

Analysis of T Cell Stimulation by Superantigen Plus Major Histocompatibility Complex Class II Molecules or by CD3 Monoclonal Antibody: Costimulation by Purified Adhesion Ligands VCAM-1, ICAM-1, but Not ELAM-1

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Summary

Many ligands of adhesion molecules mediate costimulation of T cell activation. The generality of this emerging concept is best determined by using model systems which exploit physiologically relevant ligands. We developed such an "antigen-specific" model system for stimulation of resting CD4⁺ human T cells using the following purified ligands: (a) major histocompatibility complex class II plus the superantigen Staphylococcus enterotoxin A, to engage the T cell receptor (TCR); (b) adhesion proteins vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and endothelial leukocyte adhesion molecule 1 (ELAM-1), to provide potential cell surface costimulatory signals; and (c) recombinant interleukin 1 β (rIL-1 β)/rIL-6 as costimulatory cytokines. In this biochemically defined system, we find that resting CD4⁺ T cells require costimulation in order to respond to TCR engagement. This costimulation can be provided by VCAM-1 or ICAM-1; however adhesion alone is not sufficient since ELAM-1 mediates adhesion but not costimulation. The cytokines IL-1 β and IL-6 by themselves cannot mediate costimulation, but augment the adhesion ligand-mediated costimulation. Direct comparison with the model of TCR/CD3 engagement by CD3 monoclonal antibody demonstrated comparable costimulatory requirements in both systems, thereby authenticating the commonly used CD3 model. The costimulation mediated by the activation-dependent interaction of the VLA-4 and LFA-1 integrins with their respective ligands VCAM-1 and ICAM-1 leads to increased IL-2R α (CD25) expression and proliferation in both CD45RA⁺CD4⁺ and CD45RO⁺CD4⁺ T cells. The integrins also regulate the secretion of IL-2, IL-4, and granulocyte/macrophage colony-stimulating factor. In contrast the activation-independent adhesion of CD4⁺ T cell to ELAM-1 molecules does not lead to T cell stimulation as measured by proliferation, IL-2R α expression, or cytokine release. These findings imply that adhesion per se is not sufficient for costimulation, but rather that the costimulation conferred by the VLA-4/VCAM-1 and LFA-1/ICAM-1 interactions reflects specialized accessory functions of these integrin pathways. The new finding that VLA-4/VCAM-1 mediates costimulation adds significance to observations that VCAM-1 is expressed on a unique set of potential antigen-presenting cells in vivo.

T cell stimulation mediated by antigen requires specific engagement of the TCR/CD3 complex with antigenic peptides presented by MHC molecules. In general these interactions alone are not sufficient to stimulate T cells, but

require additional costimulatory signals provided by the APC to achieve T cell activation and differentiation (see for review references 1 and 2). mAb blocking studies in APC-dependent T cell proliferation models have been instrumental in

defining accessory molecules mediating these costimulatory signals (see reference 1 for review). These types of studies are, however, limited by the fact that multiple costimulatory interactions between T cell/APC may occur simultaneously. To reduce the complexity, model systems have been explored where T cell proliferation is induced by combinations of CD3 or TCR mAb (to provide TCR crosslinking) and individual putative costimulatory ligands co-immobilized on a solid substrate. The model in which the OKT3 mAb is immobilized has been a de facto standard because it reproduces the requirement for additional costimulation. However, this requirement is not an obligate requirement for all CD3 mAb (3–5). We designed an alternate model of “antigen-specific” stimulation using purified ligands. We utilized the intrinsically higher precursor frequency of T cells responsive to well-defined superantigens, such as Staphylococcus enterotoxin A (SEA)¹ (see reference 6 for review), to elicit an antigen-specific response from unprimed resting T cells that is sufficiently strong to be measured in a primary in vitro culture. By using the capacity of purified HLA DR1 molecules to bind SEA in vitro (7), albeit probably outside the conventional peptide-binding groove (8), we generated a specific antigen-presenting MHC molecule that enabled in vitro engagement of TCR/CD3 complex. This new DR1/SEA model system can be viewed as a closer physiological correlate of antigen-specific T cell stimulation than CD3 mAb-mediated systems.

Many molecules that have been shown to provide costimulation are adhesion molecules (1, 9). This may suggest a common molecular mechanism in which T cell adhesion alone might be sufficient for costimulation. To investigate this possibility we studied a group of adhesion molecules that are all members of well-established adhesion pathways used by T cells for interacting with other cells. We chose to investigate in detail T cell interactions with three different ligands, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and endothelial leukocyte adhesion molecule-1 (ELAM-1), each of which is expressed on activated endothelium and involved in T cell adhesion to endothelium (10–13). T cell interactions with endothelial cells are critical for migration of T cells into normal tissue, inflammatory sites, and secondary lymphoid organs (14, 15). More important for the present analysis, endothelial cells can act as APCs and may play an important role in activating T cells as they migrate through (16–18).

Although the ligands studied are of particular relevance to endothelial cells, VCAM-1 and ICAM-1 are selectively expressed on other tissues and thus have additional relevance to T cell activation. The three adhesion pathways examined consisted of T cell adhesion via: (a) an undefined ligand on T cells binding to ELAM-1 (19–22); (b) very late antigen 4 (VLA-4 or CD49d/CD29) binding to VCAM-1 (see reference 23 for review); and (c) lymphocyte function-associated

antigen-1 (LFA-1 or CD11a/CD18) binding to ICAM-1 (CD54) (see reference 23 for review). The costimulatory capacity of two of these ligands, ELAM-1 and VCAM-1 has not been previously studied. However, ELAM-1 can activate integrin binding on neutrophils (63) and VLA-4 can mediate costimulation via binding through a distinct site on the VLA-4 molecule to the extracellular matrix molecule fibronectin (11, 24–26). In contrast, LFA-1/ICAM-1 interactions have been inferred to mediate costimulation by various approaches including costimulation by ligand immobilized with CD3 mAb (27).

We investigated and compared in detail (a) adhesion of CD4⁺ T cells to the molecules ICAM-1, VCAM-1 and ELAM-1; and (b) the role of these molecules in two T cell activation models; (a) a CD3 mAb system, and (b) a new antigen-specific activation system with DR1 and SEA. We also examined the role of ICAM-1, VCAM-1 and ELAM-1 in regulation of the cell-surface expression of the α chain of the IL-2 receptor (CD25) and in the regulation of release of the cytokines IL-2, IL-4, and granulocyte macrophage colony-stimulating factor (GM-CSF) by CD4⁺ T cells. A complementary role in costimulation for the recombinant cytokines rIL-1 β and rIL-6 in these model systems was also investigated.

Materials and Methods

Cells. Human PBMCs from normal donors were separated by Ficoll-Hypaque density-gradient centrifugation. Resting T lymphocytes were subsequently obtained by rigorous immunomagnetic negative selection with Advanced Magnetic Particles (Advanced Magnetic, Cambridge, MA) or Dynabeads (Dynal Inc., Fort Lee, NJ) both bound to goat anti-mouse IgG. Negative selection was performed as described (28) using a cocktail of mAbs consisting of anti-HLA class II mAb (IVA12), CD20 mAb (1F5), CD16 mAb (3G8) CD11b mAb (NIH11b-1), CD14 mAb (MMA), CD8 mAb (B9.8), and mAb against glycophorin (10F7). For isolation of the reciprocal subsets of CD45RO⁺ and CD45RA⁺ CD4⁺ T cells the CD45RA mAb (G1-15) and CD45RO mAb (UCHL-1) respectively were added to the cocktail of mAbs. Purity of the isolated cells was more than 98%. The selected CD4⁺ T cells were free of monocytes based on the criterion that there be no proliferative response to optimal concentrations (1/200 dilution) of PHA (M form) (Gibco Laboratories, Grand Island, NY) (29).

Adhesion Assays. Binding assays were performed as previously described (21, 24, 30). Briefly, 96-well flat-bottomed microtiter plates (for ELAM-1 Nunc Immunoplate MaxiSorp F96 #439454; PGC Scientific, Gaithersburg, MD; for ICAM-1 and VCAM-1 Costar #3596; Costar, Cambridge, MA) were precoated with the indicated amounts of adhesion ligand in a total volume of 50 μ l of PBS and incubated overnight at 4°C. Plates were subsequently washed twice with PBS, 50 μ l of PBS/2.5% BSA was added to each well to block nonspecific binding sites, and plates were incubated for an additional 2–3 h at 37°C. Unbound BSA was removed by washing three times with PBS, and 50,000 ⁵¹Cr-labeled CD4⁺ T cells were added in a final volume of 0.1 ml PBS/0.5% HSA; for PMA activation, T cells were added to wells containing 10 ng/ml PMA (Sigma Chemical Co., St. Louis, MO). After 1 h settling at 4°C, plates were rapidly warmed to 37°C for 10 min, nonadherent cells removed by washing plates five times with PBS, and the percentage of bound cells determined by lysing the well contents with detergent and

¹ Abbreviations used in this paper: ELAM-1, endothelial leukocyte adhesion molecule 1; GM-CSF, granulocyte/macrophage colony-stimulating factor; ICAM-1, intercellular adhesion molecule 1; LFA-1, leukocyte function-associated antigen 1; SEA, Staphylococcus enterotoxin A; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late antigen 4.

counting gamma emissions. Blocking by mAb was assessed in the continuous presence of the indicated mAbs.

Proliferation Assays. Proliferation assays are performed using standard techniques. Briefly 40,000 purified CD4⁺ T cells/microtiter well are cultured in 96-well tissue culture clusters with flat bottom wells (Costar) for 3 d in culture medium (RPMI 1640 [Hazleton Biologics Inc., Lenexa, KS] supplemented with 20 mM glutamine [Hazleton Biologics, Inc.], 10% heat inactivated FCS [Biofluids, Rockville, MD] and 100 IU/ml of penicillin, 100 µg/ml streptomycin) under various conditions as mentioned in the experiment description and pulsed (25 µl/well) with a [³H]thymidine solution (5 mCi/ml, 2 mCi/mmol specific activity; New England Nuclear, Boston, MA) during the last 8 h before being harvested on glass fiber filters. Incorporation of radioactive label is measured by liquid scintillation counting. Results are expressed as the arithmetic mean cpm of triplicate cultures. Due to the low precursor frequency of SEA-reactive CD4⁺ T cells, the cell number in the superantigen experiments was increased to 80,000/well, and cells from DR1-positive healthy donors were used to avoid any possible alloresponse. For studying ELAM-1-induced proliferation, we used 96-well Nunc-ImmunoPlate MaxiSorp F96 flat-bottomed wells Nunc #439454; (PGC Scientific, Gaithersburg, MD) pretreated with ethanol for sterility, these plates allow optimal immobilization of ELAM-1 protein. Monocyte-independent CD4⁺ T cell proliferation was obtained by the combination of PMA (10 ng/ml) and PHA (1/200 dilution) (Gibco Laboratories).

Antibody Reagents and Other Reagents. Monoclonal antibodies are used as purified immunoglobulin derived from ascites fluid unless indicated otherwise in the following listing. CD11a mAb: MHM24 (IgG1); CD18 mAb: MHM23 (IgG1) (both Dr. J.E. Hildreth, Johns Hopkins University, Baltimore, MD) (31); CD54 mAb: 84H10 (IgG1) (Dr. P. Mannoni, INSERM unit 119, Marseille, France) (32); CD58 mAb: TS2/9 (IgG1) (American Type Culture Collection) (33); CD2 mAb: 95-5-49 (IgG1) (Dr. R.R. Quinones, George Washington University, Washington, DC) (34); CD3 mAb: OKT3 (IgG2a) (ATCC) (35); anti-HLA class II mAb: IVA12 (IgG1) (Dr. J.D. Capra, South-Western School, Dallas, TX); CD20 mAb: 1F5 (used as dilutions of ascites fluid) (Dr. J.A. Ledbetter, Oncogen, Seattle, WA); CD16 mAb: 3G8 (IgG1) (used as dilutions of ascites fluid) (Dr. D.M. Segal, National Cancer Institute, Bethesda, MD); CD14 mAb: MMA (used as dilutions of ascites fluid) (ATCC); glycophorin mAb: 10F7 (used as dilutions of ascites fluid) (ATCC); CD8 mAb B9.8 (used as dilutions of ascites fluid) (B. Malissen, Marseilles, France); VCAM-1 mAb: 2G7 (19); ELAM-1 mAb: 7A9 (19); CD49d mAb: L25 (36, 37); CD29 mAb: 4B4 (Coulter Electronics, Hialeah, FL); CD45RA mAb: G1-15 (used as dilutions of ascites fluid) (J.A. Ledbetter) (38); CD45RO mAb: UCHL1 (used as dilutions of ascites fluid) (P. Beverly, London, UK) (39); CD44 mAb: NIH44-1 (IgG1) (40).

Recombinant IL-1β was generously supplied by Dr. J. Oppenheim (National Cancer Institute, Frederick, MD) and recombinant IL-6 (sp. act. 10⁶ U/mg) (Genetics Institute, Cambridge, MA) was a gift from Dr. J. Mule (National Cancer Institute, Bethesda, MD). The super-antigen SEA was a gift from S. Burger (National Cancer Institute, Bethesda, MD) and was used as indicated in the figure legends.

HLA class II DR1 molecules were affinity-purified from an EBV-transformed B cell line, LG-2, as described (41). Affinity-purified ICAM-1 was isolated from a Hodgkin's lymphoma cell line, L428, as described (27). A truncated version of ELAM-1 containing 420 amino acids (ELAM-1-420, but referred to in text as ELAM-1) of

the amino-terminal end of the mature form, along with the signal sequence, was constructed by the PCR using appropriate oligonucleotide primers. ELAM-1-420 contains the lectin and EGF domains and a portion of the complement regulatory domain. An ELAM-1-420 containing plasmid was transfected into the DHFR⁻ CHO cell line, and methotrexate-resistant (600 nM) transfectants were isolated. Details of construction and isolation of the ELAM-1-420 plasmid will be described (W. Newman, L. D. Beall, C. W. Carson, G. G. Hunder, N. Graber, Z. I. Randhawa, T. Polte, and T. V. Gopal, manuscript submitted for publication). The ELAM-1-420 molecule was purified from the culture supernatants of transfected cells by sequential affinity chromatography on Con A-Sepharose and ELAM-1 mAb 7A9-coupled Affigel. As a final purification step, the material was eluted as a homogeneous peak from C-18 reversed phase chromatography. Protein concentration was established from amino acid analysis. Amino acid sequencing showed tryptophan as the NH₂ terminus in accord with the published sequence (20); purity was estimated as at least 90%.

For isolation of VCAM-1 a truncated form of the full-length VCAM-1 cDNA was constructed making amino acid 698 the carboxy-terminus, thus deleting the transmembrane and cytoplasmic domains (42). CHO cells producing sVCAM-1 were grown in the presence of 50 mM methotrexate and 48 h conditioned medium from confluent cells was collected. Protein was isolated by a combination of Con-A-Sepharose and VCAM-1 mAb 2G7 affinity chromatographies, followed by a C-18 reversed phase separation. The final material was shown by sequencing of the NH₂-terminal five amino acids to contain the predicted sequence (43) and to be 34% pure.

The purified proteins DR1, ICAM-1, VCAM-1, ELAM-1, and the CD3 mAb OKT3 were immobilized on the plastic well bottom by dilution in PBS and overnight incubation at 4°C, where after wells were washed with PBS. The amount of purified protein applied to each well is indicated in the figures.

Cytokine Analysis. To obtain culture supernatant for cytokine analysis CD4⁺ T cells were cultured in culture medium (see above) (10⁶/ml, final volume 2 ml) in 24-well flat bottom tissue culture plates (#3524; Costar). The proteins were preimmobilized in the wells as described for 96-well plates, but added at 10-fold higher amounts to adjust for the increased well-surface of 24-well plates. The culture supernatants were harvested after 24 h and 48 h of culture. Proliferation was measured in parallel cultures with 40,000 cell/well in 96-well plates as described before. IL-2 activity was defined in a bioassay using the CTLL-2 indicator cell line (American Type Culture Collection) modified after Gillis et al. (44) (minimum detection level was 0.05 U of IL-2/ml). IL-4 and GM-CSF levels were determined in ELISA assays as described (45) (sensitivity of the assays were >24 pg/ml for IL-4 and >0.4 ng/ml for GM-CSF).

Flow Microfluorometry (FMF). Cells were cultured in 24-well tissue culture cluster plates (#3524; Costar) as described above for detection of cytokine production. After 24 h of culture cells were harvested (0.5 × 10⁶/sample). Cells were incubated with Sulfo-NHS-Biotinylated (Pierce Chemical Co., Rockford, IL) CD25 mAb TAC, a gift from Dr. T.A. Waldmann (National Cancer Institute, Bethesda, MD), at saturating concentrations for 30 min at 4°C, washed twice with HBSS (Hazleton Biologics, Inc.) containing 0.2% HSA and 0.2% sodium azide, and stained with Streptavidin-FITC conjugate (SA1001; Caltag, San Francisco, CA) for another 30 min at 4°C. Finally, the cells were washed twice and analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA).

Results

CD4⁺ T Cells Can Specifically Adhere to Immobilized ICAM-1, VCAM-1, and ELAM-1. The three affinity-purified adhesion molecules ICAM-1, VCAM-1, and ELAM-1 can all mediate binding of CD4⁺ T cells, but they show different activation requirements for efficient adhesion (Fig. 1, A–F). Binding by the T cell integrins LFA-1 and VLA-4 to their respective ligands ICAM-1 and VCAM-1 is dependent on activation of the T cell (Fig. 1, A and B) (30, 46), and can be specifically inhibited by mAbs (Fig. 1, D–E). The specific binding of CD4⁺ T cells to immobilized ELAM-1 is, in contrast, not dependent on activation of the T cell and is not increased by T cell activation (Fig. 1, C and F) (21).

Both ICAM-1 and VCAM-1, but not ELAM-1 Can Provide Costimulation for Superantigen-specific HLA Class II-dependent Activation of Resting CD4⁺ T Cells. We analyzed the roles of the adhesion molecules ICAM-1, VCAM-1, and ELAM-1 in a T cell activation model that uses the superantigen and a purified HLA class II molecule DR1, which is known to bind and present SEA (7). Fig. 2 A shows results of systematic analysis of the requirements for induction of proliferation. A range of SEA concentrations up to 1,000 ng/ml was investigated since that concentration gives maximal response in the presence of monocytes (data not shown). No response is observed to the combined stimuli (SEA, DR1, rIL-1 β /

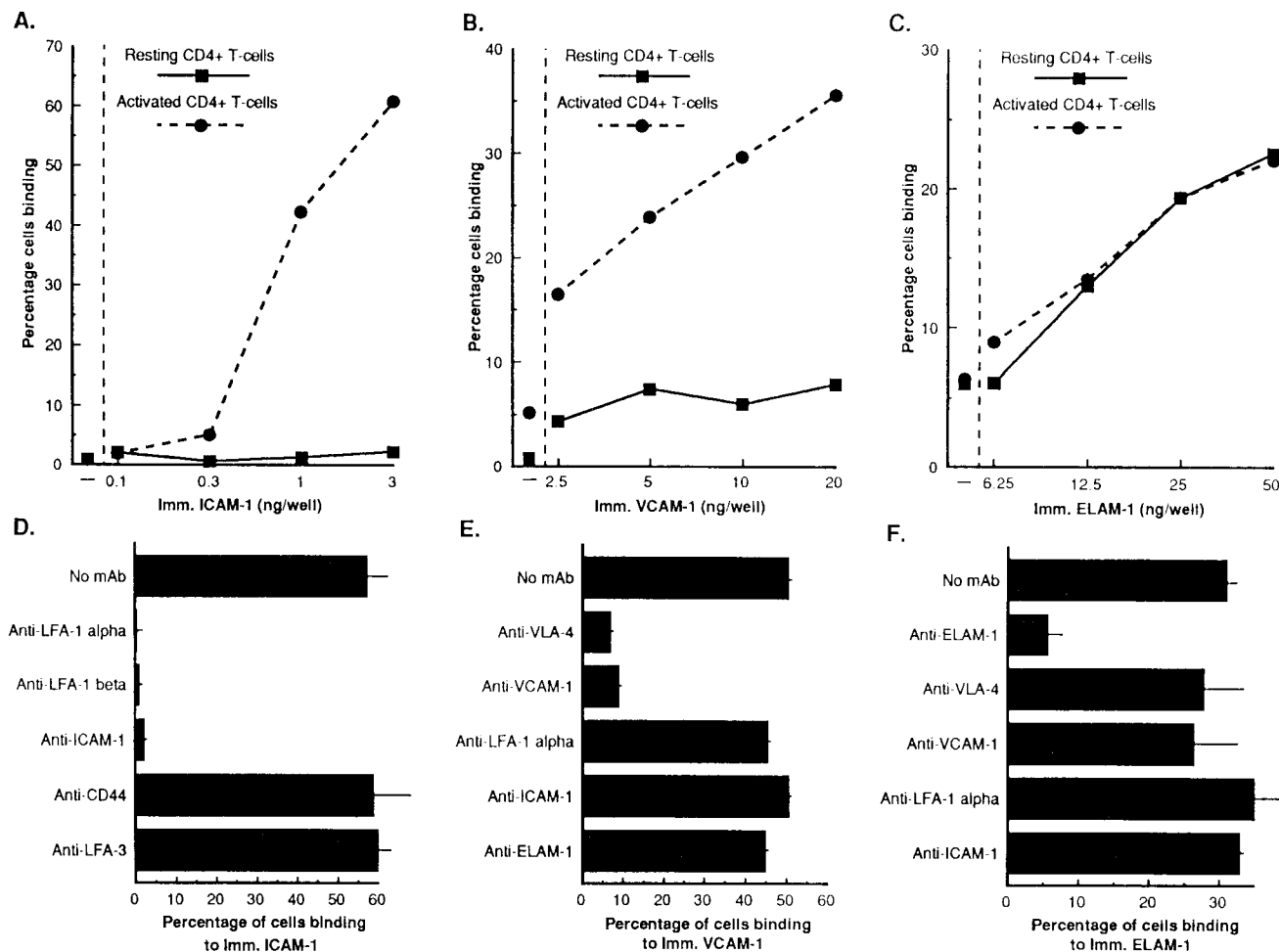


Figure 1. Binding of ⁵¹Cr-labeled CD4⁺ T cells to (A) ICAM-1, (B) VCAM-1, and (C) ELAM-1 immobilized on plastic was assessed as described in Materials and Methods. Adhesion of resting CD4⁺ T cells (—■—) and CD4⁺ T cells activated for 10 min at 37° C with PMA (—●—) to the indicated concentrations of ligand is shown. Background binding of resting and PMA-activated CD4⁺ T cells in the absence of adhesion ligand was determined with a control protein (bovine serum albumin) shown on the left of the interrupted line in each figure and has not been subtracted from the data. Binding of PMA-activated CD4⁺ T cells to (D) ICAM-1, (E) VCAM-1, and binding of resting CD4⁺ T cells to (F) ELAM-1 was assessed in the continuous presence of the following mAbs: the anti-VLA-4 mAb L25, the anti-VCAM-1 mAb 2G7, the anti-LFA-1 α chain mAb MHM24, the anti-LFA-1 β chain mAb MHM23, the anti-ICAM-1 mAb 84H10, the anti-ELAM-1 mAb 7A9, the anti-LFA-3 mAb TS2/9, and the anti-CD44 mAb NIH44-1. All mAbs were used as purified Ig at 10 μ g/ml. Binding of CD4⁺ T cells to a negative control protein (type IV collagen) was <3% and has not been subtracted from the values shown. Data are expressed as the mean per cent of cells binding from three replicate wells with bars representing standard error of the mean. Results presented are representative of three independent experiments using CD4⁺ T cells isolated from different donors.

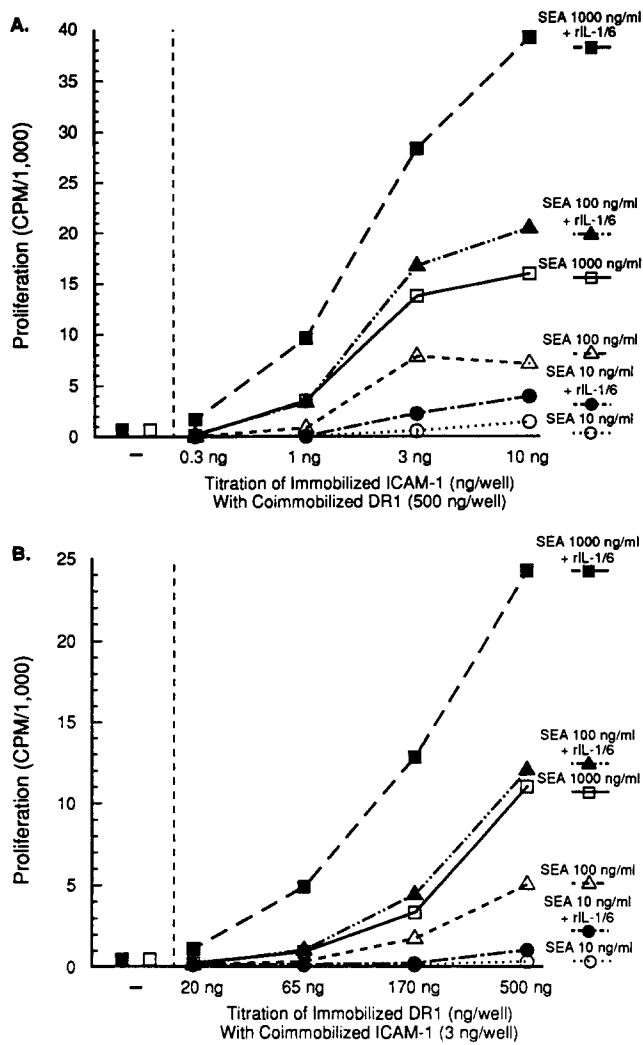


Figure 2. Proliferation of 80,000 CD4⁺ T cells/well is measured as described in Materials and Methods. Purified DR1 and ICAM-1 are immobilized on the plastic of the wells as described in Material and Methods. (A) Proliferation with various amounts of immobilized ICAM-1 (as indicated) in combination with a fixed amount of coimmobilized DR1 (500 ng/well) and a standard concentration of rIL-1 β (10 U/ml) and rIL-6 (10 U/ml) added to the culture, in the presence of various concentrations of SEA added to the culture (as indicated). Left of the interrupted line is only immobilized DR1 (500 ng/well) present in the various conditions. (B) Proliferation with various amounts of immobilized DR1 (as indicated) in combination with a fixed amount of coimmobilized ICAM-1 (3 ng/well) and various concentrations of SEA added to the culture (as indicated), in the presence or absence of the cytokine combination rIL-1 β and rIL-6 (each 10 U/ml). Left of the interrupted line only immobilized ICAM-1 (3 ng/well) is present in the various conditions.

rIL-6) in the absence of ICAM-1. Coimmobilized ICAM-1 however provide potent costimulation with DR1/SEA, which is augmented by the cytokines rIL-1 β and rIL-6 (Fig. 2, A and B).

The experiments depicted in Fig. 3 show a comparison between the OKT3 mAb-mediated and DR1/SEA-mediated activation systems in which ICAM-1, VCAM-1, and ELAM-1

were analyzed for their costimulatory capacities. ICAM-1 and VCAM-1 can both provide concentration-dependent costimulation in either system (Fig. 3, A and D, and B and E, respectively), that is augmented by the combination of cytokines, rIL-1 β and rIL-6. Coimmobilized ELAM-1, however, fails to provide costimulation capable of inducing CD4⁺ T cell proliferation.

The specificity of the costimulation by ICAM-1 and VCAM-1 in both the OKT3 (data not shown) and DR1/SEA (Fig. 4) was established with mAb blocking experiments. The ICAM-1 mediated costimulation was dependent on the interaction between T cell LFA-1 and the immobilized ICAM-1 (Fig. 4) while the costimulation by VCAM-1 is dependent on the interaction of T cell VLA-4 with the purified VCAM-1 (Fig. 4). Costimulation by both ICAM-1 and VCAM-1 is dependent on TCR/CD3 interaction with the HLA class II DR1 molecules as suggested by the complete inhibition of both systems with anti-HLA class II mAb or CD3 mAb (Fig. 4).

The combined results suggest a similar requirement for the costimulatory signals provided by ICAM-1 or VCAM-1 in both CD3 mAb- and DR1/SEA-mediated activation of resting CD4⁺ T cells. In contrast, T cells do adhere to ELAM-1 (Fig. 1), but ELAM-1 does not provide costimulation.

Resting CD45RA⁺ ("Naive") and CD45RO⁺ ("Memory") CD4⁺ T Cells Can both Be Stimulated by Coimmobilized OKT3 mAb and ICAM-1 or VCAM-1, but not ELAM-1. Since CD4⁺ T cells do adhere to ELAM-1, the lack of costimulation by ELAM-1 demonstrates that adhesion alone is not sufficient for costimulation. The negative results however could be due to the fact that in contrast to LFA-1 and VLA-4 mediated adhesion, only cells in the CD45RO⁺ "memory" subset of CD4⁺ T cells can bind to ELAM-1 (21, 22). Thus, the memory cell subpopulation in some donors might be too small to cause detectable proliferation in the system tested. We therefore investigated the costimulatory capacities of adhesion ligands using purified populations of CD45RA⁺ "naive" and CD45RO⁺ "memory" CD4⁺ T cells. To avoid the possibility of subset-specific expression of SEA-reactive TCR, we used the OKT3 mAb activation model. As shown in Fig. 5 C ELAM-1 still failed to provide costimulation, even for memory CD4⁺ T cells. In contrast, ICAM-1 (Fig. 5 A) and VCAM-1 (Fig. 5 B) costimulated both naive and memory cells. The level of costimulation of memory and naive cells by ICAM-1 or VCAM-1 varied between donors, and was not always directly correlated with the levels of receptor expression (i.e., LFA-1 and VLA-4) on these subsets (30) (data not shown).

Coimmobilized OKT3 mAb and ICAM-1 or VCAM-1, but not ELAM-1 Can Induce IL-2R α (CD25) Expression on Resting CD4⁺ T Cells. Proliferation is the result of a complete set of activation signals which, when combined, lead to cell division. Coimmobilized ELAM-1 and OKT3 mAb may result in partial activation which is not adequate for T cell proliferation. Since expression of the high affinity IL-2 receptor expression is one of the required steps leading to T cell proliferation, we measured expression of IL-2R α (CD25) after

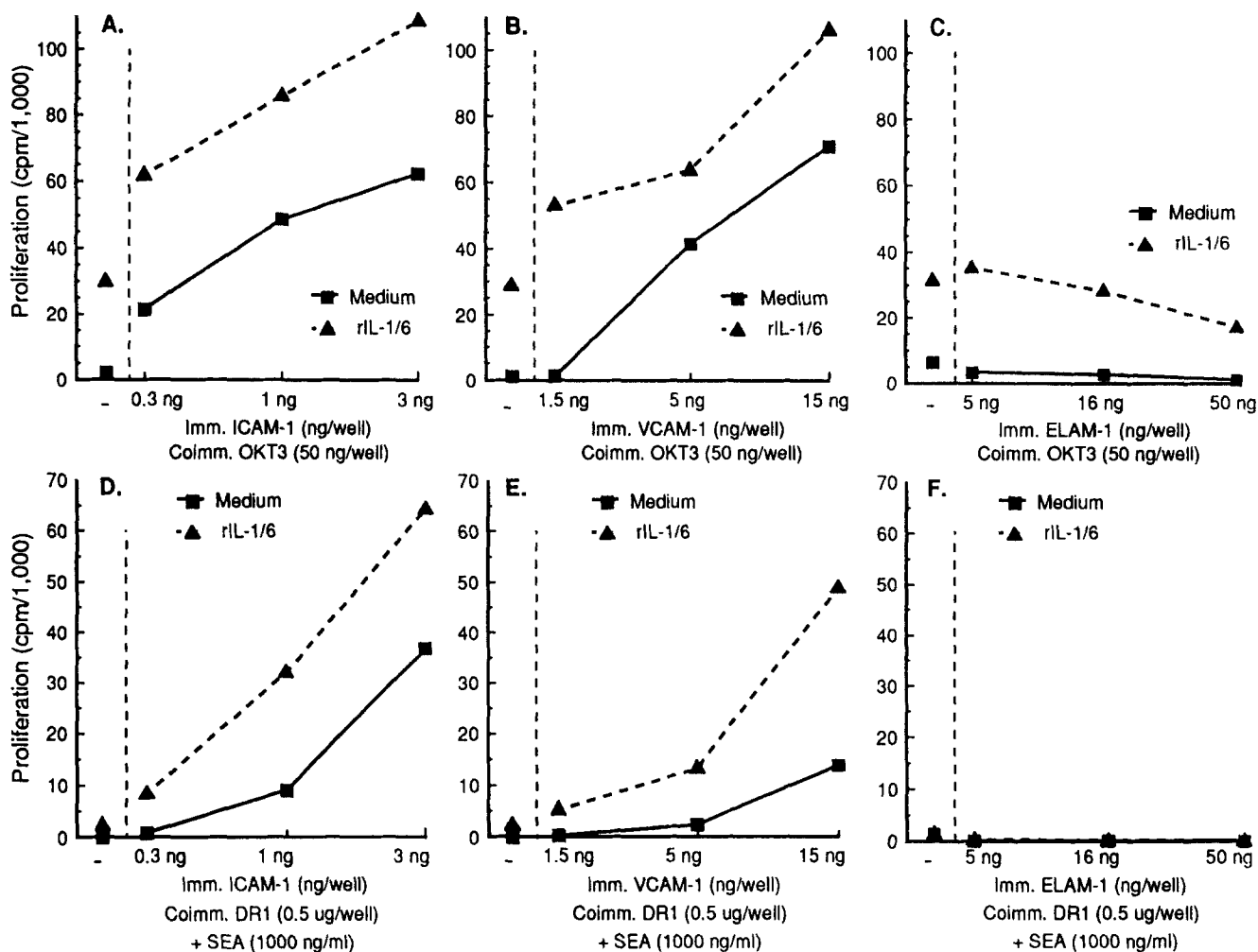


Figure 3. (A–C) Proliferation of 40,000 CD4⁺ T cells/well is measured as described in Materials and Methods. Culture conditions are with titrations of immobilized (A) ICAM-1, (B) VCAM-1, or (C) ELAM-1 in combination with a fixed amount of coimmobilized OKT3 mAb (50 ng/well), in the presence or absence of the cytokine combination rIL-1 β and rIL-6 (each 10 U/ml). Left of the interrupted lines only immobilized OKT3 (50 ng/well) is present and tested with and without rIL-1 β and rIL-6 (as indicated). (D–F) Proliferation of 80,000 CD4⁺ T cells is measured as described in Materials and Methods. Culture conditions are with titrations of immobilized (D) ICAM-1, (E) VCAM-1, or (F) ELAM-1 in combination with a fixed amount of coimmobilized DR1 (0.5 μ g/well) and standard concentration of SEA added to the culture (1,000 ng/ml), in the presence or absence of the cytokine combination rIL-1 β and rIL-6 (each 10 U/ml). Left of the interrupted lines only immobilized DR1 (500 ng/well) is present with SEA (1,000 ng/well) and tested with and without rIL-1 β and rIL-6 (as indicated). OKT3 mAb, DR1, ICAM-1, VCAM-1, and ELAM-1 are immobilized on the plastic of the well as described in Materials and Methods.

24 h of culture as an indicator of partial T cell activation. The results show that culture with only immobilized OKT3 mAb led to a small increase in IL-2R α expression on a subpopulation of CD4⁺ T cells (mainly CD45RO⁺ cells, data not shown), which is not accompanied by proliferation (Table 1). Costimulation with ICAM-1 or VCAM-1 induces IL-2R α expression on most cells (both on CD45RA⁺ and CD45RO⁺ CD4⁺ T cells, data not shown), which is comparable with that generated by the mitogenic combination of PHA and PMA (Table 1). These latter three culture conditions all result in significant proliferative responses (Table 1). In contrast, coimmobilized ELAM-1 and OKT3 mAb, does not result in increased IL-2R α expression over that induced by OKT3 mAb alone (Table 1).

Costimulation by ICAM-1 or VCAM-1, but not ELAM-1 Regulates IL-2, IL-4, and GM-CSF Release of Resting CD4⁺ T Cells. Cytokine release by T cells is also a consequence of specific stimulation. Therefore we studied the three adhesion molecules for their capability to regulate levels of the cytokines IL-2, IL-4, and GM-CSF. The partial activation induced by immobilized OKT3 mAb in presence of rIL-1 and rIL-6 leads to minimal levels of IL-2 and low levels of GM-CSF, but not IL-4 (Table 2). This costimulation with ICAM-1 or VCAM-1, in the absence or presence of rIL-1 and rIL-6, regulates release of significant amounts of IL-2, IL-4 and GM-CSF (Table 2). The levels of IL-4 produced under these conditions are significantly higher than that with the culture condition of PHA/PMA (Table 2). ICAM-1 and VCAM-1 have no effect

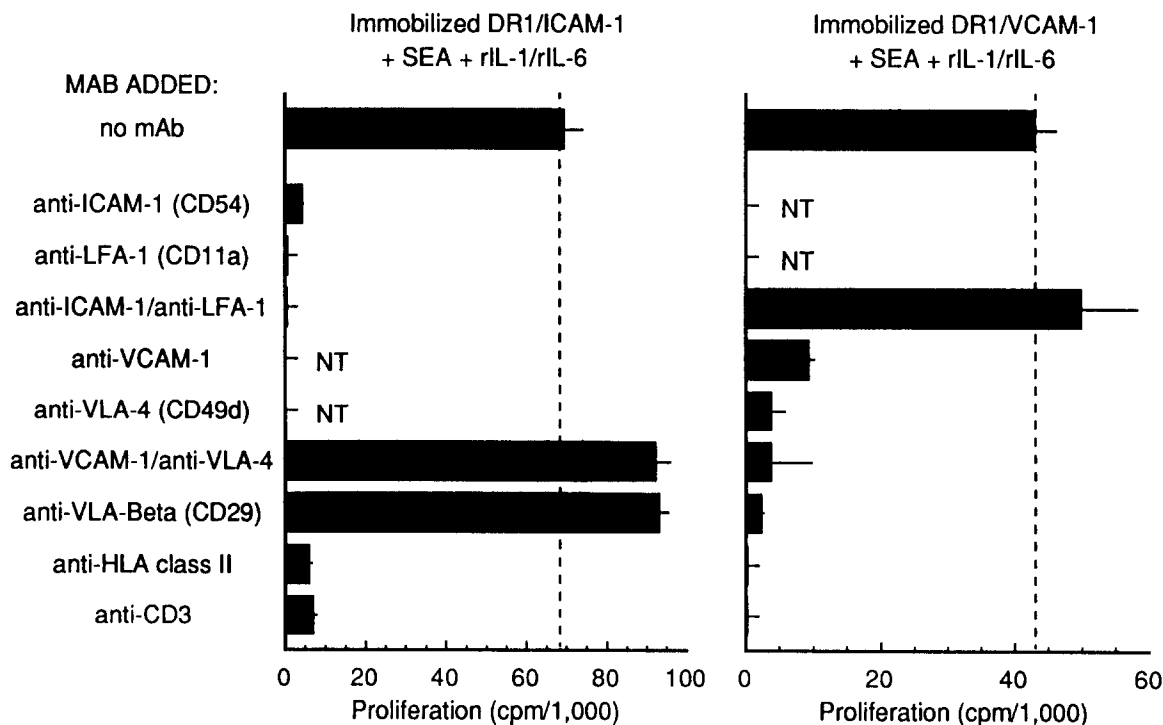


Figure 4. (Left) mAb blocking of proliferation induced by coimmobilized DR1 (0.5 $\mu\text{g}/\text{well}$) and ICAM-1 (3 ng/well), with SEA (1,000 ng/ml) and the cytokines rIL-1 β and rIL-6 (each 10 U/ml) added to the well. (Right) mAb blocking of proliferation induced by coimmobilized DR1 (0.5 $\mu\text{g}/\text{well}$) and VCAM-1 (15 ng/well), with SEA (1,000 ng/ml) and the cytokines rIL-1 β and rIL-6 (each 10 U/ml) added to the well. In both panels is the proliferation of 80,000 cells CD4 $^+$ T cells/well measured as described in Materials and Methods. Purified DR1, ICAM-1, and VCAM-1 were immobilized on the plastic of the well as described in Materials and Methods. mAbs were added as purified Ig at a final concentration of 10 $\mu\text{g}/\text{ml}$.

when applied in the absence of CD3 mAb. Again coimmobilized OKT3 mAb and ELAM-1 do not alter the cytokine release induced by OKT3 mAb in isolation (Table 2).

Discussion

Various T cell adhesion pathways also function as signal transducing pathways regulating T cell activation (see references 1 and 9 for review). We have compared the T cell adhesion pathways mediated by the molecules ICAM-1, VCAM-1 and ELAM-1 for their ability to costimulate antigen-specific responses of resting human CD4 $^+$ T cells. To examine the costimulatory potential of these molecules we developed a biochemically-defined system that can be viewed as a closer correlate of a primary antigen-specific response than the commonly used CD3 mAb-mediated T cell response. This new in vitro T cell activation model utilizes purified immobilized HLA DR1 and the superantigen SEA. We show that similar to CD3 mAb-mediated activation models, this DR1/SEA system is critically dependent on certain adhesion-ligand-mediated costimulatory signals which are distinct from the recombinant cytokines rIL-1 β and rIL-6. We further demonstrate that specific adherence of CD4 $^+$ T cells alone is not sufficient to provide such costimulation. While CD4 $^+$ T cells can bind to various adhesion molecules through either activation-independent (ELAM-1) or activation dependent

pathways (ICAM-1 and VCAM-1), only the integrin-mediated pathways (LFA-1/ICAM-1 and VLA-4/VCAM-1) can provide the costimulation required in both the CD3 mAb-mediated and the antigen-specific DR1/SEA-mediated CD4 $^+$ T cell activation models (Figs. 2, 3, and 4). The specific interaction of LFA-1/ICAM-1 and VLA-4/VCAM-1 (Fig. 4) not only leads to T cell proliferation, but also regulates the production of IL-2 and IL-4, and augments the levels of secreted GM-CSF (Table 1).

T cell adhesion to ELAM-1 is strikingly different from adhesion mediated through the integrin pathways LFA-1/ICAM-1 and VLA-4/VCAM-1 in at least three fundamental aspects. First, ELAM-1-mediated adhesion occurs without prior activation of the T cell and activation does not alter the degree of binding (Fig. 1) (21). Second, CD4 $^+$ CD45RO $^+$ "memory" T cells but not CD4 $^+$ CD45RA $^+$ "naive" T cells bind to ELAM-1 (21, 22). Third, as demonstrated in this study, T cell binding to ELAM-1 does not generate the same type of costimulatory signals as induced by T cell adhesion via LFA-1/ICAM-1 and VLA-4/VCAM-1 (Figs. 3 and 5, Tables 1 and 2). In fact, we have failed to observe costimulatory signals mediated by ELAM-1, even when the response was tested with memory T cells, in which the ELAM-1 adherent population is present in a higher frequency (Fig. 5). Furthermore, changes which reflect partial activation, such as increased IL-2R α expression (Table 1), were not evident

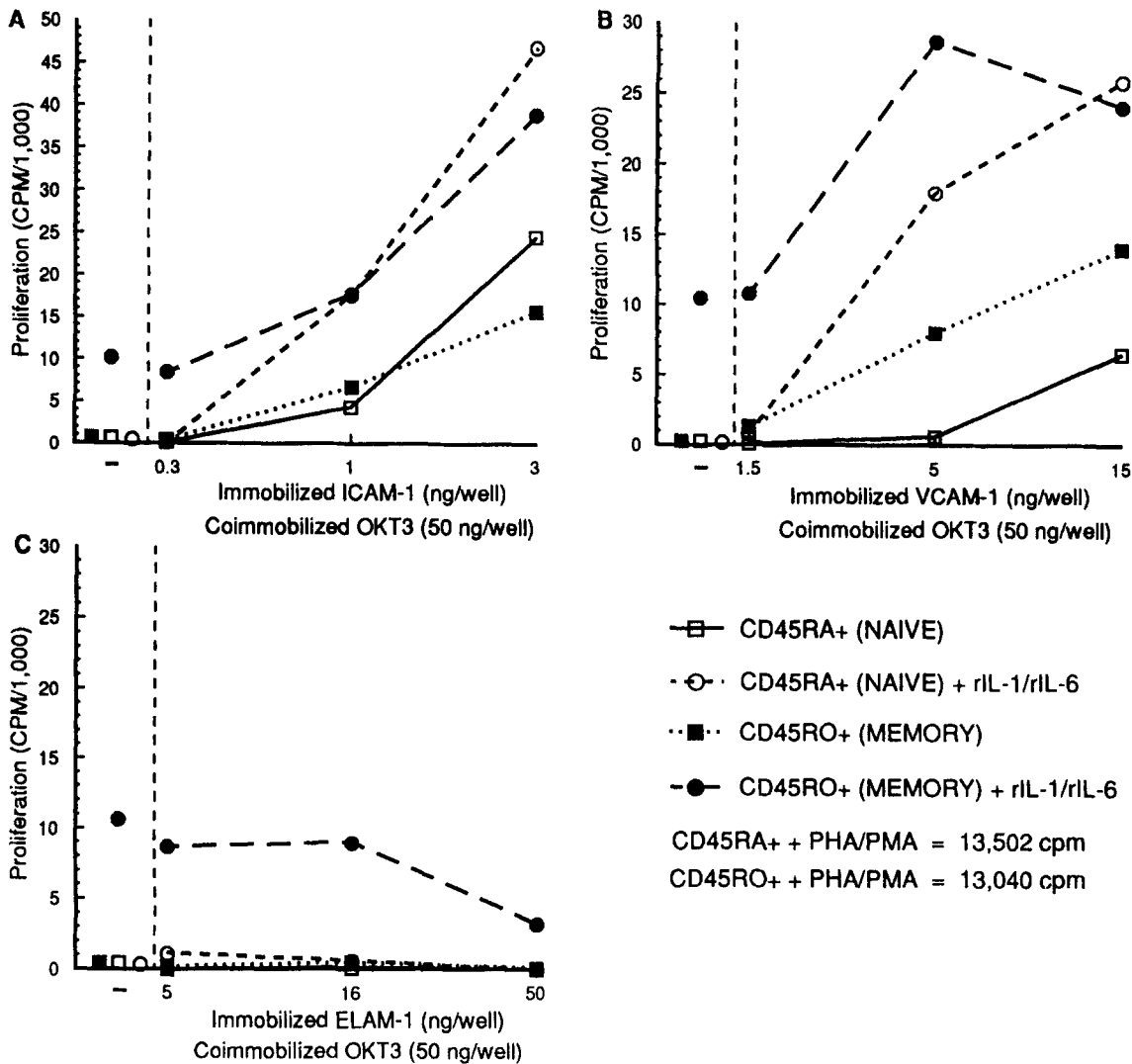


Figure 5. Proliferation of 40,000 CD45RA⁺CD4⁺ and CD45RO⁺CD4⁺ T cells/well is measured as described in Materials and Methods. Culture conditions are with titrations of immobilized (A) ICAM-1, (B) VCAM-1, or (C) ELAM-1 in combination with a fixed amount of coimmobilized OKT3 mAb (50 ng/well), in the presence or absence of the cytokine combination rIL-1 β and rIL-6 (each 10 U/ml). Left of the interrupted lines only immobilized OKT3 (50 ng/well) is present and tested for the various conditions (as indicated). Proliferation induced by PHA/PMA (as described in Materials and Methods) was for CD45RA⁺CD4⁺ and CD45RO⁺CD4⁺ T cells, respectively, 13,502 cpm and 13,040 cpm.

with purified ELAM-1. This negative result is particularly striking, given the large number of adhesive interactions, mediated either by natural ligand or mAb specific for a T cell surface molecule, that have been shown to facilitate T cell proliferation in similar in vitro systems (see references 1 and 9 for review). Our results with ELAM-1 suggest that the molecular mechanisms mediating these various costimulatory signals may not be triggered by adhesion alone. Furthermore this suggests that adhesion pathways can be divided into two major classes: (a) those that provide adhesion, and (b) those that provide both adhesion and costimulation.

The fact that ELAM-1, ICAM-1, and VCAM-1 expression on endothelium is augmented with inflammation (47, 48) has implications both for adhesion and for costimulation. The implications for adhesion are more efficient cap-

ture of T cells by endothelium at sites of inflammation. ELAM-1 is unique in its activation-independent binding, which would be expected to be critical in capture of resting T cells from the circulation (21); for reasons not yet fully defined this mechanism may predominate in migration of T cells into skin (22). Although resting T cells do not adhere particularly efficiently via their VLA-4/VCAM-1 or LFA-1/ICAM-1 pathways, these pathways are presumably important either: because the low level is enough for capture, or because other molecular interactions trigger their function during the process of T/endothelial cell capture. Not only do T cells have to contact endothelial cells during entry into tissue, but also the cuffing of lymphocytes around vessels in inflammatory infiltrates suggests sustained contact.

A variety of lines of evidence suggest that endothelial cells

Table 1. Coimmobilized ICAM-1 and VCAM-1, but not ELAM-1 Dramatically Upregulates OKT3-induced IL-2R Expression on Resting CD4⁺ T Cells

	% Cell positive*		Proliferation**
	Control	IL-2R (CD25)	
			<i>cpm/1,000</i>
Medium	5.6	6.8	0.1
Imm. OKT3	7.9	24.6	1.1
Imm. OKT3/ICAM-1	9.1	82.3	135.1
Imm. OKT3/VCAM-1	9.1	76.3	122.5
Imm. OKT3/ELAM-1	6.7	19.7	0.1
PHA/PMA	16.1	89.6	68.4

* IL-2R (CD25) expression after 24-h culture.

** Proliferation after 72-h culture.

IL-2R (CD25) expression and proliferation are measured as described in Materials and Methods. mAb OKT3 (applied at 0.5 μ g/well), ICAM-1 (applied at 30 ng/well), VCAM-1 (applied at 150 ng/well) and ELAM-1 (500 ng/well) were immobilized on the plastic of the 24-well tissue culture plate as described in Materials and Methods. As background fluorescence control levels were cells taken that were stained with only Streptavidin-FITC conjugate.

may play important roles in antigen presentation (16). The present study demonstrates that two of its activation-induced surface molecules, VCAM-1 and ICAM-1 are potent costimulators of T cell activation. VCAM-1 is only expressed on endothelial cells which have been stimulated with inflammatory cytokines such as IL-1 and TNF- α (48), a condition that also further augments ICAM-1 expression. Endothelial cells, which normally express HLA class I molecules, are induced to express HLA class II by IFN- γ (49), that also enhances HLA class I and ICAM-1 expression (50). Furthermore endothelial cells constitutively release low levels of IL-6 (51) and can be induced to produce high levels of both IL-1 (52) and IL-6 (51). Foreign antigens will be abundant on endothelium in allografts, and potentially in infectious diseases and autoimmune disorders. Thus, endothelial cells are endowed with the necessary components to effectively stimulate T-cells and are guaranteed contact with T cells; moreover, in vitro endothelial cells can function efficiently as APC (16, 53, 54). In addition to potential roles for VLA-4/VCAM-1 and LFA-1/ICAM-1, other molecules engaged during T/endothelial interactions may also provided costimulation; Pober and coworkers have demonstrated a role for CD2/LFA-3 in a model of system of endothelial cell costimulation of T cell activation (55). All these processes may have exaggerated importance in allogeneic organ transplantation where endothelial

Table 2. Induction of Cytokine Release

	IL-2*	IL-4 [†]	GM-CSF [‡]	Proliferation [§]
	<i>U/ml</i>	<i>pg/ml</i>	<i>ng/ml</i>	<i>cpm/1,000</i>
Medium	<0.05	<24	<0.4	0.06
Medium + rIL-1/rIL-6	<0.05	<24	<0.4	0.06
Imm. OKT3	<0.05	<24	3.1	0.20
Imm. OKT3 + rIL-1/rIL-6	0.3	<24	5.9	2.40
Imm. OKT3/ICAM-1	1.4	1,036	12.8	60.70
Imm. OKT3/ICAM-1 + rIL-1/rIL-6	7.9	1,819	14.6	65.50
Imm. ICAM-1 + rIL-1/rIL-6	0.4	<24	2.4	0.06
Imm. OKT3/VCAM-1	0.8	225	14.0	42.60
Imm. OKT3/VCAM-1 + rIL-1/rIL-6	7.4	1,048	14.3	55.50
Imm. VCAM-1 + rIL-1/rIL-6	<0.05	<24	<0.4	0.05
Imm. OKT3/ELAM-1	<0.05	<24	2.2	0.07
Imm. OKT3/ELAM-1 + rIL-1/rIL-6	0.3	<24	6.7	0.50
Imm. ELAM-1 + rIL-1/rIL-6	<0.05	<24	<0.4	0.05
PHA/PMA	1.8	166	16.5	29.00

Cytokine levels and proliferation are measured as described in Materials and Methods. mAb OKT3 (applied at 0.5 μ g/well), ICAM-1 (applied at 30 ng/well), VCAM-1 (applied at 150 ng/well), and ELAM-1 (500 ng/well) were immobilized on the plastic of the 24-well tissue culture plate as described in Materials and Methods. Cytokines rIL-1 β and rIL-6 were added at a final concentration of 10 U/ml.

* After 24-h culture.

† After 48-h culture.

§ After 72-h culture.

cells are both target cells and APCs, and may mediate antigen-specific recruitment of T cells (14–16).

Many groups have postulated that T cell activation requires both a first and second signal (for review see reference 2). The first signal is thought to be provided by specific engagement of the TCR/CD3 complex, and must be accompanied by a second signal(s) to allow for full activation. Failure to receive the second signal has been correlated with the induction of clonal anergy (for review see reference 2). The nature of the second signal(s) is poorly defined, but is shown to be: (a) dependent on direct cell-cell contact (56, 57); and (b) mediated by a cell surface molecule(s) expressed on cytokine stimulated, but not unstimulated APC's (57–59). Both ICAM-1 and VCAM-1 are not only capable of costimulation, but also fulfill many of the other requirements postulated for second signal(s). First, in regard to the cell-contact nature of the signal, the cell adhesion promoting capacities of ICAM-1 and VCAM-1 have been studied extensively (see references 23 and 60). Second, cell surface expression of both ICAM-1 and VCAM-1 is regulated by inflammatory cytokines (10, 43, 48, 50, 61, 62). ICAM-1 is also widely expressed on cell types known to be involved in antigen-presentation such as dendritic cells, monocytes and B cells. Activation-induced augmentation of ICAM-1 expression is correlated with increased APC function of fixed monocytes (59).

We and others have shown previously that activation-dependent adhesion of the VLA-4 receptor to its ECM ligand FN can provide costimulation in the OKT3 mAb model (24–26) (and in the DR1/SEA system, data not shown). While a role the VLA-4/FN interaction in extravasation and migration through tissue seems likely, the relevance of FN as a costimulatory molecule in antigen-specific T cell responses remains speculative. However, the identification of VCAM-1 as a cell surface adhesion ligand for VLA-4 (11) and the present data on VCAM-1 costimulation suggest an antigen-specific costimulatory role for VLA-4 molecules. The recent finding that VCAM-1 is not only expressed on activated endothelial cells, but also on lymphoid dendritic cells of tonsil, spleen and peripheral lymph node (47, 64), certain macrophages present in spleen and thymus (47), and those associated with T cells in the skin (W. Sterry, personal communication) may prove to be important in finding such a physiological role for VCAM-1 on APCs.

Recently, it has been demonstrated that the B cell activation antigen B7, a ligand for CD28, functions as an adhesion molecule (65), and that immobilized purified B7 antigen can provide costimulation (66). Consequently, there are multiple receptor/ligand interactions (CD2/LFA-3, CD28/B7, LFA-

1/ICAM-1 and VLA-4/VCAM-1) which fit many of the requirements for a "second signal". Why then is there such a redundancy of molecules capable of providing adhesion-mediated costimulation? We favor the concept that the multiplicity of molecular interactions between T cell and APC determines the specifics of activation and subsequent differentiation of the T cell and APC (9). Quantitative and qualitative differences in the expression of adhesion receptors on different T cell subsets and their respective ligands on various types of APC's may then cause differential responses via selective utilization of the various costimulatory pathways. Different combinations of costimulatory pathways may induce different responses from the T cells, for example cytokine release. Evidence for such differential cytokine release has recently been published by Cerdan et al. (67) who showed induction of IL-1 α by T cells by the combinations of CD2/CD28 mAbs and CD3/CD28 mAbs, but not by any of these mAbs in isolation, although they were able to induce T cell proliferation and TNF- α secretion. We show that costimulation by ICAM-1 and VCAM-1 both induce release of the cytokines IL-2, IL-4, and GM-CSF (Table 2). This similarity may reflect the close structural relation between LFA-1 and VLA-4 as members of the same integrin superfamily. Further analysis is required to establish whether these cytokines are produced by stimulation of one homogeneous population or by a mixture of subpopulations of CD4⁺ T cells, analogous to the murine Th1 and Th2 subsets of CD4⁺ T cells (68, 69).

In conclusion, we have analyzed the costimulatory potential of the adhesion molecules ICAM-1, VCAM-1, and ELAM-1 using a newly described T cell activation system of purified HLA-DR1 molecules and the superantigen SEA; this system closely mimics the antigen-specific response of resting CD4⁺ T cells. Our results demonstrate that while ICAM-1, VCAM-1 and ELAM-1 all mediate efficient T cell adhesion, only ICAM-1 and VCAM-1 mediate costimulation and regulate cytokine release. The inability of ELAM-1 to mediate even a partial costimulatory signal demonstrates that adhesion alone is not sufficient to generate costimulation in these *in vitro* systems. Furthermore, our demonstration of the costimulatory potential of VCAM-1, coupled with expression of VCAM-1 on unique APCs, suggests an important role for the VLA-4/VCAM-1 interaction in the regulation and modification of specific T cell responses. These results show the importance of adhesion molecules in facilitating T cell activation and suggest the mediation of specific costimulatory signals by the LFA-1 and VLA-4 integrins after interaction with their natural ligands.

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