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Autocrine Nitric Oxide Modulates CD95-induced Apoptosis in γδ T Lymphocytes*

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γδ T lymphocytes play an important early role in the defense against pathogens. Their function is terminated by acquisition of susceptibility to CD95-triggered apoptosis. Here we show that the regulation of this process depends on the activity of the endothelial NO synthase expressed by γδ T lymphocytes, which is modulated in an activation-dependent way. The effects of nitric oxide thus generated, mediated via cGMP generation, are exerted at at least two sites along the CD95 signaling cascade: one at, or upstream, and the other downstream of ceramide generation. At either site, nitric oxide/cGMP action is sufficient for protection from apoptosis. The effect of NO is selective for apoptosis induced by CD95 cross-linking, since it does not affect apoptotic program triggered by other *stimuli*. The evidence here reported demonstrates a new physiological role for nitric oxide, acting as a survival factor for T lymphocytes.

Various cell populations act coordinately in immune responses, under the control of intra- and extracellular messengers. One of these, nitric oxide (NO), is believed to play important roles as yet not completely clarified (1, 2). Among the cells involved in the first line of defense against pathogens, relevant is the contribution of activated T lymphocytes expressing the Vγ9/Vδ2 T cell receptor (TcR)[1] (3), whose function may be terminated by apoptotic cell death triggered via activation of CD95 (Fas, APO-1), a member of the tumor necrosis factor/nerve growth factor receptor superfamily (4–6). In T lymphocytes the CD95 signaling cascade can be triggered either via activation of the TcR, with ensuing expression and secretion of the CD95 physiological ligand, or via direct cross-linking of the CD95 receptor with specific monoclonal antibodies (mAbs) (7–10). Despite the fact that γδ T cells express constant levels of CD95 *in vitro*, their susceptibility to CD95-triggered apoptosis is known to change as a function of their state of activation (5, 6). The molecular mechanisms underlying T cell switch in sensitivity to CD95-triggered apoptosis are still unknown. We have investigated this problem taking advantage of normal human lymphocyte clones bearing the Vγ9/Vδ2 TcR, already characterized for their sensitivity to CD95-induced apoptosis in our laboratory (4, 6). The results obtained demonstrate that these cells express the constitutive, endothelial NO synthase (ecNOS) and that NO generated by this enzyme regulates their susceptibility to apoptosis modulating the CD95-triggered signal transduction cascade.

EXPERIMENTAL PROCEDURES

Cell Culture and Characterization—Human γδ T cell clones were established by limiting dilution, propagated in RPMI plus 10% fetal calf serum (Life Technologies, Inc., Basel, Switzerland) by cyclic restimulation with interleukin-2 (50 units/ml; EuroCetus, Amsterdam, The Netherlands), and characterized as described (4, 6).

Apoptosis Induction and Analysis—γδ T cells were incubated for 5 h with anti-CD95 mAb (clone CH-11 IgM isotype, 100 ng/10^6 cells, Kamiya Biomedical Company, Seattle, WA), anti-TcR mAb (TiγA, 1 μg/10^6 cells, a gift of T. Hercend) cross-linked with goat anti-mouse IgG Abs (Zymed, San Francisco, CA), thapsigargin (250 nM; Calbiochem, San Diego, CA), etoposide (20 μM, Bristol, United Kingdom), or C_2-ceramide (10 μM) (Biomol, Bremen, Germany) at 37 °C. In the experiments in which the caspase inhibitor acetyl-Tyr-Val-Ala-Asp-chloromethyl ketone (ac-YVAD-CMK; 200 μM; Calbiochem) was employed, γδ T cells were preincubated for 2 h at 37 °C. In the experiments with *S*-nitrosoacetylpenicillamine (SNAP; 100 μM), isosorbide dinitrate (200 μM), hemoglobin (50 μM), L-*N*-(iminoethyl)-ornithine (L-NIO; 300 μM), L-N^{ω}-arginine methyl ester (L-NAME; 300 μM), 8-Br-cGMP (500 μM), 8-Br-cAMP (500 μM) or H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ; 1 μM), cells were preincubated for 15 min at 37 °C. Apoptotic cells were identified by flow cytometry with two different protocols (4, 6): in the first, used for the experiments illustrated in Fig. 1*A*, unfixed cells were incubated for 30 min in phosphate-buffered saline (PBS) containing 50 μg/ml propidium iodide (PI), 100 μg/ml RNase A, 0.01% Nonidet P-40, for 60 min at 37 °C. The staining of apoptotically cleaved DNA gives rise to a typical sub-G1 peak in single parameter DNA histograms. Conversely, to obtain a quantitative esteem of the fraction of cells undergoing apoptosis, phosphatidyl serine exposure was monitored by staining with FITC annexin V for 15 min at room temperature, in the presence of divalent cations. Furthermore, the loss of the ability to exclude vital dyes was assessed using PI in an isotonic buffer (5 μg/ml in PBS). Early apoptotic cells exclude the dye but are stained with annexin V, whereas non-apoptotic cells are negative for both stainings. Cells were analyzed using a fluorescence-activated cell sorter (FACStar Plus, Becton Dickinson, Sunnyvale, CA). Apoptosis protection by different treatments was evaluated as the percent inhibition calculated as follows: 1 − (% of annexin V^+ cells after CD95 cross-linking in the presence of the protective drug/% of annexin V^+ cells after CD95 cross-linking) × 100.

Analysis of NOS Expression—The expression of the various NOS enzymes was assessed by flow cytometry and Western blot, using spe-

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[1] The abbreviations used are: TcR, T cell receptor; mAb, monoclonal antibody; ecNOS, endothelial nitric oxide synthase; SNAP, *S*-nitrosoacetylpenicillamine; acYVAD-CMK, ac-Tyr-Val-Ala-Asp-chloromethyl ketone; ODQ, H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; L-NAME, N^{ω}-nitro-L-arginine methyl ester; L-NIO, L-*N*-(iminoethyl)-ornithine; PBS, phosphate-buffered saline; PI, propidium iodide; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; NOS, nitric oxide synthase; 8-Br, 8-bromo; Ab, antibody; FITC, fluorescein isothiocyanate.

cific Abs (Transduction Laboratories, Lexington, KY) according to the procedures described (11, 12). For cytometric analysis, cells were fixed for 5 min at room temperature with paraformaldehyde (4% in PBS). After extensive washing, cells were permeabilized with 0.05% Nonidet P-40 in PBS and stained using 1 μg/10^6 cells of the relevant primary Ab (20 min at 4 °C) and a 5-fold excess of FITC-conjugated secondary Ab (goat anti-mouse for ecNOS and inducible NOS, and goat anti-rabbit for brain NOS). The fluorescence background was calculated in the absence of the primary Abs. For Western blot analysis, cell pellets were washed with ice-cold PBS and then solubilized for 15 min at 4 °C in a lysis buffer containing 1% Triton X-100 (v/v), 10% glycerol, 100 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Na_3VO_4, 4 mM phenylmethylsulfonyl fluoride, 20 mM sodium pyrophosphate, 5 mM EGTA, 20 μM leupeptin, 0.007 unit/ml aprotinin, 50 mM HEPES, pH 7.5. The lysates were clarified by centrifugation (14,000 × *g*) for 15 min at 4 °C, and protein content assayed by the bicinchoninic acid procedure (BCA; Pierce). After addition of sodium dodecyl sulfate and β-mercaptoethano,l the samples were boiled and 250 μg of protein/lane were loaded into the slots of 8% sodium dodecyl sulfate-polyacrylamide gels, which were run as described elsewhere (12). As internal controls for ecNOS, brain NOS, and inducible NOS, 35 μg of human endothelial cells, rat pituitary, and mouse macrophage lysates (Transduction Laboratories), respectively, were loaded into the same gels with the γδ T cell homogenate. High efficiency transfer of proteins onto nitrocellulose membranes was carried out at 200 mA for 18 h in a buffer containing 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3. After transfer both the gels and the blots were routinely stained with Ponceau red. The nitrocellulose sheets were processed at room temperature, first for 1 h with PBS + 3% bovine serum albumin, then for 2 h with appropriate concentrations of the specific anti-NOS Abs in an incubation buffer containing 150 mM NaCl, 50 mM Tris-HCl, 0.05% Tween 20, 5% powdered milk, pH 7.4. After washing five times for 5 min with the incubation buffer, the blotted bands were incubated with the relevant horseradish peroxidase-linked IgG for 1 h in the same buffer. After extensive washing immunostaining was recorded photographically, using an enhanced chemiluminescence kit from Amersham (Buckinghamshire, United Kingdom).

Measurements of cGMP Levels—1 × 10^6 cell samples were resuspended in phosphate-buffered saline supplemented with 500 μM 3-isobutyl-1-methylxanthine, with or without either L-NIO (300 μM) or L-NAME (300 μM). NOS activity was stimulated by exposing cell suspensions to anti-TcR mAb for 20 min at 37 °C. The reaction was terminated by addition of ice-cold trichloroacetic acid (final concentration: 7.5%). After ether extraction, cGMP levels were measured using a radioimmunoassay kit (DuPont) and normalized on cellular proteins as described (13).

Ceramide Measurements—2 × 10^6 cell samples were incubated in 80 μl of phosphate-buffered saline with anti-CD95 mAb (100 ng/10^6 cells) for 15 min at 4 °C, in the presence or absence of either SNAP (100 μM) or 8-Br-cGMP (500 μM), then quickly shifted at 37 °C. Incubation was stopped at various times by addition of 300 μl of ice-cold $CH_3OH/CHCl_3$ (2/1, v/v). Samples were then supplemented with 100 μl of $CHCl_3$ and 100 μl of NaCl (1 M). Phopholipids were extracted, dried under nitrogen, and resuspended in a mixture containing 5 mM cardiolipin, 1 mM diethylenetriaminepentaacetic acid, 7.5% octyl-β-glucopyranoside (Sigma). Diacylglycerol kinase assay was performed as described (14) and ceramide phosphate isolated by thin layer chromatography (Silica gel 60, Merck, Milan, Italy) using $CHCl_3/CH_3OH/CH_3COOH$ (65/15/5, v/v/v) as solvent. Authentic ceramide 1-phosphate was identified by autoradiography at R_F 0.25.

$[Ca^{2+}]_i$ Measurements—Cell suspensions were loaded in Krebs-Ringer-Hepes medium containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 6 mM glucose, 25 mM Hepes-NaOH (pH 7.4) with the Ca^{2+}-sensitive dye, fura-2 (Calbiochem), for 30 min at 25 °C, administered as acetoxymethyl ester at the final concentration of 3 μM. In the experiments in which the effects of anti-CD95 mAb were analyzed, cell aliquots (2 × 10^6 cells) were incubated with 200 ng of the mAb for 15 min at 4 °C. Cell samples were subsequently centrifuged and resuspended in a Krebs-Ringer-Hepes medium supplemented with 250 μM sulfinpyrazone (to prevent dye leakage) from which $CaCl_2$ had been omitted and 10 μM EGTA added (Ca^{2+}-free medium; estimated extracellular $[Ca^{2+}] < 10^{-8}$ M). In these conditions, Ca^{2+} release only can be detected. Cells were then transferred to a thermostatted cuvette (37 °C), maintained under continuous stirring, and analyzed in a Perkin Elmer LS-5B fluorimeter as described (13). Treatments with NO donors or 8-Br-cGMP were performed as described above. C_2-ceramide (10 μM) was added to the samples when indicated.

Statistical Analysis—The results are expressed as means ± S.E. *n* represents the number of different experiments. Statistical analysis was performed by Student's *t* test for unpaired data (two-tailed). A value of *p* less than 0.05 was considered to be statistically significant.

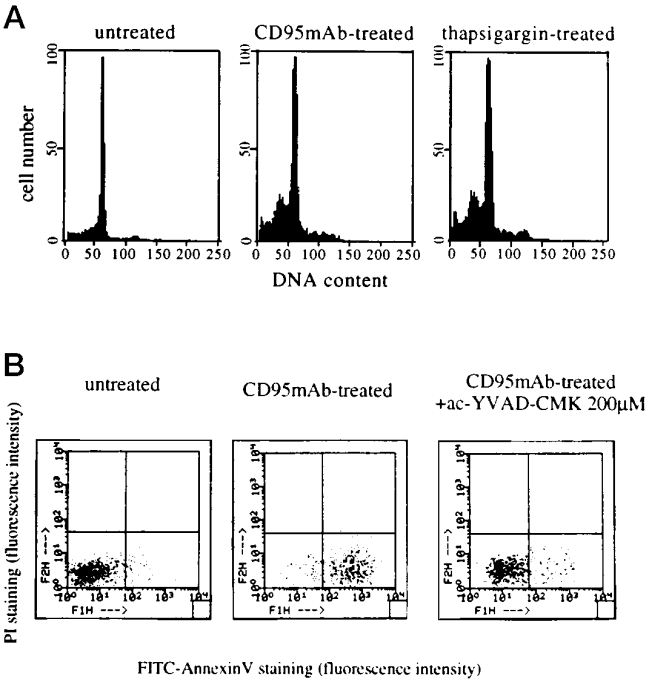

FIG. 1. **CD95-induced apoptosis of γδ T lymphocytes.** *A*, γδ T lymphocytes undergo apoptosis after CD95 cross-linking or blockade of the endoplasmic-sarcoplasmic reticulum Ca^{2+}-ATPases with thapsigargin, as demonstrated by the flow cytometric analysis of DNA content performed on untreated cells, and after 5 h of either CD95 cross-linking or incubation with thapsigargin (250 nM). *x* axis, DNA content, assessed by the quantitative binding of the intercalating red fluorescent drug PI (arbitrary units). *y* axis, relative cell number. The result of one out of five representative experiments is depicted. *B*, caspase inhibition results in the blockade of the CD95-triggered apoptotic pathway. An early feature of apoptosis, *i.e.* the exposure of the anionic phospholipid phosphatidyl serine, was assessed using FITC-labeled annexin V (*F1H*; *x* axis). On the *y* axis, the late apoptotic cells that incorporated the vital dye PI in an isotonic buffer are reported (*F2H*). *Vertical* and *horizontal lines* in the graphs are designed based on the autofluorescence of untreated, control cells. Cross-linking of the CD95 receptor resulted in phosphatidylserine exposure by most γδ T cells (*lower right panel* of the *middle plot*: 69.5%). Preincubation with the caspase inhibitor ac-YVAD CMK substantially protected γδ cells from CD95-triggered apoptosis (*lower right panel* of the *left plot*).

RESULTS AND DISCUSSION

Normal human lymphocyte clones bearing the Vγ9/Vδ2 TcR were propagated by cyclic *in vitro* restimulation (4). These cells are susceptible to apoptosis triggered by various *stimuli* (4, 6). We evaluated the apoptotic effects of cross-linking of the CD95 membrane receptor, of the treatment with the topoisomerase inhibitor etoposide, and of increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which was obtained with thapsigargin, a drug that blocks the sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPases (15). Cells were treated for 5 h with either drug, and then analyzed by flow cytometry after DNA staining with propidium iodide (6). All of the treatments were capable to induce apoptosis, as demonstrated by the appearance of the hypodiploid DNA peak at the *left* of the G1 peak (Fig. 1, *A*; data not shown). Etoposide and thapsigargin were able to induce apoptosis of γδ T cell clones throughout the entire culture period (data not shown); by contrast, sensitivity to CD95-induced cell death is acquired progressively (4, 6). The death signals of CD95 are mediated via activation of members of the caspase family of proteases (16). We analyzed the effects of a tetrapeptide, ac-YVAD-CMK, a well characterized inhibitor of caspases involved in CD95-triggered apoptosis, *i.e.* caspases 1 and 3 (16).

Cells were incubated for 2 h with 200 μM ac-YVAD-CMK before addition of either anti-CD95 mAb or anti-TcR mAb. Early apoptotic cells were identified based on the annexin V staining of exposed phosphatidylserine residues, a method that allows the quantitative analysis of the results obtained. Ac-YVAD-CMK treatment resulted in inhibition of both CD95- and TcR-induced apoptosis (65.3 ± 4.2 and 58 ± 3.4%, respectively; $n = 5$; Fig. 1*B*).

It has been recently demonstrated that T cells express the endothelial NOS isoform (17). We wondered whether endogenous production of NO, with ensuing generation of cGMP (1, 2), was responsible for the activation-dependent shift in susceptibility to CD95-mediated apoptosis. To investigate the problem, experiments were carried out first with NO donors, SNAP and isosorbide dinitrate, or with the membrane-permeant cGMP analogue, 8-Br-cGMP. In addition, since high levels of cGMP may cross-activate protein kinase A in certain cell systems (18), the effect of the membrane-permeant cAMP analogue, 8-Br-cAMP, was also evaluated. Both NO donors and 8-Br-cGMP were found to inhibit substantially CD95- and TcR-triggered apoptosis, while cell treatment with 8-Br-cAMP did not result in appreciable inhibition. Neither NO donors nor 8-Br-cGMP had any detectable effect on γδ cell death induced by different *stimuli*, including etoposide and thapsigargin (Table I; data not shown).

The effect of endogenous NO on CD95-mediated apoptosis was next investigated. Expression of the NOS enzymes, cGMP generation, or the effect of removing endogenous NO were analyzed at different times after *in vitro* restimulation of γδ T cells. Fig. 2 shows the analysis of the expression of the NOS isoenzymes at different times (14, 21, and 28 days) after restimulation, both by flow cytometry and Western blotting. Neither the brain nor the inducible isoforms were ever detected, whereas ecNOS was highly expressed after restimulation and decreased thereafter during *in vitro* propagation.

The activity of ecNOS was next investigated, as measured by cGMP generation. Activation of the TcR and the ensuing increases in $[Ca^{2+}]_i$ (whose amplitude remains unchanged during γδ T cell propagation *in vitro*; see Ref. 6) constitute a physiological *stimulus* for ecNOS (1). As shown in Fig. 3*A*, high levels of cGMP were found upon TcR activation 14 days after *in vitro* restimulation, with progressive reduction at 21 and 28 days. This effect was exclusively mediated via NO, since pretreatment with two NOS inhibitors, L-NIO and L-NAME (1), completely prevented any increased cGMP generation at all times considered (Fig. 3*A*; data not shown).

The decrease of NOS activity was inversely associated with increased susceptibility to CD95-induced apoptosis (Fig. 3, compare *black columns* in *A* and *B*). To investigate a causal relationship between the two processes, the effects of NO removal by a scavenger, hemoglobin, and of the inhibition of NOS activity by L-NIO were analyzed. As shown in Fig. 3*B*, both the blockade of NO generation via inhibition of NOS activity and the removal of NO conferred sensitivity to CD95-induced apoptosis even during the early phases after *in vitro* restimulation, *i.e.* when the cells in normal culture conditions are resistant. Similar results were obtained using the guanylyl cyclase specific inhibitor ODQ (Ref. 19; data not shown). Taken together, these data suggest that endogenously-generated NO/cGMP protects against CD95-triggered apoptosis. Whether the reduction in ecNOS protein levels fully accounts for reduced NOS activity, or whether biochemical modifications of the enzyme (20) are also involved, remains to be investigated.

To identify the mechanism(s) and the sites of action of NO in the CD95 signal transduction pathway we studied apoptosis-sensitive γδ T lymphocytes (28 days after *in vitro* restimulation). Among the signals induced by CD95 cross-linking is the hydrolysis of membrane sphingomyelin with ensuing genera-

TABLE I

Effects of NO on apoptosis of γδ T lymphocytes

Results are expressed as % of inhibition of annexin V staining (mean ± S.E.; $n = 5$) with respect to cells treated with the corresponding relevant mAb, etoposide (20 μM) or thapsigargin (250 nM). Incubations with apoptosis-inducing agents, alone or combined with either SNAP (100 μM), 8-Br-cGMP (500 μM), and 8-Br-cAMP (500 μM) were for 5 h. See "Experimental Procedures" for details. ND, not determined.

Treatment	Apoptosis inhibition (%)		
	+ SNAP	+ 8-Br-cGMP	+ 8-Br-cAMP
Anti-CD95 mAb	64 ± 2.9	41 ± 2.1	3 ± 04
Anti-TcR mAb	55 ± 2.6	35 ± 1.3	2.7 ± 0.2
Etoposide	0	0	ND
Thapsigargin	0	0	ND

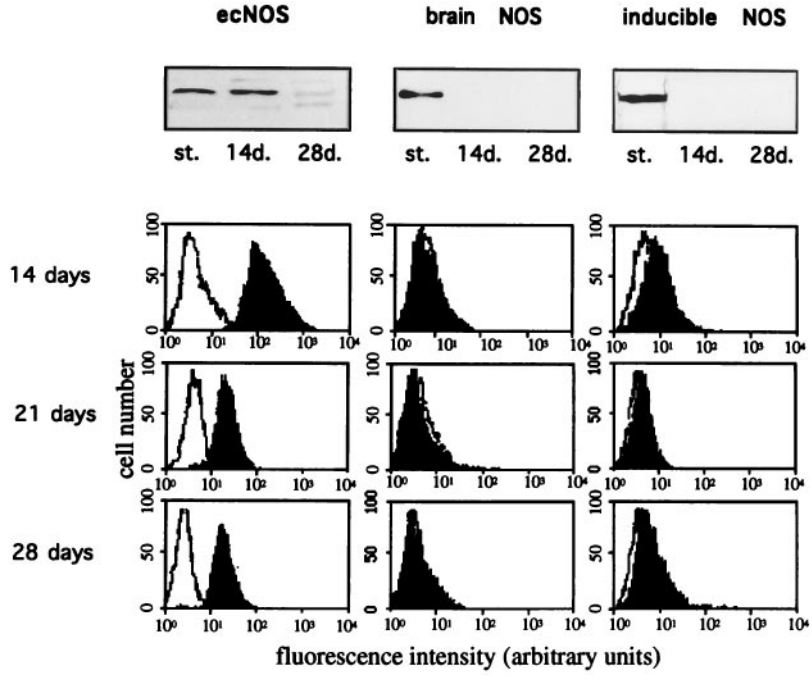

FIG. 2. **Analysis of NOS isoenzyme expression in γδ T lymphocytes.** *Upper panels* show Westen blot analysis with specific Abs of NOS expression in 250 μg of total cell lysates obtained from γδ T lymphocytes after 14 and 28 days since cell restimulation. As internal controls for ecNOS, brain NOS, and inducible NOS, 35 μg of human endothelial cells, rat pituitary, and mouse macrophage lysates, respectively, were loaded into the same gels. *st.*, positive control; *14d.*, 14 days; *28d.*, 28 days. The *lower panels* show flow cytometric analysis of the expression of ecNOS, brain NOS, and inducible NOS after 14, 21, and 28 days since cell restimulation. Results shown are representative of three separate experiments.

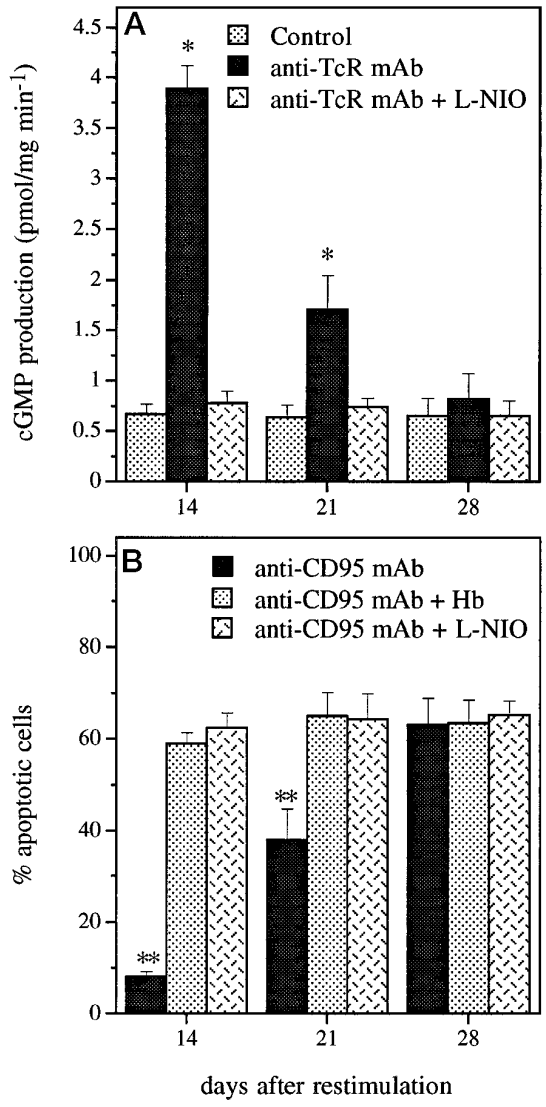

FIG. 3. **ecNOS activity determines γδ cell susceptibility to CD95-mediated cell death.** *A*, cGMP accumulation was evaluated in control untreated cells, after TcR cross-linking, and after TcR cross-linking in the presence of the NOS inhibitor L-NIO (300 μM). Data are from results obtained in five experiments run in triplicate (mean ± S.E.). *B*, apoptotic cells were revealed by flow cytometry as annexin V^+ cells after 5 h of CD95 cross-linking in the absence or presence of the NO scavenger hemoglobin (*Hb*, 50 μM), or the NOS inhibitor L-NIO (300 μM). Experiments were performed at the times indicated after *in vitro* restimulation. Results are reported as percent of apoptotic cells (annexin V^+ cells) in the different experimental conditions. Data shown are from results obtained in five experiments (mean ± S.E.). 10,000 cells were analyzed by flow cytometry per each experimental sample. *, $p < 0.01$ with respect to control; **, $p < 0.01$ with respect to cells treated with anti-CD95 mAb plus either Hb or L-NIO.

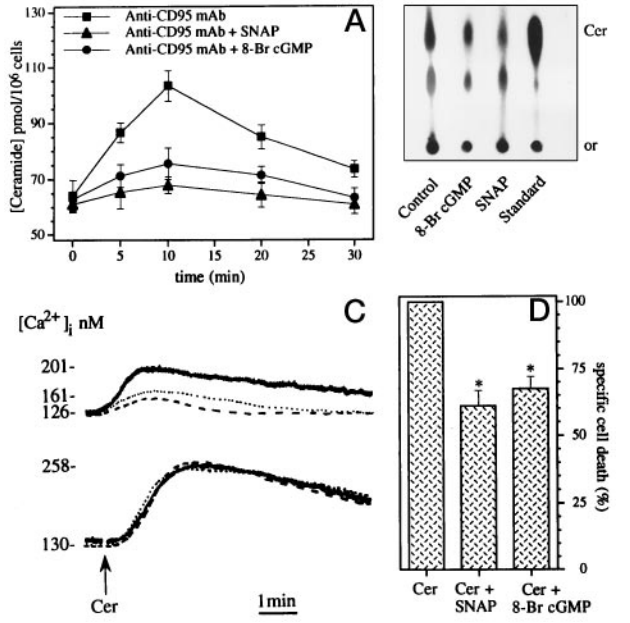

FIG. 4. **NO inhibits CD95-triggered ceramide generation and Ca^{2+} release and protects from ceramide-induced cell death.** *A*, ceramide levels were assessed by thin layer chromatography at the indicated times after CD95 cross-linking, in the absence or in the presence of either the cell permeant cGMP analogue 8-Br-cGMP (500 μM) or the NO donor SNAP (100 μM). Results shown are mean ± S.E. of five experiments run in triplicate. *B*, autoradiography of the 10-min incubation point sample is shown together with the 250-pmol authentic ceramide standard. *Control*, cells treated with the anti CD95 mAb alone; *SNAP and 8-Br-cGMP*, cells treated with the Ab in the presence of either SNAP (100 μM) or 8-Br-cGMP (500 μM), respectively. *C*, $[Ca^{2+}]_i$ variations were assessed by fluorimetric analysis of fura-2-loaded cells challenged with the anti-CD95 mAb (*upper trace*, *solid*) or the membrane-permeant analogue of ceramide, C_2-ceramide (10 μM; *Cer*, *lower trace*, *solid*) in the presence of either SNAP (100 μM; *hatched lines*) or 8-Br-cGMP (500 μM; *dotted lines*). Traces shown are representative of eight consistent experiments. *D*, cells were incubated with C_2-ceramide (10 μM) either alone or in the presence of SNAP (100 μM) or 8-Br-cGMP (500 μM). The percentage of dead cells was evaluated by flow cytometry after annexin V staining (6). Specific cell death was calculated as percent with respect to the cell death induced by ceramide alone (100%). Results are from five consistent experiments (mean ± S.E.) run in triplicates. *Cer*, ceramide. *, $p < 0.01$ *versus* ceramide alone.

tion of ceramide (21, 22), a metabolite that, when administered exogenously, triggers programmed death in cells belonging to various lineages, including lymphocytes (23). The kinetics of ceramide accumulation after CD95 ligation in the presence of either SNAP or 8-Br-cGMP were therefore evaluated by thin layer chromatography. As illustrated in Fig. 4*A*, either treatment resulted in an important reduction of CD95-induced ceramide accumulation at all time points considered (70 and 85% reduction of the 10-min peak of activity, respectively; see Fig. 4*B*).

Ceramides, via their conversion to sphingosine 1-phosphate, induce the release of Ca^{2+} from the endoplasmic reticulum (24–26). We have recently demonstrated in γδ T cells that, following CD95 cross-linking, Ca^{2+} release occurs and is critical for apoptosis induction (6). This process appears to take place as a consequence of ceramide accumulation since inhibition by the competitive inhibitor, DL-*threo*-dihydrosphingosine (26, 27), of sphingosine kinase, the enzyme that converts ceramides into sphingosine 1-phosphate, completely prevented Ca^{2+} release induced by CD95-ligation (data not shown). Whether the NO/cGMP effects on ceramide generation resulted in reduced Ca^{2+} release from intracellular stores was thus investigated. Fluorimetric analysis of γδ cells loaded with the Ca^{2+}-sensitive dye, fura-2, revealed a consistent increase in $[Ca^{2+}]_i$ upon both CD95 cross-linking and administration of 10 μM membrane-permeant ceramide (+58.3 ± 4.2% and +95 ± 5.8%, respectively, $n = 8$) (Fig. 4*C*). When the cells were incubated with either SNAP (100 μM) or 8-Br-cGMP (500 μM), CD95-triggered Ca^{2+} release was dramatically reduced (−52.3 ± 4.7% and −46.5 ± 4.3%, respectively; $n = 8$) (Fig. 4*C*, *upper traces*). The effects of NO/cGMP on Ca^{2+} release were entirely due to the inhibition of ceramide accumulation, since neither SNAP nor 8-Br-cGMP had any appreciable effect when the experiments were performed using membrane-permeant ceramide as Ca^{2+} releasing agent (Fig. 4*C*, *lower traces*).

Taken together, these results demonstrate that NO protects from apoptosis interfering with ceramide accumulation. To in-

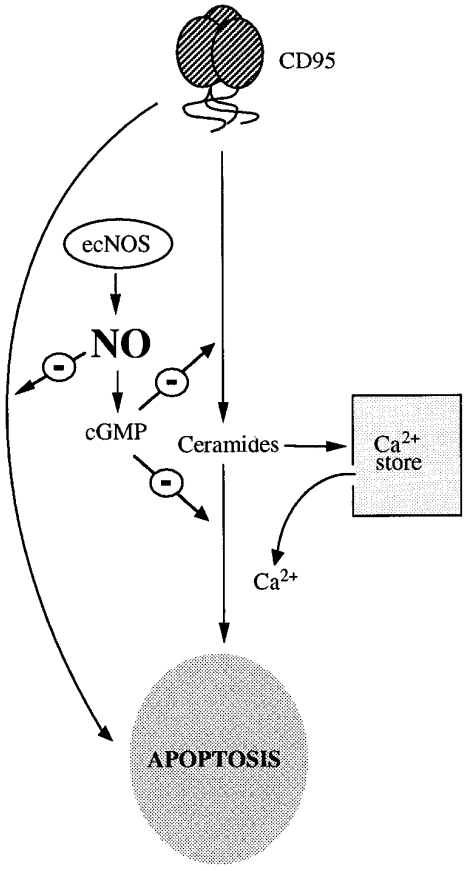

FIG. 5. **Schematic representation of the NO-mediated modulation of the CD95 apoptotic pathways.** CD95 cross-linking results in the switch on of various, possibly related signaling pathways converging to apoptosis (16). NO, generated by ecNOS activation, exerts a protective effect interfering with these pathways at different levels. NO can act either via cGMP-dependent pathways, located upstream and downstream of ceramide generation, and via cGMP-independent pathways yet to be defined. An additional consequence of the reduced accumulation of ceramides, *i.e.* inhibition of the release of Ca^{2+} from intracellular stores, contributes to the protective effect of NO on apoptosis (6).

vestigate whether this is the only site of action of NO, γδ cells were treated with membrane-permeant ceramide and the NO/cGMP effects on ceramide-induced apoptosis were analyzed. As shown in Fig. 4*D*, apoptosis protection was still detected, indicating that a second site of NO action exists downstream of sphingomyelin hydrolysis.

The results we have obtained demonstrate the existence of regulated, endogenous generation of NO in γδ T lymphocytes and implicate NO in cell protection from the CD95-triggered apoptosis. The effect of NO is specific for this signaling pathway, since the messenger has no effect on programmed death induced by *stimuli* working via different intracellular pathways. The molecular levels at which NO exerts its actions appear multiple, located upstream and downstream of ceramide generation. Of importance, the protective effect induced by SNAP against CD95-induced apoptosis is almost 2-fold with respect to that observed with 8-Br-cGMP (see Table I). This observation may underlie the existence in T lymphocytes of molecular targets regulated by NO in a cGMP-independent way. Recently, suppression of apoptosis by NO in endothelial cells was demonstrated to be mediated via *S*-nitrosylation of caspases (28). Whether this, or other, as yet unidentified mechanisms, are operating also in T lymphocytes remains to be established. A model illustrating the multiple sites of NO action along the CD95-activated signal transduction pathways in γδ T lymphocytes is proposed in Fig. 5.

The observations reported here have important implications for the overall regulation of immune response. Expression of CD95 ligand by T lymphocytes is among the physiological responses triggered by TcR activation. This event, however, does not immediately result in CD95-dependent apoptosis, but allows multiple rounds of activation and the clonal expansion of stimulated lymphocytes (29). To account for this paradox, it was proposed that signaling pathways exist that dynamically regulate the susceptibility of activated lymphocytes to die in response to CD95 activation (29). Our data identify autocrine generation of NO as the early signaling event in the pathway that regulates γδ T lymphocyte apoptosis.

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REFERENCES

1. Gross, S. S., and Wolin, M. S. (1995) *Annu. Rev. Physiol.* **57,** 737–769
2. Moncada, S., and Higgs, E. A. (1995) *FASEB J.* **9,** 1319–1330
3. Kaufmann, S. H. E. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 2272–2279
4. Ferrarini, M., Heltai, S., Toninelli, E., Sabbadini, M. G., Pellicciari, C., and Manfredi, A. A. (1995) *J. Immunol.* **154,** 3704–3712
5. Nagata, S., and Golstein, P. (1995) *Science* **267,** 1449–1456
6. Rovere, P., Clementi, E., Ferrarini, M., Heltai, S., Sciorati, C., Sabbadini, M. G., Rugarli, C., and Manfredi, A. A. (1996) *J. Immunol.* **156,** 4631–4637
7. Alderson, M. R., Tough, T. W., Davis-Smith, T., Braddy, S., Falk, B., Scholley, K. A., Goodwin, R. G., Smith, C. A., Ramsdell, F., and Lynch, D. H. (1995) *J. Exp. Med.* **181,** 71–77
8. Brunner, T., Mogil, J. R., LaFace, D., Yoo, N. J., Mahboubi, A., Echeverri, F., Martin, S. J., Force, W. R., Lynch, D. Y., Ware, C. F., and Green, D. R. (1995) *Nature* **373,** 441–444
9. Dhein, J., Walczak, C., Baumler, K. M., Debatin, P. H., and Krammer, P. H. (1995) *Nature* **373,** 438–441
10. Ju, S., Panka, D. J., Cui, H., Ettinger, R., El-Khatib, M., Sherr, D. H., Stanger, B. Z., and Marshak-Rothstein, A. (1995) *Nature* **373,** 444–448
11. Ferrarini, M., Heltai, S., Pupa, S. M., Menard, S., Zocchi, M. R. (1996) *J. Natl. Cancer Inst.* **88,** 436–441
12. Rooney, E., and Meldolesi, J. (1996) *J. Biol. Chem.* **270,** 29304–29311
13. Clementi, E., Sciorati, C., Riccio, M., Miloso, M., Meldolesi, J., and Nisticò, G. (1995) *J. Biol. Chem.* **270,** 22277–22282
14. Cifone, M. G., Roncaioli, P., De Maria, R., Camarda, G., Santoni, A., Ruberti, G., and Testi, R. (1993) *J. Exp. Med.* **177,** 1547–1552
15. Inesi, G., and Sagara, Y. (1992) *Arch. Biochem. Biophys.* **298,** 313–317
16. Nagata, S. (1997) *Cell* **88,** 355–365
17. Reiling, N., Kroncke, R., Ulmer, A. J., Gerdes, J., Flad, H. D., and Hauschildt, S. (1996) *Eur. J. Immunol.* **26,** 511–516
18. Forte, L. R., Thorne, P. K., Eber, S. L., Krause, W. J., Freeman, R. H., Francis, S. H., and Corbin, J. D. (1992) *Am. J. Physiol.* **263,** C607–C615
19. Garthwaite, J., Southam, E., Boulton, C. L., Nielsen, E. B., Schmidt, K., and Mayer, B. (1995) *Mol. Pharmacol.* **48,** 184–188
20. Garcìa-Cardeña, G., Fan, R., Stern, D. F., Liu, J., and Sessa, W. C. (1996) *J. Biol. Chem.* **271,** 27237–27240
21. Cifone, M. G., Roncaioli, P., De Maria, R., Camarda, G., Santoni, A., Ruberti, G., and Testi, R. (1995) *EMBO J.* **14,** 5859–5868
22. Skowronski, E. W., Kolesnick, R. N., and Green, D. R. (1996) *Cell Death Diff.* **3,** 171–176
23. Spiegel, S., and Merrill, A. H. (1996) *FASEB J.* **10,** 1388–1397
24. Kim, S., Lakhani, V., Costa, D. J., Sharara, A. I., Fitz, J. G., Huang, L.-W., Peters, K. G., and Kindman, L. A. (1995) *J. Biol. Chem.* **270,** 5266–5269
25. Mao, C., Kim, S. H., Almenoff, J. S., Rudner, X. L., Kearney, D. M., and Kindman, L. A. (1996) *Proc Natl. Acad. Sci. U. S. A.* **93,** 1993–1996
26. Choi, O. H., Kim, J. H., and Kinet, J.-P. (1996) *Nature* **380,** 634–636
27. Buehrer, B. M., and Bell, R. M. (1992) *J. Biol. Chem.* **267,** 3154–3159
28. Dimmeler, S., Haendeler, J., Nehls, M., and Zeiher, A. M. (1997) *J. Exp. Med.* **185,** 601–607
29. Boise, L. H., and Thompson, C. B. (1996) *Science* **274,** 67–68

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