IFN-γ Negatively Regulates CpG-Induced IL-10 in Bone Marrow-Derived Dendritic Cells[1]

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Dendritic cells (DCs) are important players in the regulation of Th1- and Th2-dominated immune responses. In these studies we showed that IFN-γ, the key mediator of Th1 immunity, actively suppressed the production of IL-10 in murine DCs when activated with LPS or CpG. Our analysis revealed that both LPS and CpG induced IL-10 and IL-12 production but that the presence of IFN-γ, in a dose-dependent manner, suppressed the production of IL-10 while enhancing that of IL-12. The observed inhibition of IL-10 production was independent of IL-12. Experiments performed with STAT-1 knockout mice demonstrated that the primary production of IL-12 induced by CpG was STAT-1 dependent, whereas the production of IL-10 was not. This finding was confirmed by the observation that CpG-induced IL-12 production could be inhibited by anti-IFN-β Abs, whereas CpG-induced IL-10 production could not be inhibited. These data also demonstrated that the inhibitory effect of IFN-γ on IL-10 expression was STAT-1 dependent and transcriptionally regulated. Thus, DCs respond to CpG by producing proinflammatory and anti-inflammatory cytokines such as IL-12 and IL-10, respectively, and IFN-γ acts to not only enhance IL-12 but also to inhibit IL-10 production. The current data demonstrate a novel pathway for IFN-γ-mediated immunoregulation and suggest that IFN-γ-dependent suppression of IL-10 production by DCs may be involved in the antagonism between Th1 and Th2 patterns of immune reactivity. *The Journal of Immunology,* **2007, 178: 211–218.**

Dendritic cells (DCs)[3] are APCs that provide a link between the innate and adaptive immune systems. Immature DCs reside in peripheral tissue and sample the environment for pathogens. The uptake of pathogens and the secretion of inflammatory cytokines induce DC maturation and migration to draining lymph nodes, where DCs encounter Ag-specific T cells (1). During this encounter, DCs present Ag, in the form of MHC-peptide complexes, and also provide costimulatory signals that induce T cell activation.

Cytokines secreted by DCs have a profound impact on subsequent Th cell differentiation (2, 3). IL-12 and/or IL-18 contribute significantly to the development of Th1 cells, whereas IL-6 and IL-10 stimulate the development of Th2 or Tr1 cells (2–5). In addition, the CD40-CD40L interaction plays an important role in stimulating IL-12 production (6), which is augmented in the presence of IFN-γ (7, 8). Recent work by Mailliard et al. (9, 10) has shown that IFN-γ, produced by activated NK cells or $CD8^+$ T cells, can skew the development of DCs toward a type I phenotype, characterized by their ability to secrete high levels of IL-12p70 following CD40 ligation and to stimulate Th1 differentiation.

The innate response by DCs is mediated by receptors that recognize conserved molecular structures on pathogens known as pathogen-associated molecular patterns. The recognition of these patterns is performed by pattern recognition receptors that include lectins (DC-SIGN, DEC-205, and the mannose receptor) (11, 12) and TLRs of which there are 11 human and 10 murine (13). TLR2, TLR4, and TLR6 form multisubunit complexes and preferentially respond to bacterial structures. Other TLRs such as TLR3, TLR7, and TLR9 respond to and detect intracellular pathogens. TLR9 is found in endosomal vesicles within the cell, and recent studies have shown that activated TLR9 signals from these vesicles (14, 15). TLR9 binds unmethylated DNA structures containing a C-G dinucleotide motif, known as CpG. Activation of TLR9 induces DC maturation, characterized by increases in the expression of costimulatory markers and MHC class II molecules (16), as well as the secretion of cytokines such as TNF-α, IL-1, IL-6, IL-10, IL-12, or IFN-αβ, IP-10, and RANTES, depending on which DC subset is activated (17).

Due to its ability to stimulate DC activation, CpG has been considered for use as an adjuvant in immunotherapy for various conditions such as allergies, infections, or tumors (18, 19). In a murine model of atopic asthma, mice sensitized with methacholine, along with CpG, had reduced eosinophilic infiltration of the lung, decreased serum levels of IgE, and decreased airway hyperreactivity (20). Atopic mice treated with CpG experienced an increase in IL-12 and IFN-γ detected in the bronchoalveolar lavage fluid. Similar effects of CpG have been shown in infectious models such as *Leishmania major* (19, 21). In another study, mice injected with a tumor cell line were treated with CpG resulting in the infiltration of the tumor by DCs expressing/producing high levels of costimulatory molecules and IL-12. This stimulated a Th1 response complete with tumor-specific cytolytic activity (22–24). These results have led to the use of CpG in a phase I clinical trial testing a cancer vaccine (25).

In the present study, we analyzed the impact of IFN-γ, a key mediator of Th1-dominated immunity, upon the ability of different TLR ligands to induce the production of DC cytokines, IL-12 and

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[3] Abbreviations used in this paper: DC, dendritic cell; BMDC, bone marrow-derived DC; KO, knockout; WT, wild type; IRF, IFN regulatory factor.

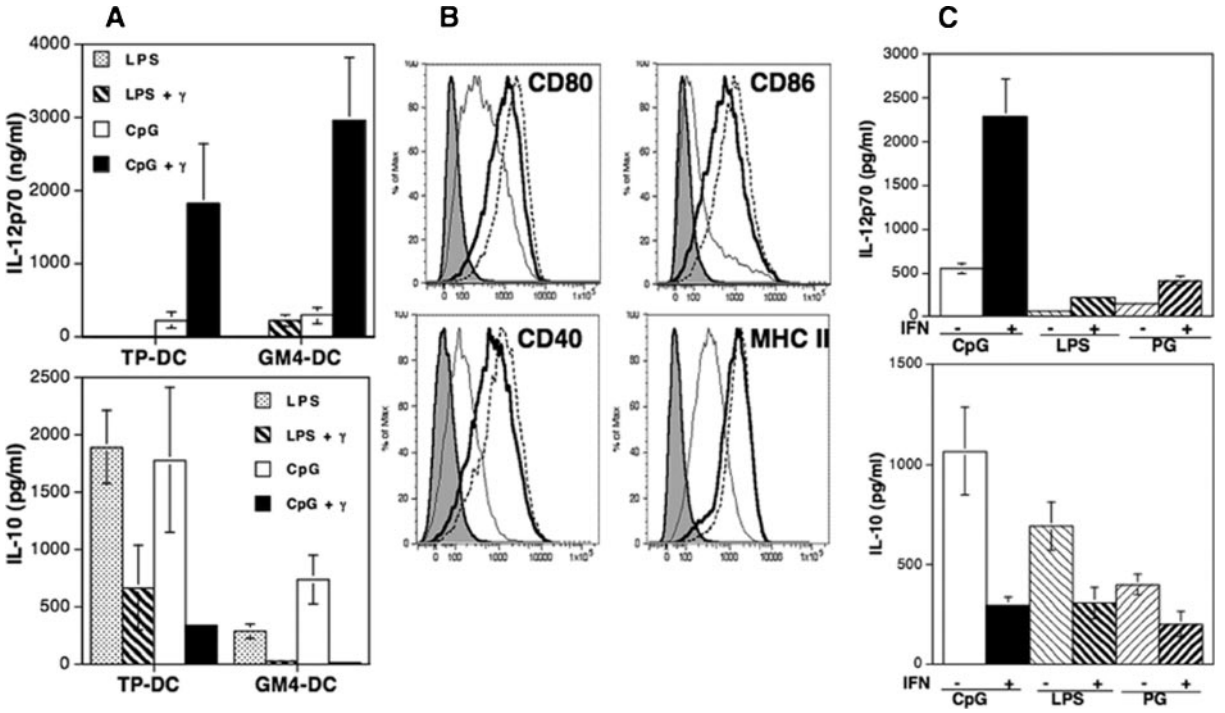

FIGURE 1. IFN-γ enhances IL-12 but suppresses the production of IL-10 in CpG activated BMDCs. Bone marrow cells grown in GM-CSF alone and then matured in the presence of TNF-α (10 ng/ml) and PGE_2 (TP DCs) and GM4 DCs were generated as described in *Materials and Methods* from BALB/c mice. *A*, TP DCs and GM4 DCs were stimulated with LPS (100 μg/ml) with or without IFN-γ (10 ng/ml) or CpG 1668 phosphothiolated (10 μg/ml) with or without IFN-γ for 24 h. The supernatants were collected and the presence of IL-10 and IL-12p70 was detected using a cytokine-specific ELISA. The results shown represent the mean ± SE of three to six independent experiments. *B*, GM4 DCs were treated with CpG with or without IFN-γ as in *A*. DCs were stained with FITC-labeled Abs specific for CD80, CD86, MHC class II, and CD11c and PE-labeled Abs specific for CD11c and CD40. The histograms depict the expression of the indicated marker on $CD11c^+$ DC. Unstimulated DCs (thin line histogram), CpG-stimulated DCs (thick line histogram), and CpG plus IFN-γ-stimulated DCs (dashed histogram) are represented. The results shown are representative of three experiments. *C*, GM4 DCs were generated from 129/J mice and stimulated with LPS (100 μg/ml) with or without IFN-γ (10 ng/ml), CpG 1668 (10 μg/ml) with or without IFN-γ, or peptidoglycan (PG, 10 μg/ml) with or without IFN-γ for 24 h. The supernatants were collected and the presence of IL-10 and IL-12p70 was detected using a cytokine-specific ELISA. The results shown represent the mean ± SE of three to six independent experiments.

IL-10, which drive the differentiation of the Th1 and Th2/Th3, respectively. We demonstrate that although CpG induced bone marrow-derived DCs (BMDCs) to secrete both IL-10 and IL-12, IFN-γ strongly suppressed the production of IL-10 while simultaneously enhancing the production of IL-12. These data demonstrate an additional level of cross-regulation of the inflammatory vs noninflammatory immune responses mediated by IFN-γ-IL-10 antagonism.

Materials and Methods

Mice

BALB/c mice were purchased from The Jackson Laboratory and 129 SvEv (STAT-1 wild type (WT)) and STAT-1 knockout (STAT-1 KO) mice were purchased from Taconic Farms. The mice were maintained at the animal facilities at the University of Pittsburgh School of Medicine.

Generation of BMDCs

BMDCs were generated from the culture of bone marrow progenitors as previously described (26, 27). Briefly, bone marrow (taken from the femurs) was depleted of RBC by high salt buffer treatment, and of T and B lymphocytes and MHC class II-expressing cells by mAb and complement treatment. The remaining cells were washed extensively and cultured in RPMI medium containing RPMI 1640 supplemented with 10% FCS at a density 0.5×10^6 cells/ml in a 6-well plate. Bone marrow cells were grown in the presence of GM-CSF (5 ng/ml) with or without IL-4 (1 ng/ml). In some cases, bone marrow cells were grown for 4 days in the presence of GM-CSF alone and then matured in the presence of TNF-α (10 ng/ml; Sigma-Aldrich) and PGE_2 (TP DCs, 10^{-6} M; Sigma-Aldrich) for an additional 18 h. GM4 DCs were obtained by growing bone marrow cells in GM-CSF plus IL-4 for 5 days. On day 5, TP DCs and GM4 DCs were harvested using an anti-CD11c microbead MACS column (Miltenyi Biotec) according to the manufacturer's instructions. The BMDCs were >90% $CD11c^+$. Both GM-CSF and IL-4 were generated in-house by transfection of Chinese hamster ovary cells with murine GM-CSF and IL-4 pNGVL3 vectors, both gifts from Dr. L. Falo (University of Pittsburgh, Pittsburgh, PA), using the FuGene (Roche Diagnostics) transfection system. Supernatants were collected after 48 h, and GM-CSF- and IL-4-specific ELISA tests (BD Pharmingen) were performed to determine the concentration of each cytokine.

FACS analysis of BMDCs

Two-color flow cytometry was performed on purified BMDCs. BMDCs were stained with FITC-conjugated mAb specific for CD80, CD86, or MHC class II (BD Biosciences) and PE-conjugated anti-CD11c (BD Biosciences) and incubated for 30 min on ice. Afterward, the cells were washed and fixed in 2% paraformaldehyde. The stained BMDCs were analyzed on an LSR II (BD Biosciences). Analysis using FlowJo (Tree Star) was performed on gated $CD11c^+$ cells.

Functional analysis of stimulated BMDCs

Microbead-purified BMDCs were washed extensively and seeded into 48-well plates at a density 1×10^6 cells/ml. The BMDCs were stimulated with CpG 1668 phosphothiolated TCCATGACGTTCCTGATGCT (10 μg/ml; DNA Synthesis Facilities, Department of Biological Sciences, University of Pittsburgh), LPS (100 μg/ml; Sigma-Aldrich), peptidoglycan (10 μg/ml; InvivoGen), poly(I:C) (10 μg/ml), or loxiribine (100 μM; InvivoGen) in the presence or absence of increasing doses of IFN-γ (R&D Systems). In some experiments, neutralizing Abs specific for IFN-α (clone 1PL17) and/or IFN-β (clone 8.S.415; U.S. Biological) were added (10 μg/ml) at the start of the culture. Supernatants were collected at various time points, and ELISA was performed to determine the levels of IL-6, IL-10, IL-12p70, and TNF-α produced. The ELISA mAb pairs were purchased from BD Pharmingen and used according to the manufacturer's instructions.

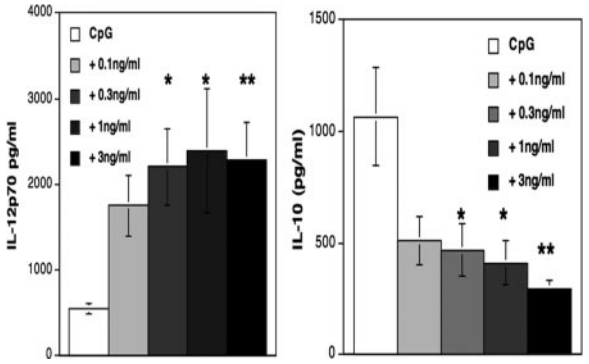

FIGURE 2. The effect of IFN-γ on IL-10 and IL-12p70 production is dose dependent. GM4 DCs were generated from 129/J mice and stimulated with CpG 1668 phosphothiolated (10 μg/ml) with or without IFN-γ at the indicated doses for 24 h. The supernatants were collected and the presence of IL-10 (*right*) and IL-12p70 (*left*) was detected using a cytokine-specific ELISA. The results shown represent the mean ± SE of three to six independent experiments. *, $p < 0.05$; **, $p < 0.01$.

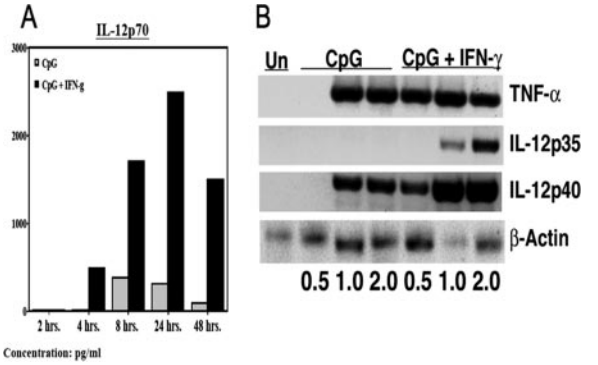

FIGURE 3. CpG induces IL-12p70 production and the response is enhanced in the presence of IFN-γ. *A*, BALB/c GM4 DCs were stimulated with CpG with or without IFN-γ for 2, 4, 8, 24, and 48 h. At the specified times, the supernatants were collected and analyzed for the presence of IL-12p70. *B*, GM4 DCs were stimulated with CpG with or without IFN-γ for 0.5, 1, and 2 h or left unstimulated (Un). The cells were collected and RNA was isolated. RT-PCR for the indicated cytokines was performed as described in *Materials and Methods*. Results shown are representative of three independent experiments. Similar results were obtained from DC generated from 129 SvEv mice (data not shown).

RT-PCR analysis of DC cytokine expression

Purified BMDCs were stimulated with CpG with or without IFN-γ for various periods of time. The cells were washed extensively in PBS and RNA was purified using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The RNA was treated with DNase (Invitrogen Life Technologies) and cDNA was prepared from 2 μg of RNA by reverse transcription using a first strand cDNA synthesis kit (Qiagen). PCR analysis for β-actin, IL-10, IL-12p35, IL-12-p40, TNF-α, and IFN-γ was performed using a HotStart DNA polymerase kit (Qiagen). The PCR amplification conditions were 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C for 30 cycles. Specific primer sets were as follows: β-actin (forward) TT CTACAATGAGCTGCGTGTG, (reverse) TTCATGGATGCCACAGGA TTC; IL-10 (forward) CTGCTATGCTGCCTGCTCTT, (reverse) CTGG AGTCCAGCAGACTCAAT; IL-12p35 (forward) ACCTGCTGAAGAC CACAGAT, (reverse) AGCAGGATGCAGAGCTTCAT; IL-12p40 (forward) CGCCTGAAGAAGATGACATC, (reverse) TGAAGAAGCT GGTGCTGTAG; TNF-α (forward) GTTCTATGGCCCAGACCCTCA CA, (reverse) TCCCAGGTATATGGGCTCATACC; and IFN-γ (forward) AACGCTACACACTGCATCTG, (reverse) CGCCTTGCTGTTG CTGAAGAA. PCR products were electrophoresed on a 2% agarose gel and visualized using a Kodak Gel Logic system (formerly EDAS 290 Gel Dock).

Cytopathic inhibition assay for IFN

A cytopathic inhibition assay for IFN was performed on supernatants stimulated with CpG or LPS as previously described (28). The supernatants were plated in 96-well plates and serially diluted for eight consecutive rounds. Vesicular stomatitis virus-sensitive L cells (4×10^4/well) were added to each well and incubated for 24 h at 37°C. The plates were read for cytopathic effect in case of toxicity of the samples, and 5000 PFU/50 μl of vesicular stomatitis virus was added to each well. The wells were analyzed at 48 h for the presence of cytopathic effect. The IFN titer was determined to be the highest dilution that protected 50% of the cells (arbitrary units).

Statistics

The differences in cytokine production between DC stimulated in various ways were assessed using one-way ANOVA analysis with Dunn's post test. All analyses were performed using GraphPad Prism version 3.0a for Macintosh. Values for $p < 0.05$ are defined as statistically significant. Values for $p < 0.01$ are defined as statistically very significant.

Results

TLR ligands induce both IL-12p70 and IL-10, whereas IFN-γ suppresses the production of IL-10

To assess the effect of TLR ligands on DCs, both immature and mature BMDCs from BALB/c mice were generated and subsequently stimulated with LPS and CpG 1668. As shown in Fig. 1*A*, DCs grown in the presence of GM-CSF and then matured in the presence of TNF-α and PGE_2 (TP DCs) and GM4 DCs responded to LPS and CpG by secreting IL-12p70 and IL-10. The secretion of IL-12p70 induced by LPS and CpG was enhanced by the presence of IFN-γ. However, IL-10 induced by LPS and CpG was suppressed in the presence of IFN-γ (Fig. 1*A*, *bottom*). Although the enhancement of IL-12p70 production by IFN-γ is well documented, the IFN-γ-mediated suppression of IL-10 production in DCs has not been extensively studied. Incubation of GM4 DCs with CpG or CpG plus IFN-γ resulted in a marked increase in the expression of CD80, CD86, CD40, and MHC class II (Fig. 1*B*). The addition of IFN-γ did not further increase the expression of these molecules (Fig. 1*B*). Similar results were obtained with a different mouse strain (129/J) and following stimulation with the TLR2 ligand peptidoglycan (Fig. 1*C*). No changes in levels of IL-6

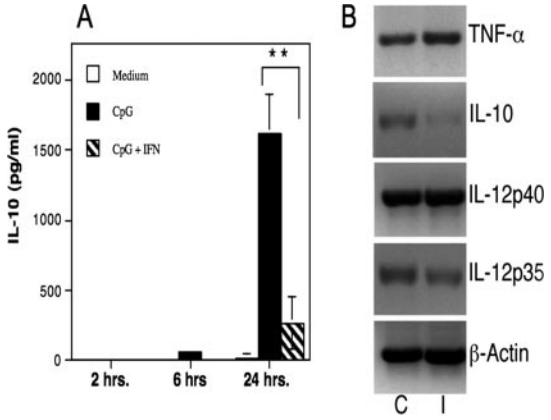

FIGURE 4. CpG induces the expression of IL-10 and this expression decreases in the presence of IFN-γ. GM4 DCs were generated from 129 SvEv mice and harvested on day 5 using an anti-CD11c microbead column. *A*, The GM4 DCs were stimulated with CpG with or without IFN-γ for 2, 6, and 24 h. Supernatants were collected at the indicated times and assayed for the presence of IL-10. **, $p < 0.01$. *B*, GM4 DCs were stimulated for 24 h with CpG (C) or CpG with IFN-γ (I). The cells were collected and RNA was extracted using an RNeasy kit, and RT-PCR for the indicated cytokines was performed as described in *Materials and Methods*. The results shown are representative of three independent experiments. Similar results were obtained using GM4 DC generated from BALB/c mice (data not shown).

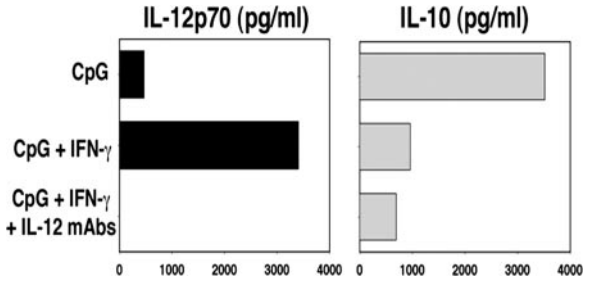

FIGURE 5. The suppression of IL-10 by IFN-γ is independent of IL-12. BALB/c DCs matured in the presence of TNF-α (10 ng/ml) and PGE_2 were stimulated with CpG, CpG with IFN-γ, or CpG with IFN-γ plus anti-IL-12 mAb (10 μg/ml). The supernatants were collected 24 h later, and the presence of IL-10 (*right*) and IL-12p70 (*left*) was detected using a cytokine-specific ELISA. The results shown are representative of two independent experiments.

or TNF-α production were induced by the addition of IFN-γ to CpG-stimulated DC (data not shown).

To determine whether the observed effect was dependent on the dose of IFN-γ, we performed additional experiments using increasing doses of IFN-γ. Doses as low as 0.3 ng/ml IFN-γ induced significant suppression of IL-10 production and significantly enhanced IL-12p70 production. (Fig. 2).

Inhibition of IL-10 by IFN-γ occurs at the transcriptional level

We looked at the kinetics of IL-12p70 and IL-10 production following CpG activation in the presence or absence of IFN-γ. For this set of experiments, GM4 DCs were stimulated with CpG with or without IFN-γ, and the supernatants were collected after 2, 4, 8, 24, and 48 h. In addition we determined whether this production was controlled at the transcriptional level by performing RT-PCR for IL-12p35, IL-12p40, and IL-10 at various time points following activation. Activation of DCs in the presence of CpG alone resulted in the production of low levels of IL-12p70 beginning 8 h after activation (Fig. 3*A*). When IFN-γ was added, the levels of IL-12p70 were significantly increased and were also detected much earlier (Fig. 3*A*). This finding correlated with the expression of IL-12p35 mRNA, which was undetectable when the DCs were stimulated with CpG alone (Fig. 3*B*), although it was detectable within 1 h of activation in the presence of IFN-γ. Similar results were seen with IL-12p40 mRNA with levels detectable as early as 30 min when IFN-γ was present (Fig. 3*B*).

In the case of IL-10, we observed that the protein was detectable at 24 h following activation with CpG alone (Fig. 4*A*). The addition of IFN-γ essentially prevented IL-10 production (Fig. 4*A*). RT-PCR analysis of samples taken at 24 h after activation revealed that the reduction in IL-10 levels was mediated at the transcriptional level (Fig. 4*B*). No IL-10 mRNA was detected before 24 h of activation.

IFN-γ suppression of IL-10 is independent of IL-12

The results from the kinetic experiments suggested that the earlier secretion of IL-12 induced by IFN-γ might be responsible for the suppression of IL-10. Much is known about the regulation of IL-12 expression by IL-10 (29–32) but it is unknown whether IL-12 regulates the expression of IL-10. To address this question, we performed experiments using anti-IL-12p40/p70 mAb to neutralize IL-12p70 function. TP DCs were stimulated with CpG both in the presence or absence of IFN-γ. Additionally, TP DCs were stimulated with CpG with IFN-γ plus anti-IL-12p40/70 mAb. CpG induced the secretion of IL-12p70 that was enhanced in the presence of IFN-γ, and as expected the addition of anti-IL-12 mAb neutralized IL-12p70 (Fig. 5, *left*). IL-10 was secreted by DCs stimulated with CpG and this secretion was suppressed by IFN-γ. However, IL-10 remained suppressed following stimulation by CpG with IFN-γ despite the presence of anti-IL-12 mAb (Fig. 5, *right*). Thus, these data demonstrate that the suppression of IL-10 production in the presence of IFN-γ is not mediated indirectly by IL-12.

The suppression of IL-10 is IFN-γ-dependent

We then determined whether the timing of the signals would alter the overall pattern of cytokine secretion. To determine the role for IFN-γ in the inhibition of IL-10 production we first treated GM4 DCs with CpG and delayed the addition of IFN-γ for 2, 4, 6, and 8 h. The levels of IL-10 and IL-12p70 produced were measured at 24 h. As shown in Fig. 6*A*, GM4 DCs pretreated with CpG produced optimal IL-12p70 levels when IFN-γ was added close to the CpG stimulus. The addition of IFN-γ at time 0 and 2 h following

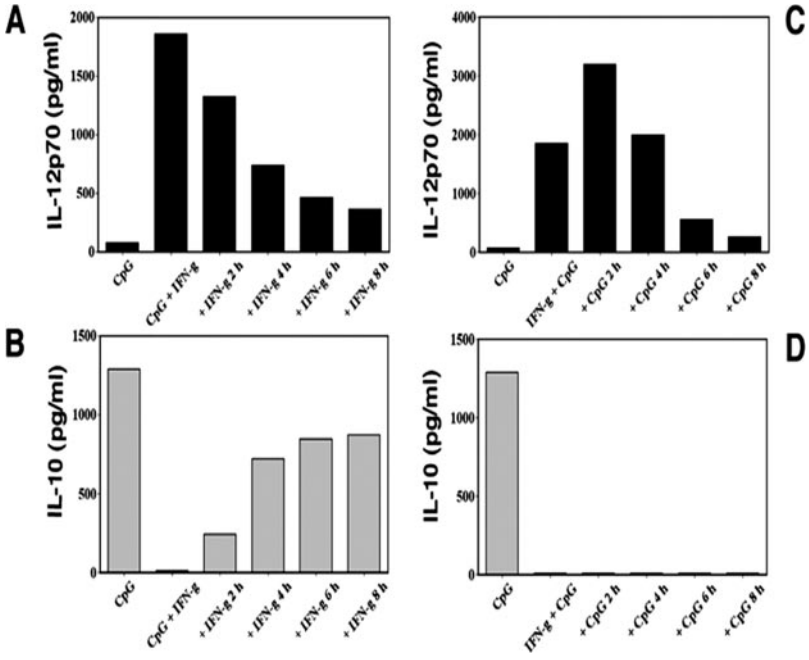

FIGURE 6. The suppression of IL-10 is directly mediated by IFN-γ. GM4 DCs were either pretreated with CpG (*A* and *B*) or IFN-γ (*C* and *D*) before adding the other stimuli at 0, 2, 4, 6, and 8 h. The supernatants from each treatment were all collected following a total of 24 h incubation and the presence of IL-12p70 (*A* and C) and IL-10 (*B* and *D*) was detected using a cytokine-specific ELISA. The results shown are representative of two independent experiments.

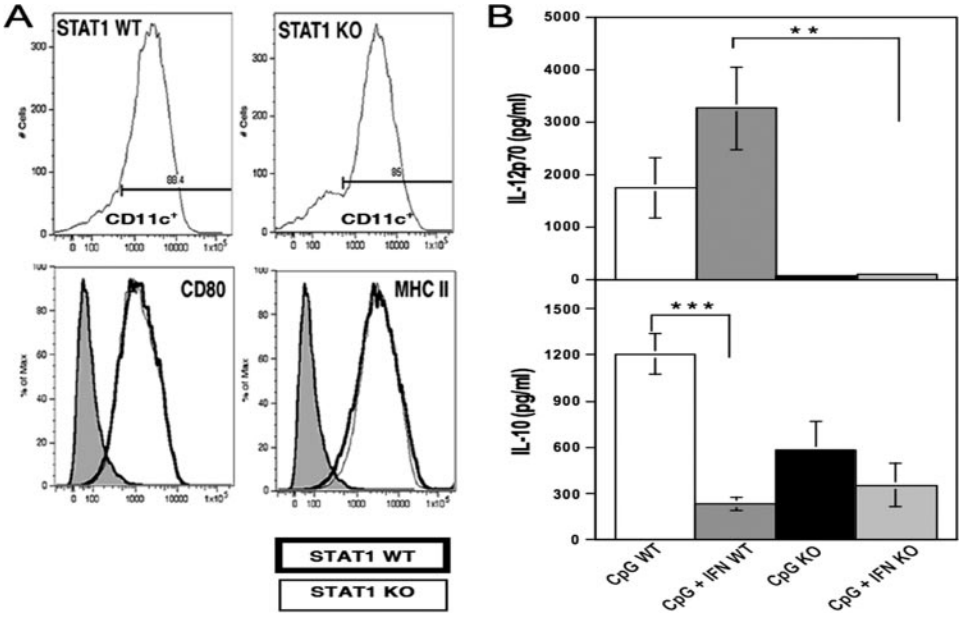

FIGURE 7. The induction of IL-12p70 by CpG and IFN-γ-induced suppression of IL-10 require STAT-1. GM4 DCs were generated from STAT-1 KO and the STAT-1 WT mice. *A*, DCs generated from STAT-1 WT and STAT-1 KO mice uniformly expressed CD11c (*top panels*). GM4 DCs from STAT-1 WT (thick line histogram) and STAT-1 KO (thin line histogram) were analyzed for the expression of CD80 and MHC class II by flow cytometry (*bottom panels*). *B*, GM4 DCs from STAT-1 WT and STAT-1 KO mice were stimulated with CpG with or without IFN-γ for 24 h. The supernatants were collected 24 h later and the presence of IL-12p70 (*top panel*) and IL-10 (*bottom panel*) was detected using a cytokine-specific ELISA. The data represent the mean and SD of eight independent experiments. **, $p < 0.01$; ***, $p < 0.001$.

CpG priming resulted in similar levels of IL-12p70, whereas delay beyond 2 h reduced the levels of IL-12 produced (Fig. 6*A*). Similarly, the greater the delay between CpG pretreatment and the addition of IFN-γ, the more IL-10 was produced and secreted (Fig. 6*B*).

In the reverse experiment, DCs were first incubated with IFN-γ before the addition of CpG, at 2, 4, 6 and 8 h (Fig. 6, *C* and *D*). The pretreatment of GM4 DCs with IFN-γ for 2 h followed by stimulation with CpG induced higher levels of IL-12p70 when compared with BMDCs stimulated with IFN-γ and CpG at time 0 (Fig. 6*C*). However, if the addition of CpG was delayed beyond 4 h the levels of IL-12p70 were substantially reduced (Fig. 6*C*). The pretreatment of BMDCs with IFN-γ suppressed the secretion of IL-10 induced by CpG at all time intervals tested (Fig. 6*D*). Pretreatment of DC with IFN-γ thus completely abrogated the ability of CpG to induce IL-10 and this resulted in a marked increase in IL-12p70 production.

STAT-1 is required for the optimal production of IL-12 but not IL-10

The results of the IFN-γ pretreatment experiments suggested that the suppression of IL-10 was dependent on IFN-γ receptor signaling. To confirm this effect, we performed experiments using GM4 DCs derived from STAT-1 KO mice. Signaling through the receptors for IFN-α, IFN-β, and IFN-γ has been shown to require STAT-1 (33, 34). GM4 DCs were generated from STAT-1 KO and STAT-1 WT mice. There were no significant differences in the number or phenotype of DCs generated from STAT-1 WT or STAT-1 KO mice (Fig. 7*A*). The GM4 DCs were then stimulated with CpG with or without IFN-γ overnight and the 24 h supernatants analyzed for IL-12p70 and IL-10. GM4 DCs generated from the STAT-1 WT mice stimulated with CpG produced IL-12p70, which was enhanced in the presence of IFN-γ (Fig. 7*B*, *top*). Consistent with previous experiments, the production of IL-10 was suppressed by IFN-γ in STAT-1 WT BMDCs (Fig. 7*B*, *bottom*). The levels of IL-12p70 by STAT-1 KO GM4 DCs stimulated with CpG or CpG with IFN-γ were significantly lower than levels of IL-12p70 produced by STAT-1 WT mice (Fig. 7*B*, *top*). The production of IL-12p70 induced by CpG and the enhancement by IFN-γ both appear to be STAT-1-dependent (Fig. 7*B*, *top*). In contrast, the levels of IL-10 produced by CpG-stimulated GM4 DCs from STAT-1 KO mice were not significantly lower than those produced by DCs from STAT-1 WT mice. However, the addition of IFN-γ to the CpG-stimulated STAT-1 KO DCs failed to reduce the levels of IL-10 produced, demonstrating the direct role of signaling via the IFN-γ receptor in the inhibition of IL-10 production.

These results demonstrate a requirement for STAT-1 in the production of IL-12p70 following CpG stimulation, which was surprising. This circumstance would suggest that CpG induced the production of IFN (α, β, and γ). We tested the supernatants from GM4 DCs stimulated with CpG for total IFN activity using a viral protection assay. Significant antiviral activity in supernatants from CpG stimulated DCs from both STAT-1 WT (704 U/ml) and STAT-1 KO (293 ± 11) mice. Simultaneously, no IFN-γ was detected by ELISA in the same supernatants.

To confirm this observation, we stimulated GM4 DC with CpG in the presence of neutralizing mAbs specific for IFN-α and/or IFN-β (Fig. 8). The addition of anti-IFN-α did not significantly reduce the production of either IL-12p70 or IL-10 (Fig. 8). In contrast, the addition of anti-IFN-β significantly reduced the levels

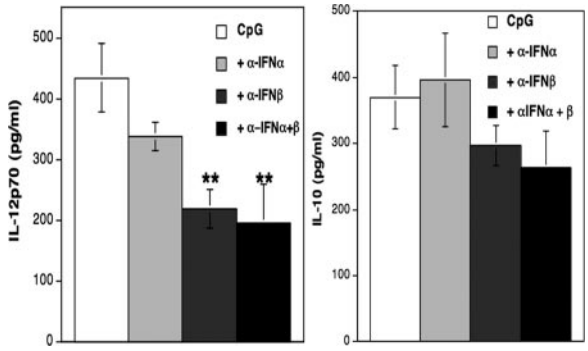

FIGURE 8. The production of IL-12p70 by CpG-activated DC is inhibited by anti-IFN-β mAbs. GM4 DCs were generated from 129/J mice and stimulated with CpG 1668 phosphothiolated (10 μg/ml) in the presence of anti-IFN-α and/or anti-IFN-β (10 μg/ml) for 24 h. The supernatants were collected and the presence of IL-10 (*right*) and IL-12p70 (*left*) was detected using a cytokine-specific ELISA. The results shown represent the mean ± SE of three to six independent experiments. **, $p < 0.01$ compared with CpG alone.

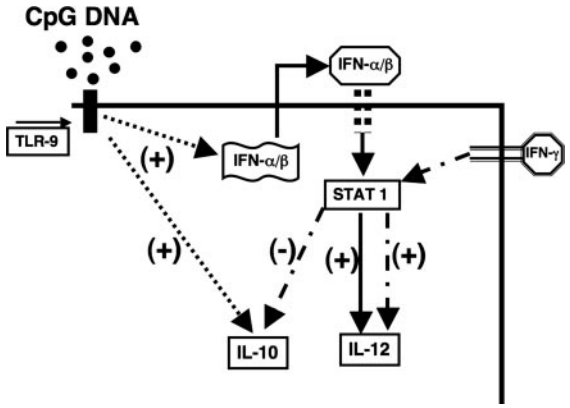

FIGURE 9. Model developed to explain CpG induces type I IFNs, which may be responsible for the induction of IL-12p70, whereas IL-10 production appears to be independent of type I IFNs. CpG interacts with TLR9 and directly stimulates the production of IFN-$\alpha\beta$ and IL-10 (dotted lines). IFN-$\alpha\beta$ is secreted and induces the production of IL-12p70 in a STAT-1-dependent manner (solid line). The addition of IFN-γ enhances the production of IL-12p70 and inhibits the production of IL-10 in a STAT-1-dependent manner (dashed line with dots).

of IL-12p70 induced by CpG, but had no effect on IL-10 production in the same cultures. The combination of both Abs did not further decrease IL-12p70 production, suggesting that IFN-β is mainly responsible for this effect (Fig. 8). Thus we conclude that CpG induces type I IFNs and that this may be responsible for the induction of IL-12p70. In contrast IL-10 production appears to be independent of type I IFNs. Fig. 9 outlines a potential model that describes these results.

Discussion

IL-10 is an important immunoregulatory cytokine that acts to limit the inflammatory response induced by Th1 cells. In this report, we demonstrate that the canonical Th1 cytokine, IFN-γ, directly inhibits the production of IL-10 in a dose-dependent and STAT-1-dependent manner. In addition, the induction of IL-12p70 production by the TLR ligand CpG was STAT-1-dependent, whereas CpG-induced IL-10 production was not. We further demonstrated that CpG-induced IL-12p70 production could be inhibited in the presence of anti-IFN-β mAb. The well-established ability of IFN-γ to enhance IL-12 production (35) may be, in addition to known effects on IL-12p40 promoter activation (36), mediated through a direct inhibition of IL-10.

It is well established that the ability of DCs to produce optimal levels of IL-12p70 when stimulated via TLR ligands is greatly enhanced in the presence of IFN-γ (35). The molecular mechanism for this enhancement has been controversial but recently a novel complex between NFAT and IFN regulatory factor (IRF)-8 was shown to bind to the IL-12p40 promoter following LPS plus IFN-γ macrophage activation (36). IL-10 is known to inhibit IL-12 production by inhibiting IL-12p40 gene expression at the level of transcription (29), and Zhu et al. (36) demonstrated that IL-10 interfered with the formation of the IRF-8/NFAT complex. Recent data have demonstrated that pretreatment of BMDCs with IFN-γ resulted in increased levels of expression of TLR9 (37). This led to enhanced IL-12p40 and IL-6 mRNA and increased levels of IL-12p70 protein. These results are consistent with our findings that pretreatment of DC with IFN-γ led to enhanced CpG-induced IL-12p70 production. In contrast pretreatment of cells with CpG did not lead to enhanced IL-12p70 production, which could be due to the previously reported inhibitory effect of CpG on IFN-γ signaling through the induction of suppressors of cytokine signaling-1 (38).

Previous studies have demonstrated a role for STAT-1 in mediating some of the effects associated with TLR4 ligation (39, 40). Recently, studies have shown a similar role for STAT-1 in TLR9 signaling pathway, the effects of which appear to be mediated through the induction of type I IFNs (41–44). In one study, Hoshino et al. (43) demonstrated that activation via TLR9 leads to IFN-β production, and that this production was MyD88-dependent while STAT-1-independent (43). This report demonstrated that the induction of CD40 by CpG was dependent on STAT-1 but did not assess cytokine production. We confirmed that in our system, CpG induced the production of type I IFNs by performing a viral protection assay, and further that anti-IFN-β mAbs inhibited the production of IL-12p70 but not IL-10 induced by CpG. The induction of IFN-$\alpha\beta$ following TLR9 ligation has recently been shown to depend on a signaling complex consisting of MyD88, IRF-7, TNFR-associated factor-6, and IL-1R-associated kinase 1 (42, 44). This response is distinct from the MyD88-independent induction of IFN-$\alpha\beta$ following TLR4 ligation, which involves IRF-3 and TRIF (45). Recent work has also implicated IRF-8 in the induction of TNF-α and IL-6 following TLR9 stimulation (46), and it is possible that IL-10 production could also be mediated via this pathway. More recent data have demonstrated that the addition of IFN-γ to CpG results in an IRF-1-dependent induction of IL-12p35 and IFN-β message, results that are consistent with the data described in this study (47). Thus, our data demonstrate that activation of DCs via TLR9 results in STAT-1-dependent production of IL-12p70 and STAT-1-independent production of IL-10 (Fig. 8).

LPS, CpG, and peptidoglycan were each capable of inducing mature and immature DCs to secrete moderate amounts of IL-10. Additional TLR ligands tested including poly(I:C) (TLR3 ligand) and loxiribine (TLR7 ligand) failed to induce significant IL-10 production, suggesting that this phenomenon may not be universal. The control of IL-10 production in myeloid cells remains poorly understood, although some recent publications have revealed new information concerning transcription factors important for IL-10 production. It was recently shown that macrophages and DCs have a unique DNase hypersensitive site in the IL-10 promoter that contains an NF-κB binding site (48). The transcription factor c-Maf is induced by IL-10 and acts to up-regulate IL-10 production and to inhibit IL-12p40 and IL-12p35 expression (49). Most recently the presence of c-Maf was shown to be essential for optimal IL-10 production by macrophages (50). Interestingly the addition of IL-4 in this system resulted in enhanced c-Maf and IL-10 expression (50). In contrast, recent research has demonstrated that IL-4 can also inhibit IL-10 production by CpG-activated DC in a STAT-6-dependent manner (51). These results are consistent with our unpublished observations that the addition of IL-4 to CpG-stimulated DCs causes enhanced IL-12p70 secretion. Our results demonstrating that IL-10 production is independent of STAT-1 are consistent with the studies by Saraiva et al. (48) suggesting that NF-κB plays an important role in IL-10 expression in macrophages and DCs.

It is well established that IL-10 negatively regulates the expression of IL-12 (30, 52), but little is known about the cytokine regulation of IL-10 production. The suppressive effect of IFN-γ on monocyte IL-10 has been reported in the literature (53), but it has not been extensively studied. In this study adherent PBMC were stimulated with *Mycobacterium leprae* extracts, and the addition of IFN-γ resulted in increased IL-12p70 and reduced IL-10 production. The reduction of IL-10 induced by IFN-γ in this situation appeared to be secondary to the presence of IL-12 (53). These previous studies did not use purified DC and it is possible that multiple cell types are

responding to the presence of IFN-γ. Our data demonstrate that IFN-γ inhibits IL-10 production, and this effect was seen both at the protein and mRNA level. The suppressive effect of IFN-γ on IL-10 expression could have been mediated directly by IFN-γ or indirectly through a secreted protein. The kinetics of IL-10 and IL-12 secretion showed that IL-12 was secreted before IL-10 following stimulation with CpG or CpG plus IFN-γ raising the possibility that IL-12 could inhibit IL-10 production. The presence of the neutralizing IL-12 mAb did not alleviate the suppression induced by IFN-γ, suggesting that IFN-γ may act directly to inhibit IL-10 production. The fact that IFN-γ failed to inhibit IL-10 production in DCs from STAT-1 KO mice further supports this interpretation. The mechanism by which IFN-γ mediates this effect is unknown, but it is possible that it may act to reduce c-Maf levels, and this area is research we are actively pursuing. Alternatively, it has recently been shown that the IκB protein Bcl-3 negatively regulates IL-10 production in macrophages (54) and it is possible that IFN-γ could influence this pathway.

The direct effect of IFN-γ on IL-10 suppression was further supported by the pretreatment experiments that demonstrated that treatment of DCs with IFN-γ, before the addition of CpG, resulted in a complete suppression of IL-10 production while dramatically enhancing the production of IL-12p70. Thus, it is possible that the inhibition of IL-10 by IFN-γ is an important mechanism in the IFN-γ-induced enhancement of IL-12p70. These results have implications for the use of CpG as a cancer vaccine adjuvant (55) because CpG is being used in several protocols as an important maturation factor for DCs. CpG stimulation results in the production of both proinflammatory (IL-12) and anti-inflammatory (IL-10) cytokines. The CpG ODN 1668, used in our studies, is a type B CpG that has been shown to either enhance (56) or have no effect (57) on tumor rejection, depending on the tumor model. The action of CpG ODN type B in the situation when tumor rejection was enhanced was found to depend on early NK cell activation (57), which might be expected to be sources of IFN-γ. Thus, the addition of IFN-γ to cancer vaccine protocols would not only enhance IL-12p70 production but also dramatically reduce IL-10 production.

In conclusion, our data indicate that the effect of IFN-γ on maturing DCs is 2-fold. IFN-γ induces maturation of DCs by enhancing the production of IL-12 while suppressing the production of IL-10 in a STAT-1-dependent mechanism. This conclusion suggests the existence of an additional level of cross-regulation between the inflammatory type (Th1 dominated) and noninflammatory type (Th2/Th3 dominated) patterns of immune response. These data presented in this study may have implications for vaccine protocols that aim to boost a type I response. The incorporation of CpG and IFN-γ, as adjuvants, into immunotherapeutic vaccines can boost immunity toward viral/bacterial pathogens and cancer.

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Disclosures

The authors have no financial conflict of interest.

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