Protein Tyrosine Kinase p56-Lck Regulates Lymphocyte Function–associated 1 Adhesion Molecule Expression, Granule Exocytosis, and Cytolytic Effector Function in a Cloned T Cell

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Summary

Elevated levels of p56-Lck kinase activity were achieved in an interleukin 2 (IL-2)–dependent cloned cytolytic T cell CTLL-2 through gene transfer approaches. CTLL-2-Lck cells remained dependent on IL-2 for growth and survival in culture but exhibited markedly elevated, IL-2–independent cytolytic activity against a variety of tumor targets. This immune cell effector function was similar to the non-major histocompatibility complex–restricted cytolytic activity previously described for lymphokine activated killer (LAK) cells, and involved a cytolytic mechanism that was independent of protein synthesis in either the T cells or the tumor targets. Characterization of CTLL-2-Lck cells revealed markedly elevated levels of both the α (CD11a) and β (CD18) chains of the cell adhesion molecule lymphocyte function–associated 1 (LFA-1) and increased binding of these T cells to a recombinant protein representing the extracellular domain of the LFA-ligand, intercellular adhesion molecule 1 (ICAM-1). Antibodies to CD11a partially abrogated cytolytic killing of tumor target cells by CTLL-2-Lck cells, suggesting that the upregulation in LFA protein levels potentially accounts at least in part for the phenotype of these T cells. Gene transfer–mediated elevations in p56-Lck kinase activity in an IL-3–dependent myeloid cell clone 32D.3 also resulted in increased LFA-1 expression, demonstrating that the findings are not unique to CTLL-2 cells. In addition to upregulation of LFA-1 expression, CTLL-Lck cells also exhibited more efficient exocytosis of cytotoxic granules upon activation with Ca^{2+}-ionophore and phorbol ester, relative to control transfected and untransfected CTLL-2 cells. The findings functionally link the Lck kinase to a T cell effector pathway involved in cell-mediated cytotoxicity.

Lck is a member of the Src family of nonreceptor protein tyrosine kinases (PTKs)[1] and is normally expressed exclusively in lymphocytes, primarily in T cells and NK cells (reviewed in 1). The p56-Lck PTK has been found in physical association with the cytosolic domains a variety of cell surface receptors in T cells including CD2, CD4, CD8, and the β chain of the IL-2 receptor complex (2–6). Furthermore, triggering of most of these receptors with specific antibodies or ligands induces elevations in Lck kinase activity suggesting an important role for this PTK in some aspect of lymphocyte function. Though it is clear from investigations of *lck* knockout mice that p56-Lck is critical for the thymic development of T cells (7), knowledge of the functional significance of Lck kinase activation for the responses of mature, fully differentiated T cells remains incomplete.

In T cell hybridomas, gene transfer–mediated elevations in p56-Lck kinase activity have been shown to augment production of the lymphokine IL-2 in response to certain T cell activation signals and in some cases can lead to constitutive IL-2 production (8, 9). In addition, a Lck-deficient mutant of the CD4$^+$ T cell leukemia line Jurkat has been described that has several defects in TCR-initiated signal transduction, all of which are corrected by gene transfer–mediated restoration of p56-Lck production (10). These findings suggest that Lck can regulate signal transduction pathways involved in TCR signal transduction and lymphokine gene expression under at least some circumstances. Consistent with this notion, T cells expressing mutants of CD4 that fail to bind to p56-Lck also are defective in IL-2 production when stimulated with antigen and antigen-presenting cells under conditions where MHC–class II interaction with CD4 is required for T cell

[1] *Abbreviations used in this paper:* BLT, *N*-α-benzyloxycarbonyl-L-lysine thiobenzyl ester; FI, fluorescence intensity; PTK, protein tyrosine kinase; sICAM-1, soluble intercellular adhesion molecule 1; TIA, T cell intracellular antigen; TIL, tumor infiltrating lymphocytes.

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activation (11, 12). Taken together, these results suggest a role for Lck in regulating at least one key event associated with helper T cell function, namely IL-2 production.

In cytolytic T cells, increases in p56-Lck kinase activity can be transiently stimulated by antibody-mediated cross-linking of CD8 (2, 13). Furthermore, cytosolic domain mutants of CD8 that fail to associate with p56-Lck are defective in mediating signal transduction responses upon stimulation with alloantigen or anti-CD3-CD8 conjugate antibodies (14, 15), suggesting that Lck plays an important role in TCR-mediated responses at least under circumstances where co-stimulation through CD8 is also required. However, association of CD8 and p56-Lck does not appear to be essential for generation of allospecific CTL effector cells in vivo, nor for CTL-mediated lysis of allogenic cells in vitro (16).

In addition to CD8, signaling through receptors for IL-2 can also stimulate increases in p56-Lck kinase activity in cytolytic T cells, suggesting a role for Lck in some aspect of IL-2 signal transduction in these cells (17, 18). Gene transfer studies using a constitutively active version of p56-Lck (*lck*F505) have demonstrated that high levels of Lck kinase activity can result in IL-2–independent activation of phosphatidyl-inositol-3′-kinase, but have no effect on IL-2 requirements for cell proliferation, growth, and survival (18, 19). Moreover, IL-2R-β mutants that fail to bind p56-Lck also fail to stimulate elevations in p56-Lck kinase activity in response to stimulation with IL-2 and are defective in induction of *c-fos* and *c-jun* mRNA accumulation but not in stimulation of cellular proliferation (20). These observations suggest that IL-2R–mediated activation of p56-Lck plays an important role in some signal transduction pathways regulated by this lymphokine, but not necessarily those involved in cell growth.

Both CD8 and IL-2 have been shown to be capable of either directly or indirectly enhancing the cytolytic activity of killer T cells, raising the possibility of a functional connection between p56-Lck and regulation of cell-mediated killing. Similarly, stimulation of NK cells through CD16 (FcR-γ-IIIA) has been shown to induce Lck kinase activation and trigger cytolytic responses by these immune cells (21), again suggesting a link between p56-Lck and cell-mediated cytotoxicity. Indeed, studies of a variant subclone of the $CD3^+$/$CD8^+$ T cell CTLL-2 that lacks p56-Lck revealed a defect in cytolytic effector function when tested in antibody-redirected cytolysis assays using a hybridoma target cell that displays anti-CD3 monoclonal antibodies on its surface (22). Furthermore, gene transfer–mediated restoration of Lck production in these T cells was associated with markedly improved cytolytic effector function (22). Additional evidence implicating p56-Lck in cytolytic T cell responses has come from *lck* knock-out mice where the few T cells that survive the thymic selection process without Lck and reach the periphery as mature T cells have been shown to mount defective CTL responses to viruses (23). The mechanism by which Lck might modulate signal transduction pathways involved in cytolytic activity however has not been previously addressed.

Here we demonstrate that gene transfer–mediated elevations in p56-Lck kinase activity significantly enhance the cytolytic activity of CTLL-2 T cells against tumor target cells. Furthermore, the findings suggest that Lck upregulates cytolytic effector function through mechanisms that involve enhanced adhesion of T cells to target cells and more active exocytosis of cytotoxic granules. These observations could have relevance to mechanisms of immune surveillance against neoplasms, inasmuch as reductions in *lck* gene expression have been previously reported in the circulating T cells of animals with solid tumors (24), and in the tumor-infiltrating lymphocytes (TIL) of patients with renal carcinoma (25).

Materials and Methods

Cells and Cell Culture. CTLL-2 and 32D.3 cell clones were maintained in IL-2– or IL-3–containing culture media as described previously (18, 26, 27). CTLL-N-LCK cells are a polyclonal population of G418-resistant cells that were transfected with an expression plasmid pGSE1731-LCK encoding normal human p56-Lck (18). CTLL-A-LCK is a clone of CTLL-2 cells that expresses at high levels a plasmid pCD2-MT-LCK(F505) encoding a murine p56-Lck kinase with a Tyr → Phe substitution at position 505 (18, 28). The derivation and characterization of these *lck*-transfected CTLL-2 cells has been described in detail previously (18). CTLL-NEO is a randomly chosen clone of CTLL-2 cells that underwent electroporation (900 μF; 750 V/cm) with 25 μg of NdeI-linearized pZIP-NEO plasmid DNA (29) and subsequent selection in medium containing IL-2 and 400 μg/ml G418 (active concentration) (GIBCO BRL, Gaithersburg, MD). 32D-NEO and 32D-LCK cells are polyclonal populations of G418-resistant 32D.3 cells that underwent electroporation (double pulse of 40 μF; 2,000 V/cm; 74 ohms followed by 1,500 μF; 250 V/cm; 74 ohms) with 25 μg of linearized pLXSN or pLXSN-*lck*(F505) plasmid DNA (9), respectively, followed by selection in media containing IL-3 and ~600 μg/ml active G418.

Indirect Immunofluorescence Assays. Indirect immunofluorescence detection and flow cytometric analysis of the cell surface antigens CD2, CD3, CD11a, CD18, VLA-4, and NK1.1 was performed as described previously (30) using a FACScan Plus (Becton Dickinson & Co., Mountain View, CA) and the following monoclonal antibodies, most of which were obtained either generously provided by James Ryan (University of California at San Francisco, San Francisco, CA [UCSF]) or from the American Type Culture Collection (ATCC; Rockville, MD): RM2-5 (rat IgG2b; CD2); 145-2C11 (hamster IgG; CD3-ϵ); PK136 (mouse IgG2a; NK1.1); FD4.4.1 (rat IgG2b; CD11a); M18.5 (rat IgG2b; CD18), PS/2 (rat IgG2a; VLA-4); M17/4.4.11.9 (rat IgG2a; CD11a), M18/2.a.12.7 (rat IgG2a; CD18). Negative control rat IgG2a, rat IgG 2b, and mouse IgG2b monoclonal antibodies were obtained from Coulter Immunology, Inc. (Hialeah, FL). Fluorescein-conjugated antisera to mouse, rat, or hamster IgG were purchased from TAGO, Inc. (Burlingame, CA).

Cytolytic Assays. ^{51}Cr release assays were performed essentially as described by Wunderlich and Schearer (31). Briefly, 2–6 × 10^6 target cells were labeled with 150 μCi ^{51}Cr in ~0.4 ml of complete medium (RPMI with 10% FCS, 1 mM L-glutamine, 100 U/ml penicillin, and 50 μg/ml streptomycin) for 1.5 h at 37°C, then washed 3× with HBSS (GIBCO BRL) and resuspended in 1 ml of complete medium for 0.5 h at room temperature to allow for spontaneous release before washing once with complete medium and resuspending at 10^5 cells/ml. T cells were stripped of IL-2 by incubation for 0.5–1 min in 1 ml of 10 mM sodium-citrate (pH 4.0)/140 mM NaCl, followed immediately by dilution into 50 ml of HBSS. After two additional washes in HBSS, T cells were resuspended in complete medium at 10^6 cells/ml with various con-

centrations (0–100 U/ml) of purified recombinant IL-2 (gift of Chiron, Inc., Emeryville, CA). Various numbers of T cells (10^4, 5×10^4, 10^5, 2×10^5, 4×10^5) in 0.1 ml were added to round-bottom wells of 96-well microtiter plates and incubated in 37°/5% CO_2 for 4 h to allow for dissipation of IL-2–generated signals in IL-2–deprived T cells. ^{51}Cr-labeled target cells (10^4 in 0.1 ml) were then added and after 4 h ^{51}Cr release into culture supernatants was measured for triplicate samples. The percent specific lysis was calculated relative to the total amount of ^{51}Cr released by treatment with 1% NP-40, after substraction of spontaneous release from tumor targets incubated without T cells as described (31). In some cases, saturating amounts (100 μg/ml) of purified monoclonal antibodies directed against CD11a (M17) or CD18 (M18/2.a.12.7) were added to T cells for 15–20 min before addition of targets at an E/T ratio of 10 or 20.

Intercellular adhesion molecule 1 (ICAM-1) Binding Assays. A soluble fragment of mouse ICAM-1 representing the extracellular domain of the protein (sICAM-1) was produced in Chinese hamster ovary cells and purified as described previously (32). Various amounts of sICAM-1 or BSA control protein were incubated in flat-bottom 96-well plates overnight at 4°C, followed by washing 3× with PBS containing 0.1 wt/vol BSA and preblocking of the wells with 1% BSA in PBS at 37°C for at least 2 h. RPMI medium (30 μl) with or without 100 nM PMA (CalBiochem-Novabiochem, San Diego, CA) was added to each well. CTLL-2 cell were cultured for 4 h in complete medium containing 25 U/ml recombinant IL-2 and 5 μCi/ml [^{3}H]thymidine, washed 3× in complete medium, resuspended at 10^6–10^7 cells/ml, and 30 μl added per well. Binding to sICAM-1 was allowed to proceed at 37°C for 15 min, after which the plates were rapidly inverted and the wells washed 3× with prewarmed medium. Adherent cells were recovered into Ecolumne scintillation fluid (ICN Chemicals, Inc., Costa Mesa, CA) for counting. Data are expressed as the percentage of cpm/well obtained for adherent cells relative to the total cpms per well determined from duplicate uninverted plates.

RNA Blotting. Total cellular RNA was isolated from cells and 10 μg aliquots were size-fractionated in 1% agarose/6% formaldehyde gels and transferred to nylon membranes (Gene Screen; New England Nuclear/Dupont, Inc., Boston, MA). RNA was fixed to filters by UV-irradiation and then prehybridized, hybridized with ^{32}P-labeled DNA probes, washed, and analyzed by autoradiography essentially as described previously (33). Hybridization probes consistent of cDNAs specific for murine perforin and granzyme A (gift from Ed Podack; University of Miami, Miami, FL), murine TNF-α, LT-α, GM-CSF, IFN-γ (provided by James Ryan; UCSF), and murine β_2-microglobulin (gift from Jane Parnes; UCSF).

Immune Complex Kinase and Immunoblot Assays. Relative levels of p56-Lck kinase activity were measured in 32D-NEO and 32D-LCK cells by immune complex kinase assays performed exactly as described previously (34) using a rabbit polyclonal antisera raised against a synthetic peptide (RNGSEVRDPLVTYEGSLPPAC) corresponding to residues 39–58 of the mouse p56-Lck kinase with a COOH-terminal cysteine added to facilitate conjugation to maleimide-activated ovalbumin (Pierce, Rockford, IL).

Secretion Assays. Granule exocytosis was monitored based on β-glucuronidase release into culture media essentially as described by Taffs and Sitkovsky (35), with minor modifications. Briefly, 50 μl of CTLL-2 cells at 4×10^6 cells/ml in complete medium were added to round-bottom 96-well microtiter plates followed by 50 μl of media with or without the combination of 10 ng/ml PMA and 0.5 μg/ml A23187 (Calbiochem-Novabiochem). After culturing for 4 h at 37°C/5%CO_2, the supernatants were recovered and assayed spectrophotometrically for β-glucuronidase using phenolphthalein glucuronic acid as the substrate. The percentage release of β-glucuronidase was calculated relative to the total cellular content of the enzyme obtained upon lysis with 1% Triton X-100, after subtraction of spontaneous background release that was comparable for parental and all transfected CTLL-2 cells and always equaled <5% of the total cellular enzyme activity.

Total cellular and secreted esterases were spectrophotometrically detected by use of the *N*-α-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) substrate method (35).

Results

A Model T Cell for Studies of Lck and Cytolytic Effector Function. CTLL-2 is an IL-2–dependent cytolytic T cell line that was originally established in culture from C57Bl/6 mice immunized with allogenic tumor cells, and that displays MHC nonrestricted cytolytic activity against allogenic and syngenic tumor cells (26). Previously we demonstrated that IL-2 induces rapid, transient increases in the specific activity of p56-Lck kinase in CTLL-2 cells, in a concentration-dependent manner (18). IL-2–inducible increases in the activity of the only other Src-like PTK present at significant levels in these cells, p59-Fyn, were not detected. In an effort to explore the functional significance of IL-2–mediated increases in p56-Lck kinase activity, we stably introduced into a clone of CTLL-2 cells expression plasmids encoding either the normal Lck kinase (N-LCK) or a mutant version that has a tyrosine → phenylalanine substitution at position 505 (F505-Lck). This mutant form of Lck removes an in vivo site of tyrosine phosphorylation that normally downregulates Lck kinase activity, thus locking the kinase in a constitutively active conformation (A-LCK) (28). Despite (*a*) ∼20-fold elevations in the levels of p56-Lck kinase activity in both CTLL-N-LCK and CTLL-A-LCK cells; (*b*) constitutive activation of phosphatidylinositol 3′-kinase in CTLL-A-LCK cells; and (*c*) striking elevations in the phosphorylation of a variety of cellular proteins on tyrosines, particularly in CTLL-A-LCK cells, these genetically modified T cells remained completely dependent on IL-2 for their growth and survival in culture (18, 19).

In the absence of an effect of gene transfer–mediated elevations in Lck kinase activity on the IL-2–dependent growth and survival of CTLL-2 cells, we next tested the cytolytic activity of these genetically modified T cells against a variety of tumor targets. Target cells were loaded with ^{51}Cr, mixed with effector cells at an E/T ratio of 20, and specific ^{51}Cr release was measured in standard short-term (4-h) cytolytic assays. As shown in Fig. 1, the parental CTLL-2 cells lysed the murine thymoma YAC-I, the human T cell acute leukemia line JURKAT, and the mouse leukemia line RLσ1 in an IL-2–dependent manner. Specific lysis of a variety of other tumor cells lines was not detected at significant levels (>5%) above background, including U937, K562, Daudi, C1498, IC-21, P815, and B16F10 cells. There was no correlation between lysis and NK sensitivity among these target cell lines.

Both CTLL-N-LCK and CTLL-A-LCK cells (also referred to as Y505Lck and F505Lck, respectively) displayed the same specificity as the parental untransfected CTLL-2 cells in these

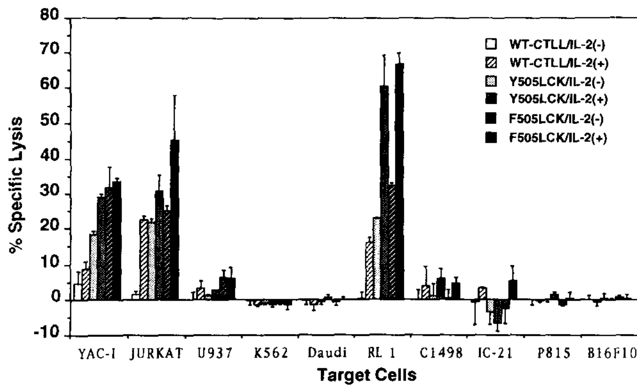

Figure 1. Analysis of cytolytic activity of CTLL-2 cells against various tumor target cells. Parental CTLL-2 cells (designated WT, for wild-type), CTLL-2 cells transfected with an expression plasmid that produces normal p56-Lck kinase or a mutant version containing a Tyr505 → Phe substitution (designated Y505-Lck and F505-Lck, respectively) were cultured with ^{51}Cr-labeled target cells for 4 h at an E/T ratio of 10 in the presence or absence of 100 U/ml recombinant IL-2 as indicated. The percent specific lysis was calculated as described (31). Data represent mean ± SD for triplicate determinations and are representative of several experiments.

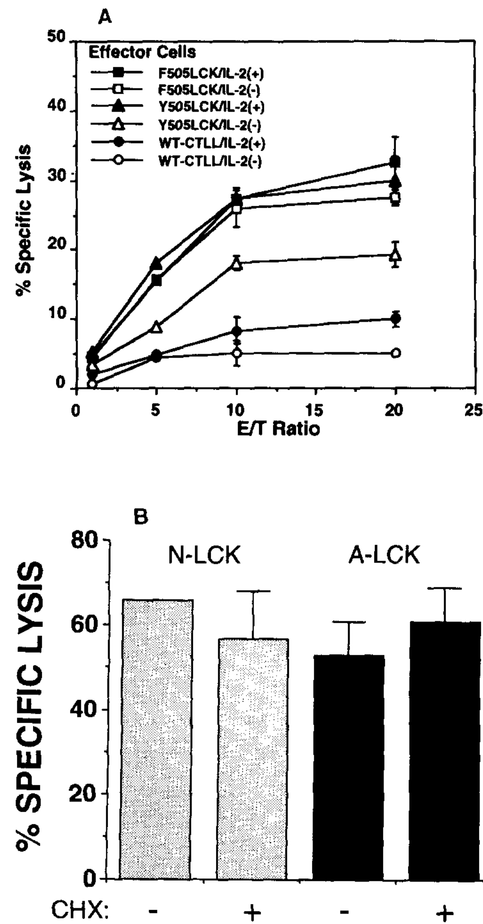

Figure 2. Characterization of cytolytic effector activity of *lck*-transfected CTLL-2 cells. In *A*, parental WT, *lck*(Y505)-transfected, and *lck*(F505)-transfected CTLL-2 cells were incubated with ^{51}Cr-labeled YAC-1 targets for 4 h at various E/T ratios in the presence or absence of 100 U/ml recombinant IL-2. The percent specific lysis was calculated and the data presented as mean ± SD for triplicate determinations. In *B*, CTLL-N-LCK and CTLL-A-LCK cells were treated with (+) or without (−) 20 μg/ml cycloheximide for 20 min in medium containing 100 U/ml recombinant IL-2, before addition of ^{51}Cr-labeled JURKAT cells at an E/T ratio of 20. ^{51}Cr release was measured 4 h later and percent lysis calculated (mean ± SD for triplicate determinations). Though representative data for only one tumor target cell line are shown in each of the panels, similar results were obtained with all three of the susceptible target cells (YAC-1, JURKAT, RLσ1) (not shown).

assays, inducing lysis of the same three target cell lines YAC-1, JURKAT, and RLσ1 that were lysed by the parental CTLL-2 cell clone but not of the seven other tumor cell lines that the untransfected CTLL-2 clone failed to lyse (Fig. 1). In contrast to parental CTLL-2 cells, however, CTLL-N-LCK and CTLL-A-LCK induced lysis of a greater percentage of target cells (Fig. 1). Induction of lysis by these T cells that contain elevated levels of p56-Lck kinase activity also was far less IL-2 dependent than for parental CTLL-2 cells. Even in the absence of IL-2, CTLL-N-LCK and CTLL-A-LCK cells exhibited cytolytic activity that was equal to or greater than that obtained with parental CTLL-2 cells that had been supplied with optimal concentrations of IL-2 (Fig. 1). This enhanced cytolytic activity of CTLL-N-LCK and CTLL-A-LCK cells relative to parental CTLL-2 cells was not due to improved survival or continued cellular proliferation in the absence of IL-2 (18).

Characterization of the Cytolytic Activity of Lck-transfected CTLL-2 Cells. The enhanced cytolytic activity of CTLL-N-LCK and CTLL-A-LCK cells, relative to parental CTLL-2 cells was apparent over a wide range of E/T ratios (Fig. 2 *A*). The lytic activity of these T cells was also dependent on cell–cell contact, as determined by cold target inhibition studies (not shown). Preincubation of CTLL-N-LCK and CTLL-A-LCK cells with the protein synthesis inhibitor cycloheximide had no effect on lytic activity, implying that gene expression and new protein synthesis are not required for the killing mechanism used by these cytolytic T cells (Fig. 2 *B*). Consistent with previous investigations of mechanisms of CTL-induced cell death (38), treatment of the tumor target cells with cycloheximide also did not impair cytolysis (not shown), implying that induction of new gene expression is also not required in the target cells. Furthermore, release of ^{51}Cr was preceded by prelysis genomic DNA digestion (as determined by quantitative DNA fragmentation assays [36]), and was partially suppressible by overexpression of the apoptosis-blocking oncoprotein Bcl-2 in the tumor target cells (36a), suggesting that an apoptotic rather than necrotic cytolytic mechanism was primarily involved (37).

Lck Upregulates Expression of the LFA-1 Adhesion Molecule. Probably the first step in T cell–mediated killing of target cells involves formation of cell–cell contact through the interactions of specific cell surface glycoproteins. For most cytolytic T cells, LAK cells, and TIL cells, perhaps the most quantitatively important cell adhesion molecules is LFA-1 (38–40). LFA-1 is a member of the integrin family of adhe-

sion molecules, and consists of a heterodimer representing α- and β-chain proteins, termed CD11a and CD18, respectively (41). LFA-1 can bind specifically to members of the ICAM family of *trans*-plasma membrane glycoproteins, including ICAM-1, ICAM-2, and related proteins (41).

We therefore compared the relative cell surface levels of CD11a and CD18 on parental CTLL-2 cells and control transfected CTLL-2-NEO cells with CTLL-N-LCK and CTLL-A-LCK cells using an indirect immunofluorescence assay. As shown in Fig. 3 *A*, relative levels of both CD11a and CD18 were markedly elevated on CTLL-N-LCK and CTLL-A-LCK cells. This increase in CD11a and CD18 expression was specific in that levels of CD2 and VLA-4, two other cell surface markers believed to play a role in the binding of T cells to other cells or to the extracellular matrix (reviewed in 41) were not elevated on genetically modified CTLL-2 cells that contained high levels of p56-Lck kinase activity (Fig. 3 *B* and our data). In fact, levels of some plasma membrane proteins such as CD3 and NK1.1 were actually lower on CTLL-N-LCK and CTLL-A-LCK cells than on parental untransfected CTLL-2 cells and the control transfected CTLL-2-NEO cell clone (Fig. 3 *B* and data not shown). CD3, for example, was essentially undetectable on both CTLL-N-LCK and CTLL-A-LCK cells. In addition, NK1.1 surface expression was slightly reduced on CTLL-N-LCK cells and markedly lower on CTLL-A-LCK cells (Fig. 3 *B*). Thus, high levels of p56-Lck kinase activity may either directly or indirectly downregulated the expression of CD3 and NK1.1 on CTLL-2 cells, while upregulating the levels of CD11a and CD18.

Lck-transfected CTLL-2 Cells Exhibit Enhanced Adhesion to Recombinant ICAM-1. To explore the functional status of LFA-1 molecules on CTLL-2 cells, we performed binding assays where T cells were tested for binding to a recombinant protein representing the extracellular domain of the LFA-1 ligand, ICAM-1. For these assays, the ICAM-1 fragment was adsorbed to wells of plastic microtiter culture plates, and T cells were allowed to bind for 15 min. T cells were tested for binding before and after activation with PMA, an agent known to induce an increase in the affinity of LFA-1 for binding to its specific ligands (41, 42). Compared with parental CTLL-2 cells, a higher percentage of both CTLL-N-LCK and CTLL-A-LCK cells bound to the recombinant fragment of ICAM-1 (Fig. 4). Enhanced binding was observed for both unstimulated and PMA-activated CTLL-N-LCK and CTLL-A-LCK cells. In fact, unstimulated CTLL-N-LCK and CTLL-A-LCK cells bound as well or better to the ICAM-1 fragment as PMA-activated parental CTLL-2 cells. Furthermore, upon PMA-induced activation, binding of CTLL-N-LCK and CTLL-A-LCK cells to the ICAM-1 fragment was as much as four- to sixfold higher than that obtained for parental CTLL-2 cells, depending on the concentration of ICAM-1 employed. The increased binding of CTLL-N-LCK and CTLL-A-LCK cells to the recombinant ICAM-1 fragment was specific, since background binding to BSA was not significantly higher for these cells (Fig. 4).

Antibodies to LFA-1 Abrogate Cytolytic Activity of Lck-transfected CTLL-2 Cells. To explore the relevance of LFA-1

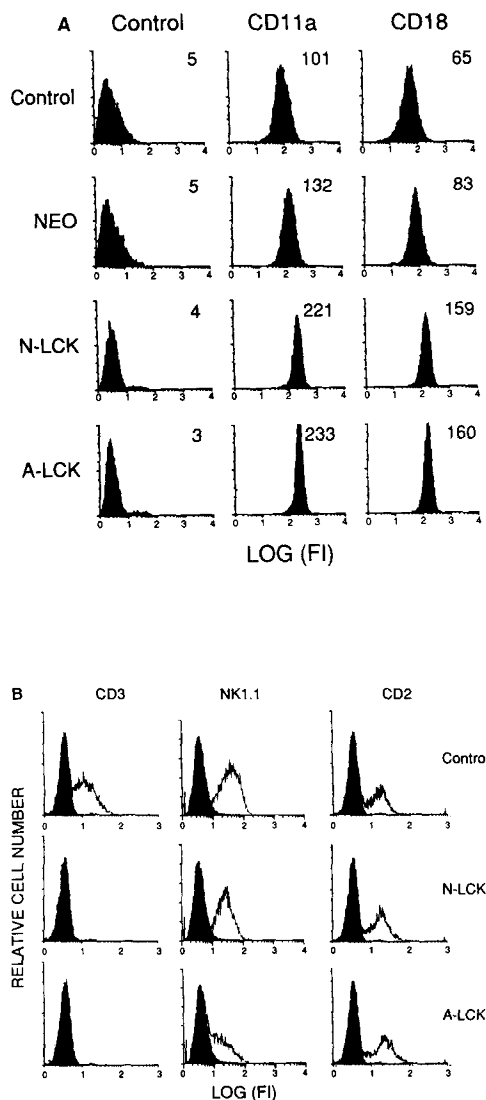

Figure 3. Transfected CTLL-2 cells with increased p56-Lck kinase activity have elevated levels of LFA-1 expression. In *A*, parental CTLL-2 cells (control) and CTLL-2 cells that had been stably transfected with expression plasmids encoding either normal Lck (N-LCK) or the Y505 mutant of Lck (A-LCK) were analyzed by indirect immunofluorescence assay for expression of CD11a and CD18. As an additional control for any nonspecific effects of the transfection procedure, a CTLL-2 cell clone was prepared that contained a neomycin-phosphotransferase (G418-resistance) without *lck* sequences (NEO). Cells were incubated with saturating amounts of the anti-CD11a antibody (FD4.4.1) (rat IgG2b), the anti-CD18 antibody (M18.5) (rat IgG2b), or a rat IgG2b negative control monoclonal antibody, followed by FITC-conjugated goat anti-rat IgG. Cells were analyzed using a flow cytometer and the data presented as histograms with relative cell number on the ordinant and relative fluorescence intensity (FI) on the abscissa in log-scale. The mean fluorescence channel is indicated for each sample. Similar data were obtained using alternative monoclonal antibodies to CD11a (M17/4.4.11.9) (rat IgG2a) and to CD18 (M18/2.a.12.7) (IgG2a) (not shown). In *B*, parental CTLL-2 cells (control), CTLL-N-LCK and CTLL-A-LCK cells were immunostained for CD3, NK1.1, and CD2 using specific monoclonal antibodies as described above. FACS® data are presented as histograms, with the results for negative control antibodies (isotype and subclass matched) in *black* and the results for monoclonal antibodies specific for CD3, NK1.1, and CD2 in *white*.

for the cytolytic effector function of CTLL-N-LCK and CTLL-A-LCK cells, we attempted to block killing by using monoclonal antibodies specific for CD11a. For these experiments, CTLL-N-LCK and CTLL-A-LCK cells were preincubated with saturating amounts of the monoclonal antibody M17, which recognizes a functionally important epitope on CD11a. As a control for any nonspecific effects of antibody binding, CTLL-N-LCK and CTLL-A-LCK cells were also preincubated with a monoclonal antibody M18/2.a.12.7 that recognizes a nonneutralizing epitope on CD18. As shown in Fig. 5 *A*, anti-CD11a antibody markedly reduced cytolytic killing of tumor target cells by both CTLL-N-LCK and CTLL-A-LCK cells. This effect of the antibody was largely specific, since the control antibody that recognizes a nonneutralizing epitope on LFA-1 had corresponding less effect on cytolytic function. Furthermore, cytolytic killing was not blocked by the anti-NK1.1 monoclonal antibody PK136 (Fig. 5 *B*), again demonstrating the specificity of the results and suggesting that NK1.1 is not involved in the cytolytic mechanism used by CTLL-2 cells. These data thus argue that LFA-1 plays an important role in the cytolytic mechanisms used by CTLL-N-LCK and CTLL-A-LCK cells. Though murine LFA-1 cannot recognize human ICAM-1 (43), the anti-LFA-1 antibody M17 did inhibit lysis of human JURKAT cells induced by CTLL-N-LCK and CTLL-A-LCK cells (not shown). Thus, murine LFA-1 may mediate binding of CTLL-2 cells to this human leukemic line via interactions with other isoforms of ICAM, such as ICAM-2 or ICAM-3/ICAM-R.

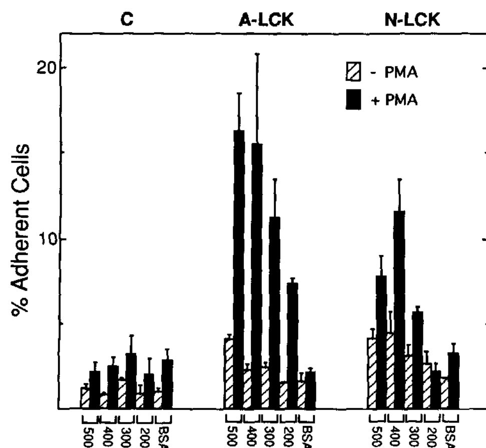

Figure 4. CTLL-2 cells with gene transfer–mediated elevations in p56-Lck kinase activity exhibit enhanced binding to a recombinant fragment of ICAM-1. Various amounts of purified recombinant protein (0–500 ng) representing a soluble fragment from the extracellular domain of ICAM-1 (sICAM-1) or 500 ng of BSA were absorbed to plastic microwells. Parental CTLL-2 cells (*C*), CTLL-N-LCK or CTLL-A-LCK cells were metabolically labeled with [^{3}H]thymidine, washed, and incubated in sICAM- or BSA-containing wells for 15 min in the presence (*solid bars*) or absence (*hatched bars*) of PMA. Nonadherent cells were removed and the percentage of adherent cells calculated as follows: percent adherent cells = [cpm from bound cells/(cpm from total input cells)] × 100. Data represent mean ± SD for three determinations.

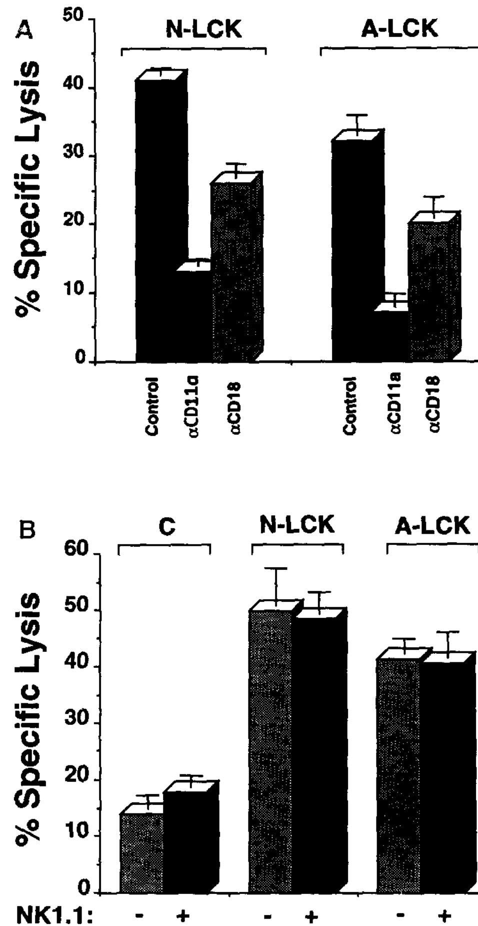

Figure 5. Anti-CD11a monoclonal antibody blocks cytolytic killing by *lck*-transfected cells. In (*A*), CTLL-2 cells that had been stably transfected with expression plasmids encoding normal Lck kinase (Y505Lck) or activated Lck containing a Tyr505 → Phe substitution (F505Lck) were incubated with either no antibody (control) or 100 μg/ml of a neutralizing anti-CD11a antibody M17/4.4.11.9 (rat IgG2a) or a nonneutralizing anti-CD18 antibody M18/2.a.12.7 (rat IgG2a). ^{51}Cr-labeled RLσ1 cells were then added at an E/T ratio of 10 and specific ^{51}Cr release was measured 4 h later. Data represent mean ± SD for three determinations. In (*B*), parental CTLL-2 cells (C), *lck*-transfected CTLL-2 (N-LCK), and *lck* (Y505)-transfected CTLL-2 cells (A-LCK) cells were incubated briefly with (+) or without (−) 10 μg/ml purified anti-NK1.1 antibody PK136 and then ^{51}Cr release was measured from JURKAT target cells 4 h later and the percent specific lysis (± SD) determined for triplicate samples. Similar data were obtained when RLσ1 cells were employed as the target (not shown).

Elevations in Lck Kinase Activity also Upregulate LFA-1 Expression in 32D.3 Cells. As a first attempt to determine whether elevations in LFA-1 expression were a general characteristic of cells that contain high levels of p56-Lck kinase activity versus a feature specific to CTLL-2 cells, we also stably introduced an expression plasmid encoding the p56-Lck(F505) protein into an IL-3–dependent myeloid cell clone, 32.D.3 (27). As a control, the same expression vector lacking *lck* sequences was stably introduced into 32D.3 cells, thus creating the line 32D-NEO. In vitro immune complex kinase assays confirmed the presence of markedly elevated levels of p56-Lck kinase activity in 32D-LCK(F505) cells relative to 32D-

NEO cells (Fig. 6 *A*). FACS® analysis of cells immunostained with antibodies specific for CD11a or CD18 revealed increased levels of both CD11a and CD18 on 32D-LCK(Y505F) cells relative to 32D-NEO (Fig. 6 *B*). These findings thus argue that the ability of p56-Lck to stimulate elevations in LFA-1 expression is not limited to CTLL-2 cells.

Lck Does Not Cause Constitutive Elevations in the Expression of Several Genes Associated with Cytolytic Killing. The observation that CTLL-2 cells which have gene transfer–mediated elevations in Lck kinase activity are more potent at killing target cells prompted us to examine the expression of a variety of genes whose encoded proteins have been associated either directly or indirectly with induction of cell death by CTLs. Furthermore, the finding that protein synthesis is not required for cytolytic killing by CTLL-N-LCK and CTLL-A-LCK cells (Fig. 2 *B*) argued that if increases in the expression of other genes were important, then the expression of these genes would have to be constitutively elevated as opposed to becoming activated after binding of T cells to specific target cells. In addition, since CTLL-N-LCK and CTLL-A-LCK cells kill in a far less IL-2–dependent manner than parental CTLL-2 cells, it was possible that genes whose expression was normally IL-2 inducible would be expressed in an IL-2–independent fashion in CTLL-N-LCK and CTLL-A-LCK cells. Relative levels of mRNAs were therefore compared in T cells that had been deprived of IL-2 for 4 h in an effort to detect any Lck-mediated alterations in gene expression that might account for the superior cytolytic activity of the CTLL-N-LCK and CTLL-A-LCK cells relative to parental CTLL-2 cells.

As shown in Fig. 7 *A*, no constitutive elevations in the levels of mRNAs encoding TNF-α, LT-α, or IFN-γ were detected in CTLL-N-LCK and CTLL-A-LCK cells. Relative levels of GM-CSF mRNA, a gene whose expression is IL-2 inducible (44), were also not constitutively increased in CTLL-N-LCK and CTLL-A-LCK cells despite their high levels of p56-Lck kinase activity. Stimulation of parental CTLL-2 cells with either IL-2 or the combination of the Ca^{2+} ionophore ionomycin and PMA served as a positive control for these Northern blot assays, inducing the accumulation of lymphokine mRNAs to easily detectable levels (Fig. 7 *A*).

It is interesting to note that steady-state levels of mRNAs for perforin, a protein with homology to the C9 component of complement (45), and granzyme A, a serine protease (46), were markedly reduced in CTLL-N-LCK and CTLL-A-LCK cells relative to parental CTLL-2 cells. Thus, CTLL-2 cells genetically modified to have increased levels of p56-Lck kinase activity are able to kill efficiently despite reductions in the relative amounts of expression of these two genes whose encoded proteins are stored in the cytotoxic granules of T cells and have been implicated directly in cytolytic effector mechanisms (47).

Another cell death–inducing protein that is stored in the cytotoxic granules of killer T cells is the RNA-binding protein apoptosin, also known as nucleolysin and T cell intracellular antigen (TIA) (48). Since antibodies that recognize the mouse form of TIA-1 have not been described to date, parental and transfected CTLL-2 cells were examined for expression of TIA-1 by Northern blot assay using a mouse TIA-1 cDNA hybridization probe (generously provided by Mark Boothby; Vanderbilt University, Nashville, TN). TIA-1 mRNA however was only barely perceptible, even when polyadenylate-selected mRNA was employed (not shown). Reverse transcriptase-PCR analysis under semiquantitative conditions confirmed the presence of at least low levels of TIA-1 mRNA in parental and transfected CTLL-2 cells but no consistent difference in the relative levels of TIA-1 mRNA was detected between untransfected CTLL-2 cells and the trans-

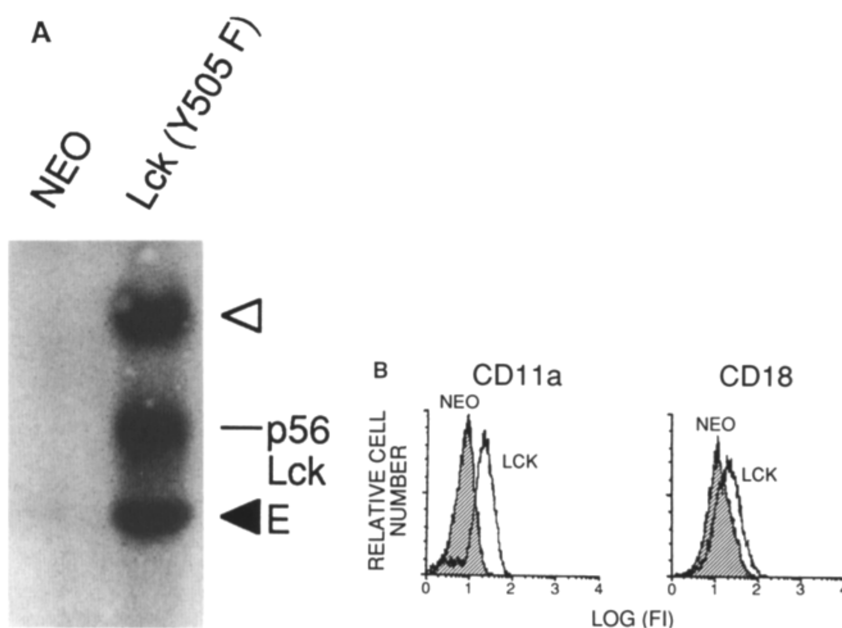

Figure 6. Production of activated p56-Lck(Y505F) kinase in 32D.3 cells upregulates LFA-1 expression. The IL-3–dependent myeloid clone 32D.3 was stably transfected with either pLXSN or pLXSN-*lck*(Y505F) plasmids and the resulting G418-resistant cells were analyzed for production of p56-Lck kinase activity (*A*) and expression of LFA-1 (*B*). In *A*, 1% NP-40 lysates were prepared from 2 × 10^6 cells and subjected to immunoprecipitation using an antiserum specific for p56-Lck. Immune complexes were incubated with a kinase reaction buffer containing γ-[^{32}P]ATP and the exogenous substrate enolase (*E*). The reaction products were analyzed by SDS-PAGE followed by autoradiography. (Data shown represent a 5-min exposure to X-ray film). An unidentified additional coprecipitating band was routinely observed in Lck immune complex kinase assays performed using 32D.3 cells (*open arrow*) but not other cells (not shown). In *B*, 32D.3 cells containing either pLXSN (NEO) or pLXSN-*lck*(Y505F) [Lck(Y505F)] were analyzed for surface expression of CD11a and CD18 by indirect immunofluorescence assay with flow cytometric analysis as described for Fig. 4. Results for 32D-NEO and 32D-Lck(Y505F) cells are presented as *shaded* and *unshaded* histograms, respectively.

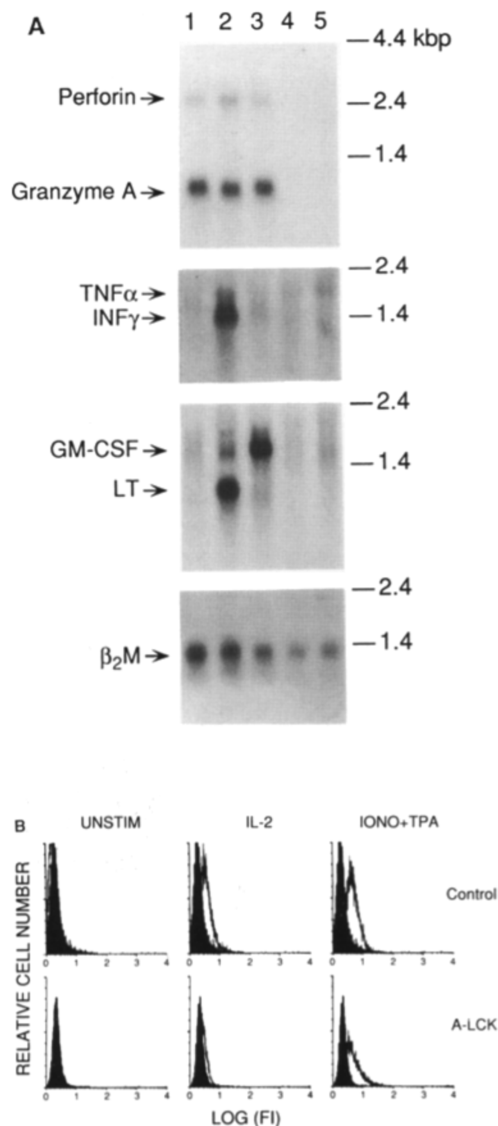

Figure 7. Analysis of expression of various mediators of CTL-induced cell death in CTLL-2 cells. In *A*, parental CTLL-2 cells (lanes *1–3*), CTLL-N-LCK cells (lane *4*), and CTLL-A-LCK cells (lane *5*) were stripped of IL-2 and rested for 4 h. Cells were then restimulated for 2 h with 100 U/ml recombinant IL-2 (lane *2*), or 0.5 μM ionomycin and 100 ng/ml PMA (lane *3*). Total cellular RNA was isolated and 10 μg aliquots analyzed by Northern blotting using probes specific for murine perforin, granzyme A, TNF-α, LT-α, INF-γ, GM-CSF, and β_2-microglobulin (β_2M). The 2-h stimulation time was empirically determined to be optimal for induction of all cytokine mRNAs in CTLL-2 cells (data not shown). The positions of selected molecular weight markers (RNA ladder BRL-GIBCO) are indicated. In *B*, untransfected CTLL-2 and CTLL-A-LCK cells were cultured for 2 d with or without IL-2 or the combination of PMA and A23187 and then incubated with TNF-R/Fc fusion protein followed by fluorescein-conjugated goat anti–human IgG antibody. Relative levels of immunofluorescence were measured by FACS® analysis and the data displayed as histograms with relative cell number (y-axis) vs. relative fluorescence intensity (FI) in log-scale (x-axis).

fected CTLL-N-LCK and CTLL-A-LCK cells, regardless of whether the cells were quiescent versus stimulated with IL-2 or with Ca^{2+}-ionophore and PMA (our unpublished observations).

In addition to cytotoxic proteins stored in secretory granules (TIA-1, proteases, perforins) and expression of genes encoding cytotoxic lymphokines (TNF-α, LT-α), signals for cell death can be delivered during cell–cell contact by receptor–ligand interactions that occur at the plasma membrane (49). We also therefore examined the cell surface levels of TNF-α, Fas-ligand, CD27-ligand, and CD30-ligand on parental and genetically modified CTLL-2 cells by indirect immunofluorescence assays, using recombinant chimeric proteins consisting of the extracellular domains of TNF-α, Fas, CD27, or CD30 fused with an immunoglobulin Fc region generously provided by Drs. Craig Smith and Ray Goodwin of Immunex, Inc. (Seattle, WA) and Carl Ware of University of California (Riverside, CA). Cell surface expression of TNF-α was present at levels slightly above background but was not appreciably elevated on CTLL-N-LCK and CTLL-A-LCK cells relative to control CTLL-2 cells, regardless of whether or not the cells were stimulated with IL-2 or the combination of PMA and Ca^{2+}-ionophore. Stimulation with PMA and Ca^{2+}-ionophore however did induce an increase in cell surface TNF-α but the relative levels of immunofluorescence were not higher for CTLL-A-LCK cells than control CTLL-2 cells and in fact tended to be somewhat lower (Fig. 7 *B*). FACS® analysis of the ligands for Fas, CD27, and CD30 produced levels of immunofluorescence that were not detectably above background on parental and genetically modified CTLL-2 cells even after stimulation with IL-2 or with PMA and Ca^{2+} ionophore (not shown). Thus, while it is possible that TNF-α or LT-α may participate in the cytotoxic mechanism by which CTLL-N-LCK and CTLL-A-LCK cells kill target cells, p56-Lck–mediated alterations in the expression of these genes do not appear to account for the superior cytolytic activity of CTLL-2 cells that contain gene transfer–mediated elevations in Lck kinase activity.

Lck Enhances Activation-induced Secretion by CTLL-2 Cells. During T cell–mediated cytolytic killing, granules are released by the T cells through an exocytosis process, thus delivering perforins, proteases, apoptosins, Ca^{2+}, ATP, and probably other potentially cytotoxic substances to the target cell. Also contained within these granules is β-glucuronidase, an enzyme whose activity can be measured by a simple colorimetric assay (50).

To determine whether Lck influences the process of granule exocytosis, parental CTLL-2 cells, as well as control transfected CTLL-NEO cells, and the CTLL-N-LCK and CTLL-A-LCK cells were cultured for 4 h in the presence or absence of PMA and the Ca^{2+}-ionophore, A23187. The combination of PMA and A23187 is known to trigger granule exocytosis in a process that recapitulates many of the biochemical events that occur during normal T cell activation (35). Both parental and genetically modified CTLL-2 cells displayed very little spontaneous secretion of β-glucuronidase regardless of their levels of Lck kinase activity. Activation-induced secretion, however, was much higher (~7–14-fold) for CTLL-A-LCK cells which contain the activated Tyr505 → Phe version of Lck kinase, compared with parental CTLL-2 and CTLL-NEO cells (Table 1). CTLL-N-LCK cells, which overproduce normal Lck kinase, secreted at levels intermediate to those

Table 1. *Lck Enhances Activation-induced Exocytosis*

	Experiment 1		Experiment 2		Experiment 3	
			β-glucuronidase			
	Percent	Total	Percent	Total	Percent	Total
CTLL-2	0 ± 0.6	0.63 ± 0.02	0.8 ± 0.4	0.39 ± 0.01	1.4 ± 0.3	0.18 ± 0.0
CTLL-NEO	1.6 ± 1.0	0.49 ± 0.01	2.6 ± 0.3	0.36 ± 0.01	3.7 ± 0.0	0.13 ± 0.02
CTLL-N-LCK	12.1 ± 1.2	0.39 ± 0.03	11.2 ± 1.0	0.24 ± 0.07	16.8 ± 3.1	0.13 ± 0.0
CTLL-A-LCK	28.5 ± 0.8	0.39 ± 0.01	19.7 ± 10.5	0.19 ± 0.0	42.0 ± 1.7	0.16 ± 0.01
			BLT-esterase			
	Secreted	Total	Secreted	Total	Secreted	Total
CTLL-2	0.004 ± 0.004	0.137 ± 0.036	0 ± 0	0.631 ± 0.068	0.008 ± 0.01	0.409 ± 0.034
CTLL-NEO	ND	ND	ND	ND	0.002 ± 0.002	0.245 ± 0.025
CTLL-N-LCK	0.004 ± 0.001	0.006 ± 0.007	0 ± 0	0.004 ± 0.004	0 ± 0	0 ± 0
CTLL-A-LCK	0.003 ± 0.002	0.004 ± 0.002	0 ± 0	0 ± 0	ND	ND

CTLL-2, CTLL-NEO, CTL-N-LCK, and CTLL-LCK cells were cultured for 4 h in medium with or without the combination of PMA and A23187. Culture supernatants were then assayed for relative levels of β-glucuronidase or BLT esterase enzyme activities (mean ± SD; $n = 3$). Total cellular associated β-glucuronidase and BLT esterase enzyme activity was also measured using cell lysates prepared from an equal number of unstimulated cells. In the top half, the percent of cellular β-glucuronidase activity released into culture supernatants was calculated, after subtraction of spontaneous release that occurred in the absence of stimulation with PMA and A23187 (always ≤5% of total cellular β-glucuronidase). The total cellular levels of β-glucuronidase were also compared among control and transfected CTLL-2 cells and the data expressed as OD 550 nm units. In the bottom half, relative amounts of secreted and total BLT esterase activity were measured and the data expressed as OD 412 nm units. Secreted BLT esterase activity represents the difference between PMA and A23187-induced and spontaneous released. Spontaneous BLT esterase release was comparable for all cell lines (not shown).

observed for CTLL-A-LCK and parental CTLL-2 cells (Table 1). The increased levels of β-glucuronidase release from CTLL-2 cells that contained elevated levels of p56-Lck kinase activity was not due to induction of cell lysis by PMA and A23187, since >95% of cells continued to exclude trypan blue dye at the end of the 4-h assay (not shown).

Though CTLL-N-LCK and CTLL-A-LCK cells released more β-glucuronidase into the medium than parental CTLL-2 and CTLL-NEO cells after stimulation, the total cellular levels of this enzyme were not higher in CTLL-2 cells that contain gene transfer–mediated elevations in Lck kinase activity compared to control cells (Table 1). Also, the approximate number and size of granules was not increased in CTLL-A-LCK cells relative to parental CTLL-2 cells, based on conventional light and electron microscopic analyses (our unpublished observations). Thus, high levels of p56-Lck kinase activity are associated with a marked increase in the efficiency or rate of activation-induced exocytosis rather than due to elevations in the size of the cytotoxic granule pool. Though increased levels of p56-Lck kinase activity clearly enhanced exocytosis induced by PMA and Ca^{2+}-ionophore, we have been unable to confirm these results using tumor targets cells in place of pharmacological T cell activating agents, because of the complication of β-glucuronidase release from dying targets.

In contrast to β-glucuronidase, CTLL-2 cells did not secrete *N*-α-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) esterases after stimulation with PMA and A23187. In fact, lysates prepared from parental and genetically modified CTLL-2 cells contained little BLT esterase activity, suggesting that these cells are relatively deficient in this class of enzymes commonly used as a measure of degranulation of CTLs (Table 1).

Discussion

Increases in the specific activity of the p56-Lck kinase can be induced in lymphocytes via a number of plasma membrane receptors whose cytoplasmic domains can interact directly or indirectly with this PTK, including CD4, CD8, CD16, and the β-chain of the IL-2 receptor complex (2–6). Here we used gene transfer approaches to explore the function of Lck in an IL-2–dependent cytolytic T cell clone derived from the CTLL-2 cell line. Gene transfer–mediated elevations in the levels of p56-Lck kinase activity markedly enhanced the cytolytic effector function of these T cells (Figs. 1 and 2) without altering their dependence on IL-2 for growth and survival in culture (18). The events involved in CTL effector function can be divided into at least four discrete steps: (*a*) binding of T cells to target cells; (*b*) triggering of the T cells through appropriate receptors to induce a variety of intracellular biochemical events broadly termed T cell "activation"; (*c*) exocytosis of granules that release toxic proteins and other substances onto the surface of the target cell; and

in some cases (*d*) delivery of additional cell death signals to the target cell via membrane-associated or secreted cytokines and other proteins such as Fas-ligand (reviewed in 49). Lck appears to regulate at least two of these four steps: binding and exocytosis.

Both CTLL-2 and 32D.3 cells that contained gene transfer–mediated elevations in Lck kinase activity displayed higher surface levels of CD11a and CD18, the α- and β-subunits of the β_2 integrin LFA-1. The affinity of LFA-1 for its ICAM counter-receptors becomes strikingly increased upon T cell activation through a poorly understood mechanism that appears to depend on protein kinase C and that can be emulated by stimulation of LFA-1–expressing cells with the phorbol esters such as PMA (41, 42). Thus, LFA-1 can potentially participate in two phases of the cell adhesion process involved in CTL function. First, low-affinity binding of LFA-1 to its receptors may help to increase the time of interaction between effector and target, thus allowing for triggering of other receptors on the T cell that can generate signals for T cell activation (e.g., TCR with MHC–class I/antigen). After T cell activation, LFA-1 then mediates high-affinity binding of T cell and target, an event that also indirectly facilitates TCR interaction with MHC/antigen in many cases, and thus potentiates the TCR-mediated activation process. In CTLL-2 cells, gene transfer–mediated elevations in p56-Lck kinase activity increased both constitutive and PMA-induced binding of these T cells to a recombinant fragment of ICAM-1 (Fig. 4), suggesting that the elevated levels of LFA-1 on CTLL-2 cells may enhance both pre- and postactivation adhesion to tumor target cells. Though it remains to be determined whether a general role for Src-like kinases in the regulation of cell adhesion will be found, we have also observed elevations in LFA-1 expression in Lyn-transfected myeloid cell lines (Torigoe, T., J. Millan, and J. C. Reed, manuscript in preparation), which are known to undergo specific elevations in their levels of Lyn kinase activity in response to IL-3 and GM-CSF (51).

In addition to upregulation of LFA-1 expression, gene transfer–mediated elevations in Lck kinase activity resulted in markedly enhanced granule secretion by CTLL-2 cells. Previously, pp60-c-Src has been implicated in the regulation of exocytosis in bovine chromaffin cells, where it is found in association not only with the plasma membrane but also with the membranes of chromaffin granules which contain norepinephrine and other secretory products (52, 53). Furthermore, pp60-c-Src has been found in direct association with synaptophysin, and can induce phosphorylation of this 38-kD protein which is a major constituent of synaptic vesicles and is thought to play a critical role in the exocytosis of synaptic vesicles in neuroendocrine cells (54). In CTLL-2 cells, secretion of β-glucuronidase, a lysosomal protein that is stored in the cytotoxic granules of cytolytic T cells (50), did not occur constitutively but rather required activation signals which could be provided by the combination of PMA and A23187 (Table 1). Lck appeared to potentiate those signals for exocytosis of granules, with the constitutively activated F505 version of Lck having greater effect than over-production of normal Lck kinase. It remains to be determined whether Lck directly regulates degranulation in cytolytic T cells by analogy to pp60-c-Src that associates with and phosphorylates proteins associated with secretory vesicles in neuroendocrine cells.

Within the cytotoxic granules of CTLs are at least three substances that have been reported to induce rapid DNA fragmentation and apoptosis in target cells: TIA-1, proteases, and ATP. Both TIA-1 and proteases require perforin or perforin-like proteins to gain access to the cytosol of target cells and thus exert their cell death–inducing effects. ATP, in contrast, can exert its effect extracellularly, perhaps via purinergic receptors or ectokinases (55). Though perforin mRNA levels were very low in CTLL-N-LCK and CTLL-A-LCK cells, it is possible that enough perforin is made by these T cells to provide at least a transient passage through the plasma membrane of target cells for entry of TIA-1 or proteases (56). Similarly, though granzyme A mRNA was undetectable in CTLL-N-LCK and CTLL-A-LCK cells and BLT esterase activity was very low in these cells, granzyme A represents only one of many different serine proteases that can be present within the granules of CTLs and the BLT esterase assay does not detect some classes of proteases such as cysteine proteases (57). Thus, a perforin- or protease-dependent mechanism for induction of cell death cannot be excluded from the data available thus far. Alternatively, the CTLL-2 cells may use mechanisms for induction of cell death that do not depend on injection of molecules across the plasma membrane of the target cell, such as those involving ATP release or interactions of plasma membrane-associated TNF-α, LT, and Fas-ligand with their specific receptors on target cells. Preliminary attempts to block the cytolytic function of parental and *lck*-transfected CTLL-2 cells using neutralizing anti-TNF-α antibodies however have been unsuccessful (our unpublished observations). We have also found that *lck*-transfected CTLL-2 cells do not lyse the TNF-sensitive target, WEHI-164 (our unpublished observations). Whatever the cell death mechanism used by CTLL-2 cells to induce target cell lysis, our findings are in agreement with those of other investigators who have noted a lack of correlation between cytolytic activity and the perforin and BLT esterase content of T cells and their granules (39, 58, 59).

Analysis of CTLL-N-LCK and CTLL-A-LCK cells revealed reduced levels of granzyme A mRNA relative to parental CTLL-2 cells, suggesting that p56-Lck downregulates expression of this serine protease-encoding gene. Velotti et al. (60) previously demonstrated that interaction of target cells with NK cell and LAK cells induces transient decreases in granzyme A mRNA levels. Thus, if p56-Lck kinase activation occurs during the stimulation of NK and LAK cells by appropriate target cells, it could be that p56-Lck regulates a negative feedback loop that involves downmodulation of granzyme A. We cannot exclude the possibility however that the reductions in granzyme A, as well as those in CD3 and NK1.1, seen in *lck*-transfected CTLL-2 reflect a selection process as opposed to direct downregulation by a p56-Lck–dependent signal transduction pathway. For example, the ex-

pression of CD3, NK1.1, and granzyme A could somehow be detrimental to the growth or survival of T cells that have constitutively high levels p56-Lck kinase activity. In this regard, cross-linking of CD4 on the surface of T cells (a procedure known to transiently activate p56-Lck [2]) prior to stimulation through CD3 has been shown to induce apoptosis of T cells (61), suggesting that constitutively high levels of p56-Lck kinase activity could be deleterious for CD3-bearing T cells.

The receptor(s) used by CTLL-2 cells for target cell recognition and triggering of T cell activation and exocytosis are unknown. Though cytolytic killing by CTLL-2 cells was clearly not MHC restricted, there was a specificity to the killing in that only 3 of 10 tumor lines were effective targets for these T cells. It seems unlikely that either CD3 or NK1.1 are involved in this process, since Lck gene transfer markedly downregulated the expression of these cell surface antigens that are known to be capable of mediating cytolytic killing in antibody-redirected cytolysis assays. Consistent with previous studies of LFA-1/ICAM-1 interactions (41), the expression of ICAMs on the surface of the target cell is also insufficient for triggering cytolysis by CTLL-2 cells, since FACS® analysis demonstrated high relative levels of ICAM-1 on the surface of some tumor cell lines which CTLL-2 cells failed to lyse (our unpublished observations). Though the specific recognition receptors involved are unknown, probably the cytolytic activity of parental and genetically modified CTLL-2 cells can be enlikened to that of LAK cells, which are functionally characterized by their ability to lyse a variety of NK-sensitive and -resistant tumor cell targets in an MHC-nonrestricted fashion (62). Consistent with this notion, the original report of CTLL-2 cells described cytolytic activity against both syngenic and allogenic tumor cells (26). The presence of both a classical T cell marker CD3 and the NK cell marker NK1.1 on parental CTLL-2 cells is also reminiscent of LAK cells, since immunophenotypic analysis of these cells has sometimes revealed subpopulations of cells that express combinations of T cell and NK cell markers (63).

Recent investigations of lymphocytes recovered from the spleens of animals that bear solid tumors have demonstrated a decrease in the relative levels of p56-Lck and some other signal transducing proteins, suggesting that tumor-induced defects in T cell signal transduction pathways may provide an explanation for the failure of the immune system to respond to and eliminate cancer cells in many cases (24). Similarly, reduced levels of p56-Lck as well as the TCR-ζ chain have been described in the TILs of patients with renal carcinoma (25). The findings presented here which link p56-Lck to the regulation of specific events involved in cytolytic effector function may therefore provide additional insights into the molecular mechanisms of tolerance induction by tumors. Further in vitro and in vivo studies are required however to assess the overall significance of p56-Lck in the complex series of molecular and cellular events that determine the outcome of host immune responses to tumors.

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