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This information is current as of July 19, 2018.

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J Immunol 2000; 164:4649-4658; ;
doi: 10.4049/jimmunol.164.9.4649
<http://www.jimmunol.org/content/164/9/4649>

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Thymic Dendritic Cells Express Inducible Nitric Oxide Synthase and Generate Nitric Oxide in Response to Self- and Alloantigens¹

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Thymocytes maturing in the thymus undergo clonal deletion/apoptosis when they encounter self- or allo-Ags presented by dendritic cells (DCs). How this occurs is a matter of debate, but NO may play a role given its ability of inducing apoptosis of these cells. APC (a mixed population of macrophages (M ϕ) and DCs) from rat thymus expressed high levels of inducible NO synthase (iNOS) and produced large amounts of NO in basal conditions whereas iNOS expression and NO production were very low in thymocytes. Analysis by FACS and by double labeling of cytocentrifuged preparations showed that DCs and M ϕ both express iNOS within APC. Analysis of a purified preparation of DCs confirmed that these cells express high levels of iNOS and produce large amounts of NO in basal conditions. The capacity of DCs to generate NO was enhanced by exposure to rat albumin, a self-protein, and required a fully expressed process of Ag internalization, processing, and presentation. Peptides derived from portions of class II MHC molecules up-regulate iNOS expression and NO production by DCs as well, both in self and allogeneic combinations, suggesting a role of NO in both self and acquired tolerance. We also found that NO induced apoptosis of rat double-positive thymocytes, the effect being more evident in anti-CD3-stimulated cells. Altogether, the present findings might suggest that DC-derived NO is at least one of the soluble factors regulating events, in the thymus, that follow recognition of self- and allo-Ags. *The Journal of Immunology*, 2000, 164: 4649–4658.

The immune system maintains self-tolerance by deleting autoreactive lymphocytes (1) and for T cells this step occurs in the thymus, where developing T cells first express their Ag-specific receptor (1). Acquired tolerance in experimental autoimmunity (2) and transplantation (3, 4) can be thymus dependent and, indeed, in the rat, intrathymic injection of allogeneic cells or allo-Ags induced unresponsiveness to subsequent pancreatic islet (5), cardiac (6), and renal allografts (7, 8). Our group reported that thymic recognition of immunogenic class II MHC synthetic allopeptides induced a state of donor-specific tolerance to allograft and prolonged the survival of kidney allografts in high-responder Wistar-Furth (WF,³ RT1^u) to Lewis (RT1^b) rat strain combinations (7, 9).

Despite the growing number of studies in the area, the cellular and molecular mechanisms underlying the induction of intrathymic tolerance are unclear. Some of these data support clonal de-

letion (10–13), which physically eliminates reactive T cells by a process of programmed cell death, or apoptosis, as the main mechanism, although clonal anergy, rendering T cells unresponsive to Ags, also plays a part (14). In favor of the clonal/deletion hypothesis are very recent data in transgenic mice expressing a TCR specific for the class I MHC Ag H-2K^b. Intrathymic injection of H-2^b spleen leukocytes reduced the CD8-positive thymocyte population by 80% nine days after thymic delivery (15). Double-positive thymocytes were also deleted (15). On the same line, intrathymic injection of OVA in mice transgenic for the TCR specific for peptide 323-339 of OVA led to apoptosis of thymocytes starting as early as 3 h and persisting up to 7 days after injection (16).

Thymic epithelial cells can induce T cell clonal deletion, at least in certain experimental systems (17), but today it seems more likely that dendritic cells (DCs) are the cell population most consistently involved in T cell-negative selection (18, 19). The negative selection potential of DCs implies that the Ag is internalized, processed, and presented to the TCR (20, 21). The Ag retention capacity of thymic DCs is also instrumental to this specialized function (22).

Soluble factors are indispensable to drive either the suppression or the maturation of a given clone to a functionally mature cell. The observation that maturing thymocytes, in the earliest stage of T cell development but not later on, undergo apoptosis when exposed to the L-arginine derivative NO (23) suggested that NO released by thymic stromal cells may take part in the cellular events involved in T cell development. This was supported by the finding that fetal mouse thymi express mRNA for the inducible type of NO synthase (iNOS) with levels of iNOS mRNA peaking around gestation day 18 and declining before birth (24). Moreover, thymic stromal cells from mouse fetuses spontaneously release NO in certain culture conditions (24), although it is not known which cell in

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Received for publication April 19, 1999. Accepted for publication February 16, 2000.

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¹ S.A. was a recipient of a fellowship from the Alfredo Leonardi Fund and G. L. Pfeiffer Foundation.

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³ Abbreviations used in this paper: WF, Wistar-Furth; DC, dendritic cell; iNOS, inducible NO synthase; DP, double positive; M ϕ , macrophage; AML, amiloride; ChlQ, chloroquine; BfA, brefeldin A; SNAP, S-nitrosoacetylpenicillamine; ABC, avidin-biotin complex; EU, endotoxin unit.

the thymus is mainly involved in NO synthesis. Reports that bone marrow-derived DCs were induced to produce NO by the iNOS inducers IFN- γ and LPS (25) aroused our interest in exploring the possibility of resident DCs being involved in NO formation in the thymus.

Overall, our findings indicate that 1) rat thymic DCs express iNOS and generate NO; 2) exposure to either self- or allo-Ags results in up-regulation of iNOS expression and enhances NO production by thymic DCs; 3) the latter phenomenon depends on an intact Ag processing and presenting pathway; and 4) rat immature thymocytes undergo apoptosis when exposed to NO.

Materials and Methods

Reagents

[3 H]L-arginine (56.4 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Dowex AG 50 WX-8 was obtained from Bio-Rad (Richmond, CA) and HEPES was from Merck (Darmstadt, Germany). Collagenase type IV, brefeldin A (BfA), chloroquine (ChlQ), amiloride (AML), and all other chemicals were purchased from Sigma (St. Louis, MO). Culture medium was RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with antibiotics, 2 mM glutamine, 50 μ M 2-ME, and 10% heat-inactivated FCS.

Rat serum albumin was obtained from Sigma. Synthetic WF polymorphic class II MHC peptides were synthesized in the Protein/Nucleic Acid Laboratory, Department of Medicine, Brigham and Women's Hospital (Boston, MA). We selected RT1.B β and RT1.D β distal domains of RT1^u (WF) and synthesized two overlapping peptides of 25 aa (1–25 and 20–44) for each locus using published sequences (26). Rat albumin and class II MHC peptides were tested for endotoxin content by the *Limulus* ameocyte lysate assay (Sigma) and were found to contain between <15 and 25 endotoxin units (EU) of endotoxin/mg peptides and 200 and 300 EU of endotoxin/mg rat albumin. These extremely low levels do not affect NO production in DCs, as documented by preliminary experiments with endotoxin from two different sources (0111:B4 and 055:B5 serotypes). As many as 10,000 EU endotoxin/ml were needed to significantly stimulate NO production in DCs.

Mouse mAbs specific for rat determinants included Abs specific for CD3 (IF4; Serotec, Oxford, U.K.), TCR- $\alpha\beta$ (R73; Serotec), TCR- $\gamma\delta$ (V65; Serotec), CD4 (W3/25; Serotec), CD8 β (341.1; Serotec), CD8 α (PE-conjugated OX8; Serotec), Ig κ -chain (OX12; Serotec), CD45RA (OX33; Serotec), CD45RC (OX22; Serotec), class I MHC (OX18; Serotec), class II MHC (OX6; Serotec), rat tissue macrophage Ag (ED2; Serotec), rat macrophage sialoadhesin (ED3; Serotec), and rat DC-restricted Ag (OX62; Serotec). Rabbit polyclonal Ab against mouse macrophage iNOS was purchased from Transduction Laboratories (Exeter, U.K.). FITC-conjugated F(ab')₂ goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), FITC-conjugated F(ab')₂ goat anti-rabbit IgG (Caltag, Burlingame, CA), Cy-3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) were used as secondary Abs.

Animals

Thymi from Lewis (RT1^l, 150–175 g) or WF rats (RT1^u, 150–175 g; Charles River Italia, Calco, Italy) were used. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (Decreto Legislativo no. 116, Gazzetta Ufficiale, Suppl. 40, February 18, 1992, circolare no. 8, G.U., July 14, 1994) and international laws and policies (European Economic Community Council Directive 86/609, OJL 358, December 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

Cells

Thymi (10–20 for each experiment) were cut into small fragments and digested with collagenase (400 U/ml) for 15 min at 37°C, filtered through a stainless steel screen, and washed in PBS to obtain a total thymocyte suspension (27, 28).

Double-positive CD4⁺/CD8⁺ (DP) thymocytes were enriched by removing class I MHC-positive cells since previous studies have shown that most DP thymocytes lack class I MHC expression (29). Briefly, rat anti-mouse IgG-coupled magnetic beads (Dynabeads; Dynal, Oslo, Norway) were coated with anti-polymorphic class I MHC Ab (OX18) and used to deplete class I MHC-positive cells at a 6:1 bead:cell ratio. Class I MHC-positive cells were removed magnetically and the resulting class I MHC-negative cells were then analyzed by flow cytometry. Double labeling with

anti-CD8-PE-conjugated and anti-CD4 Abs followed by FITC-conjugated F(ab')₂ goat anti-mouse IgG showed that the isolated cells consisted of 95% CD4⁺/CD8⁺ thymocytes, on average.

Macrophages (M ϕ) and DCs were obtained as previously reported (23, 28), with some modifications. The total thymocyte suspension obtained after collagenase digestion and stainless steel screen passage was washed twice in PBS, resuspended in RPMI 1640 supplemented with 10% FCS, and then cultured in 100-mm petri dishes (Falcon; Becton Dickinson, Lincoln Park, NJ) for 120 min at 37°C in a humidified 5% CO₂ in air incubator. Nonadherent cells were removed by extensive washing with warm PBS. Under phase-contrast microscopy, the adherent cells comprised two populations: some cells showed the characteristics of DCs in that they had dendrite-like processes and rapidly changing shape; other cells, M ϕ , were circumferentially spread, ruffled cells with many vesicles.

The adherent cells were cultured overnight in RPMI 1640 10% FCS at 37°C with 5% CO₂. After the overnight culture, the floating cells were collected. Cells that detached after the overnight culture consisted of a large cell population that was MHC I⁺, MHC II⁺⁺, CD4⁻, CD8^{-/+}, CD45RA⁻, CD3⁻, and OX62⁺, consistent with the expected profile of DCs (23, 28, 30). However, a considerable number were ED2⁺ and ED3⁺, suggesting that some M ϕ also detached during the overnight culture. Thus, we called these cells thymic APC. Some (10–15% on average) contaminating thymocytes (CD3⁺, CD4⁺, CD8⁺, MHC I^{+/+}, MHC II⁻, ED2⁻, ED3⁻, and OX62⁻) were also found. Most thymic M ϕ remained adherent after the overnight culture (28). In selected experiments, M ϕ were collected, by treating the petri dishes with EDTA 30 mM, and analyzed by FACS.

Purified DCs were obtained as described previously (30), with some modifications. Thymic APC were centrifuged on 55% Percoll (Pharmacia LKB, Uppsala, Sweden) solution for 20 min at 4°C, and the low-density fraction was collected and subjected to two rounds of plastic adherence for 30 min at 37°C. The final enrichment for DCs was routinely performed by removing T cells and M ϕ using magnetic beads. Briefly, cells were incubated with a mixture of appropriate dilutions of R73, V65, OX12, OX22, OX33, 341.1, ED2, and ED3 mAbs for 30 min at 4°C, washed three times, and then incubated with rat anti-mouse IgG-coated magnetic beads for 30 min at 4°C in agitation. After three rounds of magnetic depletion, the final population contained 85–95% DCs.

DNA fragmentation

Double-positive CD4⁺/CD8⁺ thymocytes were washed once with PBS and resuspended at 2×10^6 cells/ml in RPMI 1640 with 10% FCS with or without the NO-generating agent *S*-nitrosoacetylpenicillamine (SNAP, 10 μ M, 100 μ M, and 1 mM; Cayman Chemicals, Ann Arbor, MI) for 8, 15, and 24 h at 37°C. Dexamethasone-treated thymocytes (1 μ M) served as positive controls. In negative control experiments, a SNAP solution that had been preincubated overnight at 37°C was used. To evaluate the effect of TCR engagement on NO-induced apoptosis, DP thymocytes were cultured for 15 h in anti-CD3-coated petri dishes and then harvested and recultured in the presence or absence of either 100 μ M or 1 mM SNAP for 8 h.

A modified form of the TUNEL assay (31) was used to detect fragmented DNA in apoptotic DP thymocytes (32). Briefly, DP thymocytes were formaldehyde fixed, permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate, and labeled with dUTP-FITC by TUNEL reaction using a cell death detection kit (Boehringer Mannheim, Mannheim, Germany). Labeled cells were visualized by flow cytometry. The apoptosis values were presented after subtracting the percentage of spontaneous apoptosis (with medium alone) which averaged 15–20% at 8 h, 20–25% at 15 h, and 30–37% at 24 h. For the experiment with anti-CD3-coated plates, the apoptosis values were presented after subtracting the percentage of apoptosis in DP thymocytes cultured for 15 h with medium and recultured for 8 h with medium (32–38%).

Flow cytometry

Cell surface immunophenotypic analysis was performed by cytofluorography using the FACSort (Becton Dickinson, Mountain View, CA). Cells were incubated with optimal concentrations of primary Abs for 30 min at 4°C in PBS containing 5% FCS, washed twice with the same buffer, and incubated with FITC-conjugated F(ab')₂ goat anti-mouse IgG for unconjugated primary Abs.

For detection of iNOS, cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate. A rabbit polyclonal Ab against mouse macrophage iNOS (that also recognizes the corresponding rat Ag (33)) was used, followed by FITC-conjugated F(ab')₂ goat anti-rabbit IgG as secondary Ab. All staining included negative controls from which the primary Abs were omitted.

Light-scattering parameters and propidium iodide staining gates were set to exclude dead cells and debris.

Immunohistochemical examination of rat thymus

Thymic frozen sections (8- μ m thick) were cut on a cryostat (HM500-O; Microm, Zeiss Oberkochen, Germany). Sections were air dried, incubated for 1 h with 0.3% H₂O₂ in methanol to quench endogenous peroxidase, and processed for light microscopy immunohistochemistry using an avidin-biotin HRP complex technique (ABC method, ABC-Elite; Vector Laboratories, Burlingame, CA). Slides were blocked by 30-min incubation with non-immune sera (goat serum for anti-iNOS or horse serum for OX6) and then incubated overnight at 4°C in a moist chamber with the primary Abs (anti-iNOS, 1:150; OX6, 1:150) in PBS/1% BSA (Miles, Milan, Italy), followed by the secondary Abs (biotinylated goat anti-rabbit IgG or biotinylated horse anti-mouse IgG), ABC solution, and finally developed with diaminobenzidine for anti-iNOS and diaminobenzidine-nickel (Vector Laboratories) for OX6, as described elsewhere (33). The sections were counterstained with Harris hematoxylin (Biooptica, Milan, Italy). Negative controls were obtained by omitting the primary Ab on a second section present on all of the slides.

To evaluate whether administration of substances capable of up-regulating iNOS expression in several tissue types can modify iNOS expression and distribution in rat thymus, two animals received 20 mg/kg LPS by i.p. injection. The animals were sacrificed 18 h later and the thymus was removed and treated as above.

Intracellular localization of iNOS

Thymic APC and purified DCs were washed with PBS and cytospun into glass slides (Shandon, Cheshire, U.K.). Cells were fixed and permeabilized with ice-cold methanol for 15 min before staining with anti-iNOS or anti-class II MHC Abs (in PBS/5% FCS) followed by Cy-3-conjugated goat anti-rabbit IgG and FITC-conjugated F(ab')₂ goat anti-mouse IgG (in PBS/5% FCS), respectively. Double labeling anti-iNOS and anti-class II MHC was also performed. All staining included negative controls from which the primary Abs were omitted.

To study intracellular iNOS localization, immunoblot analysis was performed on DC whole lysate, cytosol, and particulate fraction. Purified DCs were pelleted, resuspended in 500 μ l lysis buffer (50 mM β -glycerolphosphate, 2 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100, 0.5% Nonidet P-40, 1 mM DTT, 1 mM pepabloc, 20 μ M pepstatin, 20 μ M leupeptin, and 1000 U/ml aprotinin) and sonicated. An aliquot of the whole lysate was stored at -70°C. The remaining lysate was centrifuged at 100,000 \times g for 30 min at 4°C to separate cytosol and particulate fraction (34). The cytosol was stored as for the whole lysate and the pellet was resuspended and again centrifuged at 100,000 \times g for 30 min at 4°C. The pellet was resuspended and stored at -70°C until assayed. Protein concentration was determined for each sample (whole lysate, cytosol, and particulate fraction) using the Bradford method (Bio-Rad). The proteins (10 μ g for each lane) were separated on denaturing a 7.5% SDS-polyacrylamide gel by electrophoresis and then blotted on nitrocellulose membrane by wet electroblotting for 90 min. Blots were blocked overnight at 4°C with 5% nonfat dry milk in TBS-T at pH 7.5 (20 mM Tris base, 137 mM NaCl, and 0.1% Tween 20) and then incubated for 2.5 h with anti-iNOS (1/1000) followed by the secondary Ab (biotinylated goat anti-rabbit IgG), ABC solution, and finally developed with diaminobenzidine (Vector Laboratories).

Conversion of [³H]L-arginine to [³H]L-citrulline

To evaluate NO production in the different thymic cell populations, total thymocytes, APC, and purified DCs (about 2 \times 10⁵/ml) were incubated for 24 h in RPMI 1640/10% FCS in the presence of [³H]L-arginine (0.5 μ Ci). Total thymocyte incubation was performed with 10 \times 10⁶ cells/ml to overcome the assay's detection limit.

Incubations were stopped by adding one volume of ice-cold 15% TCA. TCA-treated samples were centrifuged at 10,000 \times g to precipitate proteins. The supernatant was extracted five times with one volume of water-saturated ether, vacuum lyophilized, and resuspended in 2 ml HEPES (pH 5.5) and applied to 2-ml wet-bed volumes of Dowex AG 50 WX-8 (100–200 mesh, Li⁺ form), followed by 2 ml of water. [³H]L-citrulline was quantitated by liquid scintillation counting in the 4-ml column effluent and identified by TLC as described previously (35).

For each experiment, aliquots of RPMI1640/10% FCS containing [³H]L-arginine were incubated without cells, as blanks.

Effect of self- and non-self-Ags on iNOS expression and NO production in DCs

To establish the effect of self- or non-self-Ags on iNOS expression and NO production by DCs, *in vitro* pulsing experiments were performed using 100 μ g/ml of either rat serum albumin or sperm whale myoglobin. Since DCs mature in culture in 12–18 h after isolation and lose their Ag-processing capacity (36–38), either rat albumin or sperm whale myoglobin was added to adherent cells from WF or Lewis thymi during the overnight culture (16 h). In selected experiments, the effect of shorter exposure to a self-Ag was evaluated after 2.5- and 5.5-h incubation with rat albumin. APC floating after the incubation period were collected and DCs were purified and assayed for iNOS expression, by FACS and immunoblot, and NO production (see above). To assess the effect of Ag processing on the induction of NO synthesis in thymic DCs, we used AML (50 μ M), which inhibits Ag uptake by blocking macropinocytosis (39, 40), ChIQ (15 μ M), which inhibits endocytic function and Ag processing (41, 42), or BfA (1 μ g/ml), which blocks the endoplasmic reticulum-Golgi egress of nascent class II MHC molecules (43–46).

The effect of self- or allopeptides on iNOS expression and NO production by DCs was assessed by *in vitro* pulsing experiments using the two WF RT.1B^u and the two RT.1D^u peptides (25 μ g/ml each). The peptide mixture was added to adherent cells from WF (self-pulsing) or Lewis (allopulsing) thymi during the overnight culture. After the overnight culture, DCs were purified and assayed for iNOS expression and NO production. In some experiments, Ag-processing inhibitors (AML, ChIQ, and BfA) were added in combination with peptides.

Statistical analysis

Data are means \pm SE. The two-tail Student *t* test was used for statistical analysis of NO production and iNOS expression in different thymic cell populations and in DCs incubated with self- or allo-Ags. One-way ANOVA was used to analyze NO-induced apoptosis. Statistical level of significance was defined as *p* < 0.05.

Results

iNOS expression in rat thymus

To evaluate the expression and tissue localization of iNOS, the only NOS isoform identified in the rodent thymus (24), immunohistochemical analysis was done on cryostat sections of Lewis rat thymi using anti-iNOS Ab. Results showed iNOS intense staining at the corticomedullary junction and medulla (Fig. 1A). The same regions were strongly stained by anti-class II MHC Ab (Fig. 1, C and D). At higher magnification, iNOS staining was mainly localized on large cells surrounded by a number of negative smaller thymocytes (Fig. 1B). No iNOS expression was detected in the cortex of normal thymus (Fig. 1A). However, in thymic sections from rats receiving a single i.p. injection of LPS, some specific iNOS staining was also found focally in the cortex (data not shown), suggesting that also cortical cells, maybe M ϕ or epithelial cells, can be induced to express iNOS.

Cellular localization of iNOS and NO production within the thymic cell population

To determine the cell type(s) within the thymus that express iNOS and produce NO in basal conditions, rat thymocytes and thymic APC (DCs and M ϕ) were isolated from the thymus by differential adherence, as described in *Materials and Methods*. FACS analysis of iNOS in thymocytes and thymic APC showed specific high staining in the latter cell population (Fig. 2A), whereas in thymocytes iNOS staining was very low (Fig. 2A). As shown in Fig. 2B, *ex vivo* NO production in unstimulated total thymocytes, measured as conversion of [³H]L-arginine to [³H]L-citrulline after a 24-h incubation, was very low (0.009 \pm 0.002 nmol/10⁵ cells, *n* = 4). APC produced large amounts of NO in basal conditions (1.39 \pm 0.38, nmol/10⁵ cells, *p* < 0.01 vs thymocytes, *n* = 6), confirming that these cells express the enzymatic machinery for NO synthesis.

After overnight culture, thymic APC appeared as DCs and M ϕ

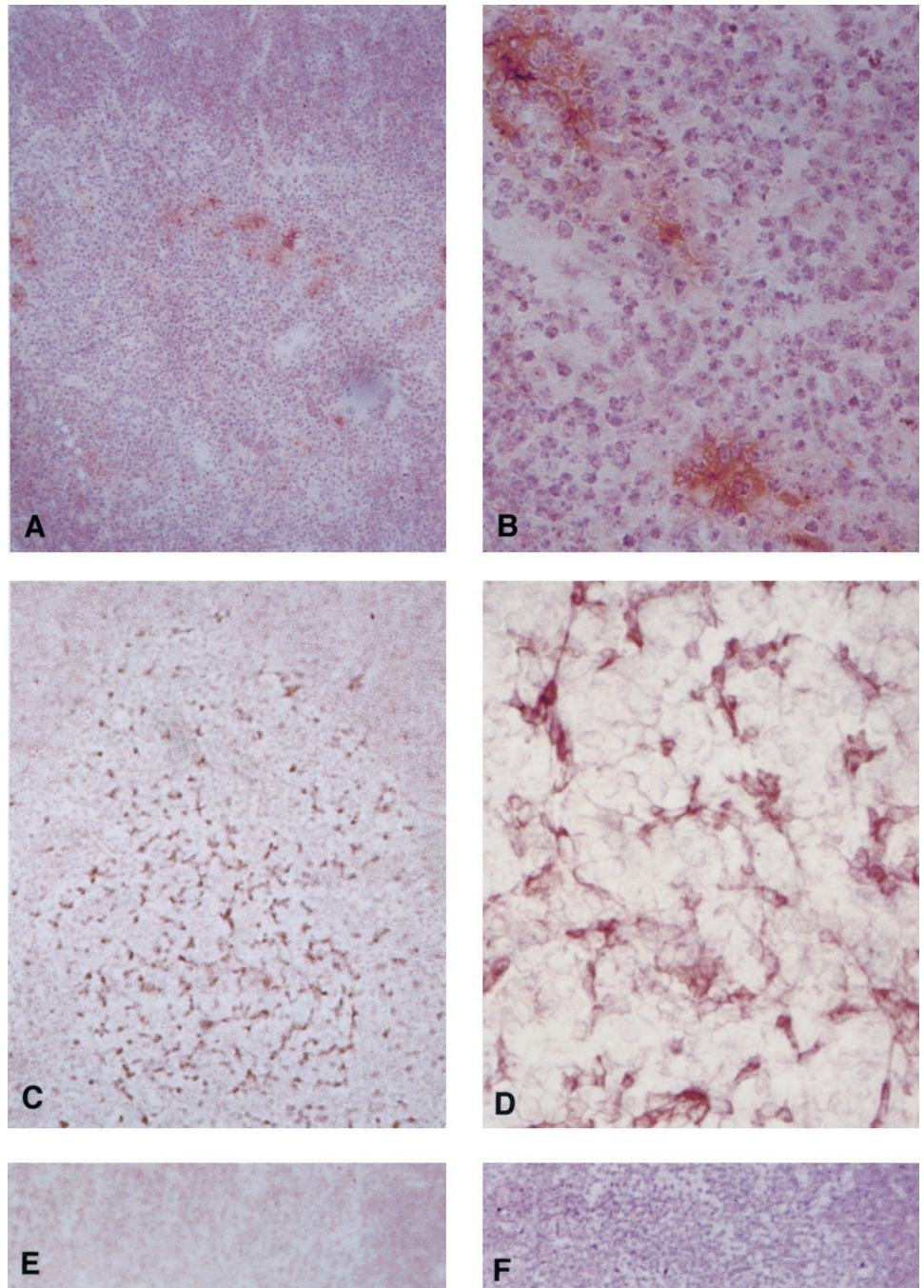


FIGURE 1. Detection of iNOS and class II MHC expression in Lewis rat thymus. Cryostat sections were stained with anti-iNOS (A and B) or anti-class II MHC (OX6, C and D) Ab followed by biotinylated secondary Abs, ABC solution, diaminobenzidine (A and B) or diaminobenzidine/nickel (C and D). No staining was seen when primary Abs were omitted (E for class II MHC and F for iNOS). Harris-hematoxylin counterstaining. Original magnification: A, C, E, and F, $\times 100$; original magnification: B and D, $\times 400$.

when viewed on cytospin slides. Double labeling of cytocentrifuged APC showed two cell populations expressing high iNOS: the majority of iNOS-positive cells had the exact morphology of DCs (high nuclear:cytoplasmic ratio and distinct long cytoplasmic processes or dendrites) and was class II MHC highly positive (Fig. 3, A and B). However, we also found unequivocal iNOS-positive staining in a second cell population that was clearly class II MHC negative or had a low MHC positivity (Fig. 3, A and B). This cell population was classified as M ϕ on the basis of the FACS finding of low class II MHC, moderate ED2, and high iNOS in a cell preparation enriched with M ϕ from rat thymi (Fig. 4).

Using the protocol as described in *Materials and Methods*, we obtained 85–95% purified thymic DCs. These cells have a typical DC morphology, express high levels of class II MHC, and are not stained by ED2 and ED3 mAbs; most of them (>60%) express a

low to moderate level of the Ag recognized by OX62 mAb (Fig. 5). Nearly 80% of purified DCs were iNOS positive by FACS when the staining was done on unstimulated cells (Fig. 5).

To confirm iNOS expression in DCs, we repeated experiments with immunofluorescence analysis on cytospin preparations. Almost all of the purified DCs were positive as for both iNOS and class II MHC staining (Fig. 3, C and D). To determine iNOS intracellular distribution in DCs, anti-iNOS immunoblots were performed on soluble and particulate fractions of DC sonicates. As shown in Fig. 6, immunoblots revealed two forms with apparent molecular masses of 135 and 130 kDa, respectively, both in the soluble and in the particulate fractions. This is consistent with previous data in primary M ϕ showing the existence of two iNOS variants both found in the cytosolic and in the membrane compartments of the cell (34). Confocal microscopy analysis on

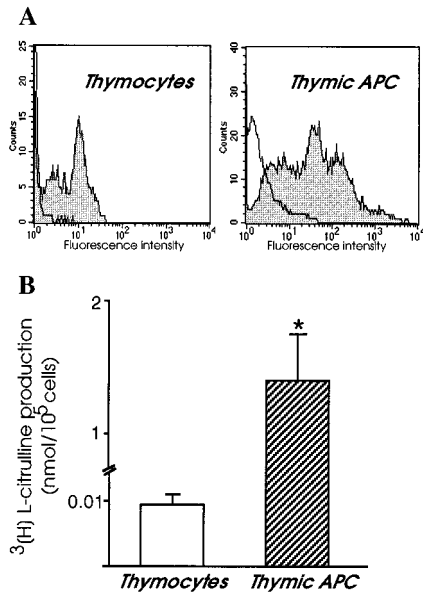


FIGURE 2. iNOS expression and NO production in thymocytes and thymic APC. *A*, Flow cytometric detection of iNOS expression (closed histograms) in thymocytes (*left panel*) and thymic APC (*right panel*). Control staining (open histograms) was performed without primary Ab. Results are representative of four similar experiments. *B*, [^3H]L-citrulline release by thymocytes and thymic APC after 24-h incubation. Data are means \pm SE. *, $p < 0.05$ vs thymocytes.

purified DCs confirmed that iNOS immunoreactivity was present both in the cytosol and in association with membrane structures (data not shown).

Consistent with the expression data, purified thymic DCs produced significant amounts of NO in basal conditions (1.74 ± 0.20 , $n = 23$ experiments). In the latter experiments, DCs from either Lewis rats ($n = 12$ experiments) or WF rats ($n = 11$ experiments) were used; no difference was found in DC-NO production from the two strains.

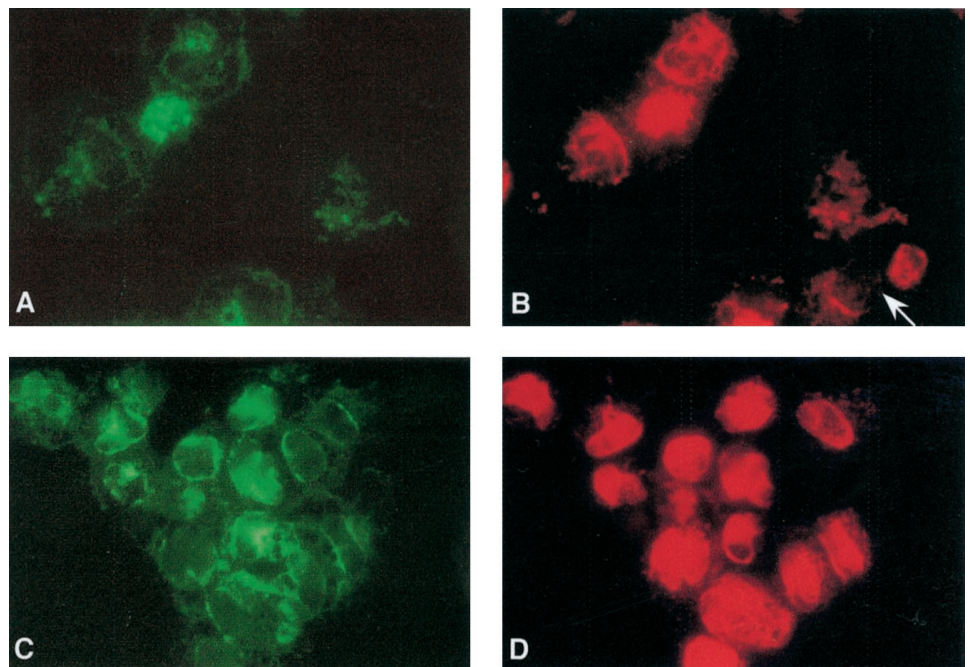
Induction of iNOS expression and NO production by thymic DCs after incubation with either self- or non-self-Ags

To establish whether engagement with self-Ags induced iNOS expression in thymic DCs, rat serum albumin was added to thymic-adherent cell preparations that were cultured for 2.5, 5.5, and 16 h, respectively. At the end of incubation period, floating cells were collected and processed as described in *Materials and Methods*. FACS analysis revealed that a 2.5-h exposure to rat albumin did not modify iNOS expression (mean FITC-fluorescence intensity, rat albumin-pulsed DCs: 375, unpulsed DCs: 325, $n = 2$), whereas iNOS expression was almost doubled by a 5.5-h exposure (mean FITC-fluorescence intensity, rat albumin-pulsed DCs: 472, unpulsed DCs: 287, $n = 2$). Maximal induction was observed after a 16-h exposure to albumin (mean FITC-fluorescence intensity, rat albumin-pulsed DCs: 652 ± 28 , unpulsed DCs: 262 ± 52 , $n = 4$, $p < 0.01$, Fig. 7*A*). Densitometric analysis of immunoblots revealed an increased immunoreactivity of protein extracts from 16-h rat albumin-pulsed DCs over unpulsed cells, either in cytosolic (150%) or in particulate fraction (200%) (Fig. 6).

As shown in Fig. 7*B*, 16-h rat albumin pulsing significantly raised [^3H]L-citrulline release compared with unpulsed cells (2.95 ± 0.55 nmol/ 10^5 cells, $n = 9$, $p < 0.01$ vs unpulsed 1.45 ± 0.32 nmol/ 10^5 cells, $n = 9$ (Lewis DCs, $n = 5$ experiments; WF DCs, $n = 4$ experiments)), indicating an increased NO biosynthesis. NO production in albumin-pulsed DCs corresponded to a concentration of NO of about 10–20 μM which is equivalent to that generated by 100 μM SNAP (23, 47).

Additional experiments were performed to find whether NO synthesized in response to DC pulsing with self-Ags reflected an aspecific effect of albumin itself or implied activation of the process of Ag processing and presentation. To this purpose AML, which inhibits macropinocytosis in DCs (39, 40), or ChIQ, which blocks presentation of Ags by neutralizing intracellular acidic compartments (41, 42), or BfA, which interferes with the egress of newly synthesized MHC molecules (43–46), was added before and maintained throughout the Ag pulsing. As shown in Fig. 7*B*, all of the above inhibitors significantly abrogated NO production by Ag pulsing.

FIGURE 3. Class II MHC and iNOS expression in cytocentrifuged thymic APC and purified DCs. Cells were stained with anti-class II MHC (green) and anti-iNOS (red) mAbs. In the thymic APC, note two cells (white arrow) that are iNOS positive but negative for class II MHC (*A* and *B*). In the purified DC preparation, all iNOS-positive cells (*D*) highly express class II MHC molecules (*C*). Negative controls performed by omitting primary Abs showed no signal (data not shown). Original magnification, $\times 1000$. Results are representative of four similar experiments.



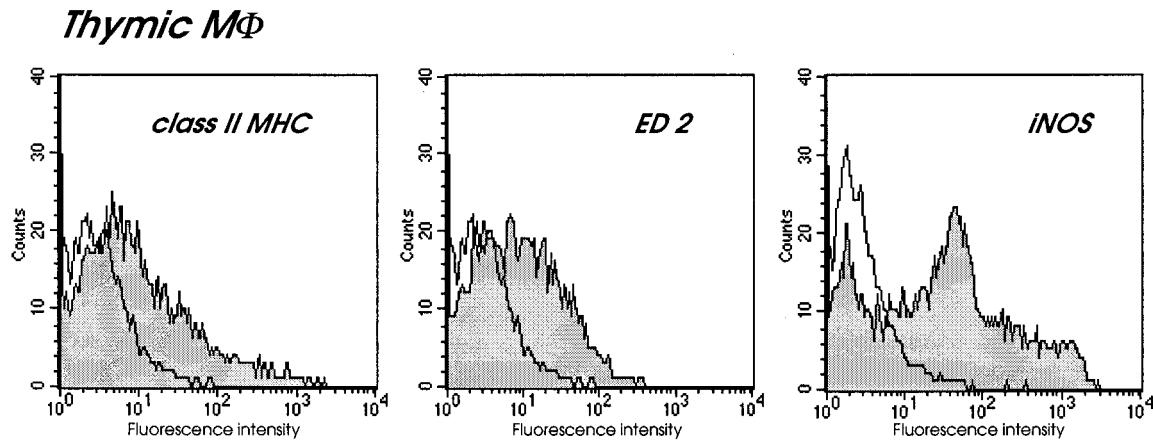


FIGURE 4. Cytofluorometric analysis of thymic MΦ. Thymic MΦ were prepared by adhesion as described in *Materials and Methods*. Cells were stained for expression of the indicated molecules (filled histograms) by using primary Abs followed by FITC-conjugated secondary Ab. Control staining (open histograms) was performed by omitting primary Ab.

To evaluate whether non-self-Ags were capable as well to induce NO synthesis in DCs, the conversion of [³H]L-arginine to [³H]L-citrulline was evaluated in sperm whale myoglobin-pulsed DCs. Results showed a 2-fold increase of NO production in myoglobin-pulsed vs unpulsed DCs (4.01 vs 1.70 nmol/10⁵ cells).

To establish whether loading with peptides from self-class II MHC molecules induced NO biosynthesis in DCs, NO production was also evaluated in WF DCs pulsed in vitro with WF class II MHC peptides. WF class II MHC peptide loading stimulated NO release, as documented by a significant increase in [³H]L-citrulline compared with unpulsed DCs (4.02 ± 0.84 nmol/10⁵ cells, *p* < 0.05 vs unpulsed 2.11 ± 0.41 nmol/10⁵ cells, *n* = 7, Fig. 8, *left panel*). To evaluate whether allogeneic class II molecules could up-regulate iNOS expression and NO production in DCs as well, DCs from Lewis rats were pulsed with WF class II MHC peptides. WF class II MHC allopeptides induced up-regulation of iNOS expression in Lewis DCs (mean FITC-fluorescence intensity, allopeptide-pulsed DCs: 565, unpulsed DCs: 336; mean of two experiments) and NO synthesis (4.19 ± 0.95 nmol/10⁵ cells, *p* < 0.05 vs unpulsed 1.78 ± 0.29 nmol/10⁵ cells, *n* = 7, Fig. 8, *right panel*) as compared with unpulsed DCs.

ChIQ and BfA both completely prevented the effect of WF class II MHC peptides on NO synthesis in WF and Lewis DCs (ChIQ,

1.26 ± 0.37; BfA, 0.94 ± 0.63, nmol/10⁵ *n* = 3), whereas AML had only a partial inhibitory effect (data not shown).

NO-induced apoptosis in DP thymocytes

The kinetics of apoptosis induced by different concentrations of the NO donor is shown in Fig. 9. After an 8-h incubation, only the highest (1 mM) SNAP concentration induced evaluable apoptosis (after subtracting the percentage of spontaneous apoptosis). After 15 and 24 h, a significant specific apoptosis was found with all three SNAP concentrations (Fig. 9). Dexamethasone-treated DP thymocytes, which served as positive control, showed an average of 55% apoptosis. Negative control experiments in which DP thymocytes were incubated with SNAP solution (1 mM), deactivated overnight at 37°C (23), showed a percentage of apoptosis comparable with untreated cells.

To investigate the effect of TCR engagement on the apoptotic effect of NO, DP thymocytes cultured for 15 h in anti-CD3-coated plates were harvested and recultured in the presence or absence of either 100 μM or 1 mM SNAP for 8 h. DP thymocytes cultured for 15 h with medium and recultured for 8 h with medium served as controls. Results are shown in Fig. 10. As expected, an 8-h incubation with 100 μM or 1 mM SNAP induced no or very little

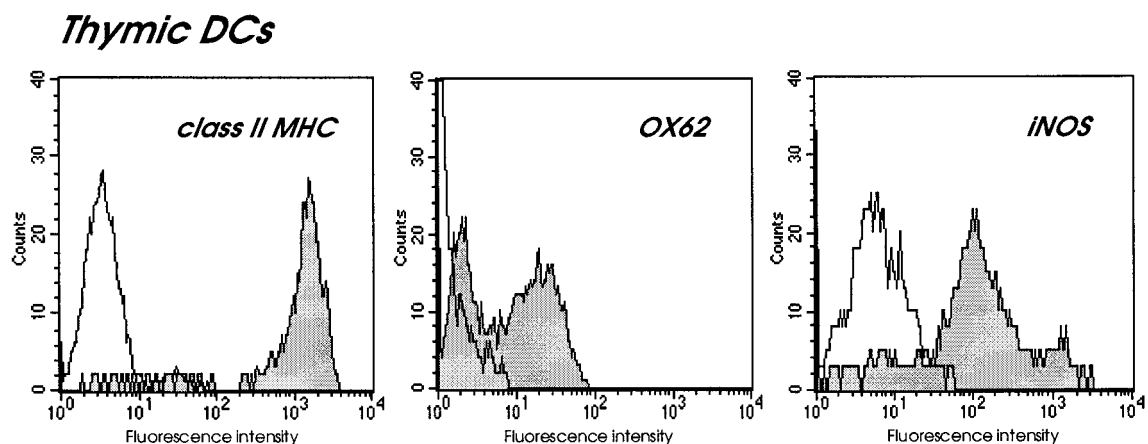


FIGURE 5. Cytofluorometric analysis of thymic purified DCs. Purified DCs were prepared as described in *Materials and Methods*. Cells were stained for expression of the indicated molecules (filled histograms) by using primary Abs followed by FITC-conjugated secondary Ab. Control staining (open histograms) was performed without primary Ab. Results are representative of four similar experiments.

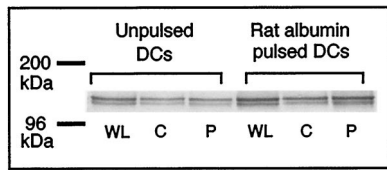


FIGURE 6. Western blot analysis of iNOS in DC lysates. Unpulsed and rat albumin-pulsed DCs were purified, sonicated in lysis buffer, and centrifuged to obtain soluble and particulate fraction. Aliquots of whole lysates (WL), cytosol (C), and particulate fraction (P) were subjected to SDS-PAGE (10 μ g for each lane) and immunoblotted with anti-iNOS Ab. The blot is representative of two similar experiments. The migration of molecular mass markers in kDa is indicated on the left.

specific apoptosis in cells pre-exposed to medium alone. By contrast, an 8-h reculture of anti-CD3-stimulated thymocytes with 100 μ M or 1 mM SNAP dose-dependently increased the percentage of apoptotic cells as compared with that of DP thymocytes treated with anti-CD3 and recultured with medium. Taken together, these

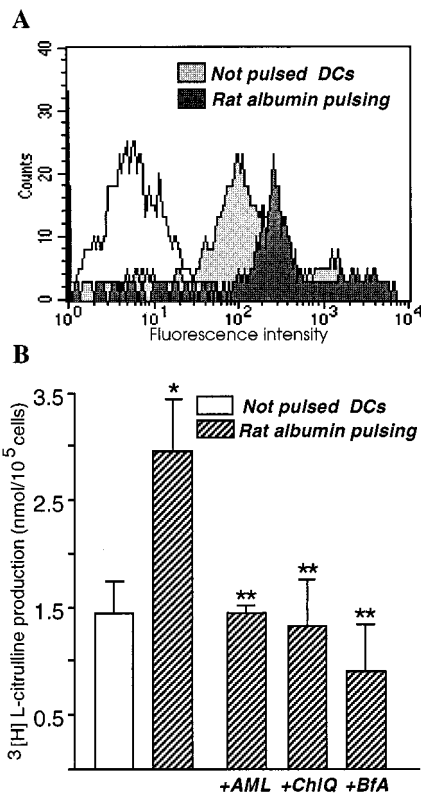


FIGURE 7. Effect of rat serum albumin on iNOS expression and NO production by purified thymic DCs. *A*, iNOS FACS analysis in rat albumin-pulsed and unpulsed DCs. Rat albumin (100 μ g/ml) was added to thymic-adherent cells during overnight culture (16 h). Cells that detached from the plates during the incubation period were collected and purified. Cells were stained for expression of iNOS (filled histograms). Control staining (open histogram) was performed without the primary Ab. Results are representative of four similar experiments. *B*, Effect of rat albumin pulsing on [3 H]L-citrulline release by purified DCs. After a 16-h incubation with rat albumin, cells were purified and incubated for 24 h with medium alone in the presence of [3 H]L-arginine. Sixteen-hour self-Ag-pulsing significantly increased the production of NO by DCs compared with unpulsed cells. AML (50 μ M), ChIQ (15 μ M), and BfA (1 μ g/ml), given before the Ag pulsing, completely prevented the induction of NO synthesis. Vehicles (DMSO for AML, medium for ChIQ, and ethanol for BfA) did not show any effect. Results are the means \pm SE of nine experiments. *, $p < 0.01$ vs basal; **, $p < 0.05$ vs rat albumin pulsing.

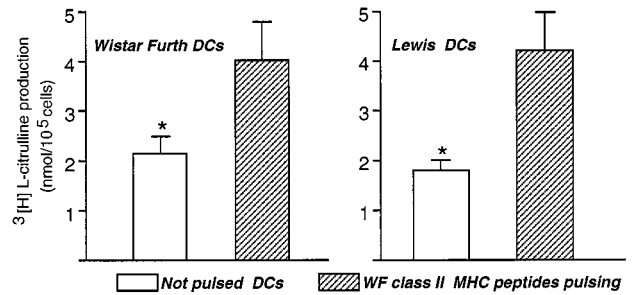


FIGURE 8. Effect of self- and allopeptides on NO production by purified thymic DCs. *Left*, Effect of self-peptide pulsing on [3 H]L-citrulline release by purified DCs from WF thymi. A mixture of the four WF class II MHC peptides (B and D distal domains of the β -chain of RT1 u , 25 μ g/ml for each peptide) was added to WF thymic-adherent cells during overnight culture. Cells that detached from the plates during the incubation period were collected and purified. Purified DCs were incubated for 24 h with medium in the presence of [3 H]L-arginine. Overnight self-peptide pulsing significantly increased the production of NO by DCs compared with unpulsed cells. Results are the means \pm SE of seven experiments. *, $p < 0.05$ vs self-peptide pulsing. *Right*, Effect of allopeptide pulsing on [3 H]L-citrulline release by purified DCs from Lewis thymi. A mixture of the four WF class II MHC peptides (B and D distal domains of the β -chain of RT1 u , 25 μ g/ml for each peptide) was added to Lewis thymic-adherent cells during overnight culture. Cells that detached from the plates during the incubation period were purified and incubated as above. Overnight allopeptide pulsing significantly increased the production of NO by DCs compared with unpulsed cells. Results are the means \pm SE of seven experiments. *, $p < 0.05$ vs allopeptide pulsing.

results suggest that TCR engagement renders DP thymocytes more sensitive to NO-induced apoptosis.

Discussion

Clonal deletion, which occurs in the thymus upon recognition of self- or allogeneic peptides by maturing T cells, is essential to the process of self (2, 10) and acquired transplant tolerance (3, 5–8). However, the nature of humoral mediator(s) involved in this process is still elusive.

We have now provided evidence that thymic DCs possess the enzymatic machinery for synthesizing NO and are actually a major source of NO within the thymus. The capacity of DCs to generate NO was enhanced by exposure to a self-protein and required a

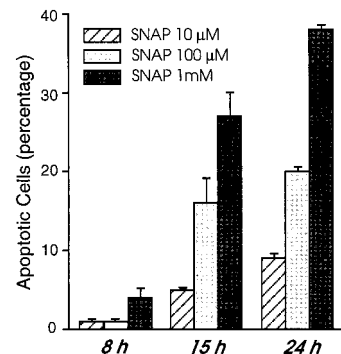


FIGURE 9. NO-induced apoptosis in DP thymocytes. DP thymocyte apoptosis induced by 8-, 15-, and 24-h incubations with the NO donor SNAP at three different doses. Dexamethasone (1 μ M) induced 55% apoptosis. Values are presented after subtracting spontaneous apoptosis in untreated thymocytes (17% at 8 h, 22% at 15 h, and 35% at 24 h on average). Results are the means \pm SE of three individual experiments.

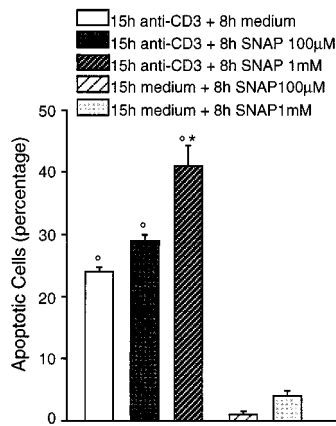


FIGURE 10. Enhanced NO-induced apoptosis in DP thymocytes pre-exposed to anti-CD3. DP thymocytes cultured for 15 h in anti-CD3-coated petri dishes or in medium alone were harvested and recultured in the absence or presence of SNAP (100 μ M or 1 mM) for 8 h. Values are presented after subtracting spontaneous apoptosis in DP thymocytes cultured for 15 h with medium and recultured for 8 h with medium. Results are the means \pm SE of four individual experiments. *, $p < 0.01$ vs 15-h anti-CD3 + 8-h medium and 15-h anti-CD3 + 8-h SNAP (100 μ M). \circ , $p < 0.01$ vs 15-h medium + 8-h SNAP (100 μ M and 1 mM).

fully expressed process of Ag internalization, processing, and presentation. Peptides derived from portions of self-class II MHC molecules up-regulate iNOS expression and NO production by DCs as well. Exactly the same was found with an allogeneic combination of class II MHC molecules and DCs, suggesting a possible role of NO in both self and acquired tolerance. The functional implication of these findings in thymic pathophysiology rests on additional data that exogenous NO induced apoptosis of rat DP thymocytes in a dose- and time-dependent fashion.

NO appears to be an ideal messenger for cell to cell interactions within the thymic parenchyma according to the following arguments: 1) It is synthesized and released upon cell activation (48, 49) and has a very short half-life (50). This would confine the effect of NO in the thymus to cells close enough to the ones initially activated. 2) Induction of apoptosis by NO in DP thymocytes (Refs. 23 and 24 and present data) is prompt, whereas single-positive mature T cells are resistant (23). 3) TCR engagement renders DP thymocytes more sensitive to the apoptotic effect of NO (Ref. 24 and present data). Thus, the effect of NO is maximal on cells ready to be clonally deleted in response to self- or allogeneic peptide-TCR-mediated recognition, as a function of its different impact depending on the T cell maturation step and TCR engagement.

Previous data (23, 24, 50) on the cell source of NO within the thymus are fragmentary and inconclusive. Immunohistochemistry analysis in this study showed that iNOS was expressed in the normal adult rat thymus and the signal was mostly localized in the corticomedullary and medullary regions. Further analysis of different thymic cell populations showed that APC, largely represented by DCs and $M\phi$, express iNOS and generate NO even constitutively soon after isolation. By contrast, NO production by thymocytes is negligible and occasionally undetectable. Two different phenotypes were identified within APC, both iNOS positive. The prevailing phenotype was class II MHC highly positive and had a high nuclear:cytoplasmic ratio and long cytoplasmic processes (dendrites), recapitulating the main characteristics of DCs (30, 51, 52). Less common were cells with immunophenotype and morphologic characteristics of resident $M\phi$ (53).

DCs represent a unique cell population in the thymus constitutively designed to express class I and class II MHC at high levels

(28, 54, 55). Functionally, these cells, in contrast to $M\phi$ which simply capture Ags, process self- and allogeneic Ags and present them in a self-restricted MHC fashion (18, 56, 57). These functional properties, unique to DCs in the thymic parenchyma, render them instrumental to the complex process of negative selection of maturing T cell. Thus, in fetal thymic reaggregation culture from $C5^{-}$ TCR transgenic mice, addition of DCs from $C5^{+}$ mice drastically reduced $CD4^{+}/CD8^{+}$ DP thymocytes, indicating that self-Ag presented by DCs induced clonal deletion of TCR-specific thymocytes (53). That thymic DCs actually induce negative selection events in vivo has been confirmed by data that targeted expression of class II MHC I-E molecules specific to DCs do actually negatively select I-E-reactive T cell clones (19).

Although it is well established that internalization and processing of Ags by thymic DCs are indispensable for negative selection, no studies have investigated whether Ag handling generates messages within the DC itself which eventually trigger cell activation. In this study, we show that thymic DC exposure to a self-circulating Ag, albumin, potently induces iNOS expression and enhances its capacity to release NO in vitro. The ability of Ag-pulsed DCs to enhance their NO synthetic capacity was completely prevented by AML, ChIQ, and BfA, three agents that effectively block presentation of diverse Ags by DCs (38–40, 45). AML inhibits macropinocytosis, a process that endows DCs with a high capacity of a nonsaturable mechanism for capturing any soluble Ag (39, 40). ChIQ, by neutralizing intracellular acidic compartments, inhibits endocytic function, Ag processing, and invariant chain cleavage from class II MHC molecules following peptide binding (41, 42), whereas BfA interferes with the egress of newly synthesized class II molecules from the endoplasmic reticulum (43–46). Thus, our data indicate that induction of iNOS and NO release by rat albumin is dependent on processing of the self-Ag by DCs, although it cannot be established which event along the Ag-processing pathway triggers iNOS induction. According to our data, iNOS up-regulation occurs at a late stage during Ag processing; indeed, maximal iNOS expression in DCs was found after a 16-h exposure to albumin, when Ag processing has been completed and Ag peptides are being presented on class II MHC molecules (58). Unpulsed thymic DCs express iNOS although at a lesser degree than Ag-pulsed DCs and immunohistochemistry findings showed iNOS-positive cells, with dendritic morphology, in the normal rat thymus. This finding can be taken as to suggest that iNOS expression in DCs is induced in vivo by endogenous thymic Ags.

When thymic DCs were exposed to either self- or allogeneic class II MHC peptides, up-regulation of iNOS and NO release was observed to an extent comparable to that elicited by albumin pulsing. Again, ChIQ and BfA completely blocked NO synthesis up-regulation by self- and allogeneic peptides. Recent work with mouse, rat, and human peptides representing portions of the polymorphic regions of class I and II MHC molecules indicate that exogenous self-peptides and allopeptides are taken up by APC and presented in a self-MHC binding site for recognition after endogenous pinocytosis, processing in the Golgi, and transport to the cell surface (59–63). In an earlier study, we found that thymic recognition of class II MHC allopeptides is sufficient for induction of tolerance in the rat renal allograft model (7). Further studies on the mechanisms of acquired thymic tolerance by class II MHC allopeptides showed that the induction phase depended on the presence of an intact thymus and at least partially on a process of T cell anergy, whereas the maintenance phase implied clonal deletion of specific alloreactive T cell clones (64).

TCR-mediated recognition of self-MHC-peptide complexes is instrumental to negative selection so that thymocytes that express TCR with high avidity for MHC/peptide undergo apoptosis (65).

However, it is also clear that additional signals, other than the TCR-mediated ones, are required to determine whether thymocytes will undergo clonal deletion or maturation and that such signals are provided by APC (65). That NO produced by thymic DCs upon Ag processing and presentation may function as a molecule that delivers such an additional signal is supported by the finding that administration of exogenous NO greatly increased apoptosis in anti-TCR Ab-stimulated DP thymocytes, whereas DP thymocytes that did not have their TCR engaged were less sensitive. If this were true, it is possible to hypothesize that thymocytes, that express TCR with high avidity for a given presented Ag, respond to NO and undergo negative selection whereas those with low affinity are spared.

NO-mediated apoptosis was reported in several cell types including macrophages (66), lymphocyte cell lines (67, 68), pancreatic β cells (69), and mouse thymocytes (23). Although mechanisms of NO-mediated apoptosis are still controversial, activation of tumor suppressor protein p53 (70), inhibition of the proteasome (70), impairment of mitochondrial function and energy depletion (71), direct DNA damage by deamination and cross-linking of DNA (72), and activation of caspase cascade (67, 68), all have been proposed. Preliminary experiments in our laboratory showing that exposure of DP thymocytes to exogenous NO activates caspases 3 and 6 and particularly caspase 8 (S. Aiello, M. Noris, and G. Remuzzi, unpublished observations) support a role for caspase cascade activation in NO-induced DP thymocyte apoptosis. Consistently, treatment of DP thymocytes with z-VAD-fmk (a broad-spectrum caspase inhibitor) partially prevented NO-induced apoptosis (Aiello et al. unpublished observations). On the other hand, other studies in rat hepatocytes (73) and human mature lymphocytes (74) have shown that NO can even suppress apoptosis via a direct inhibition of caspases activation (75, 76), indicating that not all cell types are equally sensitive to the apoptotic effect of NO.

A recent study showed that thymocyte apoptosis and animal survival after induction of polymicrobial abdominal sepsis is significantly less in animals lacking iNOS as compared with wild-type mice, providing in vivo evidence that NO has indeed a role in mediating thymocyte apoptosis and selection (77).

In summary, the information from the present study and from few other studies (23, 24) can be used to support the possibility that, upon self- and alloantigen processing, DCs up-regulate iNOS and enhance NO synthesis. Engagement of TCR with specific Ags sensitizes reactive T cell clones to the apoptotic properties of paracrinally released NO.

Acknowledgments

We are grateful to Dr. Lorenzo Gallon for helpful technical advice, Nadia Azzollini and Angela Pezzotta for expert animal care, Dr. Mario Bontempelli for invaluable help with FACS analysis, and Dr. Judy Baggott for editing this manuscript.

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