Synergism of nitric oxide and maturation signals on human dendritic cells occurs through a cyclic GMP-dependent pathway

Clara Paolucci,* Samuele E. Burastero,* Patrizia Rovere-Querini,* Clara De Palma,* Sestina Falcone,*,† Cristiana Perrotta,*,‡ Annalisa Capobianco,* Angelo A. Manfredi,* and Emilio Clementi*,‡

*Department Neuroscience and Department Immunology, DIBIT-H San Raffaele Institute, Milano, Italy; †Department Pharmacology, University of Milano, Italy; and ‡Department Pharmaco-Biology, University of Calabria, Rende, Italy

Abstract: Nitric oxide (NO), generated by phagocytes at inflammation sites, contributes to regulate immune responses through autocrine and paracrine actions on bystander cells. Among the latter are dendritic cells (DCs). Little is known about regulation of DC function by NO, especially in the human system. We exposed human monocyte-derived DCs to the NO donor (z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino] diazen-1-ium-1,2 diolate (DETA-NO) during their maturation process induced by treatment with tumor necrosis factor α or lipopolysaccharide or by CD40 activation. We report here that after exposure to DETA-NO, DCs exhibit a significantly increased ability to activate T lymphocytes stimulated by mycobacterial antigens, *Staphylococcus aureus* Cowen strain B, allo-antigens, or cross-linking of the CD3–T cell receptor complex. This effect persists after removal of DETA-NO, depends on the generation of cyclic guanosine 5′-monophosphate, and is a result of enhanced release by DCs of soluble factors, in particular interleukin (IL)-12. This modulation of DC function is a result of a synergism between NO and the various maturation stimuli, as neither enhanced T cell activation nor IL-12 release was observed after DC exposure to DETA-NO only. These results provide the first evidence that NO acts as a cosignaling molecule regulating human DC response to maturation stimuli. *J. Leukoc. Biol.* 73: 253–262; 2003.

Key Words: *guanylate cyclase · IL-12 · T cell activation · TNF-α · CD40 · lipopolysaccharide*

INTRODUCTION

Nitric oxide (NO) has been recognized as one of the most versatile players in the immune system [1]. This short-lived messenger is generated during immune responses, mainly through the inducible isoform of the NO synthase (iNOS), expressed in phagocytes after their activation by cytokines and bacterial products [2]. Because of its diffusible nature, NO acts in an autocrine and paracrine manerr on neighboring cells [3]. NO endows activated macrophages and microglial cells with antimicrobial and cytotoxic activity, enhances the function of T and natural killer cells, and regulates the generation of cytokines and chemokines at the site of infection [3–9]. Targeted deletion of iNOS renders mice more susceptible to infections [10].

Increasing evidence indicates that NO also modulates the function of dendritic cells (DCs), which are professional, antigen-presenting cells involved in the initiation and in the maintenance of immune responses. Immature DCs capture antigens at the site of inflammation and process and present them to T lymphocytes in secondary lymphoid organs [11]. These events are accompanied by DC maturation and are orchestrated by proinflammatory signals generated at the site of infection [11].

Experiments in rodents show that DCs can express iNOS and that this expression results in a reduced antigen-presenting function [12, 13]. In addition, generation of NO by iNOS-expressing DCs contributes to the clonal deletion of thymocytes and to the suppression of autoreactive T cells during experimental, allergic encephalomyelitis [14–16].

The role of NO in regulating human DC function is less clear. In liver diseases characterized by chronic inflammation of peripheral tissues, namely hepatocellular carcinoma and primary biliary cirrhosis, DCs have been found to express iNOS, and this correlates with their reduced antigen-presenting function [17, 18]. DCs from healthy donors, however, do not express iNOS when immature nor after exposure to lipopolysaccharides (LPS) and cytokines [18, 19]. Nevertheless, at the inflammation site, these DCs are exposed to NO diffusing from other NO-generating, immune cell types, together with proinflammatory molecules [4]. Recent studies in various cell types have shown that this paracrinely generated NO regulates specifically and selectively the signal transduction pathways activated by one such proinflammatory molecule, tumor necrosis

Correspondence: Dr. Emilio Clementi, DIBIT-H San Raffaele Institute, via Olgettina 58, 20132 Milano, Italy; E-mail: clementi.emilio@hsr.it

Received September 10, 2002; revised October 21, 2002; accepted November 19, 2002; doi: 10.1189/jlb.0902447.

factor α (TNF-α) [19–21]. This opens the possibility that paracrinely generated NO regulates maturation of human DCs induced by TNF-α and possibly other maturation signals, thus contributing to the antigen-presenting function of these cells during immune responses. So far, however, such a possibility has not been investigated.

To address this question, we studied the exposure of human DCs to NO during the TNF-α-induced maturation process [19, 22]. To this end, we used the NO donor (z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino] diazen-1-ium-1,2 diolate (DETA-NO), which releases NO constantly and at defined concentrations [23]. In our model, DETA-NO was added only during the DC maturation process and was subsequently removed. Our results show that exposure of maturing DCs to NO increases their ability to activate T lymphocytes. This effect of NO is persistent and independent of antigen uptake and processing. In addition, it requires activation of guanylate cyclase and generation of cyclic guanosine 5′-monophosphate, (cGMP) and is mediated through the release by DCs of interleukin (IL)-12. Similar cGMP-dependent, enhancing effects of NO on T cell activation and IL-12 release were also observed when DC maturation was obtained by treatment with LPS or by CD40 cross-linking. These results suggest that NO exerts a general, regulatory function during human DC maturation.

MATERIALS AND METHODS

Reagents

The following reagents were purchased as indicated: fluorescein isothiocyanate (FITC)-labeled mouse monoclonal antibodies (mAb) anti-human CD1a, CD40, CD80, CD83, CD86, and major histocompatibility complex (MHC) class I and class II from Caltag (Burlingame, CA); mAb anti-human CD28 from Becton Dickinson (San José, CA); recombinant human (rh)TNF-α, DETA-NO, and H-[1,2,4] oxadiazolo [4,3-α]quinoxalin-1-one (ODQ) from Alexis Italia (Florence); rhIL-4, IL-12, and granulocyte macrophage-colony stimulating factor (GM-CSF) from Strathmann Biotech GMBH (Hannover, Germany), Genzyme (Milano, Italy), and Mielogen-Schering Plough (Milano, Italy), respectively; Fycoll-Paque from BioChrom (Berlin, Germany); methyl-^{3}H thymidine from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK); *Mycobacterium tuberculosis*-derived tuberculin-purified protein derivative (PPD) from Statens Serum Institut (Copenhagen, Denmark); magnetic Dynabeads M-450 goat anti-mouse immunoglobulin G (IgG) from Dynal (Oslo, Norway); the anti-human IL-12 neutralizing mAb (clones 17F7 and 20C2, mouse IgG1); and the isotype-control mAb, kindly provided by Lars Rogge (Roche Milano Ricerche, Milan, Italy). The anti-CD40 mAb, purified from the 626.1 hybridoma, was kindly provided by Donata Vercelli (University of Arizona, Tucson, AZ). The mAb anti-human CD3 was purified from the hybridoma (OKT3) obtained from American Type Culture Collection (Manassas, VA). The reagents for tissue culture were from Gibco (Basel, Switzerland), except for fetal calf serum (FCS; clone III), obtained from Hyclone-Celbio (Milan, Italy); 8 Br-cGMP, *Staphylococcus aureus* Cowen strain B (SEB), and all of the other reagents were from Sigma Chemical Co. (Milan, Italy).

The conditions of use of DETA-NO, 8 Br-cGMP, and ODQ were selected on the basis of previous experiments in human DCs and in cell lines derived from monocytes and macrophages [19, 23, 24]. In particular, at the concentrations used in this study, these compounds appear not to be toxic and are still able to trigger biological effects [19, 23, 24].

All solutions were prepared endotoxin-free. Endotoxin contamination in all drug solutions was routinely assessed by the Lymulus amoebocyte gelification test, performed according to the manufacturer's instructions (PBI, Milan, Italy) before administration to DCs. All reagents, including the medium, scored negative.

Preparation and characterization of DCs and lymphocytes

Peripheral blood mononuclear cells (PBMC) from healthy blood donors were obtained from buffy coats (kindly provided by the Blood Transfusion Department of The DIBIT-H San Raffaele Institute, Milan, Italy) or heparinized blood drawn by venipuncture. Standard gradient separation procedures on Fycoll-Paque were used [19]. PBMC were resuspended in RPMI 1640 containing 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, from hereon referred to as "complete medium," and were allowed to adhere on six-well plates (Costar, Cambridge, MA) for 1 h at 37°C. Nonadherent cells were discarded. To derive immature DCs, PBMC were then cultured for 7 days in complete medium in the presence of human GM-CSF (50 ng/ml) and IL-4 (1000 U/ml) [19]. Depletion of residual T lymphocytes from immature DCs was routinely performed by incubating the cell preparations with the anti-CD3 mAb (1 μg/10^6 cells) and goat anti-mouse IgG-coated Dynabeads M-450. Anti-CD3 mAb-bound T cells were removed with a magnet according to the manufacturer's instructions. Purified DC preparations were routinely checked for contaminating lymphocytes by flow cytometry using FACScan Plus (Becton Dickinson, Sunnyvale, CA). Residual lymphocytes accounted for less than 1% of total cells (not shown). DCs were then incubated for 48 h with (mature DCs) or without (immature DCs) human TNF-α (50 ng/ml), LPS (1 μg/ml), or an anti-CD40-activating mAb (10 μg/ml) in the presence or absence of DETA-NO (50 μM), 8-Br-cGMP (3 mM), or ODQ in various combinations, as specified in Results below. Before the coculture with lymphocytes, DCs were washed twice in complete medium to remove all the various compounds and were then irradiated (2500 rad). Removal of the NO donor was routinely checked by verifying absence of NO generation in washed DC suspensions using a NO electrode (World Precision Instruments, Sarasota, FL) [23]. To assess DC phenotype, cells (5×10^5) were incubated for 30 min at 4°C in phosphate-buffered saline (PBS) containing 1% FCS with FITC-conjugated mAb specific to MHC class I and class II, CD1a, CD40, CD80, CD83, or CD86. Expression of the various molecules was evaluated by flow cytometry [19]. Data were analyzed with Cell Quest software (Becton Dickinson, Sunnyvale, CA). DC viability after the various treatments was assessed by propidium iodide/Annexin-V staining, exactly as described [19].

T cell-enriched preparations were obtained as follows: PBMC (5×10^6), obtained from PPD-positive or -negative donors, were washed in RPMI-1640 medium and incubated in complete medium containing 0.5% carbonyl iron for 60 min at 37°C on a rotating wheel. Phagocytic cells were removed with a magnet as described [25]. With these procedures, residual monocytes accounted for less than 1% of the cell preparations, as checked with anti-CD14 mAb by flow cytometry (not shown).

Measurement of cGMP generation

Immature DCs (1×10^6 cells/sample) were incubated for 15 min at 37°C in PBS with the phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine (0.5 mM) and were then exposed for an additional 30 min to increasing concentrations of ODQ (0–5 μM) in the presence or absence of DETA-NO (50 μM). The reaction was terminated by addition of ice-cold trichloroacetic acid (final concentration: 7.5%). After ether extraction, cGMP levels were measured using a radioimmunoassay kit and were normalized on cellular proteins, determined by the bicinchoninic acid assay procedure (Pierce, Rockford, IL).

Lymphocyte proliferation assays

For mixed lymphocyte reactions (MLRs), DCs, pretreated as described above, were cocultured at different ratios on 96-well flat-bottom plates together with heterologous lymphocytes, which were added at the constant concentration of 10^5 cells/well. DC-to-lymphocyte ratios ranged from 1:1000 to 1:5. For antigen-driven proliferation assays, DCs (2×10^4 cells/well) were incubated with autologous lymphocytes (10^5 cells/well) in the presence of 1 μg/ml PPD, 0.2 μg/ml SEB, or 0.1 μg/10^6 cells anti-CD3 mAb. In some experiments, lymphocytes were stimulated with SEB (0.2 μg/ml) on plates precoated by a 3-h incubation (37°C) with anti-CD28 mAb (5 μg/ml) or on plates precoated with the anti-CD28 mAb and anti-CD3 mAb (1 μg/ml) in the presence of a DC-conditioned culture medium (1:1). This DC-conditioned medium was

freshly prepared the day of the experiment by collecting supernatants conditioned for 48 h by the various DC preparations described above. The supernatant was filtered through a 0.2 μm Millipore filter before use. T cell proliferation was evaluated by measuring incorporation of methyl-^{3}H thymidine (0.8 μCi/well), which was added to the cocultures during the last 6 h. To this end, cells were harvested, and radioactivity was measured using a Wallac LKB β-counter.

Cytokine assays

T cells were cocultured with DCs (10^5 T cells plus 2×10^4 DCs) and were pretreated as described above on 96-well plates in 200 μl medium. IL-2, IL-4, and interferon-γ (IFN-γ) concentrations were determined in the medium 48 h after stimulation using commercially available enzyme-linked immunosorbent assay (ELISA) kits specific for each cytokine (R&D Systems, Space Import Export, Milan, Italy). IL-12p70 was measured in supernatants conditioned for 48 h by DCs pretreated with or without TNF-α, LPS (1 μg/ml), or the anti-CD40 mAb (10 μg/ml) in the presence or absence of DETA-NO using the Quantikine High-Sensitivity kit (detection limit: 0.5 pg/ml), following the manufacturer's instructions (R&D Systems).

Statistical analysis

The results are expressed as means ± SEM; n represents the number of individual experiments. Statistical analysis was performed using the Student's t-test for unpaired variables (two-tailed). In the figures, ** and *** refer to statistical probabilities (P) of <0.01 and <0.001, respectively, measured in the various experimental conditions as detailed in the legends to figures.

RESULTS

NO enhances T lymphocyte activation by TNF-α-treated DCs

Immature DCs were treated for 2 days with or without TNF-α (50 ng/ml) in presence or absence of the NO donor DETA-NO (50 μM) and were then washed free of the cytokine and the NO donor. At the concentration used, this NO donor releases a constant flux of 50 ± 2.3 nM NO (n=4), as measured by a NO electrode [23] and does not affect DC viability (see also ref. [19]). Thus, antigen-presenting function of pretreated DCs was evaluated by measuring DC-induced proliferation of allogeneic T lymphocytes in MLRs (**Fig. 1A**) or autologous T lymphocytes stimulated with SEB (0.2 μg/ml), PPD (1 μg/ml), or anti-CD3 mAb (0.1 μg/10^6 cells; Fig. 1B). In all these experimental settings, the pretreatment with DETA-NO alone resulted in no changes in DC-induced T cell proliferation. By contrast, when administered to DCs together with TNF-α, the NO donor increased DC ability to stimulate T cells. T cell proliferation was significantly higher than that induced by untreated DCs or DCs pretreated with TNF-α alone, in MLRs (Fig. 1A), and in autologous reactions in the presence of SEB, anti-CD3 mAb, or

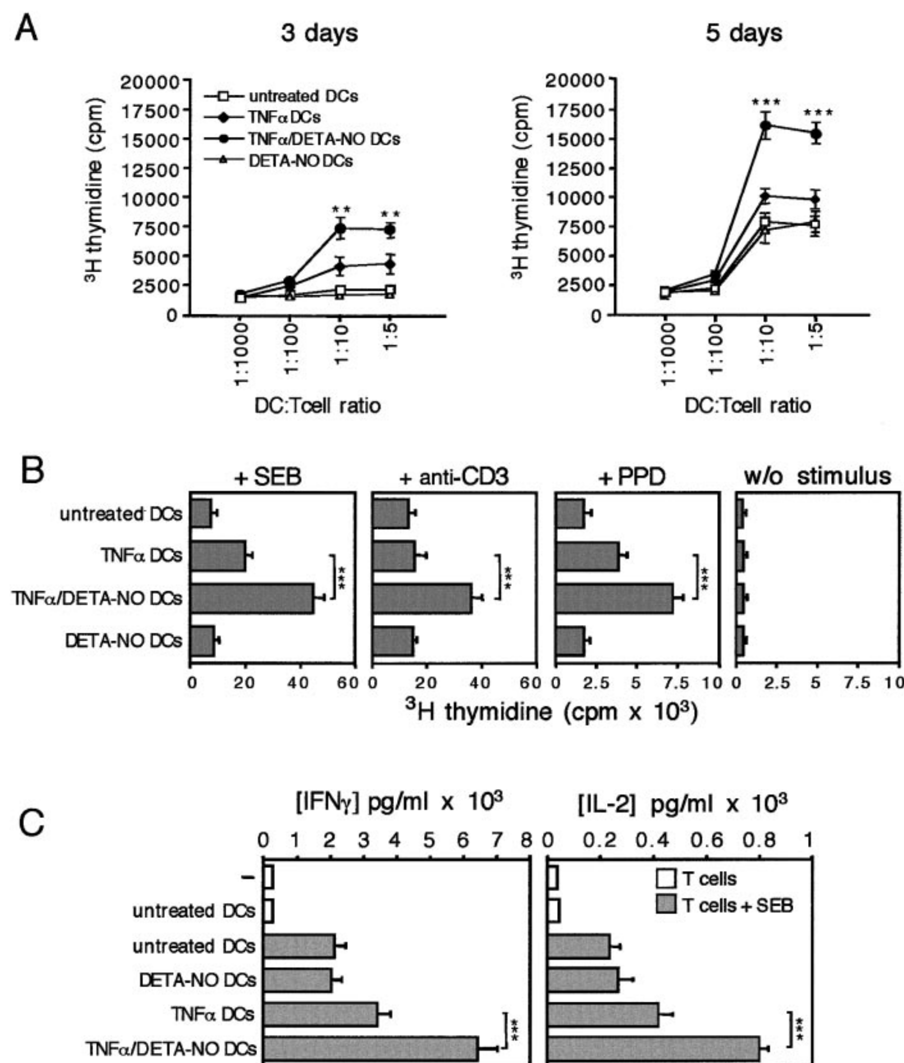

Fig. 1. NO enhances the ability of TNF-α-treated DCs to induce T cell activation. Immature DCs were treated for 2 days with or without TNF-α (50 ng/ml) in the presence or absence of DETA-NO (50 μM). (A) These pretreated DCs were cocultured at the indicated ratios with a fixed number of allogeneic T cells (10^5 cells/well); proliferation (^{3}H-thymidine incorporation in T cells) was measured after 3 and 5 days of DC/T cell coculture. (B) Pretreated DCs (2×10^4) were cocultured with 10^5 autologous T cells in the presence of SEB (0.2 μg/ml), anti-CD3 (0.1 μg/10^6 cells), and PPD (1 μg/ml) or without any stimulus. Proliferation was measured after 3 days. (C) IFN-γ and IL-2 secretion were measured in the supernatant from 3 days coculture of 10^5 autologous T cells with or without 2×10^4 pretreated DCs and in the presence or absence of SEB. Asterisks indicate statistical significance, measured as indicated in Materials and Methods, of TNF-α/DETA-NO DCs versus TNF-α-treated DCs (n=8).

PPD (Fig. 1B). Regardless of the pretreatment, DCs alone induced no significant proliferation of unstimulated, autologous T cells (Fig. 1, A and B).

The concentration of IFN-γ, IL-2, and IL-4 in the medium under the various experimental conditions described above was assessed. Figure 1C shows the values measured for IFN-γ and IL-2 using SEB-stimulated T cells. Concentrations of IFN-γ and IL-2 detected in the presence of DCs pretreated with DETA-NO alone were similar to those observed with immature, untreated DCs. Pretreatment of DCs with TNF-α resulted in increased release of cytokines, which was further, significantly enhanced in the presence of the NO donor. By contrast, release of IL-4 was not modified by DC pretreatment with DETA-NO. (Using SEB-stimulated T cells, values were 3.2±0.11 and 3.4±0.19 pg/ml with untreated DCs and DCs pretreated with TNF-α, respectively, and 2.9±0.12 and 3.1±0.09 pg/ml with DCs pretreated with DETA-NO and DETA-NO plus TNF-α, respectively; $n=3$). In the absence of SEB, no significant release of IFN-γ, IL-2, and IL-4 was observed, regardless of whether lymphocytes were cultured alone or in the presence of untreated, immature DCs.

The effect of NO on T lymphocyte activation by TNF-α-treated DCs is dependent on the generation of cGMP

NO biological effects are mediated through signaling pathways, dependent and independent of their ability to activate guanylate cyclase and generate cGMP [2]. Recently, we have shown that NO triggers cGMP generation in immature human DCs and that the cyclic nucleotide contributes to regulate endocytosis in these cells [19]. In the present experimental setting, administration of DETA-NO resulted in generation of cGMP, which was inhibited in a concentration-dependent way by ODQ, a specific guanylate-cyclase inhibitor [26] (**Fig. 2**). Therefore, we investigated whether the NO-dependent enhancement of DCs antigen-presenting ability was mediated through cGMP. To this end, immature DCs were pretreated with or without TNF-α in the presence or absence of the membrane-permeant cGMP analog 8 Br-cGMP (3 mM), of DETA-NO, or of DETA-NO, together with increasing concentrations of ODQ (0–5 μM). DCs treated with 8 Br-cGMP and TNF-α induced proliferation of T cells and secretion of IFN-γ and IL-2 higher than those induced by TNF-α-treated DCs and with a pattern similar to that observed with DCs treated with DETA-NO and TNF-α (**Fig. 3, A–C**, left). Pretreatment with ODQ, although ineffective in DCs treated with TNF-α only, reversed in a concentration-dependent way the effect induced by coadministration of TNF-α and DETA-NO on the proliferation of T cells and the secretion of cytokines (Fig. 3, A–C, right).

A soluble factor released by DCs treated with TNF-α and NO is responsible for the enhanced T lymphocyte activation

TNF-α-induced differentiation of DCs toward a mature, antigen-presenting phenotype involves the coordinated up-regulation of proteins on the plasma membrane, including MHC classes I and II and costimulatory molecules, as well as the release of soluble factors [27].

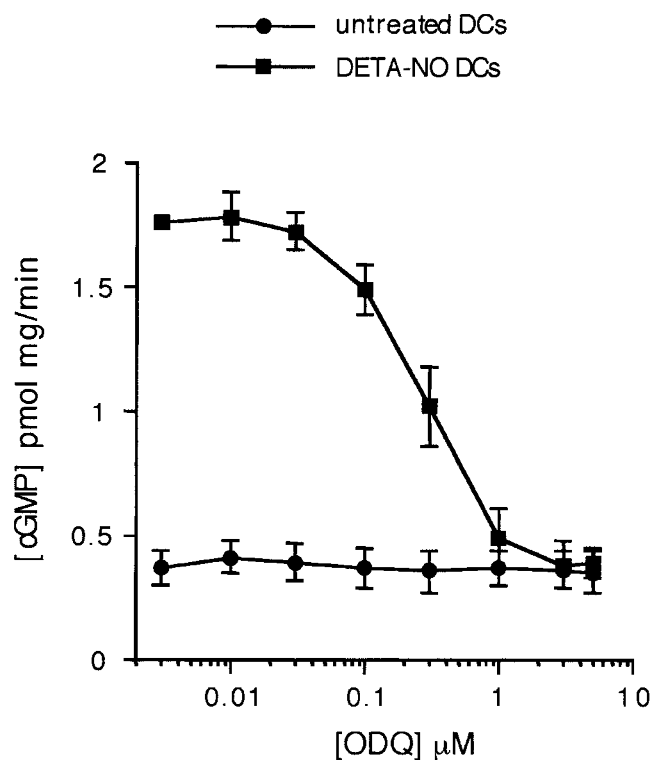

Fig. 2. Generation of cGMP by DCs. Immature DCs were pretreated for 15 min with the phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine (0.5 mM) and were then incubated for 30 min with increasing concentrations of ODQ (0–5 μM) in the presence or absence of DETA-NO (50 μM). cGMP generation in the various experimental conditions was measured using a radioimmunoassay specific for the cyclic nucleotide.

We examined whether NO modulates plasma membrane expression of MHC classes I and II, CD1a, CD83, and costimulatory CD40, CD80, and CD86 molecules. The pattern of expression of these molecules in immature and TNF-α-treated DCs is shown in **Figure 4**. Treatment of immature DCs for 48 h with DETA-NO did not change expression of MHC classes I and II nor any of the costimulatory molecules investigated. Likewise, the NO donor did not modify the pattern of expression of these molecules elicited by a 48-h maturation with TNF-α (Fig. 4).

Whether release of soluble factors accounted for the effects of NO-treated DCs on T cell proliferation was next investigated. Immature DCs were treated for 2 days with or without TNF-α in the presence or absence of DETA-NO, were washed free of the cytokine and the NO donor, and were maintained for a further 2 days in culture. Culture medium was then removed and assayed for its ability to modulate proliferation of T lymphocytes in an activation system consisting of a CD28-activating mAb administered together with SEB or anti-CD3 mAb. Both of these activation systems triggered significant T cell proliferation (**Fig. 5A**). Addition of the supernatant from cultures of untreated DCs or DCs treated with only DETA-NO did not modify this activation. By contrast, the supernatant from TNF-α-treated DCs increased T cell proliferation (more evidently in the anti-CD28/SEB model). In both of the activation systems analyzed, this effect was significantly greater using supernatants from DCs coincubated with TNF-α and DETA-NO (Fig. 5A).

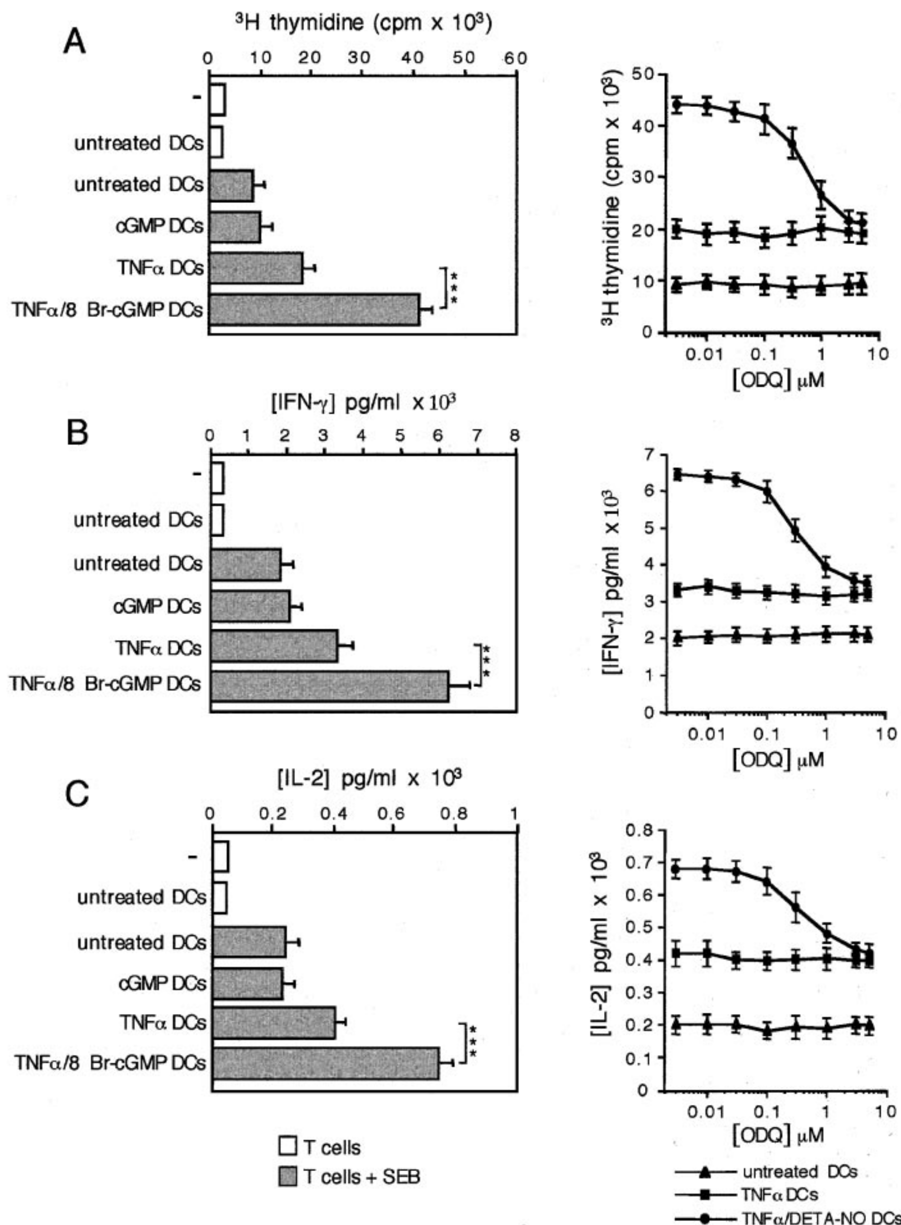

Fig. 3. The NO-dependent enhancement of the ability of DCs to induce T cells activation is mediated through cGMP. Immature DCs were incubated for 2 days with or without TNF-α (50 ng/ml) in the presence or absence of DETA-NO (50 μM), the membrane-permeant cGMP analog 8 Br-cGMP (3 mM), or the inhibitor of soluble guanylate cyclase ODQ (0–5 μM), as indicated in the keys to the various panels. Autologous T cells (10^5) were then cocultured with or without 2 x 10^4 pretreated DCs in the presence or absence of SEB (0.2 μg/ml). T cell activation was evaluated as proliferation (A) and secretion of IFN-γ and IL-2 (B and C) after 3 days. Asterisks indicate statistical significance, measured as indicated in Materials and Methods (n=8).

Increased release of IL-12 is responsible for the enhanced T cell activation by NO/TNF-α-treated DCs

Among the proinflammatory cytokine released by DCs, IL-12 has been shown to play pivotal roles in T cell activation [28]. We thus evaluated whether NO regulates release of IL-12. As shown in Figure 5B, release of IL-12 was slightly increased by pretreatment of DCs with TNF-α and was further, significantly enhanced when DETA-NO or 8 Br-cGMP was coadministered with TNF-α. The dependence on cGMP generation of the effect of DETA-NO was further confirmed by the observation that administration of ODQ (3 μM) together with DETA-NO and TNF-α gave rise to IL-12 generation similar to that observed in DCs pretreated with TNF-α alone. Basal IL-12 release was not modified by DC pretreatment with DETA-NO, 8 Br-cGMP, or ODQ alone. To evaluate whether this increased secretion of IL-12 was involved in stimulation of T lymphocyte proliferation by DCs, we tested the effect of a neutralizing anti-IL-12 Ab cocktail [29]. Inhibition of IL-12 did not significantly modify T cell proliferation induced by the supernatant from untreated and TNF-α-pretreated DCs. This applied to anti-CD28/SEB and anti-CD28/anti-CD3-stimulated T cells (Fig. 5C). In contrast, the neutralizing anti-IL-12 Ab cocktail abolished the enhancing effect on T cell proliferation induced by DETA-NO when combined with TNF-α, giving rise to proliferation values similar to those observed with TNF-α alone. These effects appeared to be specific, as they were not observed in the presence of an isotype-control Ab.

NO enhances T lymphocyte activation by DCs matured with LPS or an anti-CD40 mAb through cGMP-dependent IL-12 release

To assess whether the effect of NO and the mechanism of its action were specific for TNF-α-induced DC maturation or a more general phenomenon, we investigated DC maturation induced by LPS and activation of CD40. Immature DCs were

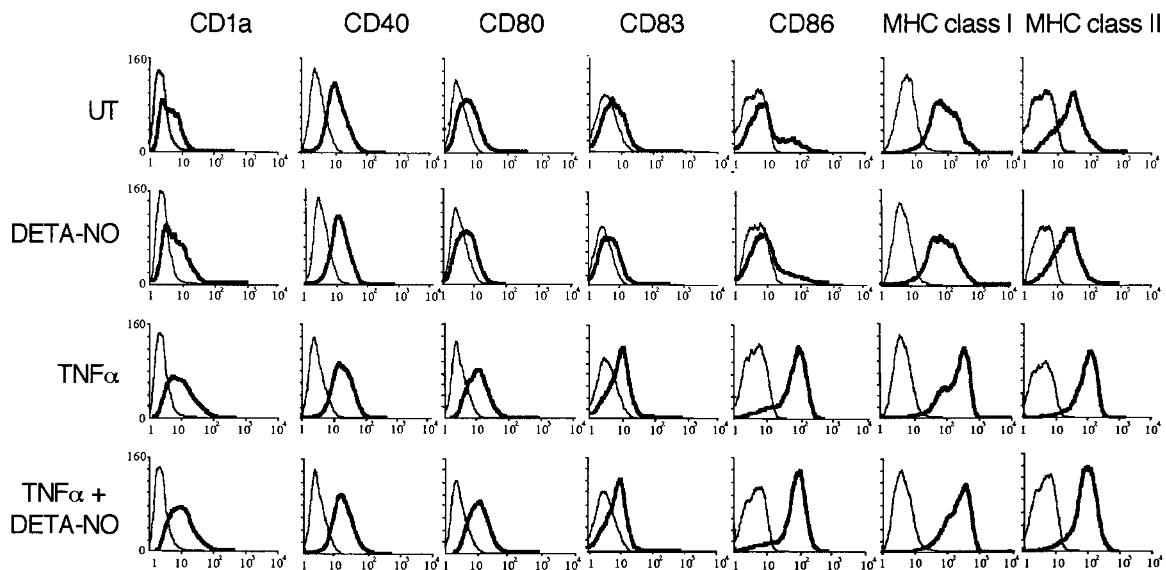

Fig. 4. Phenotypic characterization of DCs induced to mature with TNF-α in the presence or absence of NO. Immature DCs were treated for 2 days with or without TNF-α (50 ng/ml) in the presence or absence of DETA-NO (50 μM). Cell preparations were analyzed by flow cytometry after staining with FITC-conjugated Ab specific for the surface antigens specified at the top of each column (solid-line histograms). Cell autofluorescence is indicated by the superimposed, thin-line histograms. The results shown are from 1 experiment representative of 10 consistent ones. UT, Untreated.

treated for 2 days with or without LPS (1 μg/ml) or an anti-CD40-activating mAb (10 μg/ml) in the presence or absence of DETA-NO, 8 Br-cGMP, and ODQ (3 μM) in various combinations. The cells were then washed free of all the treatments. LPS treatment and CD40 cross-linking triggered DC maturation, as assessed by increased expression of CD80, CD83, CD86, and MHC class II, none of which was modified by cotreatment with DETA-NO (**Table 1**). When administered to DCs, together with LPS or the anti-CD40 mAb, however, the NO donor increased DC ability to stimulate T cells in MLRs (**Fig. 6, A** and **B**) and in autologous reactions in the presence of SEB (Fig. 6, C and D). As these effects of NO were similar to those observed with TNF-α-treated DCs, we evaluated whether they were a result of IL-12 release. As already reported [30], treatment of DCs with LPS or anti-CD40 Ab resulted in the release of concentrations of IL-12 higher than those observed after DC treatment with TNF-α. These values were further, significantly increased by the coincubation of DCs with DETA-NO (**Fig. 7, A** and **B**).

We then investigated the effects on T cell proliferation in the anti-CD28/SEB system of supernatants obtained from 2 days of culture of DCs with LPS or anti-CD40 mAb, prepared as described above for the experiments with TNF-α. Both conditions gave rise to T cell proliferation, which was significantly increased when supernatants were obtained from DCs also incubated with DETA-NO (Fig. 7, C and D). Administration of the neutralizing anti-IL-12 Ab cocktail reduced T cell proliferation induced by the supernatant from LPS and anti-CD40-pretreated DCs and abolished the enhancing effect on T cell proliferation induced by DETA-NO.

Similar to the experiments with TNF-α, also in the experiments with LPS- and anti-CD40-treated DCs, we found that all the effects of NO were dependent on cGMP generation, as they were mimicked by 8 Br-cGMP and reversed by ODQ (Figs. 6, C and D, and 7, A and B).

To further evaluate the role of released IL-12, we measured the effect of adding increasing concentrations of exogenous IL-12 to T lymphocytes in the anti-CD28/SEB experimental system. As shown in Figure 7E, IL-12 increased T cell proliferation in a concentration-dependent manner. The effect of exogenous IL-12 appeared in the range of that induced by supernatants containing comparable concentrations of endogenously generated IL-12 (cf. Fig. 7E with C and D and with Fig. 5C).

DISCUSSION

In inflamed peripheral tissues, DCs are exposed simultaneously to a variety of signals, among which is NO [1, 4]. A role for NO in regulating DC functions had already been proposed, however, based mostly on results obtained in the murine system [1, 4]. The latter is different from the human system. In fact, at variance with their murine counterparts, DCs from healthy donors are unable to express iNOS [19]. Thus, they are exposed, not to autocrine but only to paracrine NO, i.e., the one generated at the site of inflammation by neighboring, NOS-competent cells.

Our experimental protocol was designed to expose DCs to NO only during their maturation, which was triggered with three different stimuli, TNF-α, LPS, and an activating, anti-CD40 mAb. This approach allowed us not only to mimick the effects of an exposure of human DC to paracrine NO but also to dissect those effects of NO that are accounted for by a direct action on DCs. Under these conditions, we found that NO, although ineffective when administered alone, synergizes with each of the three tested stimuli to trigger a modified DC maturation program, resulting in an enhanced ability to stimule T lymphocyte proliferation. This effect of NO was found to possess three main characteristics. The first is that it is inde-

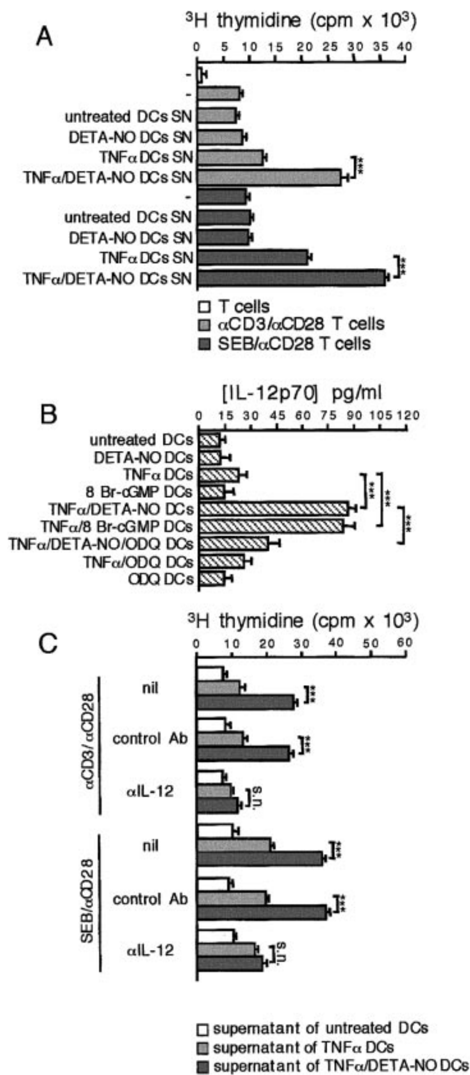

Fig. 5. Release of IL-12 is crucial to enhanced T lymphocyte activation by DCs treated with TNF-α and NO. Supernatants were collected from DCs pretreated for 2 days with or without TNF-α (50 ng/ml) in the presence or absence of DETA-NO (50 μM), 8 Br-cGMP (3 mM), or ODQ (3 μM). (A) T cell proliferation in the presence or absence of the various supernatants, as indicated on the left, was stimulated for 3 days with anti-CD3 (1 μg/ml)/anti-CD28 (5 μg/ml) mAb or SEB (0.2 μg/ml)/anti-CD28 mAb, as detailed in Materials and Methods. Control experiments with unstimulated T cells were run in parallel. (B) Concentration of IL-12p70 in the supernatants, evaluated by ELISA. (C) Experiments were performed as in A but in the absence (nil) or presence (αIL-12) of an IL-12 neutralizing mAb cocktail, as described in Materials and Methods. Experiments with an isotype-control Ab were performed in parallel. Asterisks indicate statistical significance, measured as indicated in Materials and Materials; s.n., Statistically not significant (n=6).

pendent of antigen uptake/processing, as it was observed in MLRs; in autologous reactions in the presence of SEB, anti-CD3 mAb, or PPD; and in the presence of the DC-conditioned medium only. Second, the enhanced DC ability of stimulating T lymphocyte proliferation persists after removal of NO, suggesting that priming with NO in peripheral tissues may be sufficient to exert a licensing effect on antigen presentation in lymphoid organs. Finally, the effect of NO appears independent of the stimulus that triggers DC maturation, as it was equally observed with LPS, TNF-α, or the anti-CD40 mAb. This, together with the observation that the increased ability to activate T cells by NO was only observed when NO and each of the above stimuli were coadministered to DCs, indicate that NO acts through regulation of specific, transductional events activated by these maturation stimuli. Paracrine NO, therefore, appears to function as a cosignal influencing the cross-talk between DCs and T cells.

We investigated the possible mechanisms through which NO exerts its action on maturing DCs and found it to depend in all cases on activation of guanylate cyclase and generation of cGMP, as demonstrated by experiments with the membrane-permeant cGMP analog 8-Br-cGMP and the guanylate-cyclase inhibitor ODQ. Although DC exposure to NO did not modify the maturation-regulated expression of MHCs and major co-stimulatory molecules, it greatly affected release to the extracellular milieu. In particular, with all three maturation stimuli tested, we identified increased release of IL-12 as relevant to the cGMP-dependent effect of NO. The role of IL-12 was investigated by two experimental approaches. First, we added an IL-12 neutralizing Ab to the supernatants from the various DC cultures. Under these conditions, the enhancing effect of NO on T cell proliferation disappeared. Second, we studied the effect of exogenous IL-12 added to T lymphocytes in the anti-CD28/SEB system and found T cell proliferation stimulated in a concentration-dependent way, as already reported in a similar system [31]. This effect was in the range of that induced by supernatants containing comparable concentrations of IL-12 produced by DCs exposed to NO, together with LPS, TNF-α, or the anti-CD40 mAb. From these experiments, we conclude that increased release of IL-12 explains the observed effect of NO/cGMP-treated DCs on T lymphocyte proliferation.

Activation of guanylate cyclase and generation of cGMP mediate many effects of NO in a variety of cells, including signaling events activated by TNF-α and LPS (see, e.g., refs. [20, 21, 32–34]). Despite the relevant role played by NO/cGMP

TABLE 1. Phenotypic Characterization of DC Maturation Induced by LPS or by CD40 Cross-linking in the Presence or Absence of NO

	CD80	CD83	CD86	MHC class II
Untreated DCs	1.70 ± 0.08	1.02 ± 0.06	14.9 ± 0.82	11.5 ± 0.32
LPS DCs	3.21 ± 0.25	2.10 ± 0.16	28.2 ± 1.12	21.5 ± 1.24
LPS/DETA-NO DCs	3.31 ± 0.27	2.06 ± 0.13	28.4 ± 0.92	21.6 ± 1.45
αCD40 DCs	7.80 ± 0.32	8.31 ± 0.29	54.8 ± 2.12	27.9 ± 1.33
αCD40/DETA-NO DCs	7.84 ± 0.24	8.40 ± 0.42	55.1 ± 2.66	28.1 ± 1.42

Immature DCs were treated for 2 days with or without LPS (1 μg/ml) or an anti-CD40 mAb (10 μg/ml) in the presence or absence of DETA-NO (50 μM). Cell preparations were analyzed by flow cytometry after staining with FITC-conjugated Ab specific for CD80, CD83, CD86, and MHC class II. Values shown are the relative fluorescence intensities ± SEM measured for each FITC-conjugated Ab versus cell autofluorescence (n=6).

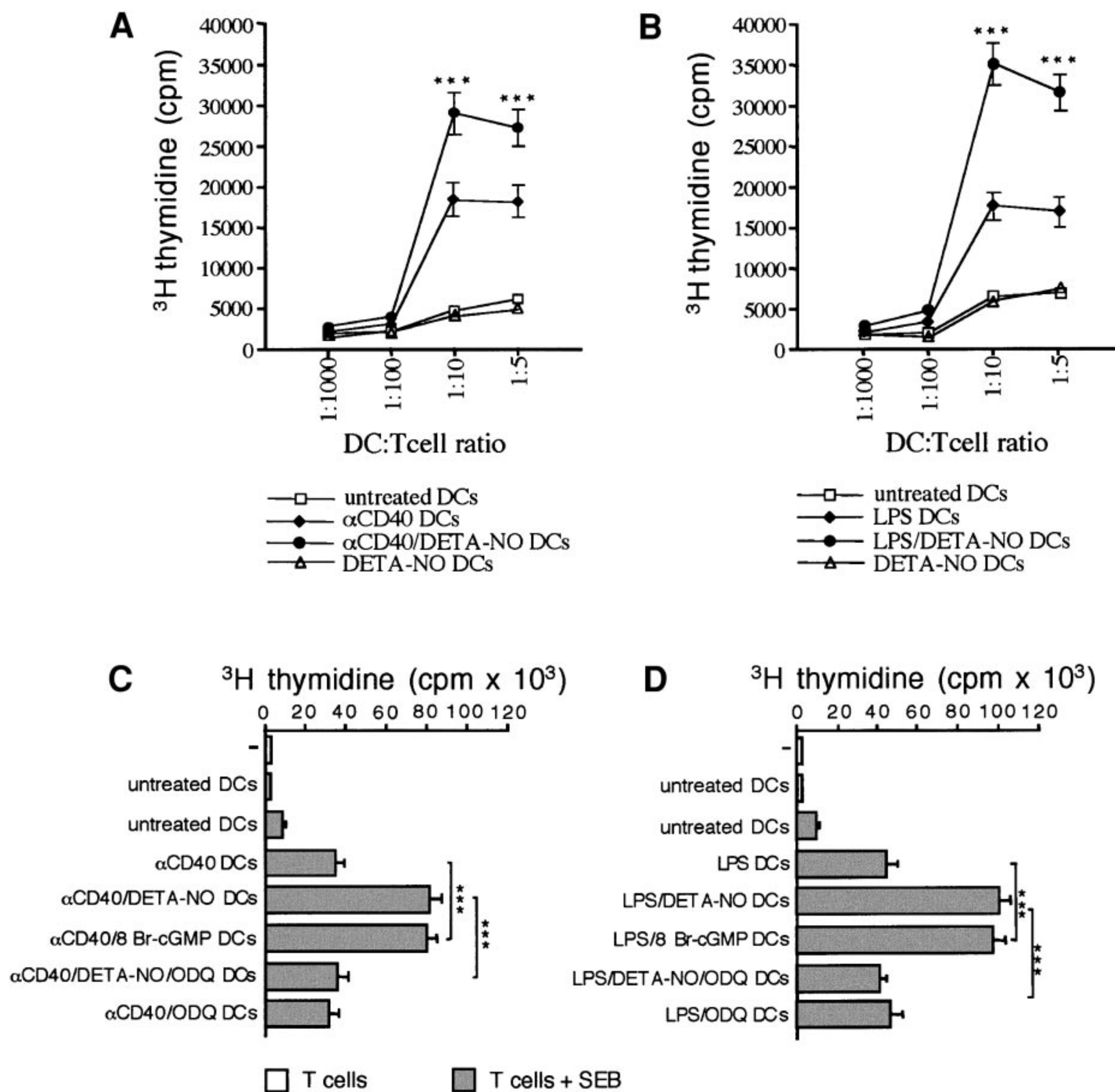

Fig. 6. NO enhances the ability of LPS- and anti-CD40 mAb-treated DCs to induce T cell activation. Immature DCs were treated for 2 days with or without LPS (1 μg/ml, A and C) or an anti-CD40 mAb (10 μg/ml, B and D) in the presence or absence of DETA-NO (50 μM), 8 Br-cGMP (3 mM), or ODQ (3 μM). (A and B) These pretreated DCs were cocultured at the indicated ratios with a fixed number of allogeneic T cells (10^5 cells/well); proliferation (^{3}H-thymidine incorporation in T cells) was measured after 5 days of DC/T cell coculture. (C and D) Pretreated DCs (2×10^4) were cocultured with 10^5 autologous T cells in the presence of SEB (0.2 μg/ml) or without any stimulus. Proliferation was measured after 3 days. Asterisks indicate statistical significance, measured as indicated in Materials and Methods (n=6).

in immune responses, however, evidence about cGMP regulating maturation signal-transduction pathways in DCs is still limited to a recent report by our group, showing a cGMP-dependent reduction by NO of the levels of ceramide observed after DC treatment with TNF-α [19]. The identification in three distinct signal-transduction pathways of IL-12 as the mediator of the NO/cGMP action may provide a first clue toward elucidating the molecular events regulated by the cyclic nucleotide during DC maturation.

Generation of IL-12 by DCs is a tightly regulated event [28]. Production of this cytokine can be elicited by most pathogens and increased further by activated CD40 ligand-positive T cells [35]. DC maturation, however, does not necessarily comprise IL-12 secretion. For example, DCs matured in the presence of cholera toxin or CD95 ligand fail to secrete IL-12 [36]. Similarly, proinflammatory cytokines, such as IL-1β and TNF-α, appear poor activators of IL-12 secretion [37]. Our results provide evidence that the TNF-α-induced phenotype is reversed in the presence of NO.

Sustained generation of NO as a consequence of iNOS up-regulation in competent immune cells is among the effects of LPS, TNF-α, and CD40 ligand [1]. NO, therefore, appears to be part of a feed-forward loop aimed at amplifying immune responses through an increased antigen-presenting function of DCs. Of importance, we found that NO increases secretion by T cells of IFN-γ and IL-2 but not of IL-4. This novel function of NO might, therefore, contribute to explain the mechanisms by which cooperation among IL-12, NO, and other T helper cell

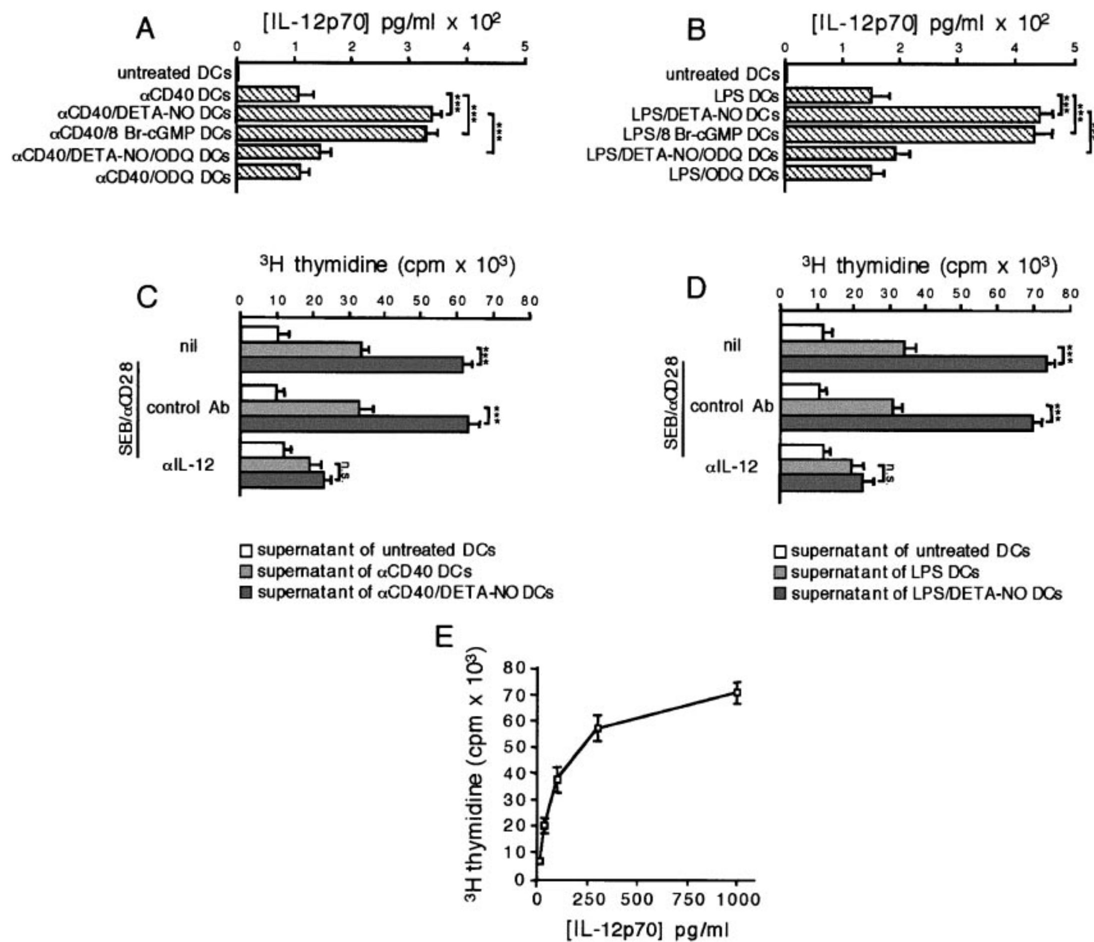

Fig. 7. Release of IL-12 is crucial to the enhanced T lymphocyte activation by DCs treated with LPS or anti-CD40 mAb in the presence of NO. Supernatants were collected from DCs pretreated for 2 days with or without LPS (1 μg/ml, A and C) or an anti-CD40 mAb (10 μg/ml, B and D) in the presence or absence of DETA-NO (50 μM), 8 Br-cGMP (3 mM), or ODQ (3 μM). (A and B) Concentration of IL-12p70 in the various supernatants, evaluated by ELISA. (B and D) T cell proliferation in the presence or absence of the various supernatants was stimulated for 3 days with SEB (0.2 μg/ml)/anti-CD28 mAb, in the absence (nil) or presence of an IL-12 neutralizing mAb cocktail (αIL-12), as described in Materials and Methods. Experiments with an isotype-control Ab were performed in parallel. Asterisks indicate statistical significance, measured as indicated in Materials and Methods (n=6). (E) T cell proliferation was stimulated for 3 days with SEB (0.2 μg/ml)/anti-CD28 mAb in the presence of increasing concentrations of IL-12 (0–1000 pg/ml; n=5).

type 1 cytokines contributes to healing infections in vivo [38–41].

ACKNOWLEDGMENTS

This work was supported by grants from Italian Association for Cancer Research (to E. C. and to A. A. M) and cofinanziamento 2001 from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (to E. C.). C. Paolucci is supported by a fellowship from The Italian Association for Cancer Research. We thank Jacopo Meldolesi and Daniele D'Ambrosio for their critical revision of the manuscript and Donata Vercelli and Lars Rogge for providing the anti-CD40 mAb and the anti IL-12 mAb cocktail, respectively.

REFERENCES

1. Bogdan, C. (2001) Nitric oxide and the immune response. *Nat. Immunol.* **2,** 907–916.
2. Moncada, S., Higgs, E. A. (1995) Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB J.* **9,** 1319–1330.
3. Sciorati, C., Rovere, P., Ferrarini, M., Paolucci, C., Heltai, S., Vaiani, R., Clementi, E., Manfredi, A. A. (1999) Generation of nitric oxide by the inducible nitric oxide synthase protects γδ T cells from Mycobacterium tuberculosis-induced apoptosis. *J. Immunol.* **163,** 1570–1576.
4. MacMicking, J., Xie, Q. W., Nathan, C. (1997) Nitric oxide and macrophage function. *Annu. Rev. Immunol.* **15,** 323–350.
5. Huang, F. P., Niedbala, W., Wei, X. Q., Xu, D., Feng, G. J., Robinson, J. H., Lam, C., Liew, F. Y. (1998) Nitric oxide regulates Th1 cell development through the inhibition of IL-12 synthesis by macrophages. *Eur. J. Immunol.* **28,** 4062–4070.
6. Diefenbach, A., Schindler, H., Donhauser, N., Lorenz, E., Laskay, T., MacMicking, J., Rollinghoff, M., Gresser, I., Bogdan, C. (1998) Type 1 interferon (IFNα/β) and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. *Immunity* **8,** 77–87.
7. Diefenbach, A., Schindler, H., Rollinghoff, M., Yokoyama, W. M., Bogdan, C. (1999) Requirement for type 2 NO synthase for IL-12 signaling in innate immunity. *Science* **284,** 951–955.
8. Niedbala, W., Wei, X. Q., Piedrafita, D., Xu, D., Liew, F. Y. (1999) Effects of nitric oxide on the induction and differentiation of Th1 cells. *Eur. J. Immunol.* **29,** 2498–2505.
9. Bingisser, R. M., Tilbrook, P. A., Holt, P. G., Kees, U. R. (1998) Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway. *J. Immunol.* **160,** 5729–5734.

10. Wei, X. Q., Charles, I. G., Smith, A., Ure, J., Feng, G. J., Huang, F. P., Xu, D., Muller, W., Moncada, S., Liew, F. Y. (1995) Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* **375,** 408–411.
11. Banchereau, J., Steinman, R. M. (1998) Dendritic cells and the control of immunity. *Nature* **392,** 245–252.
12. Holt, P. G., Oliver, J., Bilyk, N., McMenamin, C., McMenamin, P. G., Kraal, G., Thepen, T. (1993) Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages. *J. Exp. Med.* **177,** 397–407.
13. Lu, L., Bonham, C. A., Chambers, F. G., Watkins, S. C., Hoffman, R. A., Simmons, R. L., Thomson, A. W. (1996) Induction of nitric oxide synthase in mouse dendritic cells by IFN-γ, endotoxin, and interaction with allogeneic T cells: nitric oxide production is associated with dendritic cell apoptosis. *J. Immunol.* **157,** 3577–3586.
14. Aiello, S., Noris, M., Piccinini, G., Tomasoni, S., Casiraghi, F., Bonazzola, S., Mister, M., Sayegh, M. H., Remuzzi, G. (2000) Thymic dendritic cells express inducible nitric oxide synthase and generate nitric oxide in response to self- and alloantigens. *J. Immunol.* **164,** 4649–4658.
15. Downing, J. E., Virag, L., Jones, I. W. (1998) NADPH diaphorase-positive dendritic profiles in rat thymus are discrete from autofluorescent cells, immunoreactive for inducible nitric oxide synthase, and show strain-specific abundance differences. *Immunology* **95,** 148–155.
16. Xiao, B. G., Huang, Y. M., Xu, L. Y., Ishikawa, M., Link, H. (1999) Mechanisms of recovery from experimental allergic encephalomyelitis induced with myelin basic protein peptide 68-86 in Lewis rats: a role for dendritic cells in inducing apoptosis of CD4+ T cells. *J. Neuroimmunol.* **97,** 25–36.
17. Yamamoto, K., Akbar, S. M., Masumoto, T., Onji, M. (1998) Increased nitric oxide (NO) production by antigen-presenting dendritic cells is responsible for low allogeneic mixed leucocyte reaction (MLR) in primary biliary cirrhosis (PBC). *Clin. Exp. Immunol.* **114,** 94–101.
18. Ninomiya, T., Akbar, S. M., Masumoto, T., Horiike, N., Onji, M. (1999) Dendritic cells with immature phenotype and defective function in the peripheral blood from patients with hepatocellular carcinoma. *J. Hepatol.* **31,** 323–331.
19. Paolucci, C., Rovere, P., De Nadai, C., Manfredi, A. A., Clementi, E. (2000) Nitric oxide inhibits the tumor necrosis factor α-regulated endocytosis of human dendritic cells in a cyclic GMP-dependent way. *J. Biol. Chem.* **275,** 19638–19644.
20. Bulotta, S., Barsacchi, R., Rotiroti, D., Borgese, N., Clementi, E. (2001) Activation of the endothelial nitric-oxide synthase by tumor necrosis factor-α. A novel feedback mechanism regulating cell death. *J. Biol. Chem.* **276,** 6529–6536.
21. De Nadai, C., Sestili, P., Cantoni, O., Lievremont, J. P., Sciorati, C., Barsacchi, R., Moncada, S., Meldolesi, J., Clementi, E. (2000) Nitric oxide inhibits tumor necrosis factor-α-induced apoptosis by reducing the generation of ceramide. *Proc. Natl. Acad. Sci. USA* **97,** 5480–5485.
22. Nelson, E. L., Strobl, S., Subleski, J., Prieto, D., Kopp, W. C., Nelson, P. J. (1999) Cycling of human dendritic cell effector phenotypes in response to TNF-α: modification of the current 'maturation' paradigm and implications for in vivo immunoregulation. *FASEB J.* **13,** 2021–2030.
23. Clementi, E., Brown, G. C., Feelisch, M., Moncada, S. (1998) Persistent inhibition of cell respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. *Proc. Natl. Acad. Sci. USA* **95,** 7631–7636.
24. Orsi, A., Beltràn, B., Clementi, E., Hallén, K., Feelisch, M., Moncada, S. (2000) Continuous exposure to high concentrations of nitric oxide leads to persistent inhibition of oxygen consumption by J774 cells as well as extraction of oxygen by the extracellular mileu. *Biochem. J.* **346,** 407–412.
25. Burastero, S. E., Cutolo, M., Dessi, V., Celada, F. (1990) Monoreactive and polyreactive rheumatoid factors produced by in vitro Epstein-Barr virus-transformed peripheral blood and synovial B lymphocytes from rheumatoid arthritis patients. *Scand. J. Immunol.* **32,** 347–357.
26. Garthwaite, J., Southam, E., Boulton, C. L., Nielsen, E. B., Schmidt, K., Mayer, B. (1995) Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. *Mol. Pharmacol.* **48,** 184–188.
27. Banchereau, J., Schuler-Thurner, B., Palucka, A. K., Schuler, G. (2001) Dendritic cells as vectors for therapy. *Cell* **106,** 271–274.
28. Ma, X., Trinchieri, G. (2001) Regulation of interleukin-12 production in antigen-presenting cells. *Adv. Immunol.* **79,** 55–92.
29. Rogge, L., Sinigaglia, F. (1997) Early events controlling T-helper cell differentiation: the role of the IL-12 receptor. *Chem. Immunol.* **68,** 38–53.
30. Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., Alber, G. (1996) Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J. Exp. Med.* **184,** 747–752.
31. Kubin, M., Kamoun, M., Trinchieri, G. (1994) Interleukin 12 synergizes with B7/CD28 interaction in inducing efficient proliferatioon and cytokine production of human T cells. *J. Exp. Med.* **180,** 211–222.
32. Harbrecht, B. G., Wang, S. C., Simmons, R. L., Billiar, T. R. (1995) Cyclic GMP and guanylate cyclase mediate lipopolysaccharide-induced Kupffer cell tumor necrosis factor-α synthesis. *J. Leukoc. Biol.* **57,** 297–302.
33. Wilkinson, M. F., Earle, M. L., Triggle, C. R., Barnes, S. (1996) Interleukin-1β, tumor necrosis factor-α, and LPS enhance calcium channel current in isolated vascular smooth muscle cells of rat tail artery. *FASEB J.* **10,** 785–791.
34. Browning, D. D., Windes, N. D., Ye, R. D. (1999) Activation of p38 mitogen-activated protein kinase by lipopolysaccharide in human neutrophils requires nitric oxide-dependent cGMP accumulation. *J. Biol. Chem.* **274,** 537–542.
35. Schulz, O., Edwards, A. D., Schito, M., Aliberti, J., Manickasingham, S., Sher, A., Reis, E., Sousa, C. (2000) CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells in vivo requires a microbial priming signal. *Immunity* **13,** 453–462.
36. Rescigno, M., Piguet, V., Valzasina, B., Lens, S., Zubler, R., French, L., Kindler, V., Tschopp, J., Ricciardi-Castagnoli, P. (2000) Fas engagement induces the maturation of dendritic cells (DCs), the release of interleukin (IL)-1β, and the production of interferon-γ in the absence of IL-12 during DC-T cell cognate interaction: a new role for Fas ligand in inflammatory responses. *J. Exp. Med.* **192,** 1661–1668.
37. Lanzavecchia, A., Sallusto, F. (2001) Regulation of T cell immunity by dendritic cells. *Cell* **106,** 263–266.
38. Perez-Santos, J. L., Talamas-Rohana, P. (2001) In vitro indomethacin administration upregulates interleukin-12 production and polarizes the immune response towards a Th1 type in susceptible BALB/c mice infected with Leishmania mexicana. *Parasite Immunol.* **23,** 599–606.
39. Das, L., Datta, N., Bandyopadhyay, S., Das, P. K. (2001) Successful therapy of lethal murine visceral leishmaniasis with cystatin involves up-regulation of nitric oxide and a favorable T cell response. *J. Immunol.* **166,** 4020–4028.
40. Wilhelm, P., Ritter, U., Labbow, S., Donhauser, N., Rollinghoff, M., Bogdan, C., Korner, H. (2001) Rapidly fatal leishmaniasis in resistant C57BL/6 mice lacking TNF. *J. Immunol.* **166,** 4012–4019.
41. Uzonna, J. E., Bretscher, P. A. (2001) Anti-IL-4 antibody therapy causes regression of chronic lesions caused by medium-dose Leishmania major infection in BALB/c mice. *Eur. J. Immunol.* **31,** 3175–3184.