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Murine Plasmacytoid Dendritic Cells Produce IFN-\(\gamma\) upon IL-4 Stimulation\(^1\)

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IL-4 plays a key role in inducing IL-4 production in CD4\(^+\) T cells, functioning as an important determinant for Th2 cell differentiation. We show here that IL-4 induces IFN-\(\gamma\)-production in B220\(^+\) plasmacytoid dendritic cells (PDCs). By searching for cell populations that produce IFN-\(\gamma\) upon IL-4 stimulation, we found that PDCs were a major IFN-\(\gamma\)-producing cell upon IL-4 stimulation in wild-type and Rag-2\(^{-/-}\) splenocytes. Isolated PDCs, but not CD11b\(^+\) DCs or CD8\(^+\) DCs, produced IFN-\(\gamma\)-upon IL-4 stimulation. In vivo, the depletion of PDCs by anti-Ly6G/C Ab prevented IFN-\(\gamma\)-production induced by IL-4 administration. We also found that IL-4 induced IFN-\(\gamma\)-production, but not IL-12 or IFN-\(\alpha\)-production, in PDCs and also strongly enhanced CpG oligodeoxynucleotide-induced IFN-\(\gamma\)-production, but not CpG oligodeoxynucleotide-induced IL-12 or IFN-\(\alpha\)-production. However, IL-4 did not induce IFN-\(\gamma\)-production in Stat6\(^{-/-}\) PDCs. Moreover, IL-4 induced Stat4 expression in PDCs through a Stat6-dependent mechanism, and only the Stat4-expressing PDCs produced IFN-\(\gamma\). Furthermore, IL-4 did not induce IFN-\(\gamma\)-production in Stat4\(^{-/-}\) PDCs. These results indicate that PDCs preferentially produce IFN-\(\gamma\)-upon IL-4 stimulation by Stat6- and Stat4-dependent mechanisms. The Journal of Immunology, 2005, 175: 5681–5689.

Cytokine environment is critical for the differentiation and commitment of immune cells. For example, IL-4, a representative Th2 cytokine, induces further Th2 cell differentiation, whereas a Th1 cytokine IFN-\(\gamma\)-in coordination with IL-12 induces Th1 cell differentiation (1–3). Although these positive feedback mechanisms are essential for the profound differentiation of Th cells, the immune system also has a number of intrinsic and extrinsic machinery to antagonize the excessive differentiation of immune cells (4, 5).

Dendritic cells (DCs)\(^3\) are a migratory group of bone marrow-derived leukocytes with at least three subtypes in mouse spleen: CD8\(^+\) DCs, CD11b\(^+\) DCs, and B220\(^+\) DCs (‘plasmacytoid DCs’) (PDCs) (6, 7). Although CD8\(^+\) DCs and CD11b\(^+\) DCs express high levels of MHC class II molecules and costimulatory molecules such as CD80 and induce T cell proliferation, PDCs express MHC class II molecules at very low levels, do not express CD80, and fail to stimulate T cell proliferation (8, 9). These findings suggest that PDCs are immature DCs with a weak ability as APCs. On the other hand, PDCs localize in the T cell zone of lymphoid tissues and produce a large amount of type I IFNs upon bacterial or viral infection (10–12). Therefore, it is suggested that PDCs play a key role in innate immune responses.

Recently, a number of experiments have suggested that innate immune responses contribute significant polarizing influences on Th differentiation (13). The global view is that TLR activation of APCs such as DCs induces cytokine production that favors Th1-type immune responses and prevents the development of deleterious Th2 responses (13). On the other hand, a recent study has shown that PDCs inhibit Th2 responses even in the absence of TLR signaling (14). However, the role of PDCs in Th differentiation is still largely unknown.

In this study, by searching for cell populations that produce IFN-\(\gamma\)-upon IL-4 stimulation, we found that PDCs were a major IFN-\(\gamma\)-producing cell upon IL-4 stimulation and that IL-4 preferentially induced IFN-\(\gamma\)-production in PDCs by a Stat6-dependent mechanism. We also found that IL-4 induced Stat4 expression in PDCs through a Stat6-dependent mechanism and that only the Stat4-expressing PDCs produced IFN-\(\gamma\). Furthermore, we found that Stat4-deficient PDCs did not produce IFN-\(\gamma\)-upon IL-4 stimulation. Our results highlight a unique function of IL-4-induced IFN-\(\gamma\)-production in PDCs in the immune regulation of cytokine networks.

Materials and Methods

Mice

BALB/c mice were purchased from Charles River Laboratories. Stat6-deficient (Stat6\(^{-/-}\)) mice (15) and Rag-2\(^{-/-}\) mice were backcrossed for more than eight generations onto BALB/c mice. Stat4\(^{-/-}\) mice on a BALB/c background were purchased from The Jackson Laboratory. OVA-specific DO11.10 TCR transgenic (DO11.10\(^{10\%}\)) mice (16) were backcrossed over 10 generations onto BALB/c mice. All mice were housed in microisolator cages under specific pathogen-free conditions and all experiments were performed according to the guidelines of Chiba University.

Reagents

Mouse IL-2, IL-4, IL-7, IL-9, IL-13, and IL-15 were purchased from PeproTech. Phosphorothioate-stabilized CpG oligodeoxynucleotide (ODN) 1668 (TCCATGACGTTCCTGATGCT) was purchased from Hokkaido System Science.

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\(^3\) Abbreviations used in this paper: DC, dendritic cell; PDC, plasmacytoid DC; ODN, oligodeoxynucleotide; WT, wild type.
Flow cytometric analysis

Cells were stained and analyzed on a FACSCalibur (BD Biosciences) using CellQuest software. The following Abs were purchased from BD Pharmingen: anti-CD4 FITC, PE (H129.19), anti-CD8 FITC, PE (53-6.7), anti-CD220 FITC, PE, allophycocyanin, PerCP, biotin (RA3-6B2), anti-CD3 PE (145-2C11), anti-CD19 PE (ID3), anti-CD11b (Mac-1) PE (M170), anti-CD11c FITC (HL3), anti-Ly6G/C PE (RB6-8C5), anti-erythroid PE (TER-119), anti-pan NK PE (DX5), anti-CD80 PE (16-10A1), anti-CD86 PE (GL-1), and anti-I-A^d PE (AMS-32.1). Before staining, FcRs were blocked with anti-CD16/32 Ab (2.4G2; BD Pharmingen). Negative controls consisted of isotype-matched, directly conjugated, nonspecific Abs (BD Pharmingen).

Isolation of DC subtypes

Splenic DCs were prepared using OptiPrep (Axis Shield) according to the manufacturer’s instructions. In brief, spleens were cut into small fragments and then digested with collagenase A (0.5 mg/ml; Roche) for 10 min at 37°C with continuous agitation. Digested fragments were filtered through a stainless steel sieve, and cells were resuspended in 3 ml of HBSS and then mixed with 1 ml of OptiPrep to make 15% iodoxalin solution (density 1.085 g/ml). Cell suspension was overlaid with 5 ml of 12% iodoxalin solution (density 1.069 g/ml) and 1 ml of HBSS. Low-density cells were collected by centrifugation at 600 × g for 15 min at room temperature. Low-density cells were stained with anti-CD11c FITC and FITC-stained cells were positively collected using anti-FITC microbeads (Miltenyi Biotec), according to the manufacturer’s instructions. The resultant cells were routinely >95% pure CD11c^+ cells by FACS analysis.

To isolate PDCs, low-density cells were prepared from wild-type (WT), Stat6^−/− splenocytes, or Stat4^−/− splenocytes and then stained with a mixture of PE-labeled Abs to CD3, CD11c, DX5, and TER-119. After PE-stained cells were depleted using anti-PE microbeads (Miltenyi Biotec), cells in flow-through were stained with anti-B220 biotin and subsequently B220^+ cells were positively collected using streptavidin microbeads (Miltenyi Biotec). Alternatively, PDCs were purified using a PDC isolation kit according to the manufacturer’s instructions (Miltenyi Biotec). In both cases, the resultant cells were >95% pure B220^+CD19^− cells by FACS analysis.

For CD11b^+ DCs purification, low-density cells were prepared from WT splenocytes and stained with a mixture of PE-labeled Abs to CD3, B220, DX5, TER-119, and CD8. After PE-stained cells were depleted using anti-PE microbeads, cells in flow-through were stained with anti-CD11c FITC and CD11c^+ cells were positively collected using anti-FITC microbeads. The resultant cells were routinely >95% pure CD11b^+CD8^−CD11c^+ cells by FACS analysis.

For CD8^+ DCs purification, low-density cells were prepared from WT splenocytes and stained with a mixture of PE-labeled Abs against CD3, B220, DX5, TER-119, and CD8. After PE-stained cells were depleted using anti-PE microbeads, cells in flow-through were stained with anti-CD11c FITC and CD11c^+ cells were positively collected using anti-FITC microbeads. The resultant cells were routinely >95% pure CD11b^+CD8^+CD11c^+ cells by FACS analysis.

Cell culture

Isolated PDCs, CD11b^+ DCs, or CD8^+ DCs were cultured (5 × 10^4/ml) in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 50 μM 2-ME, 2 mM l-glutamine, and antibiotics (complete RPMI 1640 medium) at 37°C for 2 h in the presence or in the absence of IL-4 (20 ng/ml). In some experiments, PDCs were stimulated with CpG ODN (10 μg/ml) for 48 h to 72 h. PDCs were also stimulated with IL-2, IL-7, IL-9, IL-13, or IL-15 (20 ng/ml each) for 72 h to determine whether these cytokines induce IFN-γ production from PDCs. In other experiments, anti-IL-12 (p40/p70) Ab (10 μg/ml, clone C17.8; BD Pharmingen), anti-IL-2R β-chain Ab (10 μg/ml, clone TM-β1; BD Pharmingen), or anti-IL-18 Ab (5 μg/ml, clone 93-10C; MBL) was added to neutralize IL-12, IL-2 or IL-15 or IL-18, respectively. A mixture of anti-murine IFN-α Ab (20 μg/ml, clone 4E1A) (17), anti-murine IFN-β Ab (20 μg/ml, clone 7DF3) (17), and anti-type I IFN receptor antisera (10 μg/ml, R&D Systems) was used to neutralize type I IFNs.

ELISAs

The amounts of IFN-γ and IL-12 in the culture supernatant were measured by the enzyme immunoassay using murine IFN-γ and IL-12 (p70) ELISA kits from BD Pharmingen. The amounts of IFN-γ in the culture supernatant were measured by an IFN-α ELISA kit from PBL. The assays were performed in duplicate according to the manufacturers’ instruction. The minimum significant values of these assays were 31.3 pg/ml IFN-γ, 62.5 pg/ml IL-12, and 12.5 pg/ml IFN-α.

Intracellular staining for IFN-γ

Cells were stimulated with IL-4 (20 ng/ml) in the complete RPMI 1640 medium for the indicated periods (48 or 72 h). Monensin (2 μM; Sigma-Aldrich) was added for final 4 h to prevent cytokine release. After surface staining, cells were fixed with IC FIX (BioSource International), permeabilized with IC PERM (BioSource International), and stained with anti-IFN-γ allophycocyanin (XMg1.2; BD Pharmingen) as described previously (18).

Intracellular Stat4 staining

Intracellular staining for Stat4 was performed as described elsewhere (19) with minor modifications. In brief, isolated PDCs from WT splenocytes or Stat6^−/− splenocytes were cultured for 48 h in the presence or in the absence of IL-4 (20 ng/ml). Cells were harvested, washed with PBS, fixed with IC FIX, and permeabilized with 90% methanol and subsequently with IC PERM. Cells were then incubated with anti-Stat4 Ab (Zymed) or control rabbit IgG (Serotec) for 30 min at room temperature. After washing, cells were incubated with Alexa Fluor 647-conjugated anti-rabbit IgG Ab (Molecular Probes) and analyzed on a FACSCalibur. In the case of double staining, the cells were positively collected using anti-FITC microbeads (Miltenyi Biotec) according to the manufacturer’s instructions. The resultant cells were routinely >95% pure CD11c^+ cells by FACS analysis.

[FIGURE 1. B220^+ PDCs produce IFN-γ upon IL-4 stimulation. A. Splenocytes from Rag-2−/− mice were cultured with or without IL-4 (20 ng/ml) for 3 days with monensin added for the final 4 h. After cells were stained with anti-B220 and either anti-CD11c or anti-DX5, intracellular staining for IFN-γ was performed. Representative FACS profiles of anti-CD11c vs anti-IFN-γ staining (left panels) and anti-CD11c vs anti-IFN-γ staining gated on either IFN-γ^+ cells or IFN-γ^− cells (right panels) are shown. B. CD11c^− low-density WT splenocytes were isolated from WT mice as described in the Materials and Methods. Cells were then cultured with or without IL-4 for 3 days and analyzed for the expression of CD11c, B220, CD19, CD811b, Ly6G/C, and I-A^d together with the intracellular IFN-γ. Shown are representative FACS profiles of anti-CD11c vs anti-IFN-γ staining from four independent experiments (left panels). Representative FACS profiles of anti-CD19 vs anti-B220, anti-CD3 vs anti-B220, and anti-CD8 vs anti-B220 staining on IFN-γ^+ cells, as well as histograms for anti-Ly6G/C, anti-CD11b, and anti-I-A^d staining on IFN-γ^+ cells are shown in the right panels. Dashed lines indicate the staining with isotype-matched control Abs.]

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intracellular staining for Stat4 and IFN-γ, FITC-conjugated anti-rabbit IgG Ab (Zymed) was used as a second Ab.

RT-PCR analysis

Total cellular RNA was prepared and RT-PCR analysis was performed as described previously (20). The primer pairs for Stat4 were 5'-CTGGGTTGACACATGTGGAA-3' and 5'-TGGCTTTGAGACTTCGCAGC-3'. The primer pairs for GATA3 and T-bet were described elsewhere (21). RT-PCR for β-actin was performed as a control. All PCR amplifications were performed at least three times with multiple sets of experimental RNAs.

Taqman PCR analysis

The expression levels of Stat4 mRNA were determined by real-time PCR using a standard protocol on ABI PRISM 7000 instrument (Applied Biosystems). PCR primers and fluorogenic probes for Stat4, T-bet, and GATA3 were described previously (22). The levels of Stat4 mRNA were normalized to the levels of GAPDH mRNA (Applied Biosystems).

Effect of in vivo depletion of PDCs on IFN-γ production induced by IL-4 administration

To deplete PDCs in vivo, anti-Ly6G/C Ab (500 μg/mouse; BD Pharmingen) was injected i.p. to Rag-2−/− mice as described previously (12). As a control, purified rat IgG2b (BD Pharmingen) was injected to Rag-2−/− mice. In some experiments, 120G8 Ab (500 μg/mouse; a gift from Drs. G. Trinchieri and D. La Face, Schering-Plough Research Institute, Dardilly, France) (23) was injected to Rag-2−/− mice to deplete PDCs. Twenty-four hours later, rIL-4 (10 μg/mouse) or saline (as a control) was injected i.v. in the retro-orbital vein of the mice. The levels of IFN-γ in sera were determined by ELISA using a highly sensitive mouse IFN-γ ELISA kit (AN-18; BD Pharmingen) at 24 and 48 h after IL-4 injection. The minimum significant value of this assay was 3 pg/ml IFN-γ.

Th1 and Th2 cell differentiation

Splenic CD4+ T cells from DO11.10 mice were purified (>90% pure by flow cytometry) using T cell enrichment columns (R&D Systems) and stimulated with plate-bound anti-CD3ε mAb (5 μg/ml, clone I45-2C11; BD Pharmingen) plus anti-CD28 mAb (5 μg/ml, clone 37.51; BD Pharmingen) at 37°C for 48 h in the presence of IL-12 (7.5 ng/ml; R&D Systems) (Th1 condition) or IL-4 (15 ng/ml; R&D Systems) and anti-IFN-γ mAb (15 μg/ml, clone XMG1.2; BD Pharmingen) (Th2 condition) as described previously (18).

Data analysis

Data are summarized as mean ± SD. The statistical analysis of the results was performed by the unpaired t test. Values of p < 0.05 were considered significant.

FIGURE 2. PDCs but not CD11b+ or CD8+ DCs produce IFN-γ upon IL-4 stimulation. A, PDCs, CD11b+ DCs, and CD8+ DCs were prepared from WT splenocytes as described in Materials and Methods. Each DC subtype was cultured with or without IL-4 for 3 days, and the amounts of IFN-γ in the supernatants were measured by ELISA. Representative FACS profiles of isolated each DC subtype are shown in the left panels. Data are means ± SD from four independent experiments. ND = not detectable. B, PDCs produce IFN-γ upon IL-4 stimulation in vivo. Rag-2−/− mice were injected i.p. with anti-Ly6G/C Ab (500 μg/mouse) or rat IgG2b (as a control). Twenty-four hours later, rIL-4 (10 μg/mouse) or saline (as a control) was injected i.v. in the retro-orbital vein of mice. The levels of IFN-γ in sera were determined by ELISA using a highly sensitive mouse IFN-γ ELISA kit (AN-18; BD Pharmingen) at 24 and 48 h after IL-4 injection. Data are means ± SD for four mice in each group. ND, not detectable.

FIGURE 3. IL-4 induces IFN-γ but not IL-12 or IL-10 production in PDCs. Isolated PDCs from WT splenocytes were cultured with IL-4 (20 ng/ml) and/or CpG ODN (10 μg/ml) for 3 days, and the amounts of IFN-γ, IL-12, and IFN-α in the supernatants were measured by ELISA. Data are means ± SD from four independent experiments. ND, not detectable. *, p < 0.001.
FIGURE 4. IL-4 induces IFN-γ production in PDCs by a Stat6-dependent mechanism. A, Stat6 is essential for IL-4-induced IFN-γ production in PDCs. Representative anti-B220 vs anti-CD11c staining on CD3−CD11b−CD19−DX5−TER-119− splenocytes from WT mice and Stat6−/− mice are shown in the left panels (n = 6 mice for each genotype), indicating normal development of PDCs in Stat6−/− mice. Isolated PDCs from WT splenocytes or Stat6−/− splenocytes were cultured with or without IL-4 for 3 days, and the amounts of IFN-γ in the supernatants were measured by ELISA. Data are means ± SD from four independent experiments. B, IL-13 does not induce IFN-γ production in PDCs. Isolated PDCs from WT splenocytes were cultured with IL-4 and/or IL-13 (20 ng/ml) for 3 days, and the amounts of IFN-γ in the supernatants were measured by ELISA. Data are means ± SD from four independent experiments. ND, not detectable. C, Other γc-dependent cytokines do not enhance IL-4-induced IFN-γ production from PDCs. Isolated PDCs from WT splenocytes were cultured with IL-2 (20 ng/ml), IL-7 (20 ng/ml), IL-9 (20 ng/ml), or IL-15 (20 ng/ml) in the presence or in the absence of IL-4 (20 ng/ml) for 3 days, and the amounts of IFN-γ in the supernatants were measured by ELISA. Data are means ± SD from four independent experiments (Figure legend continues).
Results

**B220⁺ PDCs produce IFN-γ upon IL-4 stimulation**

To examine the negative-feedback regulation of cytokine networks, we searched for cell populations that produce IFN-γ upon IL-4 stimulation. We found that ~5% of IL-4-stimulated Rag-2⁻/⁻ splenocytes became positive for intracellular IFN-γ staining (Fig. 1A, left panels). Multicolor FACS analyses revealed that the majority of IL-4-induced, IFN-γ-producing cells expressed CD11c at low levels, expressed B220 at high levels, but lacked the expression of DX5 (Fig. 1A, right panels). In contrast, the majority of IFN-γ-nonproducing cells in IL-4-stimulated Rag-2⁻/⁻ splenocytes were positive for DX5 but negative for B220 (Fig. 1A), suggesting that these IFN-γ-nonproducing cells are NK cells.

To further characterize cell populations that produce IFN-γ upon IL-4 stimulation, CD11c⁺ low-density splenocytes were isolated from WT mice and then stimulated with IL-4. Again, the majority of IL-4-induced, IFN-γ-producing cells expressed B220 at high levels and expressed CD11c at low levels (Fig. 1B). IFN-γ-producing cells were also positive for anti-Ly6G/C staining (Fig. 1B). Moreover, IFN-γ-producing cells expressed class II MHC molecules (I-A<sup>+</sup>) at very low levels but lacked the expression of CD19, CD3, CD8, and CD11b (Fig. 1B). These results suggest that the IL-4-induced, IFN-γ-producing cells are very similar to type I IFN-producing PDCs (10–12). PDCs but not CD11b⁺ DC or CD8⁺ DCs produce IFN-γ upon IL-4 stimulation

To determine whether PDCs specifically produce IFN-γ upon IL-4 stimulation, isolated PDCs, CD11b⁺ DCs, and CD8⁺ DCs were examined for their ability of IFN-γ production upon IL-4 stimulation. Consistent with the data obtained by intracellular IFN-γ staining (Fig. 1), isolated PDCs produced a considerable amount of IFN-γ upon IL-4 stimulation (625.5 ± 79.9 pg/ml, means ± SD, n = 4) (Fig. 2A). On the other hand, IL-4-stimulated CD8⁺ DCs produced little IFN-γ (74.3 ± 32.2 pg/ml, n = 4) and IL-4-stimulated CD11b⁺ DCs did not produce IFN-γ (Fig. 2A). Together with the data shown in Fig. 1, these results indicate that among DC subtypes, PDCs specifically produce IFN-γ upon IL-4 stimulation.

We also examined whether PDCs produced IFN-γ upon IL-4 stimulation in vivo. As shown in Fig. 2B, when IL-4 was administered i.v. to Rag-2⁻/⁻ mice, a considerable amount of IFN-γ was detected in the serum after 24 and 48 h. Importantly, the depletion of PDCs with preinjection of anti-Ly6G/C Ab significantly decreased the IL-4-induced IFN-γ production (n = 4 mice, p < 0.01) (Fig. 2B). A similar trend was observed with 120G8 Ab, which depletes PDCs more specifically (23), although statistical significance was not achieved due to the limited number of mice examined (data not shown). These results suggest that PDCs produce IFN-γ upon IL-4 stimulation in vivo.

IL-4 preferentially induces IFN-γ production in PDCs

PDCs have been identified as a potent producer of IFN-α and IL-12 upon viral or bacterial infection (8–12). Therefore, we examined whether IL-4-induced IFN-α and IL-12 production in isolated PDCs. However, IL-4 did not induce the production of IFN-α or IL-12 (p>0.01) in PDCs (n = 4) (Fig. 3). In contrast, PDCs produced considerable amounts of IFN-γ, IFN-α, and IL-12 upon CpG ODN stimulation, a potent stimulator of PDCs through TLR9 (24, 25) (Fig. 3). Furthermore, IL-4 strikingly enhanced CpG ODN-induced IFN-γ production ~10-fold but not CpG ODN-induced IFN-α or IL-12 production in PDCs (n = 4, p < 0.001) (Fig. 3). These results indicate that IL-4 preferentially induces IFN-γ production in PDCs.

**IL-4 induces IFN-γ production in PDCs by a Stat6-dependent mechanism**

It is well established that IL-4 uses Stat6 as a signaling molecule (26). Therefore, we next studied whether Stat6 was required for IL-4-induced IFN-γ production in PDCs using Stat6-deficient (Stat6⁻/⁻) mice. The number of PDCs (CD19⁻ B220⁺ CD11clow cells) in spleen was similar between Stat6⁻/⁻ mice and WT mice (Fig. 4A), suggesting that Stat6 is not essential for the development of PDCs. However, when isolated PDCs were stimulated with IL-4, WT PDCs but not Stat6⁻/⁻ PDCs produced IFN-γ (Fig. 4A). On the other hand, CpG ODN-induced IFN-γ production was similarly observed between WT PDCs and Stat6⁺/⁺ PDCs (data not shown). These results indicate that among signaling molecules under IL-4, Stat6 is essential for IFN-γ production in PDCs. We also examined the effect of IL-13, which shares type II IL-4R with IL-4 and activates Stat6 (26), on IFN-γ production in PDCs. However, IL-13 did not induce IFN-γ production in PDCs (Fig. 4B) nor enhance IL-4-induced IFN-γ production in PDCs (Fig. 4B), suggesting that type I IL-4R but not type II IL-4R is involved in IL-4-induced IFN-γ production in PDCs. Moreover, another representative Th2 cytokine, IL-5, did not induce IFN-γ production nor enhance IL-4-induced IFN-γ production in PDCs (data not shown).

Other γc-dependent cytokines do not induce IFN-γ production nor enhance IL-4-induced IFN-γ production in PDCs

To determine whether other γc-dependent cytokines induce IFN-γ production in PDCs, isolated PDCs were stimulated with IL-2, IL-7, IL-9, and IL-15 in the presence or in the absence of IL-4 for 3 days. As shown in Fig. 4C, none of γc-dependent cytokines, except for IL-4 induced IFN-γ production in PDCs (Fig. 4C). In addition, none of them significantly enhanced IFN-γ production in IL-4-stimulated PDCs (Fig. 4C).

IL-4 does not alter the maturation state of PDCs

It has been shown that the ability of DCs for cytokine production depends on their maturation state (27, 28). We then examined whether IL-4 changed the maturation state of PDCs and thus induced IFN-γ-producing ability. Consistent with previous reports (8–12), isolated PDCs expressed I-A<sup>+</sup> at very low levels, and lacked the expression of CD80 (Fig. 4D) and CD86 (data not shown). IL-4 did not alter the expression levels of I-A<sup>+</sup>, CD80, and CD86 of PDCs (Fig. 4D and data not shown). In contrast, when PDCs were stimulated with CpG ODN, the expression levels of I-A<sup>+</sup> and CD80 were significantly increased (Fig. 4D). In addition,
**FIGURE 5.** IL-4 induces Stat4 expression in PDCs by a Stat6-dependent mechanism. A, Isolated PDCs from WT splenocytes were cultured with or without IL-4 (20 ng/ml) for 16 h. As a control for Stat4-expressing cells, Th1-polarized cells were prepared from DO11.10 mice as described previously (15). Shown are representative data of RT-PCR analysis for Stat4 and β-actin mRNA from four independent experiments (left panels). Taqman PCR analysis for Stat4 and GAPDH (as a control) mRNA was performed, and the levels of Stat4 mRNA were normalized to the levels of GAPDH mRNA (middle panel). Data are means ± SD from four independent experiments. *, Significantly different from the mean value of control response (PBS); p < 0.01. Isolated PDCs from WT splenocytes or Stat6−/− splenocytes were stimulated with or without IL-4 (20 ng/ml) for 48 h, and the expression levels of Stat4 were evaluated by intracellular staining. Shown are representative FACS profiles from four independent experiments (right panels). B, IL-4-induced, Stat4-expressing PDCs produce IFN-γ. Isolated PDCs from WT splenocytes were cultured with or without IL-4 (20 ng/ml) for 48 h. Intracellular (Figure legend continues)
IL-4 did not change the morphology of PDCs, whereas CpG ODN changed PDCs to dendritic morphology (data not shown). These results suggest that IL-4 does not change the maturation state of PDCs.

**T-bet is not induced by IL-4 in PDCs**

T-bet plays an important role in inducing IFN-γ production in CD4⁺ T cells (29). Recent findings using T-bet-deficient mice have also suggested that T-bet is vital for IFN-γ production from CD11c⁺ DCs upon IL-12 stimulation (30). To examine the possible involvement of T-bet in IL-4-induced IFN-γ production in PDCs, we examined the expression of T-bet mRNA in PDCs in the presence or in the absence of IL-4 stimulation. The expression of GATA3, an important negative regulator of IFN-γ production (4), was also examined in parallel. As shown in Fig. 4E, unstimulated PDCs expressed GATA3 mRNA but not T-bet mRNA. IL-4 did not induce the expression of T-bet mRNA nor alter the expression levels of GATA3 mRNA (Fig. 4E). T-bet mRNA was not detected by Taqman PCR analysis even after IL-4 stimulation (data not shown). These results suggest that T-bet may not be involved in IL-4-induced IFN-γ production in PDCs.

**IL-4 induces Stat4 expression in PDCs through a Stat6-dependent mechanism and Stat4-expressing PDCs produce IFN-γ**

It has been demonstrated that Stat4 is required for IFN-γ production in many cell types (31, 32). Stat4 expression has also been demonstrated to be correlated with IFN-γ-producing ability in CD8⁺ DCs (33). Therefore, we next examined the expression levels of Stat4 in IL-4-stimulated PDCs. As shown in Fig. 5A, in the absence of IL-4 stimulation, isolated WT PDCs did not express Stat4 mRNA (lane 1). However, Stat4 mRNA was significantly up-regulated in WT PDCs upon IL-4 stimulation (lane 2), although the expression level was still lower than that in Th1-polarized cells (lane 3) (Fig. 5A, left panel). Stat4 mRNA induction by IL-4 stimulation was confirmed by real-time PCR analysis (Fig. 5A, middle panel). We also examined the expression levels of Stat4 at protein levels using intracellular staining in WT PDCs and Stat6⁻/⁻ PDCs and found that IL-4 significantly induced Stat4 expression in ~50% of WT PDCs but not in Stat6⁻/⁻ PDCs (Fig. 5A, right panel). These results indicate that Stat6 is essential for IL-4-induced Stat4 expression in PDCs. In addition, although IFN-γ has been shown to induce Stat4 expression in some cell types (34), anti-IFN-γ Ab did not affect IL-4-induced Stat4 expression in PDCs (data not shown).

We then examined the correlation between Stat4 expression and IFN-γ production at single-cell levels by double intracellular staining. Interestingly, Stat4-expressing PDCs but not Stat4-nonexpressing PDCs produced IFN-γ upon IL-4 stimulation (Fig. 5B). We also examined whether Stat4 was essential for IL-4-induced IFN-γ production in PDCs using Stat4⁻/⁻ mice. Although PDCs normally developed in Stat4⁻/⁻ mice (data not shown), IL-4 did not induce IFN-γ production in Stat4⁻/⁻ PDCs (Fig. 5C). Taken together, these results indicate that the induction of Stat4 by IL-4-Stat6 signaling is required for IFN-γ production in PDCs.

**IL-4-induced IFN-γ production does not depend on IL-12 or type I IFNs**

To determine whether endogenously produced cytokines from PDCs are a major IFN-γ-producing cell upon IL-4 stimulation and that IL-4 preferentially induces IFN-γ production in PDCs by a Stat6-dependent mechanism. Moreover, IL-4 induces Stat4 expression in PDCs through a Stat6-dependent mechanism and the IL-4-induced, Stat4-expressing PDCs produce IFN-γ. Furthermore, Stat4⁻/⁻ PDCs do not produce IFN-γ upon IL-4 stimulation. These results suggest that PDCs produce IFN-γ upon IL-4 stimulation by Stat6- and Stat4-dependent mechanisms.

We demonstrate that PDCs are a major IFN-γ-producing cell upon IL-4 stimulation. By searching for Rag-2⁻/⁻ splenocyte populations that produce IFN-γ upon IL-4 stimulation, we found that the majority of IL-4-induced, IFN-γ-producing cells expressed B220 at high levels and CD11c and Ly6G/C at low levels (Fig. 1). We also found that IL-4 induced IFN-γ production from isolated B220⁺ PDcs but not from CD11b⁺ DCs or CD8⁺ DCs (Fig. 2A) and that the depletion of PDCs by anti-Ly6G/C Ab prevented IL-4-induced IFN-γ production in vivo (Fig. 2B). Inhibition of IL-4-induced IFN-γ production was similarly observed with the administration of 120G8 Ab, although statistical significance was not achieved due to the limited number of mice examined. On the other hand, IL-4 did not induce IFN-α or IL-12 production in PDCs (Fig. 3). IL-4 also strongly enhanced CpG ODN-induced IFN-γ production but not CpG ODN-induced IFN-α or IL-12 production in PDCs (Fig. 3). Taken together, these results indicate that PDCs are a major IFN-γ producer upon IL-4 stimulation and that IL-4 preferentially induces IFN-γ production in PDCs.

**Discussion**

In this study, we show that PDCs are a major IFN-γ-producing cell upon IL-4 stimulation and that IL-4 preferentially induces IFN-γ production in PDCs by a Stat6-dependent mechanism. Moreover, IL-4 induces Stat4 expression in PDCs through a Stat6-dependent mechanism and the IL-4-induced, Stat4-expressing PDCs produce IFN-γ. Furthermore, Stat4⁻/⁻ PDCs do not produce IFN-γ upon IL-4 stimulation. These results suggest that PDCs produce IFN-γ upon IL-4 stimulation by Stat6- and Stat4-dependent mechanisms.

It is well recognized that Stat6 plays a critical role in the production of IL-4 in CD4⁺ T cells upon IL-4 stimulation through the induction of GATA3, a master regulator of Th2 cell differentiation (4). In contrast, we show here that IL-4 induces IFN-γ production...
in PDCs by a Stat6-dependent mechanism (Fig. 4A). We also show that IL-4 does not alter the expression levels of GATA3 (Fig. 4E) nor induce the expression of T-bet, a key molecule for IFN-γ production in CD4 T cells (29), in PDCs (Fig. 4E). Therefore, in contrast to CD4+ T cells, the expression levels of T-bet and GATA3 may not be causatively associated with the production of IFN-γ in PDCs.

Our results show that IL-4, but not other γc-dependent cytokines, induces IFN-γ production from PDCs (Fig. 4C). In contrast, it has been demonstrated recently that IL-4 synergistically enhances IL-2-induced IFN-γ production from NK cells, but IL-4 itself does not induce IFN-γ production from NK cells (35). It has also been shown that although IL-4 enhances IL-12-induced IFN-γ production from CD8+ DCs, IL-4 itself does not induce IFN-γ production from CD8+ DCs (36). Moreover, we found that bone marrow-derived PDCs generated with fms-like tyrosine kinase-3 ligand did not produce IFN-γ upon IL-4 stimulation (data not shown). Thus, the IL-4 signaling pathway for IFN-γ production may differ depending not only on cell types but also on the maturation state of the cells.

We also show that IL-4 induces Stat4 expression in PDCs by a Stat6-dependent mechanism (Fig. 5A), that only the Stat4-expressing PDCs produce IFN-γ at single-cell levels (Fig. 5B), and that Stat4−/− PDCs do not produce IFN-γ upon IL-4 stimulation (Fig. 5C). Therefore, it is indicated that Stat4 is required for IL-4-induced IFN-γ production in PDCs. Interestingly, we also found that when PDCs were stimulated with Cpg ODN for 48 h, Stat4 induction was detected by intracellular FACS analysis (data not shown). This finding may account for the synergistic effect of Cpg ODN on IL-4-induced IFN-γ production in PDCs (Fig. 3).

The mechanisms leading to Stat4 activation could not be yet identified. Indeed, the phosphorylation status of Stat4 could not be clearly defined in IL-4-stimulated PDCs presumably for technical reasons (data not shown). However, as tyrosine phosphorylation is required for the activity of STAT proteins (37), a Stat4-activating cytokine seems to be involved in IL-4-induced IFN-γ production in PDCs. Because IL-12 is a representative cytokine that activates Stat4 (38) and because it has been reported that IL-4 enhances IL-12 production from CD11c+ DCs (39) or CD8α+ DCs (40) in some situations, it was suggested that IL-12 might be responsible for activating Stat4 in PDCs. However, we found that IL-4 by itself does not induce IL-12 production from PDCs (Fig. 3) and that a neutralizing Ab against IL-12 did not inhibit IL-4-induced IFN-γ production in PDCs (Fig. 5D), suggesting that IL-12 is not responsible for the activation of Stat4 in PDCs.

Recently, it has also been demonstrated that in addition to IL-12, type I IFNs activate Stat4 and induce IFN-γ production in some cell types such as CD8+ T cells (41). However, again, we found that IL-4 by itself did not induce IFN-γ production from PDCs (Fig. 3) and that neutralizing Abs against type I IFNs did not inhibit IL-4-induced IFN-γ production in PDCs (Fig. 5E). These findings suggest that type I IFNs are not responsible for the activation of Stat4 in PDCs. Recent studies have also demonstrated that IL-23 (42) and IL-21 (43) use Stat4 as a signaling molecule in some cell types. Therefore, IL-23, IL-21, or an undefined Stat4-activating cytokine may function as a Stat4-activating cytokine and then may contribute to IL-4-induced IFN-γ production in PDCs. Further studies that identify the cytokine responsible for Stat4 activation are required for the understanding of the mechanism leading to IL-4-induced IFN-γ production in PDCs.

The effect of IL-4 on the expression of Stat4 in DCs seems different depending on the subtypes of DCs, as well as the maturation state of each DC subtype. Recently, Fukao et al. (33) have shown that when IL-4 is present during the maturation of CD11c+ DCs, IL-4 suppresses Stat4 induction and subsequent IL-12-induced IFN-γ production in CD11c+ DCs. On the other hand, the same group has shown that IL-4 does not alter the expression levels of Stat4 in mature CD8+ DCs (36). However, we showed that IL-4-Stat6 signaling induced Stat4 expression in PDCs (Fig. 5). Moreover, we found that the maturation state of PDCs, assessed by the expression levels of CD80 and I-Ad, was similar between Stat4-expressing PDCs and Stat4-nonexpressing PDCs (data not shown). Therefore, our results indicate that IL-4 specifically induces Stat4 expression and IFN-γ-producing ability in PDCs without affecting their maturation state.

In the present study, we showed that a typical Th2 cytokine IL-4 induced the production of a typical Th1 cytokine IFN-γ in PDCs in BALB/c mice. IL-4-induced IFN-γ production in PDCs was also observed in C57BL/6 mice (data not shown), suggesting that this phenomenon is a general one observed beyond strain differences. Because IL-4 is produced in an early phase in immune responses by NK T cells (44) and/or basophils (45, 46), the IL-4-induced IFN-γ production by PDCs may function in the negative-feedback regulation against a Th2-type deviation in an early phase of immune responses. In this regard, de Heer et al. (14) have demonstrated recently that PDCs inhibit typical Th2 responses such as IgE production and allergic airway inflammation. Although the authors indicated the induction of regulatory T cells as the mechanism underlying the PDC-mediated Th2 cell suppression (14), IL-4-induced IFN-γ production in PDCs may also contribute to the PDC-mediated inhibition of allergic airway inflammation because IFN-γ inhibits Ag-induced Th2 cell differentiation (1–3) and allergic airway inflammation (47).

In conclusion, we have shown that PDCs preferentially produce IFN-γ upon IL-4 stimulation by Stat6- and Stat4-dependent mechanisms. Although further studies are required to address the physiological importance of IL-4-induced IFN-γ production in PDCs, our results would give a new insight into PDC-mediated immune regulation of cytokine network.

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Disclosures

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