IFN-γ, as Secreted during an Alloresponse, Induces Differentiation of Monocytes into Tolerogenic Dendritic Cells, Resulting in FoxP3+ Regulatory T Cell Promotion

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Assia Eljaafari, Yin-Ping Li, and Pierre Miossec

IFN-γ has been shown to inhibit monocyte (Mo) differentiation into mature dendritic cells (DC). Because IFN-γ also plays a role in tolerance induction, we asked whether this could be related to generation of tolerogenic DC (Tol-DC). Toward this aim, we cultured Mo with GM-CSF plus IL-4 in the presence or absence of IFN-γ for 6 days and induced their maturation with TNF-α for 2 additional days. We showed that IFN-γ deviated Mo differentiation from mature DC toward Tol-DC. Indeed, IFN-γ-generated DC 1) expressed moderate levels of costimulatory molecules, but high levels of Langerin and CD123 molecules, 2) were maturation resistant, and 3) were unable to efficiently present alloantigen to T cells. More interestingly, naïve CD4+ T cells primed with IFN-γ-generated DC expressed FoxP3 mRNA at high levels and exerted regulatory functions upon secondary stimulation with alloantigen. To address whether endogenously secreted IFN-γ mediates a similar effect, we used the alloreaction as a model. We showed that cell-free supernatant harvested from an HLA-mismatched, but not HLA-identical, alloresponse induced differentiation of Mo into Tol-DC able to promote regulatory T cell generation. Moreover, when supplemented with GM-CSF plus IL-4, HLA-mismatched cell-free supernatant inhibited differentiation of Mo into mature DC. Finally, by adding Abs directed against inflammatory cytokines, we demonstrated that IFN-γ plays a preponderant role in this inhibition. In conclusion, our results clearly demonstrate that exogenous or endogenous IFN-γ, as well, induces differentiation of Mo toward Tol-DC, which results in FoxP3+ regulatory T cell promotion. The Journal of Immunology, 2009, 183: 2932–2945.

Interferon-γ has recently been implicated in tolerance induction either 1) by acting as an inducer of Th2 responses, which counteracts the activity of Th1, or 2) by allowing regulatory T cell (Treg) function and/or expansion, or 3) by inducing the tolerogenic activity of APC, notably through increased expression of inducible NO synthase and/or induction of the immunomodulatory IDO enzyme activity (1–4). Moreover, the positive effect of IFN-γ on tolerance induction has also been demonstrated in vivo by using animal models. Thus, in a skin transplant model, ex vivo exposure of CD4+ T cells to allogeneic bone marrow-derived dendritic cells (DC) cultured in the presence of IFN-γ has been shown to result in the promotion of a Treg population that prevents allograft rejection (5). In another study, it has been shown that administration of IFN-γ plus M-CSF-mediated macrophages promotes the clinical and histological resolution of experimental chronic colitis, with enrichment in CD25+FoxP3+ T cells (6).

Delneste et al. have previously reported that IFN-γ inhibits commitment of monocyte (Mo) into DC (7). Indeed, cells treated with IFN-γ plus GM-CSF plus IL-4 (GM/IL-4) expressed high levels of CD64, CD86, and HLA molecules. Upon maturation, those cells did not acquire CD83 expression and were unable to up-regulate HLA class II, CD80, CD86, and CD40 expression. Moreover, these cells displayed reduced T cell costimulatory properties and expressed no IL-12 mRNA. Although it was proposed in that study that IFN-γ induces a switch of Mo differentiation toward macrophages, the accurate analysis of their results together with the help of recent knowledge on DC plasticity suggest that those cells were not macrophages, but rather maturation-resistant DC. Maturation-resistant DC have been indeed defined as tolerogenic dendritic cells (Tol-DC), as reported by Morelli and Thomson (8). Those DC share common characteristics, such as moderate or high expression of surface MHC molecules, in association with a low ratio of costimulatory to inhibitory signal and an impaired ability to secrete Th1 cell-driving cytokines, such as IL-12.

Because maturation-resistant DC exert tolerogenic functions on the one hand, and because IFN-γ leads to tolerance induction in some experimental models on the other hand, we investigated whether IFN-γ by itself could induce commitment of GM/IL-4-treated Mo into Tol-DC. Our results demonstrated that priming naïve T cells with such DC resulted in the generation of FoxP3+ Treg. Moreover, use of alloreaction as a model led us to demonstrate that endogenously secreted IFN-γ plays an equivalent role.

Materials and Methods

Generation of DC

Peripheral blood was harvested from healthy volunteers, and mononuclear cells were isolated by Ficoll-Paque (1.077 g/ml) density gradient centrifugation. Mo were purified by using the MACS CD14 positive selection kit (StemCell Technologies), resulting in >90% purity. Purified Mo were cultured at a concentration of 1 × 10^6 cells/ml in 3 ml of RPMI 1640 in 6-well plates, supplied with 10% AB serum, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, and 1.5 mg/ml NaHCO₃, GM-CSF (200 U/ml) and IL-4 (500 U/ml) were used, when indicated. Medium was changed by...
removing 1.5 ml of medium and adding back 1.5 ml of fresh medium with cytokines at day 2 and day 5. At day 6, cells were transferred into Teflon wells and cultured at a density of 0.5 × 10^6 cells/well in RPMI 1640 with TNF-α (200 U/ml) for another 2 days to induce maturation.

**Production of HLA-mismatched and HLA-identical MLR cell-free supernatant (CFS)**

PBMC collected from healthy volunteer donors were irradiated at 30 Gy and used as stimulating cells. PBMC (1 × 10^6) collected from HLA-DR-mismatched or HLA-identical MLR sibling donors were used as responder cells and cultured with 1 × 10^5 stimulating cells in a 2-ml final volume for 5 days. Supernatants from four to five different pairs were pooled, filtrated, and supplemented with 10% AB serum, l-glutamine, and antibiotics. Then, pH was adjusted to 7.4 with NaHCO₃.

**FACS analysis**

At day 6 and day 8, cells were stained with FITC- or PE-conjugated mouse mAbs. The following Abs were used: anti-CD1a, anti-CD14, anti-CD16, anti-CD80, anti-CD86, anti-CD83, anti-DR, anti-CD40, anti-CD54, anti-CD32, and anti-CD64, as well as relevant isotypic controls.

For intracellular detection of IFN-γ-producing T cells, purified CD3⁺ T lymphocytes were harvested at day 5 of a primary MLR, washed in PBS, and suspended in fresh RPMI 1640 medium supplemented with 10% FBS at the concentration of 5 × 10^5 cells/ml. They were then stimulated with 50 ng/ml PMA and 1 μM ionomycin for 6 h at 37°C. For the last 4 h, GolgiStop (BD Pharmingen) was added at a concentration of 2 μM to block cytokine secretion. Cells were washed and stained with anti-CD25, anti-CD28, and anti-CD4 mAbs (BD Pharmingen) for 20 min at 4°C. After further washing, cells were stained with anti-IFN-γ Ab (BD Pharmingen) for 30 min at room temperature. At least 1 × 10^6 cells were analyzed in live gate with FACSScan (BD Medical Systems). For intracellular staining of FoxP3, lymphocytes were washed and suspended in PBS, and membrane staining of CD4 was performed. Intracellular staining (CliniSciences) was performed, as described above for IFN-γ staining. Analyses were performed using FACSanto and Partec Software (Dako).

**Endocytosis**

Endocytosis of cells was measured by the cellular uptake of FITC-dextran. Briefly, cells were collected and suspended in Dulbecco’s PBS; they were preincubated for 15 min at 37°C or 4°C, as negative control. Then, they were incubated with 1 mg/ml FITC-dextran for 30 min at the same temperature conditions. Cold PBS was added to stop endocytosis, and cells were then washed twice. When needed, cells were fixed with 1% formalin. The quantitative uptake of FITC-dextran by cells was analyzed by FACS.

**Allogenic MLR assay**

**Primary MLR.** Naive CD4⁺ CD45RA⁻ T cells (10^6/ml) were purified with anti-CD4/CD45⁺ magnetic beads (Miltenyi Biotec) and stimulated with irradiated (30 Gy) DC (10^6/ml) for 5 days, in 24-well plates, in RPMI 1640 supplemented with 10% AB serum, antibiotics, and l-glutamine.

**Secondary MLR.** On day 5, lymphocytes were washed, purified with the help of anti-CD3 magnetic beads (Miltenyi Biotec). Lymphocytes (1 × 10^6) were stimulated with 10^5 irradiated mononuclear cells harvested from the same donor as the DC used for priming (APC) or from a third-party blood donor (APC). T cells were cultured for 3 days in complete RPMI 1640 medium with 10% human AB serum. Thymidine incorporation was then measured following an 18-h pulse with 1 μCi of [³H]thymidine.

**Measurement of the regulatory function of alloreactive T cells**

Primary MLR were performed by stimulating 10^6 CD4⁺ CD45RA⁻ naive T cells with 10^6 irradiated DC. Alloreactive CD3⁺ T cells collected from primary MLR were purified with anti-CD3 magnetic beads and were added to unprimed naive T cells, at the concentrations of 1 primed T cell for 2 unprimed T cells (1:2), 1:8, or 1:32. Then, cells were cultured in microtiter plates for 5 days, and thymidine incorporation was measured following an 18-h pulse with 1 μCi of [³H]thymidine.

Where specified, T cell proliferation was also measured by the cell surface stain CFSE. Alloreactive T lymphocytes (5 × 10^6/ml) were stained with 5 μM CFSE. After 10 min, cells were washed twice in PBS with 10% FCS. After 5 days, cells were harvested before acquisition on FACScan.

**Real-time RT-PCR**

Total RNA was then extracted from 1 × 10^6 total cells using TRIzol reagent (Invitrogen). One microgram of RNA was reverse-transcribed using the ThermoScript RT-PCR system (Invitrogen), and PCR amplification was performed using a LightCycler instrument with a FastStart DNA Master SYBR Green I real-time RT-PCR kit (Roche Molecular Biochemicals). Specific amplification of genes of interest (i.e., FoxP3, IL-10, IL-2, TGF-β, CD3ε, cyclophilin B (CPB)) was performed using the LightCycler primer set kits, according to the manufacturer’s instructions (Roche Molecular Biochemicals). CPB was used as a housekeeping gene, as previously reported (9). Thermocycling was performed with 45 cycles of amplification (10 s at 95°C, 10 s at 68°C, and 16 s at 72°C). The levels of expression of each mRNA and their estimated crossing points were determined relative to the standard preparation using the LightCycler computer software. Results are based on the ratio of genes of interest to CPB or to CD3ε mRNA amplification. CD3ε was used to normalize the concentration ratios of mRNA expressed by T cells, such as FoxP3, IL-2, and IFN-γ. Indeed, as previously reported by others (10), we observed that CD3ε did not increase upon stimulation of T cells with PHA, CD3 plus CD28, or allomixtum.

**IL-10 and TGFβ mRNA levels were normalized to CPB, because these two genes are expressed by T cells and APC, as well.**

**Statistical analysis**

Data were expressed as means ± SD. The two-tailed Student’s t test was performed to compare two or more mean values. A probability of null hypothesis <0.05 (p ≤ 0.05) was considered statistically significant (indicated by *).

**Results**

**GM/IL-4 plus IFN-γ-generated DC express a maturation-resistant phenotypic profile**

To address whether IFN-γ induces differentiation of DC into ToltDC, we cultured Mo in the presence of GM/IL-4 with or without IFN-γ for 6 days. We then exposed cells for 2 additional days to TNF-α to test their capacity to mature. As shown in Fig. 1A, GM/IL-4-treated Mo differentiated into classical CD14low/CD16 CD123low/CD14low/CD16 low. Following TNF-α treatment, almost no maturation marker appeared, and costimulatory molecules were higher than those of GM/IL-4-DC. Following TNF-α exposure (Fig. 1C), the phenotypic profile of cells cultured with GM/IL-4 plus IFN-γ was quite different. Indeed, at day 6, they were CD14low CD16 DC-SIGN but CD1a⁻. Moreover, they expressed at high levels CD123 and Langerin molecules, two markers of plasmacytoid DC and Langherhans DC, respectively (Fig. 1A). Moreover, their levels of expression CD86, HLA-DR, CD54 and CD40 molecules were higher than those of GM/IL-4-DC. Following TNF-α treatment, almost no maturation marker appeared, and costimulatory molecule expression levels did not increase (Fig. 1B). In correlation with this maturation profile, their capacity to endocytose dextran particles was profoundly downregulated following TNF-α exposure (Fig. 1C).

**GM/IL-4 plus IFN-γ DC exert tolerogenic functions**

To address whether these cells exerted a tolerogenic function, we primed naive T cells with GM/IL-4 plus IFN-γ-DC or GM/IL-4-DC during 5 days, washed cells extensively, purified CD3⁺ T cells with magnetic beads, and stimulated them with APC from the same donor as DC, or from a third party donor, for 3 additional days. As shown in Fig. 2A, T cells primed with GM/IL-4 plus IFN-γ-DC were weakly responsive to allogeneic APC harvested from the same donor as the DC used for priming. Following
secondary stimulation, they expressed higher levels of FoxP3 and IL-10, but lower levels of IL-2 mRNA, than did T cells primed with GM/IL-4-DC. This cytokine profile was more specifically triggered by donor APC than by third-party APC. TGFβ was expressed at levels almost similar to those induced by GM/IL-4-DC (Fig. 2B). Consistent with this tolerogenic mRNA pattern, T cells primed with GM/IL-4 plus IFN-γ-DC exerted potent regulatory functions on unprimed alloreactive T cells (Fig. 2C). This regulatory function, however, was not specific for the primary donor alloantigen.

Thus, these results indicate that GM-IL-4 plus IFN-γ-DC promote regulatory functions on primed naive T cells.

**Mismatched MLR cell-free supernatant induces differentiation of Mo into maturation-resistant DC**

We have previously reported that the proinflammatory cytokines secreted during an alloresponse activate immature DC and induce their maturation (11). Because IFN-γ was involved in this effect, we then asked whether IFN-γ secreted during the alloresponse could induce Mo differentiation toward either mature or maturation-resistant DC.
FIGURE 2. GM/IL-4 plus IFN-γ DC are tolerogenic. A, CD4⁺ naive T cells were cultured for 5 days with GM/IL-4-DC (black histograms) or GM/IL-4 plus IFN-γ DC (hatched histograms), then purified using CD3 magnetic beads and cultured with mononuclear cells harvested from the same donor as that of DC (APCb), or from a third-party donor (APCc). MLR experiments were performed for 3 days. Results are expressed as [³H]thymidine incorporation (cpm).

B, Expressions of IFN-γ, IL-2, FoxP3, IL-10, and TGFβ mRNA levels were measured 24 h poststimulation and are expressed relative to CD3ε or CPB mRNA copy numbers, as mentioned in Materials and Methods. Results represent means of three independent experiments.

C, Unprimed naive T cells (black histograms) were allostimulated in the presence of CD3⁺ T cells previously primed with mmCFS-DC (hatched histograms) at the ratios of 1:2, 1:8, or 1:32 in MLR experiments. Stimulating APC were harvested from the same donor as that of DC (APCb) or from a third-party donor (APCc). This figure is representative of three independent experiments.
With this aim, Mo were cultured for 6 days either with 1) cell-free supernatant harvested from HLA-mismatched MLR (mmCFS) or 2) from HLA-identical MLR (idCFS), as negative control, or 3) a combination of GM-CSF and IL-4 (GM/IL-4), as positive control. As shown in Fig. 3A and as compared with idCFS, mmCFS induced increased expression of HLA-DR, CD54, CD40, CD80, and CD86 molecules on Mo at levels similar to, or even higher than, those induced by GM/IL-4. However, exposure of DC generated in mmCFS (mmCFS-DC) to TNF-α for 2 additional days did not result in the significant appearance of CD83, a marker of mature DC, or in a significant up-regulation of CD80, CD86, and HLA-DR molecules, as compared with GM/IL-4-DC (Fig. 3B). Additionally, as compared with idCFS-treated cells, CD14 and FcγRs, that is, CD16, CD32, and CD64 molecules, which are classical markers of the Mo/macrophage lineage, were down-regulated but not abrogated (Fig. 4A). Therefore, to investigate whether mmCFS-treated cells should be considered macrophages or DC, expression of CD11c and DC-SIGN molecules was analyzed. As shown in Fig. 4A, CD11c was equally expressed by the three different cell subsets, which did not allow discrimination between macrophages or DC. DC-SIGN, which is a specific marker of DC, was highly expressed by both GM/IL-4-DC and mmCFS-DC but not idCFS-treated cells (Fig. 4A) or Mo (data not shown). Thus, these results strongly suggest that mmCFS induces differentiation of Mo into maturation-resistant DC.

Analysis of mmCFS-DC morphology and cytokine secretion profile

The analysis of the forward and side light scatters in cyt fluorometry reflects size and granularity patterns of cells, respectively. As shown in Fig. 4B, the forward/side light scatter patterns suggested that, at the morphological level, mmCFS-DC were much closer to GM/IL-4-DC than to idCFS-treated Mo.

Because DC are known to secrete IL-10 at the immature state, but IL-12 upon maturation, we then investigated the effect of mmCFS on the expression of these two cytokines at both day 6 and day 8 of culture. As shown in Fig. 4C, GM/IL-4-DC fulfilled criteria of mature DC, while low IL-12 levels but high IL-10 mRNA levels were expressed at both day 6 and day 8 in mmCFS-DC. Therefore, these results support that mmCFS-DC are indeed unable to mature.

Day 8 mmCFS-DC exert endocytic functions but weak allostimulatory capacity

We then analyzed the functions of mmCFS-DC at both day 6 and day 8. During differentiation, the ability to capture Ag is a function shared by Mo and immature DC, but it is lost by mature DC. As expected at day 6, both GM/IL-4-DC and mmCFS-DC were able to endocytose dextran particles (Fig. 5A). However, following exposure to TNF-α, only mmCFS-DC remained able to exert this function, thus supporting their failure to mature.

During maturation, DC are known to increase their capacity to stimulate T cells by up-regulating their Ag-presenting cell function. Thus, to investigate this function, we tested the allostimulatory capacity of day 8 mmCFS-DC in MLR experiments. As shown in Fig. 5B, GM/IL-4-DC induced a strong alloresponse, while the response of T cells stimulated with mmCFS-DC was weak, and even lower than that induced by idCFS-treated Mo. Additionally, T cells primed with mmCFS-DC for 5 days secreted almost no IFN-γ upon secondary stimulation with PMA plus ionomycin. Accordingly, following secondary stimulation, low levels of CD25 and CD28 molecules, two activation markers, were expressed as compared with the high levels in T cells primed with GM/IL-4-DC (Fig. 5C).

Thus, these results indicate that mmCFS-DC exert weak allostimulatory functions but sustained endocytic capacity. mmCFS-DC are tolerogenic

Because maturation-resistant DC are known to exert tolerogenic functions (8), we then investigated whether the inability of mmCFS-DC to stimulate an alloresponse could be due to the induction of a tolerogenic response. Toward this aim, we primed naïve T cells with mmCFS-DC or GM/IL-4-DC during 5 days, washed cells extensively, purified CD3+ T cells with magnetic beads, and stimulated them with APC from the same donor for 3 additional days. As shown in Fig. 6A, we observed that T cells primed with allogeneic mmCFS-DC were induced to express high levels of IL-10 and FoxP3, with low IFN-γ and no IL-2 mRNA levels, following secondary stimulation with APC originated from the same donor as DC. TGFβ was expressed at levels almost similar to those induced by GM/IL-4-DC. Consistent with this tolerogenic mRNA pattern, T cells primed with mmCFS-DC exerted potent regulatory functions on unprimed alloreactive T cells (Fig. 6B). Moreover, ILT3, a receptor that is mainly expressed on tolerogenic APC (12), was up-regulated at day 8 in mmCFS-DC but not in GM/IL-4-DC (Fig. 6C). Thus, our results clearly show that mmCFS induces differentiation of Mo into Tol-DC, which are able to promote Treg.

idCFS-Mo do not induce tolerance

Because idCFS-treated Mo also exerted weak stimulatory functions in MLR experiments, we then asked whether these cells could also exert tolerogenic functions and promote Treg generation. To answer this question, we measured the expression of IL-2, IFN-γ, FoxP3, IL-10, and TGFβ mRNA in T cells initially primed with GM/IL-4-DC, mmCFS-DC, or idCFS-Mo, and secondarily stimulated with allogeneic APC originated from the same donor as Mo or DC. As shown in Fig. 7A, in cells primed with idCFS-Mo, we observed that the levels of mRNA expression of the genes mentioned above were between those of mmCFS-DC and GM/IL-4-DC. Similar results were obtained in MLR experiments (Fig. 7B). However, a clearcut difference was observed when T cells primed with idCFS-Mo, mmCFS-DC, or GM/IL-4-DC were added to unprimed alloreactive T cells in MLR experiments. Indeed, as shown in Fig. 7C, only T cells primed with mmCFS-DC were able to exert a potent regulatory activity on unprimed T cells, as assessed by the blocking of T cell division in CFSE-labeled cells. As expected, GM/IL-4-DC highly improved the alloresponse of unprimed T cells, whereas idCFS-Mo did not induce any significant modification.

Thus, collectively, these results indicate that mmCFS, but not idCFS, induces differentiation of Mo into Tol-DC.

The inability of mmCFS to induce differentiation of Mo into immunocompetent DC is related to the presence of an inhibitory factor

We then asked whether, as for exogenous IFN-γ, mmCFS could inhibit GM/IL-4-mediated mature DC generation. To answer this question, mmCFS was added to GM/IL-4 from the beginning of Mo cultures. Then, cells were treated with TNF-α for 2 additional days to induce their maturation. As assessed by CD80, CD86, and CD83 expression, maturation was induced in GM/IL-4-DC, but not in mmCFS plus GM/IL-4-DC (Fig. 7). Thus, these results suggest that the failure of mmCFS to induce full DC maturation is likely related to the presence of an inhibitory factor in mmCFS.

IFN-γ is the major cytokine responsible for the inhibition of mismatched MLR-CFS-mediated Mo differentiation into mature DC

To investigate whether endogenously secreted IFN-γ contributed to the inhibition of DC maturation, we tried to overcome the inhibitory effect of mmCFS on GM/IL-4-mediated DC differentiation by adding neutralizing Abs directed against IFN-γ. As assessed by increased expression of CD80, CD86, and CD83
FIGURE 3. mmCFS induces generation of maturation-resistant DC from Mo precursors. A, Phenotype of Mo (dotted-line histograms) purified from blood donors and cultured either in the presence of GM/IL-4, mmCFS, or idCFS for 6 days (black histograms) is shown. B, TNF-α was added for 2 additional days. Phenotype of cells cultured for 2 additional days with TNF-α (black histograms) is represented and compared with that of day 6 cells (dotted-line histograms). This figure is representative of four different experiments.
FIGURE 4. mmCFS-DC phenotype, morphology, and cytokine mRNA expression profile. A. Membrane expression of CD14 and FCγR in day 8-cultured cells (black histograms) is represented. Dotted-line histograms represent isotypic control. B. Forward vs side light scatter patterns of day 8-cultured cells are shown and are representative of four different experiments. C. IL-10 and IL-12 mRNA level expression in GM/IL-4-DC (white histograms) or mmCFS-DC (black histograms) was measured by quantitative RT-PCR and expressed as a ratio to CPB mRNA levels. Results represent means of three independent experiments.
FIGURE 5. Functions of day 8 mmCFS-DC. A, GM/IL-4-DC and mmCFS-DC were tested at both day 6 and day 8 for their capacity to endocytose FITC-labeled dextran particles at either 37°C (black histograms) or 4°C, as negative control (dotted-line histograms). This phenotype is representative of two independent experiments. B, The allostimulatory capacity of GM/IL-4-DC (■), mmCFS-DC (x), or idCFS-DC (△) was tested using alloreactive T cells, as responder cells, in MLR experiments. Results are expressed as [3H]thymidine incorporation (cpm) and are representative of three independent experiments. C, T cells primed with GM/IL-4-DC and mmCFS-DC for 5 days were purified using anti-CD3 magnetic beads and stimulated with PMA plus ionomycin for 6 h. Intracellular IFN-γ secretion and CD25 and CD28 membrane expression were measured by cytofluorometry, as described in Materials and Methods.
we observed that anti-IFN-\(\gamma\)/H9253 Ab was able to increase expression of each DC maturation marker, but at lower levels than those observed in control mature DC. The expression of DC-SIGN in cells treated by GM/IL-4, GM/IL-4 plus mmCFS, or GM/IL-4 plus mmCFS plus anti-IFN-\(\gamma\) indicated that these three subsets of cells were indeed DC. Abs directed against other proinflammatory

![Graph A](image)

Figure 6. mmCFS-DC are tolerogenic. A, CD4\(^+\) naive T cells were cultured for 5 days with GM/IL-4-DC (open bars) or mmCFS-DC (filled bars), then purified using CD3 magnetic beads and cultured with mononuclear cells harvested from the same donor as that of DC. Expression of FoxP3, TGF\(\beta\), IL-10, IL-2, and IFN-\(\gamma\) mRNA levels were measured 24 h poststimulation and are expressed relative to CD3e or to CPB mRNA copy numbers, as mentioned in Materials and Methods. B, CD3\(^+\) T cells previously primed with mmCFS-DC were added or not (open bars) to unprimed naive T cells at the ratios of 1:2 (filled bars), 1:8 (hatched bars), or 1:32 (dotted bars) in MLR experiments with stimulating mononuclear cells harvested from the same donor as that of DC (APCb) or from a third-party donor (APCc). This figure is representative of two independent experiments. C, ILT3 mRNA level expression in GM/IL-4-DC (open bars) or mmCFS-DC (filled bars) was measured by quantitative RT-PCR at day 6 and day 8 and is expressed as a ratio to CPB mRNA levels. Results represent means of two independent experiments.
FIGURE 7. idCFS-Mo are not tolerogenic. T cells were primed for 5 days with GM/IL-4-DC (filled bars), idCFS-Mo (hatched bars), or mmCFS-DC (open bars), then purified using CD3 magnetic beads and cultured with mononuclear cells harvested from the same donor as that of DC (APCb). A, Expressions of IL-2, IFN-γ, FoxP3, IL-10, and TGFβ mRNA levels were measured 24 h poststimulation with APCb and expressed relative to CD3ε or CPB mRNA copy numbers, as mentioned in Materials and Methods. B, Secondary MLR experiments were performed for 3 days. Results are expressed as [3H]thymidine incorporation (cpm). C, Unprimed naive T cells (white histograms), previously labeled with CFSE, were allostimulated in the presence of CD3ε T cells previously primed with GM/IL-4-DC, idCFS-Mo, or mmCFS-DC (black histograms), at the ratio of 1:2, in MLR experiments. At day 5, expression of CFSE was measured by cytofluorometry. Indeed, after each round of T cell division, the intensity of CFSE is reduced and can be quantitated using cytofluorometry. This figure is representative of two independent experiments.
cytokines, such as TNF-α, TNFβ, and IL-6, were then used to analyze their putative effect on DC maturation recovery following treatment with GM/IL-4 plus mmCFS (Table I, set 1). As already shown above (Fig. 8B), IFN-γ inhibition by mAbs resulted in increased expression of CD80, CD86, and CD83. Anti-TNFR I+II Ab, which neutralized both TNF-α and TNF-β cytokine activities, restored CD86 expression only, while anti-IL-6 Ab weakly increased CD83 expression. In parallel, we performed reciprocal experiments in which IFN-γ, IL-6, TNF-α, or TNFβ cytokines were added to GM/IL-4. As already shown in Fig. 1B and as shown in

FIGURE 8. mmCFS inhibits GM/IL-4-mediated commitment of Mo into mature DC. A, mmCFS was added to GM/IL-4 in monocyte cultures for 6 days. TNF-α was added for 2 additional days, and expressions of CD80, CD86, and CD83 were analyzed by cytofluorometry (black histograms). GM/IL-4-DC and mmCFS-DC phenotypes are shown as controls. Dotted-line histograms represent phenotype of cells labeled with isotypic controls. B, In cells treated with GM/IL-4 plus mmCFS, Abs directed against IFN-γ were added or not from the beginning of cultures. Expressions of CD80, CD86, CD83, and DC-SIGN were measured by cytofluorometry (black histograms). GM/IL-4-DC phenotype is shown as control. Dotted-line histograms represent phenotype of cells labeled with isotypic controls.

-Black Histograms:
Day 8 cells

-White Histograms:
Isotypic controls
Table I. mmCFS inhibits GM/IL-4-mediated differentiation of Mo into DC due to IFN-γ secretion

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<td>57.97</td>
</tr>
<tr>
<td>GM/IL-4 + HLA-mmMLR-Sn + A/TNF-1 II</td>
<td>2.88</td>
<td>62.84</td>
<td>66.39</td>
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</tbody>
</table>

Set 2

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>CD80</th>
<th>CD86</th>
<th>CD83</th>
</tr>
</thead>
<tbody>
<tr>
<td>% MFI</td>
<td>% MFI</td>
<td>% MFI</td>
<td>% MFI</td>
</tr>
<tr>
<td>GM/IL-4</td>
<td>35.07</td>
<td>27.95</td>
<td>67.99</td>
</tr>
<tr>
<td>GM/IL-4 + IFN-γ</td>
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<td>60.14</td>
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<tr>
<td>GM/IL-4 + IL-6</td>
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<td>86.89</td>
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<tr>
<td>GM/IL-4 + TNF-α</td>
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<td>30.89</td>
<td>55.82</td>
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<tr>
<td>GM/IL-4 + TNF-β</td>
<td>51.77</td>
<td>23.71</td>
<td>63.67</td>
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</tbody>
</table>

* Set 1, mmCFS was added to GM/IL-4 in Mo cultures for 6 days. Anti-IFN-γ, anti-IL-6, and anti-TNFRII polyclonal Abs were used to neutralize the effects of the correspondent cytokines in GM/IL-4 plus mmCFS cultures. TNFRII was added for 2 additional days, and expression of CD80, CD86, and CD83 were analyzed by cytometry. Set 2. Exogenous IFN-γ, IL-6, TNF-α, or TNFβ was added to GM/IL-4 in Mo cultures for 6 days. TNFα was added for 2 additional days, and expression of CD80, CD86, and CD83 were analyzed by cytometry. MFI indicates mean fluorescence intensity.

Table I, set 2, supplementing GM/IL-4 with IFN-γ resulted in the inhibition of DC maturation, as assessed by the strong down-regulation of CD80 and CD83 molecules. Addition of TNF-α, but not TNF-β, induced a weak decrease in the expression of CD86, and addition of IL-6 resulted in a decrease in CD80 but not CD83 expression. Thus, these results indicate that IFN-γ is the major cytokine responsible for the inhibition of DC maturation by mmCFS.

**Discussion**

Increasing evidence supports the notion that IFN-γ can induce tolerance. Herein we have extended these findings by reporting a new mechanism of IFN-γ-mediated tolerance induction, where IFN-γ deviates differentiation of GM/IL-4-treated Mo from mature DC toward maturation-resistant and tolerogenic DC. Indeed, we have shown that IFN-γ-treated DC express DC markers such as DC-SIGN, Langerin, and CD123, but that they do not mature following exposure to TNF-α (Fig. 1). Moreover, those cells induced regulatory T cell generation when used to prime naive T cells (Fig. 2). However, Treg suppressive function was not specific to donor alloantigens, consistent with other reports (13, 14). Delneste et al. previously reported that IFN-γ induces a switch of Mo differentiation from DC to macrophages (7). However, our results provide a different conclusion, since we report that IFN-γ commits GM/IL-4-treated Mo to differentiate into Tol-DC. Indeed, we have observed that IFN-γ plus GM/IL-4-DC did not express CD16 and CD14 (Fig. 1A), two markers of the macrophage/Mo lineage. Moreover, in the Delneste et al. paper, IFN-γ plus GM/IL-4-treated cells were unable to phagocytose latex beads and did not express any specific esterase activity, which are two key functions of macrophages. Furthermore, like us, those authors found that following induction of maturation, endocytosis was maintained, whereas Ag presentation was weak, which are the characteristics of maturation-resistant DC. Finally, we clearly demonstrated that those DC exerted tolerogenic functions.

In a previous report, we demonstrated that the allosresponse activates immature DC into mature DC (11). Because IFN-γ is heavily secreted during a mismatched allosresponse, we then asked whether it would induce differentiation of Mo toward Tol-DC. This was indeed the case since we have shown that mismatched allogeneic reaction, but not HLA-identical alloreaction, induces generation of cells displaying characteristics of maturation-resistant DC at both the phenotypical and functional levels. These cells expressed low levels of CD14 molecules, moderate levels of co-stimulatory molecules, including CD80 and CD86, but high levels of CD40 and CD54. They were unable to express maturation markers, such as CD83 or DC-LAMP (data not shown), upon induction of maturation with TNF-α (Fig. 3). That these cells were DC was supported by their expression of DC-SIGN. Indeed, this marker has been shown to be expressed by DC and to initiate contact with resting T cells through ICAM3 (15). It is expressed by immature DC, but does not increase upon maturation, which led Steinman to suggest a possible involvement of DC-SIGN in induction of tolerance (16). However, DC-SIGN can also be found at the surface of some specialized macrophages located in tissues, such as lung or placenta, where they are thought to act as receptors of viruses such as HIV or hepatitis C (17, 18). Nevertheless, DC-SIGN is not expressed by peripheral blood cultured macrophages (18), which agrees with our results, since we found DC-SIGN expression in GM/IL-4-DC or mmCFS-DC, but not idCFS-DC (Fig. 4A) or fresh Mo (not shown).

At the functional level, mmCFS-DC expressed low levels of IL-12, but high levels of IL-10 mRNA (Fig. 4C). Accordingly, their ability to stimulate allosreactive T cells was severely impaired, and endocytosis, which is a feature of immature DC but not of mature DC, was maintained following TNF-α treatment (Fig. 5). Finally, mmCFS-DC-primed naive T cells acquired Treg markers and function, as shown in Fig. 6. Whereas idCFS-Mo presented lower stimulatory activity than did GM/IL-4-DC (Fig. 5B), this could not be related to an ability to promote tolerance, as shown in Fig. 7. These results therefore indicated that the acquisition of a tolerogenic function by DC treated with mmCFS is related to the cytokines secreted upon the mismatched allosresponse from which mmCFS was harvested, but not to a particular property of cultured Mo.

Tol-DC induce immune tolerance through several pathways, including clonal deletion of allosreactive T lymphocytes, anergy, deviation of Th differentiation, or generation of Treg (19, 20). Among Treg, several CD4⁺ subpopulations have been identified, such as 1) the naturally occurring CD4⁺CD25⁺ Treg, which co-express inhibitory molecules, such as CTLA-4 (21) and FoxP3 (22–24); 2) IL-10⁺ type 1 Treg (Tr1), which do not express FoxP3 (25, 26); and 3) TGFβ⁺ type 3 helper T cells (Th3) (27, 28). However, in a recent report, it has been shown that inducible Treg...
can express both IL-10 and FoxP3 cells (29). In our study, we detected increased expression of FoxP3 and IL-10, but not TGFβ mRNA, by T cells primed with mmCFS-DC following secondary stimulation with donor APC (Fig. 6). However, whether a single T cell was able to express both IL-10 and FoxP3 expression remains to be investigated.

The mechanisms underlying the generation of Tol-DC by mmCFS were then investigated. With this in mind, we postulated that the inability of mmCFS-DC to mature could result from IFN-γ secretion. Indeed, when mmCFS was added to GM-CSF and IL-4 in Mo cultures, we observed an inhibitory effect on GM/IL-4-mediated Mo-DC differentiation (Fig. 8). By using several Abs directed against IFN-γ and other inflammatory cytokines, we demonstrated that IFN-γ was preponderantly involved in this inhibition. Indeed, anti-IFN-γ Abs led to partial recovery of CD83 and CD80 molecules and increased expression of CD86 in GM/IL-4 plus mmCFS-DC (Table I). In reciprocal experiments, when added to GM/IL-4, exogenous IFN-γ, but not TNF-α or IL-6, inhibited the neo-expression of CD83, a maturation marker (Fig. 1 and Table I), thus supporting the preponderant contribution of IFN-γ in mmCFS-mediated Tol-DC generation.

The mechanisms leading to tolerance induction in organ transplantation are very complex. Indeed, while immature DC or Tol-DC subsets have been shown to prolong allograft survival (30), other reports have demonstrated the positive impact of the alloimmune response on immune regulation by showing that alloantigen, but not syngeneic, mature DC are more effective than geneic, but not syngeneic, mature DC are more effective than.

Tol-DC subsets have been shown to prolong allograft survival (30), or DC genetically modified to coexpress IL-10 (38) or CCR7 (INSERM and UCB Lyon) for help in cytometry analysis.

Disclosures

The authors have no financial conflicts of interest.

References


