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Neuronal Nitric Oxide Synthase Modulates Maturation of Human Dendritic Cells

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Nicole Bacher,* Andreas Friebe,‡ Ellen I. Closs,†,2 and Kerstin Steinbrink*1,2

Dendritic cells (DCs) are the most potent APCs of the immune system. Understanding the intercellular and intracellular signaling processes that lead to DC maturation is critical for determining how these cells initiate T cell-mediated immune processes. NO synthesized by the inducible NO synthase (iNOS) is important for the function of murine DCs. In our study, we investigated the regulation of the arginine/NO-system in human monocyte-derived DCs. Maturation of DCs induced by inflammatory cytokines (IL-1β, TNF, IL-6, and PGE2) resulted in a pronounced expression of neuronal NOS (nNOS) but only minimal levels of iNOS and endothelial NOS were detected in human mature DCs. In addition, reporter cell assays revealed the production of NO by mature DCs. Specific inhibitors of NOS (N-nitro-1-arginine methyl ester) or of the NO target guanylyl cyclase (H-(1,2,4)-oxadiazolo[4,3-a] quinoxalin-1-one) prevented DC maturation (shown by decreased expression of MHC class II, costimulatory and CD83 molecules) and reduced IL-12 production) and preserved an immature phenotype, indicating an autocrine effect of nNOS-derived NO on human DC maturation. Notably, inhibitor-treated DCs were incapable of inducing efficient T cell responses after primary culture and generated an anergic T cell phenotype. In conclusion, our results suggest that, in the human system, nNOS-, but not iNOS-derived NO, plays an important regulatory role for the maturation of DCs and, thus, the induction of pronounced T cell responses. The Journal of Immunology, 2010, 184: 6025–6034.

Nitrıc oxide, a free radical gas, is known as an important regulator and mediator of a wide range of physiological processes, including blood vessel relaxation, neurotransmission, inflammation, apoptosis, cytotoxicity and immune processes (1–3). In the latter, NO exerts a protective function against various infections through its cytotoxic activity. In the murine immune system, NO is also involved in the regulation of adaptive immunity by affecting the balance between Th1/Th2 responses or other T cell-regulated immune processes (2, 4, 5). However, the role of NO in adaptive immunity in humans is less clear. Increased expression of NO synthesis (NOS) has been demonstrated in psoriasis, an inflammatory skin disease characterized by skin-homing T cells expression of NO synthase (NOS) has been demonstrated in psoriasis, an inflammatory skin disease characterized by skin-homing T cells with a strong impact on subsequent efficient T cell activation.

Materials and Methods

Culture medium
IMDM supplemented with 2.5% autologous plasma was used for generation of DCs. T cells were cultured and stimulated in X-VIVO-20 (both from Cambrex, Taufkirchen, Germany). A673 cells were grown in Iscove’s...
modified DMEM supplemented with 10% FBS and 4 mM glutamine. ECV304 cells in DMEM, supplemented with 4 mM glutamine and 10% FBS, and RFL-6 cells in F-12 medium with 15% FBS and 4 mM glutamine. HUVECs were expanded in Earl’s medium 199 supplemented with 20% FBS, penicillin, streptomycin, and 5.3 mM/l glutamine.

**Cell lines**

The human cell line A673 (neuroepithelioma, established from a patient with a possible primary rhabdomyosarcoma) expressing nNOS, the human cell line VEC-304 (a variant of the T-24 bladder carcinoma cell line) as a negative control for nNOS expression, and the rat lung fibroblast cell line RFL-6 were obtained from American Type Culture Collection (Manassas, VA). HUVECs were isolated as previously described (25), expanded on fibronectin-coated dishes and used in the third passage. All cell lines were regularly tested for mycoplasma infection using DAPI (Roche Molecular Biochemicals, Mannheim, Germany). No contamination could be detected.

For incubation of cells with amino acids, only the L-isomers were used.

**Abs**

For flow cytometry, Abs against human CD2 (6F10.3), CD14 (RM052), CD19 (J4.119), CD80 (MAB104), CD83 (HB15A) (all Beckmann Coulter, Krefeld, Germany), CD86 (B6U3, Serotec, Oxford, U.K.), HLA-DR (YDI/63.4.10, Serotec), nNOS (nNOS-AB, A-11, Santa Cruz Biotechnology, Santa Cruz, CA), CD40 (5c3), CD208-APC (110-1112) (all Becton Dickinson, CCR7-APC (150503; R&D Systems, Wiesbaden, Germany), mouse (MOPC-31C, MOPC-173, 27-35), and rat (R35-95, MOPC-21) subclass-specific isotopes (all Beckmann Coulter) were used. Conjugated secondary reagents: FITC-conjugated goat–anti–IgG (Biozol, Eching, Germany), PE-conjugated donkey anti–mouse-IgG (Jackson ImmunoResearch Laboratories, Suffolk, U.K.), for myeloid DC: anti-CD1c-PE (M241, Ancell, Cologne, Germany). Staining of MACS-sorted T cells: FITC-conjugated CD4 (13B8.2) or CD8 (B9.11), FITC-conjugated mouse-IgG (697.1Mc7) (all Beckmann Coulter).

**Generation of DCs**

DCs were generated as described (26). PBMCs were isolated from buffy coats using Ficol gradients and suspended in culture dishes for 45 min. Non-adherent cells were rinsed off the plates and remaining cells were cultured in 3 ml IMDM supplemented with 400 U/ml GM-CSF (Leukine, Berlex, Richmond, CA), 1000 U/ml IL-4 (Strathmann, Hamburg, Germany), and 2.5% autologous plasma. At day 6, nonadherent cells were collected, re-suspended in IMDM supplemented with 2.5% autologous plasma, 400 U/ml GM-CSF, and 1000 U/ml IL-4, and additionally stimulated with 2.5 mg/ml IL-1β, 2.5 mg/ml TNF-α, 250 U/ml IL-6 (Strattmann), and 0.5 mg/ml PGE₂ (Minprostin, Pharmacia-Upjohn, Erlangen, Germany) for 2 days to induce mature DCs (mDCs). In some experiments, IFN-γ 100 U/ml (Strattmann) and/or IL-10 100 ng/ml (Sigma-Aldrich, St. Louis, MO) were used.

H-(1,2,4)-oxadiazolo [4,3-a] quinoxalin-1-one (ODQ: 50–200 μm) or N-nitro-L-arginine methyl ester (L-NAME) 0.1–9.0 mM (both Alexis, San Diego, CA) were added to the DC culture in concentrations as indicated. Viability of DCs was tested by propidium-iodide staining in every experiment. Analyses revealed a slightly higher percentage of dead cells after inhibitor treatment (L-NAME, 11.47 ± 3.52%; ODQ, 19.78 ± 7.42%) as compared with control DCs (7.73 ± 4.64%) that was adjusted by using the same number of viable DCs in coculture experiments with T cells.

Myeloid DCs were obtained from peripheral blood using the BDCA-1® DC isolation kit (Milteny Biotec, Bergisch-Gladbach, Germany) according to the manufacturer’s protocol. Purity (~>85%) was controlled by flow cytometry (CD1c⁺, HLA-DR⁺, lineage⁻, CD86⁻, CD83⁻). Maturation was induced by 2.5 μg/ml TNF-α, 250 U/ml IL-6 (Strattmann), and 0.5 mg/ml PGE₂ (Minprostin, Pharmacia-Upjohn). In some experiments, DCs were additionally stimulated with IFN-γ 100 U/ml (Strattmann) and LPS 100 ng/ml (Sigma-Aldrich).

**Isolation of CD4⁺CD45RA⁺ T cells**

CD4⁺CD45RA⁺ T cells were prepared from buffy coats using Negative CD4⁺ T Cell Isolation Kit II (MACS systems; Milteny Biotec) (purity ~>95%).

**Induction of an allogeneic T cell response by DCs and restimulation experiments**

DCs and CD4⁺CD45RA⁺ T cells were prepared as described previously. For primary culture, 5 × 10⁵ of the respective DCs per well in 3 ml X-VIVO 20 supplemented with 0.5% autologous plasma and 2 U/ml IL-2 for 4 d. For proliferation experiments, serial dilutions starting at 0.25 × 10⁵ of the respective DCs as indicated were cultured with 0.25 × 10⁵ CD4⁺CD45RA⁺ in 200 μl X-VIVO 20 supplemented with 0.5% autologous plasma and 2 U/ml IL-2 for 4 d, and proliferation was measured at day 4 by [3H]thyidine incorporation. For restimulation experiments, 0.25 × 10⁵ T cells were stimulated with anti-CD3 (0.5 μg/ml) and anti-CD28-mAb (1 μg/ml). T cell proliferation was detected 72 h after activation.

**Flow cytometric analysis**

Phenotype of DCs was analyzed by flow cytometry (FACScalibur, CellQuest software, Becton Dickinson, Heidelberg, Germany). Intracellular staining was performed by use of Cytofix/Cytoperm Kit (Becton Dickinson).

**Cytokine analysis**

For assessment of cytokine production, supernatants were collected after coculture of DCs and T cells after 4 d of primary culture, or 24 and 72 h after restimulation and stored at −70°C. The amounts of IL-2, IFN-γ, IL-5, and IL-13 were assessed by ELISA, using commercially available Abs and standards according to the manufacturer’s protocols (BD Pharmingen, Heidelberg, Germany). For assessment of IL-10 production, human IL-10 ELISA was used (Immunotools, Friesothe, Germany). For analysis of DCs cytokine profile, supernatants were harvested at day 8 of culture and ELISA for IL-10 (Immunotools), IL-12p40, IL-12p70 (Becton Dickinson) and IL-23 (BioSource International, Camarillo, CA) production were performed, according to the manufacturer’s protocols.

**Immunoblotting**

Membranes were probed with anti-nNOS-Ab (Clone: pAb, BD Transduction Laboratories, Heidelberg, Germany) and anti-ε2F2Bc (H290; Santa Cruz Biotechnology) overnight at 4°C. Detection was performed by incubation with HRP-conjugated Abs (goat anti-rabbit, Cell Signaling Technology, Frankfurt, Germany). Proteins were visualized by ECL Plus (Amersham Pharmacia, Freiburg, Germany) using Hyperfilm (Amer sham).

**RNA isolation**

RNA isolation was used by RNAeasy Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer and quantified by the absorption at 260 nm. Total RNA from human brain tissue was obtained from Clontech Laboratories (Mountain View, CA).

**Quantitative RT-PCR**

One-step RT-PCR was performed with the QuantiTect RT-PCR Kit (Qiagen) in 25 μl reactions in a 96-well spectrophotometric thermal cycler (iCycler, Bio-Rad, München, Germany) (dNTPs 400 μmol/l each). For real-time PCR (MgCl₂, 5.3 mmol/l, 94˚C 15 s, 60˚C 60 s), the oligonucleotides as listed below served as sense and antisense primers (0.8 μmol/l each). Taqman hybridization probes (0.4 μmol/l, Table I) were double-labeled with FAM as the reporter fluorophore and carboxytetramethylrhodamine (TAMRA) as the quencher. Fluorescence was monitored at each 60˚C annealing/extension step.

**RFL-6 reporter cell assay**

RFL-6 reporter cells were grown to confluence in 6-well plates (diameter 35 mm) and washed twice in Locke’s solution (LS; composition, 154 mmol/l NaCl; 5.6 mmol/l KCl; 2 mmol/l CaCl₂; 1 mmol/l MgCl₂; 10 mmol/l HEPES; 3.6 mmol/l NaHCO₃; and 5.6 mmol/l glucose). RFL-6 cells were then preincubated 30 min in LS supplemented with 200 U/ml superoxide dismutase (Roche Molecular Biochemicals, Mannheim, Germany), 50 μM 3-ethyl-1-methylxanthine, and 100 μM ODQ (where indicated). DCs were incubated for 10 min in 100 μM arginine and 200 U/ml superoxide dismutase. For NO measurement, DCs (4 × 10⁵ per well) were transferred onto trans-well plates and incubated for 4 min on the RFL-6 reporter cells at 37°C.

After the 4-min incubation, the DC-containing transwells and the LS were removed from the RFL-6 cells, 1 ml ice-cold sodium acetate buffer (20 mmol/l, pH 4.0) was added and the cells were rapidly frozen in liquid nitrogen. The cGMP content of the RFL-6 cells was determined by RIA as described previously (27). Basal cGMP values measured in RFL-6 cells incubated under the same conditions but not exposed to DCs were subtracted from the experimental values. In some experiments, ODQ (100 μM) was used to demonstrate the functional relevance and specificity of the results. DCs were collected and used for RNA isolation.

**Statistical analyses**

Statistical significances of differences between experimental groups were evaluated by repeated measure ANOVA and Dunnett’s multiple comparison test with the GraphPad Prism 5 software package (GraphPad, San Diego, CA). Values of p < 0.05 were considered significant.
Results

Regulation of NOS isoform expression during maturation of monocyte-derived DCs

We generated human DCs from peripheral monocyct progenitor cells according to a protocol that has been established some years ago enabling the reproducible generation of large amounts of donor-specific myeloid DC under GMP-conditions and opening the way for the clinical use of DCs as natural adjuvants in clinical vaccination trials (13, 28).

Involvement of NO in DC-related immune processes has been described in both, the murine and human system, but so far, no information was available on the regulation, expression, and function of NOS isoforms in the course of maturation. Thus, we addressed the question if human DCs express any of the three NOS isoforms, eNOS, nNOS, or iNOS during maturation. We found a moderate expression of eNOS but only minimal levels of iNOS or nNOS mRNA in iDCs at day 6 of culture (Fig. 1A). In contrast, a high nNOS expression was observed in mDCs at day 8 accompanied by only minimal levels of eNOS and iNOS (Fig. 1A). Kinetic analyses revealed detectable nNOS expression at day 6 that was found to increase 250-fold at day 8 during DC maturation (related to nNOS expression in human brain tissue [100%]) (Fig. 1B). Our experiments demonstrated a complete downregulation of eNOS in mDCs and a small induction (3-fold) of iNOS mRNA during maturation of human DCs treated with cytokines (Fig. 1A) or other immune mediators (LPS alone, LPS and IFN-γ, data not shown). However, absolute iNOS expression was very low; maximal levels of iNOS were 500-fold lower than maximal levels of nNOS in mDCs (day 8). Considering the minimal expression of iNOS and eNOS, we assume no major contribution of these isoforms to total NO production in mDCs (Fig. 1A).

Myeloid DCs from blood of healthy donors have been characterized as immature APCs (10, 11). RT-PCR analyses revealed that neither iNOS nor nNOS isoforms were detected in myeloid immature CD11c+ DCs obtained directly from peripheral blood (data not shown). Maturation of myeloid DCs in the presence of a cytokine mixture (IL-1β, TNF-α, IL-6, PGE2 ≥ IFN-γ/LPS) did not induce a significantly increase in nNOS expression (data not shown), indicating that nNOS-related pathways may not contribute to myeloid DC function.

Production of NO by human mDCs

NO has been reported to be a versatile player of the immune system with various effects on several immune cells, including DCs and T cells (2). Our experiments revealed the novel finding of an increased and high expression of nNOS in human mDCs. Thus, in our study, we focused on the analyses of the functional relevance of this observation.

To verify the high expression of nNOS mRNA in mDCs on the protein level, Western blot and flow cytometry experiments were performed. In accordance to RT-PCR results, we found a very low expression of nNOS in iDCs and a high expression in mDCs, suggesting a functional role of nNOS during the final phase of DC maturation (Fig. 2A, 2B).

To determine whether mDCs produce bioactive NO, we incubated these cells in transwells with a confluent layer of RFL-6 reporter cells. The latter express high amounts of the NO-GC and thus produce cGMP proportional to the amount of NO they are exposed to (27). Notably, mDCs induced significant amounts of cGMP in reporter cells (Fig. 2C). The mDC-induced cGMP production was completely inhibited by treatment with a specific inhibitor of NO-GC (ODQ), demonstrating specificity of the results (Fig. 2C). In contrast, in experiments using iDCs, which did not express nNOS, we found a significantly reduced NO production as compared with mDCs (data not shown). These data demonstrate relevant NO production by mDCs, indicating that nNOS is functionally active in these cells.

Unaltered expression of arginase and cationic amino acid transporters during differentiation of human DCs

An important factor that may determine NOS activity is the availability of its substrate, L-arginine. Arginase is provided to cells by specific carrier proteins. Among these, the so-called cationic amino acid transporters (CATs) are considered to be the major route of uptake in most cells and tissues (29, 30). Individual CAT isoforms have been postulated to be important for the substrate supply of the different NOS isoforms: CAT-1 for eNOS, CAT-2B for iNOS, and CAT-3 for nNOS. To find out if changes in NO expression were accompanied by changes in transporter expression, we quantified CAT mRNA by quantitative RT-PCR. DCs exhibited a strong expression of human CAT-1 mRNA. CAT-2B expression was in the range of 20–30% of CAT-1 (Fig. 3A). In contrast, no substantial expression of the low affinity splice variant CAT-2A (data not shown) and only a very low expression of CAT-3 were found. This expression pattern did not change during DC maturation and maturation (Fig. 3A).

Substrate supply to NO may be limited by other arginine-consuming enzymes through competition for the common substrate arginine (31). In murine DCs, it has been speculated that arginase participates in the regulation of NO synthesis (32). However, in human DCs, no arginase expression or activity has been detected (33). In accordance with these findings, we found only very low expression or modulation of arginase I or II at any time point of DC culture (Fig. 3B).

Our data indicate that modulation of arginase supply by membrane transport or consumption by arginase does not play an important role in the maturation and NO-related processes in human monocyte-derived DCs.

Table I. Primer pairs for RT-PCR analyses

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense Strand (5′–3′)</th>
<th>Antisense Strand (5′–3′)</th>
<th>TaqMan Probe (5′–3′) 6FAM-[TAMRA]</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>GTGCTGTGATCAGTATGCCT</td>
<td>CATAGTGTGACTTGGCACTTGGC</td>
<td>AGTGGAAAATACCGGCTAGCTG</td>
</tr>
<tr>
<td>nNOS</td>
<td>GTGATGACATATAGTCCGTG</td>
<td>GCAGAGCGTACTTCCGTCAGAG</td>
<td>TGGCAAGCAGGGTCAGCTG</td>
</tr>
<tr>
<td>CAT-1</td>
<td>CATGGCTGTGACTTGAGAC</td>
<td>ATCCTCTGACTGCTGCTCAGAT</td>
<td>TGGCAAGCAGGGTCAGCTG</td>
</tr>
<tr>
<td>CAT-2A</td>
<td>CATGGCTGTGACTTGAGAC</td>
<td>ATCCTCTGACTGCTGCTCAGAT</td>
<td>TGGCAAGCAGGGTCAGCTG</td>
</tr>
<tr>
<td>CAT-3</td>
<td>CATGGCTGTGACTTGAGAC</td>
<td>ATCCTCTGACTGCTGCTCAGAT</td>
<td>TGGCAAGCAGGGTCAGCTG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CATGGCTGTGACTTGAGAC</td>
<td>ATCCTCTGACTGCTGCTCAGAT</td>
<td>TGGCAAGCAGGGTCAGCTG</td>
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</tbody>
</table>
Inhibition of NOS results in diminished maturation of human DCs

To elucidate the functional relevance of nNOS in DC immunology, we performed inhibition experiments by culturing DCs in the presence of the NOS-specific inhibitor L-NAME during days 1–6 (generation of iDC, Fig. 4A), days 6–8 (maturation of mDC, Fig. 4B), or the total culture period (days 1–8, Fig. 4C). If given at days 1–6 of DC differentiation, when no nNOS expression was detectable, the treatment with L-NAME did not change the phenotype and maturation state of iDCs as assessed by the expression pattern of MHC class II, the costimulatory molecules CD80 and CD86 and the human DC maturation marker CD83 (Fig. 4A, 4D). In contrast, NOS inhibition during cytokine-induced DC maturation (day 6–8), when a strong nNOS expression developed, induced an impaired maturation and differentiation of mDCs as demonstrated by a more immature phenotype with reduced expression of critical features of maturation (CD80, CD83, and HLA-DR) (Fig. 4B, 4D). Similar results were obtained when NOS was inhibited during the entire culture period (days 1–8, Fig. 4C, 4D). Analyses of the viability of DCs after L-NAME treatment revealed slightly higher numbers of dead cells as compared with control DCs, indicating only a low toxicity of the inhibitor, even in the highest concentration used (see Materials and Methods).

Statistical analyses revealed a significant reduction in the expression of CD80, CD83 and the additionally analyzed molecule CD208/DC-LAMP (a membrane protein specifically expressed in the later stages of maturation) in DCs generated in the presence of L-NAME during day 6–8 of maturation (Fig. 4E). The percentage of CD40 and chemokine receptor CCR7-positive DCs was unaffected by L-NAME treatment (Fig. 4E).

DC-related cytokines are critically involved in the induction and polarization of resulting T cell responses. IL-12 is known to be required for Th1/Tc1 cell development and T cell activation, IL-10 has been shown to have immunosuppressive functions and may play a pivotal role in the induction and function of regulatory T cells and IL-23 contributes to the generation of Th17 T cells (34).

To evaluate the cytokine profile of mDCs after inhibition of nNOS activity during maturation, supernatants of DCs were analyzed for IL-12p40, IL-12p70, IL-10, and IL-23. No significant production of IL-12p40 was observed in iDCs (data not shown) in contrast to mDCs producing high amounts of this cytokine (Fig. 4F). The levels of IL-12p40 were significantly reduced in experiments where the nNOS activity was blocked by the inhibitor L-NAME during maturation (Fig. 4F). Induction of IL-12p70, IL-10, and IL-23 secretion was not observed in any of the approaches performed with iDCs and mDCs (data not shown).

Thus, we found an impaired DC maturation after inhibition of nNOS, indicating that nNOS-derived NO contributes to maturation of human monocyte-derived DCs.

The effect of NO on human DC maturation is cGMP-dependent

Effects of NO are known to be mediated through both, cGMP-dependent and -independent signaling pathways. In human DCs, the
depicted as mean fluorescence intensity (MFI), was reduced on the amount of surface molecules of CD40, CD80, CD83, and CD86, CD83, CCR7, and CD208 on inhibitor-treated DCs. In addition, the DCs, demonstrated by a significantly reduced expression of CD80, very clearly illustrated that ODQ blocked the maturation of human DCs (see highest concentration used, excluding a toxic effect of the inhibitor where the NO-GC–specific inhibitor ODQ was present during DC culture) inhibition of cGMP production had no effect (Fig. 5A, 5B). At primary culture, we did not find significant alterations in the secretion of IFN-γ (Th1 cytokine) or IL-5/IL-13 (Th2 cytokines) by stimulation with inhibitor-treated DCs as compared with untreated mDCs (data not shown).

To analyze the differentiation of T cell populations primed by L-NAME DCs and ODQ DCs, we performed restimulation experiments. In contrast to T cells cocultured with untreated mDCs, we observed a significant reduction in proliferation of T cells primarily activated by either L-NAME DCs or ODQ DCs (Fig. 6C). This is an indication for the induction of a state of T cell anergy. To formally prove T cell hyporesponsiveness and to exclude a higher rate of apoptosis in T cells induced by inhibitor-treated DCs, high amounts of IL-2 (100 U/ml) were added in the restimulation experiments. The addition of IL-2 completely overcame the anergic state and restored proliferation to the extent of control effector T cells generated by primary activation with mDCs (Fig. 6D). These data confirm the result of anergy induction as IL-2 is known to overcome the anergic state of T cells.

Analyses of the T cell cytokine profile revealed a significant reduction in IFN-γ, IL-5, and IL-13 in culture supernatants of T cells induced by inhibitor-treated DCs in contrast to control T cells generated by primary activation with mDCs (Fig. 6E). IL-10 has been described to be involved in the induction and function of tolerogenic DCs and anergic and regulatory T cells (26, 39, 40), yet no relevant levels of this cytokine were detected at restimulation regardless of the conditions of primary activation (data not shown).

These results demonstrate that T cell populations primed by DCs in which NO production had been inhibited during final maturation, display features of anergic T cells, such as low proliferation and reduced cytokine secretion.

Discussion

DCs are the most potent APCs, and play important roles both in immunity and tolerance induction. In the last decade, protocols for the generation of human monocyte-derived DCs have been established, allowing for analysis of the immunological features of these important APC populations and the successful translation of this knowledge into DC-targeted vaccination studies in various diseases (28, 41, 42).

The major and novel finding of our study is that human monocyte-derived DCs express nNOS on maturation and that nNOS-derived NO increases DC maturation in an autocrine fashion that leads to efficient T cell activation. Many studies have documented important regulatory effects of NO in murine models of inflammation and infections. However, the role of NO in adaptive immunity in humans is less clear. Clinical studies have demonstrated that NO is increased in psoriasis suggesting that NO may be involved in T cell-related immune responses in humans (6–9). In psoriasis, iNOS-expressing DCs were found, and treatment with an anti–CD11a-mAb strongly reduced infiltration by these DCs in patients clinically responding to this agent (7). However, the presence of NO-GC and NO-stimulated cGMP generation has been reported (35–37). To determine whether the effect of NO on DC maturation is mediated by cGMP, DCs were treated with the NO-GC–specific inhibitor ODQ during generation of iDCs (days 1–6 of culture, Fig. 5A), during the maturation phase (mDCs, days 6–8, Fig. 5B), or the total culture period (days 1–8, Fig. 5C). Flow cytometry analysis revealed that during generation of iDC (day 1–6 of culture) inhibition of cGMP production had no effect (Fig. 5A, 5D).

In contrast, DC maturation was markedly inhibited in experiments where the NO-GC–specific inhibitor ODQ was present during DC maturation as shown by reduced expression of costimulatory molecules CD80 and CD86, as well as MHC class II and CD83 (Fig. 5B–D). Extensive additional analyses of six unrelated donors very clearly illustrated that ODQ blocked the maturation of human DCs, demonstrated by a significantly reduced expression of CD80, CD83, CCR7, and CD208 on inhibitor-treated DCs. In addition, the amount of surface molecules of CD40, CD80, CD83, and CD86, depicted as mean fluorescence intensity (MFI), was reduced on DCs generated in the presence of ODQ (Fig. 5E).

The viability of DCs after treatment with the NO-GC inhibitor ODQ was only slightly decreased, even in the presence of the highest concentration used, excluding a toxic effect of the inhibitor on DCs (see Materials and Methods).

Furthermore, a diminished production of IL-12p40 after NO-GC inhibition was observed (Fig. 5F). No secretion of the Th1/Tc1-inducing cytokine IL-12p70, the immunosuppressive cytokine IL-10 or IL-23 was detected in control or inhibitor-treated DCs (data not shown). These results are in line with our data obtained from NOS inhibition experiments, indicating that nNOS-derived autocrine NO and activation of NO-CG is required for APC maturation during the final phase of culture when a strong nNOS expression and significant NO production is observed.

Inhibition of NO signaling in DCs during maturation impairs their capacity for efficient T cell activation

In the murine system, low NO concentrations were shown to preferentially induce Th1 immune responses, whereas high amounts of NO are immunosuppressive (4, 38). It has been reported that NO acts directly on DC and/or T cells and in synergy with IL-12 produced by APCs (2).

To evaluate the role of nNOS-derived NO during DC maturation for subsequent T cell stimulation, we performed allogeneic coculture experiments with CD4+CD45RA+ T cells and DCs, which had been pretreated with the NOS-specific inhibitor L-NAME (L-NAME DCs) or the NO-GC–specific inhibitor ODQ (ODQ DCs) during maturation (days 6–8) when nNOS is highly expressed (Figs. 1B, 2A). Primary culture with L-NAME DCs as well as ODQ DCs resulted in a significantly impaired DC-induced T cell proliferation as compared with untreated mDCs (Fig. 6A, 6B).
nNOS-related NO is involved in maturation of human DCs. A–D, iDCs and mDCs were generated as described in Materials and Methods. The NOS specific inhibitor L-NAME was added at the indicated concentrations and time intervals (A, day 1–6; B, day 6–8; C, day 1–8). Phenotype of DCs was analyzed by flow cytometry. A–C, Percentage of HLA-DR-CD80, -CD83, and -CD86 double-positive cells and MFI of costimulatory molecules and CD83 are demonstrated as dot blots. Similar results were obtained in five independent experiments. D, MFI of CD80, CD83, and CD86 expression of DCs from one representative experiment plotted against the respective concentration of L-NAME (n = 5). E and F, DCs were generated as described in the presence or absence of 9 mM L-NAME as indicated and the phenotype was analyzed by flow cytometry. E, Pooled data of the percentage of positive DCs and the respective MFI of CD40, CD80, CD83, CD86, CCR7, and CD208 of six independent experiments are shown, the respective median and range are given and the significance is indicated. *p ≤ 0.05. F, Levels of IL-12p40 were assessed in supernatants of different DC populations. Pooled data of four independent experiments are shown (mean ± SD, *p ≤ 0.05).
authors did not show if iNOS expressing DCs produce significant amounts of NO in vivo or in vitro. In our study, we found only very low levels of iNOS mRNA in DCs, indicating that in monocyte-derived DCs iNOS does not play a major role. This is supported by data from Paolucci et al. (35), who did neither detect any iNOS protein in immature nor TNF-α matured human monocyte-derived DCs. In contrast, significant levels of nNOS (500-fold more than iNOS and comparable to nNOS expression in

FIGURE 5. NO-induced signaling in human DCs is guanylyl cyclase dependent. A–D, ODQ, a specific NO-GC inhibitor, was added to DC culture in various concentrations as indicated and during different periods (A, day 1–6; B, day 6–8; C, day 1–8). Untreated (medium) DCs served as controls. A–C, Percentage of HLA-DR-CD80, -CD83, and -CD86 double-positive DCs and MFI of costimulatory molecules and CD83 are demonstrated by dot blots. D, MFI of CD80, CD83, and CD86 plotted against the respective concentrations of ODQ present during the indicated culture period (one representative experiment of five). E and F, DCs were generated as described in the presence or absence of 200 μM ODQ and the phenotype was analyzed by flow cytometry. E, Pooled data of the percentages of positive DCs and the respective MFI of CD40, CD80, CD83, CD86, CCR7, and CD208 of six independent experiments are shown, the respective median and range are given and the significance is indicated. *p ≤ 0.05; **p ≤ 0.01. F, Levels of IL-12p40 were assessed in supernatants of different DC populations. Pooled data of four independent experiments are shown (mean ± SD, **p ≤ 0.01).
brain tissue) were expressed in mDCs after maturation with TNF-α, IL-6, IL-1, and PGE2. High nNOS expression was verified on the protein level and by production of bioactive NO. In addition, myeloid DCs directly purified from the blood of control individuals and characterized as a population of immature human DCs did not express nNOS, confirming our data that nNOS was only expressed by mDCs.

High levels of bioactive NO was detected only in supernatants of mDCs but not in iDCs. These data are in line with the work of Fernandez-Ruiz et al. (23) demonstrating that monocyte-derived DCs generated from healthy volunteers produce NO. They detected iNOS expression in iDCs and mDCs and thus concluded that iNOS was responsible for NO secretion by human DCs (23). However, they did not analyze the expression of the two other isoforms, eNOS or nNOS. Another group reported no detectable NO production of human DCs, neither CD34⁺-derived nor immature monocyte-derived DCs that supports our finding that in iDCs, expressing low amounts of nNOS, no relevant production of NO is detectable, and that nNOS-derived NO provides for maturation-related processes (14). In addition, we used the RFL-6 reporter cell assay to measure bioactive NO, a method at least 100 times more sensitive than the Griess reagent used by Nishioka et al. (14).

Our discovery of endogenous NO production through nNOS in mDCs suggests an autocrine function through activation of the NO–GC pathway present in these cells as reported by Paolucci et al. (35, 36). In fact, inhibition of either NOS or the downstream effector NO-GC reduced the maturation of DCs, indicating a critical role for endogenous nNOS-derived NO, and NO-induced cGMP, respectively, in DC maturation. These results are in line with reports by others showing increased maturation of human DCs induced by exogenous NO, reflected by high expression of maturation-specific surface molecules and cytokine production, such as IL-12 (14, 24, 36). Furthermore, Paolucci et al. (35) found that NO inhibits TNF-regulated endocytosis of human DCs. However, they FIGURE 6. Inhibition of NO signaling during maturation of DCs impairs their efficacy for T cell activation. A and B, DCs treated with L-NAME (9000 μM) or ODQ (200 μM) during the maturation period of culture (days 6–8) or untreated control DCs were cocultured with CD4⁺CD45RA⁺ T cells for 4 d in primary cultures. T cell proliferation was assessed by [³H] thymidine incorporation after 96 h of primary culture. Median and range are given and significance is indicated. **p ≤ 0.01; ***p ≤ 0.001. C and D, After primary culture with mDCs, L-NAME DCs, or ODQ DCs, T cells were restimulated with anti-CD3/anti-CD28, and T cell proliferation was determined. C, Pooled relative proliferation data of five experiments (normalized to proliferation of T cells generated by primary culture with mDCs = 100). Median and range are given and the significance is indicated. **p ≤ 0.01; ***p ≤ 0.001. D, The effect of high-dose IL-2 (100 U/ml) on T cell proliferation at restimulation (anti-CD3/anti-CD28) was analyzed. One representative experiment of five is shown. **p ≤ 0.01. The respective primary activation of T cells in A–E was as indicated.
did not detect any endogenous NO production of DCs and thus assumed that at the site of inflammation, exogenous NO may work through cGMP and prolong the ability of human DCs to internalize Ags (35).

Some studies demonstrate that cytokine- or pathogen-induced maturation of murine DCs correlates with endogenous iNOS expression and NO production, indicating that iNOS expression increases during maturation of murine DCs (27, 43). In addition, treatment of DCs with NO donors or overexpression of iNOS in DCs induced maturation of murine DCs as shown by enhanced surface expression of MHC class II and costimulatory molecules. Consistently, NO donor-treated murine DCs were capable of enhancing T cell proliferation (21). These observations in murine DCs are consistent with our results demonstrating that endogenous NO also induces the maturation of human DC. However, a different NOS isoform appears to play a role in the human system; nNOS, but not iNOS-derived, NO seems to be critical for maturation of human monocyte-derived DCs. These still somehow controversial results of NO impact on immune responses may be due to differences in the properties of murine and human DCs and of various DC subpopulations.

NO is synthesized by NOS using arginine as substrate. Some studies report that arginine activation can limit the availability of arginine as a substrate for NOS and thereby negatively regulate its enzymatic activity (31). In our study, we found very low levels of arginase I and II and no maturation-dependent regulation of the expression of either arginase isoforms or of the arginine transporters CAT1 and -2B in human DCs. These results indicate that neither arginine supply by membrane transport nor metabolism by arginase is regulated by maturation-dependent processes in human DCs. However, we did not analyze if arginine concentrations in DCs were unaltered during maturation. In contrast, Fernandez-Ruiz et al. (23) detected arginase II but not arginase I isoforms in human monocytes and DCs but they did not find a maturation-related regulation of the enzymes either.

In this study, we describe that autocrine NO is involved in human DC maturation resulting in increased IL-12 production by mDCs and a strong subsequent T cell activation. Furthermore, DCs matured under conditions of nNOS inhibition induced an anergic T cell population characterized by low proliferation and reduced IFN-γ, IL-5, and IL-13 secretion. This indicates that DCs after nNOS inhibition exhibit a tolerogenic phenotype that prevents the generation of effector T cells by induction of anergy.

In our study, we used two different blocking reagents to analyze the impact of autocrine NO production on DC maturation and APC function: 1) the NOS-specific inhibitor L-NAME, and 2) the NOGC-specific inhibitor ODQ. To further prove the functional relevance of our data in a more sophisticated setting, we performed knock-down experiments by transfection of DCs with nNOS-specific small interfering RNA (siRNA). Transfection of siRNA resulted in a partial reduction of nNOS protein and mRNA levels up to 60%. However, no significant changes in NO production were found and, consequently, an unaffected DC phenotype was observed (data not shown).

Previous studies in the murine system have shown that NO exhibits a biphasic function in immune regulation and immune homeostasis; high concentrations of NO induce apoptosis in T cells or suppress T cell proliferation, whereas low amounts of NO preferentially activate Th1 cells by upregulation of cGMP that selectively induces the expression of IL-12Rβ2 (4, 38, 43–45). In our study, inhibition of NO by L-NAME during coculture of mDCs and T cells did not result in a modulated or diminished T cell activation, excluding a direct effect of human mDC-derived NO on T cell activation (data not shown).

Understanding the immune mechanisms that result in maturation of human DCs is important for determining how these cells initiate T cell-related immune processes. Our study indicates that, in contrast to the murine system, in human mDCs nNOS but not iNOS expression is crucial for their Ag-presenting function and T cell stimulatory capacity. As a novel finding, we demonstrate that human nNOS-derived NO, critically involved in autocrine DC maturation and DC-mediated induction of T cell activation, contributes to the fine-tuning of immune processes in health and disease. These findings may be of relevance for the development of therapeutic tools targeting NOS-related pathways in DCs to modulate the resulting immune response.

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Disclosures

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References


