IL-12/IL-18-Dependent IFN-γ Release by Murine Dendritic Cells

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IL-12/IL-18-Dependent IFN-γ Release by Murine Dendritic Cells

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Dendritic cells (DC) develop in GM-CSF-stimulated cultures from murine bone marrow progenitors in serum-free (or low serum) medium. CD11c<sup>+</sup> myeloid DC from 7-day cultures stimulated with TNF-α, IFN-α, IFN-γ, or LPS up-regulated surface expression of CD40 and CD86 costimulator and MHC class II molecules, did not up-regulate the low “spontaneous” release of IL-12, and did not release IFN-γ. Stimulation of in vitro-generated DC with exogenous IL-12 and IL-18 (but not with IL-4 or LPS plus IL-18) induced IFN-γ expression and release in 15–20% of the DC (detectable by FACS analyses or ELISA). Endogenous IL-12 p70 produced by DC in response to ligation of CD40 stimulated IFN-γ release when exogenous IL-18 was supplied. In vivo-generated, splenic CD8α<sup>+</sup> and CD8α<sup>+</sup> DC (from immunocompetent and immunodeficient H-2<sup>d</sup> and H-2<sup>b</sup> mice) cultured with IL-12 and IL-18 released IFN-γ. The presence of LPS during the stimulation of DC with IL-18 plus endogenous (CD40 ligation) or exogenous IL-12 did not affect their IFN-γ release. In contrast, splenic DC pretreated in vitro or in vivo by LPS strikingly down-regulated IFN-γ release in response to stimulation by IL-12 and (endogenous or exogenous) IL-12. Hence, DC are a source of early IFN-γ generated in response to a cascade of cytokine- and/or cell-derived signals that can be positively and negatively regulated. The Journal of Immunology, 2001, 167: 957–965.

Specific activation of alternative effector functions in an immune response is known as polarization. T cell priming depends on professional APCs that deliver early polarizing signals to naive T cells (reviewed in Ref. 1). Dendritic cells (DC) are the most potent APC known (reviewed in Refs. 2 and 3). When immature, tissue-resident DC encounter a pathogen, they capture its Ags in a microenvironment loaded with signals originating from either the pathogen or nonspecific tissue responses to the pathogen. This stimulates their migration and maturation into presentation-competent APC. In lymph nodes (remote from the site of pathogen invasion), DC prime naive T cells, thereby initating the polarization of the emerging T cell response (1). DC thus seem to be not only key mediators for delivering Ag-specific information to T cells but also convey to them information on the protective effector functions required to eliminate a pathogen. In addition to their role of specifically stimulating T cell responses, the modulation of the Th1- or Th2-promoting capacity of DC has gained considerable interest.

DC of the myeloid lineage (mDC) are potent stimulators of T cell responses. Murine and human bone marrow (BM) precursors develop in vitro into mDC in cultures supplemented with GM-CSF and other cytokines, e.g., IL-4, TNF-α, or Flt3 ligand (FL, fms-like tyrosine kinase 3 ligand) (4–9). DC generated in this system can be used to study the cascade of signals that modulate the phenotype of immature, or activated DC with Th1- or Th2-promoting or -inhibiting capacity. IL-12 is required to stimulate immune responses of IFN-γ-producing CD4<sup>+</sup> T cells (10–14) and CD8<sup>+</sup> CTL (15–18). This is evident by the deficient Th1 immunity in STAT4<sup>−/−</sup>, IL-12α<sup>−/−</sup>, or IL-12β<sub>2</sub> knockout (KO) mice (19–21). DC-derived IL-12 plays a key role in priming Th1 T cell responses (reviewed in Refs. 11 and 22). A major signal that triggers IL-12 release is the ligation of CD40 (23–27). Signaling through CD40 induces IL-12 secretion that synergizes with IL-2 for the induction of IFN-γ production by T cells (23). Signaling of immature DC in vivo is required to induce their competence to respond to CD40 ligation with IL-12 release (28). Th1 polarization has been shown to depend on APC-derived IL-12 and T cell-derived IFN-γ (12, 29). As T cell-derived IFN-γ usually appears only 2–4 days after the initiation of a response, an alternative early source for this cytokine would facilitate early polarization of T cell responses. The cytokine IL-18 facilitates priming of Th1 immune responses in synergy with IL-12 (30–32). IL-18 and IL-12 synergistically stimulate IFN-γ production by T cells (33–35), B cells (36), NK cells (37), macrophages (38, 39), and DC (40, 41). Both major regulator and effector cytokines for Th1 immune responses, IL-12 as well as IFN-γ, can thus be produced by DC. We studied the signals that regulate IFN-γ production by murine DC either generated in vitro from BM progenitors or generated in vivo and isolated as splenic DC.

Materials and Methods

Mice

BALB/cJ (H-2<sup>b</sup>) mice, C.B-17<sup>−/−</sup> and C.B-17<sup>−/−</sup> (SCID) (H-2<sup>b</sup>) mice, and C57BL/6J (H-2<sup>b</sup>) mice were bred and kept under standard pathogen-free conditions in the animal colony of the University of Ulm (Ulm, Germany). Breeding pairs of these mice were obtained from Bomholtgard (Ry, Denmark). BALB/c STAT4<sup>−/−</sup> KO mice were provided by M. J. Grusby (Department of Immunology and Infectious Diseases, Harvard School of Public Health and Department of Medicine, Harvard Medical School, Boston, MA) (19). A breeding colony of these mice was established in Ulm. Female mice were used at 10–16 wk of age.
FIGURE 1. Surface phenotype of CD11c+ DC harvested from 7-day BMC cultures. Nonadherent cells were harvested from serum-free day 7 B6 BMC cultures. CD11c+ DC were purified from this cell population by MACS sorting, stained with Abs to CD11c, CD54, CD40, CD80, CD86, I-A+ (MHC class II), K+ (MHC class I), and NLDC-145 (DEC-205), and analyzed by FACScan. Dead cells were excluded by propidium iodide. A representative example of seven independent analyses is shown.

FIGURE 2. Surface phenotype of cytokine-stimulated, BM-derived CD11c+ DC. Purified CD11c+ DC were harvested from day 7 BMC cultures and restimulated in vitro for another 2 days (at a density of 106 cells/well) in GM-CSF/FL-containing medium supplemented with no cytokines (none) or 20 ng/ml IL-4, IL-12, IL-18, TNF-α, IFN-γ, 500 U/ml IFN-γ type 1, or 1 μg/ml LPS. The cell surface phenotype of the DC was analyzed by flow cytometry.

Generation of DC from BM

The in vitro generation of DC from murine BM has been described (4). Briefly, BM cells (BMC) prepared from femurs were depleted of CD4+ CD8+ B220+ lymphocytes and MHC class II+ cells (cat. no. 492-01, 494-01, 521-01, 524-01; Miltenyi Biotec, Bergisch Gladbach, Germany) by MACS following the manufacturer’s instructions. These BMC depleted of T cells, B cells, and maturing myeloid cells were cultured at a density of 106 cells/ml (cat. no. 150 229; Nunc, Wiesbaden, Germany) in UltraCulture medium (BioWhittaker, Verviers, Belgium) supplemented with 5 ng/ml GM-CSF and 10 ng/ml FL (cat. no. 315-03 and 300-19; PeproTech, Rocky Hill, NJ), 2 mM glutamine, and antibiotics. DC developed from C57BL/6 (B6)-derived BMC in serum-free cultures; growth of DC from BALB/c-derived BM progenitors required 0.5% v/v FCS supplements to the medium. Cultures were incubated at 37°C in humidified air with 5% CO2.

Flow cytometry analyses of DC

Cells were suspended in PBS/0.3% w/v BSA supplemented with 0.1% w/v sodium azide. Nonspecific binding of Abs to FcR was blocked by preincubating cells with the anti-CD16/CD32 mAb 2.4G2 (1 μg/106 cells; cat. no. 01240D; PharMingen, Hamburg, Germany). Cells were incubated with 0.5 μg/106 cells of the relevant mAb for 30 min at 4°C, washed twice, and subsequently incubated with a second-step reagent for 15 min at 4°C. Cells were washed twice and analyzed on a FACScan (BD Biosciences, Mountain View, CA). Dead cells were excluded by propidium iodide staining. The following reagents and mAbs from PharMingen were used: PE-conjugated anti-I-A+I-E+ (cat. no. 06345A); biotinylated anti-H-2D b,d,k (cat. no. 06232D); PE-conjugated anti-I-Ad/I-E d (cat. no. 06345A); PE-conjugated anti-I-A b (cat. no. MCA949) from Biozol (Eching, Germany), the FITC-conjugated IgG1 mAb R3-34 (cat. no. 20614A), and streptavidin-Red670 (cat. no. 19543-024; Life Technologies, Carlsbad, CA). Dead cells were excluded by propidium iodide. A representative example of seven independent analyses is shown.

CD4+ CD8+ T cells were harvested, washed twice in staining buffer with 0.5% w/v BSA, 0.1% w/v sodium azide, incubated (15 min, 4°C) with 2.4G2 Ab to block nonspecific binding of Ab to FcR, washed with staining buffer, resuspended in HBSS (cat. no. 24020-091; Life Technologies), surface stained with a conjugated anti-CD11c Ab, washed twice with HBSS, resuspended in 100 μl Cytofix/Cytoperm solution for 20 min at 4°C, and washed twice in 1 ml 1× Perm/Wash solution. Fixed and permeabilized cells were resuspended in 50 μl 1× Perm/Wash solution and stained for 30 min at 4°C with either the FITC-conjugated anti-IFN-γ Ab (cat. no. 18114A) or FITC-conjugated rat IgG1 isotype control Ab (cat. no. 20611A). Cells were washed twice in 1× Perm/Wash solution and suspended in staining buffer. Cells (106) were analyzed by flow cytometry using a FACScan equipped with a 15-mW argon laser (BD Biosciences) and CellQuest software (BD Biosciences). Analysis gates were set on CD11c+ cells.

Splenic DC

Splenic DC were purified as described by Fukao et al. (41). Briefly, spleens were cut into small pieces and incubated for 45 min in RPMI 1640 medium containing collagenase I (0.5 mg/ml) and DNase (0.1 mg/ml). EDTA (at a final concentration of 10 mM) was added for the last 3 min of the incubation period. The tissue pieces were minced through a nylon mesh. Single cells were harvested, washed, resuspended in Nycodenz overlaid with RPMI 1640 medium, and centrifuged at 4°C at 9500 × g for 20 min. Cells on the interface were collected, washed twice, and cultured for 2 h to allow DC to adhere. Nonadherent cells were removed, and adherent DC were incubated overnight to detach. From these cells, contaminating B and T cells were depleted by MACS using biotinylated anti-CD3, anti-CD4, anti-B220 mAb (PharMingen), and streptavidin-coupled microbeads. From the
CD3+ CD4+ B220− population, CD8α+ DC were positively selected by MACS. From the CD8α− fraction, CD11c+ DC were positively selected by MACS. Flow cytometric analyses demonstrated that these DC subsets contained >98% CD8α− CD11c+ cells or CD8α+ CD11c− DC. These purified DC were cultured at 5 × 10^4/10^5 well with the indicated amounts of cytokines in RPMI 1640/5% FCS in 150 μl flat-bottom microwells.

**LPS injection of mice**

Mice were injected i.v. 6 h before sacrifice with either 50 μg LPS (in 200 μl PBS) per mouse (cat. no. t.2143; Sigma, St. Louis, MO) or 200 μl PBS (solvent control).

**Cytokines and cytokine detection by ELISA**

The following recombinant mouse cytokines were obtained from Pepro-Tech: IL-4 (cat. no. 214-14), IL-18 (cat. no. 315-04), TNF-α (cat. no. 315-01A), GM-CSF (cat. no. 315-03), and FL (cat. no. 300-19). IL-12 p40 (cat. no. 499-ML) was purchased from R&D Systems (Wiesbaden, Germany). IFN-γ (cat. no. 19301T) and IL-12 p70 (cat. no. 19361V) were obtained from PharMingen. Universal type I IFN (human IFN-α A/D, Bg-III) was obtained from PBL Biomedical Laboratories (New Brunswick, NY).

Cytokines in supernatants were detected by conventional double-sandwich ELISA. For detection and capture, the following mAbs (from PharMingen) were added at 6.6 μg/ml: IFN-γ (cat. no. 499-ML) was purchased from R&D Systems (Wiesbaden, Germany). IFN-γ (cat. no. 499-ML) was purchased from R&D Systems (Wiesbaden, Germany) using the EasyWin software (TECAN). The detection limits of ELISA. Mean values of triplicates (±SD) of one of three experiments are shown.

Results

**The generation of mDC from BM progenitors**

We generated DC from (T cell−, B cell−, mature myeloid cell-depleted) BM progenitors of BALB/cJ, BALB/c STAT4−/−, or B6 mice in cultures supplemented with GM-CSF and FL. DC were generated from B6-derived BMC in serum-free cultures; growth of DC from BALB/c-derived BM progenitors required 0.5% v/v FCS supplements to the medium. Of the nonadherent cells harvested from 7-day cultures, 50–70% were CD11c+. The CD11c+ cells were CD11b− NLDC-145− CD54− CD4+ CD8− and expressed readily detectable levels of MHC class I molecules and CD80 (Fig. 1 and data not shown). Expression of the macrophage-specific markers CD14 or F4/80 was not detected (data not shown). This phenotype resembles that previously described for immature mDC (42–44). In CD11c+ mDC populations from day 7 cultures, a subset of 20–30% of the cells showed up-regulated surface expression of MHC class II, CD40, and CD86 molecules (Fig. 1). Incubating mDC for another 2 days in GM-CSF and FL increased the fraction of DC with up-regulated surface expression of costimulator and MHC class II molecules (Figs. 1 and 2 and data not shown). Purified mDC (from all mouse strains tested) cultured with GM-CSF/FL “spontaneously” released low amounts of IL-12 p40 and IL-18, but no IFN-γ into the supernatant (Fig. 3 and data not shown). Thus, the majority of in vitro-cultured mDC maintained an immature phenotype during a 7- to 9-day culture period, but a small subset of these cells spontaneously matured under serum-free culture conditions. In this system, we studied signals that trigger IFN-γ release by DC.

**Cytokine stimulation of in vitro-generated DC**

On day 7 of culture, nonadherent cells were harvested. CD11c− cells were purified by MACS (cat. no. 520-01; Miltenyi Biotec) and replated in UltraCulture medium supplemented with GM-CSF, FL, glutamine, antibiotics, and in the case of BALB/c-derived DC with 0.5% v/v FCS. In some experiments, DC were cultured for a further 2–3 days in medium to which the indicated cytokines were added at a concentration of 20 ng/ml; LPS was added at 1 μg/ml, and IL-12 was added at 500 U/ml. On day 9–10 of culture, CD11c+ cells were analyzed by flow cytometry or used in further studies. CD11c+ DC were washed three times and seeded into round-bottom microwells at 2 × 10^4 cells/200 μl in UltraCulture medium with GM-CSF, FCS, and antibiotics. These cultures were stimulated with the indicated amounts of IL-12 p70, IL-18, and/or IL-4. In some experiments, irradiated (18,000 rad) CD40 ligand (CD40L)-transfected J558L cells (or nontransfected J558L control cells) were added at 6.6 × 10^5 cells/milliliter. After a 48-h incubation period, supernatants were harvested and tested for cytokines by ELISA.

![FIGURE 3. IL-12 release by cytokine-stimulated CD11c+ DC. CD11c+ DC purified from day 7 BMC cultures were cultured in vitro in GM-CSF/FL-containing medium (at 10^6 cells/well) for 2 days with 20 ng/ml IL-4, IL-18, TNF-α, IFN-γ, 500 U/ml IFN-γ type 1, 1 μg/ml LPS, or J558/CD40L transfectants (3.3 × 10^5/ml). IL-12 p70 and IL-12 p40 were detected in supernatants of DC cultures by conventional double-sandwich ELISA. Mean values of triplicates (±SD) of one of three experiments are shown.](http://www.jimmunol.org/content/jimmunol/176/12/959/F3.large.jpg)
spontaneous IL-18 release, and did not induce IFN-γ release by DC.

Synergistic action of IL-12 and IL-18 on DC stimulates IFN-γ release

IFN-γ was not released by DC stimulated with cytokines or LPS. In contrast, stimulation with IL-12 plus IL-18 efficiently induced IFN-γ release by DC (Fig. 4A). This confirms a recent report (41). IL-4 codelivered with either IL-12 or IL-18 (over a broad dose range) did not trigger IFN-γ release by DC, as evident in four independent experiments using different mouse strains. (Fig. 4, A and B and data not shown). This observation is in contrast to previously reported data (41). IL-12 responsiveness of DC was required to generate an IFN-γ response after synergistic stimulation with IL-12 and IL-18 because DC from STAT4−/− KO BALB/c mice did not release IFN-γ after IL-12/IL-18 stimulation (Fig. 5). Titration experiments showed that fairly high doses of 20–60 ng/ml IL-18 were required to stimulate IFN-γ release by DC co-stimulated with 20 ng/ml IL-12 (Fig. 4B). IFN-γ release by DC stimulated with IL-12 and IL-18 increased substantially over a 3- to 4-day culture period (Fig. 4C). Cytoplasmic staining of DC for IFN-γ followed by flow cytometry analyses showed that 15–20% of the cells expressed IFN-γ after a 96-h stimulation with IL-12/IL-18 (Fig. 4D). The IFN-γ+ and IFN-γ− mDC subsets showed no detectable difference in surface marker expression. They were CD4−CD8+ and displayed reduced levels of CD11c, CD11b, and DEC-205 on the cell surface (data not shown). Exogenous IL-12 and IL-18 hence synergistically stimulate expression and release of IFN-γ by mDC.

IL-12 p70 released by DC stimulated through CD40 induces IFN-γ release in synergy with IL-18

As shown in Fig. 3 and in previous reports (23–25, 27, 50), CD40 ligation is a potent stimulus that triggers IL-12 release by DC. CD40 ligation (coculture with the CD154/CD40L-expressing cell line J558/LCD154) of DC produced 120 ng/ml IL-12 p40 in BALB/c mice and 25 ng/ml IL-12 p40 in B6 mice; in both mouse strains tested CD40 ligation triggered release of similar amounts 0.3–0.5 ng/ml IL-12 p70 (Figs. 3 and 6A). Release of both immunosuppressive IL-12 p40 and bioactive IL-12 p70 is thus substantially increased by CD40 ligation. In BALB/c mice, a 250-fold excess of IL-12 p40 over IL-12 p70 was released whereas in B6 mice this excess was 80-fold (Fig. 6A). This was found in seven independent experiments using BM-derived DC from BALB/c and B6 mice.

FIGURE 4. CD11c+ DC release IFN-γ after stimulation with IL-12 and IL-18. A, Purified CD11c+ DC were stimulated for 72 h with 20 ng/ml IL-12, IL-18, IL-4, or combinations of these cytokines. IFN-γ was measured by ELISA in supernatants conditioned by stimulated DC. B, IL-18, but not IL-4, induces IFN-γ release by CD11c+ DC in synergy with IL-12 (20 ng/ml). C, Kinetic of IFN-γ release by CD11c+ DC stimulated with IL-12 (20 ng/ml) and IL-18 (60 ng/ml) for 48–96 h. D, A subset of 15–20% of CD11c+ DC expresses cytoplasmic IFN-γ after stimulation with IL-12 and IL-18, as detected by flow cytometric analyses.
that is also abundantly produced by the stimulated DC) coinduces bioactive IL-12 p70 heterodimer (and not the IL-12 p40 molecule in the presence of a neutralizing anti-IL-12 mAb (group 7) that CD40 ligation and exogenous IL-18 also triggered IFN-γ (Fig. 7). Coculture of DC with the CD154-expressing cell line J558L/CD154 (group 5), the CD154− control cell line J558L (group 1), or with high doses of either IL-12 (group 2) or IL-18 (group 3) did not trigger IFN-γ release by DC. Stimulation of DC with exogenous IL-12 plus exogenous IL-18 efficiently triggered an IFN-γ response by DC (group 4). The combined stimulation by CD40 ligation and exogenous IL-18 also triggered IFN-γ release by DC (group 6) that was 3-fold lower than the IFN-γ release in response to high doses of exogenous IL-12 and IL-18. Hence, endogenous IL-12 p70 can synergize with IL-18 in stimulating IFN-γ expression by DC. IFN-γ release by DC was completely abrogated in the presence of a neutralizing anti-IL-12 mAb (group 7) that recognizes IL-12 p35 and IL-12 p70 (but not IL-12 p40). Thus, the bioactive IL-12 p70 heterodimer (and not the IL-12 p40 molecule that is also abundantly produced by the stimulated DC) coinduces the IFN-γ response of DC. Hence, ligation of CD40 on the surface of DC initiates an autocrine, IL-12/IL-18-dependent cascade that facilitates IFN-γ release by DC. Endogenous IL-12 p70 operates efficiently despite a large excess of immunosuppressive IL-12 p40 in the system.

**Autocrine IL-18 release by DC is insufficient to prime them for IFN-γ release in response to IL-12**

BM-derived DC spontaneously release low levels of IL-18 during culture. Neither the ligation of CD40 on the surface of DC nor the stimulation of DC by LPS or cytokines (TNF-α, type I or II IFNs, IL-4, or IL-12) enhanced the spontaneous IL-18 release by DC (data not shown). Furthermore, these cytokines did not enhance the spontaneous IL-18 release by DC stimulated by either LPS or surface CD40 ligation (data not shown). Exogenous IL-18 at a dose >10 ng/ml was required to trigger a detectable IFN-γ response by mDC (Fig. 4B). The IL-18 release of cultured DC was thus low and not up-regulated by the tested stimuli that induce DC maturation. This level of IL-18 release was insufficient to support autocrine induction of IFN-γ release by DC. To efficiently stimulate IFN-γ release by DC under physiological conditions, either an additional (cytokine or cell interaction) trigger is required to enhance IL-18 production by DC or IL-18 has to be supplied by an alternative source.

**Modulation of IL-12 p70 release by DC induced by CD40 ligation has little effect on their IFN-γ response**

CD40 ligation stimulates DC (from different inbred mouse strains) to release bioactive IL-12 p70 and immunosuppressive IL-12 p40 (Fig. 6A). DC pretreated with IL-4 or TNF-α before CD40 ligation showed enhanced release of IL-12 p70 as well as a 3- to 10-fold reduction of the p40:p70 ratio of the released IL-12 (Fig. 6B). Pretreatment of DC with IL-12, IL-18, or LPS had little effect on their IL-12 p70 release in response to CD40 ligation (Fig. 6B and data not shown). When IL-4, TNF-α, or LPS were present during the stimulation of DC by exogenous IL-18 and CD40 ligation, their IFN-γ release was not modulated (Fig. 6C and data not shown). Hence, these well-characterized maturation signals for DC have only a limited effect on IFN-γ release costimulated by IL-12/IL-18.

DC pretreated in vitro with LPS down-modulates IFN-γ release in response to IL-12/IL-18 stimulation

We tested whether endogenously produced IL-12 produced by DC after CD40 ligation can synergize with IL-18 to stimulate IFN-γ release (Fig. 7). Coculture of DC with the CD154-expressing cell line J558L/CD154 (group 5), the CD154− control cell line J558L (group 1), or with high doses of either IL-12 (group 2) or IL-18 (group 3) did not trigger IFN-γ release by DC. Stimulation of DC with exogenous IL-12 plus exogenous IL-18 efficiently triggered an IFN-γ response by DC (group 4). The combined stimulation by CD40 ligation and exogenous IL-18 also triggered IFN-γ release by DC (group 6) that was 3-fold lower than the IFN-γ release in response to high doses of exogenous IL-12 and IL-18. Hence, endogenous IL-12 p70 can synergize with IL-18 in stimulating IFN-γ expression by DC. IFN-γ release by DC was completely abrogated in the presence of a neutralizing anti-IL-12 mAb (group 7) that recognizes IL-12 p35 and IL-12 p70 (but not IL-12 p40). Thus, the bioactive IL-12 p70 heterodimer (and not the IL-12 p40 molecule that is also abundantly produced by the stimulated DC) coinduces the IFN-γ response of DC. Hence, ligation of CD40 on the surface of DC initiates an autocrine, IL-12/IL-18-dependent cascade that facilitates IFN-γ release by DC. Endogenous IL-12 p70 operates efficiently despite a large excess of immunosuppressive IL-12 p40 in the system.

**Autocrine IL-18 release by DC is insufficient to prime them for IFN-γ release in response to IL-12**

BM-derived DC spontaneously release low levels of IL-18 during culture. Neither the ligation of CD40 on the surface of DC nor the stimulation of DC by LPS or cytokines (TNF-α, type I or II IFNs, IL-4, or IL-12) enhanced the spontaneous IL-18 release by DC (data not shown). Furthermore, these cytokines did not enhance the spontaneous IL-18 release by DC stimulated by either LPS or surface CD40 ligation (data not shown). Exogenous IL-18 at a dose >10 ng/ml was required to trigger a detectable IFN-γ response by mDC (Fig. 4B). The IL-18 release of cultured DC was thus low and not up-regulated by the tested stimuli that induce DC maturation. This level of IL-18 release was insufficient to support autocrine induction of IFN-γ release by DC. To efficiently stimulate IFN-γ release by DC under physiological conditions, either an additional (cytokine or cell interaction) trigger is required to enhance IL-18 production by DC or IL-18 has to be supplied by an alternative source.
Splenic DC from mice pretreated in vivo with LPS strikingly down-regulate IFN-γ release in response to IL-12/IL-18

We showed that DC pretreated in vitro with LPS down-regulate IFN-γ release stimulated by IL-12/IL-18 (Fig. 6C). We tested whether the pretreatment of DC in vivo impairs their ability to produce IFN-γ when stimulated by IL-12/IL-18.

Splenic DC obtained from LPS-treated mice showed evidence of activation, i.e., up-regulation of CD40, CD86, and MHC class II surface expression (Fig. 9A). Splenic DC from LPS-pretreated mice produced lower amounts of IL-12 p40 and showed almost complete suppression of IL-12 p70 release after CD40 ligation (Fig. 9B). IFN-γ release by splenic CD11c+ DC from LPS-pretreated mice was strikingly reduced after in vitro stimulation by either IL-12/IL-18 or CD40 ligation/IL-18 when compared with IFN-γ release by splenic DC from nontreated mice (Fig. 9B). This was observed with immunocompetent BALB/c and B6 mice in three independent experiments. The presence of LPS in the in vitro stimulation cultures had no influence on the tested response pattern (Fig. 9B). DC pretreated with LPS either in vitro or in vivo thus down-regulate IFN-γ release in response to IL-12/IL-18 stimulation.

Discussion

We generated in vitro murine mDC from BM progenitors using an established system based on GM-CSF (52). The system was modified to enhance the yield of DC and to prevent in vitro DC maturation. These changes included the use of serum-free culture conditions and the exclusion of exogenous factor supplements (e.g., IL-4 or TNF-α) that trigger DC maturation. From B6-derived BM progenitors stimulated by GM-CSF and FL, immature DC developed in the culture system in the absence of serum. In contrast, BALB/c-derived BM progenitors required low serum supplements to the medium (0.5% v/v FCS) to support DC development. FL has been shown to expand DC in GM-CSF-stimulated BMC cultures but does not induce DC maturation. Other cytokines were not added to BMC cultures in which mDC were generated and expanded. Although the lineage relationships between DC subsets (e.g., monocyte-derived DC, Langerhans cells, lymphoid-related DC, BM-derived mDC) are not definitely established, the CD11b+/CD11c+ CD4+ CD8- NLDC-145/DEC-205+ CD14+ surface phenotype of the DC we generated resembles that described for mDC.

Within the cultured DC populations, a subset of 20–30% of the cells showed evidence of CD40, CD86, and MHC class II surface expression (Fig. 9A). Splenic DC from LPS-pretreated mice produced lower amounts of IL-12 p40 and showed almost complete suppression of IL-12 p70 release after CD40 ligation (Fig. 9B). IFN-γ release by splenic CD11c+ DC from LPS-pretreated mice was strikingly reduced after in vitro stimulation by either IL-12/IL-18 or CD40 ligation/IL-18 when compared with IFN-γ release by splenic DC from nontreated mice (Fig. 9B). This was observed with immunocompetent BALB/c and B6 mice in three independent experiments. The presence of LPS in the in vitro stimulation cultures had no influence on the tested response pattern (Fig. 9B). DC pretreated with LPS either in vitro or in vivo thus down-regulate IFN-γ release in response to IL-12/IL-18 stimulation.

FIGURE 6. Modulation of IL-12 p40, IL-12 p70, and IFN-γ release of DC in response to CD40 ligation by cytokines or LPS. A, Purified, BM-derived CD11c+ DC from BALB/c or B6 mice were cocultured in GM-CSF/FL-supplemented medium for 2 days with CD40L-expressing J558L/CD40L transfectants. IL-12 p40 and IL-12 p70 were determined by double-sandwich ELISA. B, Purified, BM-derived CD11c+ DC from B6 mice were cultured in GM-CSF/FL-supplemented medium for 2 days with 20 ng/ml IL-4, IL-12, IL-18, TNF-α, or 1 μg/ml LPS. The DC were washed and cocultured for an additional 2 days with CD40L-expressing J558L/CD40L transfectants. IL-12 p40 and IL-12 p70 were determined by double-sandwich ELISA. C, Purified, BM-derived CD11c+ DC from B6 mice were cultured in GM-CSF/FL-supplemented medium for 2 days with either no additional stimuli (pretreatment in vitro: none) or with 1 μg/ml LPS (pretreatment in vitro: LPS). The DC were washed and cultured for an additional 2 days with IL-18 plus IL-12, IL-18 plus the CD40L-expressing J558L/CD40L transfectants, IL-18 plus LPS, or IL-18 plus LPS plus J558L/CD40L transfectants. IFN-γ was measured by ELISA in supernatants conditioned by stimulated DC. Mean values of triplicates (±SD) of one of four independent experiments are shown.
maturation-suppressing factors was thus apparent in the system confirming previous experience with DC cultures (28). It seems difficult to control spontaneous DC differentiation using currently available DC separation and culture techniques.

IL-12 release by DC is well characterized. Stimulation of DC by GM-CSF and TNF-α or LPS (but not GM-CSF and IL-4, IL-18, or IFN) triggers IL-12 release (Fig. 3A and data not shown) (8). Although this pathway leads preferentially to IL-12 p40 release (as described in Ref. 53), we also found some release of IL-12 p70 by LPS-stimulated DC (Fig. 3A). Spontaneous IL-12 release by DC was enhanced 5- to 10-fold by TNF-α or LPS stimulation. In contrast, it was enhanced >100-fold by CD40 ligation (Fig. 3A). This confirms that CD40-dependent signals are the most potent stimuli for IL-12 release by DC (23–27). Although lymphoid-related DC have often been considered the main source of DC-derived IL-12, our data indicate that mDC are also potent producers of IL-12. We describe that BM-derived DC stimulated by CD40 ligation produce a large excess of immunosuppressive IL-12 p40. IL-12 p40 homodimers inhibit Th1-polarized immune responses by suppressing IFN-γ release from spleen cells, CTL priming, IgG2/IgG3 Ab responses, and allo-specific delayed-type hypersensitivity reactions and prolonging allograft survival (53–55). Because IL-12 p70 secreted by DC stimulated IFN-γ release (the response was blocked by an anti-IL-12 p35 Ab), its bioactivity was readily detectable in the presence of excess IL-12 p40.

IL-18 is produced by DC (35), but the stimuli that trigger IL-18 expression by DC are not well defined. Spontaneous IL-18 release by DC is detectable but is not up-regulated by stimulation with cytokines, LPS, or CD40 ligation (data not shown). IL-12 produced by mDC in response to CD40 ligation supports their IFN-γ response. In contrast, endogenous IL-18 release by mDC is insufficient to support IFN-γ expression by mDC (because exogenous IL-18 had to be provided to detect this response). The release of IL-18 by DC may be low, the secreted IL-18 may be rapidly cleared from the supernatant by DC, and/or the main stimulus that drives IL-18 release by DC may not have been identified. IL-18 is an interesting cytokine: it drives Th2-biased responses of the innate and specific immune system, but strikingly synergizes with IL-12 in driving Th1-polarized immune responses (56). Thus it is a key mediator that can switch a prevailing Th2- into a Th1-biased micromilieu if IL-12 appears.

DC do not release IFN-γ spontaneously or in response to stimulation by IFN, IL-4, IL-12, IL-18, LPS, or CD40 surface molecules (data not shown). We show in this study the expression and release of IFN-γ by murine DC stimulated with IL-12 and IL-18. This confirms and extends reports of IFN-γ production by CD8α+ lymphoid DC (40) and murine splenic DC populations (41). FACS analyses indicate that 15–20% of the stimulated CD11c+ DC population express IFN-γ (Fig. 4D). We did not detect a marker profile characteristic for IFN-γ-producing DC. In contrast to a previous

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** CD40 ligation generates endogenous IL-12 that synergizes with IL-18 in stimulating IFN-γ release by DC. Purified, BM-derived CD11c+ DC from B6 mice were stimulated for 72 h with nontransfected J558L cells (lane 1), 20 ng/ml IL-12 (lane 2), 60 ng/ml IL-18 (lane 3), both IL-12 and IL-18 (lane 4), CD40L-expressing J558L/CD40L transfectants (lane 5), J558L/CD40L cells and IL-18 (lane 6), or J558L/CD40L cells, 20 ng/ml IL-18, and 10 μg/ml anti-IL-12 p35 Ab (lane 7). IFN-γ was determined in supernatants by double-sandwich ELISA. Mean values of triplicates (±SD) of one of three independent experiments are shown.

![Figure 8](http://www.jimmunol.org/)

**FIGURE 8.** Splenic CD8α+ or CD8α− DC produce IFN-γ after IL-12/IL-18 stimulation. CD11c+ DC were isolated from the spleen of immunocompetent BALB/c or B6 mice or severely immunodeficient (SCID) C.B-17scid/scid mice. CD8α+ or CD8α− CD11c+ DC subsets were prepared as described in Materials and Methods. These cells were cultured in GM-CSF-supplemented medium for 3 days, either with no additional cytokines or with IL-12, IL-18, or IL-12 plus IL-18. IFN-γ was determined in supernatants by double-sandwich ELISA. Mean values of triplicates (±SD) of one of five independent experiments are shown.
report (41), IFN-γ release was readily inducible in CD8α+ and CD8β+ splenic DC (Fig. 8). Hence, IFN-γ is produced by many cell types stimulated with IL-12 and IL-18, including NK cells, B cells, DC, and macrophages.

The presence of LPS had no effect on the IFN-γ release of DC stimulated by IL-12/IL-18 or CD40 ligation/IL-18. In contrast, pretreating DC in vitro or in vivo with LPS strikingly down-regulated their ability to respond with IFN-γ release to stimulation by IL-12/IL-18 or CD40 ligation/IL-18. DC have been shown to produce IL-12 only transiently and to become refractory to further stimulation (57). This exhaustion of cytokine production is also evident from our data and is particularly striking for IFN-γ production. This observation has implications for T cell priming conditions in, e.g., gut or liver, where continuous LPS exposure prevails.

Our data suggest a cascade of events. Immature mDC spontaneously release low levels of IL-12, IL-18, and chemokines (but no IFN-γ); it is uncertain whether this takes place also in vivo or whether it reflects in vitro culture conditions that activate DC. Most proinflammatory cytokines tested up-regulate surface expression of MHC and costimulator molecules on the surface of DC but do not stimulate their release of Th1-promoting cytokines (IL-12, IL-18, or IFN-γ). Ligation of CD40 on the surface of DC is the most potent stimulus for IL-12 p70 release by DC but does not enhance IL-18 or IFN-γ release. IL-12 p70 confers IL-18 responsiveness to DC by inducing the IL-18 receptor. The synergistic action of IL-12 p70 and IL-18 induces IFN-γ expression in DC. Thus a cascade of cytokines and cellular interactions is required to induce and/or enhance the Th1-promoting capacity of mDC. These data may help to condition DC to enhance their efficacy in specific, adoptive immunotherapies of cancer, autoimmunity, and chronic infectious disease.

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