DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes

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Summary

Intercellular adhesion molecules play an important role in the generation of T lymphocyte-mediated immune responses.   Here, we describe a novel accessory molecule, DNAX accessory molecule-1 (DNAM-1), that is constitutively expressed on the majority of peripheral blood T lymphocytes.   DNAM-1 is a 65 KD transmembrane glycoprotein consisting of 318 amino acids including two immunoglobulin-like domains.   Anti-DNAM-1 monoclonal antibody (mAb) inhibits T and NK cell-mediated cytotoxicity against a variety of tumor cell targets and blocks cytokine production by alloantigen-specific T cells.   In addition, DNAM-1 is a tyrosine-phosphorylated signal transducing molecule that participates in primary adhesion during cytotoxic T lymphocytes (CTL)-mediated cytotoxicity.

Introduction

T lymphocyte-mediated cytotoxicity is a complex process involving engagement of the CD3/T cell antigen receptor complex (TcR), signal transduction, and subsequent cytolysis of target cells (Apasov, et al., 1993; Berke, 1994, 1995; Henkart, 1994). In addition to specific antigen recognition by the TcR, CTL require the engagement of intercellular accessory molecules on T cells by their respective ligands expressed on target cells (Springer, 1990; Phillips, et al., 1991; O'Rourke and Mescher, 1992; Rodrigues, et al., 1992). A variety of accessory molecules expressed on T cells (including CD8, CD4, CD28, LFA-1 and CD2) are involved in this process (Bierer, et al., 1989; Janeway, 1988; Kupfer and Singer, 1989; Springer et al. 1987; Dustin and Springer, 1989; Kishimoto, et al., 1987; Vives, 1994). Accessory molecules facilitate the intercellular conjugation of CTL with target cells and transduce signals which augment or synergize with signals from the TcR (Bierer, et al., 1989; Springer, 1990).

The adhesion molecules LFA-1 and CD2 are crucial for cytotoxicity mediated by many CTL (Shaw, et al., 1986; Springer, 1990). CTL-mediated cytotoxicity, however, is often not completely blocked in the presence of anti-CD2 or anti-LFA-1 mAb (unpublished observation), suggesting the existence of other adhesion molecule(s) on CTL that are involved in the cytolytic process. In the present investigation, we have identified a novel signal transducing adhesion molecule, DNAM-1, that is involved in cytotoxicity and lymphokine secretion mediated by CTL. Here, we describe the functional and molecular characteristics of DNAM-1.

Results

DNAM-1 is involved in T cell and NK cell-mediated cytotoxicity

Many CTL clones mediate "promiscuous" or "natural killer (NK)-like" lysis against a variety of tumor cell targets by an unknown mechanism that is independent of TcR engagement (TcR-independent cytotoxicity).   To identify the accessory molecules involved in this process, we generated mAbs against a human CTL clone that inhibited TcR-independent   cytotoxicity.   The mAb DX11 was selected because of its ability to block cytotoxicity against a variety of tumor cell lines (Fig. 1A).   The antigen recognized by the DX11 mAb was designated DNAX accessory molecule-1 (DNAM-1).   The DX11 mAb inhibited lysis of both hematopoietic and non-hematopoietic target cells, but was selective in that anti-DNAM-1 did not affect killing of certain target cells (e.g. the JY and 721.221 EBV-transformed B lymphoblastoid cell lines) (Fig. 1A).   Since DNAM-1 is expressed on the CTL but not on many of these tumor cell targets, these results indicated that DNAM-1 inhibits cytotoxicity at the level of the effector cell.   Similar results were obtained when CTL were precoated with DX11 mAb and washed prior to the addition of the target cell lines (not shown).   DNAM-1 is also expressed on NK cell clones and $F(ab')_2$ fragments of the DX11 mAb efficiently inhibited the NK cell-mediated cytotoxicity against these tumor cell targets, but did not affect cytotoxicity against JY or 721.221 (Fig. 1B).

DNAM-1 expression on hematopoietic cells

In peripheral blood, DNAM-1 is expressed on the majority of α/β-TcR+ T cells, $\gamma/\delta-$TcR+ T cells, NK cells (CD3-,CD56+), monocytes (CD14+), and a subset of B cells (Fig. 2), but is not on granulocytes or erythrocytes (data not shown).   DNAM-1 is also expressed on a subset of thymocytes co-expressing high surface density CD3 and on a

variety of transformed hematopoietic cells (data not shown).   DNAM-1, however, is not present on fibroblastic cell lines nor was it detected on tumor cell lines of epithelial or neuronal derivation (including the Colo-205 colon carcinoma cell line, the PA-1 ovarian teratocarcinoma cell line, the SK-N-SH neuroblastoma cell line, the MCF-7 breast carcinoma cell line, the BDMEL-1 melanoma cell line, and the CaOV3 ovarian carcinoma cell line).

DNAM-1 is involved in the antigen-specific cytotoxicity and lymphokine secretion mediated by CTL

HLA-A2-specific CD8+ CTL clones expressing cell surface DNAM-1 (Fig. 3A) lysed the HLA-A2-expressing tumor lines JY and Colo-205 and cytotoxicity was completely inhibited by anti-HLA-A2 or anti-CD3 mAbs.   Addition of the DX11 mAb to the cytotoxicity assays inhibited cytolysis of Colo-205, but had no effect on the cytolysis of JY (Fig. 3B).   Anti-CD18 mAb blocked alloantigen-specific lysis of both Colo-205 and JY, demonstrating that LFA-1 is involved in cytotoxicity against both targets whereas DNAM-1 is preferentially involved in killing of Colo-205 and not JY.

Experiments were undertaken to determine if DNAM-1 is also required for lymphokine secretion initiated by alloantigen recognition.   DX11 mAb, as well as anti-CD18 or anti-HLA-A2 mAb, significantly inhibited the secretion of TNFα and IFN-γ by the CTL co-cultured with Colo-205 (Fig. 3C).   By contrast, the cytokine secretion by the CTL clones induced by JY was not inhibited by DX11 mAb.

DNAM-1 is a signal transducing molecule.

mAb against CD2 and CD3 are able to trigger cytolytic activity when CTL are co-cultured with target cells expressing Fc receptors (Spits, et al., 1985; Mentzer, et al, 1985; Siliciano, et al., 1985; Scott, et al., 1989).   As demonstrated in Fig. 4A, the DX11 mAb is also capable of inducing antibody-mediated re-direct cytolysis when CTL

are co-cultured with the murine Fc receptor-bearing P815 mastocytoma.   The magnitude of the antibody-induced cytolysis was comparable to that observed with a mAb against CD3.   In addition to enhancing conjugate formation between the CTL and P815 targets, DNAM-1 also appears to actively participate in signaling.   As shown in Fig. 4B, engagement of DNAM-1 by DX11 mAb-coated P815 target cells resulted in tyrosine phosphorylation of DNAM-1, with maximum phosphorylation occurring about 1 min after addition of the mAb.

DNAM-1 protein purification and cDNA cloning

DNAM-1 protein was purified from lysates of peripheral blood mononuclear cells (PBMC) using lectin affinity and   DX11 mAb-affinity chromatography.   The amino acid sequences of the N-terminal and   three internal peptide fragments were obtained from the purified DNAM-1 protein (Fig. 5A).   Degenerate oligonucleotide primers were designed based on the N-terminal and an internal peptide residues and used to amplify a 100 base pair (bp) cDNA fragment from a T cell cDNA library.   The 100 bp fragment was then used as a probe to isolate a full length DNAM-1 cDNA (Fig. 5A).   The DNAM-1 cDNA is 2,603 bp and contains an open reading frame encoding a type-I transmembrane protein with an 18 amino acid (aa) leader sequence, 230 aa extracellular domain, 28 aa transmembrane domain, and 60 aa cytoplasmic region. Two pairs of cysteine residues in the extracellular domain were flanked by consensus sequences for   immunoglobulin-like domains (Fig. 5B), indicating that DNAM-1 is a member of the immunoglobulin supergene family.   Three tyrosines are present in the cytoplasmic domain, and the REDIYNYP sequence may be phosphorylated by a src kinase (on the Y just down from the EDI) and is a possible binding site for Grb2 (Cantley and Songyang, 1994). Southern blot analysis of human genomic DNA suggests that DNAM-1 is encoded by a single gene (not shown).   The DNAM-1 gene is located on chromosome 18q22.3, as determined by fluorescence <u>in</u> <u>situ</u> hybridization (Callen, et al.,

1990). DNAM-1 is unique, demonstrating no significant homology to other protein or nucleotide sequences in the GenBank, EMBL or PIR databases. Although demonstrating only 22% homology, DNAM-1 is most similar to the tactile receptor (Wang, et al., 1992) that is also expressed on CTL.

The DNAM-1 cDNA was transfected into COS-7 cells, which resulted in surface expression of DNAM-1 protein as detected by immunofluorescence using the DX11 mAb (Fig. 5C). The molecular weight of DNAM-1 immunoprecipitated from ^{125}I-labeled lysates of both PBMC and COS-7 cells transfected with DNAM-1 cDNA was ~65 KD when analyzed by SDS-PAGE under both reducing and non-reducing conditions (Fig. 5C). The mobility of DNAM-1 decreased from ~65 KD to ~35 KD after treatment with N-glycosidase F (Fig. 5D), consistent with the size of the polypeptide predicted from the DNAM-1 cDNA and the presence of 8 potential N-linked glycosylation sites in the extracellular domain (Fig. 5A).

DNAM-1 is an adhesion molecule.

The observation that DX11 mAb blocked CTL and NK lysis of some, but not all, targets suggested that certain cells may express cell surface ligands for DNAM-1. Therefore, experiments were performed to determine if cells with a putative DNAM-1 ligand, such as Colo-205, would specifically adhere to COS-7 cells transfected with DNAM-1 cDNA. As shown in Fig. 6, Colo-205 cells displayed significant binding to DNAM-1+ COS-7 transfectants and this binding was specifically inhibited by the DX11 mAb. Visual examination of these cultures also indicated substantial clustering of Colo-205 cells to DNAM-1 transfected COS-7 cells, but not untransfected COS-7 cells (not shown).

Discussion

A successful strategy to identify membrane receptor on T lymphocytes involved in CTL function has been to generate monoclonal antibodies that block cytolysis. Using this approach, LFA-1 (CD11a/CD18), CD2, and CD8 have been implicated as accessory molecules participating in cytolytic function (reviewed in Bierer and Burakoff, 1991; Berke, 1994). However, it is possible to generate CTL from patients lacking LFA-1 expression (van de Wiel-van Kemenade, et al., 1992) and from mice with disrupted CD2 genes (Killeen, et al., 1992). Also because mAb against LFA-1, CD2, and CD8 often only partially block cytotoxicity, it seemed likely that other receptors involved in CTL function exist, prompting us to search for such molecules. DNAM-1 was identified by selecting a mAb that inhibit CTL and NK cell-mediated lysis. Like CD2 and LFA-1, DNAM-1 is expressed on most T and NK cells and functions as an intercellular adhesion molecule involved in cytolytic activity and lymphokine secretion. Functional studies suggest that the ligand for DNAM-1 (DNAM-1L) is a cell surface antigen broadly expressed on both hematopoietic and non-hematopoietic tissues. Similarly, the ligands for LFA-1, i.e. CD54 (Marlin and Springer, 1987; Rothlein, et al., 1986), and CD2, i.e. CD58 (Krensky, et al., 1983; Dustin, et al., 1987), are also expressed on many cell types, allowing NK and T cells to interact with endothelial cells and epithelial tissues during immune responses. Further studies are underway to identify the DNAM-1L and determine the structural requirements involved in DNAM-1 binding.

Because essentially all T and NK cells express LFA-1, CD2, and DNAM-1 the relative contribution of these receptors may depend upon the array and density of their ligands present on the antigen-presenting cells or target cells. In some situations, blocking any single receptor-ligand interaction may only partially or fail to inhibit CTL function because other molecules are operational. Under other circumstances the

combination of several receptors may be necessary to achieve the threshold of binding or signaling required to activate effector function, in which case blocking any one of several accessory molecules may be sufficient to completely inhibit cytotoxicity.   The function of some adhesion molecules is affected by signals transmitted by the TcR. Dustin and Springer have shown that the affinity of LFA-1 for its ligand is transiently enhanced after TcR binding. (Dustin and Springer, 1989).   Thus, the role of the various adhesion molecules in lymphocyte - APC interactions may be dictated by a dynamic process and influenced by signals generated through other membrane receptors.

Intercellular adhesion molecules provide not only for cell binding, but also participate in signal transduction.   mAbs against CD2 function as agonists and can trigger CTL and NK cell cytolytic function (Siliciano, et al., 1985).   Similarly, cross-linking DNAM-1 with mAb induces cytolysis mediated by CTL and NK cells and also results in tyrosine phosphorylation of the DNAM-1 molecule.   Presently, the tyrosine kinase responsible for DNAM-1 phosphorylation is unknown and further studies are warranted to define the biochemical events accompanying DNAM-1 signal transduction.

In summary, our studies have shown that DNAM-1 is a novel receptor involved in intercellular adhesion and lymphocyte signaling.   Adhesion molecules play a critical role in a variety of immune responses including allograft rejection, tolerance (Isobe, et al., 1992), cell differentiation (Gunji, et al., 1992), lymphocyte homing (Hogg & Landis, 1993),   and viral and tumor immunity (Springer, 1990; Phillips, et al., 1991).   Since DNAM-1 is broadly expressed on a variety of peripheral leukocytes including T cells, NK cells, monocytes, and B cells, it may be involved in the wide range of immune responses.

Experimental Procedures

Hybridomas and Cell Lines.

The anti-DNAM-1 mAb DX11 (IgG1, κ) was generated by fusing the Sp2/0 myeloma cell line with splenocytes from a BALB/c mouse immunized with a human CTL clone.   Hybridomas were cloned by using a FACStar (Becton Dickinson Immunocytometry Systems, San Jose, CA).   Colo-205 is a colon carcinoma cell line, PA-1 is an ovarian teratocarcinoma cell line, SK-N-SH is a neuroblastoma cell line, MCF-7 is a breast carcinoma cell line, K562 is an erythroleukemia cell line, Jurkat is a T cell leukemia cell line, U937 is a myeloid leukemia cell line, JY and 721.221 are EBV-transformed B lymphoblastoid cell lines (American Type Culture Collection, Rockville, MD).   BDMEL-1 is a melanoma cell line generated by our laboratory. Colo-205 and JY express HLA-A2. COS-7 cells were generously provided by Dr. Brian Seed, Massachusetts General Hospital, Boston, MA.

mAbs and Flow Cytometry.

Control IgG, anti-CD3 (Leu 4 mAb), anti-CD18 (L130 mAb), anti-CD8α (Leu 2 mAb), anti-CD56 (L185 mAb), anti-CD19 (Leu 12 mAb) and anti-CD14 (Leu M3 mAb) were generously provided by Becton Dickinson Immunocytometry Systems. CyChrome-conjugated anti-CD3 was purchased from Pharmingen, San Diego, CA. Anti-phosphotyrosine mAb (4G10) was purchased from Upstate Biotechnology Inc., Lake Placid, NY.   Anti-HLA-A2 mAb was purified from MA2.1 hybridoma obtained from ATCC (McMichael, A.J., et al, 1980).   F(ab')$_2$ fragments were prepared by digesting DX11 mAb (IgG1) and anti-CD18 (IgG1) with immobilized pepsin (10 mg IgG in 10 ml of 0.2 M sodium citrate, 0.15 M NaCl buffer, pH 3.5, with 2.5 ml immobilized pepsin for 2 h at 37°C) (Pierce, Rockford, IL), and afterwards removing residual intact mAb by protein

A affinity chromatography.   Purity of the F(ab')$_2$ fragments were determined by SDS-PAGE.   Methods of immunofluorescent staining and flow cytometry have been described previously (Lanier and Recktenwald, 1991).   Lymphocytes and monocytes were identified by their characteristic forward and right angle light scatter properties, as described previously   (Lanier and Recktenwald, 1991).

CTL and NK clones.

CTL and NK clones were established from peripheral blood of healthy donors as described (Phillips, et al., 1991).   In brief, PBMC (Stanford Blood Center, Stanford, CA) were isolated by Ficoll/Hypaque centrifugation.   CD3+ T cell clones or CD3-CD56+ NK were cloned by flow cytometry and cultured using the conditions described previously (Yssel, et al., 1984).   Alloantigen (HLA-A2)-specific CTL clones were established from by mixed lymphocyte cultures using an HLA-A2+ EBV-transformed B lymphoblastoid cell line, JY, as the stimulator and PBMC from HLA-A2- donors as responders, as described (Yssel, et al., 1984; Phillips, et al., 1991).

Cytotoxicity assay.

Cell lines were labeled with ^{51}Cr and used as targets in a 4-h radioisotope release assay, as described (Lanier, et al., 1983).   Data are expressed as the means of triplicate cultures and the spontaneous radioisotope release was typically < 10% of total ^{51}Cr release.   % cytolysis was calculated as:

$$\frac{\text{cpm specific 51Cr release - cpm spontaneous release}}{\text{cpm total 51Cr release - cpm spontaneous release}} \times 100$$

Lymphokine assay.

1 x 10^5 CTL clones were incubated with 1 x 10^5 irradiated Colo-205 or JY cells in 200 μl medium in U-bottomed 96-well plates (Becton Dickinson Labware, Lincoln Park, NJ) for 18 hr in the presence of the indicated mAbs (each at a final concentration of 10

μg/ml).   Supernatants were harvested and lymphokines were measured by ELISA, as described (Chretien, et al., 1989).

Protein purification and sequencing.

2×10^{10} PBMC were lysed in 200 ml of lysis buffer (50 mM Tris, 150 mM NaCl, pH 8.0) containing 1% NP-40 and protease inhibitors (1 mM PMSF and 20 Kallikrein inhibitor U/ml aprotinin).   After removal of nuclei by centrifugation, the sample was incubated with 40 ml Con A Sepharose (Pharmacia, Uppsala, Sweden).   Glycoproteins were eluted with lysis buffer containing 0.5 M α-methyl-mannopyranoside and were passed through a column containing 10 ml of Sepharose CNBr-coupled (Sigma, St. Louis, MO) to a control murine IgG1 mAb.   DNAM-1 glycoproteins were then isolated by using a column containing 25 ml of Sepharose covalently coupled to the DX11 mAb (2 mg DX11 mAb/ml Sepharose beads) and were eluted with 50 ml of 50 mM diethylamine (pH >11.5).   The sample was concentrated to a final volume of 40 μl by using a Centricon-30 (Amicon Inc., Beverly, MA).   2X Laemmli   buffer was added and the proteins were separated by SDS-PAGE using 7% acrylamide gels (Bio-Rad, Richmond, CA) and blotted onto PVDF membranes (ProBlott, Applied Biosystems, Foster City, CA).   The membrane was stained with Coomassie blue (Bio-Rad) or Ponceau S (Sigma).   Membrane fragments containing the DNAM-1 protein were subjected to direct N-terminal sequencing at our institution or digested with trypsin for sequence determination of internal peptides at the Harvard Microsequencing Facility (Cambridge, MA).   Digested peptides were separated by reverse phase HPLC and sequencing was done with an Applied Biosystems 476A gas phase sequencer.

cDNA cloning

Primary PCR was performed with a sense degenerative primer encoding the peptide EEVLWHT (an N-terminal   peptide fragment) and an antisense degenerative

primer encoding the peptide QQDSIA (an internal peptide fragment) using a human Th0 T cell clone (MOT81) cDNA plasmid library as the template.   Using this primary PCR product as the   template, a second PCR was performed with a degenerative sense primer encoding the peptide PFAENM (another N-terminal peptide fragment) and the same antisense primer.   A 100-bp PCR product generated from the second PCR specifically hybridized with a ^{32}P-radiolabeled oligonucleotide probe 5'-TTTGCIGAIAACATGTCCCTIGAITGTGTITACCC-3' (I indicates inosine) encoding   the peptide FAENMSLECVYP in the N-terminal peptide fragment. 2×10^6 plasmid clones from the Th0 cDNA library were seeded into three 96-well plates and wells were screened by PCR for presence of the 100 bp DNAM-1 cDNA fragment.   Two positive wells were detected and the clones in these wells were screened by a standard colony hybridization method using the ^{32}P-radiolabeled 100-bp fragment as a probe.   Two positive clones containing the same cDNA insert of 2.7 kb (plasmid LL378) were isolated and sequenced.   The DNAM-1 cDNA was subcloned into the pMET7 expression vector and transfected into COS-7 cells using DEAE-dextran by the technique described previously (Aruffo and Seed, 1987).   Transfectants were stained by immunofluorescence and analyzed by flow cytometry.

Biochemistry

Cells were labeled with ^{125}I using lactoperoxidase and glucose oxidase and lysed in Tris-buffered saline (50 mM Tris, 15 mM NaCl, pH 8.0) containing 1% NP-40, protease inhibitors (1 mM PMSF and 20 Kallikrein inhibitor U/ml aprotinin) and phosphatase inhibitors (1 mM EGTA, 10 mM NaF, 1 mM $Na_4P_2O_7$, 0.1 mM β-glycerophosphate, 1 mM Na_3VO_4).   DNAM-1 glycoproteins were immunoprecipitated using the methods described previously (Lanier, et al., 1988) and treated with N-glycosidase F (Boehringer Mannheim Corp., Indianapolis, IN) using the conditions recommended by the manufactures.   Samples were analyzed by SDS-PAGE.

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For the analysis of DNAM-1 phosphorylation, 5×10^5 HLA-A2-specific CTL were incubated with 1×10^6 P815 cells and DX11 mAb in a total volume of 100 μl tissue culture medium (RPMI-1640 containing 10 % fetal calf serum) in U-bottomed 96-well plates.   At various times, the cell mixture was centrifuged, supernatants discarded, and the cells were solublized in 1% NP-40 Tris buffered saline pH 8.0 with phosphatase and protease inhibitors.   Lysates from 3×10^6 CTL were immunoprecipitated with the indicated mAbs, as described (Lanier, et al., 1988), and proteins were resolved by SDS-PAGE.   Immunoprecipitates were transferred (100 V, 1 hr in 25 mM Tris, 192 mM glycine, 20% methanol) to PVDF membranes (Immobilon-P, Millipore)   Membranes were incubated overnight in Tris buffered saline containing 0.5% Tween 20 and $MgCl_2$ (TBST) and 10% non-fat dry milk and then incubated with FITC-conjugated DX11 mAb (1 μg/ml in TBST with 10% non-fat dried milk) for 2 hr at room temperature.   Proteins were detected by using HRP-conjugated sheep anti-FITC (Amersham, Arlington Heights, IL) and developed with SuperSignal CL-HRP Substrate (Pierce, Rockford, IL). Chemiluminescence was detected by autoradiograph.

Cell adhesion assay

COS-7 cells were transfected with DNAM-1 or CD94 (Chang, et al., 1995) cDNA, as described (Aruffo and Seed, 1987).   ^{51}Cr-labeled Colo-205 cells were added to 24 well plates containing adherent COS-7 cells expressing DNAM-1 or CD94 and incubated for 60 min at 37^0C in the presence or absence of DX11 mAb (10 μg/ml). After gently washing with tissue culture medium, cells were lysed in 10% Triton-X (in water) and radioactivity was measured.   Results are expressed as mean of triplicate cultures.

Acknowledgments

References

Apasov, S., Redegeld, F., Sitkovsky, M. (1993) Cell-mediated cytotoxicity: contact and secreted factors. Curr Opin in Immunol *5*, 404-410

Aruffo, A., and Seed, B. (1987) Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. Proc. Natl. Acad. Sci. USA *84*, 8573-8577

Berke, G. (1994) The binding and lysis of target cells by cytotoxic lymphocytes: molecular and cellular aspects. Annu. Rev. Immunol. *12*, 735-773

Berke, G. (1995) The CTL's kiss of death. Cell *81*, 9-12

Bierer, B.E., Sieckman, B.P., Ratnofsky, S.E., Burakoff, S.J. (1989) The biological roles of CD2, CD4, and CD8 in T-cell activation. Ann. Rev. Immunol *7*, 579-599

Bierer, B.E., Burakoff, S.J. (1991) T cell receptors: adhesion and signaling. Adv. in Cancer Res. 56, 49-76

Callen, D.F., Baker, E., Eyre, H.J., Chernos, J.E., Bell, J.A., Sutherland, G.R. (1990) Reassessment of two apparent deletions of chromosome 16 p to an ins (11; 16)   and a t (1; 16) by chromosome painting.   Ann. Genet. *33*, 219-221

Cantley, L.C., Songyang, Z. (1994) Specificity in recognition of phosphopeptides by src-homology 2 domains. J. Cell Sci. Suppl. 18, 121-126

Chang, C., Rodriguez, A., Carretero, M., Lopez-Botet, M., Phillips, J.H., Lanier, L.L. (1995) Molecular characterization of human CD94: a type II membrane glycoprotein related to the C-type lectin superfamily. Eur. J. Immunol. 25, 2433-2437

Chretien, I., Van Kimmenade, A., Pearce, M.K., Banchereau, J., & Abrams, J.S. (1989) Development of polyclonal anti monoclonal antibodies for immunoassay and neutralization of human interleukin-4.   J. Immunol. Meth. *117*, 67-81

Dustin, M.L., Sanders, M.L., Shaw, S., Springer, T.A. (1987) Purified lymphocyte function-associated antigen 3 binds to CD2 and mediates T lymphocyte adhesion. J. Exp. Med. *165*, 677-692

Dustin, M.L., Springer, T.A. (1989) T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. Nature *341*, 619-624

Gunji, Y., Nakamura, M., Hagiwara, T., Hayakawa, K., Matsushita, H., Osawa, H., Nagayoshi, K., Nakauchi, H., Yanagisawa, M., Miura, Y., Suda, T. (1992) Expression and function of adhesion molecules on human hematopoietic stem cells: CD34+ LFA-1- cells are more primitive than CD34+ LFA-1+ cells. Blood *80*, 429-436

Henkart, P.A. (1994) Lymphocyte-mediated cytotoxicity: Two pathways and multiple effector molecules. Immunity *1*, 343-346

Hogg, N., Landis, R. C. (1993) Adhesion molecules in cell interactions. Curr Opin in Immunol *5*, 383-390

Isobe, M., Yagita, H., Okumura, K., Ihara, A. (1992) Specific acceptance of cardiac allograft after treatment with antibodies to ICAM-1 and LFA-1. Science *255*, 1125-1127

Janeway, C.A. (1988) T-cell development. Accessory or coreceptors? Nature *335*, 208-210

Killeen, N., Stuart, S.G., Littmann, D.R. (1992) Development and function of T cells in mice with a disrupted CD2 gene. EMBO J. *11*, 4329-4336

Kishimoto, T.K., O'Connor, K., Lee, A., Roberts, T.M., Springer, T.A. (1987) Cloning of the beta subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family. Cell 48, 681-690

Klensky, A.M., Sanchez-Madrid, F., Robbins, E., Nagy, J.A., Springer, T.A., Burakoff, S.J. (1983) The functional significance, distribution, and structure of LFA-1, LFA-2, and LFA-3: cell surface antigens associated with CTL-Target interactions. J. Immunol. *131*, 611-616

Kupfer, A., Singer, S.J. (1989) Cell biology of cytotoxic and helper T cell functions. Immunofluorescence microscopic studies of single cell and cell couples. Ann. Rev. Immunol. *7*, 309-337

Lanier, L.L., Le, A.M., Phillips, J. H., Warner, N.L., Babcock, G.F. (1983) Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. J. Immunol. *131*, 1789-1796

Lanier, L.L., Buck, D.W., Rhodes, L., Ding, A., Evans, E., Barney, C., Phillips, J.H. (1988) Interleukin 2 activation of natural killer cells rapidly induced the expression and phosphorylation of the Leu-23 activation antigen. J. Exp. Med. *167*, 1572-1585

Lanier, L.L. and Recktenwald, D.J. Multicolor immunofluorescence and flow cytometry. (1991) Methods: A companion to method in enzymology. *2*, 192-199

Marlin, S.D., Springer, T.A. (1987) Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). Cell *51*, 813-819

McMichael, A.J., Parham, P., Rust, N., Brodsky, F. A monoclonal antibody that recognizes an antigenic determinant shared by HLA A2 and B17. Hum. Immunol. *1*, 121-129

Menzer, S.J., Barbosa, J.A., Burakoff, S.J. (1985) T3 monoclonal antibody activation of nonspecific cytolysis: a mechanism of CTL inhibition. J Immunol *135*, 34-38

O'Rourke, A.M., and Mescher, M.F. (1992) Cytotoxic T-lymphocyte activation involves a cascade of signaling and adhesion events. Nature *358*, 253-255

Phillips, J.H., McKinney, L., Azuma, M., Lanier, L.L. (1991) A novel $\beta4,\alpha6$ integrin-associated epithelial cell antigen involved in natural killer cell and antigen-specific cytotoxic T lymphocyte cytotoxicity. J. Exp. Med. *174*, 1571-1581

Phillips, J.H., Gumperz, J.E., Parham, P., Lanier, L.L. (1995) Superantigen-dependent, cell-mediated cytotoxicity inhibited by MHC class I receptors on T lymphocytes. Science *268*, 403-405

Rodrigues, M., Nussenzweig, R.S., Romero, P., Zavala, F. (1992) The in vivo cytotoxic activity of CD8+ T cell clones correlates with their levels of expression of adhesion molecules. J. Exp. Med. *175*, 895-905

Rothlein, R., Dustin, M.L., Marline, S.D., Springer, T.A. (1986) A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. J. Immunol. *137*, 1270-1274

Scott, C.F., Boloender, S., McIntyre, G.D., Holldack, J., Lambert, J.M., Venkatesh, V.P., Morimoto, C., Ritz, J., Schrossman, S.F. (1989) Activation of human cytolytic cells through CD2/T11: Comparison of the requirements for the inducer and direction of lysis of tumor targets by the T cells and NK cells. J. Immunol. *142*, 4105-4112

Shaw, S., Ginther Luce, G.E., Quinones, R., Gress, R.E., Springer, T.A., Sanders, M.E. (1986) Two antigen-independent adhesion pathways used by human cytotoxic T-cell clones. Nature *323*, 262-264

Siliciano, R.F., Pratt, J.C., Schmidt, R.E., Ritz, J., Reinherz, E.L. (1985) Activation of cytolytic T lymphocyte and natural killer cell function through the T 11 sheep erythrocyte binding protein. Nature *317*, 428-430

Spits, H., Yssel, H., Leeuwenberg, J., DeVries, J.E. (1985) Antigen-specific T cell and antigen-specific proliferating T cell clones can be induced to cytolytic activity by monoclonal antibodies against T3. Eur. J. Immunol *15*, 88-91

Springer, T.A., Dustin, M.L., Kishimoto, T.K., Marlin, S.D. (1987) The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. Ann. Rev. Immunol. *5*, 223-252

Springer, T.A. (1990) Adhesion receptors of the immune system. Nature *346*:425-434

van de Wiel-van Kemenade, E., Te Veldet, A.A., De Boer, A.J., Weening, R.S., Fisher, A., Borst, J., Melief, C.J.M., Figdor, C.G. (1992) Both LFA-1-positive and -deficient T cell clones require the CD2/LFA-3 interaction for specific cytolysis activation. Eur. J. Immunol. *22*, 1467-1475

Vives, J. (1994) CD50 is the "signal transducer" ICAM. The Immunologist *2*, 93-96

Wang, P.L., O'Farrel, S., Clayberger, C., Krensky, A.M. (1992) Identification and molecular cloning of tactile: A novel T cell activation antigen that is a member of the Ig superfamily. J. Immunol. 148, 2600-2608

Yssel, H., de Vries, Y.E., Koken, M., van Blitterswijk, W., Spits, H. (1984) Serum-free medium for the generation and the propagation of functional human cytotoxic and helper T cell clones. J. Immunol. Meth. *72*: 219-227

<div align="center">**Figure Legends**</div>

Figure 1. **A.** Anti-DNAM-1 mAb, DX11, and anti-CD18 (LFA-1β) mAb inhibit TcR-independent cytolysis against a variety of tumor cell targets mediated by CTL clones. Colo-205, PA-1, SK-N-SH, BDMEL-1, MCF-7, and CaOV3 are non-hematopoietic tumor cells lines and do not express DNAM-1. Hematopoietic cell lines tested include K562 (DNAM-1 negative), Jurkat (DNAM-1 positive), U937 (DNAM-1 negative) and the 721.221 and JY EBV-transformed B lymphoblastoid cell lines (both DNAM-1 positive). **B.** DX11 mAb inhibits NK cell clone-mediated cytolysis against Colo-205, PA-1 and U937, but did not affect cytolysis of JY and 721.221. Data are representative of experiments using several CTL and NK clones. mAbs were used at 10 μg/ml and the effector to target ratio was 10 to 1.

Figure 2. Expression of DNAM-1 on peripheral blood mononuclear cells. DNAM-1 is expressed on CD3+CD4+ T cells (90%), CD3+CD8+ T cells (80%), CD3+TcRγδ+ T cells (99%), CD3-CD56+ NK cells (97%), CD19+ B cells (3%), and monocytes (100%) in peripheral blood. PBMC from a healthy donor were stained with phycoerythrin (PE)-conjugated DX11, CyChrome-conjugated anti-CD3, and FITC-conjugated anti-CD4, anti-CD8α, anti-CD56, anti-CD19, or anti-CD14. The cells were analyzed by flow cytometry using a FACScan. An electronic gate was used to identify CD3+ (A) and CD3- (B) lymphocytes and monocytes (C) and the FITC (x-axis) and PE (y-axis) fluorescence of these populations were displayed as contour plots (4 decade log scales). >98% of PBMC stained with FITC-conjugated control murine IgG1 and PE-conjugated control murine IgG were present in the lower left quadrant of the contour plots (not shown). Data are representative of results obtained using several different blood donors.

Figure 3. **A**.   DNAM-1 expression on an HLA-A2-specific CD8+ CTL clone. Cells were stained with PE-conjugated DX11 mAb (shaded histogram) or PE-conjugated control IgG1 mAb (open histogram) and analyzed by flow cytometry.   **B.**   DX11 mAb inhibited cytolysis of Colo-205, but not JY, mediated by HLA-A2-specific CTL clones AS547.97, AS547.93, and AS547.91.   mAbs were used at 10 μg/ml and the effector to target ratio was 5 to 1.   **C.**   DX11 mAb inhibited the secretion of GM-CSF and TNF-α by an HLA-A2-specific CTL clones AS547.97 and AS 547.93 stimulated by Colo-205, but not JY.   mAbs were used at 10 μg/ml

Figure 4. **A**.   An HLA-A2-specific CD8+ CTL clone lysed murine FcR+ P815 cells in the presence of DX11 mAb or anti-CD3 mAb, but not DX11 F(ab')$_2$ fragments.   Data are representative of experiments performed with several CTL clones.   mAbs were used at 10 μg/ml.   **B**.   HLA-A2-specific CTL were stimulated with DX11 mAb-precoated P815 cells for indicated time.   Cells were then lysed and immunoprecipitated with control murine IgG1 mAb (cIg), DX11 mAb, or anti-phosphotyrosine mAb (4G10). The immunoprecipitates were analyzed by Western blot using DX11 mAb, showing that tyrosine residues of DNAM-1 are phosphorylated after the stimulation.

Figure 5. **A**.   Nucleotide and predicted amino acid sequences of DNAM-1.   The leader and putative transmembrane domain are underlined.   Peptide sequences that were obtained from purified DNAM-1 protein are indicated by dotted underlines. Potential N-linked glycosylation sites in the extracellular domain and tyrosine phosphorylation sites in the cytoplasmic region are circled and boxed, respectively. The GenBank accession number of DNAM-1 is U56102.   **B.**   Schematic diagram of DNAM-1 protein. Leader peptide, extracellular, transmembrane and cytoplasmic regions are indicated. Two pairs of cysteine residues in the extracellular portion potentially able to participate in intrachain disulfide-bonding for the formation of Ig-like domains are

indicated.   **C.**   DNAM-1 expression on COS-7 cells transfected with pMET7 vector alone (open histogram) or with DNAM-1 cDNA in pMET7 (shaded histogram).   **D**. ^{125}I-labeled COS-7 cells transfected with the DNAM-1 cDNA or PBMC were lysed and DNAM-1 antigen was immunoprecipitated with control murine IgG1 or DX11 mAb. The immunoprecipitates were   treated with N-glycosidase F, as indicated, and analyzed by SDS-PAGE.

Figure 6. ^{51}Cr-labeled Colo-205 cell bound adherent COS-7 cells transfected with DNAM-1 cDNA and the binding was specifically blocked by the DX11 mAb.   DX11 mAb was added to assay at 5 μg/ml. ^{51}Cr-labeled Colo-205 cell did not bind to untransfected COS-7 cells (not shown).