Dendritic cells (DC) are professional antigen-presenting cells that possess specific and efficient mechanisms to initiate immune responses. Upon encounter with pathogens, immature DC will go through a maturation process that converts them to highly immunogenic mature DC. Despite the fact that nitric oxide (NO) was produced in large amounts in maturing DC, it is still unclear whether NO is the key molecule that initiates and enhances DC maturation and T cell proliferation, respectively. Here, we report that NO donor and overexpression of either nitric-oxide synthase 2 (NOS2) or nitric-oxide synthase 3 (NOS3) alone can induce surface expression of major histocompatibility complex class II (MHC II) and both the essential co-stimulatory molecules CD80 and CD86 in immature DC. Consistently, NO donor-treated immature DC were capable of enhancing T cell proliferation in vitro in the absence of lipopolysaccharide. Interestingly, NOS2 interacts with CD74 (the MHC II-associated invariant chain), and the degradation of CD74 by caspases in immature DC was inhibited upon treatment with NO donor. Because the trafficking of MHC II is CD74-dependent, the increase in cell surface localization of MHC II in maturing DC is in part due to the increase in CD74 protein expression in the presence of NOS2 and NO.

Dendritic cells (DC) are professional antigen-presenting cells that possess specific and efficient mechanisms to initiate immune responses. Major histocompatibility complex class II (MHC II) molecules bind peptides derived from internalized proteins that have entered the endocytic pathway and present them at the cell surface for the activation of CD4+ T cells. Upon encounter with pathogens, immature DC go through a maturation process that converts them to highly immunogenic mature DC. Immature DC are good at capturing pathogens or foreign antigens, but most of the intracellular MHC II molecules that can bind and present antigens to T cells are localized to the late endosomes. In mature DC, MHC II molecules are free to traffic to the cell surface together with their peptides to present to T cells. Some protein molecules play essential roles in controlling the retention and trafficking of MHC II in DC. We previously showed that in the immature DC, a number of endosomal proteins were degraded by caspases (1, 2). However, in maturing DC, protein degradation by caspases was inhibited, presumably by NO (1). NO is a central messenger molecule in vascular regulation, immunity, and neurotransmission (3, 4). Despite the fact that NO was produced in large amounts in lipopolysaccharide (LPS)-treated DC, it is still unclear whether NO alone can contribute directly to the initiation and induction of DC maturation and T cell proliferation, respectively, in the absence of LPS.

Here, we report that NO donor-treated immature DC were capable of enhancing T cell proliferation in vitro in the absence of LPS. In addition, the MHC II-associated invariant chain (CD74) was up-regulated in immature DC upon treatment with NO donor. CD74 is a type II integral membrane protein essential for proper MHC II folding (in the endoplasmic reticulum) and targeting of MHC II–CD74 complex to the endosomes (5). Degradation of CD74 is highly regulated via stepwise proteolytic events, in which only the downstream degradation pathway is well understood. Full-length CD74 (p31) bound to MHC II molecule is cleaved by proteases to form p22 and then to p10 that is further digested into CLIP (class II-associated invariant chain peptide), which then dissociates from MHC II with the help of H2-DM that facilitates the exchange of CLIP for antigenic peptides (6, 7).

Despite the identification of the various degradation products of CD74, the exact pathway of CD74 degradation remains unclear (8–10). Here, we found that CD74 was a substrate for caspases, and the degradation of CD74 was inhibited in DC treated with either LPS or NO donor (NONOate), which released NO nonenzymatically at physiological pH (11). Furthermore, NOS2 interacts and enhances the stability of CD74 in maturing DC.

**EXPERIMENTAL PROCEDURES**

*Mouse DC and Macrophage Culture*—Bone marrow-derived DC and macrophages from 6 to 7 weeks old C57/BL 6J wild type and NOS2−/− mice (I-Aα, I-Eβ) (The Jackson Laboratory) were established by the bulk culture method as previously described (1). Briefly, bone marrow cells were harvested from the long bones and plated on dishes in complete Dulbecco’s modified Eagle’s medium with 10% fetal calf serum containing either 20...
ng/ml of mouse recombinant granulocyte/macrophage-CSF (for DC) or macrophage-CSF (for macrophages). The cells were fed every 2 days with complete Dulbecco’s modified Eagle’s medium containing either 15 ng/ml granulocyte/macrophage-CSF (for DC) or macrophage-CSF (for macrophages). For immature DC and macrophages, the cells were harvested after day 7. As for mature DC, the cells were further subcultured in media containing 1 μg/ml LPS (Sigma) for 48 h.

Flow Cytometry Analysis—Untreated or DC treated with either LPS or NONOate were harvested with 2 mM EDTA and then washed with staining buffer (5% bovine serum albumin, 2 mM EDTA, 2 mM NaN₃, in phosphate-buffered saline) followed by incubation for 30 min on ice with the mouse FITC-conjugated anti-MHC class II, anti-CD80, anti-CD86, and hamster anti-CD11c antibodies (all of the antibodies were purchased from BD Transduction Laboratories). After washing with staining buffer, the cells were fixed and analyzed by flow cytometry.

Immunofluorescence—DC seeded on coverslips were treated with LPS for 48 h and fixed in chilled methanol for 4 min at −20 °C. After washing, the cells were incubated with rat anti-CD74 and rabbit anti-NOS2 antibodies and visualized with FITC-conjugated goat anti-rat and rhodamine-conjugated goat anti-rabbit IgG.

Measurement of NO—NO was assayed by measuring the concentration of stable end product NO₂⁻. NO₂⁻ production was determined by a Griess reaction. Aliquots of culture supernatant (100 μl) were incubated with 100 μl of Griess reagent (Sigma) at room temperature for 10 min. The absorbance was measured at 550 nm in an automated plate reader. Concentration was determined with reference to a standard curve of NaN₃.

Co-immunoprecipitation—Proteins were extracted from mature DC and incubated on ice for 1 h in cold lysis buffer (20 mM Tris, pH 8.0, 10 mM EDTA, 100 mM NaCl, 1 mM DTT, 1% Brij98, and protease inhibitor) followed by centrifugation at 13,000 rpm for 15 min at 4 °C. The protein extracts (1 mg) were incubated overnight with 5 μg of the corresponding antibodies bound to protein A-Sepharose beads (GE Healthcare) in lysis buffer plus 1% bovine serum albumin and 10% fetal bovine serum at 4 °C. The beads were then washed three times in buffer A (20 mM HEPES, pH 7.2, 100 mM KCl, 1 mM DTT, 0.2 mM ATP, 0.5% Brij98) and three times in buffer B (identical to buffer A except without Brij98) before being resuspended in SDS sample buffer. Immunoprecipitated proteins and 5% of the supernatant were separated on SDS-PAGE and analyzed by immunoblot.

Construct for Overexpression of CD74 and Its Mutants—Total RNA was extracted from bone marrow–derived DC using TRIzol (Invitrogen) according to the manufacturer’s conditions and used as template for reverse transcription-PCR. cDNA was generated using avian myeloblastosis virus reverse transcriptase (Promega) and oligo(dT) and subsequently amplified with PCR using forward primer (5′-GGAATTCATGGATGACCAACGCCGTCCTCTAACCAT-3′) and reverse primer (5′-CTCTGAGCTAGACTACGTCGTTGACCCAG-3′). The resulting CD74 PCR product was inserted into pDMyc vector using the XbaI and EcoRI restriction sites. Mutagenesis of Asp to Ala in CD74 was performed by PCR using same reverse primer (see above) and mutagenic forward primer (5′-GGAATTCATGGATGACCAACGCCGTCCTCTAACCAT-3′), changing Asp to Ala. Both of the constructs were checked by DNA sequencing. The constructs were transfected into DC2.4 cells using the Effectene (Invitrogen) according to the manufacturer’s conditions. After incubation for 48 h, the transfected cells were lysed for further analysis.

Caspase Activity Assay—1 × 10⁵ cells were lysed with 1 ml of lysis buffer (50 mM HEPES, 5 mM DTT, 0.1 mM EDTA, 0.1% CHAPS, pH 7.4) for 10 min at 4 °C. Protein (20 μg) was incubated at 37 °C in a buffer containing 25 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, and 10 mM DTT, with the respective colorimetric substrates (Z-YVAD-pNA, Z-WHED-pNA, and Z-DEVD-pNA) (all from Calbiochem), in a 96-well, flat-bottomed microtiter plate. The cleavage of the substrates was quantified after 2 h by using measurement of O.D.405 nm absorbance.

In Vitro Transcription of Capped mRNA and Electroporation of Primary DC—The pDMyC/NOS2 plasmids were linearized with XbaI. The in vitro transcription was performed with T7 polymerase according to the manufacturer’s instructions (Promega). DC were harvested at day 6, washed twice with serum-free Opti-MEM, and resuspended to a final concentration of 4 × 10⁷ cells/ml in Opti-MEM medium. Subsequently, 100 μl of cell suspension was mixed with 20 μg of mRNA and electroporated in a 0.2-cm cuvette (voltage, 300V; capacitance, 150 microfarads; resistance, 100 Ω) using the GenePulser II (Bio-Rad). After electroporation, the cells were transferred to fresh culture medium containing granulocyte/macrophage-CSF or induced with 1 μg/ml LPS and were cultured for 24 h to allow them to fully mature.

Mixed Lymphocyte Reaction Assay—CFSE-labeled thymocytes from BALB/c mice were incubated with DC that were cultured in medium alone (Ctrl) or stimulated for 24 h with 1 μg/ml LPS or 16 h with 100 mM NONOate. After 7 days, the T cell proliferation was analyzed by flow cytometry. Control labeled T cells (red histograms) were performed with the same protocol but in the absence of DC.

For CFSE labeling, purified thymocytes were resuspended with 0.1% bovine serum albumin in phosphate-buffered saline at a density of 5 × 10⁶ cells/ml and were labeled for 10 min at 37 °C with 0.3 μM CFSE. CFSE-labeled cells were “quenched” with phosphate-buffered saline containing 5% fetal calf serum and were washed twice. T cell division was assessed by fluorescence-activated cell sorter analysis 48 or 72 h later.

Statistics—Student’s t test was used for statistical analyses.

RESULTS

NO Donor Enhanced T Cell Activation by Immature Dendritic Cells—During DC maturation, NO was produced in large amounts presumably catalyzed by NOS2, which was also up-regulated during DC maturation. Despite the fact that NOS2 is the major NOS that catalyzes the production of NO during LPS-induced DC maturation, it is still unclear whether NOS2 is the only NOS that contributes to the production of NO in maturing DC. To examine this issue, DC derived from wild type, NOS2⁻/⁻, or NOS3⁻/⁻ mice were either uninduced or
induced with LPS prior to detection for NO production. Uninduced DC derived from wild type, NOS2−/−, and NOS3−/− mice produced very little NO, presumably because of low basal expression of NOS (Fig. 1A, group 1). On the other hand, LPS induction increased the production of NO in both wild type and NOS3−/− but not in NOS2−/− immature DC (Fig. 1A, group 3). However, the increase in NO production observed in LPS-treated NOS3−/− DC was lower as compared with LPS-treated wild type DC (Fig. 1A, group 3), suggesting that NOS3 did contribute to NO production during LPS-induced DC maturation. As control, wild type, NOS2−/−, and NOS3−/− DC were incubated with L-NMMA to inhibit all forms of NOS (Fig. 1A, group 2). Furthermore, we observed that NOS3 was up-regulated at both the mRNA and protein level in LPS-treated DC (Fig. 1B). Taken together, our results suggest that both NOS2 and NOS3 contribute to NO production in LPS-induced DC. However, NOS2 was the major NOS that catalyzed NO synthesis.

Despite the fact that NO was produced in large amounts in LPS-treated DC (data not shown), it is still unclear whether NO alone can contribute directly to the initiation and induction of DC maturation and T cell proliferation, respectively, in the absence of LPS. To examine this point, immature DC were incubated with NO donor (NONOate) alone and stained for cell surface MHC II, CD80, and CD86 by flow cytometry. Interestingly, we observed that NONOate alone was able to up-regulate cell surface expression of MHC II as well as both the essential co-stimulatory molecules CD80 and CD86 in the absence of LPS (Fig. 1C). Furthermore, an increase in surface expression of MHC II, CD80, and CD86 was also observed in DC overexpressing NOS2 and NOS3. In this experiment, LPS-treated DC was used as control. Because other than MHC II, both the co-stimulatory molecules CD80 and CD86 were also up-regulated upon NONOate treatment, we hypothesized that NONOate alone was able to enhance antigen presentation in the absence of a general maturation signal such as LPS. To examine this possibility, immature DC were either untreated or treated with NONOate or LPS (as positive control) prior to incubation with thymocytes in an mixed lymphocyte reaction assay to detect DC-induced T cell proliferation. Interestingly, DC treated with NONOate alone were able to enhance T cell proliferation, although the enhancement was slightly lower than that observed in LPS-treated DC (Fig. 1D).

NO Inhibits CD74 Protein Degradation—We have recently shown that proteases such as caspases are involved in the degradation of molecules localized to the endosomal membrane trafficking pathway in immature DC (1). In addition, the activity of caspase is partially inhibited by exogenously added NO and endogenous NO produced by the enzymatic activity of NOS2 in the mature DC (Refs. 1 and 12; Fig. 2A). Because NO can induce higher expression of MHC II on the cell surface of DC, it is interesting to determine whether NO can also regulate expression of protein molecules essential for the stability and intracellular trafficking of MHC II complex in DC. Day 5 immature DC were either untreated (control) or treated with...
Interestingly, we also observed that the expression of NOS2 was significantly increased in the presence of both LPS and NO inhibits CD74 protein degradation. A, cell extracts (300 μg) from immature DC either induced with LPS (gray bar) or NOSOate (black bar) were incubated with caspase-1 and -4 substrate (YVAD), caspase-4 and -5 substrate (WHED) and caspase-3, -6, -7, -8, and -10 substrate (DEV), respectively, for 4 h. Caspase activities were then measured according to the method stated under “Experimental Procedures.” Inhibition of caspase activity upon LPS and NONOate treatment were shown. B, immature DC derived from wild type, NOS2-/-, and NOS3-/- mice were either untreated (lane 1) or treated with l-NMMA (lane 2), LPS (lane 3), l-NMMA/LPS (lane 4), caspase inhibitor, CI (lane 5), or NONOate (lane 6). Protein extracts derived from various treatments were analyzed by SDS-PAGE and Western blot. NOS2, CD74, and β-actin were detected by the corresponding antibodies (left panel). Mouse bone marrow-derived primary macrophages were cultured in medium alone (Ctrl) or treated with either LPS or CI. Cell extracts were separated by SDS-PAGE and analyzed by Western blot analysis. CD74 and β-actin were detected by the corresponding antibodies (right panel). C, total RNA was extracted from DC treated as indicated in A; and NOS2, CD74, and β-actin mRNA level were determined by reverse transcription-PCR described under “Experimental Procedures.” D, immature DC were treated with 1, 5, 10, 50, or 100 1 μM NONOate for 16 h. The cell extracts were separated by SDS-PAGE and analyzed by Western blot. CD74 and β-actin were detected by the corresponding antibodies. The bars stand for the density of bands of CD74, which were normalized with the bands of β-actin. E, immature DC derived from wild type (gray bar) and NOS2-/- (black bar) were either untreated (control) or treated with LPS. Cell extracts were separated by SDS-PAGE and analyzed by Western blot. CD74 and β-actin were detected by the corresponding antibodies. The bars stand for the density of bands of CD74, which were normalized with the bands of β-actin.

NONOate for 12 h at 37 °C. Protein extracts from either untreated or treated DC were separated by SDS-PAGE and probed with antibody against CD74, the MHC II-associated invariant chain essential for MHC II trafficking and antigen presentation in antigen-presenting cells (5). NONOate significantly increased the expression of CD74 (at the protein level) in a dose-dependent manner (Fig. 2, B, lane 6, and D) but not at the mRNA level as shown by reverse transcription-PCR (Fig. 2C, lane 6). LPS, which induced NOS2 expression at both the transcriptional and translational levels, also increased CD74 protein expression (Fig. 2, B, lane 3, and C, lane 3). To further demonstrate the importance of NO in controlling the expression of CD74 protein, immature primary DC were either treated with l-NMMA (inhibitor of NOS1, NOS2, and NOS3 activities) alone or treated with both l-NMMA and LPS. l-NMMA treatment alone did not vary the level of CD74 protein expression in the immature DC (Fig. 2B, lane 2). However, LPS-induced CD74 expression was repressed by l-NMMA even in the presence of NOS2 (Fig. 2B, compare lanes 4 and 3). Interestingly, we also observed that the expression of NOS2 was significantly increased in the presence of both LPS and l-NMMA (LPS/l-NMMA) as compared with LPS treatment alone (Fig. 2B, lanes 3 and 4). Unexpectedly, no increase in NOS2 at the transcriptional level was observed upon LPS/l-NMMA treatment as compared with LPS treatment alone (Fig. 2C, lanes 3 and 4). Instead, a decrease in NOS2 mRNA synthesis was observed. This indicates the presence of a positive feedback machinery where the inhibition of NOS activity by l-NMMA treatment enhances the stability of the NOS2 protein.

Next, we asked the question of whether the loss of the NOS2 gene alone would enhance cleavage of CD74 in the immature DC and/or cause a decline in the cleavage inhibitory effect in LPS-treated DC. Primary DC prepared from wild type and NOS2-/- mice were either untreated or treated with LPS and checked for CD74 protein expression. As expected, in the absence of NOS2, LPS-induced CD74 protein expression in DC was significantly reduced but not totally inhibited (Fig. 2E, lanes 2 and 4).

Based on our observation that both LPS and NONOate treatment up-regulates CD74 expression in DC, we hypothesized that CD74 could be a substrate for caspases in immature DC. Immature DC were either untreated (control) or treated with 200 μM caspase inhibitor I for 4 h at 37 °C. Protein extracts from either untreated or treated DC were separated by SDS-PAGE and probed with antibody against CD74. Interestingly, both the
p31 and p41 isoforms of CD74 were significantly up-regulated in caspase inhibitor (CI)-treated DC (Fig. 2B, lane 1 and 5). This upregulation of CD74 protein expression in CI-treated DC was not due to the increase in CD74 mRNA level (Fig. 2C, lane 5). Furthermore, both LPS and CI treatment did not show significant changes to CD74 expression in the mouse bone marrow-derived primary macrophages, suggesting that the effects of LPS and CI on the expression of CD74 are DC-specific (Fig. 2B, lanes 7–9).

Caspase-1 and Caspase-4 Are Potentially Involved in the Degradation of CD74 in DC—Because DC treated with CI expressed a higher level of CD74 proteins, we next made attempts to identify the classes of caspases that potentially utilized CD74 as a substrate. Primary DC were either untreated or treated with various caspase inhibitor (200 μM): Z-VAD-FMK, Z-WHED-FMK, Z-YVAD-FMK, and Ac-DEVD-CMK. The cell extracts were separated by SDS-PAGE and transferred to nitrocellulose membrane. CD74 and β-actin were detected by the corresponding antibodies (Fig. 3A). Immature DC were either untreated (control) or treated for 4 h with various concentrations (100, 10, 1, and 0.1 μM) of Z-WHED-FMK or Z-YVAD-FMK, and then detected for the expression of CD74 proteins by Western blotting analysis. CD74 and β-actin were detected corresponding antibodies. The bars describe the density of CD74 bands (normalized with the β-actin bands). C, the amino acid sequence of CD74 cytosolic domain from different species of animals were aligned together. The red letters indicate the conserve caspase cleavage site. D, DC2.4 expressing either Myc-CD74 (panel a) or Myc-(D-A)CD74 (panel b) were fixed and stained with antibodies against c-Myc followed by FITC-conjugated anti-mouse IgG. The images were taken using an Olympus Fluoview 500 at 100× magnification. E, DC2.4 cell extracts derived from either untreated or CI-treated DC (expressing either Myc-CD74 or Myc-(D-A)CD74, respectively) were analyzed by Western blot using antibodies against c-Myc and CD74. The bars describe the density of Myc bands (normalized with the bands of β-actin). F, Myc-CD74 and Myc-(D-A)CD74 were expressed in DC2.4, immunoprecipitated using antibody against CD74, and digested with recombinant caspase-1 and caspase-4 in vitro prior to detection using anti-Myc and anti-rabbit IgG polyclonal antibodies.
NOS2 Interacts with CD74

Inhibitory effect of Z-YVAD-FMK was relatively more specific than Z-WHED-FMK based on its ability to inhibit CD74 degradation in a dose-dependent manner (Fig. 3B). Because Z-YVAD-FMK and Z-WHED-FMK are strong inhibitors of both caspase-1 and caspase-4, it is highly possible that CD74 is a substrate for either caspase-1 and/or caspase-4.

We identified a putative caspase recognition/cleavage site DQRD by screening the amino acid sequence of cytoplasmic domain of CD74 (Fig. 3C). First, we determined whether the DQRD motif was important for intracellular localization of CD74. Myc-CD74 (wild type) and Myc-(D-A)CD74 (Asp → Ala mutant) were expressed in DC2.4 and detected using specific antibody against c-Myc. Interestingly, changing the DQRD motif to the DQRA motif by point mutation did not cause mislocalization of CD74 in DC (Fig. 3D). To determine whether the DQRD motif is a caspase recognition and cleavage site, protein extracts derived from either untreated or CI-treated DC (expressing either Myc-CD74 or Myc-(D-A) CD74, respectively) were analyzed by Western blot using antibodies against c-Myc and CD74. The degradation inhibitory effect of CI on Myc-CD74 was more drastic as compared with the effect on Myc-(D-A)CD74 (Fig. 3E, compare lanes 3 and 4 with lanes 5 and 6). In addition, we consistently observed that the quantity of total Myc-(D-A)CD74 proteins was significantly higher as compared with the amount of Myc-CD74 proteins in the untreated DC. This strongly suggests that the DQRD motif is indeed a caspase cleavage site and that D-A mutation significantly enhances the stability of CD74 in DC. As expected, CI treatment increased the expression of endogenous CD74 detected by IN-1 mAb (Fig. 3E, lower panel). To further confirm that CD74 was a substrate for caspases and that the DQRD motif on the N terminus of CD74 was a caspase cleavage site, Myc-CD74 and Myc-(D-A)CD74 was expressed in DC2.4, immunoprecipitated using CD74-specific mAb (IN-1) and digested with recombinant caspase-1, and caspase-4 in vitro prior to detection using anti-Myc polyclonal antibody. Immunoprecipitated Myc-CD74 but not Myc-(D-A)CD74 was preferentially cleaved by caspase-4 (Fig. 3F, lane 3). On the other hand, Myc-CD74 was not cleaved by caspase-1 (Fig. 3F, lane 2). However, it is worth noting that a small amount of Myc-(D-A)CD74 was cleaved by caspase-1 (Fig. 3F, lane 2, lower panel). The reason for this is unclear, but there is a possibility that the D-A mutation on the DQRD motif might have created a site more receptive to caspase-1 activity.

NOS2 Forms Complexes with CD74—Nitric oxide is a labile free radical gas molecule that intervenes in a large number of fundamental cellular processes such as vascular regulation, neurotransmission, and immunity (3, 4). NO reacts in water with oxygen and its reactive intermediates to yield (i) stable anions such as NO− and NO2−, (ii) unstable higher oxides such as N2O3, and (iii) unstable peroxides (ONOO−). We showed that NO can partially initiate DC maturation based on the fact that NONOate (a NO donor) treatment and overexpression of NOS2 and NOS3 have the ability to induce the expression of cell surface MHC II, CD80, and CD86, hallmarks of DC going through maturation. In addition, the MHC II-associated invariant chain CD74 was up-regulated in DC upon treatment with NONOate. CD74 is essential for proper MHC II folding (in the ER) and targeting of MHC II-CD74 complex to the endosomes (15, 16). In the endosomes, CD74 protein undergoes degradation via several stepwise proteolytic processes (executed by various specific proteases) and formed cleavage products, p31, p22, and p10 according to their molecular weight. The final cleavage product, CLIP (a product derived from the cleavage of p10), is a short peptide of 23 amino acids still bound to peptide-binding groove of the MHC II αβ complex. Every proteolytic step is executed by specific proteases, and cleavage of proteins could be one of the methods used by cells to control protein expression level at various
stages of cell development. During DC maturation, the activities of proteases such as caspases were repressed, resulting in the accumulation of a number of endosomal proteins (1). In conjunction with this, full-length CD74 (p31) protein was also up-regulated upon DC maturation (Refs. 17 and 18 and this study). An unaltered CD74 mRNA level in DC after treatment with LPS excludes the possibility of up-regulation at the transcriptional level. Although several major proteases involved in the terminal stages of CD74 processing were identified, very little is known about how proteolytic degradation of CD74 was regulated in maturing DC. Previous report suggested that asparagine endopeptidase was involved in the degradation of CD74 protein in B

FIGURE 4. NOS2 forms complexes with CD74. A, stereo three-dimensional rendering images of NOS2 and CD74 subcellular localization in DC. Co-localization of NOS2 (red) with CD74 (green) viewed at two different angles. Z scaling = 0.49 μm; Z-stack size = 12.6 μm; number of slices = 26. B and C, X-Z/Y-Z projection of collected confocal images showing co-localization of NOS2 with CD74 at two different positions. Z scaling = 0.43 μm; Z-stack size = 14.18 μm; number of slices = 34. Stereo three-dimensional rendering and X-Z/Y-Z projection were performed using the Volocity visualization software from Improvision. D, protein extracts derived from LPS-treated DC were immunoprecipitated (IP) with CD74 or NOS2 antibodies as indicated and sequentially probed with NOS2 and CD74 antibodies as indicated. E, DC2.A were transfected with Myc-CD74 or Myc-(D-A)CD74, which was indicated under “Experimental Procedures” followed by induction with LPS. The cells were harvested, lysed, and immunoprecipitated with anti-c-Myc antibody and probed with NOS2 and CD74. The lysates were also precipitated with the corresponding isotype IgG.

FIGURE 5. Overexpression of CD74 enhances the MHC II cell surface expression and antigen presentation. DC were transfected (by electroporation) with luciferase (black line), Myc-CD74 (purple line), or Myc-(D-A)CD74 (green line) mRNA. After 24 h incubation, the cells were harvested and double-labeled with antibodies against CD80-FITC (A), CD86-FITC (B), and MHC II-FITC(C) followed by flow cytometry analysis.
cells (8). However, no difference in the processing of the CD74 could be observed between asparagine endopeptidase-deficient and wild type mice (9). Our data strongly suggest that caspase could be one of the major proteases that cleaves/degrades CD74 based on our observations that (i) inhibition of caspases activity by CI resulted in the accumulation of CD74 proteins and (ii) immunoprecipitated Myc-CD74 protein was a cleavage substrate for caspasas, in vitro. Screening of the N terminus cytoplasmic domain of CD74 identified one potential caspase recognition/cleavage site, DQRD, which is conserved among human, mouse, and rat. In addition, the motif DQRD had been shown to be a specific cleavage site for caspase-3 (19). However, a caspase-3-specific inhibitor was not able to inhibit degradation of CD74 in DC. Further experiments using recombinant caspase-1 and caspase-4 enzymes showed that Myc-CD74 but not Myc-(D-A)CD74 was preferentially cleaved by recombinant caspase-4. Furthermore, disruption of the DQRD motif by point mutation (Myc-(D-A)CD74) significantly increased the half-life of CD74 and further supported the hypothesis that the DQRD motif was a caspase recognition/cleavage site.

Our previous studies suggested that caspase activity in immature DC was relatively high, and this activity was significantly inhibited in mature DC by a mechanism closely linked to the activity of NOS2 that catalyzed the synthesis of NO that possesses the ability to S-nitrosylate the catalytic domain of active caspasas (Refs. 12 and 20; Fig. 6). This is consistent with our observation that CD74 degradation was repressed by just treating immature DC with NONOate. In LPS-induced DC, production and secretion of endogenous NO correlate with NOS2 expression (Ref. 21 and this study). Because NO is a labile free radical molecule, we hypothesized that for NO to effectively exert its inhibitory actions on caspasas via S-nitrosylation and to protect proteins/complexes from proteolytic degradation, a high localized concentration of NO is essential. Hence, it is critical for NOS (the enzyme that catalyzes NO synthesis) to localize in proximity to target proteins/complexes. Interactions between NOS and intracellular proteins had been recently reported by several laboratories (3, 13, 14). One of the reports suggested that NOS2 interaction with heat shock protein enhanced gp96-dependent production of NO in immature DC (22). Our study shows that NOS2 partially co-localizes and more interestingly, interacts with CD74 in DC. Thus, the presence of NOS2 could catalyze the production of sufficiently highly localized concentration of NO to protect CD74 from caspase-dependent degradation. Furthermore, point mutagenesis and co-immunoprecipitation studies revealed that the association of NOS2 and CD74 is dependent on the DQRD motif on the cytosolic domain of CD74. However, LPS-induced production of NOS2 without NO synthesis (in the presence of L-NMMA that inhibits the activity of all three forms of NOS) did not reduce CD74 degradation, thus excluding the possibility that NOS2 protein binding to CD74 might have prevented caspase from accessing the recognition/cleavage site (DQRD) on the N terminus of CD74 by inducing either steric hindrance or conformational change. Although the DQRD motif is essential for both recognition/cleavage by caspase and binding of NOS2 to CD74, there is a possibility that the binding of caspases to the N terminus of CD74 is independent of NOS2 binding and vice versa. In immature DC where the expression of NOS2 is low, caspase binds to the DQRD motif and cleaves CD74 efficiently (Fig. 6). However, upon induction by LPS that initiates DC maturation, the expression of NO synthesis (in the presence of L-NMMA that inhibits the activity of all three forms of NOS) did not reduce CD74 degradation, thus excluding the possibility that NOS2 protein binding to CD74 might have prevented caspase from accessing the recognition/cleavage site (DQRD) on the N terminus of CD74 by inducing either steric hindrance or conformational change. Although the DQRD motif is essential for both recognition/cleavage by caspase and binding of NOS2 to CD74, there is a possibility that the binding of caspases to the N terminus of CD74 is independent of NOS2 binding and vice versa. In immature DC where the expression of NOS2 is low, caspase binds to the DQRD motif and cleaves CD74 efficiently (Fig. 6). However, upon induction by LPS that initiates DC maturation, the expression of NOS2 increases. NOS2 then binds to the N terminus of CD74 in proximity to the DQRD motif. Although this binding of NOS2 does not interfere with the binding of caspases to the DQRD motif, it can catalyze the production of NO that inhibits caspases and prevents CD74 degradation. Thus, the NOS2-CD74 partnership is essential to keep the CD74 or its complexes intact. In fact, it is of great importance for CD74 to bind to NOS2 immediately after synthesis and to be inserted into the ER membrane. This is consistent with our
observation that NOS2 co-localizes with CD74 on the ER-like membrane structures in maturing DC.

The expression level of CD74 was low in both the wild type (NOS2+/+) and NOS2−/− immature DC. As expected, after treatment with LPS, the expression of CD74 in the wild type DC increased dramatically. Interestingly, we also observed an increase of CD74 expression in the NOS2−/− DC after LPS induction, suggesting that other LPS-inducible forms of NOS might be involved in protecting CD74 from degradation during DC maturation. In this case, NOS3 could likely be the candidate based on the following reasons: (i) currently, there are three known forms of NOS (NOS1, NOS2, and NOS3), of which only NOS2 and NOS3 had been shown to express in non-neuronal cells (3, 23) and our study showed that DC expressed NOS3 and the expression of NOS3 was inducible by LPS; and (ii) the activity of NOS2 was partially dependent on the expression of NOS3 (24, 25).

Our results showed that CD74 is a substrate for caspases and that the N terminus of CD74 contained a single caspase cleavage site. Cleavage at the N terminus of CD74 by proteases was somehow expected, based on the fact that it was the only part of the CD74 molecule exposed to the cytoplasm. Because CD74 degradation was inhibited in the presence of caspase inhibitors, it is likely that cleavage at the N terminus by caspase alone could initiate degradation of CD74. In addition, based on the localization of CD74 to the ER-like compartment co-localized with NOS2 in maturing DC (this study), there is a possibility that degradation of CD74 could be initiated by caspase-4 on the cytoplasmic surface of the ER membrane in DC. This was supported by previous reports that caspase-4 was one of the caspases inhibited during DC maturation, and caspase-4 was localized to the ER membrane (1, 26–28). Caspase-4 had been shown previously to be essential for cytokine maturation and inflammation and was reported to play key roles in ER stress- and amyloid-β-induced cell death (27, 29). Although caspase-4 was shown here to cleave and presumably to initiate CD74 degradation in DC, the mechanism that regulates such event is still unclear. Although our results indicated that the degradation of CD74 could be initiated on the ER membrane based on the ER localization of caspase-4, we could not exclude the possibility that CD74 was degraded after being targeted to the endosomes. This was supported by our observation that a CD74 construct with its N terminus deleted but still retaining the endosomal targeting dileucine motif was still able to target to the endosomes (data not shown). Further experiments along this line will shine more light on this issue.

Protection of CD74 from degradation could be one of the mechanisms that might contribute to the increase in antigen presentation efficiency in maturing DC. A previous study showed that both p31 and p41 forms of CD74 promoted MHC II antigen presentation in mouse fibroblast cells (30). This is consistent with our observation that inhibition of CD74 degradation by either exogenous NO or overexpression of NOS2 accompanied an increase in MHC II cell surface expression in DC. In addition, overexpression of CD74 by mRNA electroporation in DC increased the cell surface expression of CD80/CD86 and MHC II. This further supports our model that NO is the key molecule that inhibits caspase-dependent degradation of CD74 and other protein molecules of the endosomes during LPS-induced DC maturation. Controlling the half-life of these proteins was vital in regulating the trafficking of MHC II complex and antigen presentation during DC maturation. It was recently shown that proteolytic peptides derived from CD74 were able to modulate cell proliferation and survival (31). Thus, control of CD74 half-life by caspases could contribute significantly to the signaling cascade that controls cell proliferation and survival.

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