

(72) HAYNES, Barton F., US
(72) ARUFFO, Alejandro, US
(72) PATEL, Dhavalkumar, US
(72) BOWEN, Michael A., US
(72) MARQUARDT, Hans, US
(72) SIADAK, Anthony W., US
(71) DUKE UNIVERSITY, US
(71) BRISTOL-MYERS SQUIBB COMPANY, US
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(54) **LIGAND DE CD6**
(54) **CD6 LIGAND**

(57) La présente invention concerne en général les CD6 et plus particulièrement un ligand des CD6 présent superficiellement sur les cellules épithéliales du thymus, sur les monocytes, sur les lymphocytes T activés et sur divers autres types de cellules. L'invention concerne également, non seulement des procédés d'inhibition de l'interaction entre CD6 et le ligand des CD6, mais également des procédés de sélection de composés en fonction de leur aptitude à inhiber cette interaction. L'invention concerne enfin des anticorps ainsi que des fragments de liaison de ces anticorps, spécifiques du ligand des CD6.

(57) The present invention relates, in general, to CD6 and, in particular, to a CD6 ligand present on the surface of thymic epithelial cells, monocytes, activated T cells and a variety of other cell types. The invention further relates to methods of inhibiting the interaction of CD6 and the CD6 ligand, and to methods of screening compounds for their ability to inhibit that interaction. The invention also relates to antibodies, and binding fragments thereof, specific for CD6 ligand.

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(21) International Application Number: PCT/US97/12511 (22) International Filing Date: 17 July 1997 (17.07.97) (30) Priority Data: 08/684,594 18 July 1996 (18.07.96) US (71) Applicants: DUKE UNIVERSITY [US/US]; 230 North Building, Research Drive, Durham, NC 27708-0083 (US). BRISTOL-MYERS SQUIBB COMPANY [US/US]; 345 Park Avenue, New York, NY 10154 (US). (72) Inventors: HAYNES, Barton, F.; 4923 Wentworth Drive, Durham, NC 27707 (US). ARUFFO, Alejandro; 1012 Spruce Street, Edmonds, WA 98020 (US). PATEL, Dhavalkumar; 3012 Deerchase Wynd, Durham, NC 27712 (US). BOWEN, Michael, A.; 200 Roy Street #540, Seattle, WA 98040-4408 (US). MARQUARDT, Hans; 9222 S.E. 46th Street, Mercer Island, WA 98040-4408 (US). SIADAK, Anthony, W.; 6210 1st Avenue, N.W., Seattle, WA 98107 (US). (74) Agent: WILSON, Mary, J.; Nixon & Vanderhye P.C., 8th floor, 1100 North Glebe Road, Arlington, VA 22201-4714 (US).		(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: CD6 LIGAND (57) Abstract The present invention relates, in general, to CD6 and, in particular, to a CD6 ligand present on the surface of thymic epithelial cells, monocytes, activated T cells and a variety of other cell types. The invention further relates to methods of inhibiting the interaction of CD6 and the CD6 ligand, and to methods of screening compounds for their ability to inhibit that interaction. The invention also relates to antibodies, and binding fragments thereof, specific for CD6 ligand.		

CD6 LIGAND

This invention was made with Government support under Grant Nos. CA-28936 and AI-07217 awarded by the National Institutes of Health. The Government has
5 certain rights in the invention.

This is a continuation-in-part of Application No. 08/432,016 filed May 1, 1995, which is a continuation-in-part of Application No. 08/333,350, filed November 2, 1994, which is a continuation-in-part of Application No.
10 08/143,903, filed November 2, 1993, the entirety of these applications being incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates, in general, to CD6
15 and, in particular, to a CD6 ligand present on the surface of thymic epithelial cells, monocytes, activated T cells and a variety of other cell types. The invention also relates to a nucleic acid sequence encoding the CD6 ligand and to vectors and host cells
20 comprising same. The invention further relates to methods of inhibiting the interaction of CD6 and the CD6

ligand, and to methods of screening compounds for their ability to inhibit that interaction.

BACKGROUND

CD6 is a 130 kDa glycoprotein that is homologous to CD5 and the macrophage scavenger receptor (Aruffo et al, J. Exp. Med. 174:949 (1991)). CD6 is expressed on the surface of mature thymocytes, peripheral T cells and a subset of B cells (Kamoun et al, J. Immunol. 127:987 (1981)). CD6 appears to be involved in T cell activation since monoclonal antibodies (mabs) to CD6 augment T cell receptor (TCR) mediated activation of T cells (Gangemi et al, J. Immunol. 143:2439 (1989)), and the CD6 molecule is tyrosine phosphorylated during TCR-mediated T cell triggering (Wee et al, J. Exp. Med. 177:219 (1993)). Mabs to CD6 have been shown to be clinically useful for the deletion of T cells for allogeneic bone marrow transplantation (Soiffer et al, J. Clin. Oncol. 10:1191 (1992)).

Results obtained by screening a panel of mabs to T cell surface antigens for their ability to inhibit the binding of thymocytes to human thymic epithelial cells (TEC) suggested that CD6 is involved in the binding of thymocytes to TEC (Vollger et al, J. Immunol. 138:358 (1987)). The present invention relates to a ligand for

CD6 involved in that binding and to a nucleic acid sequence encoding same.

OBJECTS AND SUMMARY OF THE INVENTION

5 It is the general object of the invention to provide a CD6 ligand.

It is a specific object of the invention to provide a CD6 ligand in isolated form and a nucleic acid sequence encoding that ligand.

10 It is a further object of the invention to provide a method of inhibiting the binding of CD6 to a CD6 ligand.

It is yet another object of the invention to provide a method of screening compounds for their ability to inhibit CD6/CD6 ligand binding.

15 It is a further object of the invention to provide a monoclonal antibody specific for a CD6 ligand.

20 In one embodiment, the present invention relates to an isolated CD6 ligand, including both divalent cation dependent and divalent cation independent forms thereof. The invention further relates to mimetopes of such a ligand.

In another embodiment, the present invention relates to a nucleic acid sequence encoding a CD6 ligand and to a vector and host cell comprising same. The

invention also relates to a method of producing a CD6
ligand or a portion thereof that comprises culturing a
host cell comprising a CD6 ligand encoding sequence or a
portion thereof under conditions such that the sequence
5 is expressed and the CD6 ligand or portion thereof is
thereby produced.

In a further embodiment, the present invention
relates to a method of inhibiting binding of CD6 present
on the surface of a first cell to CD6 ligand present on
10 the surface of a second cell. The method comprises
contacting the CD6 present on the surface of the first
cell with a soluble CD6 ligand, or mimetope thereof,
under conditions such that the soluble CD6 ligand, or
mimetope thereof, binds to the CD6 present on the
15 surface of the first cell and thereby inhibits binding
of the CD6 ligand present on the surface of the second
cell to the CD6 present on the surface of the first
cell. Alternatively, the method comprises contacting
the CD6 ligand present on the surface of the second cell
20 with soluble CD6, or mimetope thereof, under conditions
such that the soluble CD6, or mimetope thereof, binds to
the CD6 ligand present on the surface of the second cell
and thereby inhibits binding of the CD6 present on the
surface of the first cell to the CD6 ligand present on
25 the surface of the second cell.

In another embodiment, the present invention relates to a method of screening a test compound for the ability to inhibit binding of CD6 to CD6 ligand. The method comprises: i) contacting the test compound with one of CD6 and CD6 ligand under conditions such that a complex between the test compound and the CD6 or CD6 ligand can form, ii) contacting the combination of CD6 or CD6 ligand and test compound resulting from step (i) with the other of CD6 and CD6 ligand under conditions such that complexation between the CD6 and the CD6 ligand can occur, and iii) determining the extent of complexation between the CD6 and the CD6 ligand and thereby the ability of the test compound to inhibit binding of the CD6 to the CD6 ligand.

In yet a further embodiment, the present invention relates to anti-CD6 ligand antibodies and antigen binding fragments thereof that can be produced enzymatically (eg Fab and F(ab)₂ fragments) and to CD6 ligand specific binding proteins that can be produced recombinantly (eg fusion proteins and chimeric antibodies).

Further objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Effect of divalent cations on binding of CD6-Ig (= CD6-Rg) to thymic epithelial cells (TEC). TEC were incubated with 5 μ g of CD6-Ig or CD5-Ig in buffers containing 2% BSA, Tris-HCl buffered saline (TBS) and either 5 mM CaCl_2 , 5 mM MgCl_2 or 5 mM MnCl_2 , and assayed by indirect immunofluorescence and flow cytometry. Shown is the relative binding of CD6-Ig in the various buffers. Relative binding was determined as the ratio of specific binding of CD6-Ig (fluorescence of CD6-Ig - fluorescence of CD5-Ig) in sample buffer to the specific binding in TBS. Shown is a representative of three experiments (except for TBS+Mg which was performed only once). Both CaCl_2 and MnCl_2 were able to significantly enhance the binding of CD6-Ig to TEC ($p \leq 0.1$).

Figure 2. CD6-Ig binding to TEC is enhanced by Mn^{++} . TEC were incubated with 5 μ g of CD6-Ig or CD5-Ig in buffers containing 2% BSA, TBS and various concentrations of MnCl_2 , and assayed by indirect immunofluorescence and flow cytometry. The graph shows the relative binding of CD6-Ig in buffers containing varying amounts of MnCl_2 . Shown is a representative of three separate experiments.

Figure 3. Binding to TEC is enhanced by the expression of CD6 in COS cells. COS cells that express CD6 in a stable manner (COS-CD6D) or control COS cells (COS-Neo) were incubated with calcein-AM labelled TEC at a ratio of 1:5 for 60 min at 4°C. Binding was detected by using a combination of light and fluorescence microscopy. Binding was determined to be the percentage of COS cells with 3 or more bound TEC. The expression of CD6 in COS cells enhanced the binding of COS cells to TEC, and this binding was inhibited by anti-CD6 antibody.

Figure 4. Monoclonal antibody J4-81 inhibits the binding of human CD6-Ig fusion protein to human TEC. The binding panel of 470 mabs from the V International Workshop on Human Leukocyte Differentiation Antigens and a panel of anti-integrin mabs was screened for reactivity to TE cells. Of the 154 mabs that reacted with TE cells, 126 were used in assays to inhibit the binding of CD6-Ig to TE cells. The workshop mabs that reacted with TE cells and were used in this study were: A008, A014, A023-4, A036-7, A044, A047-9, A063, A065, A070, A074, A080, A090, A092, A107, A110, A113, A124, A132, A139, A141, A145, B002, B003, B005-6, B011-2, B014, B019, B025, B027-9, B031, B046, B049, B052, B055-7, B059, B068, CB10, CB22, CB27, E001, E015, E031, E045,

E050, E053-4, E056-7, M01-2, M09, M14-5, M25, M32, M35-8, M43-4, MR1, MR4, MR6, MR9, MR12, MR14, XB001, NK32, P005, P012, P023, P036, P044, P098, P107-8, S009, S013, S023-4, S031, S075, S107, S188, S201, S241, S245, S252, S263, S271, S273-4, 5T-015, 5T-076, 5T-080, 5T-084, CD24.3, CD40.2, CD73.1, CD74.1 and CD77.1. Anti-integrin mabs used were: TS2/7, P1H6, 12F1, P1B5, P4G9, B5610, P3010, EA-1-6, 135-13C, P502, P309, K20, β 4, P3G8, P3G2, P1H12 and TS2/16. The cells were pre-incubated with mabs at a dilution of 1:100 for 15 min at 4°C followed by incubation with 5 μ g of either CD5-Ig or CD6-Ig. Cells were washed and fusion proteins detected by reaction with fluorescein conjugated antiserum specific to the Fc portion of human IgG and flow cytometry. Specific binding was determined to be the difference in fluorescence of CD6-Ig binding and CD5-Ig binding. The binding compared to control mab (P3) is shown. There were four instances where goat anti-human IgG-Fc cross reacted with the test mab (A024, B049, 5T-084 and CD73.1). Mab J4-81 (S252) inhibited CD6-Ig binding by $56 \pm 5\%$ (N=2).

Figure 5. Mab J4-81 reacts strongly with the surface of all cultured TEC. Cultured TEC were incubated with mab J4-81 (S252 from the Vth International Workshop on Human Leukocyte

Differentiation Antigens) and control mab (P3) for 30 min at 4°C. Following a wash with PBS containing 2% BSA, bound antibodies were detected by reaction with fluorescein conjugated goat anti-mouse IgG followed by flow cytometry. Shown are the fluorescence profiles of control mab (P3) and the mab against a CD6 ligand (J4-81).

Figure 6. Expression of CD6 and its ligand in postnatal human thymus. CD6 and its ligand in frozen human thymus sections (4 μ m) were labelled by indirect immunofluorescence using mabs T12 and S252 (J4-81), respectively. Panel 1 shows the pattern of reactivity to T12 and panel 2 shows the pattern of reactivity to mab J4-81. At least three different thymus tissues were analyzed with similar results. CD6 was expressed on thymocytes in the medulla (med) and moderately on thymocytes in the cortex (ctx). Mab J4-81 detected medullary thymic epithelial cells and Hassal's bodies (HB).

Figure 7. Immunoprecipitation of radioactively labelled glycoproteins from metabolically labelled TEC with CD6-Ig. Cells were labelled with 6-³H-glucosamine for 48 hours, lysed and lysates were immunoprecipitated with CD6-Ig and with CTLA4-Ig as a control. CD6-Ig

immunoprecipitation of TEC lysates was carried out with and without inclusion of 15 mM EDTA. The immunoprecipitation of a 105 kDa molecule and a 35 kDa molecule by CD6-Ig was divalent cation independent and the immunoprecipitation of an additional 90 kDa molecule by CD6-Ig was divalent cation dependent.

Figure 8. CD6-Rg (= CD6-Ig) binds to a number of human-derived cell lines. Binding of CD6-Rg (solid line) and CD5-Rg (dashed line) to a number of human cell lines was examined by flow cytometry. The cell lines were grouped into three categories based on their ability to bind CD6-Rg high binding, low binding, and no binding. The percentage binding used to group cells into these three categories is based on the number of cells whose fluorescence intensity is greater than that of cells stained with the isotype matched CD5-Rg fusion protein (vertical dash-dot line). The human cell lines examined were: (a) HBL-100, (b) H3719, (c) H3606, (d) LCL8664, (e) GM0833, (f) IMR90, (g) Jurkat, (h) Peer, (i) HUT78, (j) HPB-ALL, (k) JM, (l) H9, (m) LTR228, and (n) Raji. A total of 10^4 cells were analyzed in each experiment.

Figure 9. CD6-Rg binding is saturable and trypsin sensitive. (A) The binding of increasing concentrations

of CD6-Rg and CD5-Rg to the breast carcinoma cell line HBL-100 was examined by flow cytometry. The percentage binding (ordinate) was determined as described in Figure 8. A total of 10^4 cells were examined at each protein concentration. (B) The binding of CD6-Rg to HBL-100 cells which were pretreated with trypsin was compared to the binding of CD6-Rg and CD5-Rg to untreated HBL-100 cells by flow cytometry. This figure shows the results of a representative experiment out of three. Trypsin digests were carried out as described.

Figure 10. CD6-Rg binding is divalent cation dependent. The binding of CD6-Rg to HBL-100 cells in the presence of EDTA (+EDTA, dotted line) was compared to the binding of CD6-Rg (dash-dot line) and CD5-Rg (dashed line) by flow cytometry. The effect of EDTA on CD6-Rg binding to HBL-100 cells was also examined after the CD6-Rg/HBL-100 complexes were allowed to form (+EDTA, solid line). A total of 10^4 cells were examined in each experiment.

Figure 11. CD6-Rg binding is blocked by an anti-CD6 mAb. The ability of two different anti-CD6 mAb (MBG6 and G3-6) and an irrelevant IgM mAb to block the binding of CD6-Rg to HBL-100 cells was examined by flow

cytometry. In each case serial dilution of the antibodies was tested.

Figure 12. CD6-Rg binding is modulated by cytokines. The binding of CD6-Rg (solid line) and CD5-Rg (dashed line) to untreated HBL-100 cells or HBL-100 cells treated with the indicated cytokines was examined by flow cytometry. The percentage of HBL-100 cells whose fluorescence intensity is to the right of an arbitrary marker (dash-dot line) following CD6-Rg binding is noted in each panel. The data shown in columns (A) and (B) represent two different experiments.

Figure 13. Immunoprecipitation of CD6-Rg binding protein(s). HBL-100 cells were radiolabeled with [³H]glucosamine. Protein(s) reactive with either CD6-Rg CTLA4-Ig, or an anti-EGF receptor mAb were immunoprecipitated from cell lysates in the absence or presence of EDTA and analyzed by SDS-PAGE. The arrows point to the two radiolabeled polypeptides which bound to CD6-Rg in the absence of EDTA. Molecular masses are given in kDa.

Figure 14. CD6-Rg binds to cells in lymphoid organs. (A) CD6-Rg binding to skin. Scattered cells in the dermis are labeled. Nonspecific labeling of hair

shafts is indicated by arrows. (B) Reactivity of human IgG1 with a serial skin section. Only weak labeling of hair shafts is evident (arrows). (C) Reactivity of CD6-Rg with thymus tissue. A delicate reticular labeling pattern is present in the cortex. (D) Reactivity of human IgG1 with thymus tissue. Only faint background labeling was detected. (E) Reactivity of CD6-Rg with lymph node. Cells in the intermediate or paracortical sinuses were labeled. (F) No binding of human IgG1 to lymph node tissue was observed.

Figure 15. Human epidermal keratinocytes express a surface ligand(s) for CD6. Shown are the reactivities of cultured epidermal keratinocytes with CD6-Rg, CD5-Rg, or ELAM1-Rg. Cells were incubated with 5 μ g of fusion protein in PBS containing 2% BSA and washed. The fusion proteins were labeled with fluorescein conjugated goat antiserum to human IgG and assayed by flow cytometry.

Figure 16. CD6-CD6 ligand interactions mediate TE-thymocyte binding. Shown is a summary of 5 separate TE-thymocyte binding experiments. Panel A depicts the amount of rosette formation in the presence of specific antibodies and panel B depicts the amount of rosette formation in the presence of fusion proteins. Standard errors are indicated by bars. Both anti-CD6 antibody

and CD6-Rg fusion protein partially inhibit TE-thymocyte binding: T12 inhibited rosette formation by $49 \pm 9\%$ and CD6-Rg inhibited rosette formation by $35 \pm 9\%$. The differences in binding between control and 35.1 (p<0.003), T12 (p<0.015) and CD6-Rg (p<0.05) were statistically significant.

Figure 17. Binding of COS-CD6 to TE cells is CD6-specific. Shown are histograms of CD6 expression on COS-neo (control) cells and COS-CD6 cells as determined by indirect immunofluorescence with mAb T12 and flow cytometry. Background fluorescence (control mAb P3) is depicted by a dotted line and CD6 expression is indicated by a solid line. The bar graph depicts COS-neo and COS-CD6 binding to TE cells in the presence of either mAbs P3 or T12. Shown are the mean and SEM of three separate experiments.

Figure 18. Expression of CD6 on thymocyte subsets. Thymocytes were stained with mAb T12, fluorescein conjugated Fab fragments of goat anti-mouse IgG and a combination of CD4-PE and CD8-cychrome and analyzed on a FACStar^{Plus} flow cytometer. Shown are data representative of experiments on 3 different thymuses with histograms of CD6 expression on all thymocytes, CD4+CD8+ (immature, DP) thymocytes, CD4+CD8- (mature,

SP4) and CD4-CD8+ (mature, SP8) thymocytes. Background fluorescence (with control mAb P3) is shown in each histogram.

Figure 19. CD6 mediates the binding of both mature
5 and immature thymocytes to TE cells. CD1+ or CD1-
thymocytes were incubated with TE cells in the presence
of either control antibody (P3), anti-CD2 (35.1) or
anti-CD6 (T12) and assayed for rosette formation. The
average percentage inhibition of TE-thymocyte binding
10 and SEM (relative to control antibody) by anti-CD2 and
anti-CD6, from 3 separate experiments, is shown.
Inhibition of CD1+ (immature) thymocyte binding to TE
cells is depicted by open boxes and inhibition of CD1-
(mature) thymocyte binding is depicted by filled boxes.
15 The binding of both the mature and immature subsets of
thymocytes to TEC is inhibited by anti-CD2 and anti-CD6
antibodies. The difference in inhibition of CD1+ vs.
CD1- cells by anti-CD2 mAb 35.1 was statistically
significant ($p < 0.01$) but the difference in inhibition by
20 anti-CD6 mAb T12 was not ($p < 0.25$).

Figure 20. Thymic epithelial cells and thymic
fibroblasts express a trypsin-sensitive surface ligand
for CD6. (A) Shown are representative histograms
depicting the reactivities with CD6-Rg of thymic

epithelial cells, thymic fibroblasts, and thymocytes. Also shown in each panel are background levels of fluorescence with control fusion protein CD5-Rg.

(B) The binding of CD6-Rg to TE cells in either divalent cation containing media (- EDTA), trypsin-treated TE
5 cells in media (trypsin) or TE cells in PBS + 10 mM EDTA (EDTA) was tested. Shown are histograms of a representative experiment (from >10 performed) depicting CD6-Rg binding to TE cells as determined by indirect IF
10 and flow cytometry.

Figure 21. Effect of preincubation of J4-81 and J3-119 on CD6-Rg binding to TE cells. Shown are histograms of CD6-Rg binding to the surface of human TE cells, as detected by indirect IF and flow cytometry, in
15 the presence of mAbs P3 (control), J4-81 or J3-119 in media containing divalent cations (- EDTA) or no divalent cations (+ EDTA). The binding of control human IgG is shown in each panel. The bottom panels show composite summary histograms of CD6-Rg binding in the
20 presence of either P3, J4-81 or J3-119. Data are representative of 10 experiments in the presence of divalent cations and 2 experiments with no divalent cations.

Figure 22. MAb J3-119 blocks the binding of biotinylated J4-81 to the surface of TE cells. Shown is the specific binding of biotinylated J4-81 (in fluorescence units minus background) in the presence of increasing concentrations of mAbs A3D8, J3-119 and J4-81. Diluted ascites (1:50, 1:100, 1:250 and 1:500) was used and relative amount of mAb is presented as the inverse of the dilution $\times 10^3$. MAb A3D8, which binds strongly to the surface of TE cells did not alter the binding of J4-81 while both J3-119 and non-biotinylated J4-81 inhibited the binding of biotinylated J4-81. Data are representative of 2 experiments.

Figure 23. MAb J4-81 and CD6-Rg bind to the same 100 kDa glycoprotein on the surface of human TE cells. Shown are autoradiographs of ^{125}I -labelled TE cell surface proteins cross-linked to either mAb P3 (control), mAb J4-81, hIgG1 (human Fc control), or CD6-Rg. The cross-linked proteins were treated with either 2-ME alone (to cleave the cross-linker) or trypsin and 2-ME prior to electrophoresis. Both CD6-Rg and mAb J4-81 bound to 100 kDa proteins with identical trypsin digestion patterns. Data are representative of 2 separate experiments.

Figure 24. Expression of CD6L-100 or ALCAM in postnatal human thymus. Shown are photomicrographs (representative of experiments on 5 thymuses) of frozen sections of 2 month human thymus stained by indirect IF with mAb J4-81. Panel A shows a section of thymus stained by indirect IF with mAb J4-81. Panel A shows a section of thymus cortex and panel B shows a section of thymus medulla with Hassall's bodies (HB). The thymic capsule is indicated by dashed lines in panel A. Thymic epithelial cells (arrows), in both the cortex and in and around HB in medulla, reacted with mAb J4-81. Thymocytes did not react with mAb J4-81. An identical pattern was seen using mAb J3-119 (not shown) (400X magnification).

Figure 25. Binding of COS-CD6 to TE cells is CD6-specific. Fig. 25A. Histograms of CD6 expression on COS-neo (control) and COS-CD6 cells were determined by indirect immunofluorescence with the anti-CD6 mAb T12 (Gangemi et al, J. Immunol. 143:2439-2447 (1989)) (solid line) or a control antibody P3 (dotted line). Fig. 25B. The bar graph depicts COS-neo and COS-CD6 binding to TE cells in the presence of either the T12, J4-81 or P3 mAbs. Shown are the mean and SEM of three separate experiments.

Figure 26. Sequence analysis of the predicted amino acid sequence of the CD6 ligand designated ALCAM, Northern analysis of ALCAM mRNA expression, and ALCAM cell surface expression by activated T cells. Fig. 26A.

5 Alignment of the immunoglobulin-like extracellular domains of ALCAM (residues 35-512), BEN, neurolin, RAGE and MUC18. The lower case letter in front of the protein name designates the species (human, h, chicken, c, and fish, f). Consensus residues are those shared by

10 three or more proteins. Invariant residues are shown shaded and Cys's are highlighted with an asterisk. The numbering of the peptide sequences shown, obtained from published manuscripts, are as follows: cBEN, 8-484 (Pourquie et al, Proc. Natl. Acad. Sci. USA 89:5261

15 (1992)); neurolin, 1-466 (Lawssing et al, Differentiation 56:21 (1994)); RAGE, which contains three Ig domains, 30-307 (Neeper et al, J. Biol. Chem. 267:14998 (1992)); MUC18, 40-525 (Lehmann et al, Proc. Natl. Acad. Sci. USA 86:9891 (1989)). Fig. 26B. 15 μ g

20 of total RNA from peripheral blood monocytes and 25 μ g of total RNA from resting and PHA activated (72h) peripheral blood mononuclear cells, the T cell lymphomas CEM and MOLT4, the erythroleukemia cell line K562, the B cell lymphomas RAMOS, RAJI and DAUDI, the myelo-

25 monocytic cell lines HL60 and U937, the large granular lymphoma YT, the human breast carcinoma HBL-100 and COS

cells were used to prepare an RNA blot. Random primed ³²P-labeled ALCAM or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA's were used as probes.

Fig. 26C. Peripheral blood mononuclear cells were
5 activated *in vitro* with PHA and the ability of T cells to bind to either CD6-Rg or J4-81 was monitored for a period of ten days by two color immunofluorescence and flow cytometry. The mean channel fluorescence vs. day was plotted.

10 Figure 27. Human chromosomal mapping. Fluorescence *in situ* hybridization (FISH) of cDNA probes to R-banded chromosomes generated signals on both chromatids (indicated by arrows) on chromosome 3 at bands q13.1-q13.2. The region is shown by brackets on a
15 G-banding ideogram.

Figure 28. Binding of ALCAM to CD6. Fig. 28A. Flow cytometry histograms showing the binding of the anti-ALCAM mAb J4-81 and the CD6-Rg fusion protein to COS cell transfectants expressing ALCAM. Fig. 28B. Flow
20 cytometry histograms showing the binding of anti-CD6 mAb G3-6 and ALCAM-Rg to COS cell transfectants expressing CD6. The level of background binding of mAb's or fusion proteins to mock transfected COS cells is shown by shaded histograms.

Figure 29. ALCAM encoding sequence and predicted amino acid sequence.

Figure 30. Domains of ALCAM and truncated forms thereof.

5

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a molecule that represents one member of a binding pair, the other member of that pair being the CD6 molecule present on the surface of mature thymocytes, peripheral T cells and a subset of B cells. The CD6 ligand, as it is referred to herein, occurs in two forms - one form is independent of divalent cations for CD6 binding, the other is divalent cation-dependent. The first of these forms (the cation-independent form) comprises a 105 kDa protein (as determined by SDS-PAGE under reducing conditions) and is termed activated leucocyte-cell adhesion molecule (ALCAM). The second form is divalent cation-dependent, is a distinct molecule separate from ALCAM, and comprises a 90 kDa protein (determined by SDS-PAGE under reducing conditions). The molecular weight of ALCAM based on the predicted amino acid sequence shown in Figure 29 is about 65 kDa. The amino acid sequence of ALCAM shown in Figure 29 includes 9

potential sites for N-linked glycosylation. Thus, the glycosylated form of the ALCAM CD6 ligand can be expected to have a molecular weight of 100-105 kDa.

ALCAM consists of an N-terminal hydrophobic signal peptide, which is cleaved from the mature protein, followed by an extracellular domain, a hydrophobic transmembrane domain and a cytoplasmic domain. Single domains or combinations of domains can be used to inhibit a variety of interactions. For example, extracellular domains, separately or in combination with each other or other sequences, can be expected to be useful products for inhibiting CD6-ALCAM interactions. The extracellular domain of ALCAM can be divided into 5 immunoglobulin (Ig)-like domains. The two N-terminal domains are of the V-set, the three Ig domains that follow are of the C2-set (Pourquie et al, Proc. Natl. Acad. Sci. USA 89:5261 (1992); Tanaka et al, Neuron 7:535 (1991); Burns et al, Neuron 7:209 (1991). (See Figure 30.) These Ig-like domains can be used in isolation or in combination with other sequences.

The CD6 ligand (ALCAM) of the invention is present on a variety of tissue and cell types, including fibroblasts, skin epidermal keratinocytes, medullary TEC, gut epithelium, pancreas islet and acinar cells, as well as monocytes and activated T cells, hepatocytes and neurons of the brain cortex. The tissue distribution of

the CD6 ligand indicates that the CD6/CD6 ligand system is important for mediation of T cell interactions with monocytes and other activated T cells as well as with epithelial cells, fibroblasts and specialized cells, such as pancreatic islet cells. The expression of CD6 and CD6 ligand in brain indicates that the CD6/CD6 ligand system is involved in interactions between cells of the nervous system.

The CD6 ligand of the invention can be isolated from natural sources (see above) by immunoprecipitation, for example, with a CD6 fusion protein as described in the Examples that follows. A suitable fusion protein, the CD6-Ig (= CD6-Rg) fusion protein, can be produced by ligating the coding sequence of the extracellular portion of CD6 (Aruffo et al, J. Exp. Med. 174:949 (1991)) to the coding sequence of the Fc portion of human IgG as described by Seed and Aruffo (Proc. Natl. Acad. Sci. USA 84:3365 (1987)). The CD6-Ig fusion protein can be expressed in COS cells by transient transfection as previously described (Seed and Aruffo, Proc. Natl. Acad. Sci. USA 84:3365 (1987)).

The availability of the CD6 ligand protein from cell sources, including those noted above, makes possible the cloning of the CD6 ligand gene(s). Sequencing of all or part of the CD6 ligand protein provides the information necessary for designing nucleic

acid probes or primers suitable for use in isolating the CD6 ligand coding sequence(s), for example, from cDNA or genomic DNA libraries. The Unizap XR system from Stratagene can be used to prepare a suitable cDNA library from thymic epithelial cells. Other libraries that can be used include the brain frontal cortex and liver tumor Hep G2 cDNA libraries from Stratagene.

More specifically, the CD6 ligand can be purified by affinity chromatography. Anti-CD6 ligand mab (5-10 mg) (see, for example, Pesando et al, J. Immunol. 137:3689 (1986)) can be used to prepare an affinity column for CD6 ligand by immobilization of anti-CD6 ligand protein on Sepharose (Bowen et al, J. Immunol. 151:5891 (1993)) or CNBr-activated Sepharose 4B (Pharmacia), as described by Patel et al (Virology 149:174 (1986)). CD6 ligand from protein lysates of, for example, breast carcinoma cells (eg HBL-100), thymic epithelial cell or B lymphoma cell line lysates, can be affinity purified using the anti-CD6 ligand Sepharose column. CD6 ligand can be further purified over a C₁₈ reverse phase HPLC column (Vydac) using acetonitrile/H₂O gradients containing trifluoroacetic acid with UV detection at 240nm. (Example IV includes a description of the J4-81 immunopurification of CD6 ligand from breast carcinoma cells. Examples V and VI include a

description of the preparation of antibodies to four additional epitopes of ALCAM.)

Once purity has been established, the N-terminal amino acid sequence of intact CD6 ligand, and peptides generated with V8-protease, trypsin or other proteases, can be determined. From the amino acid sequence, degenerate oligonucleotide primers can be designed that recognize cDNA for CD6 ligand, to PCR amplify CD6 ligand cDNA. To guard against errors in the PCR amplification, ³²P-labelled PCR amplified CD6 ligand cDNA can be used as a probe to detect CD6 ligand cDNAs from an appropriate cDNA library. The nucleotide sequence of CD6 ligand cDNA can be determined using standard methodologies. (See generally, Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press (1989).)

Example IV includes a description of the analysis of purified ALCAM and CNBr fragments thereof in a pulsed-liquid protein sequencer. The amino acid sequences from intact protein and internal fragments are homologous with the chicken neuronal adhesion molecule BEN/SC-1/DM-GRASP. Example IV describes the use of DNA fragments corresponding to chicken BEN to isolate CD6 ligand cDNA. The ALCAM cDNA sequence and predicted amino acid sequence are shown in Fig. 29.

Once cloned, the CD6 ligand encoding sequence can be introduced into a host cell, for example, in an expression construct (eg, wherein the vector is a viral or plasmid vector) in operable linkage with a promoter.

5 The construct can be used to transform a procaryotic or eucaryotic host cell. Eucaryotic hosts are preferred since the CD6 ligand is a glycosylated protein. Mammalian cell systems are particularly advantageous (eg, the COS M6 system or the Chinese hamster ovary

10 (CHO) cell system). The CD6 ligand can be produced in soluble form by culturing the host cells transfected with a gene encoding CD6 ligand protein (eg, an Ig fusion protein) under conditions such that the encoding sequence is transcribed and the transcript is translated

15 into protein.

The present invention relates to the CD6 ligand in its entirety and also to portions thereof suitable, for example, for use as antigens that can be used in standard immunization protocols to generate antibodies

20 (monoclonal (see Examples V and VI) or polyclonal) (or binding fragments thereof) specific for the CD6 ligand (the ligand and the portions can be synthesized chemically or recombinantly). Such portions represent at least 5 consecutive amino acids of the CD6 ligand (eg

25 of the ALCAM sequence shown in Figure 29), preferably, at least 10 or 12 amino acids, more preferably, at least

25 consecutive amino acids, most preferably at least 40,
45 or 50 amino acids. Larger portions can also be used,
for example, portions of at least 100, 250 or 500 amino
acids. The invention includes portions of the CD6
5 ligand corresponding to the signal sequence, or the
extracellular, transmembrane or cytoplasmic domain (or
subunits of these domains such as the immunoglobulin
variable region-like domain (V) and immunoglobulin
constant region-like domain (C) subunits of the
10 extracellular domain), alone or in combination with
other CD6 ligand domain sequences or non-CD6 ligand
sequences.

The invention also relates to a CD6 ligand encoding
sequence (DNA or RNA) in its entirety and to portions
15 thereof suitable for use, for example, as probes or
primers (hybridization conditions described as standard
by Strategene and/or Gibco (eg for PCR) can be used).
Such portions represent at least 15, 20, 30, 75 or 150
nucleotides of the CD6 ligand (for example, of the
20 sequence given in Figure 29). Larger portions can also
be used, for example, portions of at least 300, 750 or
1500 nucleotides. The invention includes portions of
the CD6 ligand encoding sequence corresponding to the
signal sequence or the extracellular, transmembrane, or
25 cytoplasmic domain. Such portions can be present in

various combinations and in combination with non-CD6 ligand encoding sequences (eg, ALCAM-Rg).

Ligation of CD6 on T cells by anti-CD6 mab provides a potent co-mitogenic signal for unseparated T cells (Gangemi et al, J. Immunol. 143:2439 (1989); Morimoto et al, J. Immunol. 140:2165 (1988); Wee et al, J. Exp. Med. 177:219 (1993)), directly activates separated CD4+ T cell clones (Swack et al, J. Biol Chem. 266:7137 (1991)), and directly activates TCR $\gamma\delta$ but not TCR $\alpha\beta$ T cells (Pawlec et al, Hum. Immunol. 31:165 (1991)).

Thus, CD6 is a molecule of mature T cells that is intimately involved in TCR mediated T cell triggering; ligation of the TCR complex leads to CD6 phosphorylation (Wee et al, J. Exp. Med. 177:219 (1993)). Methods of inhibiting CD6/CD6 ligand interactions *in vivo* provides a potent immunotherapeutic strategy.

Soluble CD6 ligand (isolated from natural sources or produced recombinantly) can be used, for example, to inhibit CD6-mediated T cell activation that is dependent on T cell CD6-CD6 ligand + accessory cell contact. A portion of the CD6 ligand can also be used; for example, a domain such as the extracellular domain can be used alone or in combination with other domains or other sequences. Such an immunotherapeutic regimen is useful in treating diseases caused by activated T cells such as multiple sclerosis, inflammatory uveitis, including

Cogan's syndrome, rheumatoid arthritis, T cell mediated vasculitis syndromes, such as Wegener's granulomatosis and temporal arteritis, and organ allograft rejection. Indeed, it has been shown that CD6 mab depletion of bone marrow (BM) used for allogeneic BM transplant prevents graft versus host disease (Soiffer et al, J. Clin. Oncol. 10:1191 (1992)).

In addition to soluble CD6 ligand, soluble CD6, as well as mimetopes (mimics) of CD6 and CD6 ligand (prepared, for example, as described by Szostak (TIBS 17:89 (1992)) or Tsai et al (J. Immunol. 150:1137 (1993)), for example, from random RNA, DNA or peptide libraries), can be used as immunotherapeutic agents in regimens involving inhibiting CD6/CD6 ligand interaction. Anti-CD6 and anti-CD6 ligand antibodies (preferably monoclonal antibodies (see Examples V and VI)) can also be used in such regimens. These immunotherapeutic agents can be formulated with, for example, a pharmaceutically acceptable carrier, diluent, etc., as a pharmaceutical composition. The concentration of active agent in the composition and the amount of active agent administered will vary with the agent, the patient and the effect sought. Optimum doses can be readily determined.

The demonstration that CD6-CD6 ligand interactions mediate the adhesion of CD6 expressing cells to TE cells

indicates that CD6-CD6 ligand binding plays a role in regulating T cell maturation. Other molecules including CD2/LFA-3, ICAM-1/LFA-1, VLA-3, -4, -6, and fibronectin can serve as adhesion molecules in thymocyte-stromal cell binding (Singer et al, J. Immunol. 144:2931-2939 (1990), Giunta et al, J. Exp. Med. 173:1537-1548 (1991), Utsumi et al, Proc. Natl. Acad. Sci. USA 88:5685-5689 (1991)). The identification of a CD6 ligand permits determination of the relative contribution of CD6 in thymocyte-TE cell binding and T cell maturation. CD6-ligand may also be involved in B cell development, since studies in the chicken showed that ALCAM (BEN) is expressed on endothelial cells in the bursa of Fabricius (Pourquie et al, Development 109:743-752 (1990)), which is an organ in birds where B cells develop. Presently, there is no information on the ability of CD6 ligand to transduce intracellular signals. The finding that the anti-CD6 mAB T12 can activate T cells in the presence of accessory cells and in the apparent absence of crosslinking FcR's, indicates that activation of T cells by signaling through CD6 is important in autoimmune reactivity (Gangemi et al, J. Immunol. 143:2439-2447 (1989)). In support of this, anti-CD6 mAB's can enhance or inhibit the autologous MLR (Gangemi et al, J. Immunol. 143:2439-2447 (1989), Bott et al, Int. Immunol. 7:783-792 (1994)) (Singer, N.G. et al, J. Immunol.

(1994)). Since CD6 ligand is expressed by activated T cells and monocytes as well as by a number of T and B cell lines, CD6-CD6 ligand interactions would appear to play a role in functional interactions between CD6+T and B cells and activated leukocytes and CD6-CD6 ligand binding would appear to mediate adhesive interactions among activated leukocytes. Thus, while ALCAM may not affect the initiation of an inflammatory response, it may inhibit maintenance of a chronic inflammatory response, such as rheumatoid arthritis.

ALCAM (BEN) in the chicken is expressed predominantly during early embryonic development and in the brain (Pourquie et al, Development 109:743-752 (1990)). ALCAM functions as a homophilic adhesion molecule and supports neurite outgrowth (Tanaka et al, Neuron 7:535-545 (1991), Burns et al, Neuron 7:209-220 (1991)). Expression of human ALCAM by neurons in the brain has been reported (Patel et al, "Identification and characterization of an 100 kDa ligand for CD6 on human thymic epithelial cells", J. Exp. Med. (1994)). Interactions between the immune and nervous system are important in the pathology of certain chronic neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, and amyotrophic lateral sclerosis (Appel et al, Advances in Neurology 56:405-412 (1991), McGeer et al, Can. J. Neurol. Sci. 18:376-379 (1991),

Rowland, L.P., Advances in Neurology 56:3-23 (1991)).
The finding that CD6 and CD6 ligand are both expressed
by cells in these systems indicate that this
receptor/ligand pair functions in cellular interactions
5 between the immune and nervous systems.

For example, since inflammation requires
amplification of immune responses, blocking CD6 binding
can be expected to prevent immune response amplification
and inflammation. Further, inhibition of leukocyte
10 interactions with cells of the brain can be used to
treat chronic neurodegenerative diseases. Mutations in
ALCAM can be expected to lead to disorders of the
nervous and/or immune systems. When such disorders are
identified, the CD6 ligand nucleotide sequence or RFLP's
15 (restriction fragment length polymorphisms) identified
for the CD6 ligand can be used as markers for candidate
gene searches. For example, since ALCAM is expressed on
motor neuron's and immune cells, candidate diseases to
screen for ALCAM mutations are sporadic cases of
20 amyotrophic lateral sclerosis and multiple sclerosis.

In addition to the above, it will be appreciated
from a reading of this disclosure that soluble ALCAM,
for example, can be used to induce regeneration of
damaged or severed nerves. Induction can be effected by
25 administering soluble ALCAM at the site of nerve damage,
for example, by injection.

The CD6 ligand gene is a potential candidate for mutations in Mendelian disorders affecting the nervous system and immune system. No disease suggestive for further testing has yet been mapped to that region of human chromosome 3 to which the CD6 ligand gene has been assigned. The polymorphisms identified in the CD6 ligand cDNA can be used for candidate gene exclusion studies. (Polymorphisms noted may result from the fact that cDNAs were obtained from a PHA activated cDNA library and HL60 cells. Different donor sources often show polymorphisms in genes.)

Certain aspects of the invention are described in greater detail in the non-limiting Examples that follows.

EXAMPLE IExperimental details:

Cells and culture conditions. TEC were cultured by the explant technique in enriched media as previously described (Singer et al, Human Immunol. 13:161 (1985)). Human thymus tissue was obtained from the Department of Pathology, Duke University Medical Center, as discarded tissue from children undergoing corrective cardiovascular surgery. Contaminating thymic fibroblasts were removed by complement mediated lysis with mAb 1B10 which binds to a cell surface antigen on human fibroblasts (Singer et al, J. Invest. Dermatol. 92:166 (1989)) followed by treatment with 0.02% EDTA in PBS. 3T3 fibroblast feeder layers were removed by treatment with 0.02% EDTA in PBS prior to detachment of TEC from culture dishes with 0.05% trypsin in PBS containing 0.02% EDTA. Cells were washed 3 times prior to analysis. TEC were activated with 500 U/ml IFN- γ in DMEM containing 5% FCS, 1 mM sodium pyruvate (Gibco) 0.025 μ g/ml amphotericin B (Gibco), 100 U/ml penicillin and 1000 μ g/ml streptomycin for 48-72h at 37°C.

Thymocytes were obtained by teasing from thymus tissue, purified by centrifugation through ficoll-hypaque and washed with RPMI 1640. Thymocytes were used

immediately or frozen in media containing 20% FCS, 7.5% DMSO and 10 μ g/ml gentamicin (Scherring) in RPMI 1640. Frozen thymocytes were thawed by incubation in media containing 30% FCS, 10 μ g/ml deoxyribonuclease I
5 (Sigma), 10 μ g/ml gentamicin and 20 U/ml heparin (Upjohn) in RPMI 1640 as described (Denning et al, J. Immunol. 138:680 (1987)). Viable thymocytes were purified by centrifugation through ficoll-hypaque.

COS-M6 cells (ATCC) were grown in DMEM containing
10 10% FCS, 1 mM sodium pyruvate (Gibco), 0.025 μ g/ml amphotericin B (Gibco), 100 U/ml penicillin and 100 μ g/ml streptomycin.

Human epidermal keratinocytes (EK) were cultured from neonatal foreskins. Foreskins were incubated at
15 4°C overnight in 2.5 mg/ml trypsin type III (Sigma) in HBSS (GIBCO), the epidermis was removed, teased into a single cell suspension, seeded onto mitomycin C treated 3T3 fibroblast feeder layers and cultured as described (Hashimoto et al, J. Exp. Med. 157:259 (1983)).

20 *Detection of cell surface antigens:* Unfixed cultured cells were suspended in either PBS, TBS (0.9 M NaCl, 50 mM Tris-HCl, pH 7.3) or DMEM containing 2% BSA and 0.1% NaN₃. Cells were incubated with CD6-Rg, CD5-Rg or ELAM-Rg (from Sandro Aruffo, Bristol-Myers Squibb),
25 recombinant fusion proteins (100 μ g/ml) for 30 min at

4°C and washed with PBS containing 2% BSA and 0.1% NaN₃. FITC conjugated goat anti-human IgG₁ was used as a secondary reagent. Cells were analyzed on a FACStar Plus flow cytometer (Becton-Dickinson, Inc., Mountain View, California) and data was processed using the software program PC-Lysys (Becton-Dickinson).

TEC-thymocyte rosette binding assay: TEC-thymocyte rosette binding assays were performed as described (Singer et al, Proc. Natl. Acad. Sci. USA 83:6588 (1986)). Briefly, 10⁶ thymocytes were mixed with 2 x 10⁵ TEC in PBS or DME containing 2% BSA and 0.1% NaN₃, centrifuged for 3 min at 250g, and incubated for 60 min at 4°C. Cells were gently resuspended and counted under light microscopy. TEC which bound 3 or more thymocytes were scored as positive. The thymocytes used in these experiments were either freshly isolated, thawed from liquid nitrogen, or separated into subpopulations. Thymocytes were separated into CD1⁺ (immature, CD6^{low}) or CD1⁻ (mature, CD6^{hi}) subpopulations by indirect immunofluorescent staining with Na1/34 (anti-CD1; from Andrew McMichael, Oxford, England) and goat anti-mouse IgG (KPL) followed by fluorescence activated cell sorting using a FACStar Plus. All thymocyte subpopulations were >95% pure on reanalysis of sorted cells. TEC-thymocyte binding was inhibited by

preincubation of either thymocytes or TEC with antibodies or fusion proteins prior to binding.

Antibodies used were: P3X63 (control mab) (ATCC), 35.1 (anti-CD2; from John Hansen, Seattle, Washington (Martin et al, J. Immunol. 131:180 (1983)) and T12 (anti-CD6; from Ellis Reinherz, Boston, Massachusetts (Reinherz et al, Cell 30:735 (1982))).

COS cell transfection and binding studies: Stable lines of CD6-expressing COS cells were made by co-transfection of plasmids containing the CD6 gene under the control of the CMV promoter (A. Aruffo, Seattle, Washington, Aruffo et al, J. Exp. Med. 174:949 (1991)) and the pSVneo plasmid containing the bacterial neomycin resistance gene driven by an SV40 promoter as described by Liao et al (J. Immunol. December 1, 1993). Cells expressing the neomycin resistance gene were selected by incubation in DME/10%FCS containing 60µg/ml G418 (GIBCO). Cells expressing CD6 were identified by indirect immunofluorescence using mab T12. CD6 positive and negative cells were cloned by single cell sorting using a Becton-Dickinson FACStar^{Plus} flow cytometer.

CD6-expressing COS cells (COS-CD6D) and control COS cells (COS-Neo) were used in binding studies, with TE cells similar to those outlined above. To differentiate between COS cells and TE cells, TE cells were

metabolically labelled with 1 μ g/ml calcein AM
(Molecular Probes, Eugene, Oregon) for 15 min at 37°C in
PBS prior to harvest. Calcein AM-labelled cells are
fluorescent and can be easily differentiated from other
5 cells by fluorescence microscopy.

*Construction of the CD6-Rg (CD6-Ig) chimeric gene
and preparation of the fusion protein:* The CD6
immunoglobulin fusion gene was constructed by digesting
a plasmid containing the cDNA encoding the full-length
10 CD6 (Aruffo et al, J. Exp. Med. 174:949 (1991)) with the
restriction enzyme Esp1. The Esp1 digested plasmid was
then flushed with DNA polymerase and the appropriate
Bam HI linkers were added to it. The plasmid was then
digested with Bam HI and Nde 1. The fragment that
15 contained the extracellular domain of CD6 was then
isolated and subcloned into a vector containing a cDNA
fragment encoding the constant domain of a human IgG1
protein (Aruffo et al, Proc. Natl. Acad. Sci. USA
89:2292 (1992)). The constructions of CD5-Ig and CTLA4-
20 Ig have been described elsewhere (Linsley et al, Science
257:792 (1992) and J. Exp. Med. 174:561 (1991)). 500 μ g
of DNA of the appropriate gene was transfected into COS
cells by the DEAE-dextran chloroquine method. 18-24 hrs
later, the 10% FBS Dulbecco's modified Eagle's medium was
25 replaced by serum-free media after a rinse wash with

PBS. The cell culture was maintained 7-10 days before it was harvested. Briefly, the supernatant containing the fusion protein was centrifuged at 2500 rpm for 20 min and filtered through a 0.45 μ m Millipore filter.

5 The supernatant was passed over a 2-ml packed Protein A column at a flowrate of 1.00 ml/min. The bound fusion protein was eluted with pH 3.5, 0.1 M citric acid and was immediately neutralized with pH 9.0, 1.0 M Tris. The eluted protein was then dialyzed overnight in PBS
10 and protein determination was done using the Bradford method.

Screening of mabs for inhibition of CD6-Ig binding to TE cells: Mabs from the 5th International Workshop on Human Leukocyte Differentiation Antigens and a panel
15 of anti-integrin mabs (see description of Figure 4) were screened for reactivity to the surface of TE cells as outlined above, using fluorescein-conjugated goat anti-mouse IgG (KPL) as a secondary reagent. Of the 154 mabs that reacted to TE cells, 126 were used in this assay.
20 TE cells (100k) were incubated with ascites or purified mab at 1:100 for 15 min at 4°C. Either CD5-Ig or CD6-Ig (5 μ g) was added to this mixture and allowed to react for 2 hr at 4°C. After washing with PBS-containing 2% BSA, CD5-Ig and CD6-Ig were labelled with a fluorescein
25 conjugated goat antiserum specific for the Fc protein of

human IgG (Sigma). This reagent did not cross react with most murine mabs. To account for any cross-reactivity that may have occurred, binding was determined to be the difference in fluorescence (Δ FL) between samples containing CD6-Ig and CD5-Ig. The Δ FL of samples pre-incubated with control mab P3 was considered to represent 100% binding.

Immunoprecipitation and protein labelling
conditions: 120 μ Ci/ml glucosamine (NEN) was used in the labelling experiment. The cells were cultured in glucose-free media plus 10% dialyzed fetal calf serum for 48 hrs upon addition of the radiolabelled glucosamine. The cells were then lifted with EDTA, washed in HBSS and lysed in 20 mM Tris, pH 7.5 containing leupeptin, PMSF and pepstain (1 μ g/ml), 0.05% sodium cholate, 2% NP-40. The lysate was pre-cleared with 100 μ g/ml human IgG1 followed by 50 μ l of Protein A beads. Pre-cleared lysates were then incubated with 50 μ g/ml CD6-Rg or CTLA4-Ig or 2 μ g/ml anti-EGF receptor antibody and Protein A Sepharose. 2 mM Ca^{++} and Mg^{++} were added to the lysates (this is the concentration of divalent cations contained in the HBSS binding buffer). The immunoprecipitates were washed 3x in lysis buffer, 2x in PBS, boiled in reducing SDS-PAGE loading buffer and loaded unto a 8-10.5% SDS-PAGE gel. The gels were

fixed in 40% isopropanol, enhanced in m AMiAmplify^R reagent (Amersham), dried and autoradiographed.

Results:

5 *Anti-CD6 mabs and CD6-Ig fusion proteins inhibit*
TE-thymocyte binding: Using a suspension TE-thymocyte
binding assay (Table 1), it was found that the CD6 mab
T12 inhibited TE-thymocyte binding by $49 \pm 9\%$ (N=5).
Similarly, recombinant CD6-Ig fusion protein inhibited
TE-thymocyte binding by $35 \pm 9\%$ (N=5). This suggested
10 that human TEC express a ligand for CD6.

Table 1

Effect of monoclonal antibodies and fusion protein
on thymocyte-thymic epithelial cell binding

mab/fusion protein % Binding ^a		% Inhibition ^b	
P3	32.8 ± 5.2	0	
35.1 (anti-CD2)	8.2 ± 2.4	76.0 ± 5.4	p<0.003
T12 (anti-CD6)	17.2 ± 3.8	48.7 ± 9.2	p<0.015
ELAM-Ig	29.7 ± 3.6	0	
CD6-Ig	19.3 ± 3.2	34.5 ± 9.3	p<0.05

^a Binding was determined to be the percentage of TE cells rosettes (≥ 3 bound thymocytes). Shown is the mean and standard error of 5 separate experiments.

^b % Inhibition = $100 (\text{Binding}_{\text{control}} - \text{Binding}_{\text{exp}}) / \text{Binding}_{\text{control}}$. The controls were P3 for mabs, and ELAM-Ig for CD6-Ig.

P values represent 2 tailed Student's t-test comparing binding in the presence of mabs or CD6-Ig to control binding.

Human TE cells express a ligand for CD6: To confirm that human TEC express a ligand for CD6, TEC were incubated with recombinant CD6-Ig fusion proteins and assayed for CD6-Ig binding to TEC by indirect immunofluorescence followed by flow cytometry. Neither of the negative controls (CD5-Ig and ELAM-Ig) bound to the surface of TEC while CD6-Ig did bind to TE cells (Table 2), indicating that there is a ligand for CD6 expressed on the surface of human TE cells. CD6-Ig binding to the CD6 ligand on TE cells was enhanced by divalent cations (Ca^{++} and Mn^{++}) and partially inhibited by EDTA (Figures 1 and 2). The data suggest that there are at least two components to the binding of CD6-Ig to TEC - a component (or ligand) that is dependent upon divalent cations, and a component (or ligand) that is divalent cation-independent.

Table 2

Reactivity of fusion proteins on
human thymic epithelial cells^a

fusion protein	MFC ^b
IgG ₁	72.9
ELAM-Ig	70.5
CD5-Ig	72.1
CD6-Ig	164.9

^a Cultured TE cells were incubated with 2000 μ g/ml fusion proteins. Fusion proteins were detected by indirect immunofluorescence followed by flow cytometry.

^b Linear Mean Fluorescence Channel. Data are representative of 3 experiments.

CD6 is an adhesion molecule: The fact that T12 and CD6-Ig both partially inhibit the binding of thymocytes to TEC strongly suggested that CD6 was an adhesion molecule. The TE rosette studies, however, did not rule out the possibility that T12 and CD6-Ig inhibited TE-thymocyte binding because of steric hinderance. To

determine if CD6 was indeed an adhesion molecule, a COS cell line (COS-CD6D) was made that expressed high levels of transfected CD6. COS cells without CD6 (CS-Neo) did not bind to TEC, whereas COS-CD6D cells did bind TEC (Figure 3). Furthermore, this COS-CD6D:TEC cell binding was CD6-dependent as binding was inhibited by mab to CD6 (T12).

Mab J4-81 reacts with a ligand for CD6: To identify the CD6 ligand, a large panel of anti-integrin mabs and a blind panel of 479 mabs from the Vth International Workshop on Human Leukocyte Differentiation Antigens were screened for reactivity to TEC cells. Of the 154 mabs that reacted with TEC, 126 mabs were used in assays to inhibit the binding of CD6-Ig to TEC. As shown in Figure 4, only Workshop mab S252 (J4-81) was able to inhibit the binding of CD6-Ig to TEC. Mab J4-81, which was raised against B cell surface antigens, recognizes a 105 kDa protein on B cells (Pesando et al, J. Immunol. 137:3689 (1986)) and reacts strongly with the surface on all cultured TEC (Figure 5) and with medullary TEC in frozen sections of human thymus (Figure 6).

Immunoprecipitation of 105 kDa, 90 kDa and 35 kDa molecules from TEC with CD6-Ig: In the absence of EDTA, CD6-Ig fusion protein immunoprecipitated protein species with molecular weights of 105, 90 and 35 kDa (Figure 7, lane 1) (note approximately 150 kDa band). In the presence of EDTA, CD6-Ig immunoprecipitated only the 105 and 35 kDa species (Figure 7, lane 2) (note approximately 150 kDa band). In lane 3, in the absence of EDTA, the control fusion protein, CTLA4-Ig, did not immunoprecipitate the 105, 90 or 35 kDa protein species.

Mab J4-81 inhibits the binding of TE cells to COS-CD6D cells: A third approach has been used to define that the 105/35 kDa protein molecules detected by mab J4-81 is a ligand for CD6. As shown above, COS-CD6D:TEC binding is CD6-specific. To further demonstrate that CD6 and the 105/35 kDa molecules recognized by J4-81 form an adhesion molecule pair, COS-CD6D:TEC binding with mab J4-81 was inhibited (Table 3).

Table 3

Effect of monoclonal antibodies COS-CD6D:TEC binding

mab	% Binding ^a	% Inhibition ^b
P3	25.0 ± 1.8	0
T12 (anti-CD6)	4.5 ± 0.7	82.7 ± 2.1 p<0.0007
S252 (J4-81)	8.7 ± 0.7	65.3 ± 1.8 p<0.0008

a Binding was determined to be the percentage of COS cells rosettes (≥ 3 bound TEC). Shown is the mean and standard error of 3 separate experiments.

b % Inhibition = $100 (\text{Binding}_{\text{P3}} - \text{Binding}_{\text{exp}}) / \text{Binding}_{\text{P3}}$.

P values represent 2 tailed Student's t-test comparing inhibition in the presence of T12 or J4-81 inhibition in the presence of P3.

Reactivity of J4-81 mab with human tissue in indirect IF assay: Mab J4-81 reacted with TEC, epidermal keratinocytes, fibroblasts, acinar cells and islet cells of pancreas, gut epithelium, monocytes (10%) and activated PB T cells (21%). B cells are also positive for J4-81 (Pesando et al, J. Immunol. 137:3689 (1986)).

Taken together, these data demonstrate that the CD6-CD6 ligand system is involved in TEC-thymocyte interactions, that CD6 is an adhesion molecule and that 105/35 kDa proteins detected by mab J4-81 comprise a ligand for CD6. Moreover, that the CD6-divalent cation-independent ligand defined by mab J4-81 is expressed on a wide variety of immune and epithelial cell types suggests that the CD6/CD6 ligand system is important in immune cell interactions with other immune cells and with a wide variety of epithelial microenvironments.

EXAMPLE II

Experimental details:

Cell lines, fusion protein, and antibodies. The colon carcinoma-derived cell line H3719 and the melanoma-derived cell line H3606 were from Drs. K.E. and I. Hellström (Bristol-Myers Squibb, Seattle, WA). The human breast epithelial cell line HBL-100, the EBV-transformed human B cell line LCL, the human lung fibroblast IMR90, the adult T cell leukemia Jurkat, the cutaneous T cell lymphoma HUT78, the human peripheral blood acute leukocytic leukemia derived cell line HPB-All, the human T cell lymphoma H9, the human Burkitt lymphoma Raji, the fibroblast cell line GM0833, the B

cell lymphoblastoma Peer, the T cell leukemia JM, and the B cell lymphoblastoma LTR228 were used and obtained primarily from the American Type Culture Collection (Rockville, MD). The CD5-Rg (Aruffo et al, Proc. Natl. Acad. Sci. USA 89:10242 (1992)), ELAM1-Rg (Walz et al, Science 250:1132 (1990)), and CTLA4-Ig (Linsley et al, J. Exp. Med. 174:561 (1991)) have been previously described. The anti-CD6 mAb G3-6 (IgG) was from Dr. J. Ledbetter (Bristol-Myers Squibb, Seattle, WA) (Ledbetter et al, Proc. Natl. Acad. Sci. USA 84:1384 (1987)); the anti-CD6 mAb MBG6 (IgM) was from Dr. A. McMichael (Oxford University, Oxford, U.K.) (Bastin et al, J. Clin. Exp. Immunol. 46:597 (1981)); the anti-CD6 mAb 2H1 was from Dr. C. Morimoto (Dana-Farber Cancer Institute, Boston, MA) (Morimoto et al, J. Immunol. 140:2165 (1988)); the anti-CD6 mAb T12 has been previously described (Reinherz et al, Cell 30:735 (1982)). The DNA restriction enzymes and the DNA linkers were obtained from New England Biolabs (Beverly, MA); RPMI, HBSS, FBS, and prestained molecular weight markers were obtained from GIBCO-BRL (Gaithersburg, MD). Human epidermal keratinocytes (EK) were cultured from neonatal foreskins. Foreskins were incubated at 4°C overnight in 2.5 mg/ml trypsin type III (Sigma, St. Louis, MO) in HBSS (GIBCO-BRL), the epidermis was removed, teased into single-cell suspension, seeded onto mitomycin C-treated

3T3 fibroblast feeder layers, and cultured as described (Hashimoto et al, J. Exp. Med. 145:259 (1983)).

Preparation of CD6-Rg. The plasmid encoding the CD6-Rg containing a full-length cDNA clone encoding CD6 (Aruffo et al, J. Exp. Med. 174:949 (1991)) was digested with the restriction enzyme *EspI*, treated with the Klenow fragment of DNA polymerase I, and ligated to *BamHI* linkers (New England Biolabs, Beverly, MA). The vector was then digested with the restriction enzymes *NdeI* and *BamHI*. The *NdeI/BamHI* DNA fragment containing the extracellular domain of CD6 was subcloned into a plasmid containing a cDNA fragment encoding the constant domains (hinge, CH2, and CH3) of human IgG₁. The CD6-Rg protein was prepared by transient expression in COS cells as previously described (Aruffo et al, Cell 61(7):1303 (1990)) and purified from the supernatant of COS cell transfectants by absorption to, and elution from, a protein A column (Repligen, Cambridge, MA).

CD-6 Rg cell binding studies. Typically, 5×10^7 cells/ml in HBSS/2% FBS/20 mM Hepes (wash/stain buffer) were incubated with 50 μ g/ml of fusion protein. Adherent cells were detached from dishes with 0.5 mM EDTA in PBS (PBS/EDTA) and washed once, while nonadherent cells were washed once before incubation

with the fusion protein for 1 hr on ice. The cells were subsequently washed three times and incubated with FITC-goat anti-human IgG (10 μ g/ml, Tago, Burlingame, CA) for 1 hr on ice. The cells were washed three times, fixed
5 with 1% paraformaldehyde in PBS, and analyzed by flow cytometry (Epics V, Coulter, Hialeah, FL).

This staining procedure was used to examine if the binding of CD6-Rg to HBL-100 cells was saturable. In those experiments the CD6-Rg and control fusion protein
10 were used at the following concentrations: 10, 20, 30, 40, 50, 100, and 200 μ g/ml. To investigate the sensitivity of CD6-Rg ligand(s) to trypsin, HBL-100 cells (5×10^7) were treated with 0.05% trypsin/0.5 mM EDTA for 30 min at 37°C, washed three times with
15 wash/stain buffer, incubated with CD6-Rg and analyzed by flow cytometry as described above. To examine the divalent cation requirement for CD6-Rg binding, CD6-Rg was incubated with HBL-100 cells in the presence of 15 mM EDTA, or, alternatively, the EDTA was added to
20 HBL-100 cells which had been previously incubated with CD6-Rg and washed. For anti-CD6 blocking experiments CD6-Rg (50 μ g/ml) was incubated with a 1:50, 1:100, and 1:200 dilution of the 1 mg/ml anti-CD6 mAb G3-6 (IgG) or ascites containing the MBG6 mAb (IgM, from
25 A.J. McMichael) or an isotype matched (IgM) antibody. To examine the cytokine-induced modulation of CD6-Rg

binding to HBL-100 cells, cells were incubated with IL-1 β , TNF- α (Genzyme, Boston, MA), and IFN- γ (Upstate Biotechnology Inc., Lack Placid, NY) (10 ng/ml) individually, in pairwise combinations, or all together, for 48 hr at 37°C, 5% CO₂ prior to CD6-Rg binding studies.

Immunoprecipitation studies. For immunoprecipitation studies, cells were incubated with 120 μ Ci/ml of [6-³H]glucosamine (NEN Dupont, Boston, MA) in glucose-free RPMI containing 10% normal RPMI and 10% dialyzed FBS for 48 hr at 37°C and 5% CO₂. The cells were lifted from the culture dish with PBS/EDTA, washed in HBSS, and lysed in 20 mM Tris, pH 7.5, containing leupeptin, PMSF, and pepstatin (1 μ g/ml, Boehringer Mannheim, Indianapolis, IN), 0.05% sodium cholate, 2% NP-40 (lysis buffer). The lysates were spun to remove nuclei and precleared by incubating two times with 50 μ g/ml of human IgG1 (Sigma) and 50 μ l of protein A-Sepharose slurry (Repligen) for 30 min at 4°C. Cell lysates were then incubated with 50 μ g/ml of CD6-Rg or CTLA4-Ig or 2 μ g/ml of an anti-EGF receptor antibody (Oncogen Science, Uniondale, NY) in the presence of either 2 mM Ca²⁺ and Mg²⁺ or 15 mM EDTA with 50 μ l of protein A-Sepharose (Repligen), for 2-4 hr at 4°C. The protein A-Sepharose beads were then washed three times

with lysis buffer. The immunoprecipitated proteins were analyzed by SDS-PAGE (8-10.5% gradient gel) followed by autoradiography.

Immunohistology. Murine (Balb/c) lymphoid and
5 nonlymphoid tissues were removed and frozen in liquid nitrogen. Six-micrometer cryostat tissue sections were prepared, mounted on glass slides, and fixed with ice-cold acetone. The fixed sections were stained using
10 50 $\mu\text{g}/\text{ml}$ of CD6-Rg or human IgG1 (Sigma) in a solution of PBS containing 1 mM Ca^{2+} and Mg^{2+} , 10% BSA, and 10% normal goat serum (NGS) (staining buffer) for 1 hr at room temperature. The slides were washed three times in staining buffer and incubated with a 10 $\mu\text{g}/\text{ml}$ of
15 fluorescein-conjugated, affinity-purified goat anti-human IgG antibody (Tago) for 1 hr at room temperature. After washing three times with staining buffer, the slides were examined by fluorescence microscopy.

Results:

CD6 receptor globulin, CD6-Rg. The CD6-Rg fusion
20 gene was constructed by fusing a cDNA fragment encoding the 400-amino acid extracellular domain of CD6 (Aruffo et al, J. Exp. Med. 174:949 (1991)), including its amino acid terminal signal sequence, onto a cDNA fragment

encoding the hinge (H), CH2, and CH3 domains of human IgG₁ and subcloned into the mammalian expression vector CDM7B⁺ (Aruffo et al, Cell 61(7):1303 (1990)) as described above. The cDNA fragment encoding the IgG constant domain contains three point mutations. These amino acid substitutions result in the impaired binding of IgG Fc domains to FcRI, II, and III.

The CD6-Rg protein used in this study was obtained by transient expression in COS cells and purified by absorption to, and elution from, a protein A column (Aruffo et al, Cell 61(7):1303 (1990)). CD6-Rg is expressed as a covalent homodimer and is recognized by all anti-CD6 mAb tested in an ELISA assay (T12, 2H1, MBG6, and G3-6). The previously described CD5-Rg (Aruffo et al, Proc. Natl. Acad. Sci. USA 89:10242 (1992)), ELAM1-Rg (Walz et al, Science 250:1132 (1990)), and CTLA4-Ig (Linsley et al, J. Exp. Med. 174:561 (1991)) fusion proteins and/or human IgG1 were used as isotype matched controls in all the binding and immunoprecipitation studies.

CD6-Rg binding to human and murine cells. The binding of CD6-Rg to a number of human and murine cell lines was examined by indirect immunofluorescence using flow cytometry. CD6-Rg bound to a subset of the cell lines examined (Fig. 8). Among the cell lines which

showed the brightest fluorescence intensity following CD6-Rg staining were the human breast epithelial-derived cell line HBL-100, the human colon carcinoma-derived cell line H3719, the melanoma-derived cell line H3606, the EBV-transformed human B cell line LCL, and the human fibroblast cell lines GM0833 and IMR90. Among the cell lines exhibiting intermediate fluorescence intensity following CD6-Rg binding were the lymphoid cell lines Jurkat, Peer, and HUT78. A number of other lymphoid cell lines including HPBALL, JM, H9, LTR228, and Raji exhibited no binding to CD6-Rg. None of these cell lines exhibited significant binding to the control fusion protein CD5-Rg. The binding to HBL-100 cell line was further characterized.

Binding of increasing concentrations of CD6-Rg to the HBL-100 cell line showed that the interaction of CD6-Rg with this cell line was dose dependent and saturable (100 μ g/ml, Fig.9A). Treating these cells with trypsin abolished CD6-Rg binding (Fig. 9B), while neuraminidase or N-glycanase treatment only slightly decreased CD6-Rg binding. The binding of CD6-Rg to HBL-100 was in part EDTA sensitive when the chelator was added before the CD6-Rg/HBL-100 binding, but not after (Fig. 10). In addition CD6-Rg bound very weakly to this cell line in PBS; however, addition of either Ca^{2+} or

Mg²⁺ (2 mM final concentration) resulted in strong CD6-Rg binding to HBL-100 cells.

The ability of the anti-CD6 mAb MGB6 and G3-6 to block the binding of CD6-Rg to HBL-100 cells was examined. The MGB6 mAb was able to block CD6-Rg binding to HBL-100 in a concentration-dependent manner (Fig. 11), while the G3-6 mAb was unable to block CD6-Rg binding to HBL-100 cells at concentrations as high as 0.02 mg/ml (Fig. 11).

Effects of cytokines on CD6-Rg binding. Cytokines regulate the expression of a number of cell surface proteins. The effect of cytokines on the expression of the CD6-binding protein(s) expressed by HBL-100 cells was examined. Treatment of the cells with a mixture of IL-1 β , TNF α , and IFN- γ resulted in the downregulation of CD6 ligand(s) expression (Fig. 12). Treatment with each of the cytokines alone or with pairwise combinations of the cytokines showed that a mixture of TNF α and IFN- γ was predominantly responsible for the downregulation of CD6 ligand(s) expression by these cells.

Immunoprecipitation of a CD6 ligand. The CD6-Rg fusion protein was used to immunoprecipitate the CD6 ligand(s) expressed by HBL-100 cells. Multiple attempts to immunoprecipitate the CD6 ligand(s) following ¹²⁵I

cell surface labeling or [³⁵S]methionine/cysteine metabolic labeling from both cell lines were unsuccessful. In contrast, [³H]glucosamine labeled surface glycoproteins of ~90 and ~40 kDa were
5 immunoprecipitated from HBL-100 cell lysates with CD6-Rg (Fig. 13). It is unclear if the ~40-kDa species is a unique protein or a degradation product of the ~90-kDa protein. Similarly, CD6-Rg was able to immunoprecipitate proteins of ~90 and ~40 kDa from
10 [³H]glucosamine-labeled H3606, a melanoma-derived cell line. CD6-Rg was unable to immunoprecipitate these proteins if EDTA was present in the cell lysate.

In addition, CD6-Rg but not CTLA4-Ig immunoprecipitated a polypeptide with a molecular mass
15 of ~100 kDa from radiolabeled HBL-100 cell lysates in both the presence and the absence of EDTA (Fig. 13). This observation is consistent with cell binding studies which showed that CD6-Rg binding to HBL-100 cells was not completely blocked by EDTA (Fig. 10) but was
20 completely abolished by pretreating the HBL-100 cells with trypsin (Fig. 9B). This ~100-kDa polypeptide was also immunoprecipitated from the H3606 lysates by the CD6-Rg fusion protein.

CD6-Rg Immunohistochemistry. To identify tissues
25 which expressed high levels of CD6 ligand(s) a panel of

tissue sections obtained from murine brain, skin, liver, kidney, heart, spleen, lymph node, thymus, and small intestine were examined for CD6-Rg binding. Reactivity was observed in the skin, lymph node, and thymus tissue sections (Fig. 14). In the skin bright punctate staining of the dermis as well as staining of hair follicles was observed (Fig. 14A); however, human IgG₁ also reacted with the hair follicle, suggesting that this interaction was not mediated by the CD6 portion of the molecule. In the thymus the predominant reactivity was observed in the cortex (Fig. 14C) while in the lymph node bright staining was seen along the intermediate sinus (Fig. 14E).

Based on these observations, the binding of CD6-Rg to the murine thymic epithelial-derived cell line Z210 and to cultured human epidermal keratinocytes (EK) was examined. It was found that CD6-Rg bound to both Z210 and EK cells (Fig. 15). Binding to Z210 cells was trypsin sensitive, divalent cation-dependent, and modulated by cytokines in a similar manner to that observed with HBL-100 cells. The control fusion protein CD5-Rg did not bind to Z210 or EK cells (Fig. 15).

EXAMPLE IIIExperimental details:

Cells and culture conditions. TE cells and thymic fibroblasts (TF) were cultured by an explant technique as described (Singer et al, Human Immunol. 13:161 (1985); Singer et al, J. Invest. Dermatol. 92:166, (1989)). Human thymus tissue was obtained from the Department of Pathology, Duke University Medical Center, as discarded tissue from children undergoing corrective cardiovascular surgery, and thymocytes prepared as described (Denninget al, J. Immunol. 139:2573 (1989)). COS-M6 cells (ATCC, Rockville, MD) and HBL-100 cells (ATCC) were grown in DMEM containing 10% FCS, 1mM sodium pyruvate, 0.025 μ g/ml amphotericin B, 100 U/ml penicillin and 100 μ g/ml streptomycin.

Monoclonal antibodies. Antibodies used in this study were: P3X63/Ag8 (control mAb; ATCC), 35.1 (anti-CD2; from J. Hansen, Seattle, WA), T12 (anti-CD6; from E. Reinherz, Boston, MA), Na1/34 (anti-CD1a; from A. McMichael, Oxford, England), A3D8 (anti-CD44; (Telen et al, J. Clin. Invest. 71:1878 (1983)), J4-81 and J3-119 (Pesando et al, J. Immunol. 137:3689 (1986)), phycoerythrin conjugated anti-CD4 (CD4-PE; Dako,

Carpinteria, CA) cychrome conjugated anti-CD8 (CD8-Cy; Pharmingen, San Diego, CA), and the blind panel of 479 mAbs from the 5th International Workshop on Human Leukocyte Differentiation Antigens (Shaw, Concepts of cross-lineage (blind panel) analysis of expression of differentiation antigens. In: Leukocyte Typing, Schlossman et al, eds, Oxford University Press, Oxford (1994)).

Detection of cell surface antigens. Unfixed, cultured TE cells, TF, COS cells or HBL-100 cells were suspended in either PBS, TBS (0.9 M NaCl, 50 mM Tris-HCl pH 7.3; with or without 5 mM CaCl₂, 5 mM MgCl₂ or 5 mM MnCl₂) or DMEM (with or without 10 mM EDTA) containing 2% BSA and 0.1% NaN₃. Cells were incubated with CD6-Rg (Wee et al, Cell. Immunol. (1994)) or with CD5-Rg (Arrufo et al, Proc. Natl. Acad. Sci. USA 89:10242 (1992)), ELAM-Rg (Walz et al, Science 250:1132 (1990)), CTLA4-Rg recombinant fusion protein (Linsley et al, J. Exp. Med. 174:561 (1991)), or human IgG (Sigma, St. Louis, MO) as controls (100 µg/ml) for 30 min at 4°C and washed with PBS containing 2% BSA and 0.1% NaN₃. Fluorescein conjugated goat anti-human IgG₁ (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was used as a secondary reagent. Cells were analyzed on either a Prifile II flow cytometer (Coulter Corp., Hialeah, FL)

or a FACStar^{Plus} flow cytometer (Becton-Dickinson, Inc., Mountain View, CA) and data was processed using the software program PC-Lysys. To determine trypsin sensitivity of fusion protein interactions, cells were
5 incubated with 0.2% trypsin in PBS containing 1 mM EDTA for 30 min at 37°C and washed extensively prior to reactivity with fusion proteins. Three color immunofluorescence studies were performed.

TE-thymocyte rosette binding assay. TE-thymocyte
10 rosette binding assays were performed as described (Singer et al, Proc. Natl. Acad. Sci. USA 83:6588 (1986)). Thymocytes were separated into CD1⁻ (immature, CD6^{lo}) or CD1⁻ (mature, CD6^{hi}) subpopulations by indirect immunofluorescent staining with NaI/34 and goat anti-
15 mouse IgG followed by fluorescence activated cell sorting using a FACStar^{Plus} fluorescence activated cell sorter. Thymocyte subpopulations were >95% pure on reanalysis of sorted cells. Mabs used in assays of
TE-thymocyte binding were used at or in excess of
20 saturating binding titers.

COS cell transfection and binding studies. Stable lines of CD6-expressing COS cells were constructed by co-transfection of plasmid CD6-15 containing the CD6 gene under the control of a CMV promoter (Aruffo et al,

J. Exp. Med. 174:949 (1991)) and the pSVneo plasmid containing the bacterial neomycin resistance gene driven by an SV40 promoter as described (Liao et al, J. Immunol. 151:6490, (1993)). COS cells expressing CD6
5 were identified in indirect immunofluorescence assays using mAb T12. CD6+ and CD6- cells were cloned using a Becton-Dickinson FACStar^{plus} fluorescence activated cell sorter.

CD6-expressing COS cells (COS-CD6) and control COS
10 cells transfected with pSVneo (COS-neo) were used in suspension binding assays with TE cells. To differentiate between COS cells and TE cells, TE cells were metabolically labelled with 1 μ M calcein AM (Molecular Probes, Eugene, OR) for 15 min at 37°C in PBS
15 prior to harvest. Calcein AM-labelled cells are fluorescent and can be differentiated from non-labelled cells by fluorescence microscopy.

Screening of mAbs for inhibition of CD6-Rg binding to TE cells. Mabs from the blind panel of the 5th
20 International Workshop on Human Leukocyte Differentiation Antigens and a panel of anti-integrin mabs were screened for reactivity to the surface of TE cells. Of the 154 mAbs that reacted with TE cells, 126 were used in this assay. TE cells (10^5) were incubated
25 with ascites or purified mAb at a dilution of 1:100 for

15 min at 4°C. Either CD5-Rg (5µg) or CD6-Rg (5µg) were added to this mixture and allowed to react for 2 hrs at 4°C. After washing with PBS containing 2% BSA, CD5-Rg and CD6-Rg were labelled with a fluorescein-conjugated antiserum specific for the Fc portion of human IgG (Sigma). To account for any cross-reactivity with murine Ig that may have occurred with fluorescein-conjugated anti-human IgG, binding was determined as the difference in fluorescence (ΔFL) between samples containing CD6-Rg and CD5-Rg. The ΔFL of samples pre-incubated with control mAb P3 represented 100% binding.

Antibody blocking studies. MAb J4-81 was purified from ascites using a protein G-sepharose column (Pierce, Rockford, IL) and biotinylated with sulfo-NHS-biotin (Pierce) as recommended by the manufacturer. Biotin-J4-81 (1 µg/ml) was bound to the surface of TE cells in the presence of variable doses of mAbs A3D8, J4-81 or J3-119. After incubation with FITC-conjugated streptavidin (Southern Biotechnology Associates, Inc., Birmingham, AL), cells were washed, fixed with 0.4% paraformaldehyde and analyzed by flow cytometry.

Immunoprecipitation and protein labelling conditions. TE cells were metabolically labelled with 120 µCi/ml ³H-glucosamine (New England Nuclear, Boston,

MA), harvested and immunoprecipitated as previously described (Wee et al, Cell. Immunol. (1994)). TE cell surface proteins were labelled with ^{125}I (New England Nuclear) using lactoperoxidase as previously described (Jones, Analysis of radiolabeled lymphocyte proteins by one- and two-dimensional polyacrylamide gel electrophoresis. In: Selected methods in cellular immunology. Mishell and Shiigi, eds. W.H. Freeman and Company, New York, pp. 398-440 (1980)).

Protein cross-linking. TE cells, surface labelled with ^{125}I , were incubated with mAbs (1:100 ascites), purified immunoglobulin (200 $\mu\text{g}/\text{ml}$) or recombinant immunoglobulin fusion proteins (200 $\mu\text{g}/\text{ml}$) for 2-4 hours in DME/5% FBS. After extensive washing with cold PBS, bound immunoglobulins were cross-linked to cell surface proteins with 1 mM DTSSP (Pierce) in PBS for 60 min at 4°C. DTSSP was inactivated with 20 mM Tris-HCl (pH 8.0) and the cells were washed with cold PBS. Cells were lysed in PBS containing 1% NP-40, 1 mM PMSF, 0.1 mM TLCK and 0.1% NaN_3 . Immunoglobulin complexes were purified with protein A-sepharose beads (Sigma). Prior to SDS-PAGE, protein complexes were solubilized with SDS loading buffer (2% SD, 10 mM Tris-HCl pH 7.4, 20% glycerol, bromphenol blue) containing 2% 2-mercaptoethanol (2-ME) to cleave the cross-linker. To

confirm identity of proteins, purified protein complexes were treated with 0.2% trypsin in 1 mM EDTA for 30 min at 25°C (Patel et al, Virology 149:174 (1986)) prior to solubilization and cross-linker cleavage. After
5 electrophoresis of proteins in discontinuous SDS-polyacrylamide gels, the gels were fixed in 40% methanol, impregnated with Amplify^R reagent (Amersham, Arlington Heights, IL) dried and exposed to
10 autoradiography film or imaged using a PhosphorImager System (Molecular Dynamics, Sunnyvale, CA).

Immunohistology. Normal human tissues were obtained as discarded tissue from the Department of Pathology, Duke University Medical Center and frozen in liquid nitrogen. Indirect IF assays of mAb reactivity
15 on acetone fixed tissue sections were performed as described (Haynes et al, J. Invest. Dermatol. 76:323 (1982)).

Results:

Effect of anti-CD6 antibodies and CD6-Rg on TE-thymocyte binding. The role of CD6 in the binding of thymocytes to TE cells was determined using a TE-thymocyte suspension binding assay. Both antibody to CD6 (T12) and recombinant CD6-immunoglobulin fusion protein (CD6-Rg) inhibited the binding of thymocytes to TE cells (Figure 16). As previously reported (Patel and Haynes, Semin. Immunol. 5:283 (1993)), anti-CD2 mAb 35.1 (as a positive control) inhibited TE-thymocyte binding by $76 \pm 5\%$ ($p < 0.003$). Anti-CD6 antibody T12 inhibited TE-thymocyte binding by $49 \pm 9\%$ ($p < 0.015$). Similarly, the CD6 human Ig fusion protein, CD6-Rg, inhibited TE-thymocyte binding by $35 \pm 9\%$ ($p < 0.05$). Addition of a combination of saturating amounts of mAbs 35.1 and T12 to TE-thymocyte binding assays resulted in a level of inhibition of binding ($74 \pm 10\%$ inhibition) that was not significantly different from blocking by mAb 35.1 alone. Nonetheless, the data with anti-CD6 mAb and CD6-Rg suggested that CD6 may be an adhesion molecule that participates in the binding of thymocytes to TE cells, and that there may be a ligand for CD6 on human TE cells.

Binding of CD6-expressing COS cells to TE cells.

Although inhibition of TE-thymocyte binding with both anti-CD6 antibody and CD6-Rg was suggestive that CD6 was an adhesion molecule, inhibition of TE-thymocyte binding may have occurred because of stearic hinderance. To confirm that CD6 was an adhesion molecule, stable transfectants of CD6-expressing COS cells (COS-CD6) were constructed (Figure 17). COS cells transfected with pSVneo only (COS-neo) did not bind well to TE cells (6 ± 1% COS-neo binding TE cells), whereas COS-CD6 did bind to TE cells (25 ± 2% COS-CD6 binding TE cells, p<0.01) (Figure 17). The specificity of the COS-CD6/TE binding was examined by testing for the inhibition of COS-CD6/TE binding by CD6 mAb T12. Compared to control mAb P3 and COS-neo cells (6 ± 1% COS-neo binding TE), CD6 mAb T12 completely inhibited the binding of COS-CD6 to TE cells (5 ± 1% COS-CD6 binding TE, p<0.01) to baseline levels of binding (Figure 17), confirming that COS-CD6/TE binding was CD6-specific.

Effect of anti-CD6 mAb on the binding of thymocyte subsets to TE cells. The role of CD6 in the binding of subsets of thymocytes was determined by examining the expression of CD6 on thymocyte subsets, and by testing the binding of sorted mature versus immature thymocyte subsets to TE cells in the presence of CD6 mAbs.

Expression of CD6 on thymocyte subsets was determined by three color immunofluorescence and flow cymetry (Figure 18). While all thymocytes expressed CD6, immature CD4+CD8+ double positive (DP) thymocytes were CD6^{lo} and the mature CD4+CD8- or CD4-CD8+ single positive (SP) thymocytes were CD6^{hi}.

To determine the role of CD6 in the binding of thymocyte subsets to TE cells, thymocytes were separated into CD1+ and CD1- subsets by fluorescence activated cell sorting. CD1 was chosen because CD1+ thymocytes are the DP CD6^{lo} cells and CD1- thymocytes are the SP CD6^{hi} cells, and CD1 mAbs do not inhibit TE-thymocyte binding (Patel and Haynes, Semin. Immunol. 5:283 (1993)). Both CD1+ and CD1- thymocytes bound well to TE cells (45 ± 4% and 54 ± 6%, respectively, p=NS). CD2 mAb 35.1 inhibited the binding of immature thymocytes (77 ± 9% inhibition) to a greater degree than that of mature thymocytes (52 ± 11% inhibition) (p<0.01). CD6 mAb T12 also inhibited the binding of both immature (22 ± 7% inhibition) and mature (39 ± 17% inhibition) thymocytes to TE cells (p<0.25) (Figure 19).

CD6-Rg binding to cells of the thymic microenvironment. The ability of CD6-Rg fusion protein to bind to TE cells, thymic fibroblasts and to thymocytes was determined by indirect IF and flow

cytometry. CD6-Rg bound to the surface of TE cells as well as to thymic fibroblasts, but not to thymocytes (Figure 20A). Binding of CD6-Rg to TE cells was trypsin-sensitive and partially dependent upon divalent cations (Figure 20B). CD6-Rg bound well to TE cells in a buffer containing DME and 5% FBS, but in the presence of 10 mM EDTA, binding of CD6-Rg to TE cells was inhibited by $54 \pm 4\%$ ($N=5$, $p<0.001$, Figure 20B). To further evaluate the divalent cation-dependence of CD6-Rg binding, CD6-Rg binding was determined in buffers containing 5 mM CaCl_2 , 5 mM MgCl_2 or 5 mM MnCl_2 . While both CaCl_2 and MnCl_2 enhanced the binding of CD6-Rg to TE cells ($144 \pm 5\%$, $p=0.01$, and $318 \pm 22\%$, $P<0.01$, respectively), MgCl_2 did not affect CD6-Rg binding ($94 \pm 2\%$, $p=\text{NS}$).

Antibody-mediated inhibition of CD6-Rg binding to TE cells. To begin to identify the CD6 ligand(s), a panel of 479 mAbs from the 5th International Workshop on Human Leukocyte Differentiation Antigens was screened for reactivity to the surface of TE cells by indirect immunofluorescence and flow cytometry (Shaw, Concepts of cross-lineage (blind panel) analysis of expression of differentiation antigens. In: Leukocyte Typing, Schlossman et al, eds, Oxford University Press, Oxford (1994)). Of the 154 mAbs that reacted with the surface

of TE cells, 126 mAbs were used in assays to inhibit the binding of CD6-Rg to TE cells. Of the 122 mAbs that did not react with the secondary antiserum, only one (J4-81) inhibited the binding of CD6-Rg to TE cells. MAb J4-81
5 inhibited the binding of CD6-Rg to TE cells by $60 \pm 7\%$ ($N=10$, $p<0.001$) and to the breast cell line HBL-100 by $40 \pm 3\%$ ($N=3$, $p<0.02$) (Table 4), which has also been shown to bind CD6-Rg (Wee et al, Cell. Immunol. (1994)). In flow cytometry assays, both TE cells and HBL-100
10 cells reacted strongly with mAb J4-81.

Table 4. Mab inhibition of CD6-Rg binding to TE and HBL-100 cells.

Cell Type	mAB	Δ Fluorescence*	% Binding [‡]	% Inhibition [§]
TE cells	P3	52	100	0
	CD9	60	115	-15
	CD24	48	92	8
	CD40	48	92	8
	CD46	57	110	-10
	CD51	46	88	12
	CD54	44	85	15
	CD58	45	87	13
	CD59	52	100	0
	CD63	53	102	-2
	CD66	56	108	-8
	J4-81	21	40	60
HBL-100	P3	58	100	0
	J4-81	34	59	41

* Δ Fluorescence = Fluorescence(CD6-Rg) - Fluorescence (CD5-Rg). Shown is the binding of CD6-Rg compared to control (CD5-Rg) in the presence of selected mAbs that bind well to the surface of TE cells.

[‡]% Binding = $100[\Delta\text{FL}(\text{experimental mAb}) - \Delta\text{FL}(\text{P3})] / \Delta\text{FL}(\text{P3})$.

[§]% Inhibition = $100[\Delta\text{FL}(\text{P3}) - \Delta\text{FL}(\text{experimental mAb})] / \Delta\text{FL}(\text{P3})$.

MAB J3-119, reported to react with a second epitope on the molecule detected by J4-81 (Pesando et al, J. Immunol. 137:3689 (1986)), enhanced the binding of CD6-Rg to TE cells (Figure 21) by $48 \pm 5\%$ ($N=6$, $P<0.005$),
5 and to HBL-100 cells by $45 \pm 11\%$ ($N=3$, $p<0.1$). To confirm that mAb J3-119 recognized the same protein as mAb J4-81, the ability of mAb J3-119 to block the binding of biotinylated J4-81 to TE cells was tested. While mAb A3D8 to CD44 (which binds well to the surface
10 of TE cells had no effect on J4-81 binding, both J4-81 and J3-119 mAbs inhibited the binding of biotinylated J4-81 (99% and 82%, respectively) (Figure 22). Moreover, mAb J4-81 modulated surface expression on B cells of the antigen detected by J3-119 (Pesando et al,
15 J. Immunol. 137:3689 (1986)).

To further establish that mAb J4-81 recognized a ligand for CD6, the ability of J4-81 to inhibit the CD6-specific binding of COS-CD6 cells to TE cells was tested. MAb J4-81 significantly inhibited the binding
20 of TE cells to COS-CD6 cells ($87 \pm 1\%$, $N=3$, $p<0.001$), thus confirming that J4-81 recognized a CD6 ligand.

MAB J4-81 and CD6-Rg bind 100 kDa TE cell proteins that are identical. To identify the TE cell surface protein(s) that bound to CD6-Rg, a strategy was devised
25 whereby CD6-Rg interactions with CD6 ligand(s) on

surface ^{125}I labelled TE cells were stabilized with DTSSP, a cleavable homobifunctional cross-linking reagent reactive with free amino groups, and CD6-Rg containing complexes were purified using protein A-sepharose beads. Using this strategy, CD6-Rg specifically reacted with a 100 kDa TE cell surface protein (Figure 23). MAb J4-81 cross-linked to ^{125}I labelled TE cells also yielded an 100 kDa protein that migrated with the 100 kDa protein recognized by CD6-Rg (Figure 23). Trypsin digestion studies showed that the 100 kDa protein identified by J4-81 had an identical trypsin digestion pattern to the 100 kDa protein identified by CD6-Rg (Figure 23), indicating that both proteins were identical. MAb J4-81 also specifically immunoprecipitated an 100 kDa protein from extracts of TE cells metabolically labelled with ^3H -glucosamine.

The 100 kDa CD6 ligand is a divalent cation-independent ligand for CD6. The immunoprecipitation studies (above) showed that CD6-Rg was able to immunoprecipitate a 100 kDa protein in the presence and absence of divalent cations. Thus, the 100 kDa protein may be a divalent cation-independent CD6 ligand. Further, J4-81 only partially inhibited the binding of CD6-Rg to TE cells in the presence of divalent cations, suggesting that there may be more than one ligand for

CD6. MAb J4-81 nearly completely inhibited ($80 \pm 10\%$ inhibition, $p < 0.1$) CD6-Rg binding to TE cells in the presence of EDTA (Figure 21), confirming that the protein recognized by JA-81 is primarily involved in
5 divalent cation-independent CD6-CD6 ligand interactions. In contrast, mAb J3-119 enhanced CD6-Rg binding by $47 \pm 7\%$ ($p < 0.1$) in the absence of divalent cations (Figure 21).

Tissue distribution of mAb J4-81 reactivity. The
10 tissue distribution of the 100 kDa glycoprotein recognized by J4-81 was examined on frozen sections of a variety of human tissues by indirect IF (Table 5). The reactivity of mAb J4-81 with human tissues and cell
lines was broad. While CD6 was expressed on thymocytes
15 in postnatal human thymus, the 100 kDa CD6 ligand was expressed on cortical and medullary TE cells and Hassall's bodies (Figure 24). In tissues other than
thymus, J4-81 reacted with epidermal keratinocytes, gut
epithelium, breast epithelium, pancreatic acinar and
20 islet cells, hepatocytes, renal tubular epithelium, neurons of the cerebral cortex, and fibroblasts.

Table 5. Reactivity of mAb J4-81 in sections of normal human tissues.

Tissue (Number Tested)	J4-81 Reactivity
Thymus (5)	Hassall's bodies, epithelium, fibroblasts
Spleen (2)	Scattered mononuclear cells
Lymph Node (1)	Scattered mononuclear cells
Tonsil (2)	Pharyngeal epithelium lymphocytes
Appendix (1)	Fibroblasts, lymphoid cells, epithelium
Colon (2)	Fibroblasts, epithelium
Esophagus (2)	Basal epithelium
Breast (2)	Epithelium, fibroblasts
Liver (2)	Hepatocyte, Kupfer cells, fibroblasts
Pancreas (2)	Acinar and islet cells
Kidney (2)	Fibroblasts, subset of tubules, Bowman's capsule
Skin (2)	Perivascular fibroblasts, epithelium
Brain (2)	Neurons

The cell types reacting with mAb J4-81 in each of the tissue types are listed.

EXAMPLE IVExperimental details:

Cells lines, tissue culture, antibodies. TE cells were cultured by an explant technique as described
5 (Singer et al, Human Immunol. 13:161 (1985); Singer et al, J. Invest. Dermat. 92:166, (1989)). COS cells, breast carcinoma HBL-100, B cell lymphoma cell lines Ramos, Raji and Daudi, T cell lymphoma cell lines CEM and MOLT4, erythroleukemia cell line K562, and monocyte-
10 like cell lines HL60 and U937 were obtained from ATCC (Rockville, MD) and maintained under standard tissue culture conditions in Iscove's Modified Dulbecco's Medium plus 10% fetal bovine serum. NK-like cell line YT was obtained from E. Podack (Univ. of Miami Sch. of
15 Medicine). Antibodies used in this study were J4-81 (Pesando et al, J. Immunol. 137:3689 (1986)) as ascites, Leu4 (anti-CD3)-phycoerythrin (Becton Dickinson, San Jose, CA), G3-6 (anti-CD6 from J. Ledbetter, Bristol-Myers Squibb), anti-L6 (Bristol-Myers Squibb, ATCC), T12
20 (anti-CD6, ATCC), and P3 (ATCC).

Binding of TE cells to COS cell transfectants expressing CD6. COS-CD6 and COS-neo cells were prepared by co-transfecting a plasmid encoding CD6 (Aruffo et al,

J. Exp. Med. 174:949 (1991)), and the pSVneo plasmid (Liao et al, J. Immunol. 151:6490 (1993)). Following G418 selection, CD6+ cells were cloned using a Becton-Dickinson FACStar^{PLUS} fluorescence activated cell sorter. 5 COS-neo and COS-CD6 TE cells were used in a suspension binding assay. To differentiate between COS cells and TE cells, TE cells were labeled with 1 μ M calcein AM (Molecular Probes, Eugene, OR) for 15 min. at 37°C in PBS prior to harvest. Calcein AM-labeled cells are 10 fluorescent and can be differentiated from unlabeled cells by fluorescence microscopy. Adhesion was quantitated by scoring the percentage of COS-CD6 cells that were conjugated with TE cells. For blocking adhesion, antibodies T12, J4-81, and P3 were used at or 15 in excess of saturating binding titers.

Cloning and characterization of the gene for the antigen recognized by mAb J4-81. J4-81 immunoaffinity purification of ALCAM from HBL-100 cells ($\sim 3 \times 10^9$) was performed as described (Bowen et al, J. Immunol. 20 151:5896 (1993)). The purified protein and CNBr fragments were analyzed in a pulsed-liquid protein sequencer by previously described methods (Maresh et al, Arch. Biochem. Biophys. 311:95 (1994)). Amino-terminal sequences were screened against the SwissProt data base 25 using Genetics Computer Group (GCG) sequence-analysis

software. Chicken BEN probes were synthesized by PCR from a chicken embryo cDNA library (Clontech), random prime labeled with ^{32}P -dCTP, and used to screen a PHA activated human T cell cDNA library. A partial cDNA
5 clone was isolated; 200 bp of the 5' end of this cDNA was used to isolate a clone containing the entire coding region from a HL60 cDNA library. Database searches with the cDNA derived peptide sequence of ALCAM were
10 performed using the BLAST program, alignments and consensus sequence derivation of the four highest scoring hits were performed using the Pileup and Pretty programs from the GCG sequence analysis software.

Northern analysis. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood with
15 lymphocyte separation medium (Organon Teknika, Durham, NC) according to the manufacturer's instructions. PBMC were stimulated with 1 $\mu\text{g}/\text{ml}$ phytohemagglutinin (PHA; Boehringer Mannheim Indianapolis, IN). Total cellular RNA was prepared with TRIzol reagent (Gibco BRL,
20 Gaithersburg, MD). Monocytes were isolated from PBMC's by adherence to plastic for 2.5h. The RNA's were fractionated on a denaturing formaldehyde gel; 25 μg of RNA was loaded for all samples except for monocytes, where 15 μg was loaded. RNA was transferred to
25 nitrocellulose TC (Schleicher and Schuell, Keene, NH)

and probed with a random primed ^{32}P -dCTP labeled ALCAM or GAPDH cDNA. Filters were exposed to film at -70°C and developed.

PHA blast preparation, staining. PBMC were
5 isolated and activated with PHA as described above and
stained on selected days with mAb J4-81 ascites (1:200)
or CD6-Rg (50 $\mu\text{g}/\text{ml}$) followed by a FITC labeled second
antibody and anti-CD3-PE; cytofluorometric analysis of
J4-81 staining T cells was performed on a FACScan
10 (Becton Dickinson, San Jose, CA) by gating on CD3
positive cells.

Chromosomal mapping of the gene encoding ALCAM.
Two overlapping cDNA clones of the human ALCAM gene
containing 2.2 kb and 1.9 kb inserts in CDM7 were used
15 as probes for fluorescence *in situ* hybridization (FISH)
as described (Milatovich et al, Genomics 11:1040
(1991)). The probe was labeled with biotin-dUTP by
nick-translation and was hybridized at a concentration
of 500 ng/50 μl per slide to pre-treated and denatured
20 metaphase chromosomes from a human PHA-stimulated
lymphocyte culture. Salmon sperm DNA served as carrier
but no human genomic DNA was used as competitor. After
incubation, washing, signal detection with avidin-FITC
(Vector Laboratories) and one round of amplification

with biotinylated goat anti-avidin D antibody (Vector Laboratories), chromosomes were counterstained with propidium iodide and analyzed under an Axiophot (Zeiss) epifluorescence microscope as described (Milatovich et al, Genomics 11:1040 (1991)). Hybridization signals were considered specific only when the signal was observed on both chromatids of a chromosome. Results were recorded by digital imaging using a cooled charge-coupled device (CCD) camera (Photometrics PM512) and displayed in pseudo-colors.

Construction of ALCAM-Rg. ALCAM-Rg was constructed by PCR technology similarly to CD6-Ig (Aruffo et al, Proc. Natl. Acad. Sci. USA 89:2292 (1992)). ALCAM cDNA in pCDM8 was used as a template. A forward oligonucleotide primer (GGCCAGATATACGCGTTGACATT) encompassing an MluI site in the CMV promoter of pDCM8 and a reverse primer (TGTATCATGTGGATCCGCCTGGTCATTACCTTTTCTCT) containing a BamHI site and specific for the nucleotides encoding the extracellular membrane proximal residues of ALCAM were used to synthesize a fragment that encoded a DNA fragment encoding truncated ALCAM. This PCR product was digested with MluI and BAMHI and ligated to a vector (pCDM7) that was similarly digested and encoding the hinge, CH1 and CH2 domains of human IgG1. This resulted

in a chimeric gene encoding the extracellular region of ALCAM fused to the Fc portion of human IgG1. Protein was produced by transient expression of ALCAM-Rg in COS cells and purified by absorption to and elution from protein A Sepharose. Briefly, plasmid DNA was transfected into COS cells by the DEAE-dextran chloroquine method. 18-24 hours later, the 10% FBS Dulbecco's modified Eagle's medium was replaced by serum-free media after a rinse wash with PBS. The cell culture was maintained 7-10 days before it was harvested. Briefly, the supernatant containing the fusion protein was centrifuged at 2500 rpm or 20 min and filtered through a 0.45 μ m Millipore filter. The supernatant was passed over a Protein A Sepharose column at a flow rate of 1.00 ml/min. The bound fusion protein was eluted with pH 3.5, 0.1 M citric acid and was immediately neutralized with pH 9.0, 1.0 M Tris. The fusion protein was dialyzed against PBS and concentrated with a Certicor concentrator (Amicon).

Binding of CD6-Rg to ALCAM expressing COS cells and binding of ALCAM-Rg to CD6 expressing COS cells. COS cells were either transfected with parent vector CDM8 or with cDNA clones encoding CD6 or ALCAM by the DEAE-dextran method (Aruffo et al, Proc. Natl., Acad., Sci., USA 84:8573 (1987)). Three days post-transfection the

cells were detached with PBS/0.5 mM EDTA, washed with PBS, and resuspended in PBS/1% BSA/0.1% NaN₃ (PBA) containing the following concentrations of mAb's or fusion proteins: J4-81 (aALCAM) 1:200 dilution of ascites. G3-6 (aCD6) 5 µg/ml, CD6-Rg 50 µg/ml, and ALCAM-Rg 25 µg/ml. After 30 min. on ice, cells were washed twice with PBA and resuspended in PBA containing either goat anti-mouse or human IgG-FITC for 30 min. on ice. The cells were washed twice in PBA, once with PBS, fixed in PBS/1% formaldehyde, and analyzed by flow cytometry.

Results:

CD6 on transfected COS cells promotes TE cell adhesion. To examine directly the role of CD6 in thymocyte-TE cell adhesion, a stable COS cell transfectant expressing CD6 was prepared. COS cells expressing CD6 (COS-CD6) supported TE cell adhesion while COS cells transfected with the parent vector alone (COS-neo) did not (Fig. 25). The adhesion of TE cells to COS-CD6 cells was inhibited by pretreating the COS-CD6 cells with anti-CD6 mAb T12 or by pretreating TE cells with J4-81 mAb (Fig. 25B), indicating that CD6 mediated adhesion to TE cells involves the molecule recognized by mAb J4-81.

Cloning and characterization of the antigen recognized by mAb J4-81 (activated leukocyte-cell adhesion molecule (ALCAM)). A J4-81 affinity column was used to purify the protein from HBL-100, a breast carcinoma cell line that expressed high levels of a CD6 ligand (Wee et al, Cell. Immunol. 158:353 (1994)) and the antigen recognized by J4-81 (Patel et al, J. Exp. Med. (1994)). Amino acid sequences obtained from intact protein and internal peptide fragments were found to have substantial homology with the chicken neural adhesion molecule BEN/SC-1/DM-GRASP (Pourquie et al, Proc. Natl. Acad. Sci. USA 89:5261 (1992)); Tanaka et al, Neuron 7:535 (1991)); Burns et al, Neuron 7:209 (1991)), which was also reported to be expressed by activated chicken leukocytes (Corbel et al, Cell Immunol. 141:99 (1992)). DNA fragments corresponding to chicken BEN were obtained by PCR and used to screen a PHA activated human T cell cDNA library. A single clone that did not contain the complete coding region was isolated. A 200 bp PCR fragment derived from the 5' region of this clone was used to isolate a ~1.9 kb cDNA clone that contained the complete coding sequence from an HL60 cDNA library (the nucleotide sequence has been deposited in GenBank, accession #L38608). The two cDNA clones exhibit polymorphisms at three nucleotide positions in the region of overlap, two of which result

in differences at the protein level (G to A from HL60 to the T cell clone at position 836 results in the substitution of N231 by S; C to T from HL60 to the T cell clone at position 965 results in the substitution of M274 by T).

The predicted amino acid sequence of the human homologue of chicken BEN (ALCAM), a type I membrane protein, consists of a 27 amino acid (aa) N-terminal hydrophobic signal peptide, followed by 500 aa extracellular domain, a 24 aa hydrophobic transmembrane domain, and a 32 aa cytoplasmic domain (see Figure 29). Glycosylation of ALCAM probably accounts for much of the difference between the predicted molecular weight of ~65 kDa and the 100-105 kDa molecular weight observed by immunoprecipitation (Patel et al, J. Exp. Med. (1994)); Pesando et al, J. Immunol. 137:689 (1986)). Comparison of the aa sequence of ALCAM with others in the data base showed that it was homologous to neurolin (Lawssing et al, Differentiation 56:21 (1994)), a protein expressed by neural axons of the goldfish visual system (38% identity/55% similarity), RAGE (Neeper et al, J. Biol. Chem. 267:14998 (1992)), a receptor for advanced glycation end products (28/43%), and MUC18 (Lehmann et al, Proc. Natl. Acad. Sci. USA 86:9891 (1989)), a cell surface protein whose expression correlates with the

metastatic potential of melanoma cells (23/49%) (Fig 26A).

ALCAM is expressed by activated leukocytes. By Northern blot analysis an ALCAM cDNA probe hybridized with a ~5.2 kb mRNA expressed by mitogen activated peripheral blood mononuclear cells (PBMC) and a number of T cell, B cell, monocytic, and tumor derived cell lines; activated monocytes showed two additional minor species of ~10 and 8.5 kb (Fig. 26B). ALCAM mRNA was not detected in unactivated PBMC. To examine if the presence of ALCAM transcripts in activated T cells corresponded to the expression of a J4-81 and CD6-Rg binding protein, PBMC were activated with PHA and aliquots of the bulk culture were examined by flow cytometry for 10 days. Expression of a J4-81 and CD6 binding protein on T cells was observed two days after activation, was maximal at three days, and declined to undetectable levels eight days after activation (Fig. 26C). The binding of J4-81 and CD6-Rg to activated T cells exhibits the same expression kinetics, indicating that these two molecules recognize the same molecular target.

Assignment of human ALCAM gene to chromosome bands 3q13.1-q13.2. Fluorescence in situ hybridization

experiments using ALCAM cDNA clones as probes localized the gene to a single site on the proximal long arm of human chromosome 3. Of 25 metaphase spreads analyzed, 20 exhibited a specific fluorescent signal on both
5 chromatids at this site, and 13 of 20 had signal on both chromosome 3 homologues. The chromosomes were identified by an R-banding pattern produced by the incorporation of BrdU after synchronization of the cell culture. The ALCAM signal was assigned to bands 3q13.1-
10 q13.2 (Fig. 27).

ALCAM is a CD6 ligand. To directly examine if the ALCAM cDNA clone isolated from HL60 cells directs the expression of a protein which binds J4-81 and CD6-Rg, the cDNA was transfected into COS cells. Three days
15 post-transfection, the COS cells were examined for their ability to bind to J4-81 (aALCAM) or CD6-Rg by flow cytometry. As shown in Fig. 28A, COS cells expressing ALCAM were able to bind to both J4-81 and CD6-Rg. To further confirm that ALCAM is a CD6 ligand, a chimeric
20 gene encoding ALCAM-Rg was constructed. This protein was expressed as a covalent homodimer with a molecular mass of ~200 kDa and was recognized by mAb J4-81. To demonstrate the binding of ALCAM-Rg to CD6, COS cells were transfected with a cDNA clone encoding CD6 (Aruffo
25 et al, J. Exp. Med. 174:949 (1991)) and examined by flow

cytometry three days later. COS cells transfected with CD6 bound both G3-6 (aCD6) and ALCAM-Rg (Fig. 28B). These results demonstrate that CD6 and ALCAM are a receptor/ligand pair.

5 Chicken BEN has been reported to mediate homotypic adhesion (Pourquie et al, Proc. Natl. Acad. Sci. USA 89:5261 (1992)); Tanaka et al, Neuron 7:535 (1991)); Burns et al, Neuron 7:209 (1991)). ALCAM-Rg was able to bind COS cells that expressed ALCAM, indicating that
10 ALCAM mediates both homophillic and heterophillic adhesion.

EXAMPLE V

Production of anti-human ALCAM monoclonal antibodies. A 6-8 week old female BALB/c mouse
15 (Taconic, Germantown, NY) was immunized with a purified, recombinant fusion protein consisting of the extracellular domain of human ALCAM fused to the hinge, CH2, and CH3 domains of murine IgG2a. Primary
20 immunization was administered intraperitoneally as a 100 µl emulsion of 50 µg protein in Ribi adjuvant (R-730, Ribi ImmunoChem Research, Inc., Hamilton, MT).
Two identical immunizations were performed on days 19 and 29. On day 63, 50 µg of protein in 100 µl of PBS was administered intravascularly. Three days later,

cells were harvested from the spleen and all
identifiable lymph nodes, and fused at a 3:1 ratio of
leukocytes:myeloma cells with X63-Ag8.653 myeloma cells
(Kearney et al, J. Immunol. 123:1548 (1979)) according
5 to the method of Lane (J. Immunol. 81:223 (1985)). The
cell suspension was seeded into 15 96-well culture
plates at a density of 1.44×10^5 cells/well in the
presence of hybridoma growth medium [Iscove's Modified
Dulbecco's Medium (IMDM) supplemental with 10% fetal
10 calf serum, 2 mM L-glutamine, 100 U/ml penicillin,
100 μ g/ml streptomycin, 10% cloning factor (BM-Condimed
H1; Boehringer Mannheim, Indianapolis, IN), and HAT (100
 μ M hypoxanthine; 0.4 μ M aminopterin; 16 μ M thymidine)].

Screening of hybridoma supernatants. Hybridoma
15 supernatants were screened on day 10 using an antigen
capture based ELISA that employed a horseradish
peroxidase (HRP) conjugated goat anti-mouse IgG second
step reagent (Zymed Laboratories, South San Francisco,
CA) for the detection of bound mAb. Briefly, 50 ng of
20 fusion protein diluted in 50 mM sodium bicarbonate, pH
9.6, was adsorbed overnight at room temperature to wells
of 96 well Immulon II microtiter plates (Dynatech
Laboratories, Chantilly, VA). The wells were washed
three times with PBS/0.5% Tween-20; unreactive sites
25 were blocked with specimen diluent (Genetic Systems,

Seattle, WA) for 1 h (this and all subsequent incubations were at room temperature). The plate was washed three times and hybridoma supernatants were added for 1 h and then washed three times. The HRP conjugated second reagent, diluted 1:1000 in specimen diluent, was added to the wells for 1 h and then washed three times. EIA chromagen diluted 1:100 in EIA substrate buffer (Genetic Systems, Seattle, WA) was added, allowed to develop for approximately 15 min., and stopped with 1 N H₂SO₄. The plates were read at OD450/630 on a BioTek microtiter plate reader (BioTek Instruments, Winooski, NH).

Those supernatants that reacted with ALCAM Rg but not a similarly constructed irrelevant Rg fusion protein were further evaluated for their ability to bind to the T cell lymphoma cell line, HPB-ALL, which expressed ALCAM. Approximately 5×10^5 cells were treated with 100 μ l of supernatant for 1 h on ice, washed 3 x with PBA (PBS/1% BSA/0.1% sodium azide) and then treated for 30 min. on ice with a 1:50 dilution of goat anti-murine IgG-FITC (BioSource International, Camarillo, Calif.) in PBA. The cells were then washed 2 x with PBA, 1 x with PBS, fixed with PBS/2% formaldehyde, and analyzed by flow cytometry with a FACScan (Becton Dickinson, Mountain View, CA).

Those hybridomas that produced mAb that recognized the HPB-ALL cells were then cloned by limiting dilution in a 96 well microtiter plate in hybridoma growth medium lacking HAT. The supernatants were similarly evaluated by ELISA and FACS analysis as described above.

Characterization of anti-ALCAM hybridomas/monoclonal antibodies. Hybridoma clones were expanded and the supernatants were subjected to Protein G Sepharose affinity chromatography to obtain purified antibody. Antibody was eluted with 0.1 M sodium citrate, pH 3.0, neutralized with 1/10 volume 1M Tris, pH 9.0, and dialyzed against PBS. Antibodies were concentrated with a Centricon 30 concentrator (Amicon, Beverly, MA) and quantitated by OD280 absorbance to determine antibody concentration.

EXAMPLE VI

Determination of antibody domain specificity. ALCAM is encoded by 5 extracellular Ig like domains: 2 amino-terminal V set domains followed by 3 C2 set domains. To determine the domain specificity of the monoclonal antibodies, a series of ALCAM Rg fusion proteins that were truncated starting with the carboxy-terminal domains were constructed (described below).

The fusion proteins were expressed in COS cells and purified by Protein A Sepharose affinity chromatography. The ALCAM fusion proteins (ALCAM-, ALC VVCC-, ALC VVC-, ALC VV-, ALC V-) and the irrelevant fusion protein CD6 Rg were adsorbed to Immulon I (Dynatech Laboratories, Chantilly, VA) 96 well microtiter plates at 60 ng/well as described above. The plates were processed as above for the screening ELISA, but purified antibody diluted in specimen diluent was added at 50 ng/well. Human IgG adsorbed, HRP conjugated donkey anti-murine IgG (Jackson Immunoresearch, Malvern, PA) diluted 1:2000 in specimen diluent was used to detect antibody binding to the fusion proteins.

The results of this experiment are summarized in Table 6. Also included in this assay was the above described anti-ALCAM mAb, J4-81. From this type of analysis, a prediction as to the domain specificity of the monoclonal antibodies can be made. HAL 151 recognized V1, while J4-81 appears to recognize V2 of ALCAM, as do HAL 31.1, 33.1, 62.1, 81, and 143. HAL 192 recognizes C1, HAL 20.1, 47.1, and 145 recognize C2 and HAL 8.2 recognizes C3.

Table 6

Characterization of Domain Specificity of anti-ALCAM mAb

mAb	CD6	ALCAM	ALC WCC	ALC WC	ALC W	ALC V	Domain Specificity*
HAL 8.2	-	+	+/-	+/-	+/-	+/-	C3?
HAL 20.1	-	+	+	+/-	-	-	C2
HAL 31.1	-	+	+	+	+	-	V2
HAL 33.1	-	+	+	+	+	-	V2
HAL 47.1	-	+	+	+/-	-	-	C2
HAL 62.1	-	+	+	+	+	-	V2
HAL 81	-	+	+	+	+	+/-	V1-2
HAL 114.3	-	+	+	+	-	-	C1
HAL 143	-	+	+	+	+	-	V2
HAL 145	-	+	+	-	-	-	C2
HAL 151.2.1	-	+	+	+	+	+	V1
HAL 192	-	+	+	+	-	-	C1
J4-81	-	+	+	+	+	-	V2
J3-119	-	+	+	+	+	-	V2

* Presumed domain specificity was determined by an ELISA that measured the binding of anti-ALCAM to immobilized ALCAM fusion proteins.

The monoclonal antibodies were then isotyped by a similar assay as already described. ALCAM Rg was adsorbed to Immulon I plates and treated with the monoclonal antibodies as above. Antibody binding was
5 determined with the mIgG isotype specific HRP conjugated secondary reagents (Pharmingen, San Diego, CA) at the following dilutions: IgG1 1:15,000, IgG2a 1:100, IgG2b 1:2,000, and IgG3 1:250. The substrate was allowed to develop for 45 min. before stopping with H₂SO₄. Readings
10 at OD450/630 were taken. HAL 8.2, 31.1, 33.3, 62.1, 81, 114.3, 145, and J4-81 are IgG1, HAL 20.1 and 151 are IgG2a, and HAL 47.1, and 143, are IgG2b.

Hybridomas producing the following monoclonal antibodies were deposited at the American Type Culture
15 Collection, Rockville, MD on June 18, 1996 and assigned the indicated accession numbers:

	Antibody	Accession No.
	HAL 8.2	HB 12139
	HAL 47.1	HB 12137
20	HAL 62.1	HB 12138
	HAL 143	HB 12141
	HAL 114.3	HB 12136
	HAL 151.2.1	HB 12140.

EXAMPLE VII

Construction of ALCAM domain truncation fusion proteins. The construction of the ALCAM Rg fusion protein is described above. Complementary DNA fragments encoding single or multiple domains of ALCAM (Fig. 30) were obtained by PCR (polymerase chain reaction). The sets of primers used for the individual fragments were as follows: (1) FP3/VVCC-R, (2) FP3/VVC-R, (3) FP3/VV-R, (4) FP3/V-R. The sequences of the primers are: FP3 gtacgggccagatatagcggttgacattgatta, VVCC-R agtgtctataggatccttgccttctacaatgagagt, VVC-R agtgtctataggatccaaatccaaatagtgaactgt, VV-R agtgtctataggatcctctgtaggatagtaaataatc, V-R agtgtctataggatccagtgctttgcttacaatttcagg. The template used for the synthesis was ALCAM cDNA in pCDM8. The reactions were carried out by standard PCR protocols using Tag polymerase (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. The cycles were as follows: 1X 94°C/3min., 55°C/2min., 72°C/3min.; 30X 94°C/1.5 min., 55°C/2 min., 72°C/2.5 min., and 1X 72°C/3 min. The PCR products were digested with MLU1 and BAMH1 restriction enzymes and cloned into a vector containing the hinge, CH2 and CH3 domains of human IgG1 that had been similarly digested with the same enzymes. COS cells were transfected with

the vectors and the fusion proteins were purified by adsorption to and elution from Proteins A Sepharose as described above for antibody purification.

* * *

5 All documents cited above are hereby incorporated in their entirety by reference.

One skilled in the art will appreciate from a reading of this disclosure that various changes in form and detail can be made without departing from the true
10 scope of the invention.

WHAT IS CLAIMED IS:

1. A method of inhibiting binding of CD6 present on the surface of a first cell to CD6 ligand present on the surface of a second cell comprising contacting said CD6 ligand with an anti-CD6 ligand antibody, or binding fragment thereof, under conditions such that said antibody or binding fragment thereof binds to said CD6 ligand present on the surface of said second cell and thereby inhibits binding of said CD6 present on the surface of said first cell to said CD6 ligand present on the surface of said second cell.

2. A method of screening a test compound for the ability to inhibit binding of CD6 ligand to an anti-CD6 ligand antibody comprising:

i) contacting a test compound with one of CD6 ligand, or binding portion thereof, or anti-CD6 ligand antibody, or binding fragment thereof, under conditions such that a complex between said test compound and said CD6 ligand, or binding portion thereof, or said anti-CD6 ligand antibody, or binding fragment thereof, can form;

ii) contacting the other of said CD6 ligand, or binding portion thereof, or said anti-CD6 ligand antibody, or binding fragment thereof, with the combination resulting from step (i) under conditions such that binding of said CD6 ligand, or binding portion thereof, to said anti-CD6 ligand antibody, or binding fragment thereof, can occur; and

iii) determining the extent of binding of said CD6 ligand, or binding portion thereof, to said anti-CD6 ligand antibody, or binding fragment thereof, and thereby the ability of said test compound to inhibit binding of said CD6 ligand to said anti-CD6 ligand antibody.

3. A monoclonal antibody specific for V1, C1, C2 or C3 domain of the CD6 ligand shown in Figure 29.

4. A monoclonal antibody having the binding characteristics of HAL 8.2, 47.1, 62.1, 114.3, 143, or 151, or antigen binding fragment thereof.

5. An Ig-like domain of the extracellular domain of a molecule having the amino acid sequence shown in Figure 29.

6. The IgG-like domain according to claim 5 wherein said Ig-like domain is a V-like domain.

7. The IgG-like domain according to claim 5 wherein said Ig-like domain is a C2-like domain.

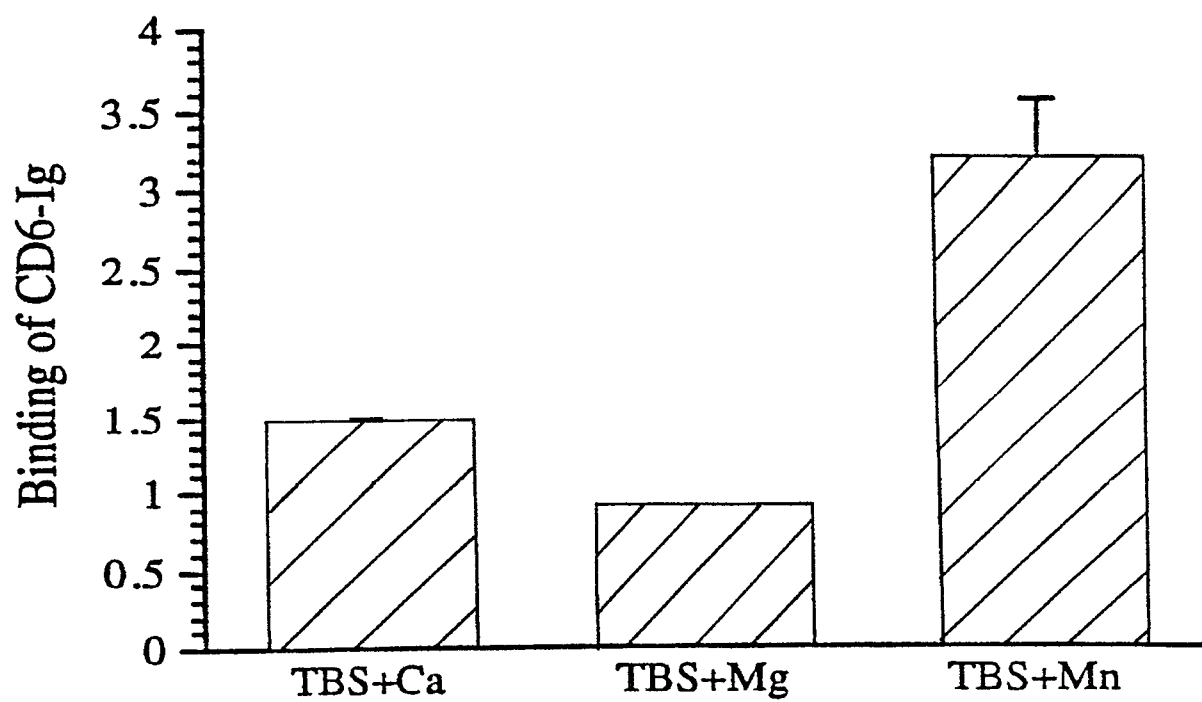
8. A hybridoma that produces the monoclonal antibody of claim 3.

9. A hybridoma that produces the monoclonal antibody of claim 4.

10. A hybridoma having ATCC accession number HB 12136, HB 12137, HB 12138, HB12139, HB 12140, or HB 12141.

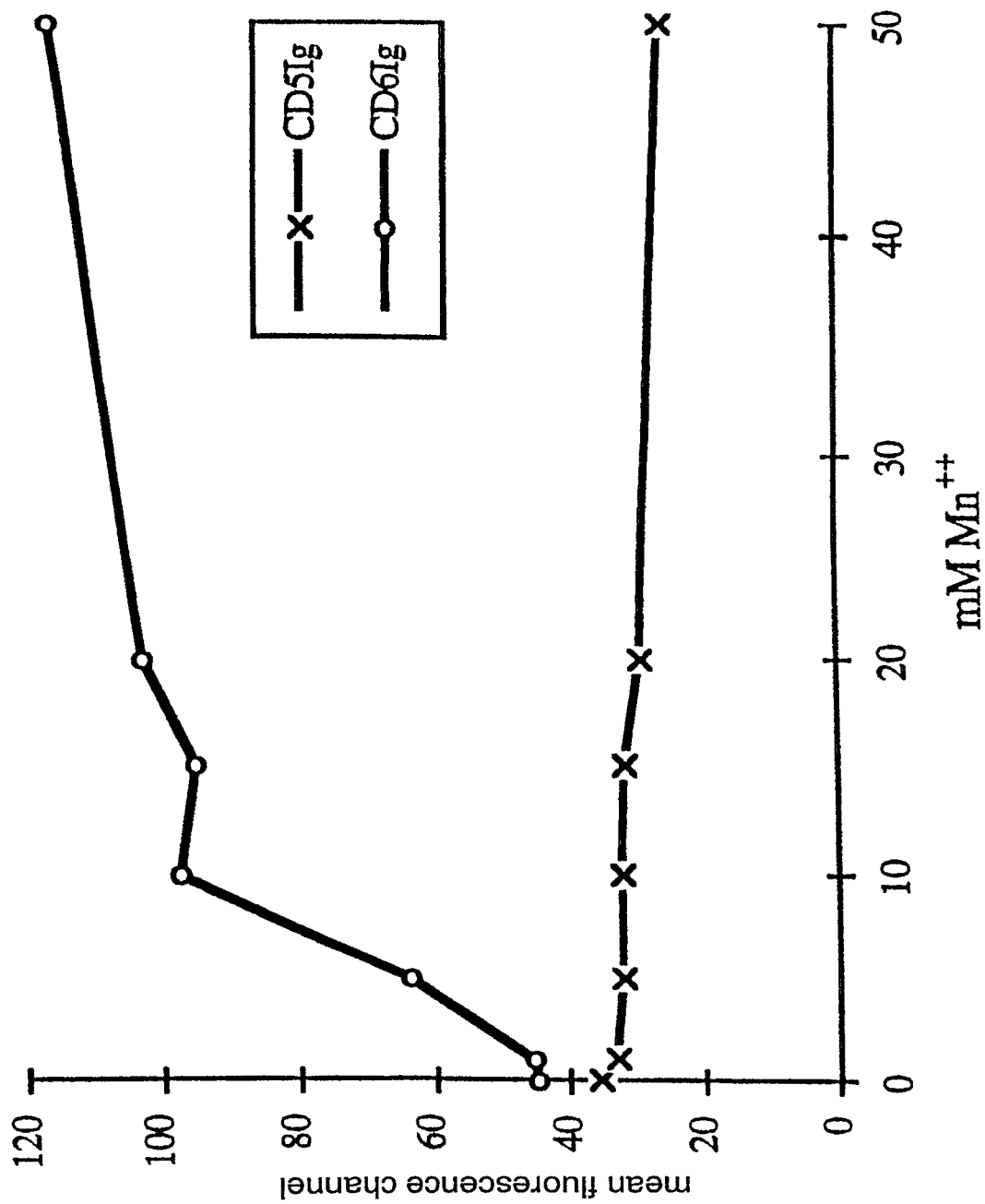
11. A recombinant binding protein comprising an antigen binding region of said monoclonal antibody of claim 3.

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**FIG. 1**

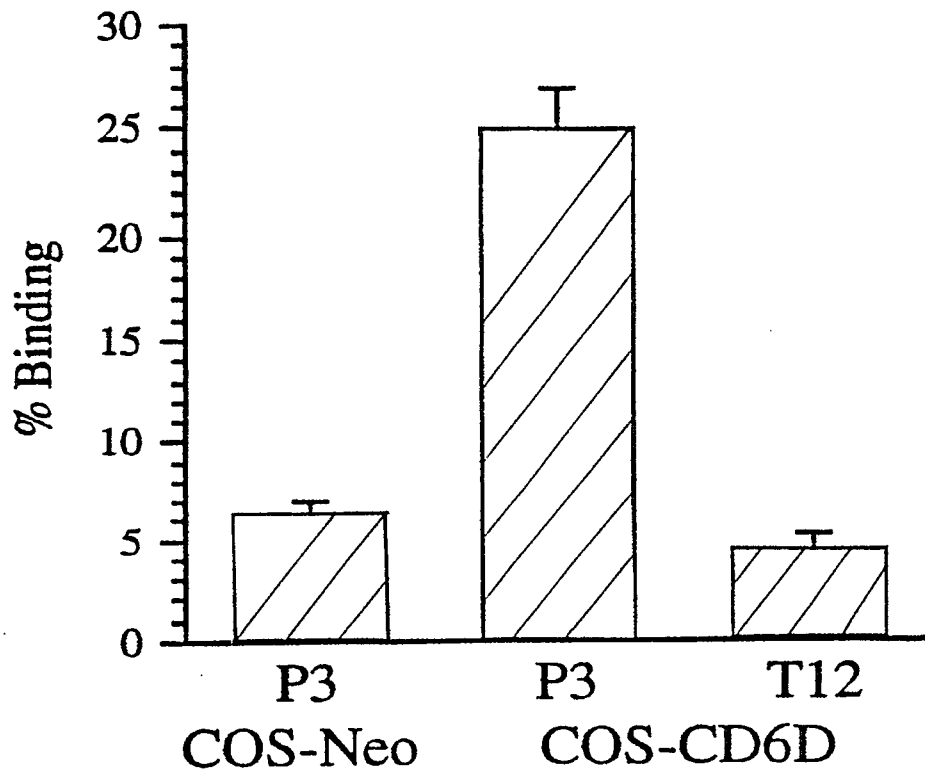
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FIG. 2

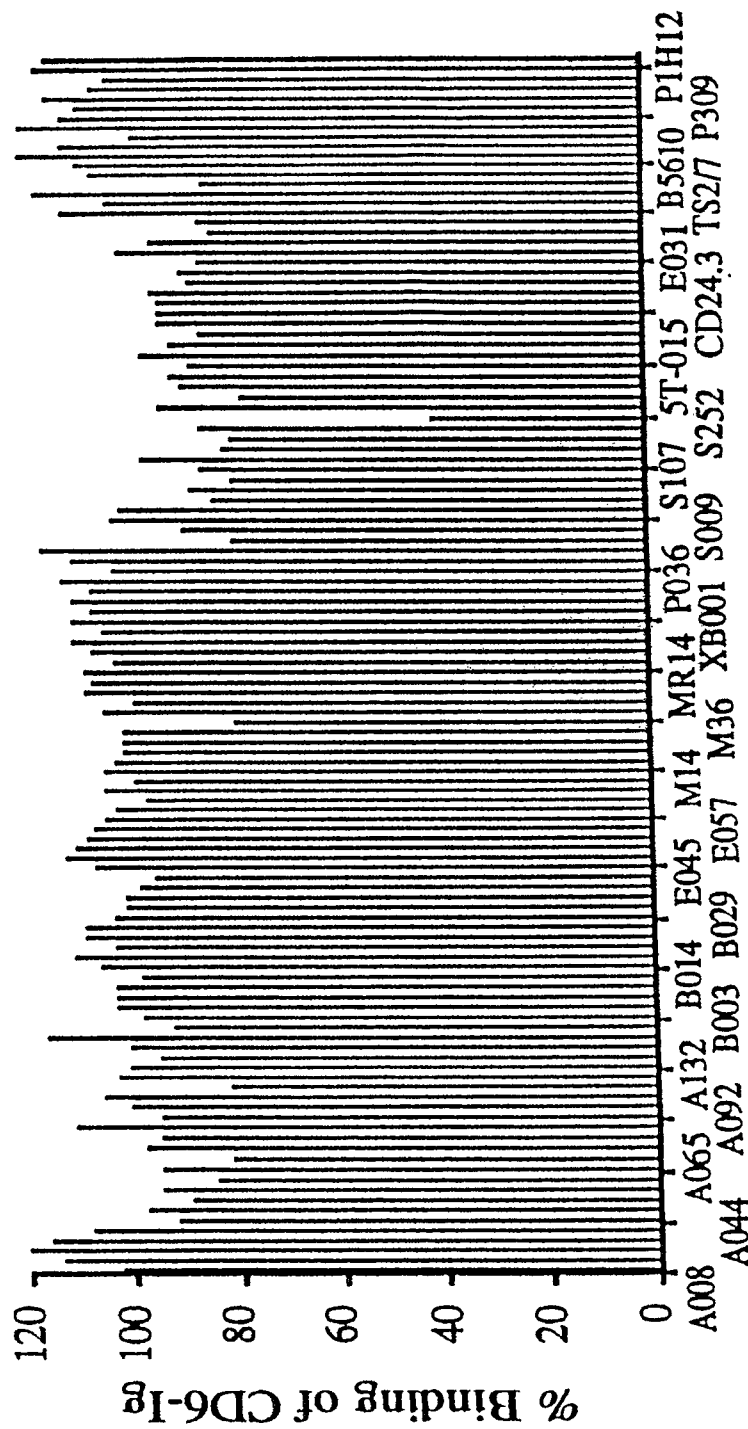


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**FIG. 3**

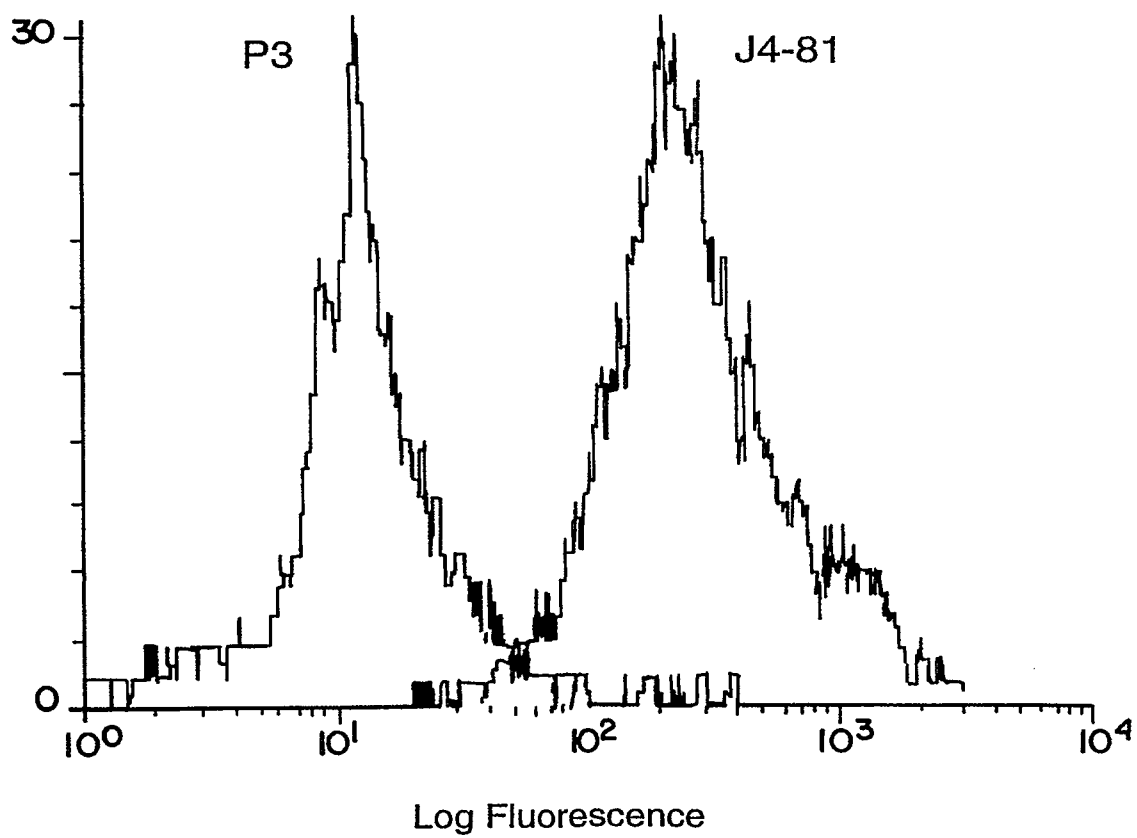
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monoclonal antibodies

FIG. 4

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FIG. 5

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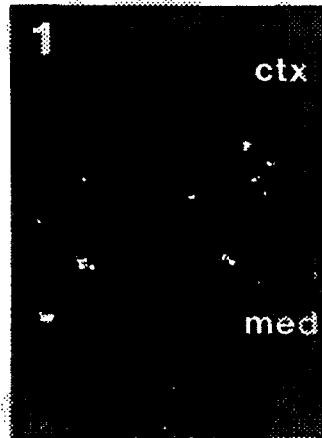


FIG. 6 A

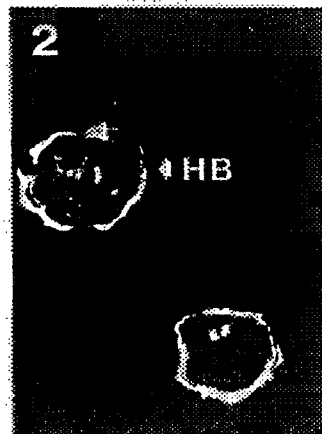
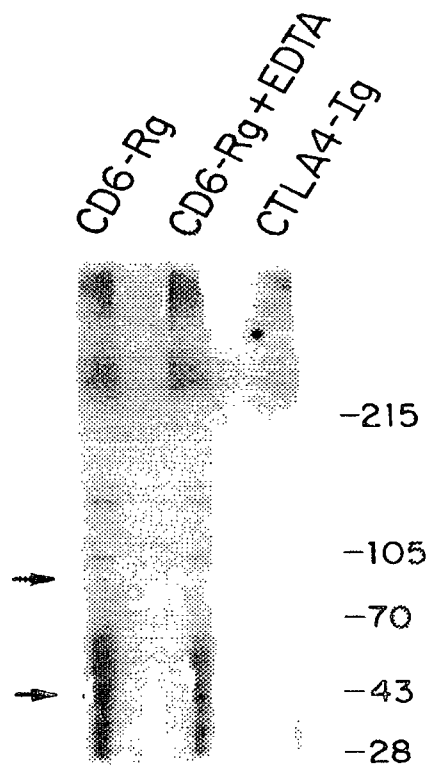


FIG. 6 B

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**FIG. 7**

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CHARACTERIZATION OF THE CD6 LIGAND(S)

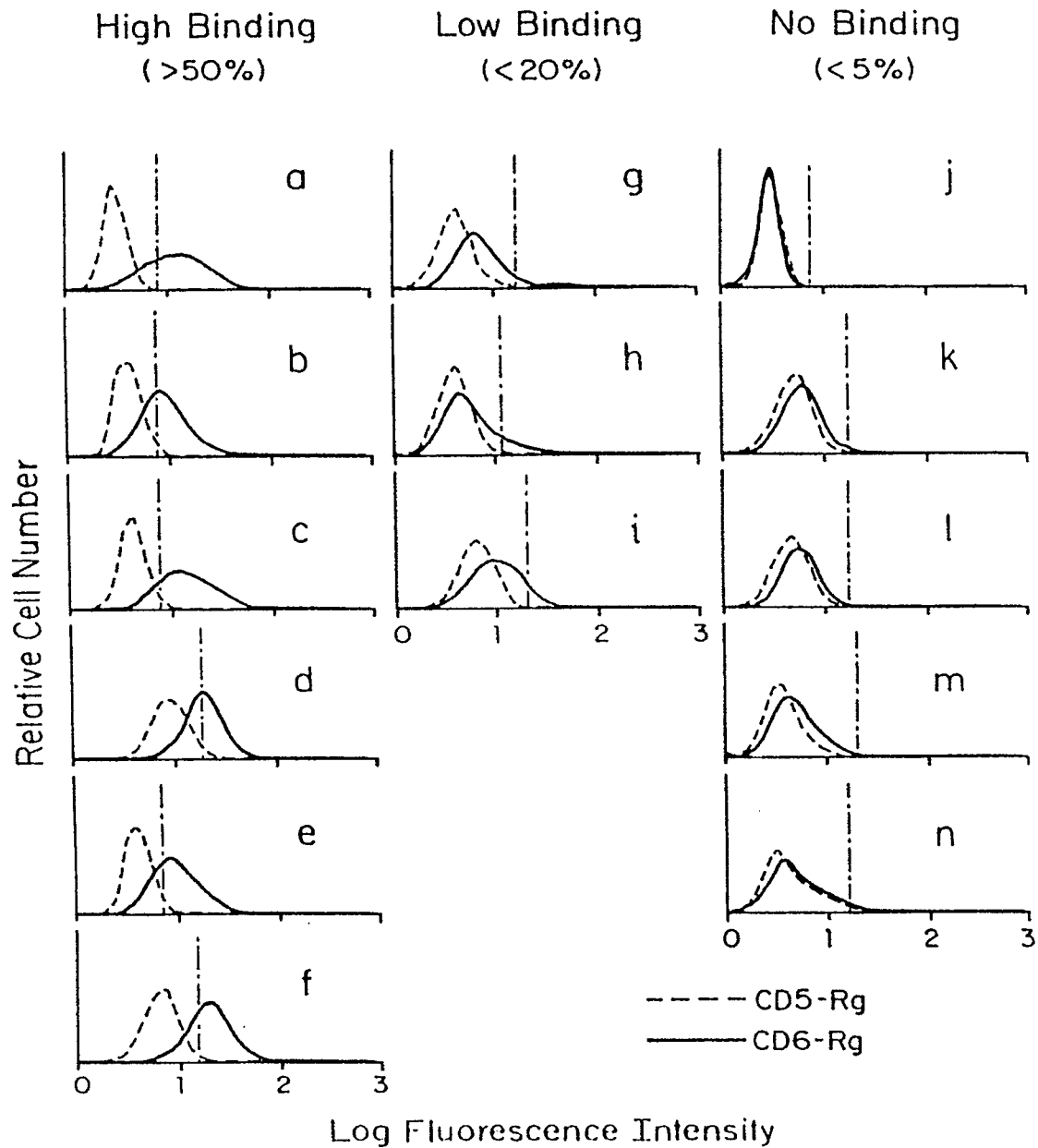
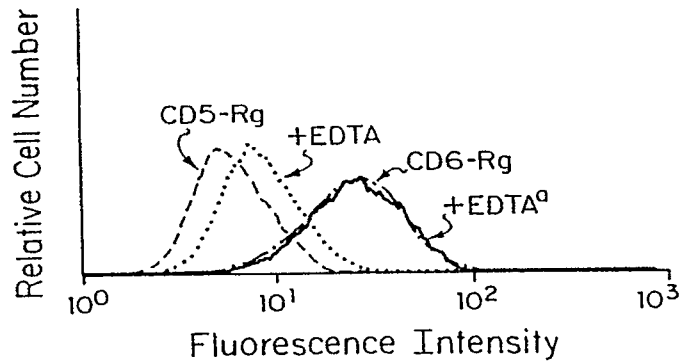
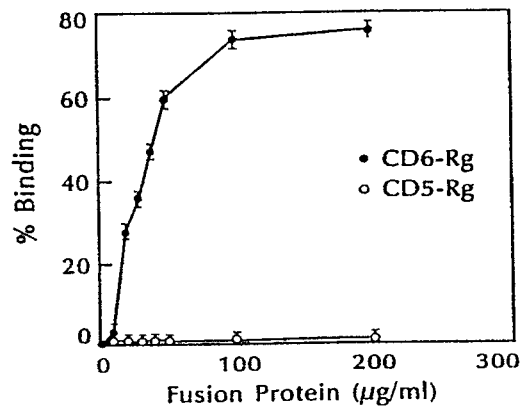
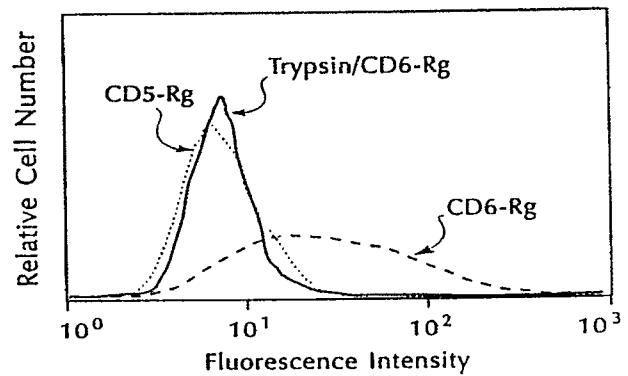


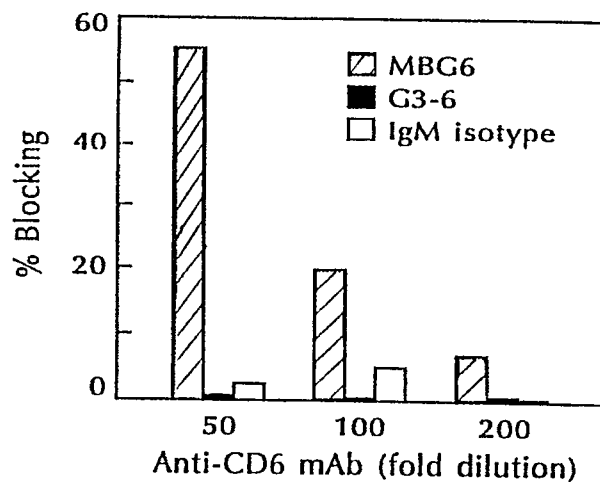
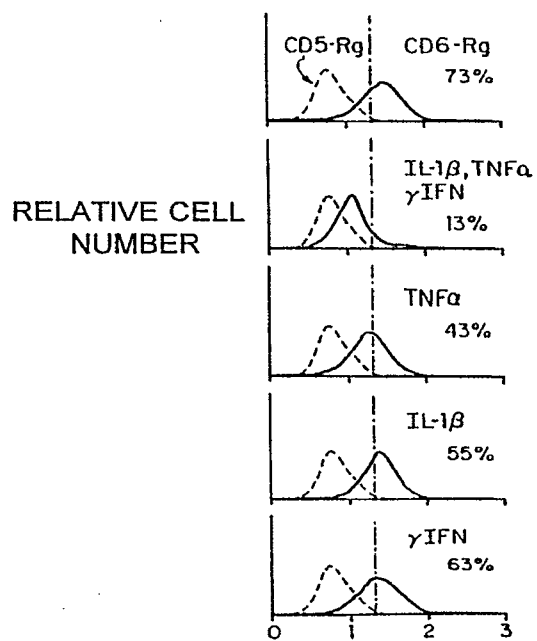
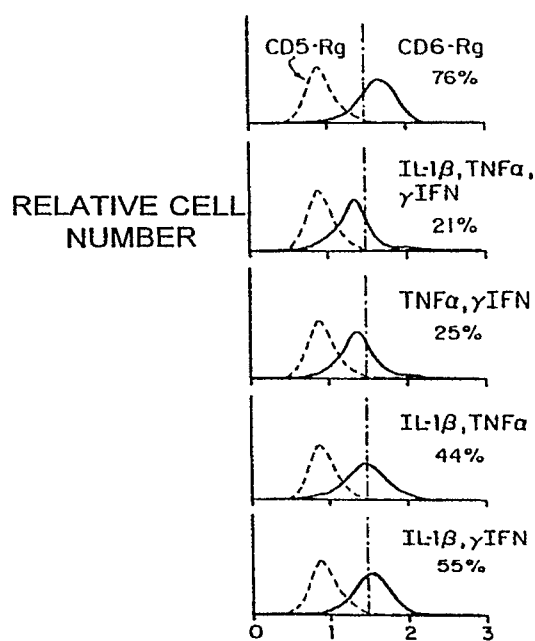
FIG. 8
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**FIG. 10****FIG. 9A****FIG. 9B**

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CHARACTERIZATION OF THE CD6 LIGAND(S)

**FIG. 11****FIG. 12A****FIG. 12B**

LOG FLUORESCENCE INTENSITY LOG FLUORESCENCE INTENSITY

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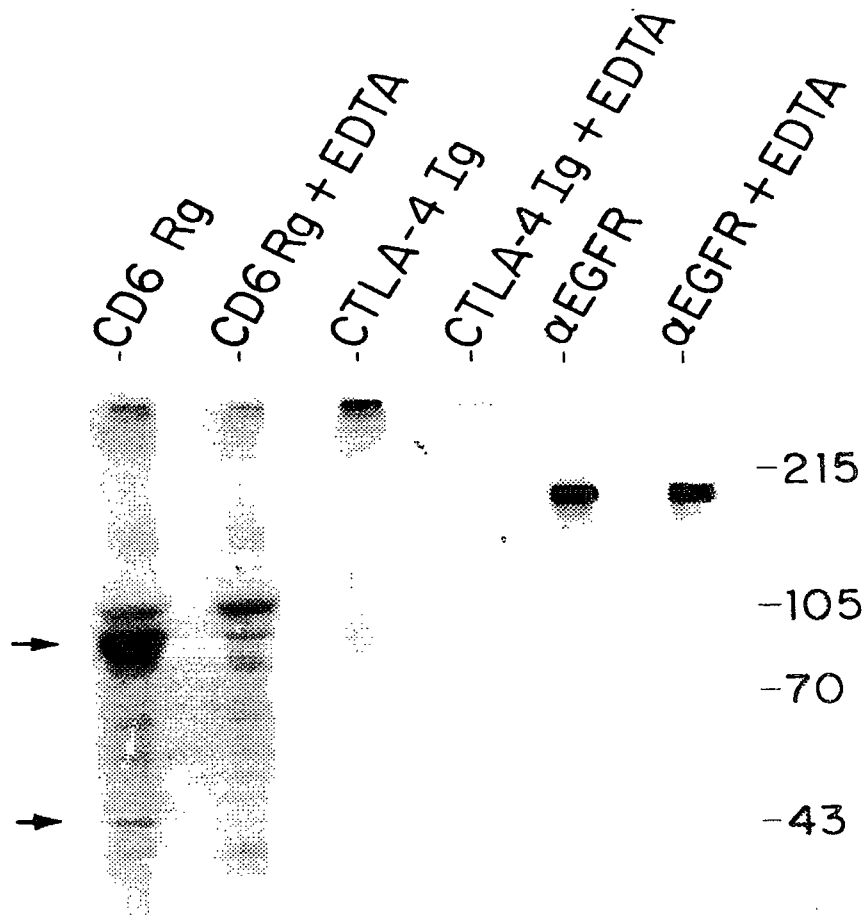


FIG. 13

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FIG. 14A

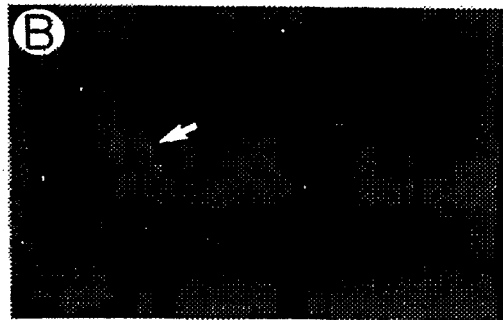


FIG. 14B



FIG. 14C



FIG. 14D

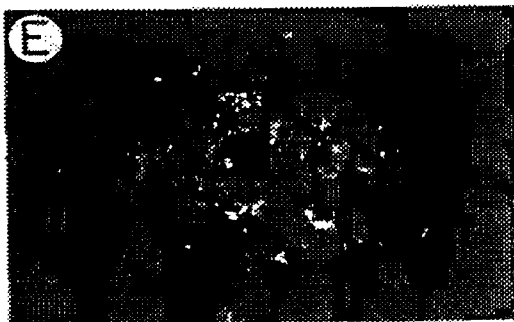


FIG. 14E

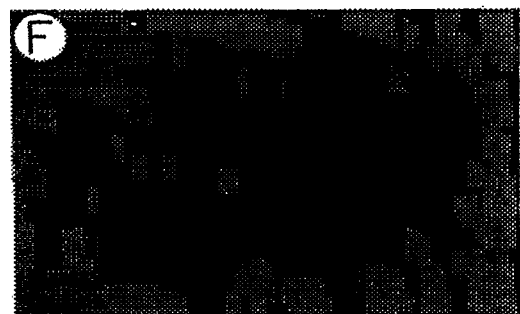


FIG. 14F

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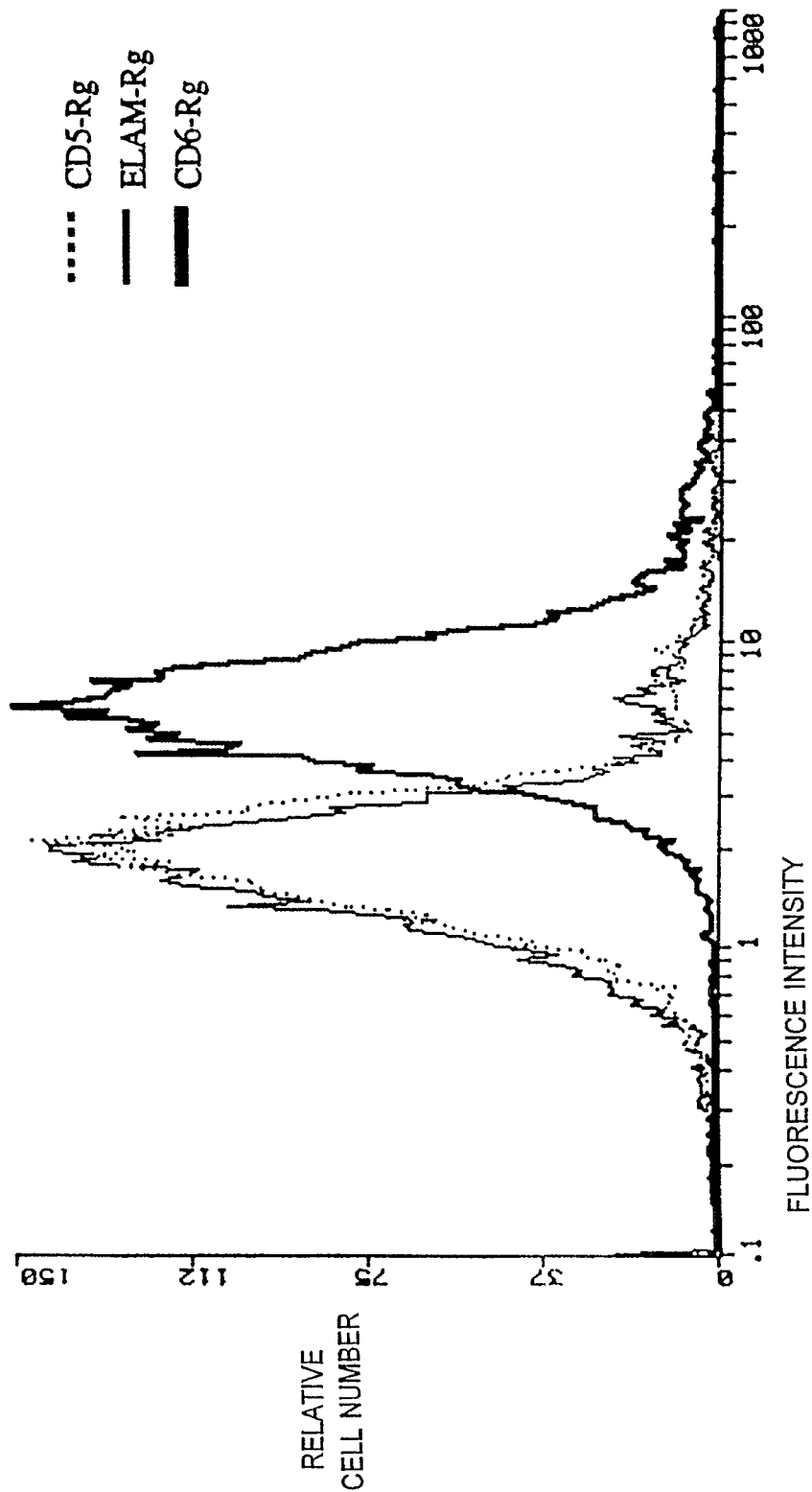
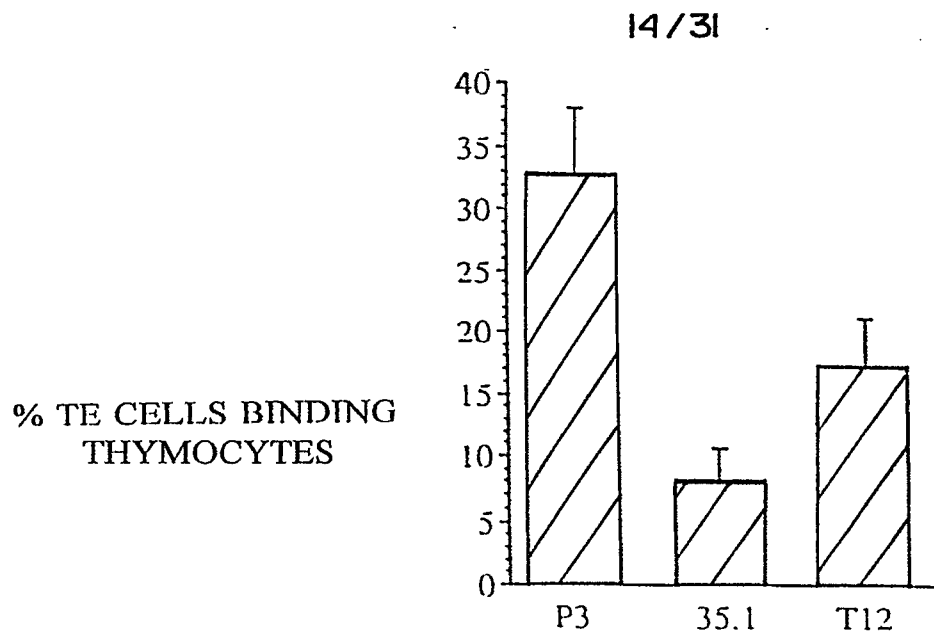
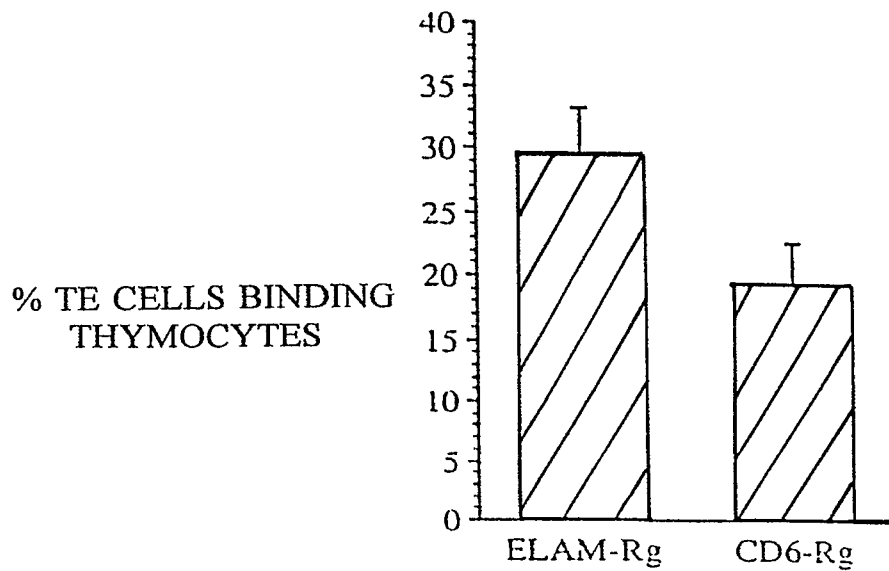
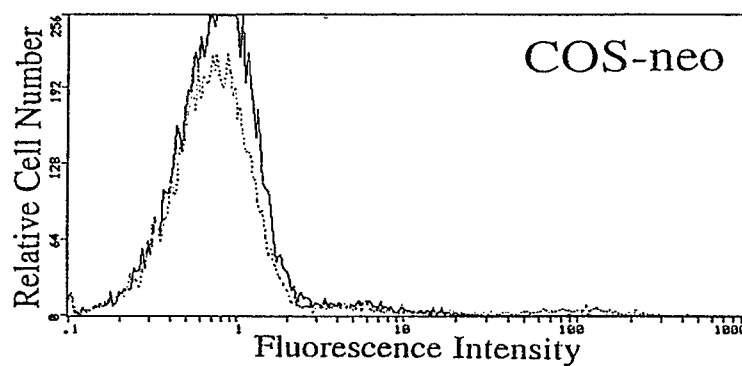
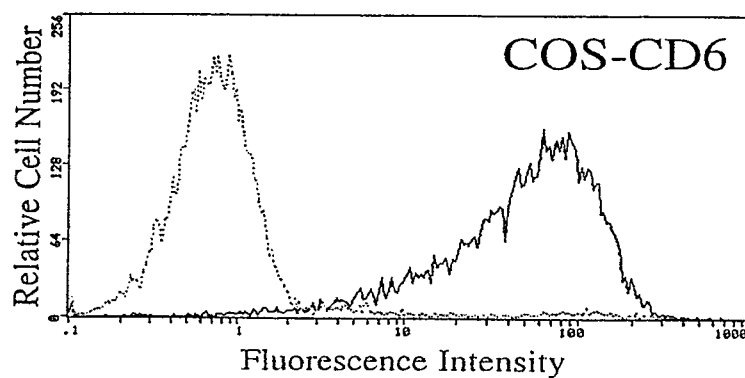
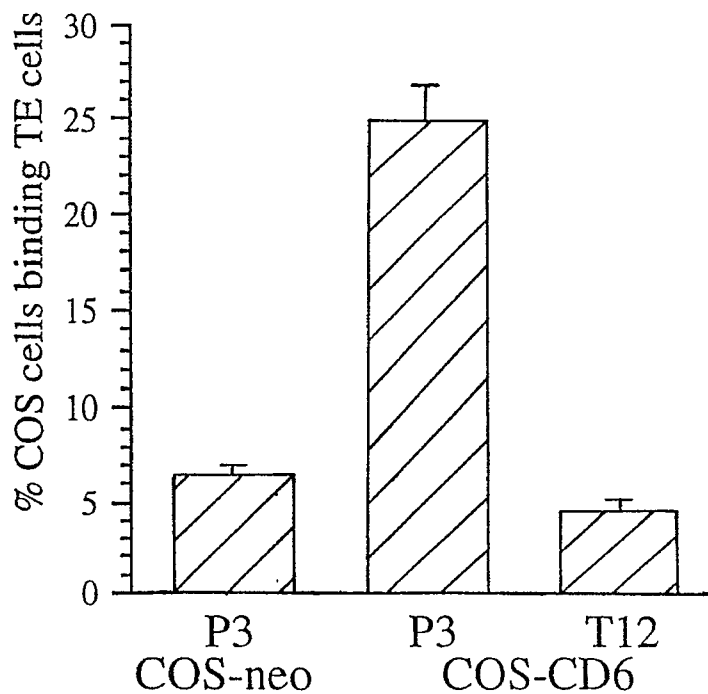


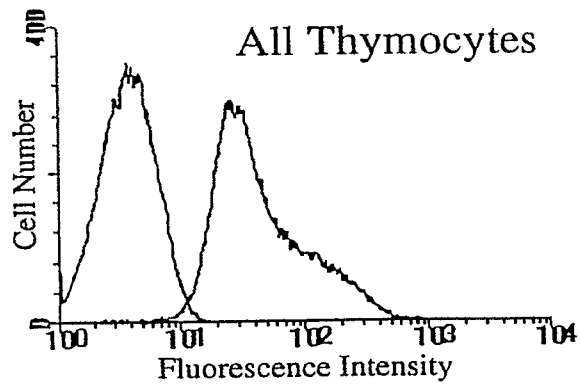
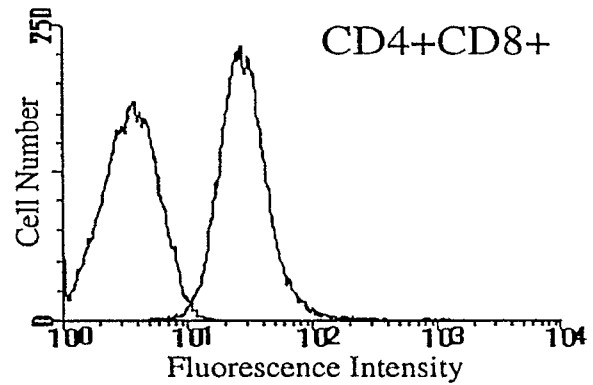
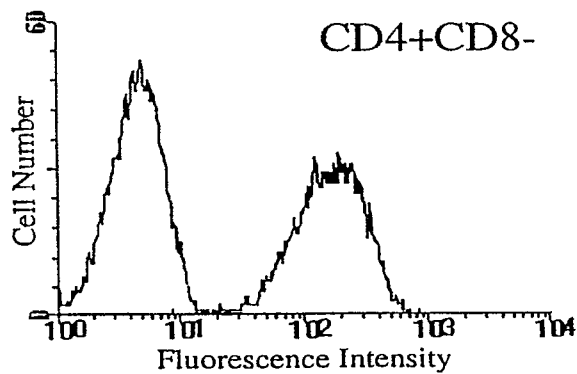
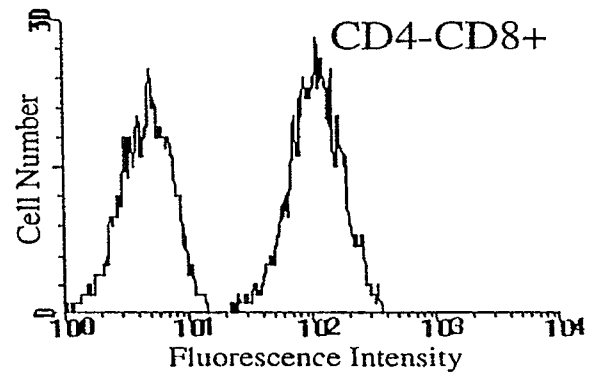
FIG. 15

**FIG. 16A****FIG. 16B**

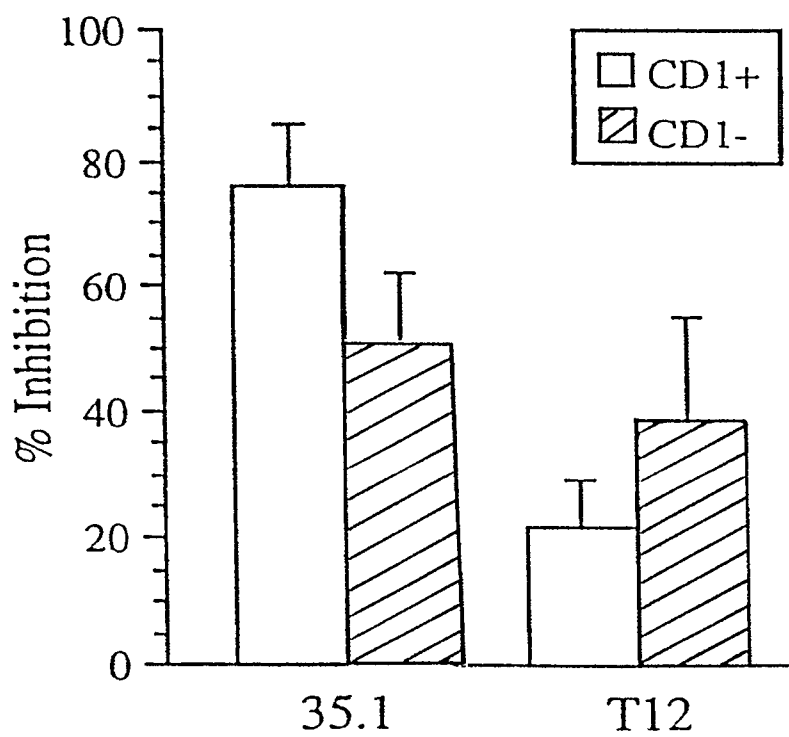
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**FIG. 17A****FIG. 17B****FIG. 17C**

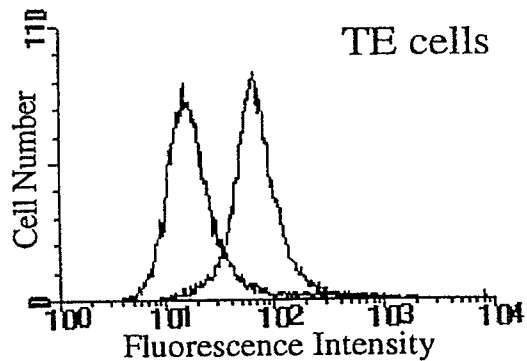
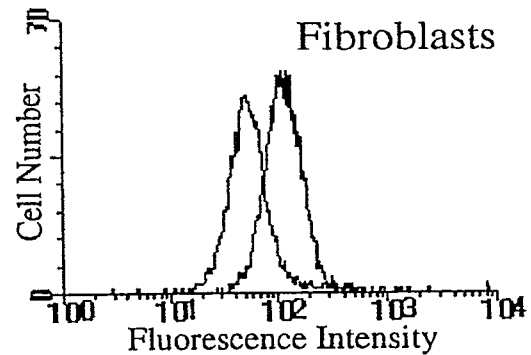
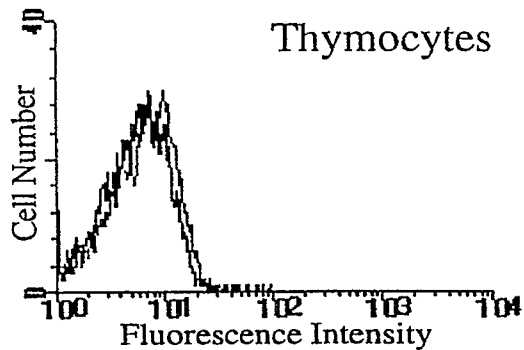
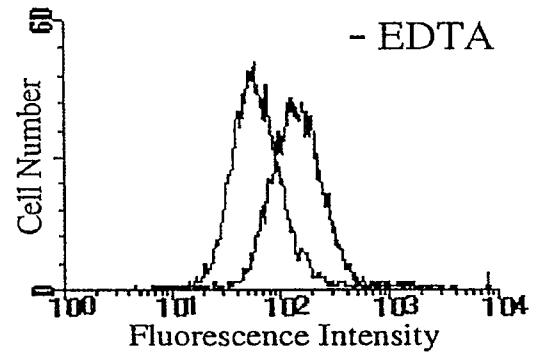
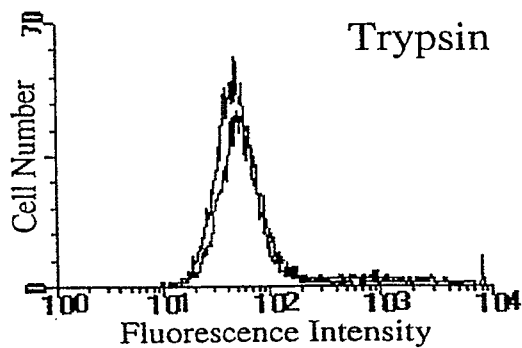
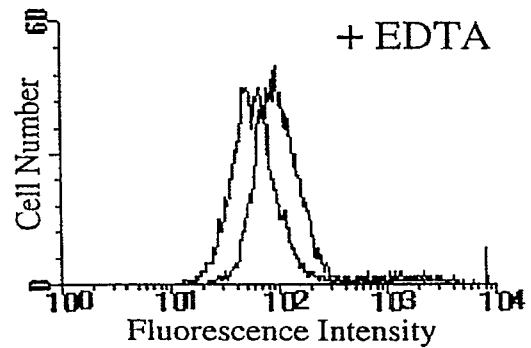
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**FIG. 18A****FIG. 18B****FIG. 18C****FIG. 18D**

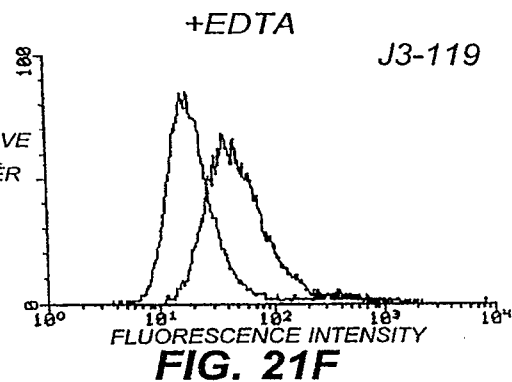
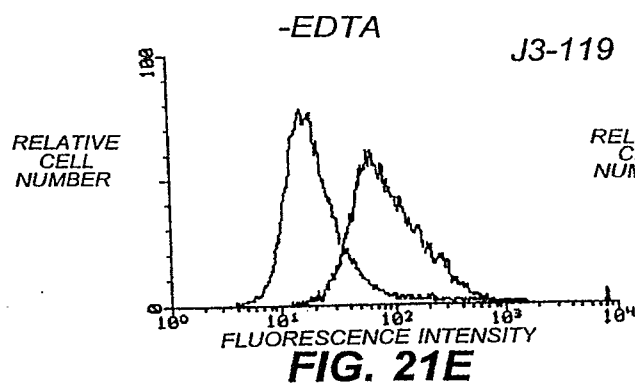
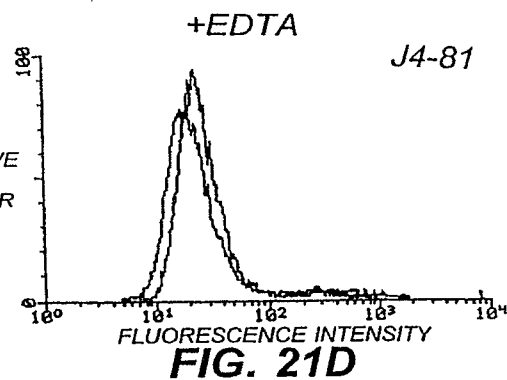
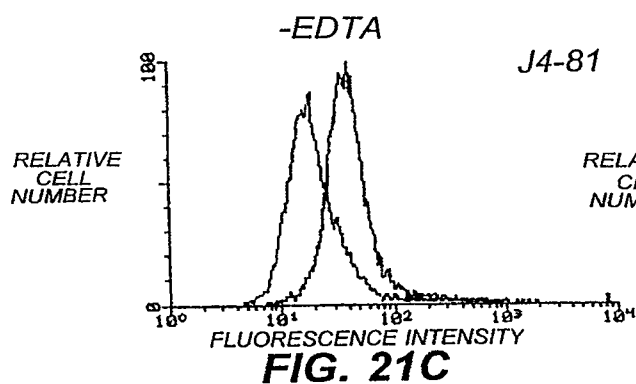
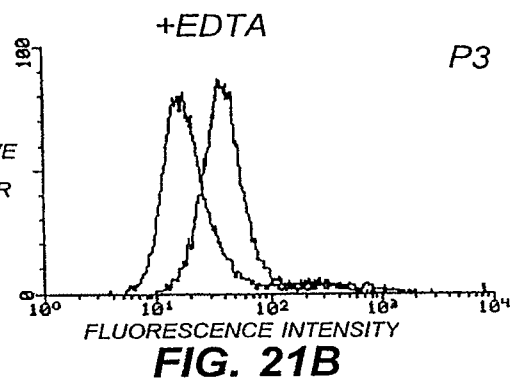
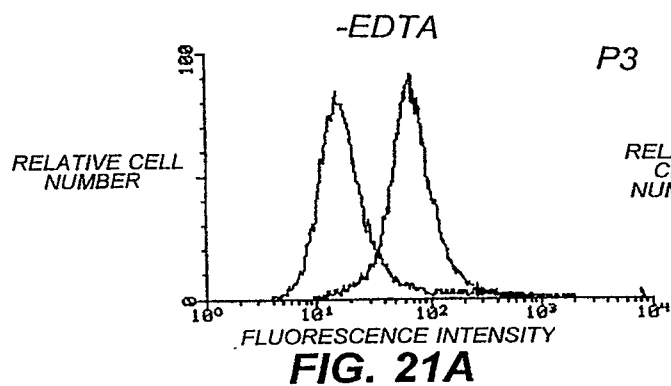
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**FIG. 19**

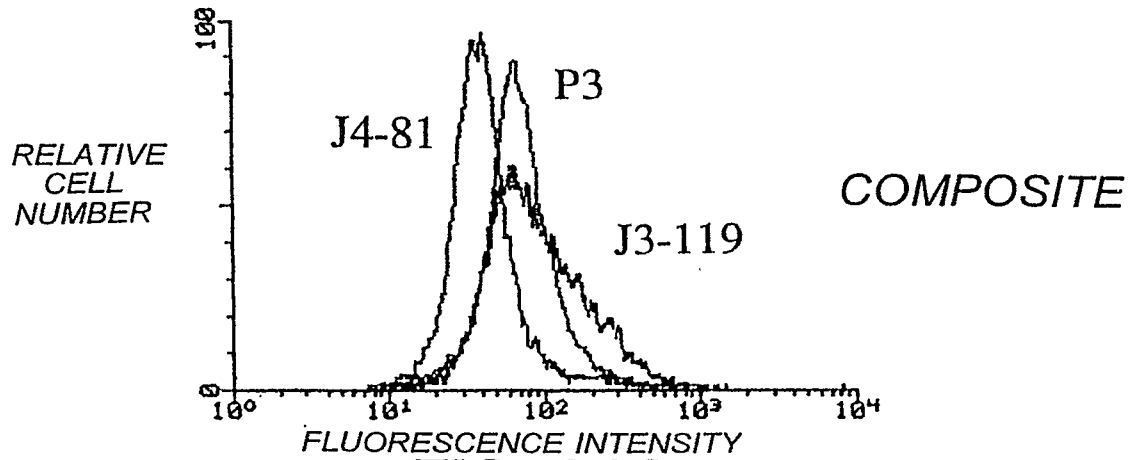
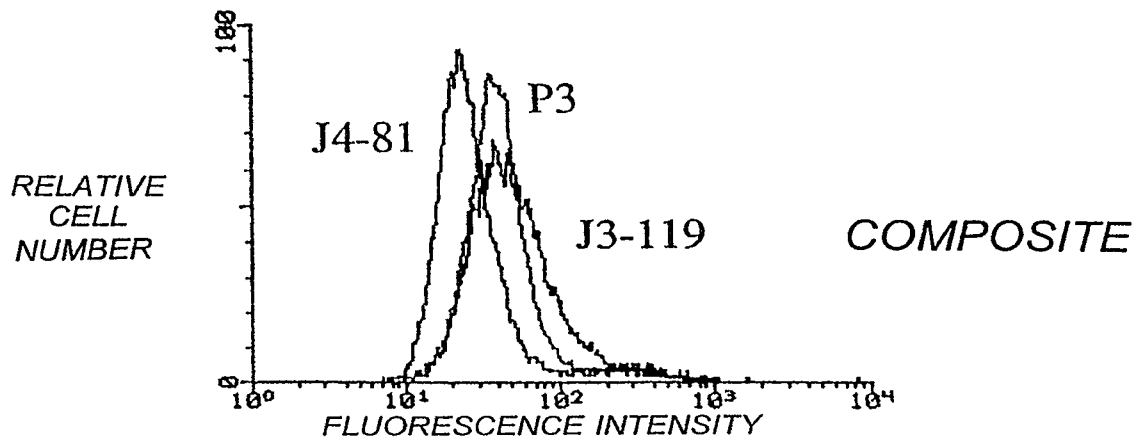
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**FIG. 20A****FIG. 20B****FIG. 20C****FIG. 20D****FIG. 20E****FIG. 20F**

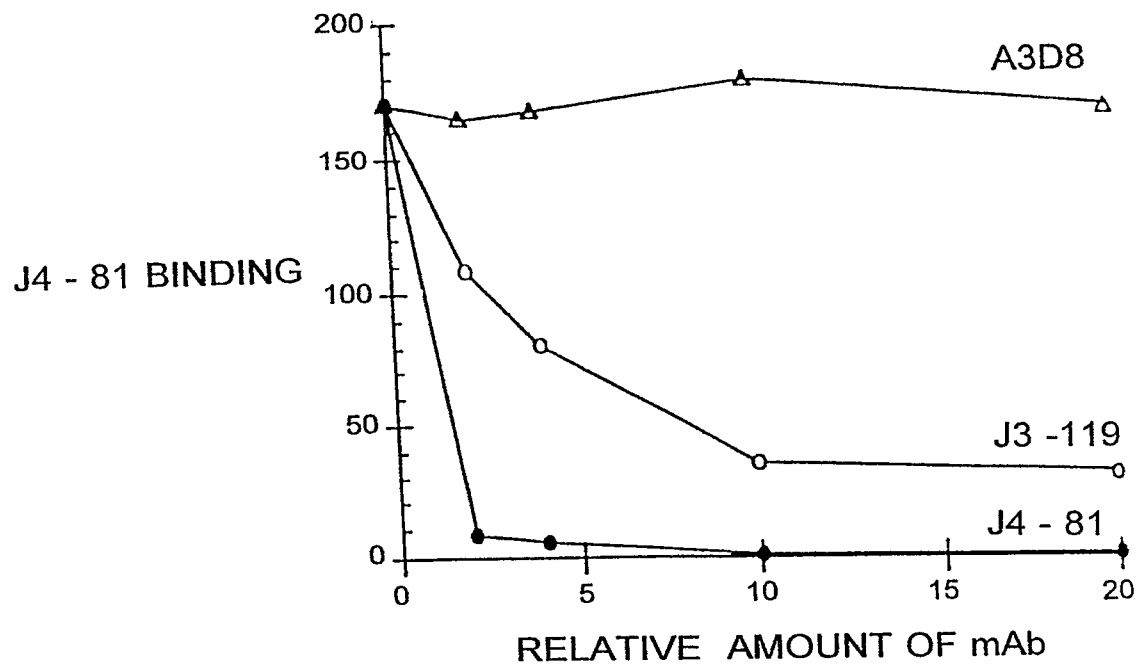
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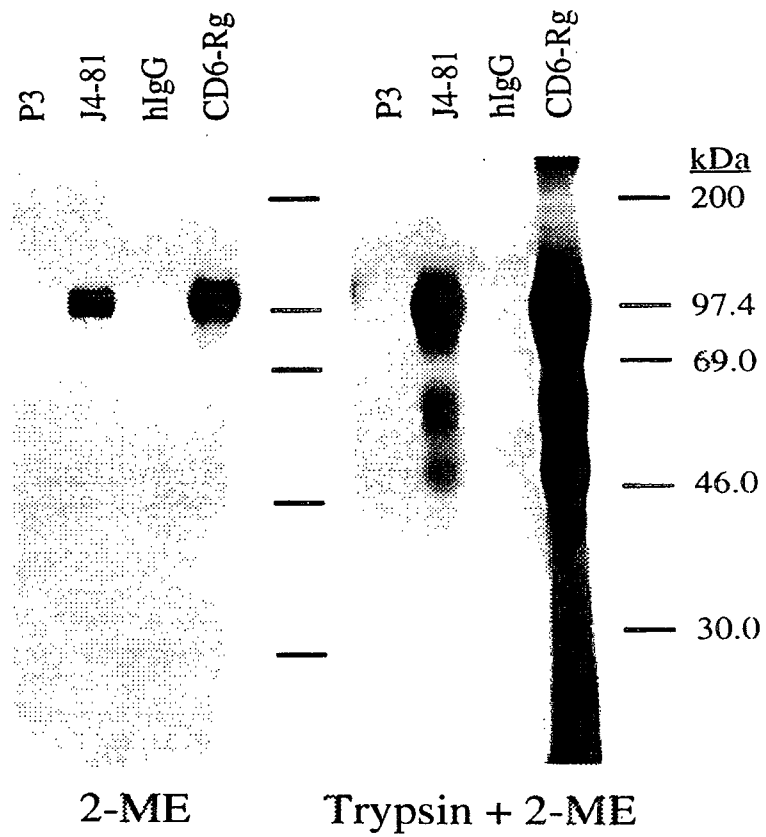
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-EDTA**FIG. 21G***+EDTA***FIG. 21H**

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FIG. 22

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**FIG. 23**

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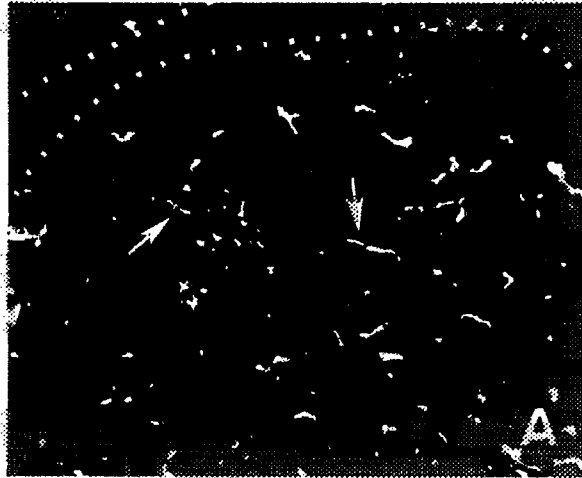


FIG. 24 A

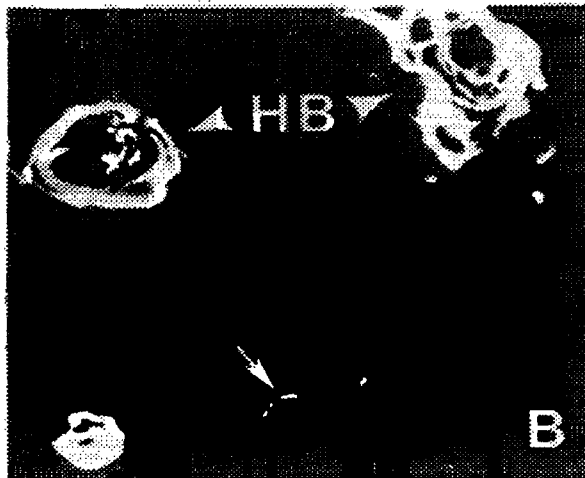


FIG. 24 B

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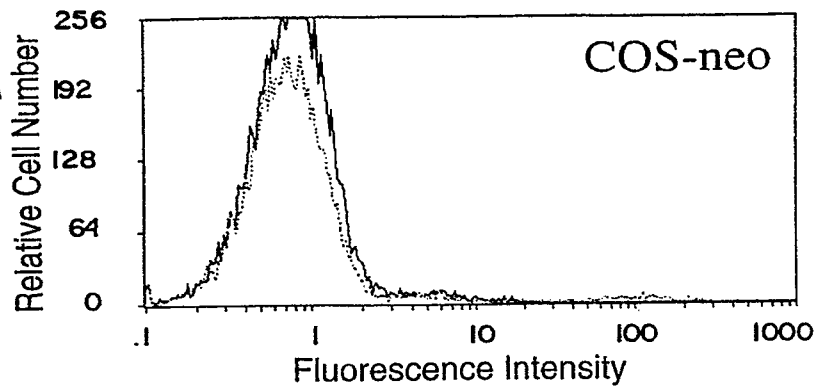
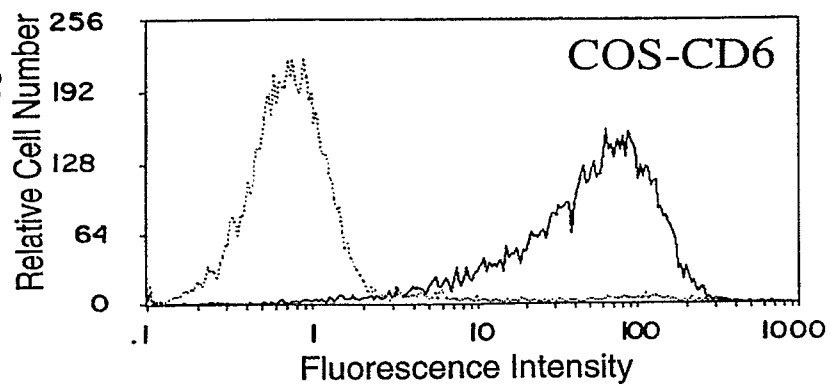
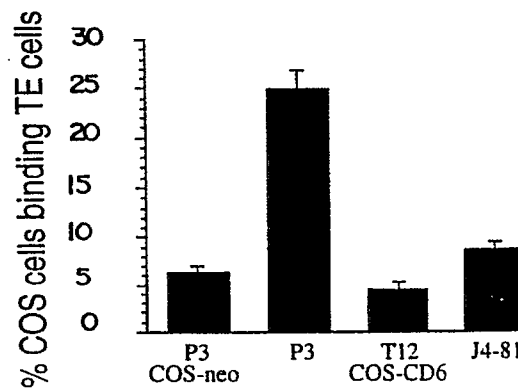
FIG. 25A**FIG. 25B****FIG. 25C**

FIG. 26A

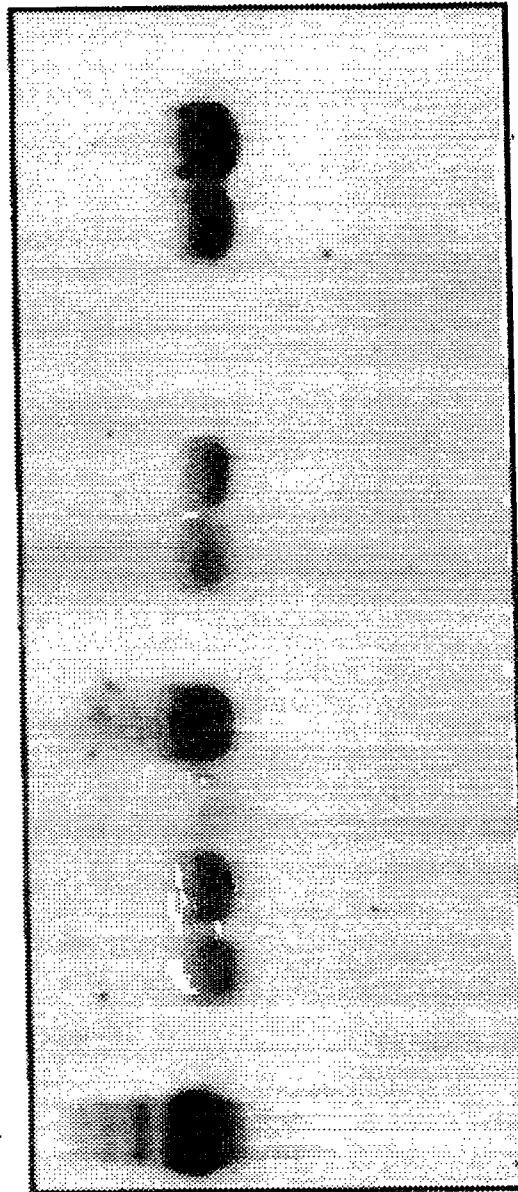
1	100
hALCM	* NARISDEKRF VCMVLTEDNV FEAPTI.VKV
cBEN	YGDITWPCR LEV..PDGLM FGKWKYEMEN GSPVFIAPRS STKKNVQYDD VPDKYDRLNL SE.NYTLIS
fNeurolin	YGETIIVPCN DGTKKPDGLI FTKWKYKDD GSPDGLLVKQ AQDEATVSA TDGKYSRISI AA.NSSLLIA RGSLLADQVF TCMVSTFNL EETSV.E.VKV
hRAGE	IGEPVLKCK GAPKPPQRL ENKLTGRTE AKVLSP...OG GGPWDSVARV LP.NGSLFLP AVGIQDEGIF RCRAMRNKG ETKSNYRVRV
hMuc18	VGSTALKCG LSQSQGNLSH VDFSVHKEK RTSSVVCARA RAR.....AN LGSTSKRLSL QDRGATALT QVTPQDERIF LCQCKRLGPR STASSASTK
Consensus	YG-TI--PC- L- ---P--L- F-KWKY-K-- GSPV---R- ---YK-RLSL ---N-TL-I- ---I-DE--F -CM-V----- -E-S---VKV
101	200
hALCM	* SKALFLETEQ LKLGDCISE DSYPDGNITW YRNGKVLHPL EGAVVIFK EMDPVTQLYT MTSTLEYKTT K.ADIQMPFT CSVTYGP..
cBEN	FKQPSQPEIL HQADFLETEK LKMLGECVVR DSYPEGNVTV YKNGRVLPV EEVVINLRK VENRSTGLFT MTSSLOMPT K.EDANAKFT CIVTVHGP..
fNeurolin	HKPSAPVIK NNAKELENGK LTQLGECVVE NANPADLIW KKNOTLNDV DGTIITSTI TKDKITGLSS TSSRLQYTAR K.EDVESQFT CTAKHVMG..
hRAGE	YQIPGKPEIV DSASELTAGV PNKVGTCVSE GSYPAAGTSLW HLDGKPLVFN EKGVSKEQT RRHETGLFT LQSELNVTPA RGGDRPTFS CSFSPGLPRH
hMuc18	LRMPNI.QVN PLGIPVNSKE PEEVATPCVGR NGYPIQVIW YKNGRPLKEE KNRVHIQSSQ TVE.SSGLYT LQSIILKAQLV K.EDKDAQFY CELANYRLP..
Consensus	-K-PS-PEI- --A--LE--- L--LG-CV-E -SYP-G---W YKNG--L-P- E--V-I-----TGL-T --S-L-Y--- K-ED-----FT C---Y--P--
201	300
hALCM	* SGQKTIHQEQ AVFDIYPTQ QVTIQVLPK SAIPEGDNIT LKCLGNPNP PE.....EFL FYLPQPEGI RSSNTVTLTD VRRNATGDYK C...SLIDK..
cBEN	SGQKTIQSEP VVFDVHYPTQ KVTIRVLSQS STIEGDNVT LKCSGNGNPP PQ.....EFL FYIPGTEGI RSSDTVMTD VRRNATGEYK C...SLID..
fNeurolin	PDQV...SEP ESFPIHYPTQ KVSQVVSQ. SPIREGDVT LKQADGNPP PT.....SFN FNKGGKVTV TDKDVTLTG VTRADSGIYK C...SLIDN..
hRAGE	RALRTAPIQ RVMEP.VPLE EVQLVVEPEG GAVPAGTIVT LTCEVPAQPS PQ.....IH WMKDGVPPLP PPSVLIILPE IGPQDQGTYS C...VATHSS
hMuc18	SGNHMKESRE VTPVFYPTQ KWLEVP.V GMLKEGDRVE IRCLADGNPP PHFSISKQNP STREAEETT NDNGVLVLEP ARKEHSGRYE CQAWNLDTMI
Consensus	SGQ-T--SEP -VFD--YPTQ KV-L-V-P-- S-IKED-VE LKCLADGNPP PHFSISKQNP STREAEETT NDNGVLVLEP ARKEHSGRYE CQAWNLDTMI
301	400
hALCM	* KSMIASTAIT VHYLDLSLNP SGEVTRQIGD ALPVSCITISA SRNATVVMK DNIRLRSSPSFSSLY QDAGNYVCET ALQVEGLKK RESLTIVEG
cBEN	KSMMDTTIT VHYLDLQTP SGEVTRQIGE ALPVSCITISS SRNATVFWIK DNTRMKTSPSFSSLOY QDAGNYICET THKEVEGLKK RKTLLKIVEG
fNeurolin	DVMESTQFT VSFLDVSLTP TGVVLKNVGE NLIVSLDKNA SSEAKVTWK DNRLDKLPDFSKLTQ SDAGLYVCDV S...LEGIKR SLSFELTVEG
hMuc18	SLISEPQELL VNYVSDVRVS PAAPERQGS SLT.ITCEAES SQDLEFOWLR EETDQVLERG PVLQHLDKR EAGGGRVCA SVPSIPGLNR TQLVKLAIFG
Consensus	--M-----T V-YLD--L-P -G-V--Q-G- -L-VSC---- S--A-V-W-K DN-----P- ---FS-L-Y -DAG-Y-C- ----EGLK- ----L-VEG
401	500
hALCM	* KPQI...KMTK KTDPSGLSKT IICHVEGFPK PAIQWITIGS GSVINQTEES PYINGRYYSK IISPEENVV LTCTAENQLE RTVNSLVSA ISISPEHDEAD
cBEN	KPQI...KMTK KTNWKMSTK IVCHVEGFPK PAVQWITIGS GSLINKTEET KYVNGFSSK IIIAPEENVV LTCTAENELE RTVTSLVSA ISISPEYDEPE
fNeurolin	IPKTI.SLTK HRSSDGRHKV LTCEAEGSPK PDVQWSYNGTNDEV SYNGKATYK LTVVPSKNLT VSCLVTKLIG EDTK..EISV FSQKNEDGTE
hMuc18	PPWMAFKERK VWVKNMVLN LSCEASGHRP PTISWVNGT ASEQDQPPQR VLSTLV... LVTPELLETG VECTASNDLG KNTSILFLEL VNLTLTPDS
Consensus	-P-I--K-TK -----K- --C--EG-PK P--QW-V-G- -S-----E- -Y-NG----K ----P--N-T -C-A-N-L- ----L--S- -S-----D----

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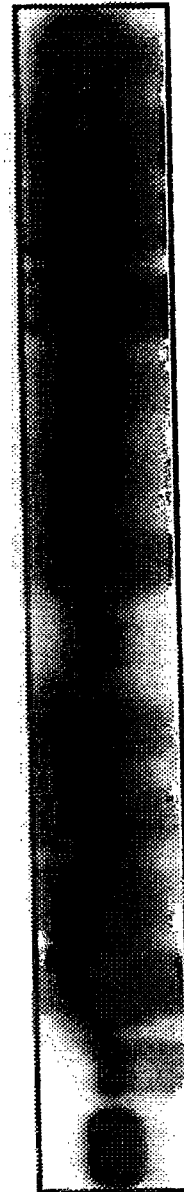
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Monocytes
PBMC
PHA B1s
CEM
MOLT4
RAMOS
RAJI
DAUDI
YT
K562
U937
HL60
HBL100
COS M6



9.5—
7.5—
4.4—
2.4—
1.4—

ALCAM



GAPDH

FIG. 26B

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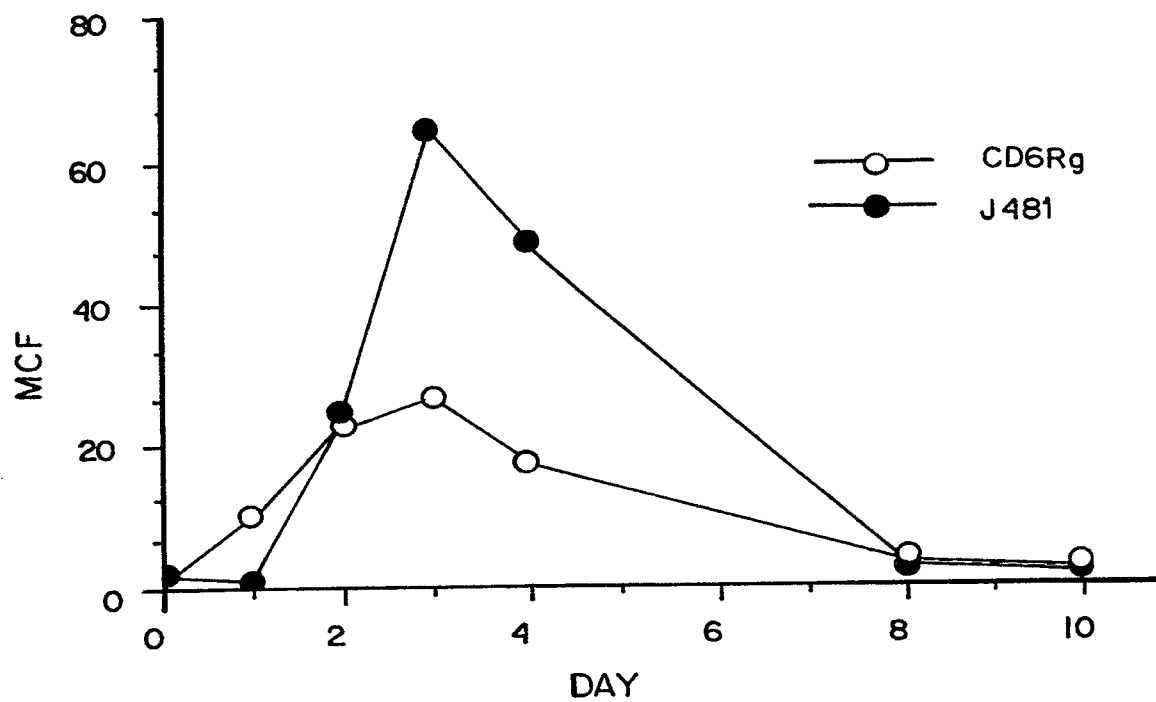
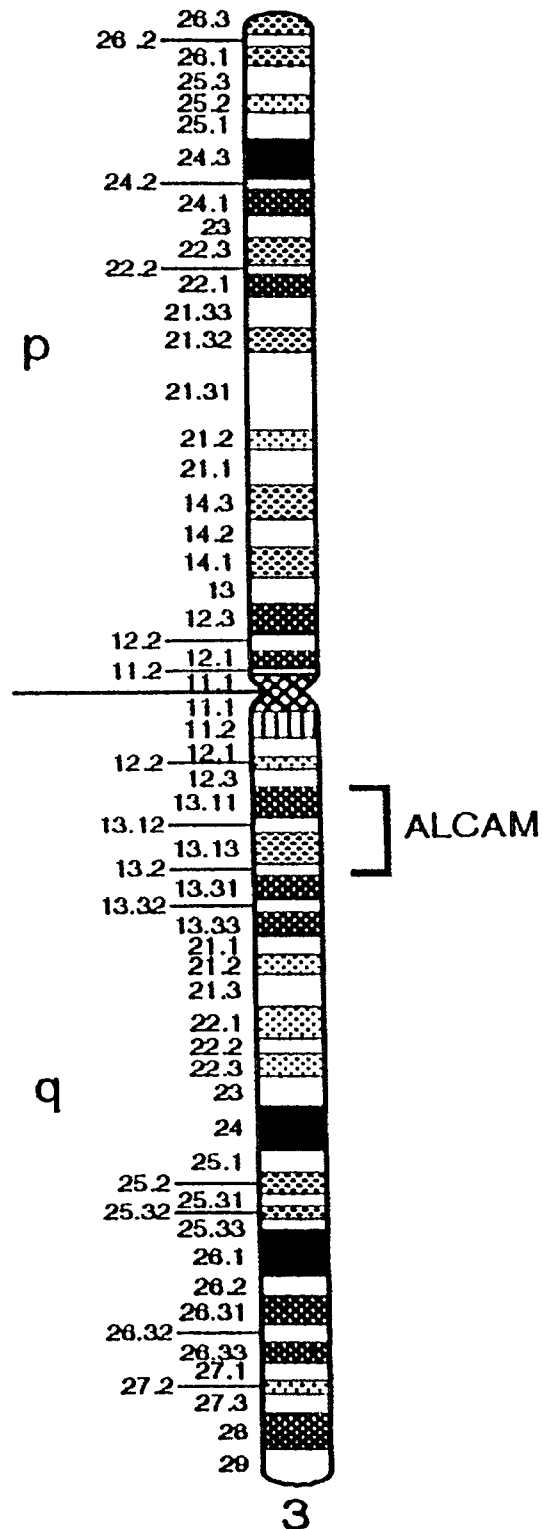
**FIG. 26C**

FIG. 27

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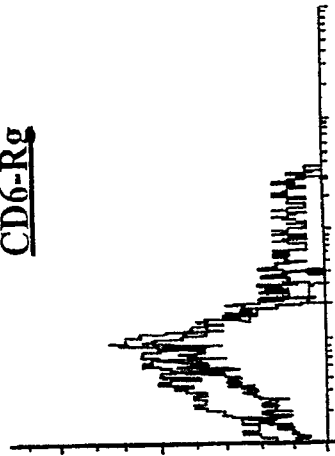
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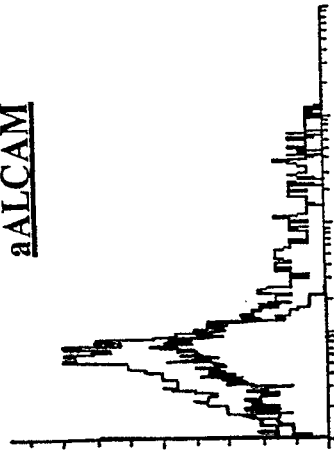
FIG. 28A

COS-ALCAM

CD6-Rg



aALCAM



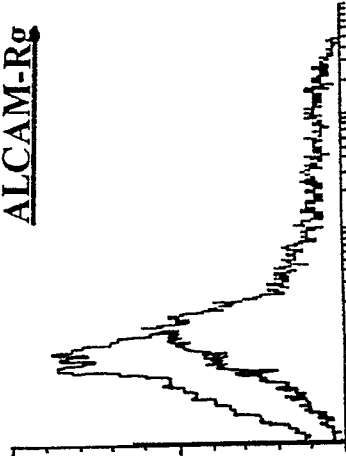
Relative Cell Number

SUBSTITUTE SHEET (RULE 26)

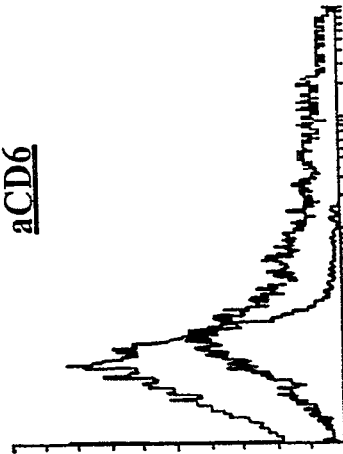
FIG. 28B

COS-CD6

ALCAM-Rg



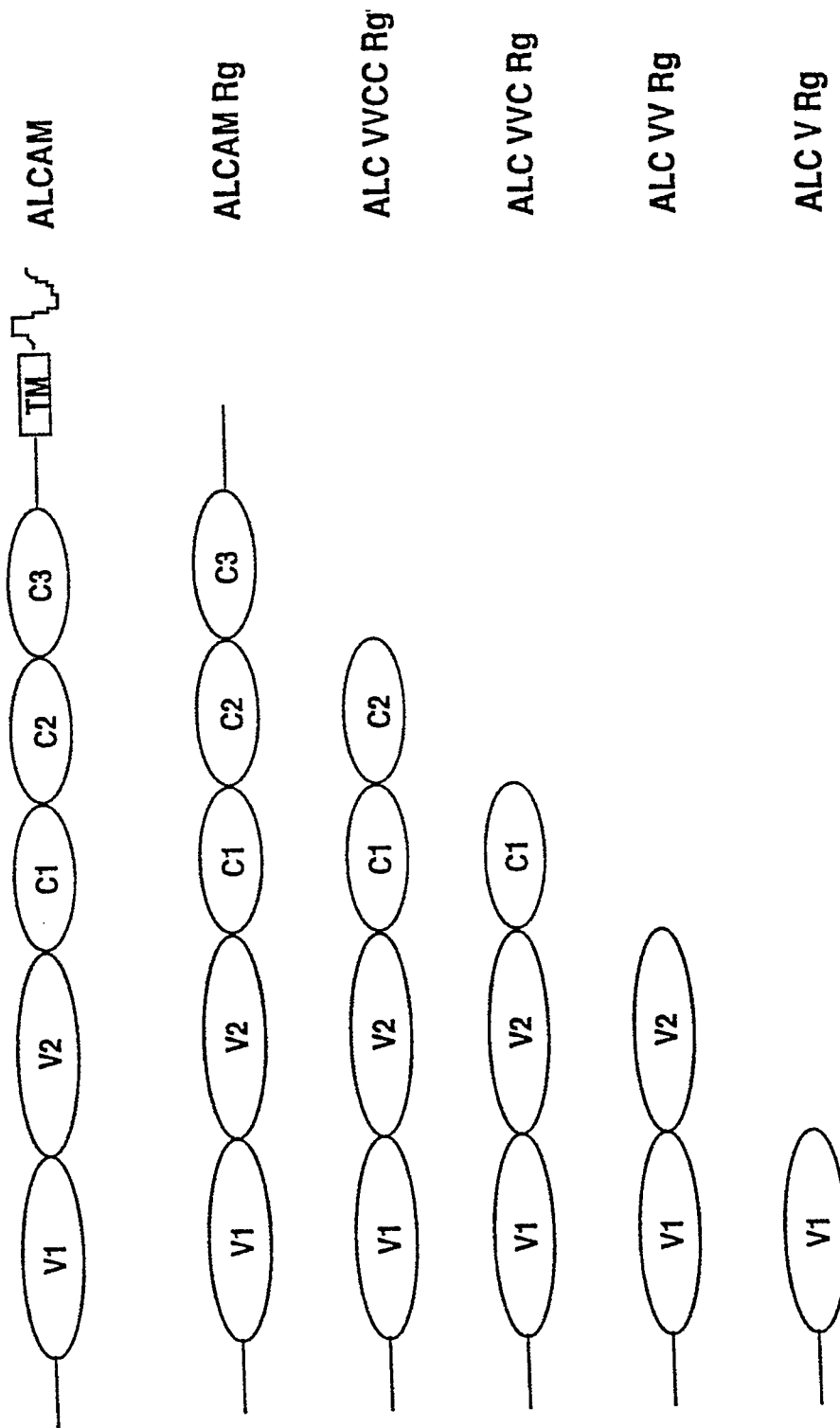
aCD6



Log Fluorescence Intensity

FIG. 29

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**FIG. 30**