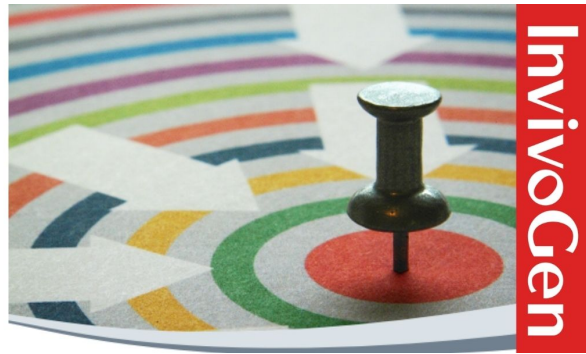


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### Nitric Oxide Inhibits IFN- $\alpha$ Production of Human Plasmacytoid Dendritic Cells Partly via a Guanosine 3',5'-Cyclic Monophosphate-Dependent Pathway<sup>1</sup> **FREE**

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# Nitric Oxide Inhibits IFN- $\alpha$ Production of Human Plasmacytoid Dendritic Cells Partly via a Guanosine 3',5'-Cyclic Monophosphate-Dependent Pathway<sup>1</sup>

Rimpei Morita,<sup>2\*</sup>† Takashi Uchiyama,\* and Toshiyuki Hori<sup>3\*</sup>

NO, a free radical gas, is known to be critically involved not only in vascular relaxation but also in host defense. Besides direct bactericidal effects, NO has been shown to inhibit Th1 responses and modulate immune responses in vivo, although the precise mechanism is unclear. In this study, we examined the effect of NO on human plasmacytoid dendritic cells (pDCs) to explore the possibility that NO might affect innate as well as adaptive immunity through pDCs. We found that NO suppressed IFN- $\alpha$  production of pDCs partly via a cGMP-dependent mechanism, which was accompanied by down-regulation of IFN regulatory factor 7 expression. Furthermore, treatment of pDCs with NO decreased production of IL-6 and TNF- $\alpha$  and up-regulated OX40 ligand expression. In accordance with these changes, pDCs treated with NO plus CpG-oligodeoxynucleotide AAC-30 promoted differentiation of naive CD4<sup>+</sup> T cells into a Th2 phenotype. Moreover, pDCs did not express inducible NO synthase even after treatment with AAC-30, LPS, and several cytokines. These results suggest that exogenous NO and its second messenger, cGMP, alter innate as well as adaptive immune response through modulating the functions of pDCs and may be involved in the pathogenesis of certain Th2-dominant allergic diseases. *The Journal of Immunology*, 2005, 175: 806–812.

Nitric oxide, a free-radical gas, is an important regulator and mediator of a wide range of physiological processes, including blood vessel relaxation, apoptosis, inflammation, and macrophage-mediated cytotoxicity for microbes and tumor cells (1–3). Most of the biological effects of NO are thought to be mediated by the cytoplasmic soluble guanylyl cyclase (GC)<sup>4</sup> that catalyzes biosynthesis of intracellular cGMP (1, 4). Evidence has indicated that NO not only exhibits protective activity against various infections but also regulates adaptive immunity by affecting the balance of Th1/Th2 responses. The inducible NO synthase (iNOS)-deficient or -mutant mice have been reported to mount significantly stronger Th1 responses than the wild-type mice with reduced virus titers, as well as pathological consequences of influenza A virus-induced pneumonia (5), whereas these mice are highly susceptible to several intracellular pathogens, including *Leishmania major*, *Mycobacterium tuberculosis*, and *Listeria monocytogenes* (6–8). Treatment with selective iNOS or NOS inhibitors has been shown to alleviate the pathological consequences not only of various virus infections, such as HSV-1-induced pneumonia and coxsackievirus B3-induced myocarditis (9, 10) but also of allergic diseases. For example, NOS inhibitors reduce the num-

ber of eosinophils infiltrated in lung tissues in sensitized rodents (2, 11, 12). Moreover, it is known that treatment with NO donors decreases IFN- $\gamma$  production in mice. These findings suggest that NO affects adaptive immunity with an apparent inclination toward inhibition of Th1 responses.

Dendritic cells (DCs) are the most potent APCs playing a pivotal role in the induction of primary immune responses (13, 14). Certain pathogen-derived compounds, cytokines, and soluble mediators have been shown to induce differentiation of immature DCs into mature DCs of a polarized phenotype toward Th1 or Th2 responses (13, 14). In humans, two distinct subsets of primary DCs are identified in peripheral blood and tonsils according to the difference in expression of CD11c (15, 16). While CD11c<sup>+</sup> myeloid DCs produce IL-12 through TLR-2 and -4 signaling, CD11c<sup>-</sup> plasmacytoid DCs (pDCs) secrete high levels of type I IFNs (IFN- $\alpha$  $\beta$ ) in response to viral infection presumably involving TLR-7 and -9 (17–19). Type I IFNs play essential roles in antiviral innate immunity by inhibiting viral replication in infected cells and by augmenting DC as well as NK cell function (20, 21). Thus, pDCs are crucial effector cells, which are the major source of type I IFNs and can modulate both innate and adaptive immunity (22).

The role of NO in adaptive immunity in humans is less clear. Nevertheless, recent clinical studies have indicated that exhaled NO is increased in certain allergic diseases such as bronchial asthma, nasal allergy, and atopic dermatitis, suggesting that NO may be involved in Th2 predominance in these disorders (23–26). Thus, it is important to dissect the cellular and molecular mechanisms by which NO affects the direction of immune response. In the present study, we focused on human pDCs and investigated whether NO altered their cytokine production and the consequent Th1/Th2 cell polarization upon stimulation with a TLR-9 ligand because pDCs have been reported to accumulate in nasal mucosa after allergic challenge in humans (27). Herewith, we show that NO suppresses the production of IFN- $\alpha$  of pDCs and polarizes them toward a Th2-promoting phenotype partly via a cGMP-dependent pathway.

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<sup>4</sup> Abbreviations used in this paper: GC, guanylyl cyclase; iNOS, inducible NO synthase; NOS, NO synthase; DC, dendritic cell; pDC, plasmacytoid DC; DETA/NO, 2,2'-(hydroxynitrosylhydrazono)bis-ethanimine; ODQ, *H*-(1,2,4)oxadiazolo(4,3- $\alpha$ )quinoxalin-1-one; db-cGMP, dibutyl-*l*-cGMP; IRF-7, IFN regulatory factor-7; OX40L, OX40 ligand; MFI, mean fluorescence intensity.

## Materials and Methods

### Media and reagents

RPMI 1640 supplemented with 10% heat-inactivated FBS (Invitrogen Life Technologies) was used throughout the experiments. 2,2'-(Hydroxynitrosodiazono)bis-ethanimine (DETA/NO) (0.5 to 50  $\mu$ M), *H*-(1,2,4)oxadiazolo(4,3- $\alpha$ )quinoxalin-1-one (ODQ) (3  $\mu$ M), 8-pCPT-cGMP ( $10^{-5}$  to  $10^{-3}$  M), and LPS (*Salmonella typhimurium*) (1  $\mu$ g/ml) were purchased from Sigma-Aldrich. Dibutyl-*l*-cGMP (db-cGMP) ( $10^{-5}$  to  $10^{-3}$  M) was purchased from Nacalai Tesque. TGF- $\beta$  (10 ng/ml), IFN- $\gamma$  (100 U/ml), and TNF- $\alpha$  (0.8  $\mu$ g/ml) were purchased from PeproTech. CpG-oligodeoxynucleotide AAC-30 (5  $\mu$ M) was synthesized by Biologica (17).

### Purification of human pDCs and their culture

Peripheral blood buffy coats were obtained from healthy human donors (kindly provided by the Kyoto Prefectural Red Cross Blood Center). PBMCs were isolated by Ficoll-Paque (Amersham Biosciences) density gradient centrifugation. pDCs were isolated from PBMCs with MACS magnetic bead columns using the BDCA-4 Cell Isolation kit (Miltenyi Biotec). The purity of pDCs was estimated as that of CD123<sup>+</sup> cells and accounted for >96% of the isolated cells. pDCs were cultured at  $5 \times 10^5$  cells/ml up to for 36 h.

### Western blot analysis of IFN regulatory factor (IRF)-7

pDCs were incubated in the culture medium with or without AAC-30, DETA/NO, ODQ, or db-cGMP ( $10^{-3}$  M) in 24-well plates at  $5 \times 10^5$  cells in 1 ml of medium/well. After 6 h, pDCs were collected, washed twice with PBS, and lysed in 60  $\mu$ l of SDS sample buffer/sample. The cell lysates were subjected to SDS-PAGE with 10% polyacrylamide gel. Then the samples were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore) and incubated sequentially with polyclonal anti-IRF-7 or polyclonal anti- $\beta$ -actin Abs (Santa Cruz Biotechnology) and with HRP-conjugated second Abs (Amersham Biosciences). IRF-7 and  $\beta$ -actin proteins were visualized using ECL detection kit (Amersham Biosciences). Relative signal intensities of IRF-7 compared with that of  $\beta$ -actin were quantified by densitometry, in which the value of freshly isolated pDCs was set as 100%.

### Flow cytometric analysis of cell surface Ags

pDCs were stimulated with AAC-30 in the presence or absence of DETA/NO. After 21 h, cells were collected and stained with the following FITC-conjugated mAbs: anti-HLA-DR (BD Biosciences), anti-CD80, and anti-CD86 (Immunotech). For the detection of OX40 ligand (OX40L), stimulated cells were incubated with biotinylated anti-OX40L mAb, ik-1 (28), for 30 min followed by PE-conjugated streptavidin. After immunofluorescence staining, cells were analyzed with a FACScan flow cytometer (BD Biosciences) using CellQuest software (BD Biosciences). The expression level of each Ag was indicated as  $\Delta$  mean fluorescence intensity ( $\Delta$ MFI), which was calculated by subtraction of MFI of the control value from that of the specific mAb.

### Apoptosis detection

After 36 h of culture in the absence or presence of the indicated stimuli in 48-well plates at  $2.5 \times 10^5$  cells in 500  $\mu$ l of medium/well, the cells were stained with propidium iodide (Molecular Probes) and FITC-conjugated annexin V (Caltag Laboratories) and analyzed with a FACScan flow cytometer (BD Biosciences) using CellQuest software (BD Biosciences).

### Analysis of intracellular cytokine production

Naive CD4<sup>+</sup> T cells were purified from umbilical cord blood mononuclear cells of healthy neonates by MACS using a MACS CD4<sup>+</sup> T Cell Isolation Kit II (Miltenyi Biotec). CD45RA<sup>+</sup>CD4<sup>+</sup> cells accounted for >95% of the isolated cells. pDCs ( $1 \times 10^5$  cells/well) that had been pretreated with the indicated reagents were washed thoroughly, irradiated (30 Gy), and cocultured with allogeneic naive CD4<sup>+</sup> T cells ( $1 \times 10^6$  cells/well) in 24-well plates for 7 days. Then cells were collected and stimulated with 50 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Calbiochem) for 5 h. Brefeldin A (10  $\mu$ g/ml) (Sigma-Aldrich) was added for the last 2 h. Cells were fixed with 2% formalin, permeabilized with PBS containing 2% FBS and 0.5% saponin, and then stained with FITC-anti-IFN- $\gamma$  mAb and PE-anti-IL-4 mAb (BD Biosciences). Stained cells were analyzed with a FACScan (BD Biosciences).

### Measurement of cytokines by ELISA

pDCs were cultured with AAC-30 in the absence or presence of DETA/NO, ODQ, or cGMP analogues in 96-well, round-bottom plates at  $1 \times 10^5$

cells in 200  $\mu$ l of medium/well. After 21 h, each supernatant was harvested, and cytokine concentrations in the supernatants were measured by the sandwich ELISA using matched paired Abs specific for IL-6, IL-10, IFN- $\alpha$ , and TNF- $\alpha$  (BioSource International), according to the manufacturer's instructions.

### Detection of iNOS expression and NO production

pDCs and human monocyte cell line THP-1 were incubated in the medium with the indicated stimuli in 48-well plates at  $2.5$  and  $1.25 \times 10^5$  cells in 500  $\mu$ l of medium/well, respectively. After 18 h, cells and the supernatants were collected. For detection of iNOS expression, cells were lysed in 80  $\mu$ l of SDS sample buffer/sample and subjected to SDS-PAGE with 10% polyacrylamide gel. Then the samples were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore) and incubated sequentially with monoclonal anti-iNOS or polyclonal anti- $\beta$ -actin Abs (Santa Cruz Biotechnology) and with HRP-conjugated second Abs (Amersham Biosciences).

NO was measured as nitrite using the Griess reaction. Briefly, 100  $\mu$ l of modified Griess reagent (Sigma-Aldrich) were added to 100  $\mu$ l of the supernatants. After 15 min, absorbance was measured at 540 nm. The nitrite content in the samples was calculated based on a standard curve read from a prepared standard solutions of sodium nitrite.

### Statistical analysis

Statistical analyses were performed by Student's *t* test, one-way ANOVA, or paired *t* test. Values of *p* < 0.05 were considered to be statistically significant.

## Results

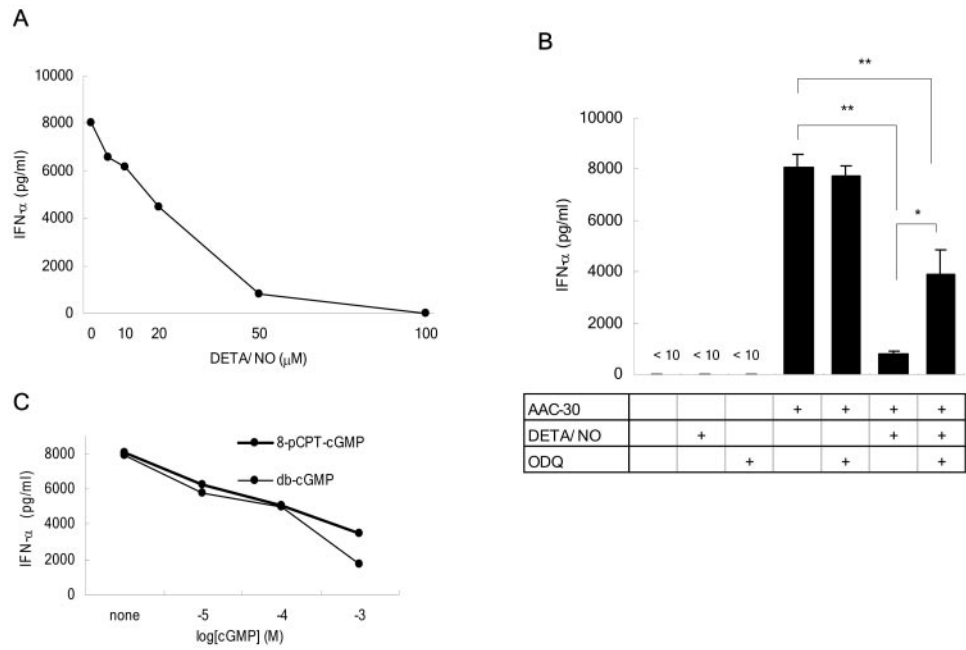
### NO suppresses IFN- $\alpha$ production of pDCs partly via cGMP-dependent mechanism

Because several reports indicated that NO was involved in the inhibition of Th1 responses (2, 3, 5, 6), first, we examined whether NO would have any influence on IFN- $\alpha$  production of pDCs. We cultured pDCs with the TLR-9 ligand, AAC-30, in the absence or presence of the NO donor, DETA/NO, for 21 h and measured IFN- $\alpha$  concentrations in the supernatants with ELISA. As shown Fig. 1A, AAC-30-stimulated pDCs produced large amounts of IFN- $\alpha$ , which was suppressed by treatment with DETA/NO in a dose-dependent manner up to 90% suppression at 50  $\mu$ M DETA/NO. The addition of a specific soluble GC inhibitor, ODQ, partly restored these cytokine productions of AAC-30 plus DETA/NO-treated pDCs (Fig. 1B). Two kinds of membrane-permeable cGMP analogues, 8-pCPT-cGMP and db-cGMP, reduced the production of IFN- $\alpha$  of pDCs in a dose-dependent manner (Fig. 1C). These results indicated that NO suppressed IFN- $\alpha$  production of pDCs, and this effect was in part mediated by cGMP-dependent mechanism.

### NO suppresses IRF-7 expression of pDCs via a cGMP-dependent mechanism

Next, we analyzed the mechanism by which NO suppresses IFN- $\alpha$  production of pDCs. We focused on the level of IRF-7 expression that is the critical determinant of the transcriptional activation of the IFN- $\alpha$  gene (29, 30) and examined the effect of NO on the expression of IRF-7 in AAC-30-treated pDCs by Western blot analysis. As previously reported, freshly isolated pDCs constitutively expressed IRF-7 to some extent, and treatment with AAC-30 for 6 h increased the expression levels of IRF-7 in pDCs (31, 32). As shown in Fig. 2, the addition of DETA/NO to AAC-30-treated pDCs decreased the expression levels of IRF-7 to the almost same levels as that of fresh pDCs, and ODQ restored the expression levels of IRF-7 in AAC-30 plus DETA/NO-treated pDCs. The addition of db-cGMP ( $10^{-3}$  M) to AAC-30-treated pDCs also decreased the expression levels of IRF-7. Because these changes coincided with the changes in IFN- $\alpha$  production of pDCs (Fig. 1, A–C), the suppression of IFN- $\alpha$  production by NO seems to be

**FIGURE 1.** Effects of NO and cGMP on production of IFN- $\alpha$  of AAC-30-stimulated pDCs. *A*, pDCs were stimulated with AAC-30 (5  $\mu$ M) in the absence or presence of DETA/NO (5–100  $\mu$ M) for 21 h. The concentrations of IFN- $\alpha$  of pDC culture supernatants were measured by ELISA. *B*, pDCs were stimulated with or without AAC-30 (5  $\mu$ M) in the absence or presence of DETA/NO (50  $\mu$ M) or ODQ (3  $\mu$ M) for 21 h. The concentrations of IFN- $\alpha$  of pDC culture supernatants were measured by ELISA. The results are shown as the mean  $\pm$  SD of three independent experiments. \*\*,  $p < 0.01$ ; and \*,  $p < 0.05$ ; Student's *t* test. *C*, pDCs were stimulated with AAC-30 (5  $\mu$ M) in the absence or presence of membrane-permeable cGMP analogues, 8-pCPT-cGMP or db-cGMP ( $10^{-5}$  to  $10^{-3}$  M), for 21 h. The concentrations of IFN- $\alpha$  were measured by ELISA. The results shown are from one representative experiment of three consistent ones.



mostly mediated by down-regulation of IRF-7 expression via a cGMP-dependent pathway.

#### NO suppresses other inflammatory cytokine production of pDCs

To investigate whether NO affects the production of inflammatory as well as anti-inflammatory cytokines of pDCs, we cultured pDCs

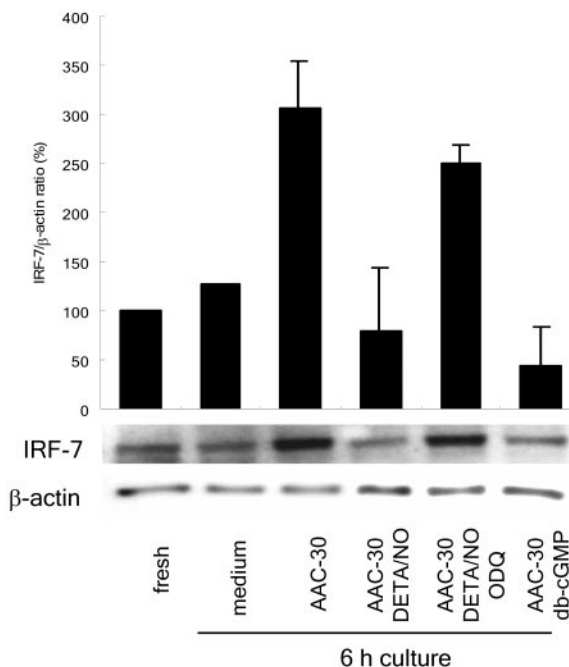
under the same conditions as Fig. 1A for 21 h and measured the concentrations of IL-6, TNF- $\alpha$ , and IL-10 in the culture supernatants by ELISA. Although unstimulated pDCs did not produce any of these cytokines, AAC-30 stimulation induced production of IL-6 and TNF- $\alpha$  but not IL-10 of pDCs as reported previously (16–18). The treatment with DETA/NO significantly reduced the production of IL-6 and TNF- $\alpha$  of AAC-30-stimulated pDCs, and the addition of ODQ partly restored the production of these cytokines (Fig. 3).

#### NO up-regulates the expression level of OX40L on pDCs in the presence of AAC-30

Next, we examined the effects of NO on the phenotypic maturation of pDCs with AAC-30. pDCs were cultured with AAC-30 in the absence or presence of DETA/NO for 21 h and then subjected to the flow cytometric analysis. The addition of DETA/NO to AAC-30 hardly changed the expression levels of CD80, CD86, or HLA-DR on pDCs (Fig. 4A). In contrast, stimulation with DETA/NO in the presence of AAC-30 resulted in significant up-regulation of OX40L (Fig. 4B), which has been reported to critically contribute to Th2 responses (33, 34).

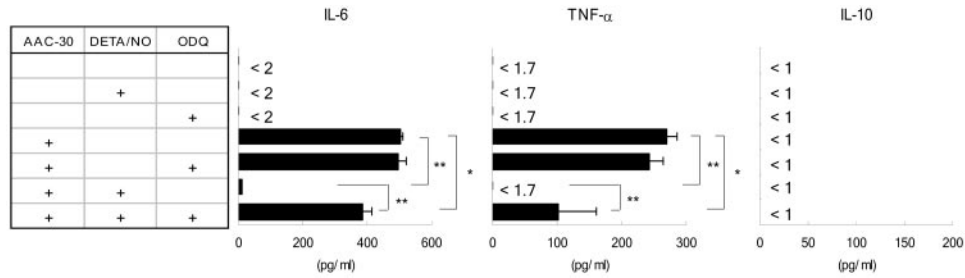
#### NO has little effect on apoptosis of pDCs in the presence of AAC-30

NO is known to have proapoptotic or antiapoptotic properties, depending on cell types (2, 35). Although it has been reported recently that NO has antiapoptotic effects on myeloid DCs stimulated with LPS (36), it is not yet clear whether NO has any effect on apoptosis of pDCs. To address this question, we incubated pDCs with or without AAC-30 in the absence or presence of a variety concentrations of DETA/NO (0.5–50  $\mu$ M) for 36 h and measured percentages of dead cells, as well as early apoptotic cells by flow cytometry. As shown Fig. 5, A and B, while the treatment with AAC-30 alone significantly reduced the percentages of dead cells of pDCs, the addition of DETA/NO hardly affected those of dead and early apoptotic cells of pDCs, indicating that NO had little proapoptotic effects on pDCs at least in the presence of AAC-30. Thus, it is likely that the decreases in cytokine productions of pDCs were caused by the decrease in the individual cellular ability to secrete cytokines rather than that in the numbers of cytokine-producing pDCs.



**FIGURE 2.** Effects of NO and cGMP on IRF-7 expression in AAC-30-stimulated pDCs. pDCs were stimulated with or without AAC-30 (5  $\mu$ M) in the absence or presence of DETA/NO (50  $\mu$ M), ODQ (3  $\mu$ M), or db-cGMP ( $10^{-3}$  M) for 6 h. Expression of IRF-7 and  $\beta$ -actin, an internal protein control, was evaluated by Western blot analysis. The data of one representative experiment of three consistent ones are shown in the lower panel. The upper bar graph indicates relative signal intensities of IRF-7. The value of freshly isolated pDCs is set as 100%. The results are shown as the mean  $\pm$  SD of the three experiments.





**FIGURE 3.** Effects of NO on production of IL-6, TNF- $\alpha$ , and IL-10 of AAC-30-stimulated pDCs. pDCs were stimulated with or without AAC-30 (5  $\mu$ M) in the absence or presence of DETA/NO (50  $\mu$ M) or ODQ (3  $\mu$ M) for 21 h. The concentrations of IL-6, TNF- $\alpha$ , and IL-10 of pDC culture supernatants were measured by ELISA. The results are shown as the mean  $\pm$  SD of three experiments. \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ ; Student's  $t$  test.

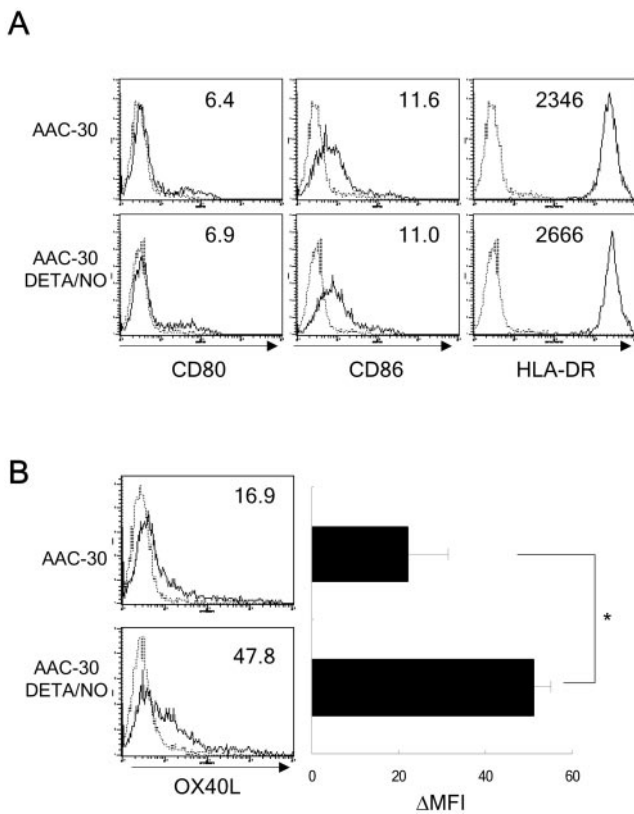
*NO polarizes pDCs toward a Th2-promoting phenotype*

Because NO suppressed IFN- $\alpha$  production of pDCs and up-regulated OX40L expression on pDCs (Figs. 1A and 4B), we next investigated whether NO could polarize pDCs toward a Th1- or Th2-promoting phenotype. To address this question, we cocultured allogeneic naive CD4<sup>+</sup> T cells with pDCs stimulated with AAC-30 in the absence or presence of DETA/NO (50  $\mu$ M). Then, CD4<sup>+</sup> T cells were analyzed for intracellular production of IFN- $\gamma$  as well as IL-4 by flow cytometry. Although coculture with AAC-30-activated pDCs generated large amounts of IFN- $\gamma$ -producing T

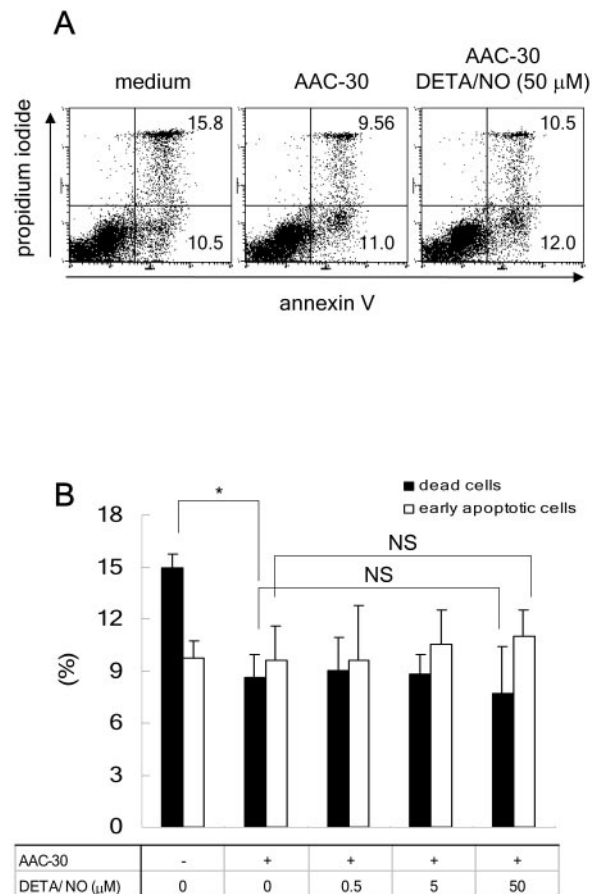
cells, the treatment of AAC-30-stimulated pDCs with DETA/NO resulted in less IFN- $\gamma$ -producing T cells and more IL-4-producing T cells (Fig. 6, A and B). These results indicated that NO polarized pDCs toward a Th2-promoting phenotype.

*Treatment with TLR ligands and cytokines induces pDCs neither to express iNOS nor to produce NO*

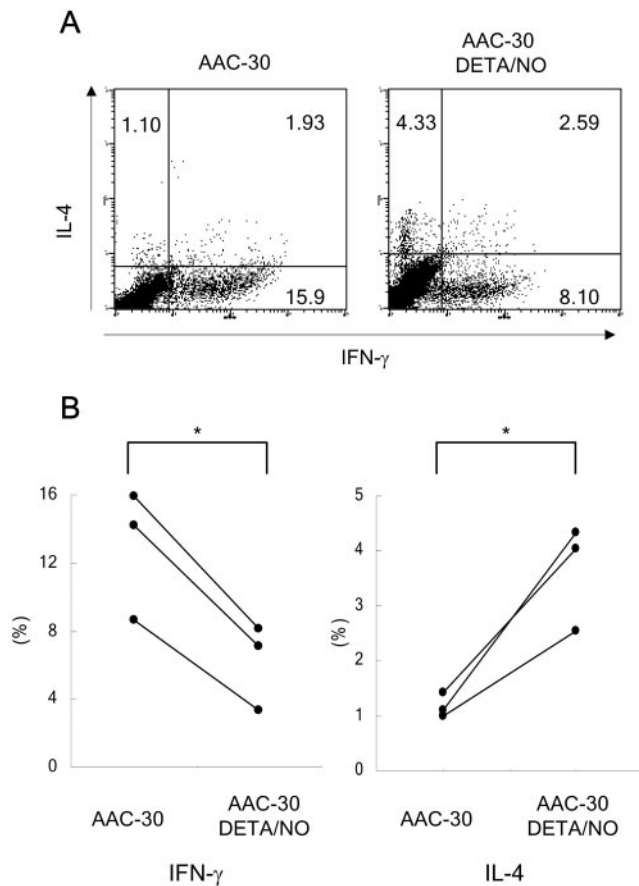
According to several reports, human myeloid DCs neither express iNOS nor produce NO in the presence of inflammatory cytokines



**FIGURE 4.** Effects of NO on phenotypic changes of AAC-30-stimulated pDCs. pDCs were stimulated with AAC-30 (5  $\mu$ M) in the absence or presence of DETA/NO (50  $\mu$ M) for 21 h, and the changes of expression of CD80, CD86, HLA-DR (A), and OX40L (B, left) were analyzed with a FACSscan. The staining profiles with specific mAbs and isotype-matched controls are shown in solid and dotted line histograms, respectively. The number in each histogram profile indicates  $\Delta$ MFI. The results shown are from one representative experiment of three consistent ones. B (right), The expression level of OX40L on pDCs is indicated as the mean  $\pm$  SD of the three experiments. \*,  $p < 0.05$ ; Student's  $t$  test.



**FIGURE 5.** Effects of NO on apoptosis of pDCs. pDCs were cultured with or without AAC-30 (5  $\mu$ M) in the absence or presence of DETA/NO (0.5–50  $\mu$ M) for 36 h. Then cells were collected, stained with propidium iodide and annexin V, and analyzed with a FACSscan. A, Dot plots of a representative experiment are shown. Numbers in the upper and lower right quadrants indicate percentages of dead and early apoptotic cells, respectively. B, Data are shown as the mean  $\pm$  SD of four independent experiments. \*\*,  $p < 0.05$ ; one-way ANOVA.

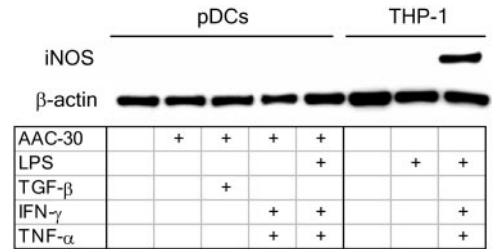


**FIGURE 6.** Effects of NO on polarization of naive CD4 T cells by pDCs. After pDCs were pretreated with AAC-30 (5  $\mu$ M) in the absence or presence of DETA/NO (50  $\mu$ M), they were irradiated and cocultured with allogeneic naive CD4<sup>+</sup> T cells for 7 days. T cells were internally stained for IFN- $\gamma$  and IL-4 and analyzed with a FACScan. **A**, The data of one representative experiment are shown. The percentage of the individual cytokine producer T cells is indicated in each dot plot profile. **B**, The assay was repeated three times, and the percentage of the individual cytokine producer T cells is shown for each sample. The differences in both IFN- $\gamma$ - and IL-4-producing cells were statistically analyzed by paired *t* test. \*, *p* < 0.05.

and/or LPS, whereas a subset of murine DCs express them (37–40). We examined whether treatment of human pDCs with TLR ligands such as AAC-30 and/or LPS together with several cytokines (32) induced expression of iNOS and subsequent generation of NO. In contrast to a human monocyte cell line, THP-1, that expressed iNOS upon stimulation with LPS and inflammatory cytokines as previously reported (41, 42), neither AAC-30 alone nor a combination of AAC30 and these inflammatory cytokines with or without LPS induced iNOS expression in pDCs (Fig. 7). Although Zhang et al. (43) have reported recently that TGF- $\beta$  is involved in the differentiation of murine DCs into NO highly producing DCs, it had no effect on iNOS expression in human pDCs. In accordance with these results, we could not detect NO production of pDCs under these conditions with the Griess reaction (data not shown).

## Discussion

In the present study, we showed that NO suppresses the production of several cytokines, especially IFN- $\alpha$  of activated pDCs partly via a cGMP-dependent pathway, up-regulates the OX40L expression on pDCs, and consequently polarizes them toward a Th2-promoting phenotype. We also demonstrated that NO inhibits the expres-



**FIGURE 7.** Effects of TLR ligands and cytokines on iNOS expression in pDCs and THP-1. pDCs and THP-1 were treated for 18 h in the absence or presence of the indicated TLR ligands: AAC-30 (5  $\mu$ M) and LPS (1  $\mu$ g/ml); and cytokines: TGF- $\beta$  (10 ng/ml), IFN- $\gamma$  (100 U/ml), and TNF- $\alpha$  (0.8  $\mu$ g/ml). Expression of iNOS and  $\beta$ -actin, an internal protein control, was evaluated by Western blot analysis. The results shown are from one representative experiment of three consistent ones.

sion of IRF-7, which may be one of the possible molecular mechanisms by which the NO/cGMP system suppresses IFN- $\alpha$  production.

NO, a water- and lipid-soluble gas, is known to be critically involved in not only vascular relaxation but also immunological host defense (1–3). The main molecular target of NO eliciting most of its downstream effects is cytoplasmic soluble GC that catalyzes biosynthesis of intracellular cGMP (1, 4). NO is produced by three kinds of NO synthases. iNOS is one among them that produces a large amount of NO for a longer time (i.e., 10–100 times more) than neuronal NOS and endothelial NOS (3), and its expression is localized in the area of inflammatory lesion such as bacterial or viral infection (2, 3). In mice, bacterial infection, LPS, or inflammatory cytokines induce iNOS expression in DCs in vivo as well as in vitro (37, 38, 43). However, previous reports indicated that human myeloid DCs do not express iNOS by stimulation with LPS and IFN- $\gamma$  (39, 40). In accordance with them, we could detect neither iNOS expression nor NO generation by pDCs stimulated with AAC-30, LPS, and several cytokines. Thus, NO-mediated regulation of human DCs is thought to depend on exogenous or paracrine NO, which is produced by activated macrophages as well as epithelial cells at inflammatory lesions (39).

IFN- $\alpha$  plays essential roles in antiviral innate immunity by directly inhibiting viral replication in infected cells and in immunoregulation by augmenting DC as well as NK cell function (20, 21). The expression of the IFN- $\alpha$  gene is largely regulated by a transcription factor, IRF-7 (29, 30). IFN- $\alpha$  is known to be produced by certain subsets of leukocytes and fibroblasts (21). Above all, pDCs produce a large amount of IFN- $\alpha$  in response to viral infection or CpG-oligonucleotides stimulation and consequently undergo differentiation into mature DCs (17–19, 44–46). Hence, they represent a unique cell lineage, which operates the two master functions of innate immunity and adaptive immune responses (22). In this context, NO/cGMP-mediated inhibition of IFN- $\alpha$  production by activated pDCs may have profound effects on the outcome of immune responses.

It is known that the balance of Th1/Th2 responses is influenced by the divergence of cytokines and costimulatory molecules of APCs (13, 14, 47). Activated pDCs produce high levels of IFN- $\alpha$  and consequently promote Th1 responses (19, 48). As in the case of histamine that has been reported to inhibit IFN- $\alpha$  production and impair the ability of pDCs to generate Th1 cells (49), NO did not alter the expression levels of CD80, CD86, and HLA-DR but suppressed IFN- $\alpha$  production. Furthermore, we observed that NO up-regulated the expression level of OX40L on AAC-30-stimulated pDCs. It should be noted that the OX40/OX40L system plays an important role in the formation of Th2 responses in both mice

and humans (33, 34). Recently, Ito et al. (50) have reported that OX40L exhibited costimulatory functions in human pDC-mediated Th2 responses. Based on these findings, polarization of pDCs toward a Th2-promoting phenotype by NO may be at least in part ascribed to the suppression of IFN- $\alpha$  production and up-regulation of OX40L expression.

Intracellular cGMP, which is not only generated by NO and its receptor soluble GC but also by natriuretic peptides and their receptors, regulates gene expression positively and negatively at transcriptional levels (4, 51). We previously reported that the receptor for atrial natriuretic peptide, GC-A, is expressed on monocyte-derived DCs and that atrial natriuretic peptide increases intracellular cGMP, inhibited LPS-induced IL-12 and TNF- $\alpha$  production, increased IL-10 production, and consequently polarized these DCs toward a Th2-promoting phenotype (52). Furthermore, our preliminary experiments showed that cGMP analogues suppressed IL-12 production by CD11c<sup>+</sup> myeloid blood DCs (data not shown). These findings may be explained partly by the reduction of NF- $\kappa$ B-binding activity by cGMP that several studies have already investigated in macrophages (51, 53). In the present study, we demonstrated for the first time that cGMP also down-regulated expression of IRF-7 in activated pDCs and consequently suppressed production of IFN- $\alpha$ , although the more detailed molecular mechanism of cGMP action is still unknown. Therefore, we hypothesize that a generalized scheme that cGMP functions as a common second messenger for the formation of Th2 responses in both myeloid and pDCs.

On the other hand, all the biological effects of NO are not mediated by cGMP (1, 3). In particular, evidence has indicated that relatively high concentrations of NO induce S-nitrosylation of certain molecules, resulting in their functional modulation. It has been reported that in murine macrophages, NO inhibits NF- $\kappa$ B and JNK1 activity by S-nitrosylation of these molecules (54, 55). Our data showed that suppression of IFN- $\alpha$  production of pDCs by DETA/NO was not completely reversed by an addition of ODQ, a specific inhibitor of soluble GC, suggesting that a cGMP-independent mechanism is also involved. It is to be determined if this cGMP-independent mechanism is mainly mediated by S-nitrosylation.

Previous studies with iNOS-deficient or -mutant mice have indicated that NO plays a critical role in the control of microbial pathogens, such as *Leishmania major*, *Mycobacterium tuberculosis*, and hepatitis B virus (6, 7, 56). Paradoxically, these mice can limit the pathological deteriorations caused by viruses such as coxsackievirus B3 and Sendai virus and their viral growth (10, 57). The iNOS-deficient mice infected with influenza A virus clear the virus from their lung and manifest less histopathologic changes in the lung than the wild-type mice (5, 57). With regard to these findings, it is suggested that the iNOS-deficient mice produce higher levels of IFN- $\gamma$  and stronger Th1 responses than wild-type mice (5). In contrast, it is also relevant to our findings that blocking OX40/OX40L interaction has been reported to alleviate manifestations of influenza A virus-induced pneumonia as well as bronchial asthma (58, 59). Although the precise mechanism by which NO inhibits Th1 responses and deteriorates viral infection is unclear, it is likely that both events are ascribed in part to inhibition of IFN- $\alpha$  production and up-regulation of OX40L expression due to NO.

Both NO, especially excessively generated by iNOS, and IFN- $\alpha$  are thought to play an important role in pathophysiology of several allergic diseases. iNOS is found to be expressed in airway epithelial cells, macrophages, and eosinophils, and increased levels of exhaled NO have been detected in asthmatic and allergic rhinitis patients (23–25). NO not only causes oxidative tissue damage but also induces eosinophil migration and cascades of PGs and wors-

ens asthma (2, 11, 12, 25, 60). Based on our results, NOS inhibitors not only reduce the tissue damages brought down by NO (60, 61) but also may be beneficial for the correction of Th2 dominant allergic reactions. In this context, it is noted that IFN- $\alpha$  production by PBMCs in ex vivo cultures is significantly lower in children with allergic than nonallergic diseases and that IFN- $\alpha$  administration has high efficacy in improving patients with severe asthma (62, 63). Furthermore, pDCs have been reported to accumulate in nasal mucosa of allergic subjects after allergen challenge, and adoptive transfer of pDCs inhibits development of asthma in a mouse model (27, 64). Although the precise mechanism of this phenomenon is unclear, it is suggested that IFN- $\alpha$  produced of these pDCs replenishes IFN- $\alpha$  levels lowered by NO generated at allergic lesion. Thus, NO and pDC-derived IFN- $\alpha$  may regulate the balance of Th1/Th2 immune responses.

In conclusion, the present study provides novel insights into the biological significance of NO and its second messenger cGMP in pDC-mediated regulation of Th1/Th2 responses. Further delineation of this aspect may lead to elucidation of the pathophysiology of allergic as well as infectious diseases and eventually to development of a novel therapy for them.

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## Disclosures

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