ILA, a Member of the Human Nerve Growth Factor/Tumor Necrosis Factor Receptor Family, Regulates T-Lymphocyte Proliferation and Survival

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ILA, a gene induced by lymphocyte activation, is a member of the human nerve growth factor tumor necrosis factor receptor family and the human homologue of murine 4-1BB. The present study analyzed the role of ILA in the regulation of human peripheral blood T-lymphocyte function. Antibodies raised against different fusion proteins recognized ILA on activated lymphocytes. These antibodies significantly increased anti-CD3-induced T-lymphocyte proliferation. When anti-CD3-stimulated cells were incubated on ILA-expressing CHO cells, proliferation was inhibited. CHO cells transfected with a control construct and not expressing ILA did not reduce T-cell proliferation. A purified fusion protein containing the extracellular domain of ILA and the constant domain of human IgG (ILA-IgG) also inhibited lymphocyte proliferation. Results obtained by 3H-thymidine incorporation were confirmed by cell cycle analysis that showed a decrease in the number of lymphocytes in S phase. Lymphocyte morphology in cultures with ILA-expressing CHO cells was suggestive of apoptosis. Flow cytometry on propidium iodide-stained cells showed a time-dependent increase in the number of hypodiploid apoptotic cells when lymphocytes were cultured on ILA-expressing CHO cells. Internucleosomal DNA cleavage was seen in these cultures, but not in the presence of ILA-negative CHO cells. Studies on the mechanism by which ILA regulates T-cell function showed that ILA-IgG inhibited anti-CD3-induced T-cell proliferation when presented in immobilized but not in soluble form. These results suggest that ILA may act by cross-linking its ligand and thereby inhibit T-cell proliferation.

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MATERIALS AND METHODS

Cells and cell culture. Human peripheral blood mononuclear cells (PBMC) were prepared and proliferation studies performed as previously described. In brief, PBMC were isolated on Ficoll-Hypaque (Sigma, St Louis, MO) density gradients from heparinized blood. Antisera to CD3, in B lymphocytes by PMA and antibodies to cell-surface Ig, and in blood monocytes by intereleukin (IL)-1β, lipopolysaccharide, and PMA. In contrast to the lymphoid-specific expression of the murine 4-1BB gene, ILA was detected in nonlymphoid cells including epithelial and hepatoma cells after stimulation with IL-1β. The ILA cDNA encodes a 30-kD protein, and antisera raised against ILA peptides or a GST-ILA fusion protein identified ILA as a cell-surface protein on activated T or B lymphocytes. The present study analyzes ILA function and shows that this receptor is involved in the regulation of proliferation and survival of T lymphocytes.
perripheral blood of healthy volunteers. The cells were cultured at 200,000 per well in 96-well flat-bottomed plates in RPMI 1640 supplemented with L-glutamine, antibiotics, and 5% heat-inactivated fetal bovine serum. Proliferation was measured as ³H-thymidine incorporation during the last 4 hours of a 72-hour culture period. In proliferation assays, each condition was tested in triplicate. CHO and COS-1 cells used in proliferation studies were grown to confluence on the culture plates and fixed with 0.25% glutaraldehyde in phosphate-buffered saline for 10 minutes at room temperature.

ILA-IGG plasmid. The coding region for the extracellular domain of ILA (nucleotides 1 to 558) was amplified by polymerase chain reaction (PCR) and inserted into the HindIII, XhoI sites of pRC/CMV (Invitrogen, San Diego, CA). The hinge-CH₂-CH₃ region of human IgG1 was also PCR-amplified and inserted at the XbaI site downstream of and in frame with the extracellular domain of ILA. Double-stranded sequencing confirmed the correctness of the respective sequences and of the ILA-IGG junction.

PCR was performed in a 50-µL vol with U Vent polymerase (Biolabs, Beverly, MA), 140 mMol/L dNTPs, 1.5 mMol/L MgCl₂, 10 mMol/L Tris, pH 8.3, 50 mMol/L KCl, and 10 mMol/L of each primer. After a 5-minute denaturation step at 94°C, the reaction proceeded in cycles of 30 minutes at 94°C, 30 minutes at 55°C, and 50 minutes at 72°C, followed by 5 minutes at 72°C. Primers used for amplification of the extracellular domain of ILA were as follows: sense, 5' GAT AAG CTG AGG AAC AGC TGT TAC; and antisense, 5' GAA GTC TAG AGG CTG CGG AGA GTG TCC TGG. Primers used for amplification of the hinge-CH₂-CH₃ domain of human IgG1 were as follows: sense, 5' TAC GTA GAG CCC AGA TCT; and antisense, 5' TTA ACT AGT TCA TTT ACC CG. This ILA-IGG vector was used as a template for in vitro transcription and translation. In vitro–translated ILA protein was prepared with the TNT-coupled reticulocyte lysate system (Promega, Madison, WI). Two micrograms of plasmid was used as a template for in vitro transcription with T7 RNA polymerase. 5'S-cysteine was included in the reaction for detection of in vitro translation products by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography. From the vector with the extracellular domain of ILA alone, before insertion of the IgG domain, a translation product of 23 kD was obtained. This is the size expected for the extracellular domain of ILA. With the ILA-IGG vector, a 55-kD protein A–binding protein was obtained, demonstrating that it was produced as a single fusion protein.

Transfection and selection of CHO and COS cells. CHO cells were transfected with pRC/RSV (Invitrogen) containing full-length cDNA in sense or antisense orientation by liposome-mediated gene transfer (DOTAP; Boehringer-Mannheim, Indianapolis, IN). The cells were cultured in the continuous presence of geneticin (1 mg/ml) for 2 months until used in the experiments.

COS-1 cells were transfected with pRe/CMV (Invitrogen) containing full-length cDNA in sense orientation or with pRe/CMV alone. The cells were cultured in the presence of geneticin (0.6 mg/ml) for 3 months until used in the experiments.

COS and CHO cells also were transfected with expression vector containing the coding sequence for the ILA-IGG fusion protein and selected as described earlier. These cells produced and secreted ILA-IGG, which was detected in the culture medium by enzyme-linked immunosorbent assay and Western blotting. When analyzed by Coomassie blue staining of polyacrylamide gels, a single band of the expected molecular mass was obtained.

Preparation of antibodies. Rabbits were immunized with protein A Sepharose–purified ILA-IGG fusion protein three times in biweekly intervals. ILA-IGG (200 ng) was resuspended in complete and incomplete (second and third immunization) Freund’s adjuvant. The antisera were purified on protein A, and reactivity to human Fc was removed by absorption on human Ig. Sera of these rabbits contained antibodies that recognized ILA in enzyme-linked immunosorbent assay. These antibody reactivities were not present in preimmune sera from the same animals. The IgG fraction of immune sera was purified, and reactivity to human Fc was deleted by absorption. Purified anti-ILA specifically stained cells transfected with the full-length ILA cDNA, as shown by immunocytochemistry and flow cytometry.

Analysis of DNA fragmentation. Cells were harvested by a 5-minute centrifugation at 2,000 rpm and resuspended in 10 mMol/L Tris, 1 mMol/L EDTA, and 0.5% Triton X-100, pH 8.0 (10² cells/50 µL buffer). Extracts were incubated on ice for 20 minutes and briefly vortexed every 5 minutes. Cell debris and high–molecular-weight DNA were removed by a 10-minute centrifugation at 14,000 rpm at 4°C. Supernatants were extracted with phenol and phenol/chloroform and precipitated with 0.5 vol 7.5 mol/L ammonium acetate and 2 vol ethanol for 2 hours at -80°C. DNA was resuspended in TE, separated on 2% agarose gels, and visualized using ethidium bromide.

Histochemical detection of DNA fragmentation. Cells were harvested, centrifuged onto glass slides, fixed with 0.25% glutaraldehyde for 1 minute, washed with water, and stained with 1 mg/ml 4',6-diamidino-2-phenylindole dihydrochloride for 10 minutes at 37°C. Slides were then washed with water, air-dried, and covered with Vectashield (Vector Labs) for analysis by fluorescence microscopy using a triple-band filter set (61000; Chroma Technology, Brattleboro, VT) on an Olympus microscope (Olympus, Tokyo, Japan).

DNA labeling for flow cytometric analysis. Cells were fixed in 70% ethanol at 4°C for 60 minutes, washed, and incubated with RNase (50 µg/mL) and propidium iodide (100 µg/mL) for 15 minutes at room temperature in the dark and kept at 4°C. Propidium iodide fluorescence of nuclei was measured by flow cytometry on a FACSscan (Becton Dickinson, Mountain View, CA) using a 560-nm dichromatic mirror and a 600-nm band-pass filter. Data are expressed as percent apoptotic (hypodiploid) nuclei.

RESULTS

ILA and lymphocyte proliferation. Lymphocytes were stimulated with suboptimal concentrations of OKT3, a mitogenic antibody to CD3. Addition of purified anti-ILA antibody but not control rabbit IgG significantly (P < .03) increased proliferation (Fig 1). Antibody to ILA did not induce proliferation in the absence of OKT3 (not shown).

In separate experimental approaches, ILA function was examined by using ILA on transfected cells or ILA-IGG fusion protein. When PBMC were cultured on glutaraldehyde-fixed ILA-expressing CHO (CHO-ILA) cells, proliferation induced by different concentrations of OKT3 was completely inhibited (Fig 2A). In contrast, lymphocytes showed high levels of proliferation in cultures that contained CHO cells not expressing ILA (transfected with a construct containing ILA in antisense orientation and selected as the ILA-expressing cells).

These experiments addressed the role of ILA in primary activation of lymphocytes. We then tested whether ILA stimulates proliferation of preactivated cells. Lymphocytes were activated with immobilized OKT3 for 3 to 10 days and then cultured on CHO-ILA or control CHO cells. As seen with freshly isolated PBMC, proliferation of preactivated lymphocytes was completely inhibited by the fixed CHO-ILA cells, as determined by ³H-thymidine incorporation (not shown).

Two additional sources of ILA protein were used to confirm these findings. Similar to CHO cells, COS-1 cells were transfected with an ILA expression vector. A different pro-
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Fig 1. Antibody to ILA costimulates lymphocyte proliferation. PBMC from 4 different donors were isolated and activated with a suboptimal concentration of anti-CD3 (OKT3). Cells were plated in 96-well plates that were either untreated (no coating) or coated with control IgG or the purified IgG fraction of ILA antiserum (anti-ILA-IgG). Coating was performed at 50 μg/mL for 2 hours at 37°C. Proliferation was determined after 3 days by a 4-hour pulse with 3H-thymidine. Each condition was tested in triplicate.

Fig 2. ILA expressed on transfected cells or ILA-IgG fusion protein inhibits lymphocyte proliferation. (A) ILA-expressing (CHO-ILA) and control CHO (CHO) cells were grown to confluence in 96-well plates and fixed with glutaraldehyde. PBMC from 3 different donors were cultured on these plates in the absence or presence of anti-CD3. (B) Identical experimental conditions as in A, but CHO and CHO-ILA cells were replaced by COS and COS-ILA cells. (C) Fifty microliters purified ILA-IgG or Fc at 280 ng/mL was used per well to coat 96-well culture plates for 2 hours at 37°C. Plates were washed twice with PBS before PBMC were added. Proliferation was determined after 3 days by a 4-hour incubation with 3H-thymidine. Triplicate determinations were performed for each condition.
CHO-ILA cells was detectable after 12 hours and increased continuously during the 72-hour culture (Fig 5). At 72 hours, the number of viable PBMC on control CHO and CHO-ILA cells was 71% and 32%, respectively.

**Immobilized ILA-IgG but not soluble ILA-IgG inhibits T-lymphocyte proliferation.** These results suggested that ILA is involved in the regulation of lymphocyte proliferation and survival. However, based on these results and findings obtained with other members of the TNF/NGF receptor family, it was possible that the observed effects either were related to inhibition of a positive signal for proliferation

Results obtained by ³H-thymidine incorporation were confirmed by cell cycle analysis. Preactivated lymphocytes were cultured on fixed CHO and CHO-ILA cells for 48 hours, stained with propidium iodide, and analyzed by flow cytometry. The percentage of dead cells and cells in G₀, S, and M phase was 16.1, 4.2, 41.3, and 38.1 for A and 34.2, 53.5, 8.7, and 2.4 for B.

**ILA and lymphocyte survival.** Lymphocytes cultured on CHO-ILA cells showed morphologic changes suggestive of apoptosis. Analysis of genomic DNA from these lymphocytes revealed internucleosomal DNA fragmentation (Fig 4). Cells were cultured on fixed CHO and CHO-ILA cells for various time intervals, stained with propidium iodide, and analyzed by flow cytometry. Apoptosis in PBMC cultured on

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**Fig 3.** ILA-expressing CHO cells reduce the number of dividing cells. Preactivated lymphocytes were cultured on glutaraldehyde-fixed control CHO (A) and CHO-ILA (B) cells for 48 hours. Lymphocytes were fixed with 7% ethanol, stained with propidium iodide, and analyzed by flow cytometry. The percentage of dead cells and cells in G₀, S, and M phase was 16.1, 4.2, 41.3, and 38.1 for A and 34.2, 53.5, 8.7, and 2.4 for B.

**Fig 4.** ILA-expressing CHO cells induce DNA fragmentation. Preactivated lymphocytes were cultured on glutaraldehyde-fixed control CHO and CHO-ILA cells for 24 hours. Genomic DNA of the lymphocytes was isolated and analyzed on 2% agarose gels.
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Time [h] | control CHO | CHO-ILA
--- | --- | ---
0 | ![control CHO](image1) | ![CHO-ILA](image2)
12 | ![control CHO](image3) | ![CHO-ILA](image4)
24 | ![control CHO](image5) | ![CHO-ILA](image6)
36 | ![control CHO](image7) | ![CHO-ILA](image8)
48 | ![control CHO](image9) | ![CHO-ILA](image10)
72 | ![control CHO](image11) | ![CHO-ILA](image12)

Fig 5. Time course of lymphocyte apoptosis. PBMC were cultured on glutaraldehyde-fixed control CHO and CHO-ILA cells for indicated times. Cells were fixed with 70% ethanol, stained with propidium iodide, and analyzed by flow cytometry.

Fig 6. Immobilized ILA-IgG but not soluble ILA-IgG inhibits T-lymphocyte proliferation. PBMC were stimulated with OKT3 on plates that had been precoated with 50 µL purified ILA-IgG or Fc (control Ig) at 280 ng/mL or in the presence of soluble ILA-IgG or Fc (control Ig) at 280 ng/mL. Proliferation was determined after 3 days by a 4-hour incubation with ³H-thymidine. Triplicate determinations were performed for each condition. Results represent 4 independent experiments with cells from different donors.

provided by ILA or were the consequence of signaling through ILA ligand, which would inhibit proliferation. To address this issue, experiments were performed with ILA-IgG either in immobilized or in soluble form. As shown earlier, when ILA-IgG was bound to tissue culture plates, it inhibited anti-CD3–induced T-cell proliferation. However, when added to the culture media and thus present in soluble form, ILA-IgG did not reduce the proliferative response (Fig 6). These results suggest that ILA may act by cross-linking its ligand and thereby inhibit T-cell proliferation.

DISCUSSION

This study suggests an important role for ILA in the regulation of T-lymphocyte proliferation and survival. ILA expressed on transfected cells or in the form of an ILA-IgG fusion protein prevented anti-CD3–induced proliferation and caused cell death by apoptosis.

T-cell proliferation in response to antibodies to the TCR or CD3 requires the presence of monocytes as accessory cells to provide costimulatory signals. In CD4+ and CD8+ primary T cells, anti-CD3 induces IL-2 receptor expression and responsiveness to exogenous IL-2, but is not sufficient to induce either IL-2 secretion or T-cell proliferation. IL-2 secretion and proliferation require both TCR cross-linking and antigen-presenting cell (APC)-derived costimulatory signals. These signals are generated either by secreted molecules or by interactions of cell-associated ligands and receptors. Several monocyte-derived cytokines, including IL-1, IL-6, TNF, and IL-12, costimulate T-cell proliferation.40-41 Stimulation by CD2 and/or CD28 antibodies defines alternative activation pathways.42 Adhesive interactions between receptors on T cells and their counterreceptors on APCs provide further costimulatory signals and involve at least four distinct adhesion molecules present on APCs. B7, ICAM-1 (CD54), LFA-3 (CD58), and VCAM-1 have been individually shown to costimulate T-cell activation.43,44
In addition to these soluble and cell-bound molecules, several members of the NGF/TNF receptor family and their ligands are now known to regulate T-cell activation. CD27 is a lymphocyte-specific receptor, and its expression is tightly regulated during T-cell ontogeny. Antibody to CD27 and recombinant soluble CD27 inhibited T-cell proliferation induced by anti-CD2, anti-CD3, mitogens, or soluble Ag, as well as PWM-driven B-cell IgG synthesis. Anti-CD27 inhibited IL-2 secretion without affecting IL-2 receptor expression. The human OX40 cell-surface antigen is a CD4+ T-cell activation marker that acts as a costimulatory receptor. Cell-bound recombinant OX40 ligand costimulates phorbol myristate acetate, PHA, and anti-CD3–induced T-cell proliferation and cytokine production.

Previous reports showed that the murine ILA homologue 4-1BB costimulates T-cell proliferation. A rat monoclonal antibody against 4-1BB enhanced anti-CD3–induced T-cell proliferation. Similar increases in proliferation of activated murine thymocytes and splenic T cells were induced by murine 4-1BB ligand. Furthermore, both monoclonal antibody to 4-1BB and cells transfected with 4-1BB ligand increased proliferative responses in mitogen-stimulated primary T cells.

Results from the present study showed that antibodies raised against an ILA-IgG fusion protein increased proliferation of anti-CD3–stimulated PBMC. When exogenous ILA in the form of ILA-IgG fusion protein was introduced into the lymphocyte cultures, proliferative responses to anti-CD3 stimulation of anti-CD3-stimulated PBMC. When exogenous ILA-IgG were dose-dependent and specificity was shown in experiments where human Ig Fc did not detectably alter lymphocyte proliferation. Furthermore, lymphocytes cultured on glutaraldehyde-fixed ILA-expressing cells did not proliferate in response to anti-CD3. Complete inhibition of lymphocyte proliferation was seen with ILA-expressing CHO, as well as COS-1, cells, and lymphocyte proliferation in the presence of fixed CHO and COS-1 cells that did not express ILA was similar to that in cultures containing only PBMC.

ILA regulates not only proliferation but also T-lymphocyte survival. ILA-IgG or ILA expressed on transfected cells induced lymphocyte apoptosis. This was shown by DNA staining and flow cytometry and by the presence of internucleosomal DNA fragmentation.

These results suggest that ILA-IgG and ILA on transfected cells regulate T-cell proliferation and survival. This could occur by at least two distinct mechanisms: ILA-IgG or ILA on transfected cells could compete with ILA expressed on lymphocytes for ligand binding, and sequestration of ILA ligand prevents ILA from providing its stimulatory signal for T-lymphocyte proliferation. Alternatively, it is possible that ILA-IgG or ILA on transfected cells bound to and cross-linked ILA ligand and thereby induced an inhibitory signal on ligand-expressing cells. Experiments to address this question showed that immobilized ILA-IgG but not soluble ILA-IgG inhibited T-cell proliferation. These results indicate that ILA ligand, when cross-linked by ILA, may deliver a signal that inhibits T-cell proliferation and induces apoptosis. Similar mechanisms for regulation of cell functions via surface-bound ligands have recently been shown for CD40, OX40, and 4-1BB.

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