Differential Regulation of Human Blood Dendritic Cell Subsets by IFNs[1]

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Based on the relative expression of CD11c and CD1a, we previously identified subsets of dendritic cells (DCs) or DC precursors in human peripheral blood. A $CD1a^+/CD11c^+$ population ($CD11c^+$ DCs), also called myeloid DCs, is an immediate precursor of Langerhans cells, whereas a $CD1a^-/CD11c^-$ population ($CD11c^-$ DCs), sometimes called lymphoid DCs but better known as plasmacytoid DCs, is composed of type I IFN (IFN-$\alpha\beta$)-producing cells. Here, we investigate the effects of IFN-$\alpha\beta$ and IFN-γ as well as other cytokines on $CD11c^+$ and $CD11c^-$ DC subsets, directly isolated from the peripheral blood, instead of in vitro-generated DCs. IFN-γ and IFN-α, rather than GM-CSF, were the most potent cytokines for enhancing the maturation of $CD11c^+$ DCs. Incubation of $CD11c^+$ DCs with IFN-γ also resulted in increased IL-12 production, and this IL-12 allowed DCs to increase Th1 responses by alloreactive T cells. In contrast, IFN-α did not induce IL-12 but, rather, augmented IL-10 production. IFN-α-primed matured $CD11c^+$ DCs induced IL-10-producing regulatory T cells; however, this process was independent of the DC-derived IL-10. On the other hand, IFN-α by itself neither matured $CD11c^-$ DCs nor altered the polarization of responding T cells, although this cytokine was a potent survival factor for $CD11c^-$ DCs. Unlike IFN-α, IL-3 was a potent survival factor and induced the maturation of $CD11c^-$ DCs. The IL-3-primed $CD11c^-$ DCs activated T cells to produce IL-10, IFN-γ, and IL-4. Thus, $CD11c^+$ and $CD11c^-$ DC subsets play distinct roles in the cytokine network, especially their responses to IFNs. ***The Journal of Immunology*****, 2001, 166: 2961–2969.**

Dendritic cells (DCs)[3] are specialized APCs that prime naive cells (1). We and others have identified distinct DC (or DC precursor) subsets in human blood using, in particular, differences in CD11c integrin expression (2). The major population is $CD1a^+CD11c^+$ immature DCs (termed fraction 1 in our previous report (2)), and the minor population is $CD1a^-CD11c^+$ DCs (previously fraction 2 (2)), and both express myeloid markers such as CD13 and CD33, and GM-CSF receptor, suggesting that these are monocyte-related DCs. $CD1a^+CD11c^+$ DCs are found as an immediate precursor of Langerhans cells. $CD1a^-CD11c^-$ cells (previously fraction 3 (2)), which are also called plasmacytoid cells or pre-DC2 (morphologically and functionally immature) are postulated to be in the lymphoid lineage (3, 4). This subset acts as the major type I IFN-producing cell upon viral infection (5, 6).

Type I IFNs (IFN-$\alpha\beta$) are important cytokines because of their beneficial use as adjuvant in antiviral or anticancer therapy. Indeed, in the immune system, type I IFNs have multiple functions, such as protection of lymphocytes from apoptosis (7), suppression of cell proliferation (8), up-regulation of MHC class I molecules (9), and modulation of Ig production (10, 11). Furthermore, type I IFNs share some biological activities with IL-12, directing Th cells to produce IFN-γ, which consequently inhibits IL-4/IL-5 synthesis (12, 13) and augments NK and CTL activities (7, 14). Thus, IFN-$\alpha\beta$ could contribute to innate immunity, activating cytotoxic effector cells and helping to eliminate microbial agents.

On the other hand, type II IFN (IFN-γ) is a product of Th1, CTL type I (Tc1), and NKT cells and is a major effector molecule in cell-mediated immunity (15). It has recently been demonstrated, however, that mouse DCs also produce IFN-γ upon activation, and that IFN-γ produced by DCs acts in an autocrine or paracrine manner to up-regulate DC production of IL-12 (16). Like IFN-$\alpha\beta$, IFN-γ has similar effects on various cell types, including macrophages and NK cells as well as lymphocytes. In contrast to IFN-γ, however, IFN-$\alpha\beta$ does not induce MHC class II expression in the mouse and sometimes counteracts the effects of IFN-γ (17).

Therefore, we attempted to clarify the effects of IFN-α and IFN-γ as well as other cytokines on the $CD1a^+CD11c^+$ DC subset (as the major population of myeloid-lineage $CD11c^+$ DCs, hereafter termed $CD11c^+$ DCs) and the $CD1a^-CD11c^-$ DC subset (hereafter termed $CD11c^-$ DCs), both of which were directly purified from the peripheral blood, instead of in vitro-generated DCs. In this study, we adopted a serum-free condition for the DC cultures to avoid the effects of serum factors. IFN-α and IFN-γ are potent maturation factors for $CD11c^+$ DCs; IFN-α induces IL-10 production by DCs, whereas IFN-γ stimulates IL-12 production. These IFN-α- and IFN-γ-primed $CD11c^+$ DCs evoke preferential production of IL-10 and IFN-γ from T cells, respectively. In contrast, $CD11c^-$ DCs do not respond well to IFN-γ, but do survive in the presence of IFN-α or IL-3. IL-3 is a far more potent maturation factor for $CD11c^-$ DCs than IFN-α in terms of the enhanced expression of MHC and costimulatory molecules and allows the DCs to induce more Th cells producing IL-10, IFN-γ, and

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[3] Abbreviations used in this paper: DC, dendritic cell; sCD40L, soluble CD40 ligand.

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IL-4. Thus, not only do different types of DCs in the blood have the capacity to induce different Th cell development, but the same types of DCs can induce different Th cell development depending on the DC maturation signals.

Materials and Methods

Media and reagents

Serum-free medium (X-VIVO 20; BioWhittaker, Walkersville, MD) supplemented with BSA (0.4%) was used throughout the experiments. Recombinant human cytokines, GM-CSF (50 ng/ml), TNF-α (2.5 ng/ml), TGF-β1 (1 ng/ml), IL-1α (10 ng/ml), IL-3 (10 ng/ml), IL-4 (50 ng/ml), IL-6 (20 ng/ml), IL-7 (20 ng/ml), and IFN-γ (1000 U/ml), were purchased from Roche (Indianapolis, IN), and IL-2 (100 U/ml), IFN-α2b (1000 U/ml), IFN-β (1000 U/ml), and soluble CD40 ligand (sCD40L; 1 μg/ml) were obtained from PeproTech (London, U.K.).

Isolation of peripheral blood DCs

Peripheral blood DCs were isolated according to the protocol previously described (2). Briefly, PBMC were incubated with anti-CD3 (HIT3a) and anti-CD14 (M5E2) mAbs (both from PharMingen, San Diego, CA), and cells binding to these mAbs were removed using sheep anti-mouse Ig-coated magnetic beads (M-450; Dynal, Oslo, Norway). $CD3^-/CD14^-$ cells were further incubated with CD4-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and the $CD4^+$ cells were then enriched through a Mini-MACS magnetic separation column (Miltenyi Biotec). The resultant DC-enriched population ($CD4^+/CD3^-/CD14^-$ cells) was stained with PE-labeled anti-CD11c (Leu M5), FITC-labeled anti-CD1a (BB-5), PE-cyanin 5.1 (PC5)-labeled HLA-DR (Immu-357), and a mixture of biotinylated mAbs against lineage markers (CD3; M2AB, binding to a different determinant from that recognized by the previous anti-CD3 mAb, CD14; UCHM1, binding to a different determinant from that recognized by the previous anti-CD14 mAb, CD16; 3G8 and CD19; HIB19) followed by RED613-streptavidin (Life Technologies, Gaithersburg, MD). The $CD1a^+/CD11c^+/lin^-/DR^+$ cells (fraction 1) and $CD1a^-/CD11c^-/lin^-/DR^+$ cells (fraction 3) were sorted by an EPICS ALTRA flow cytometer (Coulter, Hialeah, FL). For analyses of the expression of surface markers, the $CD1a^+CD11c^+$ DC subset was sorted as the $CD1a^+/lin^-/DR^+$ fraction without the staining of PE-CD11c (note that all $CD1a^+/lin^-/DR^+$ DCs are quite comparable to $CD1a^+/CD11c^+/lin^-/DR^+$ DCs (Fig. 1*A*) when analyzed after staining with anti-CD11c mAb), and $CD1a^-CD11c^-$ cells were resorted as the $CD11c^-$ fraction from the $CD1a^-/lin^-/DR^+$ population. The sorted cells were subsequently stained with the following PE-labeled mAbs: CD121a (IL-1R type 1: clone 6B5), CD25 (IL-2Rα: 2A3), CD122 (IL-2Rβ: TIC-1), CD123 (IL-3Rα: 9F5), CD124 (IL-4Rα: S4–56C9), CD125 (IL-5Rα: A14), CD126 (IL-6Rα: M91), CD130 (IL-6Rβ: AM64), CD127 (IL-7Rα: R34.34), CD114 (G-CSFR: LMM471), CD116 (GM-CSFRα: M5D12), CD117 (c-Kit: 95C3), CD135 (FLT-3: SF1.340), CD131 (common β receptor: 3D7), CD105 (TGF-β1/3R: N1-3A1), CD119 (IFN-γRα: MMHGR-1), CD120a (TNF-αR p55: 2H10), CD120b (TNF-αR p75: 4D1B10), and IFN-αRβ (MMHAR-2).

Culture of DCs

The sorted DCs were cultured in 96-well flat-bottom tissue culture plates at 4×10^4 cells in 200 μl of medium/well. In some cultures UV-irradiated Sendai virus (HVJ, Cantell strain, provided by Sumitomo Pharmaceuticals, Ehime, Japan) was added at 5 hemagglutinating units/ml (18). In culture supernatants of DCs after 24 h, the production of cytokines was determined by ELISA (kits for IL-12 p70, IL-4, and IL-10 were purchased from Immunotech (Marseilles, France) and that for IFN-α was obtained from Endogen (Woburn, MA)).

Cell viability and maturation assays

In the viability assay, viable cells were counted by trypan blue dye exclusion test after the culture and simultaneously evaluated as propidium iodide-negative and annexin V-negative fractions using the annexin V-FITC apoptosis detection kit (Genzyme, Cambridge, MA).

To evaluate cell maturation, the cells were stained with PE-labeled anti-HLA-DR (L243), HLA-DQ (1a3), CD86 (HA5.2B7), CD80 (MAB104), CD40 (MAB89), and CD83 mAb (HB15a) and analyzed by a FACScan (Becton Dickinson, Sunnyvale, CA). In the MLR assay, the cultured DCs (for 1 day) were washed and γ-irradiated at 15 Gy, and graded doses of these cells were then added to 2×10^5 allogeneic $CD4^+/CD45RA^+$ naive T cells in the medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 ng/ml streptomycin, and heat-inactivated 10% FBS (Irvine Scientific, Santa Ana, CA)) for 5 days. The cells were pulsed

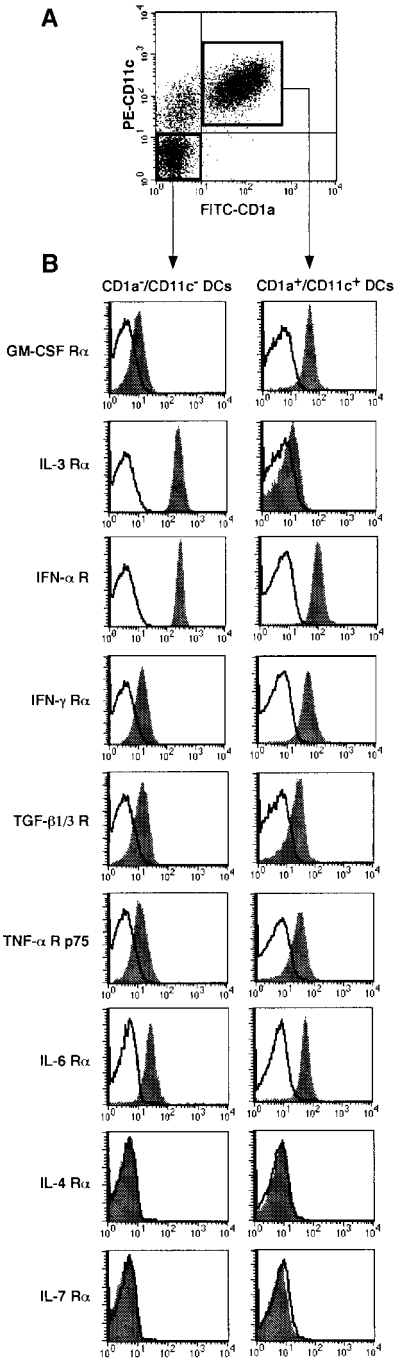

FIGURE 1. DC subsets in PBMC (*A*) and their expression of various cytokine receptors (*B*). *A*, After enriching $CD4^+$ cells from $CD3^-/CD14^-$ PBMC by MACS followed by staining with PE-CD11c, FITC-CD1a, PC5-HLA-DR, and a mixture of biotin-CD3, CD14, CD16, and CD19 with Red613-streptavidin, the cells were purified as $CD1a^+/CD11c^+$ ($CD11c^+$ DCs) and $CD1a^-/CD11c^-$ ($CD11c^-$ DCs) in the lin^-/DR^+ gated fraction as previously described (2). *B*, Expression of cytokine receptors on blood DC subsets was analyzed by staining with various PE-conjugated mAbs. In this experiment $CD1a^+/CD11c^+$ DCs were sorted as the $CD1a^+/lin^-/DR^+$ fraction without the staining of PE-CD11c (note that all $CD1a^+/lin^-/DR^+$ DCs are quite comparable to $CD1a^+/CD11c^+/lin^-/DR^+$ DCs), and $CD1a^-/CD11c^-$ DCs were resorted as the $CD11c^-$ fraction from the $CD1a^-/lin^-/DR^+$ population. The staining profiles with respective mAbs and isotype-matched control are shown by the shaded area and open area, respectively. Other mAbs for cytokine receptors, such as IL-1R type 1, IL-2Rα, IL-2Rβ, IL-5Rα, IL-6Rβ, G-CSFR, c-Kit, FLT-3, common β receptor, or TNF-αR (p55) were also analyzed, but all of them were negative (data not shown). The results shown are representative of at least three experiments.

with 1 μCi of [^{3}H]TdR during the last 8 h of the culture period. CD4$^+$/CD45RA$^+$ naive T cells, negatively selected twice by anti-CD45RO (UCHL-1), -CD8 (T8), -CD14 (M5E2), -CD16 (3G8), -CD19 (HIB19), -HLA-DR (L243), and -CD11b (ICRF44) mAbs followed by sheep anti-mouse Ig-coated magnetic beads, were obtained from allogeneic healthy volunteers. The purity of the cells was 92% or greater by reanalysis using anti-CD4 and CD45RA mAbs.

Analyses of T cell polarization stimulated by DCs

The sorted DCs precultured with the cytokine for 2 days were washed and then cocultured with allogeneic CD4$^+$/CD45RA$^+$ naive T cells in 96-well flat-bottom tissue culture plates in 200 μl of medium/well (2×10^4 cultured DCs/2×10^5 T cells). After 6 days of DC-T cell coculture with or without neutralizing polyclonal Ab to IL-12 (AB-219-NA, 20 μg/ml) or IL-10 (AB-217-NA, 20 μg/ml; both of goat polyclonal IgG from R&D Systems, Minneapolis, MN), the cells were washed and subsequently restimulated with PMA (25 ng/ml) and ionomycin (1 μg/ml) for 8 h. After restimulation, IFN-γ, IL-10, and IL-4 in the culture supernatants were analyzed by ELISA (kit from Immunotech). For intracellular cytokine analyses, brefeldin A (2 μg/ml) was added to the cultures for the last 4 h. The cultured cells were stained with PE-labeled anti-IL-10 (Caltag, Burlingame, CA) plus FITC-labeled anti-IFN-γ (Ancell, Bayport, MN) or with PE-labeled anti-IL-4 (Becton Dickinson) plus FITC-labeled anti-IFN-γ mAbs, using FIX and PERM kit (Caltag), and then were analyzed by a FACScan.

Results

Expression of cytokine receptors on DC subsets from PBMC

As shown previously, we have identified two dominant populations of immature DCs (or DC precursors) in peripheral blood based on the expression of CD1a and CD11c (2): CD11c$^+$ DC subset (CD1a$^+$/CD11c$^+$/lin$^-$/DR$^+$) and CD11c$^-$ DC subset (CD1a$^-$/CD11c$^-$/lin$^-$/DR$^+$; Fig. 1*A*). To analyze the effects of cytokines on the function of these subsets, we first examined the expression of specific receptors using mAbs. As shown in Fig. 1*B*, both subsets variably expressed receptors for GM-CSF, IL-3, IFN-α, IFN-γ, TGF-β1, TNF-α (p75), and IL-6. CD11c$^+$ DCs expressed GM-CSFR at a high level, but IL-3R at a low level, whereas CD11c$^-$ DCs expressed these receptors in a reciprocal manner. Notably, both subsets, but especially CD11c$^-$ DCs, intensely expressed IFN-αR. The rest of the receptors were comparably expressed on CD11c$^+$ and CD11c$^-$ DC subsets. IL-1R type 1, IL-2Rα, IL-2Rβ, IL-4Rα, IL-5Rα, IL-6Rβ, IL-7Rα, G-CSFR, c-kit, FLT-3, common β receptor, and TNF-αR (p55) were expressed weakly or were not detectable on both subsets (data partially shown in Fig. 1*B*).

Effects of cytokines on blood DC survival

We then evaluated the viability of DCs after 1 day of culture in the presence of a panel of cytokines using the trypan blue dye exclusion test (Table I). In the absence of cytokines, both DC subsets, especially CD11c$^-$ DCs, showed a high mortality because of the spontaneous apoptosis. GM-CSF, IL-3, IFN-α, and IFN-γ variably supported the survival of both DC subsets. As has been reported, GM-CSF best maintained the viability of CD11c$^+$ DCs, while IL-3 protected CD11c$^-$ DCs from cell death (3, 19). Intriguingly, IFN-α and IFN-γ substantially enhanced the survival of CD11c$^+$ DCs, whereas only IFN-α exhibited this effect on CD11c$^-$ DCs.

Based on these findings, we examined the viability of each subset over the longer period of 72 h. As shown in Fig. 2, GM-CSF maintained CD11c$^+$ DCs for 72 h, whereas IFN-α and IFN-γ only maintained viability for 24 h. On the other hand, the survival of CD11c$^-$ DCs was comparably maintained for 72 h by IL-3 and IFN-α, and the viabilities were not improved even in the presence of sCD40L, a potent stimulator of DC maturation.

Effects of cytokines on the maturation of blood DCs

We next examined whether cytokines that maintain DC viability also induce DC maturation. For CD11c$^+$ DCs, IFN-γ and IFN-α remarkably augmented the expression of MHC class II and maturation-associated molecules such as CD40, CD80, CD86, and CD83. GM-CSF only exerted a marginal effect (Fig. 3). However, IFN-γ was slightly more effective than IFN-α in augmenting the expression of costimulatory molecules, even though both types of IFNs were comparably efficient for MHC class II expressions. In contrast to the marginal effects of GM-CSF on CD11c$^+$ DCs, IL-3 by itself substantially up-regulated all markers on CD11c$^-$ DCs. IFN-α, however, failed to increase these molecules on CD11c$^-$ DCs. The addition of sCD40L did not up-regulate the expression of maturation markers when CD11c$^-$ DCs were cultured with IFN-α, but slightly augmented them when cultured with IL-3.

To examine whether the maturation in DC phenotype by cytokines was correlated to T cell stimulatory capacity, allogeneic MLR was performed. Each DC subset was precultured with cytokines for 1 day before the addition of allogeneic T cells. IFN-γ-treated CD11c$^+$ DCs were more powerful stimulators than those treated with IFN-α. In line with the changes in phenotype, GM-CSF only modestly increased DC function (Fig. 4*A*). For CD11c$^-$ DCs, IL-3 strongly enhanced DC function, which was increased further if sCD40L was also added (Fig. 4*B*). Even with sCD40L, IFN-α did not significantly increase DC function relative to medium alone.

T cell cytokine production in response to DCs primed with IFNs and viability-maintaining cytokines

We measured the cytokines produced from CD4$^+$ naive T cells that had been stimulated with DC subsets treated with different cytokines (Fig. 5). In general, CD11c$^+$ DCs even precultured with medium alone preferentially activated T cells to produce Th1 cytokine, i.e., high amounts of IFN-γ and low amounts of IL-10 and IL-4. IFN-γ-primed CD11c$^+$ DCs induced the largest amount of IFN-γ, but did not modulate IL-10 or IL-4 production. In contrast, IFN-α-primed CD11c$^+$ DCs induced more IL-10 and less IFN-γ without affecting IL-4 secretion, indicating that they induced the T cell subset phenotypically similar to T regulatory cells (Tr or Th3 cells).

In distinction to CD11c$^+$ DCs, CD11c$^-$ DCs precultured with medium alone induced Th2 development, i.e., the production of

Table I. *Comparative analyses of viable cell recovery with the cytokines on blood DC subsets*[a]

	No Cytokine	IL-3	GM-CSF	IFN-α	IFN-γ	TNF-α	TGF-β1	IL-6
CD11c$^+$ DCs	36.5 ± 3.4	51.0 ± 3.3*	79.5 ± 3.7**	88.3 ± 2.8**	83.8 ± 3.0**	37.5 ± 2.8	34.5 ± 2.5	38.8 ± 3.5
CD11c$^-$ DCs	19.5 ± 2.7	75.5 ± 3.3**	45.3 ± 3.2*	73.8 ± 3.1**	30.0 ± 2.7*	19.5 ± 2.3	18.8 ± 2.5	20.8 ± 2.5

[a] After 24-h culture with a panel of cytokines in the serum-free condition, the recovery of viable cells of each DC subset was counted using the trypan blue dye exclusion test. Freshly isolated cells were cultured in 96-well flat-bottom tissue culture plates at 4×10^4 cells in 200 μl of medium/well. Results are shown as percent recovery of viable cells (means ± SEM) of four separate experiments using DCs from healthy individual volunteers. Statistical significance using paired Student's *t* test (*, $p < 0.05$ and **, $p < 0.001$) is indicated.

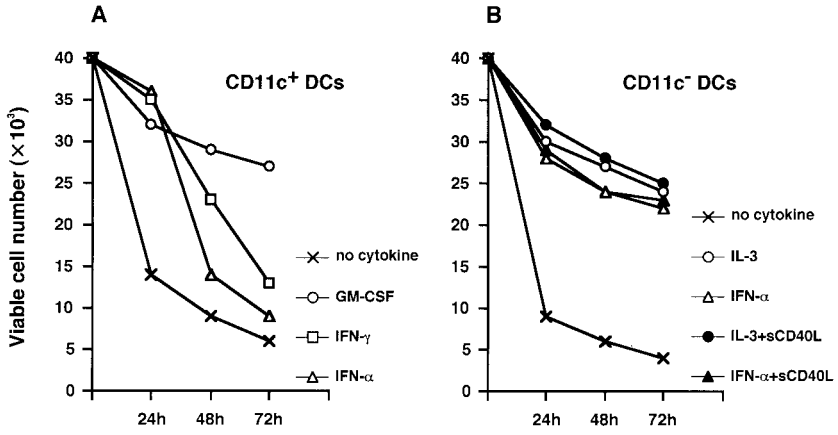

FIGURE 2. Capability of cytokines to maintain viability. In the course of a 72-h culture period, the actual number of viable cells in each DC subset was monitored by the trypan blue dye exclusion test at each 24-h point. This figure represents the results from one of four experiments.

IL-4 and IL-10. Upon priming with IL-3, the DCs induced a remarkable amount of IL-10. In contrast, DCs primed with IFN-α did not modulate T cell responses compared with the unprimed DCs.

Th1 polarization induced by IFN-γ-primed CD11c$^+$ DCs was counteracted by the simultaneous addition of IFN-α. A similar effect of IFN-α was observed in CD11c$^-$ DCs primed with IL-3.

Cytokine production of each DC subset

To elucidate the priming effect of IFN-α and IFN-γ on CD11c$^+$ DCs and IL-3 on CD11c$^-$ DCs, we analyzed cytokine production from DCs after stimulation. As shown in Fig. 6, IFN-γ treatment resulted in a marked up-regulation of IL-12 production, but not IL-10, from CD11c$^+$ DCs. In contrast, IFN-α was a poor inducer of IL-12, but dramatically induced IL-10. These findings indicated that IFN-α and IFN-γ reciprocally regulate the IL-12 and IL-10 production of CD11c$^+$ DCs. No IL-4 was detectable in supernatants of any of the DC cultures (data not shown), in agreement with the previous study by Rissoan et al. (4).

CD11c$^-$ DCs did not secrete detectable amounts of IL-12, IL-10, or IL-4 under any conditions, even though they produced abundant IFN-α after stimulation with Sendai virus (ranging from 3355 to 6006 pg/ml), confirming that CD11c$^-$ DCs are IFN-producing cells. They did not produce IFN-α under any cytokine stimulations (data not shown).

The effects of the cytokines produced by primed DCs on T cell polarization were next examined, again by examining the intracellular cytokine profiles of activated allogeneic T cells in the presence or the absence of neutralizing Ab to IL-10 and IL-12. Th1 polarization was demonstrated by CD11c$^+$ DCs that had been precultured without any cytokines (Fig. 7*A*). IFN-α-primed CD11c$^+$ DCs significantly increased the frequency of IL-10-producing cells and decreased that of IFN-γ-producing cells. The addition of neutralizing Ab to IL-10 did not change the frequency of IL-10-producing T cells, but resulted in an increase in IFN-γ-producing T cells (Fig. 7*A*). Therefore, the decrease in the frequency of IFN-γ-producing cells, but presumably not the increase in IL-10-producing cells, relates to the IL-10 secreted from IFN-α-primed DCs.

On the other hand, IFN-γ-primed DCs substantially increased the number of IFN-γ-producing cells, and this was markedly blocked by the addition of neutralizing Ab against IL-12 (Fig. 7*A*). However, anti-IL-12 Ab did not affect the induction of IL-10-producing cells. These results clearly demonstrated that Th1 skewing depended on IL-12 secreted from IFN-γ-primed DCs.

In keeping with the results reported by Cella et al. (5), CD11c$^-$ DCs cultured in the medium alone activated a substantial number of IFN-γ-producing T cells, although the frequency was far less than that of cultures with CD11c$^+$ DCs (Fig. 7*B*). These activated

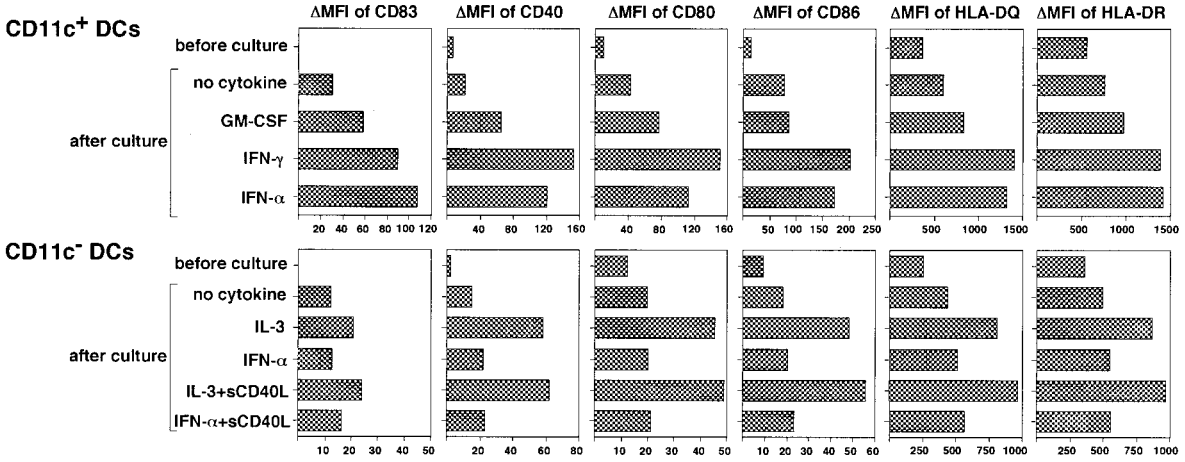

FIGURE 3. Phenotypical maturation of DCs by cytokines. After 24-h culture under serum-free conditions, the expression intensities of HLA-DR, HLA-DQ, CD86, CD80, CD40, and CD83 on each DC subset were analyzed by FACScan. The result is shown as ΔMFI (mean fluorescence intensity), which is calculated by subtraction of mean fluorescence intensity of the isotype-matched control value from that of the experimental mAb. This figure represents the results from one of four experiments.

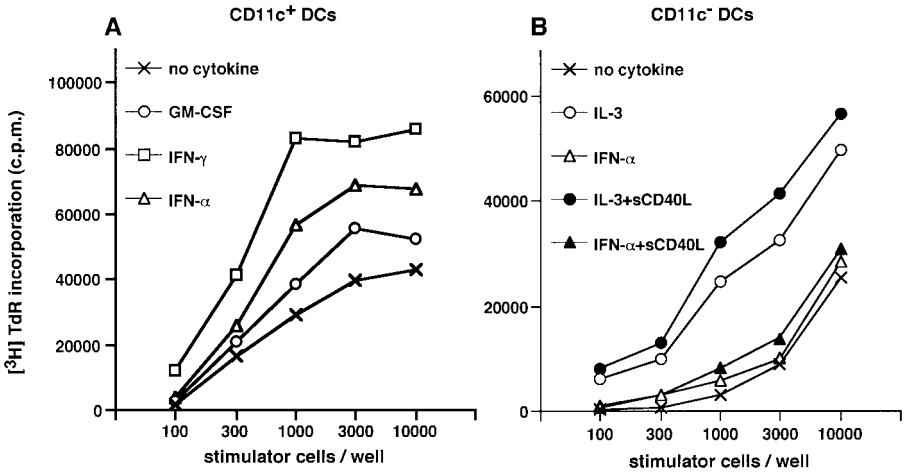

FIGURE 4. Effect of cytokine treatment on T cell stimulatory capacity of DCs. Proliferative responses of allogeneic naive $CD4^+$ T cells were measured by $[^3H]$TdR incorporation by $CD11c^+$ (*A*) and $CD11c^-$ (*B*) DCs that had been precultured with cytokine for 24 h. Results are shown as means of triplicate cultures (representative data of three experiments).

T cells, however, produced IL-10. In addition, a small but significant percentage of T cells were stained with both IL-10 and IFN-γ, indicating that some populations of activated T cells are double producers of IL-10 and IFN-γ. This type of T cell was profoundly increased in cultures stimulated with IL-3-primed $CD11c^-$ DCs. Furthermore, the number of IL-10 single-producing T cells was also increased. Anti-IL-10-neutralizing Abs did not influence the induction of IL-10 single- and double-producing T cells (Fig. 7*B*), suggesting that IL-10 does not mediate the induction of IL-10-producing cells. These results suggest that $CD11c^-$ DCs induce Th0 as well as Th2, and that IL-3 treatment potentiates such activities. To examine this possibility, T cells were stained with anti-IFN-γ and anti-IL-4 instead of anti-IL-10 Ab. As shown in Fig. 7*C*, a significant number of IL-4 single producers and double producers of IL-4 and IFN-γ increased when cultured with IL-3-primed $CD11c^-$ DCs. Therefore, IL-3-primed $CD11c^-$ DCs have the ability to stimulate not only Th2 but also Th0. On the other hand, IFN-α-primed $CD11c^-$ DCs as well as untreated DCs were incapable of preferentially inducing either Th2 or Th0, in contrast to IL-3-primed $CD11c^-$ DCs.

Finally, the counteracting effect of IFN-α on the function of IFN-γ-primed $CD11c^+$ DCs or on IL-3-primed $CD11c^-$ DCs was extended to the analyses of Th-type defined intracellular cytokine expression (Fig. 7, *right panels*).

Discussion

In the experiments presented here, we studied the effect of IFNs and relevant cytokines that are indispensable for DC development on the two subsets of immature DCs (or DC precursors) in peripheral blood; one is the $CD11c^+$ DC subset, so-called myeloid DCs or pDC1, and the other is the $CD11c^-$ DC subset, lymphoid DCs or pDC2. To comparatively study the physiological significance of the cytokines on the two types of DCs, we directly isolated them from the peripheral blood, instead of using in vitro generated DCs in the presence of GM-CSF and IL-4. We found that GM-CSF was a most effective survival factor for $CD11c^+$ DCs and that both IFN-γ and IFN-α were maturation-inducing factors rather than survival factors even though they maintain $CD11c^+$ DCs for 1 day (Figs. 2 and 3 and Table I). On the other hand, IL-3 and IFN-α, but not IFN-γ, sustained the viability of $CD11c^-$ DCs for at least 3 days (Table I and Fig. 2). In contrast to $CD11c^+$ DCs, IFN-α did not act as a maturation-inducing factor on $CD11c^-$ DCs, whereas IL-3 did. Moreover, sCD40L was ineffective in inducing $CD11c^-$ DC maturation in the presence of IFN-α (Fig. 3). These results indicate that IFN-α, which is produced in large quantities from $CD11c^-$ DCs upon infection with Sendai virus (our unpublished observation and reported previously by others (5, 6)), acts as a regulatory factor on $CD11c^-$ DCs to inhibit their maturation. This was also confirmed in the experiment with allogeneic MLR (Fig. 4).

McRae et al. (20) recently reported that IFN-$\alpha\beta$ inhibit the differentiation of monocyte-derived DCs when IFNs are added at the time of culture initiation of $CD14^+$ cells with GM-CSF plus IL-4. On the other hand, not only IFN-γ but also IFN-$\alpha\beta$ have been demonstrated to induce final maturation of in vitro generated DCs (21, 22), which is consistent with our results. The major findings of this study are 1) that both types of IFNs induced $CD11c^+$ DC

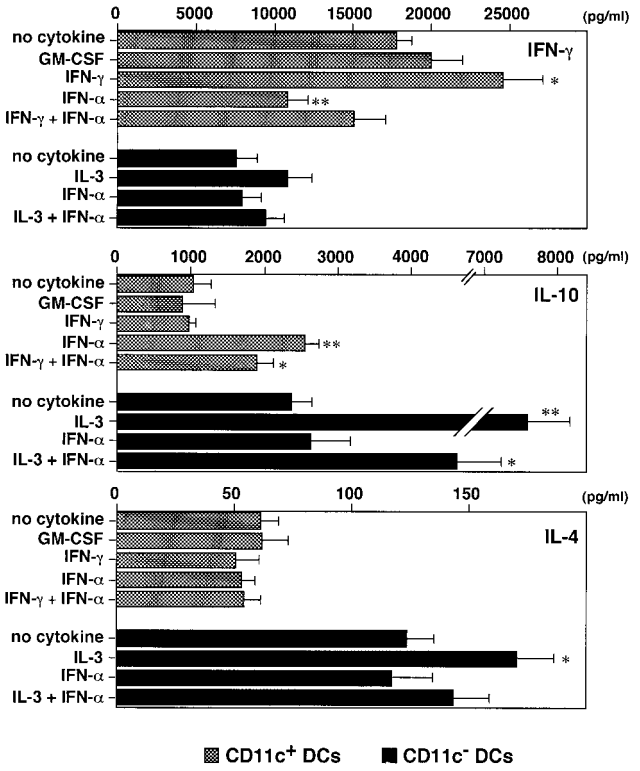

FIGURE 5. Cytokine production in allogeneic MLR by stimulation of DCs primed with cytokine. After 48-h preculture of $CD11c^+$ and $CD11c^-$ DCs with cytokine, each DC subset was mixed with allogeneic naive $CD4^+$ T cells in the serum-free medium. Six days later, the T cells were washed and restimulated with PMA and ionomycin. Supernatants were harvested at 8 h of culture and measured for the activity of IFN-γ, IL-10, and IL-4 by ELISA. Statistical significance using paired Student's *t* test (*, $p < 0.05$; **, $p < 0.01$) is indicated. Results are shown as the mean ± SEM of five experiments.

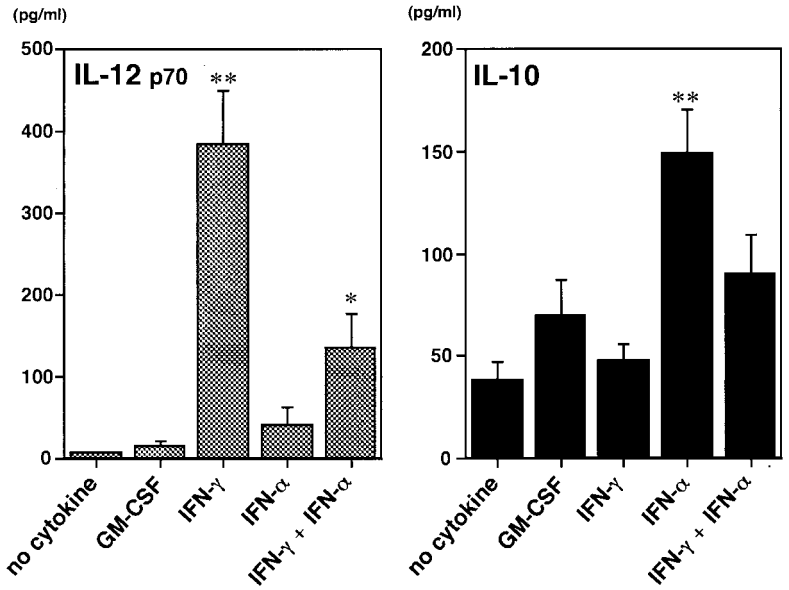

FIGURE 6. Enhanced production of IL-12p70 by IFN-γ and of IL-10 by IFN-α in CD11c$^+$ DCs. After 24-h culture of CD11c$^+$ DCs with cytokines in the serum-free condition, IL-12p70 and IL-10 secretions in the culture supernatants were measured by ELISA. Statistical significance is indicated using paired Student's *t* test (*, $p < 0.05$; **, $p < 0.01$). No IL-4 activity was detected in the supernatant of CD11c$^+$ DCs after preculture with any cytokines. Not only IL-4, but also IL-12p70 and IL-10 were detected as negative in the supernatants of CD11c$^-$ DCs with any cytokine stimulation. The data represent the mean ± SEM of five experiments.

maturation, and 2) that IFN-γ augmented the ability to induce Th1 response, whereas IFN-α stimulated an IL-10-producing Tr response along with the concomitant inhibition of IFN-γ-producing Th1 cell development (Fig. 5).

The question raised here is how CD11c$^+$ DCs treated with these two types of IFNs induced different polarized T cell responses. One possibility is that the skewed T cell responses are due to the expression level of costimulatory molecules on the DCs, since DCs stimulated with IFN-γ expressed a slightly higher level of costimulatory molecules on the surface than those stimulated with IFN-α (Fig. 3). This seems to relate to the difference in the magnitude of allogeneic T cell response induced by IFN-γ−- or IFN- α-stimulated

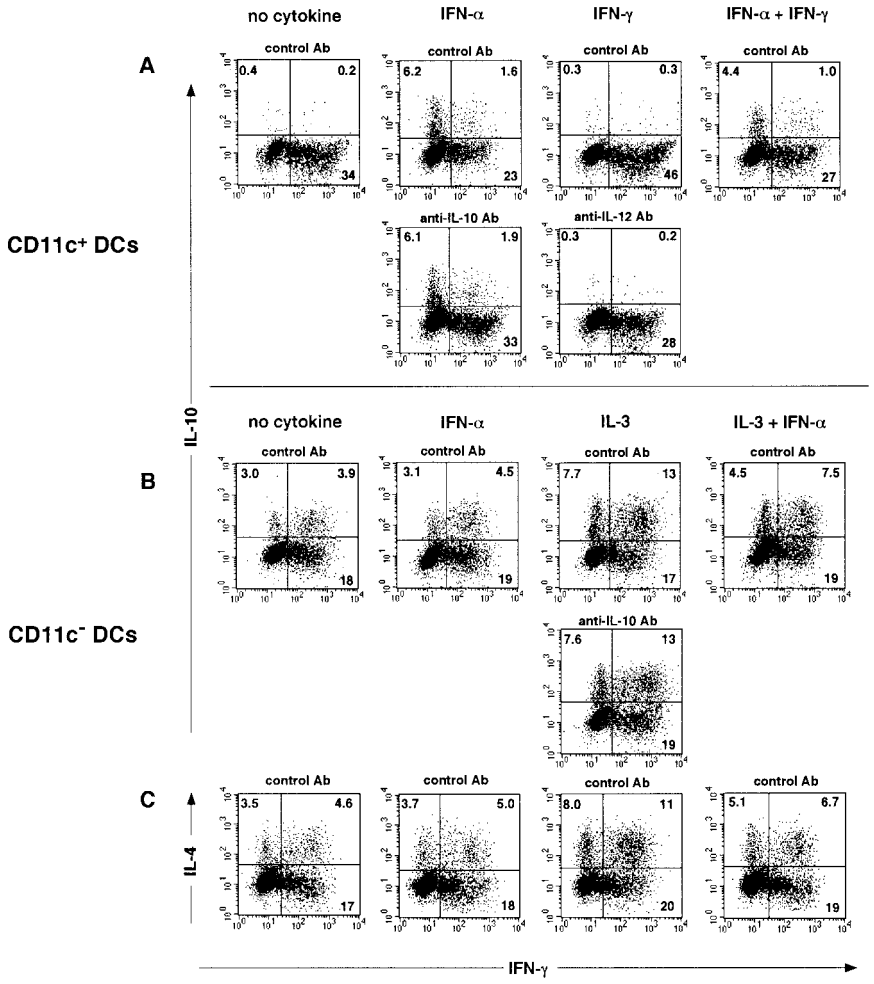

FIGURE 7. Polarization patterns of intracellular cytokine in T cells stimulated by cytokine-primed DC subsets. Allogeneic naive CD4$^+$ T cells were cultured for 6 days with cytokine-primed CD11c$^+$ (*A*) and CD11c$^-$ DCs (*B* and *C*) in the presence or the absence of neutralizing Ab against either IL-10 or IL-12. After washing, the T cells were restimulated with PMA and ionomycin for 8 h. Brefeldin A was added to the cultures for the last 4 h, and then intracellular IFN-γ with IL-10 (*A* and *B*) or IFN-γ with IL-4 (*C*) in the T cells was analyzed by FACScan. The percentages of the respective cytokine producer T cells are indicated in each dot blot profile. This figure represents the results from one of five experiments.

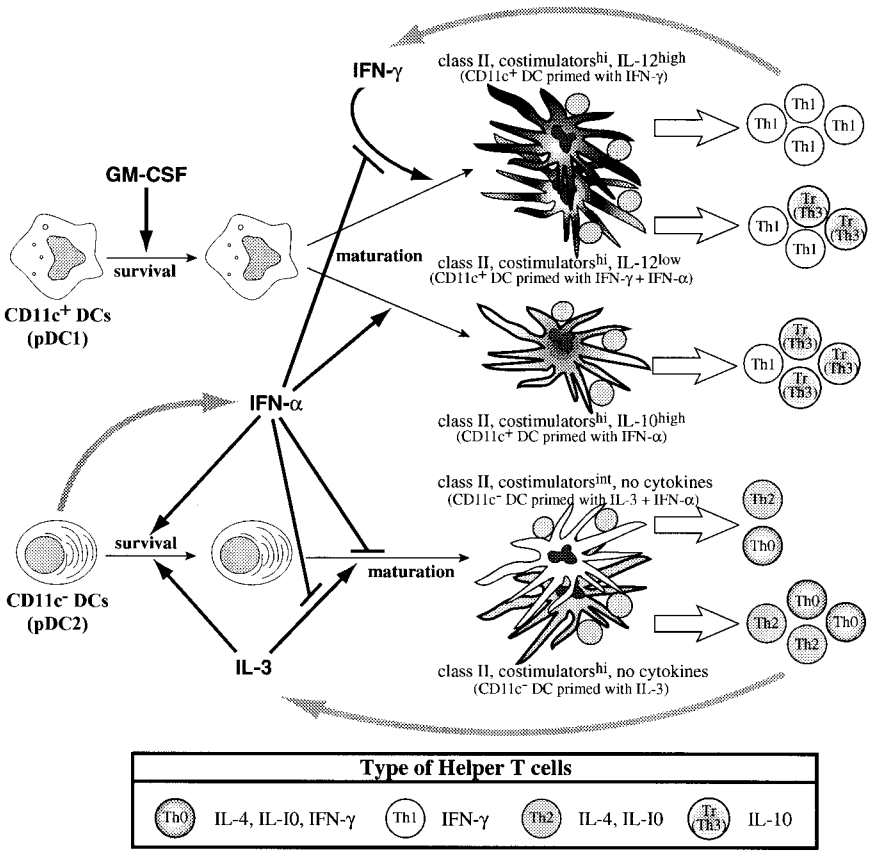

FIGURE 8. Schematic illustration of myeloid and lymphoid DCs in the cytokine network. This figure depicts the hypothesis that followed from our study. Blood DC subsets exhibit the distinct functions in cytokine network system.

DCs (Fig. 4). The other possibility is that IFN-γ and IFN-α regulate the ability of CD11c$^+$ DCs to produce different sets of cytokines. Indeed, this is also the case in our experiments. IFN-γ induced a large amount of IL-12p70 production, whereas IFN-α induced IL-10 production rather than IL-12 p70 (Fig. 6). Preferential activation of Th1 by IFN-γ-primed CD11c$^+$ DCs was shown to be mainly due to the DC-derived IL-12 because of the considerable blocking by the addition of anti-IL-12-neutralizing Ab, while IL-10-producing Tr cell development was independent of DC-derived IL-10 (Fig. 7*A*). However, the negative regulation of Th1 development by IFN-α-primed CD11c$^+$ DCs was shown to be dependent on IL-10, probably either from the DCs or the developing Tr cells, in agreement with the established idea that IL-10 is capable of inhibiting Th1 development (23–25). We also noted that a low, but significant, number of T cells produced both IFN-γ and IL-10 upon culture with IFN-α-primed, but not IFN-γ-primed, CD11c$^+$ DCs. Therefore, IFN-α may induce the production of undetermined cytokines from or surface molecules on CD11c$^+$ DCs, which would then actively facilitate IL-10-producing Tr cell development.

As for CD11c$^-$ DCs, IL-3 was a sole and effective maturation-inducing factor in terms of the expression of surface molecules involved in T cell activation. Eventually, IL-3-primed CD11c$^-$ DCs were capable of inducing a substantial allogeneic T cell response. However, no cytokine activity, such as IL-12 and IL-10, was detectable in the culture supernatant. Kadowaki et al. (26) have reported that pDC2 pretreated with IL-3 tend to induce Th2 and that a remarkable increase in Th2 cytokines, especially IL-10, is detected, as in our study (Fig. 5). This is consistent with the increase in the number of IL-10- and IL-4-producing cells (Fig. 7, *B* and *C*). In addition, the frequency of cells producing both IL-10 and IFN-γ was higher than that of cells producing either IL-4 or IL-10 alone. Such IFN-γ producer cells were also found to produce IL-4, indicating that IL-3-primed CD11c$^-$ DCs induce the activation not only of Th2 but also of Th0.

On the other hand, no apparent effect on IL-4, IL-10, and IFN-γ activities was observed in the culture with IFN-α-primed CD11c$^-$ DCs, in contrast to that with untreated DCs (Fig. 5). However, the counteracting effect of IFN-α on IFN-γ-induced-maturing CD11c$^+$ DCs was also evident when these were added simultaneously, and this was also the case with CD11c$^-$ DCs stimulated with IL-3 (Figs. 5 and 7), indicating that the effect of IFN-α on CD11c$^-$ DCs is to modulate the T cell-stimulating activity and T cell functions. IFN-β has been shown to inhibit mitogen-induced IL-12 production in bulk PBMC in an IL-10-dependent mechanism (27). In addition, IFN-$\alpha\beta$ was demonstrated to interfere with macrophage activation via a high affinity receptor for IFN-γ (17). Thus, the defeating action of IFN-α on IFN-γ-mediated stimulation may partly be ascribed to IL-10 produced from DCs and activated T cells. However, the physiological mechanism of the action of IFN-α on IL-3-mediated activation remains to be elucidated. Using IFN-β, we have obtained results similar to those for IFN-α (data not shown).

Recent progress has shown that myeloid DCs and lymphoid DCs can have distinct roles in T cell-mediated immune responses (4); monocyte-derived DCs (DC1) induce Th1 development, while DCs derived from the plasmacytoid cells (DC2) preferentially induce Th2 development. In agreement with this, two types of DCs in the blood without cytokine modification have the capacity to induce different Th cell development, as shown in our present study. However, monocyte-derived DCs treated with IL-10 or PGE_2 can, in turn, induce Th2 development (28, 29). In addition, virus-stimulated plasmacytoid DCs acquire the capacity to induce IFN-γ/IL-10-producing Th cells, but not Th2 cells (26). We have

additionally shown that 1) IFN-γ enhances CD11c$^+$ DC-induced Th1 development, while IFN-α converts this and induces Tr; and 2) IL-3 promotes CD11c$^-$ DC-induced Th2/Th0 development, while IFN-α inhibits this T cell development. Thus, not only do DCs in the different lineage have their own potential to induce respective Th responses, but even DCs in the same lineage can induce different Th responses depending on the signals or cytokine milieu involved in DC maturation.

Based on these findings, the physiological actions of growth/maturation factors such as GM-CSF, IL-3, and both types of IFNs on CD11c$^+$ and CD11c$^-$ DCs are illustrated in Fig. 8. GM-CSF mainly acts on CD11c$^+$ DCs as a factor to induce their development and to maintain their survival, but not to induce maturation in the tissue, whereas IL-3 not only sustains the survival of CD11c$^-$ DCs, but also induces their maturation. Once they encounter infection with virus, micro-organisms, or parasites, CD11c$^+$ DCs pick up the Ags, migrate to the draining lymphoid organs, and induce T cell-mediated specific immune responses to Th1 by other maturation-inducing factors, such as IFN-γ (shown in this study), or TNF-α, IL-1β, and CD40L (30). On the other hand, CD11c$^-$ DCs rapidly produce a remarkable amount of IFN-$\alpha\beta$, which stimulates CD11c$^+$ DC maturation to induce Tr and block CD11c$^-$ DC maturation. Moreover, IFN-$\alpha\beta$ intervenes in the effect of IFN-γ on CD11c$^+$ DCs and that of IL-3 on CD11c$^-$ DCs, thereby rendering them able to polarize T cells toward a Tr response and reducing the Th2/Th0 response, respectively. The effects of IFN-$\alpha\beta$ and IFN-γ depend on their relative concentrations at the local inflammatory sites and within the lymphoid tissues at the initiation and effector phases of the immune response. It can be speculated that IL-12-dependent Th1 response is down-regulated by Tr cells that directly suppress the Th1 response and by Th2 cells that indirectly modulate the differentiation of Th1. Moreover, IFN-$\alpha\beta$ may act on unprimed CD11c$^-$ DCs infiltrating inflammatory lymphoid organs in a paracrine manner to arrest their further maturation. This view seems compatible with the accumulation of plasmacytoid T cells around high endothelial venules areas in inflamed lymphoid tissues (31–33).

These findings indicate that IFN-$\alpha\beta$ is an important factor in the immune network as a negative regulatory element that limits immunopathologic effects of prolonged exposure to Th1 cytokines. The immunomodulatory effects of IFN-$\alpha\beta$ on DC-mediated T cell responses may also provide a novel mechanism for potential therapeutic benefits, for instance in viral infections such as hepatitis B or C (34, 35) and autoimmune diseases (36, 37). In addition, a recent report suggests that recipient DCs might play an important role in the pathogenesis of graft-vs-host disease in allogeneic bone marrow transplantation (38). Therefore, it is interesting to know how IFN-$\alpha\beta$ modulates the induction or severity of graft-vs-host disease through its effects on DCs.

In conclusion, distinctive DC subsets in human peripheral blood exhibit their own functions in the cytokine network, especially in the IFN milieu, resulting in the modulation of immune responses.

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