Nitric Oxide Inhibits the Tumor Necrosis Factor α-regulated Endocytosis of Human Dendritic Cells in a Cyclic GMP-dependent Way*

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Tumor necrosis factor-α (TNFα)-induced maturation of dendritic cells (DC), with down-regulation of their endocytic ability, has been reported to be mediated by the accumulation of the lipid messenger ceramide. We have now studied the effects and mechanisms of action of NO on endocytosis, investigated with fluorescein isothiocyanate-labeled dextran using human monocyte-derived DC, both immature and after treatment with TNFα. Exposure of DC to NO, released by either bystander phagocytes or NO donors, reversed the inhibition of endocytosis induced by TNFα. The intracellular accumulation of ceramide induced by TNFα was also inhibited by NO. In addition, NO was found to exert an inhibitory effect downstream of the TNFα-triggered ceramide accumulation, because NO donors reversed the inhibition of endocytosis induced by the cell-permeant C2-ceramide. These effects of NO were mimicked by the membrane-permeant cyclic GMP analogue, 8-Brcyclic GMP, and prevented by inhibition of the soluble guanylyl cyclase. At variance with rodents, the inducible isoform of the NO synthase was expressed neither in immature human DC nor after cell treatment with TNFα, interferon-γ, and lipopolysaccharide, suggesting that regulation of these cells depends on exogenous NO. NO, working through cyclic GMP, might therefore prolong the ability of human DC to internalize antigens at the site of inflammation and thus modulate the initial steps leading to antigen-specific immune responses.

Dendritic cells (DC)† are professional antigen-presenting cells involved in the initiation of immune responses (1). Immature DC capture antigens at the site of inflammation and process and present them to T cells in secondary lymphoid organs where DC prime the immune response (1). These events are accompanied by a process of DC maturation, which includes down-regulation of endocytosis, and is orchestrated by pro-inflammatory signals generated at the site of infection (1). Among these signals, an important role might be played by NO, a gaseous messenger known to modulate specific functions of cell populations involved in the immune responses (2, 3).

NO is generated intracellularly by both constitutive NO synthases (NOSs), as in the case of B and T lymphocytes (4), or by the inducible isofrom of the enzyme (iNOS), expressed by macrophages after their activation with cytokines and bacterial products (5). NO may act either in an autocrine or paracrine fashion on neighboring cells, thus contributing to a coordinate action against pathogens (5–7). Mice with a targeted iNOS deletion are more susceptible to infections (8) and show enhanced T cell activity, characterized by strong cell-mediated immune responses and tissue damage (9). Indeed, the sustained generation of NO by iNOS endows activated macrophages and microglial cells with anti-microbial and cytotoxic activity and enhances the function of bystander T cells (2, 10).

Furthermore, NO regulates the generation of cytokines and chemokines at the site of infection (discussed in Ref. 11).

Evidence obtained in the murine system indicates that NO may also modulate DC function. In particular NO, either exogenous or produced by iNOS in DC themselves, appears to inhibit their antigen presentation function (12, 13). So far, however, the mechanism of action by NO in the maturation process of DC has not been studied, nor have the intracellular targets of NO been identified.

In this study we have investigated the regulation by NO of the ability of human DC to endocytose extracellular antigens. We have used a well characterized model of DC maturation, i.e. human monocyte-derived DC exposed to tumor necrosis factor α (TNFα). This cytokine, working via its type I p55 receptor, triggers the maturation process of DC in vitro, with inhibition of their ability to endocytose soluble antigens (14–16). Our results show that NO, generated either by bystander phagocytes expressing iNOS or by NO donors, prevents in a cyclic GMP (cGMP)-dependent way the down-regulation of endocytosis induced in DC by exposure to TNFα. This effect of NO is due to the production of cGMP, which is involved in the inhibition of endocytosis by NO (1). Immature DC capture antigens at the site of inflammation and process and present them to T cells in secondary lymphoid organs where DC prime the immune response (1). These events are accompanied by a process of DC maturation, which includes down-regulation of endocytosis, and is orchestrated by pro-inflammatory signals generated at the site of infection (1). Among these signals, an important role might be played by NO, a gaseous messenger known to modulate specific functions of cell populations involved in the immune responses (2, 3).

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### EXPERIMENTAL PROCEDURES

**Materials**—The following reagents were purchased as indicated: mouse monoclonal anti-iNOS from Transduction Laboratories (Lexington, KY); horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (Abs) from Santa Cruz Biotechnology (Santa Cruz, CA); fluorescein isothiocyanate (FITC)-labeled mouse monoclonal Abs anti-human CD86, MHC class I and class II, CD80, CD86, CD40, and CD14 was from Caltag (Burlingame, CA); [γ-32P]ATP and the cGMP radioimmunoassay kit from NEN Life Science Products; the Enhanced Chemiluminescence kit from Amersham Pharmacia Biotech; recombinant human TNFα, (Z)-1-[2-(2-aminoethyl)-N-((2-ammonioethyl) amino)]diazoen-1-ium-1,2-diolate (DETA-NO), and H- (1,2,4)-oxadiazolo[4,3-\(a\)]quinolin-1-one (ODQ) from Alexis Italia (Florence, Italy); diacylglycerol kinase from BIOMOL (Hamburg, Germany); C2 ceramide, aminoguanidine, and S-nitroso-acetylpenicillamine (SNAP) from Calbiochem (Bad Soden, Germany); recombinant human interleukin-4 (IL-4) from Strathmann Biotech GMBH (Hannover, Germany); recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF) from Mielogen-Schering Plough (Milan, Italy); recombinant mouse and human interferon-γ (IFNγ) from Genzyme (Cambridge, MA); FITC-dextran (Mn = 40,000) from Sigma (Milan, Italy); Ficoll from Biochrom (Berlin, Germany); Percoll from Amersham Pharmacia Biotech; and polyed polystirene microspheres from Polyscience Inc. (Milan, Italy). The reagents for tissue culture were from Life Technologies, Inc. except for fetal calf serum. Samples were then washed three times in the same buffer and resuspended in RPMI, 10% fetal calf serum. 2 × 10^5 cell samples were incubated at either 37 or 4 °C. TNFα-induced accumulation of ceramide, a lipid messenger known to play a key role in both antigen uptake and presentation by DC (17), as well as to additional effect(s) exerted downstream of ceramide accumulation.

**Preparation of Immature and Mature Dendritic Cells**—Peripheral blood mononuclear cells were isolated from the blood of healthy donors (kindly provided by Paola Ricciardi-Castagnoli (Milan, Italy)). All other reagents were from Sigma. The N9 murine microglial cells were kindly provided by Paola Ricciardi-Castagnoli (Milan, Italy).

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Control of Dendritic Cell Endocytosis by Nitric Oxide

RPMM and stored at 4 °C, was centrifuged to remove aggregates before addition to the cells. Measurement of cGMP Generation—Immature DC (1 × 10⁶ cells/sample), incubated for 15 min at 37 °C in phosphate-buffered saline with the phosphodiesterase inhibitor hydroxy butyl methyl xanthine (0.6 mM), were incubated for an additional 15 min in the presence or absence of DETA-NO (100 μM) with or without ODQ (3 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 normalized on cellular proteins, determined by the bichinonic acid assay procedure (BCA protein assay, Pierce).

Measurement of Ceramide Concentrations—Immature DC cells (1 × 10⁶ cells/sample) were incubated in 80 μl of phosphate-buffered saline with human TNFα (200 ng/ml) in the presence or absence of either DETA-NO (100 μM), SNAP (200 μM), or 8-Br-cGMP (3 mM) with or without ODQ (3 μM) and then quickly shifted at 37 °C. At the time points indicated, incubation was stopped by the addition of 300 μl of ice-cold CH₃OH/CHCl₃ (2/1, v/v). Samples were then supplemented with 100 μl of CHCl₃, and 100 μl of NaCl (1 ×). The extracted phospholipids were incubated for 1 h at room temperature with 100 micromolar diacylglycerol kinase in the presence of 5 mg/ml cardiopain, 7.5% glycoparanesid, 1 mM diethylenetriamine pentaaetic acid, and 10 μCi of [γ-³²P]ATP (10 mCi/ml) as described (23). Under these conditions, diacylglycerol kinase is rate-limiting, and full conversion of ceramide to ceramide phosphate is thus to be expected (24). The ceramide phosphates produced were separated by thin layer chromatography (Silica gel 60, Merek, Milan, Italy) using CHCl₃/CH₃OH/CH₃COOH (65/15/5, v/v/v) as solvent. To determine the concentration of ceramide per sample, known amounts of ceramide standard were processed and loaded in parallel. The relevant spots were identified by autoradiography, and their radioactivity was estimated by microdensitometry using a Molecular Dynamics Imagequant apparatus (Buckinghamshire, UK).

Western Blotting—DC were incubated for 24 h in the culture medium with or without human TNFα (200 ng/ml), IL-1 (1000 units/ml), GMP-CSF (50 ng/ml), IFNγ (100 units/ml), and lipopolysaccharides (LPS, 10 μg/ml). Cells were then collected, washed twice with cold buffer (150 mM NaCl, 1 mM Na₂P₂O₆, 30 mM NaF, 20 mM Tris-HCl, pH 7.5), and lysed for 30 min in the same buffer containing 1% Triton X-100, 0.1 mM phenylmethyl sulfonylfluoride, 10 μg/ml aprotinin, and 10 μg/ml aprotinin. Protein content in the lysates was assayed by the bichinoninic acid procedure. After addition of SDS and β-mercaptoethanol the samples were boiled, and 50 μg of protein/lane were loaded into the slots of 10% SDS-polyacrylamide gels as described (25). High efficiency transfer of proteins onto nitrocellulose membranes was obtained by the N9 murine microglial cells activated with polystirene microspheres and IFNγ, a treatment that induces expression of iNOS and continuous generation of bioactive NO (10, 20). In the first experimental approach, immature DC were incubated in a double chamber system with or without TNFα (200 ng/ml, 48 h) in the presence or absence of activated N9 cells. DC were then resuspended in cytokine-free medium and incubated with FITC-dextran, and the internalization of the latter was measured by flow cytometry. In the TNFα-untreated, immature DC incubated at 37 °C, uptake of FITC-dextran was found to proceed linearly for 15 min and then to level off progressively. In contrast, at 4 °C no uptake was detected (Fig. 2A). Treatment of DC with TNFα resulted in a statistically significant inhibition of endocytosis induced by FITC-dextran, which was prolonged throughout the period analyzed (1 h, Fig. 2A). This effect of TNFα was not due to cytotoxicity because cell viability was 94 ± 3.8% after 48 h of treatment with the cytokine (n = 5).

In the second experimental approach, immature DC cells were incubated with TNFα (0.2–200 ng/ml) in the presence or absence of DETA-NO (100 μM) or SNAP (200 μM). TNFα induced a concentration-dependent inhibition of endocytosis (Fig. 3A) that was reversed by both NO donors (Fig. 3). Decomposed DETA-NO and SNAP, which are unable to release NO as measured by a NO-sensitive electrode, did not have any significant effect on endocytosis (Fig. 3B). Moreover, NO donors or activated N9 cells failed to induce any significant effect on endocytosis of DC that were not treated with TNFα (not shown). NO donors did not induce cytotoxicity (viability versus untreated controls was 95 ± 5.0 and 93 ± 4.3% after the 48-h incubation time with DETA-NO and SNAP, respectively, n = 3).

The Effect of NO on Endocytosis of DC Undergoing TNFα-induced Maturation Is cGMP-dependent—NO effects are known to be mediated through both cGMP-dependent and -independent pathways (5). cGMP generation in immature DC was increased by DETA-NO with respect to untreated controls (values were 2.39 ± 0.23 and 0.35 ± 0.05 pmol/mg/min, respectively), an effect that disappeared in the presence of the guanylyl cyclase inhibitor ODQ (3 μM) (values were 0.42 ± 0.07 pmol/mg/min) (n = 5). Immature DC were treated with TNFα (48 h) in the presence or absence of NO donors, either alone or in the presence of ODQ or of the membrane-permeant cGMP analogue 8-Br-cGMP (3 mM). As shown in Fig. 4, DETA-NO and SNAP prevented the effect of TNFα on endocytosis of FITC-dextran. This action of the NO donors was inhibited by ODQ and mimicked by 8-Br-cGMP. These results indicate that NO controls endocytosis regulated by TNFα through the activation of cGMP-dependent pathway(s).

Inhibition by NO of the Accumulation of Ceramide Induced by TNFα in DC Is Dependent on cGMP—Ceramide, a second messenger generated by the activation of the p55 receptor for TNFα, has been shown to mediate some of the effects of the cytokine in DC (17). The kinetics of ceramide generation by DC treated with TNFα are shown in Fig. 5A. The cytokine induced a progressive accumulation of the lipid messenger, which reached a plateau after about 6 h and remained constant thereafter. No significant ceramide accumulation was observed in cells not exposed to TNFα (Fig. 5A). When DC were incubated with the cytokine in the presence of activated N9 cells, a negative correlation was found between the amount of NO gener-
FIG. 2. Effects of NO generated by iNOS-expressing N9 cells on the endocytic activity of DC treated with TNFα. A, immature DC were cultured without TNFα (squares), with TNFα (200 ng/ml, circles), or with TNFα and iNOS-expressing N9 cells (triangles) for 48 h. Cells were then washed and suspended in fresh culture medium with or without FITC-dextran (1 mg/ml), and endocytosis at 37 °C was analyzed at the indicated time points as described under “Experimental Procedures.” As a control, endocytosis of FITC-dextran was measured also at 4 °C in cells that were not exposed to TNFα (diamonds). Endocytosis was calculated as a percentage of cells positive to FITC-dextran (DX) with respect to cells treated in the same way but not exposed to the fluorescent dye. B, immature DC were incubated for 48 h with or without TNFα (200 ng/ml), in the presence or absence of iNOS expressing N9 cells, the NOS inhibitors aminoguanidine (AG, 1 mM), and L-NAME (1 mM), as indicated in the key. The incubation medium was removed, and the nitrite concentration in it was measured as described under “Experimental Procedures.” Cells were resuspended in fresh culture medium with or without FITC-dextran for 30 min. Endocytosis was calculated as described for A, and values were expressed as percentages of those measured in cells incubated without TNFα (100%). In both panels statistical probability versus DC treated with TNFα alone is indicated by the asterisks and calculated as described under “Experimental Procedures” (n = 5).

FIG. 3. Effects of NO generated by NO donors on the endocytic activity of DC treated with TNFα. A, immature DC were incubated for 48 h with or without TNFα (0.2–200 ng/ml) in the presence (triangles) or absence (circles) of DETA-NO (100 μM), washed, and resuspended in fresh culture medium with or without FITC-dextran. B, immature DC were incubated for 48 h with or without TNFα (200 ng/ml) in the presence or absence of DETA-NO (100 μM) or SNAP (200 μM) either able or unable to release NO (decayed compounds) as indicated in the key. In both panels endocytosis was calculated as in Fig. 2B after 30 min of incubation with or without FITC-dextran. Statistical probability versus cells treated with TNFα alone is indicated by an asterisk and calculated as described under “Experimental Procedures” (n = 5).
DISCUSSION

We have investigated in DC the effects of NO and the mechanism of its action in the regulation of endocytosis. We have used immature human monocyte-derived DC that efficiently endocytose antigens in vitro (14). These cells were exposed to TNFα, which triggered an in vitro maturation process, as confirmed by the concentration-dependent reduction of endocytic ability and by the up-regulation of molecules involved in T cell activation (1, 14). The kinetics of endocytosis was studied by cell exposure to FITC-dextran. In immature DC, NO, generated either by activated N9 phagocytes or by two NO donors, DETA-NO and SNAP, did not modify endocytosis either by activated N9 phagocytes or by two NO donors, DETA-NO and SNAP, did not modify endocytosis per se. In the presence of TNFα, however, NO reversed the inhibitory effect of the cytokine, i.e. it maintained the ability of DC to internalize FITC-dextran. Because this effect of NO was prevented by ODQ, an inhibitor of the soluble guanylyl cyclase, and mimicked by the membrane permeant analogue of cGMP, 8-Br-cGMP, we conclude that NO acts via a cGMP-dependent mechanism.

The maturation process of DC is triggered by TNFα via activation of its p55 receptor (15), which in many cell types induces sphingomyelin breakdown with resulting accumulation of the lipid messenger ceramide (28, 29). The involvement of latter in the inhibition of endocytosis was documented by previous studies with immature DC, where ceramide inhibited endocytosis of various substrates, namely lucifer yellow, horse-radish peroxidase, and FITC-dextran, suggesting its role as the messenger by which TNFα down-regulates this process (17). We thus investigated whether the maintenance by NO of the endocytic ability in TNFα-treated DC was due to inhibition of ceramide accumulation. Consistent with this possibility, we found that TNFα induced a time-dependent accumulation of ceramide, which was inhibited by both NO donors and NO released by activated N9 cells. cGMP generation accounted for this effect of NO, as demonstrated by experiments with ODQ and 8-Br-cGMP.

To analyze whether inhibition of ceramide accumulation by NO was the only event responsible for its ability to reverse the effect of TNFα on the endocytic ability of DC, experiments were carried out with the membrane permeant C8-ceramide. This lipid inhibited endocytosis of FITC-dextran in a persistent way, yet NO (but not NO plus ODQ) and 8-Br-cGMP were still able to reverse this effect. This finding indicates that the action of NO on endocytosis can be explained not only by its cGMP-dependent inhibition of ceramide accumulation but also by additional effects on the signal transduction pathway activated by TNFα/ceramide, also mediated through cGMP. The molecular target(s) of this further action by NO/cGMP remain(s) to be established. These results indicate that the inhibition by NO, via cGMP, of the TNFα-induced down-regulation of endocytosis is exerted at multiple levels along the signal transduction cascade triggered by this cytokine. NO and cGMP function therefore as wide inhibitors of the action of TNFα on endocytosis rather than as selective regulators of one single transductional event.

NO generation in peripheral tissues occurs as a consequence of various stimuli (2, 5–7, 9). The events we describe, i.e. the
regulation of endocytosis by NO through cGMP, might enable DC to prolong their antigen uptake function at the site of inflammation and therefore modify the ensuing immune responses in the lymphoid organs. So far, the role of NO in the maturation process of DC has been investigated in vitro in the murine system by measuring antigen presentation as well as the ensuing T cell proliferation after cell exposure to cytokines, which results in expression of iNOS (see e.g. 13). Both these functions were found to be impaired by the generation of NO by DC themselves, suggesting an overall inhibitory role for the messenger on DC maturation (see Ref. 30 for review). These data appear consistent with the mechanism of action by NO elucidated here for human DC, because maintenance of endocytosis, characteristic of an immature phenotype, is expected to be revealed in vitro as a reduced ability to stimulate preprimed T cells.

Regulation of NO by human DC function might, however, be different. To our knowledge, iNOS expression and NO generation by these cells have not been reported, except in primary biliary cirrhosis and hepatocellular carcinoma (31, 32). Consistently, we could detect neither expression of iNOS nor NO generation by human DC exposed to various combinations of cytokines and LPS. Regulation of human DC might therefore depend on exogenous NO, generated at the site of infection by macrophages as a result of their activation by cytokine during the inflammatory response (33). Because the functional effects of TNFα on human DC appear to be reversible and reinducible (16), NO might maintain endocytosis of antigens as long as DC are confined to the inflammation site, tuning their response to this cytokine and possibly also to other maturative stimuli. After egress from the inflammation site, DC would no longer be exposed to NO and could therefore down-regulate their endocytic ability to prevent any interference by irrelevant self-antigens captured during migration to lymphoid organs (1). In conclusion, NO, acting on human DC in a paracrine fashion, may contribute to enhance immune responses, whereas disregulation of its homeostasis, with its generation under severe pathological conditions in DC (31, 32), might instead impair the immunological function of these cells.

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