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Peroxynitrite Inhibits T Lymphocyte Activation and Proliferation by Promoting Impairment of Tyrosine Phosphorylation and Peroxynitrite-Driven Apoptotic Death

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Peroxynitrite (ONOO⁻) is a potent oxidizing and nitrating agent produced by the reaction of nitric oxide with superoxide. It readily nitrates phenolic compounds such as tyrosine residues in proteins, and it has been demonstrated that nitration of tyrosine residues in proteins inhibits their phosphorylation. During immune responses, tyrosine phosphorylation of key substrates by protein tyrosine kinases is the earliest of the intracellular signaling pathways following activation through the TCR complex. This work was aimed to evaluate the effects of ONOO⁻ on lymphocyte tyrosine phosphorylation, proliferation, and survival. Additionally, we studied the generation of nitrating species in vivo and in vitro during immune activation. Our results demonstrate that ONOO⁻, through nitration of tyrosine residues, is able to inhibit activation-induced protein tyrosine phosphorylation in purified lymphocytes and prime them to undergo apoptotic cell death after PHA- or CD3-mediated activation but not upon phorbol ester-mediated stimulation. We also provide evidence indicating that peroxynitrite is produced during in vitro immune activation, mainly by cells of the monocyte/macrophage lineage. Furthermore, immunohistochemical studies demonstrate the in vivo generation of nitrating species in human lymph nodes undergoing mild to strong immune activation. Our results point to a physiological role for ONOO⁻ as a down-modulator of immune responses and also as key mediator in cellular and tissue injury associated with chronic activation of the immune system. The Journal of Immunology, 1999, 162: 3356–3366.

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3 Abbreviations used in this paper: NO, nitric oxide; ONOO⁻, peroxynitrite; O₂⁻, superoxide; SOD, superoxide dismutase; Mn-TBAP, manganese [III] tetrakis-[4-benzoic acid] porphyrin; MC-540, merocyanin 540; KLH, keyhole limpet hemocyanin; scCD3, soluble CD3; iCD3, immobilized CD3; PARS, poly(A)/D ribosyl synthetase; superoxide (O₂⁻) (17–19). The second order rate constant for the reaction between NO and O₂⁻ anion to yield ONOO⁻ is 6.7 × 10⁹ M/s (20), which is 3 times faster than the reaction of O₂⁻ with superoxide dismutase (SOD). Therefore, NO is one of the few biological molecules capable of outcompeting SOD for O₂⁻, making ONOO⁻ formation a favored reaction under in vivo conditions where cellular production of NO and O₂⁻ is increased. Peroxynitrite is a powerful oxidant (6, 17–19, 21, 22) that nitrates free and protein-associated tyrosines and other phenolic moieties (7, 23, 24). Stimulated macrophages, neutrophils, motoneurons, and endothelial cells have been demonstrated to generate peroxynitrite (23, 25–27), and as assessed by nitrotyrosine detection, recent data have provided evidence for the in vivo formation of peroxynitrite in human atherosclerosis, sepsis, human acute lung injury, and chronic inflammation (28–31). It has been demonstrated that peroxynitrite-mediated nitration of the ortho position of tyrosine residues in proteins inhibits tyrosine phosphorylation and targets proteins for degradation (32–34). In lymphocytes, the earliest of the intracellular signaling events initiated by Ag binding to the TCR/CD3 complex involves the activation of several tyrosine kinases and phosphatases that, in turn, results in the phosphorylation of several key cellular substrates (35, 36). Thus, it can be speculated that nitration of tyrosine residues on proteins could prevent T lymphocyte-dependent immune responses. It has been demonstrated that these transduction signals leading to the activation of several transcriptional factors are also strongly influenced by cellular redox status (reviewed in Refs. 37 and 38). This work was aimed to examine the effects of peroxynitrite in normal lymphocyte activation, proliferation, and survival. We have also analyzed the generation of endogenous nitrating species during normal immune responses and their consequences on activation-induced tyrosine phosphorylation and apoptotic cell death.
Materials and Methods

Materials

A stock solution of aminoguanidine was prepared from a bicarbonate salt (Sigma, St. Louis, MO) by suspending it in a minimum quantity of distilled water. Concentrated phosphoric acid was then added until no more CO₂ evolution was visualized. The pH was adjusted to 7.4 with saturated NaOH, and the final volume was adjusted with PBS to the desired stock concentration. MnO₂ powder (manganese [III] tetrakis [4-benzoic acid] porphin) was purined from Alexis (San Diego, CA). PHA, PMA, t-tyrosine, 3-amino-t-tyrosine, o-phospho-t-tyrosine, 3-nitro-t-tyrosine, and monoclonal anti-phosphotyrosine (mouse IgG1, clone PT-66) were purchased from Sigma.

Cell preparation and culture conditions

PBMC were isolated from peripheral venous blood obtained from healthy volunteers by density gradient centrifugation. T lymphocytes were purified from nonadherent cells obtained after adherence of PBMC to plastic for 2 h at 37°C. Nonadherent cells were then incubated with saturating concentrations of anti-CD14, anti-CD19, anti-CD6, and anti-CD56 murine mAbs (Dakopatts, Glostrup, Denmark) for 1 h at 4°C to completely eliminate contaminating monocytes, B lymphocytes, and NK cells. After washing, cells were incubated in the presence of magnetic beads precoated with shear anchor Abs (Dynabeads, Dynal, Oslo, Norway) to achieve a 1:1 bead to cell ratio. After incubation (1 h at 4°C) cell fractions were separated using a magnet applied to the outside of the tube. Unbound T lymphocytes were carefully aspirated, and a second round of immunomagnetic separation was performed with a bead to cell ratio of 10:1; this fraction was referred to as purified lymphocytes. Purified monocytes were isolated by adherence to plastic for 2 h at 37°C from a fraction of untreated whole PBMC that were not exposed to the Abs used in lymphocyte preparation. The purity of the subsets was evaluated by either flow cytometry or immunofluorescence, and the purity of the cell preparations used was >95%. The possibility of minor contaminant cells includes plasma cells, which, in contrast to B cells, do not react with CD19. Cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS (LPS free; Life Technologies, Grand Island, NY), 4 mM glutamine, 1 mM HEPES, 1 mM sodium pyruvate, and penicillin (100 IU/ml)-streptomycin (50 μg/ml; Life Technologies).

Synthesis of peroxynitrite

Peroxynitrite was synthesized in a quenched flow reactor as previously reported (17, 18). Briefly, solutions of 1) 0.6 M NaNO₂ and 2) 0.6 M H₂O₂ were pumped into a T junction and mixed in a glass tube. The reaction was quenched by pumping 1.5 M NaOH at the same rate into a second T junction at the end of glass tube. Excess H₂O₂ was removed by addition of MnO₂ powder. The solution was then frozen at −80°C, at which temperature peroxynitrite forms a yellow top layer due to freezing fractionation. The concentration of this layer was usually in the range of 250–400 μM as determined spectrophotometrically at 302 nm (ε₃₀₂ = 1670 M⁻¹ cm⁻¹). Working dilutions were made in 1 N NaOH.

Agarose gel analysis of DNA fragmentation

The presence of apoptotic cells was assessed by visualization of a characteristic banding pattern of internucleosomal DNA fragmentation. For this, 8 × 10⁵ cells were pelleted for 10 min at 2000 × g and resuspended in 0.5 mL of TTE buffer (10 mM Tris-HCl [pH 7.6] and 1 mM EDTA containing 0.2% Triton X-100). Fragmented DNA was separated from intact chromatin by microcentrifugation for 10 min at 13,000 × g at 4°C. Supernatants (0.5 mL) were mixed with 0.1 mL of ice-cold 5 M NaCl and vortexed vigorously. After addition of 0.7 mL of ice-cold isopropanol, DNA was precipitated overnight at −20°C. Samples were centrifuged for 10 min at 13,000 × g at 4°C and washed in ice-cold 70% ethanol. Dried pellets were solubilized in 20 μL of TE buffer (10 mM Tris-HCl [pH 7.6] and 1 mM EDTA). Samples were mixed with loading buffer, heated for 10 min at 65°C, and electrophoresed on 1% agarose gels with TBE buffer until bromophenol blue dye migrated 3–4 cm. Fragmented DNA was visualized by staining with ethidium bromide.

Flow cytometric analysis of apoptotic cells

Flow cytometric analysis was performed on an EPICS 752 FACS (Coulter, Hialeah, FL). The forward and right angle scatter signals were used to gate out cellular debris, damaged cells, and aggregates. The fluorescence emission was displayed on a log scale to yield histograms of log fluorescence intensity (x-axis) vs cell number (y-axis). The fluorescent dye merocyanine 540 (MC-540, Fluka, Ronkonkoma, NY) has been successfully used as a fluorescent probe in cytometric analysis by its ability to bind symmetric membranes with exposed phosphatidylserine on apoptotic cells (39). Staining of cells with MC-540 was performed according to the method described by McEvoy et al. (40) with minor modifications. In brief, a stock solution of MC-540 was prepared in 50% ethanol at 1 mg/ml and stored in the dark at −20°C. A working solution was freshly prepared by diluting merocyanine in HEPES-buffered salt solution with 0.1% BSA to achieve a final concentration of 2 μg/ml (10⁻⁶ M) were resuspended in 100 μl of HEPES-buffered salt solution with 0.1% BSA and immediately analyzed by flow cytometry.

Production of rabbit anti-nitrotyrosine polyclonal Abs

New Zealand rabbits were immunized with 500 μg of peroxynitrite-modified keyhole limpet hemocyanin (NO₂-KLH) emulsified with an equal volume of CFA according to the procedure of Ye et al. (41). KLH (Sigma) diluted in 100 mM PBS was nitrated in the presence of 0.5 mM peroxynitrite. Boosters were performed every 3 mo after the first injection with NO₂-KLH emulsified in IFA. Rabbits were bled 15 days after the last booster according to serum anti-nitrotyrosine titration as assessed by ELISAs against native KLH and NO₂-KLH. Pooled sera were fractionated in ammonium sulfate, and after dialysis overnight unwanted Abs against native KLH were eliminated using a Sepharose-4B column coupled to native KLH. Specific anti-nitrotyrosine Abs were affinity purified using a Sepharose-4B column coupled to 3-nitro-t-tyrosine.

ELISAs for specificity and competition analysis of anti-nitrotyrosine Abs

Polyclonal anti-nitrotyrosine Abs were screened for specificity and unwanted cross-reactions by Western blot and ELISA. Briefly, 96-well polystyrene ELISA plates were coated with either native or peroxynitrite-nitrated proteins diluted at 30 μg/ml in carbonate buffer (50 mM; pH 9.5) and incubated overnight at 4°C. After washing, plates were blocked with 5% gelatin in PBS plus 0.05% Tween-20 (v/v) for 1 h at 37°C. After washing, nitrotyrosine-peroxynitrite polyclonal Ab was added at a 1/2000 dilution (0.12 μg/ml) in PBS-0.05% Tween 2 h at 37°C. The plates were washed and then incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (Dakopatts) for 2 h at 37°C. The plates were developed with o-phenylenediamine dihydrochloride, and absorbance was read at 492 using a microplate reader. SDS-PAGE and Western blotting were performed as described below. When competition analysis was performed, the anti-nitrotyrosine Ab was preincubated for 30 min at room temperature with several concentrations of 1-t-tyrosine, 3-amino-t-tyrosine, o-phospho-t-tyrosine, and 3-nitro-t-tyrosine as competitors for Ab binding.

Nitrotyrosine and phosphotyrosine immunodetection by Western blot assays

For Western blot analysis, cell suspensions were washed twice in PBS and resuspended in hot lysis buffer (10 mM Tris-HCl [pH 6.8], 1% SDS, and 5% glycerol) followed by immediate boiling for 5 min, sonication, and direct immunoprecipitation of protein complexes by the acid method (Pierce, Rockford, IL). Samples were stored at −70°C until analysis on 10% SDS-PAGE (30 μg of protein/lane). Next, proteins were electrophoretically transferred to 0.45-μm pore size nitrocellulose membranes, and nonspecific binding sites were blocked overnight in blocking in TBS-T blocking buffer (5% BSA and 0.3% Tween in Tris-buffered saline, pH 7.5). For nitrotyrosine immunodetection, nitrocellulose filters were probed with anti-nitrotyrosine polyclonal Abs diluted 1:1000 in blocking buffer for 1 h at room temperature. After extensive washings in TBS-Tween 0.3%, blots were further incubated for 45 min with a horseradish peroxidase-linked anti-rabbit IgG polyclonal Ab raised in donkeys and developed using the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL). For phosphotyrosine immunodetection, nitrocellulose filters were blocked as described above and then probed with an anti-phosphotyrosine mAb diluted in TBS-T with 0.1% BSA. After washing in TBS-T, blots were incubated for 45 min with a horseradish peroxidase-linked anti-mouse IgG polyclonal Ab and developed as described above.

Proliferation assays

For [3H]thymidine uptake, cells were cultured in 96-well culture plates at 5 × 10⁵ cells/well in a total volume of 0.2 ml. Stimulation was conducted in the presence of immobilized anti-CD3 mAbs, PHA, and soluble (sCD3) anti-CD3 mAbs (at 5 and 10 μg/ml, respectively), or PMA (100 ng/ml) plus calcium ionophore A-23187 (0.2 μg/ml). After 3 days, cultures were pulsed with 1 μCi/well of [3H]thymidine and further cultured for 18 h.
Cells were then harvested, and radioactivity was measured in a beta scintillation counter. All samples were incubated in quadruplicate, and for each experimental condition a control with culture medium alone was included.

**Immunohistochemical studies for nitrotyrosine detection**

Immunohistochemistry to detect anti-nitrotyrosine was performed on human lymph nodes obtained from routine surgical resections for lung and colonic cancers obtained at the Hospital de Clínicas (Facultad de Medicina, Universidad de la República, Montevideo, Uruguay). Lymph nodes were fixed in 10% formalin and paraffin embedded, and 5-μm sections were mounted in silanized microscope slides. Tissue sections were probed with anti-nitrotyrosine polyclonal Abs (working dilution, 1/50) and developed with a secondary Ab coupled to biotin using a streptavidin-peroxidase kit (Sigma) and either aminoethylcarbazol or diaminobenzidine as chromogens. Histological sections were counterstained with hemalum. Controls were performed with an irrelevant polyclonal rabbit serum as the first Ab. The technical control was omission of the primary Ab. The specificity of immunostaining was always assessed by competition with soluble 3-nitrotyrosine at 5 mM.

**Results**

**Dose-dependent inhibition of T lymphocyte proliferative responses after in vitro treatment with peroxynitrite**

Fig. 1 depicts the range of nontoxic concentrations of ONOO⁻. It is shown that doses up to 1 mM ONOO⁻ result in no significant loss of lymphocyte viability after 2 h of in vitro culture as assessed by trypan blue exclusion tests. Thus, we selected a working range from 0.01 to 1 mM ONOO⁻ for further in vitro experiments. Pretreatment of purified T lymphocytes with ONOO⁻ induced a dose-dependent impairment in their proliferative activity (Fig. 2) upon activation with immobilized anti-CD3 mAbs (iCD3), PHA plus anti-sCD3 mAb, and PMA plus calcium ionophore. Compared with either iCD3 or PHA-sCD3, impairment of proliferative responses to PMA plus calcium ionophore was only observed when ONOO⁻ doses were higher than 0.25 mM. Proliferative responses to iCD3 and sCD3-PHA were significantly reduced at the lowest doses of ONOO⁻ used in this work (<0.1 mM).

**Peroxynitrite primes lymphocytes to undergo apoptotic cell death following activation in vitro**

To better define the mechanisms of peroxynitrite-induced impairment in lymphocyte proliferative responses we investigated the eventual existence of apoptotic cell death. Normal purified lymphocytes were pretreated with increasing concentrations of ONOO⁻ (ranging from 0.01 to 1 mM), and after 30 min cells were either nonactivated or activated in the presence of PMA-ionophore, iCD3, and PHA plus sCD3. Apoptotic cell death was assessed by DNA fragmentation on agarose gels or by flow cytometry using the MC-540 fluorochrome at different culture times. Fig. 3 (left panel) shows a clear pattern of DNA fragmentation in resting purified lymphocytes after 6 h of culture only at doses of ONOO⁻ higher than 0.25 mM. When peroxynitrite-treated lymphocytes were activated by anti-iCD3, DNA fragmentation started at very low concentrations (≈0.05 mM) of ONOO⁻ (Fig. 3, middle panel). Almost identical results were observed by flow cytometric analysis (Fig. 4, A and B). In contrast to CD3-mediated activation, when purified lymphocytes were stimulated by PMA plus calcium ionophore, DNA fragmentation was undetectable in the range of ONOO⁻ doses used here. The absence of apoptotic death in the case of PMA and calcium ionophores for all doses of peroxynitrite was verified by both DNA fragmentation (Fig. 3) and flow cytometry (data not shown). These results indicated that low doses of ONOO⁻ pretreatment primed purified lymphocytes to undergo apoptotic cell death upon CD3-mediated activation. The absence of DNA fragmentation after calcium ionophore plus phorbol esters in purified lymphocytes pretreated with ONOO⁻ suggested that CD3-mediated induction of apoptosis after treatment with ONOO⁻ could reflect an impairment in early protein tyrosine phosphorylation. As depicted in Fig. 4B the ratio of apoptotic cells decreased 48 h after ONOO⁻ treatment, suggesting that the apoptotic process was stopped, and a relative increase in the ratio of nonapoptotic cells was occurring at this time. Because ONOO⁻ is also a strong oxidant, a set of experiments was performed to exclude the effects of ONOO⁻-mediated changes in the redox capacity of these cells. For this, cells were treated with peroxynitrite at the indicated doses and incubated for 2 h with either N-acetylcysteine.
(10 mM) or 2-ME (50 μM) before CD3-mediated activation to restore the redox capacity of ONOO-treated cells. This treatment (data not shown) was not able to either inhibit apoptotic death or restore impaired proliferative responses.

**Peroxynitrite inhibits lymphocyte early protein tyrosine phosphorylation induced by CD3-mediated activation**

To examine the effects of ONOO⁻ on the activation-induced tyrosine phosphorylation, normal purified lymphocytes were pretreated with increasing doses of ONOO⁻ and activated after 15 min with immobilized anti-CD3 mAbs. As shown in Fig. 5, ONOO⁻ induced a progressive inhibition of early protein tyrosine phosphorylation in a dose-dependent manner. Of note, compared with peroxynitrite-untreated lymphocytes there was a relative increase in tyrosine phosphorylation over the basal values and the level after the first 5 min of activation at the lowest doses of ONOO⁻ pretreatment. Overall, these results showed that ONOO⁻ induced dose-dependent impairment of early tyrosine phosphorylation in normal lymphocytes, with almost complete inhibition at doses higher than 0.1 mM.

**Protein tyrosine nitration occurs during in vitro lymphocyte activation and depends on the presence of monocytes in cultures**

The presence of protein tyrosine nitration during immune activation in vitro was assessed by Western blot assays using specific polyclonal anti-nitrotyrosine Abs raised in rabbits. The specificity of Ab binding was assessed with either nitrated or native BSA by Western blot assays as illustrated in Fig. 6A. These results showed that anti-nitrotyrosine polyclonal Abs specifically recognized nitrated BSA but not their native form and did not cross-react with either L-tyrosine or o-phospho-L-tyrosine. In contrast, 3-nitro-L-tyrosine successfully blocked Ab immunoreactivity. Fig. 6B depicts results from competition analysis performed by ELISA using peroxynitrite-modified KLH (KLH-NO₂) and BSA (BSA-NO₂).

**FIGURE 3.** Electrophoretic analysis of DNA fragmentation in resting and activated lymphocytes after exposure to peroxynitrite. Isolated lymphocytes were either not exposed (0 mM) or were exposed to authentic peroxynitrite at the indicated concentrations. After washing, cells were cultured in complete culture medium alone (Non activated), in the presence of immobilized anti-CD3 mAbs (Immobilized anti-CD3), and in the presence of PMA plus ionophore (PMA + ionophore). Lymphocytes were harvested after 6 h and assessed for the presence of fragmented DNA by agarose gel electrophoresis.

**FIGURE 4.** Flow cytometric analysis of peroxynitrite-induced apoptotic changes in resting and activated normal lymphocytes. Isolated normal lymphocytes were resuspended in isotonic 100 mM PBS at 5 x 10⁶ cells/ml and treated with increasing concentrations of peroxynitrite (ONOO⁻ pretreatment, millimolar concentrations). Untreated cells were given reverse order addition of ONOO⁻ as described above. After 15 min at room temperature cells were washed, resuspended in complete culture medium, and cultured in 24-well plates (left panel; non-activated lymphocytes) or in plates pre-coated with immobilized anti-CD3 mAbs (right panel; CD3-activated lymphocytes). In both groups cells were harvested at different times after peroxynitrite exposure (2, 6, 12, 24, and 48 h) and analyzed by flow cytometry using merocyanin as a fluorescent probe to identify typical membrane changes associated with apoptotic death. A, Representative experiment after 6 h of cultures depicted as monoparametric histograms. B, Graphic representation of a complete kinetic analysis from a typical experiment depicting data as the ratio of apoptotic lymphocytes over total viable cell number.
Nitrotyrosine can completely block Ab binding to nitrated BSA and KLH, with a 50% inhibitory concentration (IC₅₀) of about 30 micromolar. Up to 20-mM concentrations of aminotyrosine, tyrosine, or phosphotyrosine had no effect on Ab binding to nitrated KLH or nitrated BSA (data not shown). In ELISA assays antinitrotyrosine Abs did not recognize either native KLH or native BSA (data not shown). To assess the putative role of PBMCs in producing nitrating species in vitro, additional experiments were conducted on normal purified lymphocytes cultured alone (Fig. 7B; purified T lymphocytes) or in the presence of autologous monocytes (Fig. 7A; purified T lymphocytes from whole PBMC cultures). Both cell preparations were cultured in either the presence or the absence of immobilized anti-CD3 mAbs (nonactivated and CD3-mediated activation, respectively, in Fig. 7). In all cases, lymphocytes were isolated from cultures at different times and were lysed to study the time course of the appearance of protein tyrosine nitration (30 min after activation up to 96 h in culture). Nitrotyrosine-immunoreactive bands greater than basal levels appeared after a minimum of 6–12 h following activation or after 24 h for nonactivated lymphocytes. As depicted in Fig. 7A a clear pattern of protein tyrosine nitration was observed in purified lymphocytes 12 h after activation (Fig. 7A, right panel) when isolated from whole PBMC cultures and activated by immobilized anti-CD3 mAb. Analysis of lysates from nonactivated lymphocytes isolated from whole PBMC cultures (Fig. 7A, middle panel) revealed nitrotyrosine formation but to a lesser extent than activated lymphocytes cocultured with autologous monocytes. In contrast, cell lysates from cultures of purified lymphocytes (Fig. 7B) revealed a slight pattern of nitrotyrosine-immunoreactive bands only in the case of CD3-mediated activation and no significant increase in their intensity after the observation period (Fig. 7B, right panel). No significant increase in nitrotyrosine-immunoreactive bands was observed for purified nonactivated T lymphocytes compared with their respective basal levels (Fig. 7B, left panel). These results clearly demonstrated that nitrating species were produced in vitro after immune activation as revealed by nitrotyrosine-immunoreactive bands observed in cell lysates upon CD3-mediated activation of PBMC. In addition, these experiments suggested that endogenously produced nitrating species in PBMC cultures depended on the presence of monocytes during lymphocyte activation. Further evidence of this phenomenon is depicted in Fig. 8. Cell lysates from whole PBMC cultures showed a clear pattern of protein nitration when activated by immobilized anti-CD3 mAb, PMA plus calcium ionophore, or PHA plus sCD3 mAb (Fig. 8A). Fig. 8B showed that when purified monocytes and lymphocytes were cultured alone in the absence (nil, lines 1 for each) or the presence of immobilized anti-CD3 mAb (iCD3, lines 3 for each), there was not a significant rise in nitrotyrosine over the respective basal levels. After whole PBMC cultures for 24 h in the absence or the presence of immobilized anti-CD3 mAb, monocytes and lymphocytes were isolated and analyzed separately for nitrotyrosine immunodetection. These experiments revealed that protein nitration after CD3-mediated activation was observed in monocytes or lymphocytes only when they were cocultured (Fig. 8B, line 4 for each). When cocultured monocytes and lymphocytes were analyzed separately.
in the absence of CD3-mediated activation there was not a significant increase in nitrotyrosine levels over their respective basal levels (Fig. 8B, line 2 for each). These results suggested that activated lymphocytes through soluble mediators as cytokines or cell to cell interactions were able to induce monocytes to produce nitrating species that, in turn, nitrated their own cellular proteins and also cellular proteins of the neighboring lymphocytes. Of note, nitrated lymphocytes by exogenously added ONOO\textsuperscript{−} also cellular proteins of the neighboring lymphocytes. Fig. 9 shows the pattern of nitrotyrosine-immunoreactive bands induced on the cellular proteins induced by authentic peroxynitrite were more homogeneously distributed than those observed by CD3-induced tyrosine nitration, which were concentrated at lower relative m.w. These differences might be attributed to several factors, including bolus addition of authentic ONOO\textsuperscript{−} vs continuous production at lower levels of endogenous ONOO\textsuperscript{−} and protein degradation and turnover in the case of prolonged in vitro cultures.

**Nitration of cellular proteins during immune activation in vitro was inhibited by SOD mimetics and nitric oxide synthase inhibitors**

Fig. 10 depicts the results of inhibition experiments conducted by using the cell-permeable superoxide dismutase mimetic (42) Mn-TBAP and aminoguanidine, an inhibitor of the inducible isoform of nitric oxide synthase (43, 44). Aminoguanidine at doses ranging from 1–20 mM was able to almost completely inhibit nitrotyrosine formation in whole PBMC cultures after CD3-mediated activation. Mn-TBAP was also effective in inhibiting nitrotyrosine formation in a dose-dependent manner. The combination of these two inhibitors of ONOO\textsuperscript{−} generation completely abrogated nitrotyrosine immunoreactivity of cellular proteins. These results strongly indicated that ONOO\textsuperscript{−} was at least the main nitrating agent in our experimental conditions. If we assumed that ONOO\textsuperscript{−} impairs lymphocyte activation and prime them to undergo activation-dependent cell death, it can be speculated that either aminoguanidine or Mn-TBAP, through inhibition of ONOO\textsuperscript{−} production, should enhance lymphocyte proliferation. Thus, we have studied the effects of these pharmacological treatments on lymphocyte proliferation after CD3-mediated activation. As expected, both aminoguanidine and Mn-TBAP significantly enhanced CD3-induced thymidine uptake of normal PBMCs (Fig. 11). Of note, at the higher doses of both inhibitors used here (\(>15\) mM and \(150\) \(\mu\)M for aminoguanidine and Mn-TBAP, respectively), there was a significant decrease in proliferative response compared with that of control cells.

**Demonstration of in vivo formation of nitrotyrosine residues in tissue sections from human lymph nodes undergoing nonspecific immune activation**

To evaluate the in vivo production of nitrating species during immune responses, we studied human lymph nodes obtained from radical surgical resections for lung and colonic cancers from six different patients. Lymph nodes included in this study were free of metastatic invasion and showed mild to strong reactive follicular hyperplasia and sinusoidal histiocytosis. In all tissue specimens a diffuse ganglionar immunostaining for protein nitrotyrosine was observed (Fig. 12, A–D), including interstitium, lymphocytes, macrophages, and sinuses. A weak reactivity was seen at the interstitial level between lymphocytes, drawing a ring around some of them. However, the strongest nitrotyrosine immunoreactivity
was observed principally in macrophage-rich zones, such as sinuses, perifollicular areas, and germinal centers (Fig. 12, B–D). Macrophages frequently depicted a strong intracytoplasmic immunoreactivity with a clear granular pattern (Fig. 13). In all cases controls were performed to substantiate the specificity of immunostaining, including coincubation of the first Ab with 5 mM nitrotyrosine, which completely blocked the reactivity, and using duplicated specimens incubated with irrelevant isotype-matched rabbit Abs as negative controls (data not shown).

Discussion
Peroxynitrite is a potent oxidant as well as a nitrating agent produced by the reaction of nitric oxide with superoxide and having a biological half-life of \( \leq 0.1 \text{ s} \) at pH 7.4 and 37°C (17). It readily nitrates phenolic compounds such as tyrosine residues in proteins, thereby leaving a footprint that has been detected in a number of human tissues (23, 24). As assessed by nitrotyrosine detection, peroxynitrite has been suggested to be involved in the pathogenesis of a wide range of diseases, including human atherosclerosis, human acute lung injury, chronic inflammation, endotoxic shock, and neurodegenerative diseases (28–31, 45).

In the present work we have demonstrated that authentic peroxynitrite primes normal T lymphocytes to undergo peroxynitrite-driven apoptotic death in a dose- and time-dependent manner. Recently, it has been demonstrated that ONOO\(^{−}\) induces apoptotic death in several transformed cell lines, including the promyelocytic leukemic HL-60 cell line, the human monocytic tumor cell line U-937 (46), the neuronal PC-12 cell line (47), and the murine macrophage RAW 264.7 cell line (48). With respect to nontransformed cells, Lin and co-workers (46) failed to demonstrate peroxynitrite-induced apoptotic changes in normal PBMC from healthy volunteers exposed to peroxynitrite concentrations up to 0.1 mM and analyzed 5–6 h after this treatment. In agreement with this report we failed to demonstrate significant apoptotic changes in resting peripheral lymphocytes treated with ONOO\(^{−}\) concentrations \( \leq 0.1 \text{ mM} \) and analyzed 5–6 h after treatment. In agreement with this report we failed to demonstrate significant apoptotic changes in resting peripheral lymphocytes treated with ONOO\(^{−}\) concentrations \( \leq 0.1 \text{ mM} \) and analyzed 5–6 h after treatment. In agreement with this report we failed to demonstrate significant apoptotic changes in resting peripheral lymphocytes treated with ONOO\(^{−}\) concentrations \( \leq 0.1 \text{ mM} \) and analyzed 5–6 h after treatment. In agreement with this report we failed to demonstrate significant apoptotic changes in resting peripheral lymphocytes treated with ONOO\(^{−}\) concentrations \( \leq 0.1 \text{ mM} \) and analyzed 5–6 h after treatment.

FIGURE 8. Activation-induced protein nitration in cellular lysates of monocytes and lymphocytes isolated from whole PBMC cultures. In A, PBMCs from healthy donors were either nonactivated (Basal) or activated with immobilized anti-CD3 mAbs (iCD3), PHA plus soluble anti-CD3 mAbs (PHA+sCD3), and PMA plus calcium ionophore (PMA+ion). After incubation for 24 h at 37°C, cells were harvested, and the profile of nitrotyrosine-immunoreactive bands was analyzed in cell lysates by Western blot. In B, monocytes and lymphocytes were isolated from whole PBMC and analyzed separately for nitrotyrosine contents in different experimental conditions. On the one hand, lymphocytes and monocytes were purified from PBMCs before cultures and incubated for 24 h at 37°C either in culture media alone (line 1 in Monocytes and line 1 in Lymphocytes) or in the presence of immobilized anti-CD3 mAbs (line 3 in Monocytes and line 3 in Lymphocytes). On the other hand, both monocytes and lymphocytes were purified from PBMCs after culture for 24 h at 37°C either in absence (line 2 in Monocytes and line 2 in Lymphocytes) or the presence of immobilized anti-CD3 mAbs (line 4 in Monocytes and line 4 in Lymphocytes).

FIGURE 9. Nitrilation of cellular proteins induced by authentic peroxynitrite. PBMCs (5 \( \times \) 10\(^6\)) from healthy donors were exposed to increasing concentrations of ONOO\(^{−}\) (millimolar concentrations) for 15 min at room temperature, and levels of nitrotyrosine formation were assessed in cellular lysates by Western blot using anti-nitrotyrosine Abs. Untreated BSA and peroxynitrite-treated BSA (NO\(_2\)-BSA) were used as controls.

NITRATION OF CELLULAR PROTEINS INDUCED BY PEROXYNITRITE
plus calcium ionophores. Phorbol esters in association with calcium ionophores activate purified lymphocytes and effectively bypass the early protein tyrosine phosphorylation signals associated to CD3-mediated activation.

These results suggested that peroxynitrite-induced apoptotic changes in CD3-activated lymphocytes could be related to an impairment in the tyrosine phosphorylation cascade proximal to the TCR/CD3 complex. Thus, resting normal lymphocytes appear more resistant than transformed cell lines and activated lymphocytes to peroxynitrite-mediated apoptotic death because either high doses of ONOO\(^{-}\) or extended culture times are needed to produce apoptotic death. These assumptions suggest that the activated phenotype of transformed cell lines or the activated state of normal lymphocytes could play a permissive role in the susceptibility to peroxynitrite-induced apoptotic cell death. Although the precise mechanism involved in peroxynitrite-induced apoptosis is still unclear, recent data demonstrated that peroxynitrite is a potent initiator of DNA strand breakage (49, 50), which is an obligatory stimulus for the activation of the nuclear enzyme poly(ADP ribosyl synthetase (PARS). Thus, the cellular response to peroxynitrite exposure can be influenced by the activation of PARS as a DNA nick sensor enzyme. Although the role of PARS activation in apoptosis is still controversial, it has been claimed that it can lead to the depletion of cellular energy pools and to rapid cell death by necrosis, which interferes with the execution of the apoptotic program (50–54). In addition, it has been recently reported that Bcl-2 appears to block peroxynitrite-induced apoptosis in Bcl-2-overexpressing HL-60 cells (55). Further work is needed to substantiate the putative role of either the PARS or the Bcl-2 pathways in the development of peroxynitrite-induced apoptosis.

Our results suggest that nitration of tyrosine residues could play a major role in the impairment of tyrosine phosphorylation and the enhancement of programmed cell death induced by peroxynitrite (33, 56, 57). In agreement with previous reports (32, 58), our results show that peroxynitrite at low concentrations induces a transient rise in tyrosine phosphorylation compared with that in untreated lymphocytes both basally and 5 min after CD3-mediated activation. In addition to the nitrating abilities of ONOO\(^{-}\), a large body of published data showed that peroxynitrite can induce oxidative modifications of several biomolecules, including lipids and proteins and protein fragmentation (19, 22, 59). Thus, reaction of peroxynitrite with glutathione depletes glutathione pools (18), which may sensitize cells to oxidant stress. Studies of the redox state of T lymphocytes have suggested that T cell responses and survival are affected by oxidative stress through inhibition of tyrosine phosphorylation events, calcium mobilization, and proliferative responses (60–63). However, it has been demonstrated that oxidants (when oxidant insult is not beyond the capacity of the cellular redox buffer) can elicit positive or protective responses in cells through a tyrosine phosphorylation-dependent activation of several transcription factors, including NF-κB and activating protein-1 (64, 65). In human B and T lymphocytes these responses were achieved through combined inhibition of thiol-dependent phosphotyrosine phosphatases and activation of tyrosine kinases, and the use of tyrosine kinase inhibitors provided evidence that activation of these signaling pathways are, in fact, protective responses against oxidant-induced apoptotic death (38, 61, 66, 67). The transient increase in tyrosine phosphorylation described here could be a response to a moderate oxidative insult mediated by peroxynitrite; thus, apoptotic cell death appears as a common consequence when these protective responses against oxidative stress are blocked by tyrosine nitration (68, 69). When peroxynitrite-treated lymphocytes were further activated by PMA plus calcium ionophore, we could not observe any apoptotic changes regardless of the method employed, the dose of peroxynitrite, and/or the incubation time. It can be suggested that the absence of apoptotic death could be explained because PMA and calcium ionophores effectively bypass the early events of tyrosine phosphorylation.
and, in turn, trigger protective responses against oxidative stress, which could enable to inhibit apoptotic death. In contrast, apoptotic changes observed in resting lymphocytes exposed to high doses of ONOO$^-$ could be explained by the inability to trigger these tyrosine phosphorylation-dependent protective responses as a consequence of tyrosine nitration. Decreased proliferative responses to PMA and calcium ionophores in lymphocytes pretreated with high doses of peroxynitrite were not associated with either apoptotic changes or cellular loss due to a necrotic process, which could suggest a peroxynitrite-induced anergic state.

Given the reactive nature of ONOO$^-$ in biological systems (70), one could speculate that peroxynitrite-mediated oxidative modifications could explain the impairment in lymphocyte activation and survival described here. Despite the strong oxidizing potential of ONOO$^-$, kinetic factors determine that a limited number of reactions are relevant in vivo. In particular, the reactions with thiols, metal centers, and CO$_2$ account for most of peroxynitrite consumption. Since nitration is strongly favored by metals and CO$_2$, nitration constitutes a key cellular event after peroxynitrite formation in vivo. It is unlikely that depletion of endogenous antioxidants by ONOO$^-$ could have a relevant place in explaining our results because we cannot prevent apoptotic cell death after restoration of cellular redox capacity with either N-acetylcysteine or 2-ME. In addition, the absence of apoptotic cell death after PMA-mediated activation supports the idea that oxidizing ability of ONOO$^-$ is not the main factor explaining our results. Nevertheless, further work is necessary to definitively confirm this idea.

Although other factors could play a role in peroxynitrite-mediated impairment of lymphocyte signaling proliferation and survival, our results suggest that nitration-mediated impairment of protein tyrosine phosphorylation plays a major role in this process.
through a defective coupling of the TCR-CD3 complex to proximal intracellular events such as tyrosine phosphorylation (67, 70-73). In addition, we provide strong experimental evidence for the endogenous production of nitrating species by cells of the monocyte-macrophage lineage in cultures of PBMC activated either by the CD3 pathway or in the presence of PMA plus ionophores. Generation of reactive nitrogen intermediates by human monocytes/macrophages requires the production of nitric oxide as one essential precursor for the generation of nitrating species either in vivo or in vitro. Indeed, evidence does exist for the identification of the endothelial constitutive and inducible isoforms of nitric oxide synthase mRNA, nitric oxide synthase protein, and nitric oxide production in human monocytes and macrophages (71–73). On the other hand, recent work demonstrated that nitrotyrosine is not the unique biomarker of peroxynitrite production and other NO-derived nitrating species could be formed in vivo (45). In this respect it has been recently reported that nitrite (NO$_2^-$), a major end product derived from nitric oxide metabolism, readily promotes tyrosine nitration through formation of nitryl chloride (NO$_2$Cl) and nitrogen dioxide (NO$_2$) through reaction with the inflammatory mediator hypochlorous acid (HOCl) or myeloperoxidase (74–76).

Thus, it has been proposed that the nitrite oxidation mechanisms are probably relevant during inflammatory processes, where neutrophils are recruited and activated, providing an additional pathway for the generation of reactive nitrating intermediates. Although with the present evidence, it cannot be definitively stated that the formation of nitrotyrosine in vitro is due to peroxynitrite, the absence of neutrophils in our culture conditions and the results of experiments performed with Mn-TBAP associated or not associated with aminoguanidine favor the view that under our experimental conditions formation of nitrotyrosine was dependent on the presence of superoxide and NOS activity. These arguments support the idea that peroxynitrite is the main nitrating species. Our results also showed that monocyte-derived nitrating species were able to induce long-lasting protein tyrosine nitration of their own cellular proteins and neighboring lymphocytes present in whole cultures. The persistence of nitrated proteins over time suggests that lymphocytes lack the enzymatic ability to remove nitro groups from tyrosine residues as has been demonstrated for tyrosine kinases/phosphatases.

It is presently unclear whether activated lymphocytes are able to induce the production of nitrating species by monocytes either through membrane-associated interactions or through soluble mediators as cytokines, including TNF-α (77). The pattern of nitrotyrosine proteins after in vitro activation showed that the proteins more heavily nitrated were principally concentrated <50 kDa. However, the pattern of tyrosine nitration induced by authentic peroxynitrite (Fig. 9) evolves over time to acquire a profile indistinguishable from that induced by in vitro activation (data not shown). In this respect it has been demonstrated that peroxynitrite-induced protein modifications are associated with increased degradation, which could explain our results (33). Since our results suggested that the pattern of nitrated proteins induced during immune activation might be the consequence of endogenously produced ONOO$^-$, we have showed in vivo that lymph nodes from surgical specimens with histological evidence of hyperplasia and nonspecific immune activation displayed a strong pattern of reactivity with anti-nitrotyrosine polyconal Abs. Although we cannot elucidate which species nitrated tyrosines, the results show the generation of nitrating species in vivo.

Overall, our results suggest a role for nitrating species such as peroxynitrite as major modulators of immune responses. This assumption is reinforced by the enhancement of lymphocyte proliferative responses in conditions of low to moderate inhibition of either NO or O$_2^-$ (Fig. 11), which provides experimental evidence for the physiological relevance of peroxynitrite-mediated tyrosine nitration. Our results also lead to the idea that this effect is at least in part mediated through nitration of nitrosyde residues, which result in inhibition of tyrosine phosphorylation. In this respect, one can speculate that upon activation only heavily tyrosine-phosphorylated lymphocytes will proceed to acquire adequate effector and regulator functions. Besides the potential of peroxynitrite as a down-modulator of normal immune responses, nitration of cellular proteins may be injurious via multiple mechanisms, including altered protein function, increased turnover due to enhanced proteolysis, and formation of neoantigens with eventual autoimmunity reactions. Thus, peroxynitrite generation in vivo may represent a major mediator in inflammation-mediated protein modification and tissue injury at sites of chronic or intense immune activation.

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