>Latex beads</td>
<td>0%</td>
<td>3%</td>
<td>nd</td>
</tr>
<tr>
<td><strong>Enzymatic Activity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonspecific esterase</td>
<td>-</td>
<td>+/-</td>
<td>nd</td>
</tr>
<tr>
<td><strong>Morphology</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non adherent Round/ovoid cells</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Network of elongated &amp; adherent cells</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Non adherent Round/ovoid cells*</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Network of elongated &amp; adherent cells*</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Ovoid &amp; veils*</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Monocytes were cultured in medium containing GM-CSF + IL-4, GM-CSF or M-CSF in the absence or presence of 25 ng/ml IFNγ. After 5 days, the phenotype and the uptake of FITC-labeled dextran, *Staphylococcus aureus* or latex beads was analyzed by FACS and cell morphology was determined by microscopy. Results are expressed in MFI values excepted the uptake of *Staphylococcus aureus* and latex beads expressed in percentage of positive cells. Results are representative of one out of five experiments. * CD83 expression and cell morphology were analyzed after 24 h.
incubation with 20 ng/ml LPS. nd means not done. – , +/-, + and ++ respectively mean absent, slightly detectable, detectable and strongly evidenced.

**Table II**: IFNγ prevents murine progenitor differentiation into DC.

<table>
<thead>
<tr>
<th>Surface markers</th>
<th>None</th>
<th>IFNγ</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b</td>
<td>857 ± 75</td>
<td>320 ± 95</td>
</tr>
<tr>
<td>CD11c</td>
<td>536 ± 45</td>
<td>4 ± 0.2</td>
</tr>
<tr>
<td>CD86</td>
<td>136 ± 23</td>
<td>33 ± 9</td>
</tr>
<tr>
<td>F4/80</td>
<td>140 ± 19</td>
<td>60 ± 10</td>
</tr>
<tr>
<td>IA b</td>
<td>±49% ± 10</td>
<td>978 ± 83</td>
</tr>
<tr>
<td></td>
<td>±61% ± 22</td>
<td>59 ± 13</td>
</tr>
</tbody>
</table>

| Allogenic MLR   | 41 ± 9     | 4 ± 0.8    |

Bone marrow cells from C57BL/6 mice were cultured in CM containing 5 ng/ml GM-CSF in the absence or presence of 25 ng/ml IFNγ. After five days, phenotype was analyzed by FACS. In the absence or presence of IFNγ, cells were gated on CD11c-positive or on Gr1-negative cells, respectively, as described in the Methods section. For all the markers, excepted for IA b, homogeneous populations were obtained and results are expressed in MFI values (mean ± s.d., n=4). a For IA b, two populations expressing high and low levels of IA b were observed. The percentage of cells in each population (left values; mean% ± s.d., n=4) and the MFI values of each population (right values; mean ± s.d., n=4) are mentioned. At day 5, cells were used as stimulatory cells in primary allogenic MLR. Results are expressed in cpm x 10⁻³ as mean ± s.d. of quadruplicate values and are representative of one out of three experiments.
<table>
<thead>
<tr>
<th></th>
<th>No cytokine</th>
<th>GM-CSF + IL-4</th>
<th>GM-CSF + IL-4</th>
<th>GM-CSF + IL-4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>IFN-γ</td>
<td>IFN-γ + M-CSF</td>
</tr>
<tr>
<td>CD115&lt;sup&gt;a&lt;/sup&gt;</td>
<td>nd</td>
<td>76 ± 10</td>
<td>83 ± 13</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>CD1a&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11 ± 2</td>
<td>310 ± 35</td>
<td>298 ± 29</td>
<td>358 ± 42</td>
</tr>
<tr>
<td>CD14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>56 ± 9</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>CD83&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt; 5%</td>
<td>99%</td>
<td>98%</td>
<td>99%</td>
</tr>
<tr>
<td>MLR&lt;sup&gt;d&lt;/sup&gt;</td>
<td>nd</td>
<td>2 ± 0.1</td>
<td>5 ± 0.8</td>
<td>nd</td>
</tr>
<tr>
<td>MLR&lt;sup&gt;e&lt;/sup&gt;</td>
<td>nd</td>
<td>22 ± 2</td>
<td>24 ± 3</td>
<td>nd</td>
</tr>
</tbody>
</table>

Day 5 immature DC were cultured in the absence of cytokine or in GM-CSF + IL-4 in the absence or presence of 25 ng/ml IFN-γ, 25 ng/ml IFN-γ plus 100 ng/ml M-CSF or 100 ng/ml M-CSF plus 100 ng/ml IL-6. <sup>a</sup> After 1 day, membrane CD115 expression was analyzed by FACS. <sup>b</sup> After 3 days, CD1a and CD14 expression were analyzed. <sup>a, b</sup> Results are expressed in MFI values. <sup>c</sup> After 3 days, cells were further stimulated for 24 hours with LPS and CD83 was analyzed by FACS. Results are expressed in percentage of positive cells. <sup>a, c</sup> All the data are expressed as mean ± s.d. of four separate experiments. <sup>d, e</sup> After 2 days, cells were not (d) or were (e) stimulated for 24 hours with LPS before to be used as stimulatory cells in primary allogenic MLR. Results are expressed in cpm x 10<sup>-3</sup> as mean ± s.d. of quadruplicate values and are representative of one out of three experiments. nd means not done.
Figure Legends

**Figure 1. IFN\(\gamma\) shifts monocyte differentiation from DC to macrophages.** A, Monocytes were cultured in medium containing M-CSF (circle) or GM-CSF + IL-4 (triangle) in the absence (black labels) or presence (white labels) of 25 ng/ml IFN\(\gamma\). After 5 days, cells were (right panel) or were not (left panel) stimulated for 24 hours with 20 ng/ml LPS. Cells were then irradiated and used to stimulate allogenic T cells. Results are expressed in cpm x 10\(^{-3}\) as mean ± s.d. of quintuplicate values. Results are representative of one out of three experiments. B, Monocytes were cultured in GM-CSF + IL-4 in the absence (left panel) or presence of 25 ng/ml IFN\(\gamma\) (right panel). After 5 days, cells were (lower panel) or were not (upper panel) stimulated for 24 hours with 20 ng/ml LPS and observed by microscopy (magnification x 100).

**Figure 2. IFN\(\gamma\) upregulates M-CSF and IL-6 production by IL-4 plus GM-CSF-stimulated monocytes.** A&C, Monocytes in GM-CSF + IL-4 were (white bars) or were not (black bars) stimulated with 25 ng/ml IFN\(\gamma\) at day 0 and M-CSF (A) and IL-6 (C) were quantified in the cell-free supernatants from day 1 to day 5. B&D, Monocytes were cultured in GM-CSF (grey bars) or GM-CSF + IL-4 (white bars) in the absence or presence of 1 to 50 ng/ml IFN\(\gamma\) added at day 0 and M-CSF (B) and IL-6 (D) were quantified in the 24 h supernatants (day 1). As control, monocytes were cultured in medium without cytokine (dotted bars). A-D, Results are expressed in µg/ml as mean ± s.d. of four experiments. E, Monocytes in GM-CSF + IL-4 were (口) or were not (■) stimulated with 25 ng/ml IFN\(\gamma\). At day 1, 3 and 5, membrane CD115 expression was analyzed by FACS. At day 1, intracellular CD115 expression was analyzed after cell permeabilization. Results are expressed in MFI (after subtraction of the MFI obtained with the control mAb) as mean ± s.d. of 4 separate experiments. F, Monocytes in GM-CSF + IL-4 were not (■) or were (口) stimulated with 25 ng/ml IFN\(\gamma\) in the absence or presence of neutralizing anti-M-CSF + -IL-6 mAbs or of isotype control mAbs. After 5 days, cells were stimulated with 20 ng/ml LPS and CD83 expression was analyzed by FACS. Results are expressed in MFI values as mean ± s.d., n=3.

**Figure 3. IFN\(\gamma\) enhances M-CSF and IL-6 mRNA expression by monocytes in GM-CSF + IL-4.** Freshly isolated monocytes were not (none) or were cultured in GM-CSF + IL-4 in the absence or presence of 25 ng/ml IFN\(\gamma\). After 2, 4, 8 and 16 hours, the expression of the mRNA encoding M-CSF, IL-6, IL-10, CD115 and GAPDH was analyzed by RT-PCR.
Figure 4. IFNγ does not modulate M-CSF production by immature DC. A&B, Day 5 immature DC were maintained in GM-CSF + IL-4 and 25 ng/ml IFNγ was not (black labels) or was (white labels) added at day 0, 2 or 4. After 24 hours, M-CSF (A) and IL-6 (B) were quantified in the supernatants. Results are expressed in µg/ml (mean ± s.d., n=4).
Figure 1

A

B

Non treated

LPS treated

GM-CSF + IL-4

Non

LPS

GM-CSF + IL-4 + IFN
Figure 2
Figure 3

[Image of a gel showing protein expression levels for different conditions: IFN, M-CSF, IL-6, IL-10, CD115, and GAPDH. The conditions are marked with a '-' or '+'.]
Figure 4
Interferon-γ switches monocyte differentiation from dendritic cells to macrophages

Yves Delneste, Peggy Charbonnier, Nathalie Herbault, Giovanni Magistrelli, Gersende Caron, Jean-Yves Bonnefoy and Pascale Jeannin

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