

Specific Interaction of Lymphocyte Function-associated Antigen 3 with CD2 Can Inhibit T Cell Responses

By Glenn T. Miller, Paula S. Hochman, Werner Meier, Richard Tizard, Sarah A. Bixler, Margaret D. Rosa, and Barbara P. Wallner

From Biogen, Inc., Cambridge, Massachusetts 02142

Summary

Accessory cell surface molecules, such as T cell antigen CD2 and its ligand lymphocyte function-associated antigen 3 (LFA-3; CD58), are critical costimulatory pathways for optimal T cell activation in response to antigens. Interaction of CD2 with cell surface LFA-3 not only increases T cell/accessory cell adhesion, but also induces signal transduction events involved in the regulation of T cell responses. In this report, we show that specific interactions of LFA-3 with CD2 can result in T cell unresponsiveness to antigenic or mitogenic stimuli *in vitro*. By deletion of certain regions of the extracellular domain of LFA-3, we localized the CD2 binding site to the first domain of LFA-3. We then demonstrated that a soluble, purified first domain-LFA-3/IgG1 fusion protein (LFA3TIP) interacts with CD2 and binds to the same CD2 epitope as purified multimeric or cell surface-expressed LFA-3. LFA3TIP inhibits tetanus toxoid, hepatitis B surface antigen, anti-CD3 mAb, Con A, and phytohemagglutinin P-induced T cell proliferation, as well as xenogeneic and allogeneic mixed lymphocyte reactions (MLR). Unlike anti-LFA-3 or anti-CD2 monoclonal antibodies (mAbs) which inhibit T cell responses by blocking LFA-3/CD2 binding, LFA3TIP is capable of rendering T cells unresponsive to antigenic stimuli in situations where T cell activation is independent of CD2/LFA-3 interactions. Furthermore, LFA3TIP, but not blocking anti-CD2 mAbs, is capable of inducing T cell unresponsiveness to secondary stimulation in allogeneic MLR. This inhibition of T cell responses by LFA3TIP occurs through a different mechanism from that of mAbs to LFA-3 or CD2.

Responses of the immune system are centrally controlled by T cell activity which is initiated through cellular interactions between T cells and APCs. The specificity of recognition is dictated by the TCR complex (TCR-CD3), whereas optimal T cell responses are dependent on costimulatory signals by accessory cell surface molecules, including CD2/LFA-3, LFA-1/intercellular adhesion molecule 1, CD28/B7, CD4/class II, and CD8/class I (for reviews see references 1-4). The multitude of cell surface antigens involved in T cell activation appears to be excessive and their functions redundant. However, this multiplicity of interactions represents a finely tuned regulatory mechanism of T cell activation during specific stages of the immune response, involving sequential interactions and activation of individual surface antigens and their pathways (3, 5-7), physical linkage between surface structures, and association of protein kinase and phosphatase activities (8, 9). Inhibition of any one of these costimulatory pathways with their respective mAbs is sufficient to inhibit T cell responses.

Human CD2 is a glycoprotein, expressed on all subsets of T lymphocytes, NK and LAK cells, and functions to op-

timize antigen-specific and -independent T cell responses, not only by increasing cellular adhesion between cognate partners, but also by augmenting T cell responses initiated through the TCR/CD3 engagement (1). It has also been suggested that CD2 has a regulatory role in T cell responses by transducing a negative signal to T cells (10, 11). However, so far it has not been clearly established that the inhibition of T cell responses through CD2 is independent of blocking cellular adhesion.

The CD2 ligand, LFA-3, is expressed on a large variety of cells as a transmembrane-integrated or phosphoinositol-linked surface glycoprotein (12-14). CD2/LFA-3 interactions transduce signals not only to T cells but also to interacting monocytes resulting in the secretion of cytokines capable of regulating T cell responses (15). Modulation of cell surface expression levels of both CD2 and LFA-3 has been implicated in playing a regulatory role in the antigen-specific responses of various subsets of T cells (3, 5). Purified LFA-3 in multimeric but not monomeric form potently costimulates T cell responses with mitogens or anti-CD2-specific mAbs *in vitro* (16).

The secondary structure of the CD2 and LFA-3 extracellular regions each consists of two Ig-like domains which classify them as members of the Ig superfamily (17). By mAb epitope mapping and site-specific mutagenesis, the LFA-3 binding site on CD2 has been localized to two epitopes in the first domain of CD2 (18).

We have analyzed the structural and functional requirements for interactions of LFA-3 with CD2. We mapped the CD2 binding site to the first domain of LFA-3 and constructed a dimeric first domain-LFA-3/IgG1 fusion protein. The effect of this purified fusion protein (LFA3TIP) on T cell responses was compared with that of the purified full-length monomeric or multimeric LFA-3, and that of mAbs to LFA-3 or CD2. LFA3TIP inhibits allogeneic and xenogeneic MLR, anti-CD3 mAb, or lectin-induced polyclonal, as well as antigen-specific T cell activation in vitro. We demonstrate here that inhibition of T cell proliferation by LFA3TIP is a function of its interaction with CD2, and that the mechanism of LFA3TIP inhibition is distinct from that of several CD2 or LFA-3-specific mAbs tested. LFA3TIP is capable of inhibiting T cell activation independent of blocking CD2/LFA-3 interactions.

Materials and Methods

Determination of the CD2 Binding Domain on LFA-3. Plasmid pHT16-6 containing the full-length cDNA for the transmembrane form of LFA-3 (14) was mutated using the gapped plasmid mutagenesis essentially as described (19). LFA-3 deletion mutants have the following amino acids of the mature LFA-3 deleted: M57, 51 to 60; M63, 111 to 120, M100, 11 to 70; M101, 71 to 130; and M102, 131 to 180. In all cases, Phe is being designated as the first residue of the mature LFA-3. Each deletion mutant was confirmed by DNA sequence analysis (20). Mutagenized LFA-3 cDNA was inserted into expression vector pBG368PY, or its derivative pMDR 902 (a derivative of pBG312, which carries the DHFR gene; Rosa, M. D., unpublished observations) and resulting plasmids were used to transfect Chinese hamster ovary (CHO)¹ cells which are DHFR negative (CHO-DHFR⁻) cells as described (21). Methotrexate-resistant cells were assayed for LFA-3 cell surface expression by FACS[®] analysis (FACStar Plus[®]; Becton Dickinson & Co., Mountain View, CA) using rabbit anti-LFA-3 polyclonal antibody (Rb202; Biogen, Inc.) as primary antibody and FITC-conjugated goat anti-rabbit IgG to detect Rb202 binding. Cells positive for LFA-3 expression were further tested for binding of anti-LFA-3 mAbs TS2/9 (a gift of T. Springer, Center for Blood Research, Boston, MA), 7A6, and 1E6 (Biogen, Inc.) by FACS[®] analysis with FITC-conjugated goat anti-murine IgG.

Construction and Expression of IgG1 Fusion Proteins. The human IgG1 H chain C region containing the hinge region, the C_H2 and the C_H3 sequences, were isolated with H chain-specific primers by PCR amplification of cDNA from the RNA of COS-7 cells that had been transfected with plasmids containing 3-kb genomic DNA inserts. The genomic DNA inserts had been isolated by digestion of the partial Sau3A human fibroblast genomic library EMBL/5X (a gift of Mark Pask, Biogen, Inc.). H

chain-specific oligonucleotide primers were designed so that the resulting C_H2 and C_H3 IgG1 PCR amplified sequences were flanked by a 5' SalI and a 3' NotI restriction enzyme recognition site. The NH₂-terminal 92 amino acid sequence of mature LFA-3, preceded by the LFA-3 signal sequence, was isolated by PCR amplification of pHT16-6 cDNA (14), using one primer containing a 5' NotI site and another primer containing a SalI site at the 3' end. Amplified DNA fragments of the LFA-3 and the IgG1 sequences were ligated and inserted into an expression vector pSAB132 (a derivative of pJOD-S [21]). The resulting plasmid pSAB152 contains cDNA sequences coding for the signal sequence and first 92 amino acids of LFA-3, for 10 amino acids of the hinge region, and for the full sequence of the C_H2 and C_H3 regions of IgG1, all flanked by NotI restriction sites. The NotI fragment of pSAB152, coding for the LFA-3 fusion protein, was inserted into the expression vector pMDR902, and the resulting plasmid, pMDR(92)Ig-3 was transfected into CHO-DHFR⁻ cells. M57IgG1 fusion protein was constructed by PCR amplification of the LFA-3 deletion mutant, M57 cDNA sequences coding for the first 82 amino acids of the mature M57/LFA-3. Amplified DNA was ligated to IgG1 sequences and inserted into the pSAB132 expression vector as described above for LFA3TIP. The resulting plasmid pM57IgG1 was used to transfect CHO-DHFR⁻ cells. A LFA-3/IgG1 fusion protein containing both domains of LFA-3 (LFA3FLIgG1) was constructed exactly as described for LFA3TIP, except that the signal sequence and the first 183 amino acids of the extracellular domain of LFA-3 were amplified and fused to the IgG1 sequences, as described above. The resulting plasmid pLFA3FLIgG1 was used to transfect CHO-DHFR⁻ cells. Expression levels of all LFA-3 fusion proteins were amplified by culturing transfected CHO cell lines in medium containing increasing concentrations of methotrexate. All proteins were purified from cultured medium by adsorption to protein A-Sepharose 4B (Pharmacia, Piscataway, NJ). Multimeric phosphoinositide-linked LFA-3 (PI-LFA-3) and monomeric LFA-3 were purified as described (16). The CD4IgG1 fusion protein was constructed by ligating a PCR amplified DNA fragment coding for the NH₂-terminal 180 amino acids of human CD4 to the PCR amplified fragment of the same human IgG1 sequences as had been used for LFA3TIP, followed by insertion into expression vector pSAB132. Expression in CHO cells and purification of CD4IgG1 protein was exactly as described for LFA3TIP.

Cell Binding Analysis. CHO-DHFR⁻ cells and CHO-DHFR⁻ cells transfected with mutated or wild-type LFA-3 cDNA were grown in 6-well plates (Corning Inc., Corning, NY) at 10⁵ cells/well, washed with RPMI complete medium, and incubated with 5 × 10⁶ Jurkat cells in RPMI and 10% fetal bovine serum for 4 h at 4°C. Wells were washed gently three times with RPMI medium and examined under the microscope for rosette formation.

Human (h) and sheep RBC rosetting to Jurkat cells was performed exactly as described previously (16).

LFA3TIP and mAb Binding Analysis to CD2. CD2 substitution mutants were a gift of D. Brian Seed (Massachusetts General Hospital, Boston, MA). Transfection of COS cells was carried out essentially as described (21) and CD2 expression levels were analyzed using anti-CD2 mAb GD10 (a gift of D. Hess, Biogen, Inc.). Binding of all human IgG1 fusion proteins was detected by PE-conjugated anti-human IgG, and binding of anti-CD2 mAbs TS2/18 (a gift of T. Springer, Center for Blood Research, Boston, MA) and GD10 by FITC-conjugated anti-murine IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) using FACS[®] analysis.

T Cell Proliferation Assays. PBLs from healthy volunteers were

¹ Abbreviations used in this paper: CHO, Chinese hamster ovary; h, human; HBsAg, hepatitis B surface antigen; MLR, mixed lymphocyte reaction; TT, tetanus toxoid.

isolated by Ficoll Hypaque gradient centrifugation (Pharmacia). For mixed lymphocyte reaction (MLR), stimulator cells were either allogeneic PBLs, murine B cell tumor cells A20, or human B cell tumor cells JY (gift of Tim Springer), irradiated with 2,000 rad for PBLs or 10,000 rad for tumor cells in a γ cell irradiator. For MLR with allogeneic PBLs as stimulators the responder/stimulator ratio was 1:1. With B cell tumor stimulators, a 5:1 ratio was used. Responder cells ($1-2 \times 10^5$ /well) were incubated in RPMI medium supplemented with 10% heat-inactivated FCS at 37°C in 96-well round-bottom plates (model 25850; Corning Inc.) for the indicated times, pulsed with 1 μ Ci [methyl- 3 H]thymidine (New England Nuclear, Boston, MA) for 12-15 h, and harvested using a 96-well harvester (Tomtec, Orange, CT). Anti-CD3 mAb, OKT3 (Ortho Pharmaceuticals, Raritan, NJ) was used at a final concentration of 3 ng/ml, and T11₂ and T11₃ antibodies (gift of Dr. Ellis Reinherz, Dana Farber Cancer Institute, Boston, MA) were used at a 1:900 dilution of ascites. In all T cell activation assays, isotype control mAbs at 1-5 μ g/ml were included. For murine mAbs we used MOPC21 at 5 μ g/ml, and for all fusion proteins, purified hIgG at 1 μ g/ml (Pierce, Rockford, IL). For mitogen, PHA-P, Con A (Sigma Chemical Co., St. Louis, MO), and antibody-stimulated T cell proliferation assays, PBLs were incubated for 3 d. Antigen-specific stimulated and MLR cultures were incubated for 5 d unless otherwise indicated.

Results

CD2 Binds to the First Domain of LFA-3. To analyze the structural and functional requirements of CD2/LFA-3 interactions, we identified the LFA-3 sequences involved in these interactions. Three mutant cDNAs of the extracellular domain of LFA-3 were constructed and stably expressed in CHO cells: LFA-3 mutant M100 has a deletion of the first domain, M101 a deletion of the first half, and M102 a deletion of the second half of the second domain of LFA-3 (for sequence, see reference 14). All mutants contain the LFA-3 transmembrane region intact and are therefore expressed on the cell surface of transfected CHO cells.

Expression levels of these LFA-3 deletion mutants in CHO cells were monitored through the binding of a rabbit polyclonal antibody to LFA-3, Rb202, by FACS[®] analysis (Fig. 1 *a* for all deletions). CHO cells positive for LFA-3 expression were then analyzed for binding of anti-LFA-3 mAbs 7A6 and TS2/9, mAbs that had previously been shown to block LFA-3/CD2 interactions (3, 16). Only mutants M101 and M102 (each containing a deletion in the second domain of LFA-3) showed binding of mAbs 7A6 and TS2/9. Deletion of the first domain of LFA-3 (as in M100) abolished binding of those blocking anti-LFA-3 mAbs (Fig. 1 *b*). Each LFA-3 deletion mutant expressed in CHO cells, was evaluated for its binding to CD2 by its ability to rosette CD2⁽⁺⁾ Jurkat cells. Control CHO cells and CHO cells transfected with M100 cDNA (deletion of the first domain) did not bind Jurkat cells, as was evidenced by the lack of rosette formation. CHO cells transfected with wild-type LFA-3 or with LFA-3 that had either half of the second domain deleted (M101 and M102), rosetted Jurkat cells to the same extent (data not shown). This indicates that the presence of the first domain of LFA-3 is required for the interaction of CD2 with LFA-3.

The correct conformation of the first domain of LFA-3

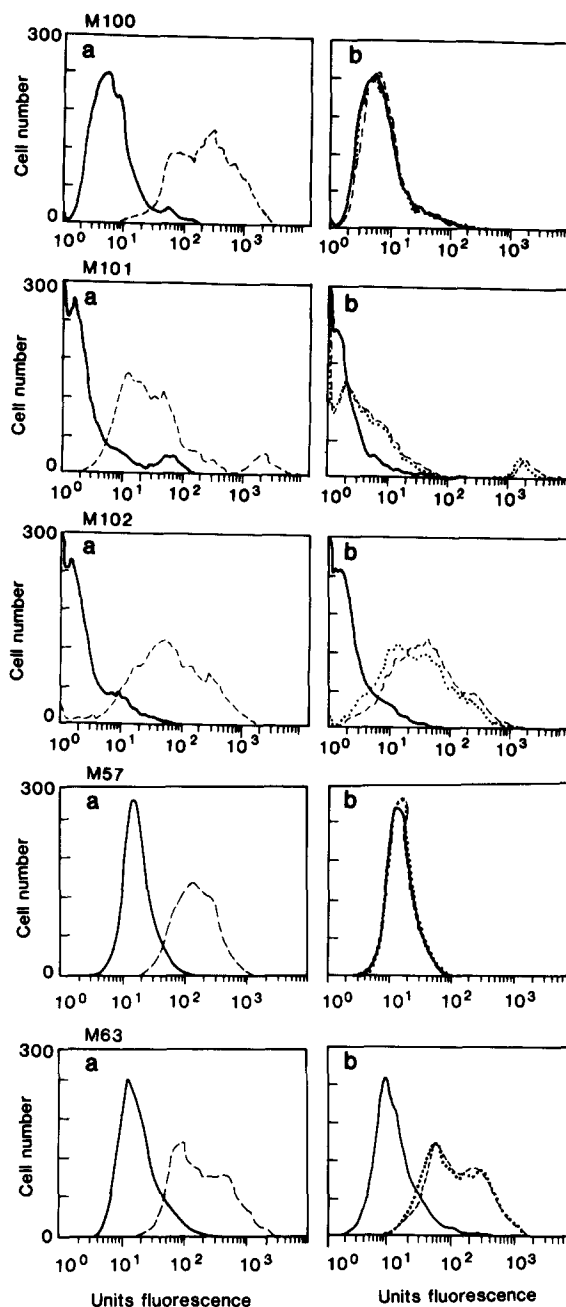


Figure 1. Anti-LFA-3 mAb binding analysis to various mutant forms of LFA-3. FACS[®] analysis of LFA-3 deletion mutants expressed in CHO cells. (Solid line) Background fluorescence of cells incubated with FITC-labeled secondary antibody. (a) Binding of rabbit polyclonal anti-LFA-3 antibody, Rb202, at a 1:100 dilution of immune serum, to demonstrate the LFA-3 expression levels (dashed line). Binding of Rb202 was detected with FITC-labeled goat anti-rabbit IgG. (b) Binding of anti-LFA-3 mAbs 7A6 (dotted line) and TS2/9 (dashed line) to the indicated cell lines, both at 10 μ g/ml. Primary antibody binding was detected with fluorescein-conjugated goat anti-mouse IgG antibodies. For each experiment, 5×10^5 cells were used.

is critical for the interaction of LFA-3 with CD2. This was suggested by the observation that a series of 10 amino acid deletions in the first domain all abolished Jurkat cell rosetting (data not shown), as well as binding of mAbs 7A6 and

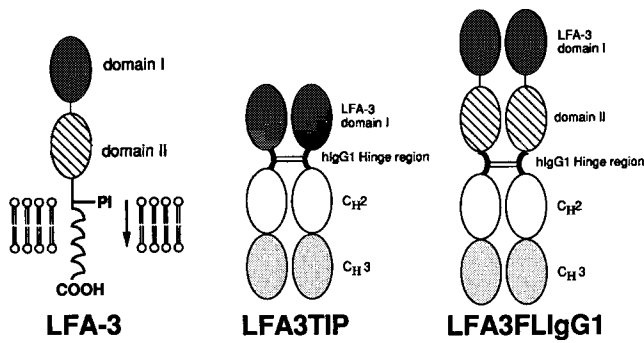


Figure 2. Schematic of the various LFA-3/IgG1 fusion proteins.

TS2/9 (a representative example, M57, which has residues 51 to 60 deleted, is shown in Fig. 1). Deletions in the second domain of LFA-3 had no effect on either mAb or Jurkat cell binding (mAb binding to one deletion, M63 with residues 111 to 120 deleted is shown in Fig. 2; rosetting data not shown).

The cDNA sequence coding for the first domain of LFA-3 (residues 1 to 92 of mature LFA-3) was fused to the human IgG1 hinge region, C_H2 and C_H3 sequences and expressed in CHO cells, where it was secreted as a dimeric molecule of 100–110 kD (a schematic is shown in Fig. 2). This fusion protein, LFA3TIP, was purified from culture medium and tested for its binding to CD2 by its ability to block hRBC or SRBC rosetting with CD2⁺ Jurkat cells. Fig. 3 shows the percent inhibition of RBC/Jurkat cell rosetting by LFA3TIP; purified mutant LFA3-M57IgG1; purified, multimeric, or monomeric LFA-3; as well as anti-CD2 or anti-LFA-3 mAbs. Rosetting of both, human and sheep RBCs with Jurkat cells is inhibited by anti-CD2 mAb TS2/18, which is specific for the LFA-3 binding epitope T11₁, and by anti-human LFA-3 mAb 7A6 (which crossreacts with sheep LFA-3). Purified PI-LFA-3 inhibits rosetting at a 300-fold lower concentration than does LFA3TIP (PI-LFA-3 inhibition data for SRBC not shown), whereas 10 times higher concentrations of monomeric LFA-3 than of LFA3TIP is required for the same IC₅₀ (Fig. 3 *a* for hRBC and *b* for SRBC). These differences in inhibitory activities between the various forms of LFA-3 are most likely due to their different

multivalent structure. We had observed previously (16) that the extent of Jurkat/RBC rosette inhibition by purified LFA-3 is directly proportional to the number of monomeric LFA-3 contained in its multimeric complex. Therefore, the level of inhibition by LFA3TIP is most likely a function of its multiple interactions with CD2, dictated by its dimeric structure. Inhibition of rosetting by LFA3TIP is due to its direct interaction with CD2 and not a function of its IgG1 sequences. This was demonstrated with control LFA-3/IgG1 fusion protein M57IgG1, which contains the same sequences as LFA3TIP except that residues 51 to 60 of LFA3TIP have been deleted. This deletion resulted in the loss of CD2 binding. M57IgG1 does not inhibit RBC rosetting (Fig. 3).

LFA3TIP but Not Purified LFA-3 Inhibits In Vitro T Cell Proliferation. Optimal activation of T cells in vitro has been shown to be strongly dependent on CD2/LFA-3 interactions, and cell surface or purified LFA-3 can transmit costimulatory T cell activation signals (1–3, 5, 22). We therefore tested the effect of purified LFA3TIP in several human in vitro T cell activation assays. In contrast with purified LFA-3, LFA3TIP inhibits specific antigen, mitogenic lectin PHA (PHA-P), and anti-CD3 mAb, OKT3 induced T cell proliferation, as well as allogeneic and xenogeneic MLRs.

Human PBLs were isolated on a Ficoll-Hypaque gradient and incubated with 0.1 μg/ml PHA-P in the presence or absence of LFA3TIP, purified multimeric LFA-3 (PI-LFA-3), or mAbs to CD2 and LFA-3. Addition of PI-LFA-3 to PHA-P activated PBLs causes a twofold increase in PBL proliferation over that seen with PHA-P activation alone. In contrast, LFA3TIP inhibits PHA-P activated PBL proliferation by 50%. Anti-CD2 mAb, TS2/18, and anti-LFA-3 mAb 1E6, both of which block CD2/LFA-3 interactions, inhibit the PHA-P stimulated proliferation by >90% (Fig. 4 *a*).

LFA3TIP is also capable of inhibiting proliferation of memory T cells in vitro in response to recall antigens. PBLs from donors that had previously been vaccinated with tetanus toxoid (TT) or hepatitis B surface antigen (HBsAg), were stimulated in vitro with TT or HBsAg. T cell proliferation to these antigens was inhibited by anti-LFA-3 mAb TS2/9 (data not shown), anti-CD2 mAb TS2/18, and LFA3TIP (Fig. 4 *b*). LFA3TIP and mAbs inhibit T cell proliferation in response to TT by 70% and to HBsAg by 85%.

As observed in the PHA-P stimulated T cell response,

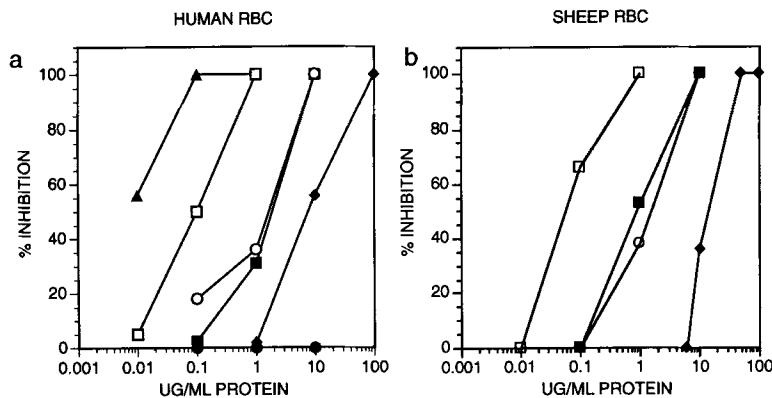


Figure 3. Inhibition of Jurkat/RBC rosetting by LFA-3 proteins and mAbs. (*a*) Human RBCs/Jurkat rosetting; (*b*) SRBC/Jurkat rosetting in the presence of M57IgG1 (●); monomeric LFA-3 (◆); LFA3TIP (■); anti-LFA-3 mAb (○); anti-CD2 mAb TS2/18 (□); PI-LFA-3 (▲) for hRBCs only. Protein concentrations in μg/ml.

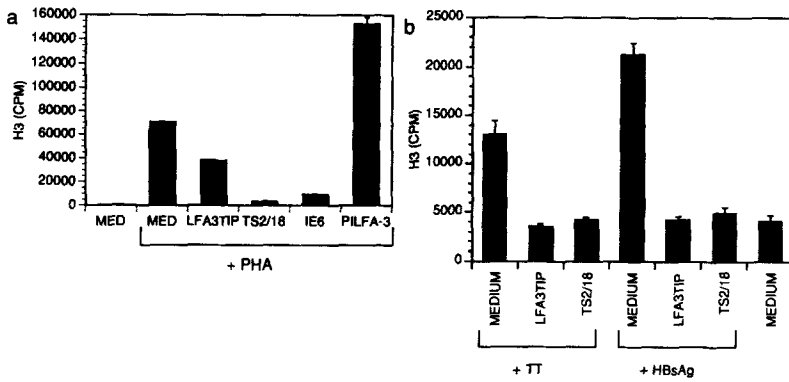


Figure 4. Effect of antibodies, PI-LFA-3, LFA3TIP2 on mitogen or specific antigen-induced T cell proliferation. (a) PHA-induced T cell proliferation. Human PBLs were incubated with 0.1 $\mu\text{g}/\text{ml}$ PHA-P in the presence or absence of LFA3TIP, PI-LFA-3, anti-CD2 mAb TS2/18, or anti-LFA-3 mAb 1E6 (all at 5 $\mu\text{g}/\text{ml}$). Incubation was carried out for 3 d and T cell proliferation determined by incorporation of [^3H]thymidine for 12 h. (b) T cell responses to TT and HBsAg. Inhibition by LFA3TIP and TS2/18 (both at 2 $\mu\text{g}/\text{ml}$) of T cell proliferation in response to TT and HBsAg. PBLs (10^5) from previously vaccinated donors were incubated with the indicated amount of antigen for 5 d, after which they were pulsed with 1 μCi [^3H]thymidine for 12 h. TT, 2 $\mu\text{g}/\text{ml}$; HBsAg, 2 $\mu\text{g}/\text{ml}$.

LFA3TIP and purified LFA-3 also have different effects on allogeneic MLR. Fig. 5 shows the effect of LFA3TIP, anti-CD2, or anti-LFA-3 mAbs, purified LFA-3, and a series of control fusion proteins on allogeneic MLR. Data was converted to percent inhibition, which allowed us to present the average results obtained in a series of separate MLRs. mAbs to LFA-3 or CD2, as well as LFA3TIP, inhibited T cell responses between 70 and 92%. Purified PI-LFA-3 or monomeric LFA-3 had no effect on T cell proliferation. Using FACS[®] analysis, we demonstrated that FITC-labeled PI-LFA-3 binds to CD2 on human PBLs during MLR (Wallner, B., and A. Frey, unpublished observation). The lack of inhibition is therefore not due to the lack of PI-LFA-3 binding. To demonstrate that the inhibitory activity of LFA3TIP is a function of its direct interaction with CD2 and is not due to its engagement of FcR on APCs, two IgG1 fusion proteins that do not bind to CD2 (M57IgG1 and CD4IgG1), as well as purified human IgG, were used as controls. CD4-IgG1 is a fusion protein consisting of the first 180 residues of CD4 attached to the same IgG1 sequences as LFA3TIP. CD4IgG1 and M57IgG1 were expressed in CHO cells and purified by the same procedure as LFA3TIP. These proteins had no effect on T cell proliferation in MLR (Fig. 5). M57IgG1 or purified human IgG1 added in excess to MLR cultures which also contained LFA3TIP had no effect on the inhibition of MLR by LFA3TIP (data not shown). As a further control, we subjected culture medium from mock-transfected

CHO cells to the same purification procedure as LFA3TIP containing culture medium. This preparation had no effect on MLR.

LFA3TIP Induces T Cell Unresponsiveness to Repeated Stimulus. In contrast with anti-CD2 mAb TS2/18, LFA3TIP is capable of inducing sustained T cell unresponsiveness in an allogeneic MLR. Allogeneic PBLs were incubated in the absence or presence of LFA3TIP or mAb TS2/18 for 2 d, PBLs were then washed to remove LFA3TIP or TS2/18 (removal was demonstrated by FACS[®] analysis as described in Materials and Methods; data not shown), and responders were restimulated with the initial stimulator cells for another 5 d in the absence of LFA3TIP or TS2/18 (in Fig. 6 a, washed). As a control, MLR cultures were incubated for 5 d in the presence of either LFA3TIP or TS2/18 (Fig. 6 a, unwashed). Both LFA3TIP and TS2/18 inhibited T cell proliferation in these cultures. In the washed cultures, which had originally been incubated with TS2/18, T cells regained their responsiveness to the stimulator cells. The extent of T cell proliferation was similar to that of the medium control of the washed cultures. However, cells originally inhibited by LFA3TIP remained unresponsive to the secondary stimulus throughout the incubation period.

The T cell unresponsiveness induced by LFA3TIP is not due to a general antiproliferative effect of LFA3TIP. Fig. 6 b shows an allogeneic MLR where PBLs were incubated with or without LFA3TIP for 5 d. LFA3TIP inhibited this MLR

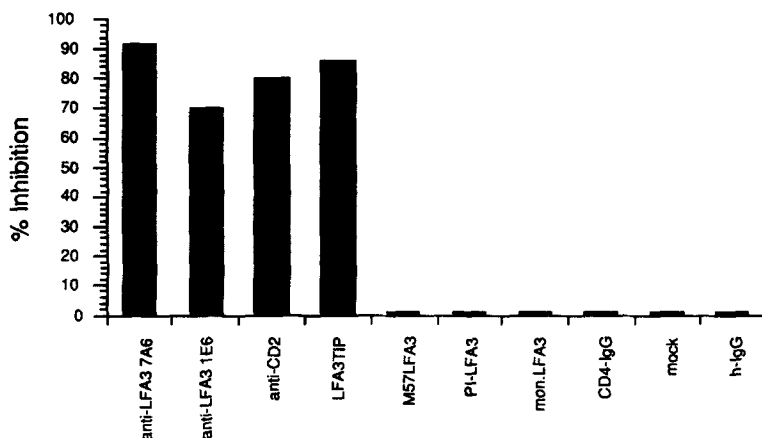


Figure 5. Effect of various forms of LFA-3 proteins or mAbs to CD2 or LFA-3 on allogeneic MLR. Responder PBLs (10^5 cells) were stimulated with irradiated allogeneic PBLs at a ratio of 1:1 for 5 d in the presence or absence of the indicated proteins: anti-LFA-3 mAbs 7A6 and 1E6; anti-CD2 mAb TS2/18; LFA3TIP; M57LFA-3 (a M57/IgG1 fusion protein); multimeric (PI-LFA3) and monomeric (mon. LFA-3) LFA-3; CD4/IgG1 fusion protein, CD4-IgG (all at 1 $\mu\text{g}/\text{ml}$); buffer control (mock) which was obtained by subjecting CHO culture medium to the same purification procedure as LFA3TIP; and purified human IgG at 1 $\mu\text{g}/\text{ml}$ (h-IgG). Percent inhibition is that over medium control.

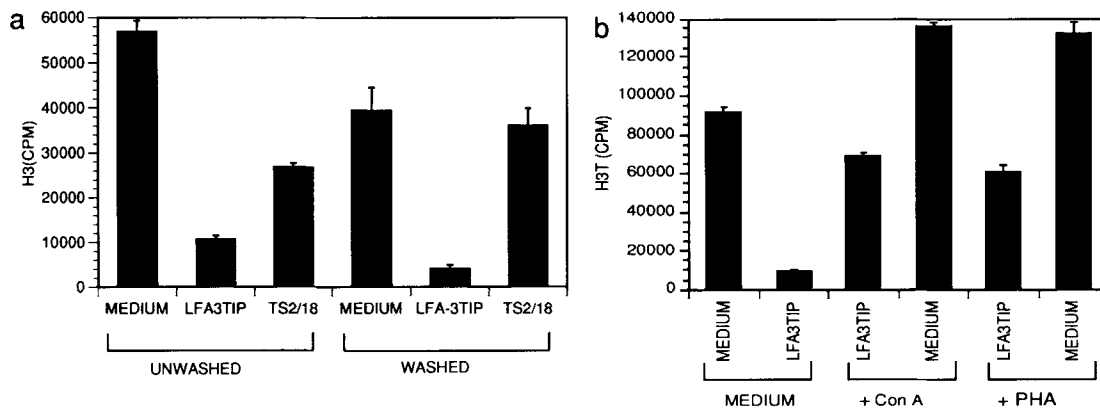


Figure 6. Induction of unresponsiveness by LFA3TIP to secondary stimuli in allogeneic MLR. (a) (*Unwashed*) Responder PBLs (10^5) were incubated with allogeneic stimulator cells at a ratio of 1:1 in the presence of LFA3TIP or TS2/18 (both at $1.5 \mu\text{g/ml}$) for 2 d, restimulated with fresh stimulator cells, and continued to be incubated for 5 d. (*Washed*) Incubations were for 2 d in the presence of LFA3TIP or TS2/18. Cultures were washed on day 2, fresh stimulator cells were added, and incubations were continued for 5 d in the absence of LFA3TIP or TS2/18. All cultures were pulsed with $1 \mu\text{Ci}$ /well, [^3H]thymidine for 12 h and harvested. (b) T cells remain responsive to PHA-P or Con A stimulus after inhibition by LFA3TIP. Responder PBLs (10^5 cells/well) were incubated with irradiated allogeneic stimulator PBLs (10^5) in the presence or absence of LFA3TIP for 5 d. At that time, Con A (at $2 \mu\text{g/ml}$) or PHA-P (at $1 \mu\text{g/ml}$) were added to cultures indicated, and incubation was continued for 2 d. Cultures were pulsed with $1 \mu\text{Ci}$ [^3H]thymidine overnight and harvested.

by 90%. After 5 d, either Con A or PHA-P were added to cultures containing LFA3TIP or medium alone for 2 d. Both PHA-P and Con A augmented T cell proliferation, even in the presence of LFA3TIP. The extent of T cell proliferation in response to PHA-P and Con A in cultures containing LFA3TIP is less than that in the medium control. This result is consistent with our previous finding that LFA3TIP inhibits PHA-P-induced T cell proliferation. Thus T cells are still responsive to mitogenic activation, indicating that LFA3TIP does not inhibit responses by rendering T cells resistant to activation in general. Furthermore, LFA3TIP added to cultures of T or B tumor cell lines did not inhibit their proliferation (data not shown).

The Mechanism by which LFA3TIP Inhibits T Cell Responses Is Different from that of mAbs. Although, both LFA3TIP and mAbs to CD2 or LFA-3 showed similar inhibitory activity of RBC/Jurkat cell rosetting, for PHA-P-induced T cell proliferation, and for primary allogeneic MLR, the ability of LFA3TIP to induce T cell unresponsiveness to secondary stimulus in allogeneic MLR indicated that its mechanisms of inhibition are different from those of mAbs. The following

experiments were conducted to obtain further evidence of its different mechanism.

Optimal stimulation of T cell responses with anti-CD3 mAbs has been shown to depend on the experimental setup and on the specific anti-CD3 mAbs used (23, 24). Here we compared the effect of LFA3TIP in solution on soluble OKT3-stimulated PBL proliferation, with the effect of anti-CD2 and anti-LFA3 mAbs. Under our experimental conditions, neither anti-CD2 mAbs, TS2/18 (Fig. 7, a and b) nor T11₁ (data not shown), nor anti-LFA-3 mAb 1E6 (Fig. 7 a) affected T cell proliferation even when added at high concentrations. In contrast, LFA3TIP inhibited the OKT3-induced T cell proliferation in a dose-dependent manner (Fig. 7 b) achieving 85% inhibition at $1 \mu\text{g/ml}$ (see Fig. 9 a). To exclude the possibility that the inhibition of the OKT3 induced T cell response by LFA3TIP is solely due to the engagement of FcRs on accessory cells by the IgG1 portion of LFA3TIP, purified human IgG was used as a control. Even at high concentrations ($10 \mu\text{g/ml}$) human IgG had no effect on OKT3-stimulated T cell proliferation (Fig. 7 b).

The difference in the inhibitory mechanisms of LFA3TIP

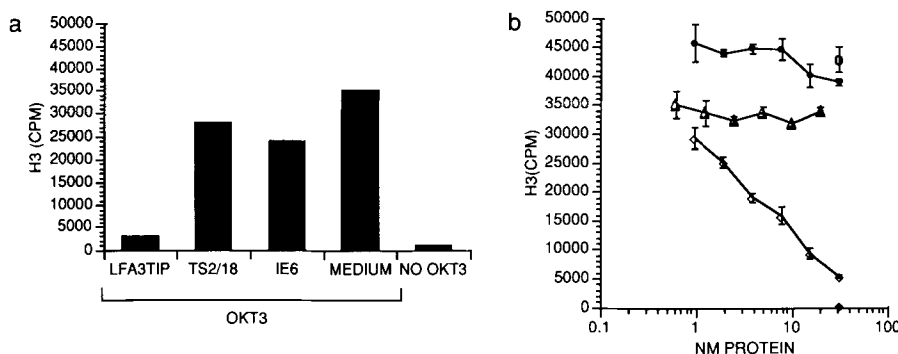


Figure 7. Effect of LFA3TIP on OKT3-induced T cell responses. (a) T cell proliferation in response to OKT3. PBLs (10^5 per well) were incubated with OKT3 (3 ng/ml) in the presence or absence of LFA3TIP ($1 \mu\text{g/ml}$), anti-CD2 mAb TS2/18, or anti-LFA-3 mAb 1E6 (both at $1 \mu\text{g/ml}$) as indicated for 3 d. On day 3, $1 \mu\text{Ci}$ [^3H]thymidine was added to each incubation for 12 h before cells were harvested. (b) Inhibition of OKT3 (3 ng/ml) stimulated T cell proliferation by increasing concentrations of LFA3TIP (\diamond), anti-CD2 TS2/18 (\bullet), purified human IgG1 (Δ), and OKT3 alone (\circ). Assay conditions were exactly as described in (a).

and mAbs was further demonstrated under conditions where T cell activation occurs independently of LFA-3/CD2 interactions. In a xenogeneic MLR, where human PBLs are stimulated with irradiated murine A20 cells (B lymphoma cell line), T cell proliferation is not dependent on the interaction of human CD2 with the mouse equivalent of LFA-3. In this system, as can be expected, neither anti-LFA-3 (which does not bind to murine A20 cells) nor anti-CD2 (which binds to human PBLs) mAbs reduce the response to A20 cells (Fig. 8 *a*; anti-LFA-3, data not shown). However, LFA3TIP inhibits this xenogeneic MLR by 80% when added on day 0 of cultures. When JY cells (human B lymphoma cell line) are used as stimulator cells in allogeneic MLR, the activation of T cell proliferation is in part facilitated by the interaction of LFA-3 with CD2. Therefore, anti-LFA-3 mAbs 1E6 or TS2/9 (data not shown) and anti-CD2 mAb TS2/18 inhibit T cell proliferation in response to JY cells. LFA3TIP also inhibits this response to the same extent as mAbs (Fig. 8 *b*). mAbs to LFA-3 (data not shown) or to CD2 are only inhibitory when added on day 0 or 1 of incubation (Fig. 8 *b*). Later additions have no effect on T cell proliferation. LFA3TIP, on the other hand, is capable of inhibiting both the allogeneic and xenogeneic MLR, even when added up to 4 d after the MLR cultures were started.

LFA3TIP Does Not Inhibit T Cell Proliferation Stimulated Directly through CD2. In all the experiments described above, LFA3TIP had an inhibitory effect on T cell responses if the stimulus to T cells occurred in part through TCR engagement. However, when T cells were stimulated to proliferate by direct interaction with the CD2 receptor by a combination of activating anti-CD2 mAbs, LFA3TIP showed no effect

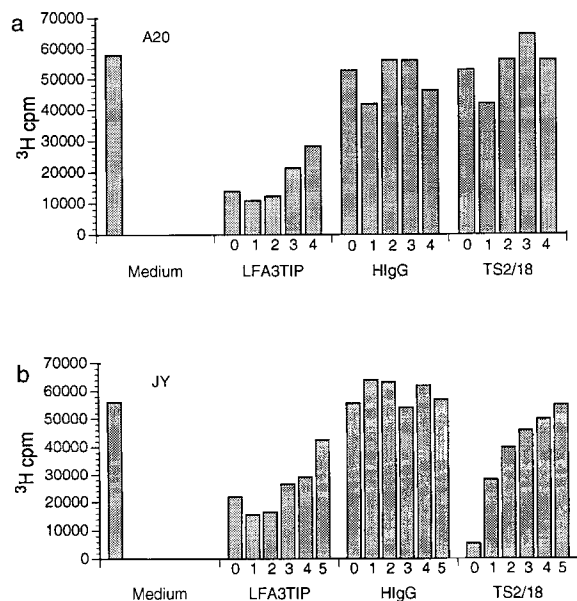


Figure 8. Inhibition of allogeneic and xenogeneic MLR by LFA3TIP and anti-CD2 mAb TS2/18. Effect of delayed additions of TS2/18, purified human IgG (HlgG), and LFA3TIP (all at 5 $\mu\text{g}/\text{ml}$) on: (a) xenogeneic MLR (A20) and (b) allogeneic MLR (JY). Numbers under each bar indicate day of addition of the respective proteins. Day 0 is the first day of MLR. MLR assay conditions were as described in Materials and Methods.

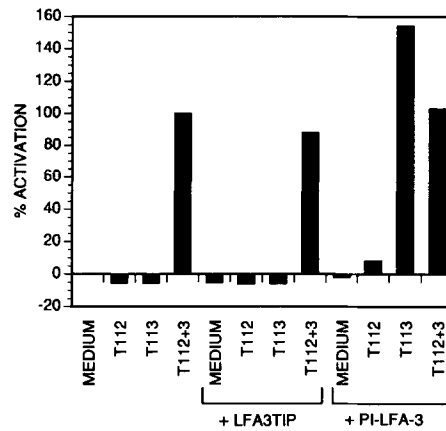


Figure 9. Percent activation of T cell proliferation in response to activating anti-CD2 mAbs. 10^5 PBLs were incubated with anti-CD2 mAbs as indicated (1:100 dilution of ascites), alone, or in combination with LFA3TIP (1 $\mu\text{g}/\text{ml}$) or PI-LFA-3 (1 $\mu\text{g}/\text{ml}$) for 3 d. T cell proliferation was detected by incorporation of [^3H]thymidine (1 $\mu\text{Ci}/\text{well}$ for 12 h). Base value is that of T cell proliferation in medium alone.

on T cell proliferation. T cells can be optimally stimulated by a combination of anti-CD2 mAbs T11₂ and T11₃ (1; and Fig. 9). Purified multimeric LFA-3 (PI-LFA-3) effectively replaces mAb T11₂ in the augmentation of T cell proliferation with mAb T11₃, but has no additional stimulatory effect on T cell proliferation in the presence of both mAbs. Even though LFA3TIP binds to the same CD2 epitope as PI-LFA-3, its binding does not costimulate T cell proliferation elicited by either mAb T11₂ or mAb T11₃ (Fig. 9). When added to cultures that had been stimulated by the combination of mAbs T11₂ plus T11₃, LFA3TIP has neither a stimulatory nor an inhibitory effect on T cell proliferation. This lack of inhibition by LFA3TIP is not due to its competition by mAbs T11₂ or T11₃ for CD2 binding sites. We demonstrated this by binding analyses shown in Fig. 10. We further confirmed by FACS[®] analysis, that LFA3TIP is bound to T cells during the antibody stimulation in these assays (data not shown).

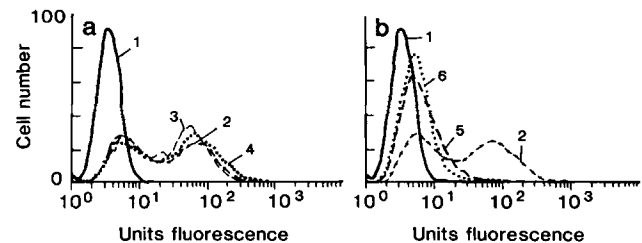


Figure 10. FACS[®] analysis of LFA3TIP and mAb binding competition to CD2⁺ Jurkat cells. (Tracings 1) PE-labeled goat anti-human IgG antibody (secondary antibody control) at 17 $\mu\text{g}/\text{ml}$; (tracings 2) binding of LFA3TIP in the absence of anti-CD2 mAbs; (tracings 3–6) binding of LFA3TIP in the presence of anti-CD2 mAbs T11₂ (3), T11₃ (4), T11₁ (5), or TS2/18 (6). mAbs T11₁, T11₂, T11₃ were at a 1:100 dilution of ascites; mAb TS2/18 at 10 $\mu\text{g}/\text{ml}$. LFA3TIP was present at 10 $\mu\text{g}/\text{ml}$. Units fluorescence are arbitrary units.

LFA3TIP Binds to the Same CD2 Epitopes as a Full-Length LFA-3/IgG1 Fusion and Cell Surface LFA-3. Since the effect of LFA3TIP on T cell activation was different from that of purified full-length LFA-3 and, in several assays, different from the effect of anti-CD2 mAbs, it was of interest to determine whether these differences were a function of the one domain structure of LFA3TIP. We therefore constructed an LFA-3/IgG1 fusion protein (LFA3FLIgG1; Fig. 2), that contained both extracellular domains of LFA-3, and compared its CD2 binding properties with those of LFA3TIP and mAbs to CD2.

It had previously been established that the interaction of CD2 with purified LFA-3 or LFA-3 expressed on human RBCs, can be blocked with anti-CD2 mAbs specific for the T11₁ epitope (mAbs T11₁ or TS2/18). Binding competition analysis of LFA3TIP with these blocking anti-CD2 mAbs to CD2 on Jurkat cells or on human PBLs indicated that

LFA3TIP binds to the same CD2 epitope as T11₁ and TS2/18. For this analysis, Jurkat cells were incubated with LFA3TIP in the absence (Fig. 10, *a* and *b*, tracing 2) or in the presence of anti-CD2 mAbs to specific CD2 epitopes (Fig. 10, *a* and *b*, tracings 3–6). LFA3TIP or LFA3FLIgG1 binding was detected by PE-conjugated anti-human IgG antibodies. Loss of PE-cell surface fluorescence in the presence of anti-CD2 mAbs was interpreted as loss of LFA3TIP binding. Coincubation of Jurkat cells with LFA3TIP and either anti-CD2 mAb TS2/18 or T11₁, both of which are specific for the LFA-3 binding epitope on CD2, reduced PE-surface fluorescence to almost background levels (Fig. 10 *b*, tracings 5 and 6). This indicated that these mAbs compete for the LFA3TIP binding site on CD2. On the other hand, mAbs to the CD2 epitope T11₂, located in the first domain, and to the epitope T11₃, located in the second domain (mAbs T11₂ and T11₃,

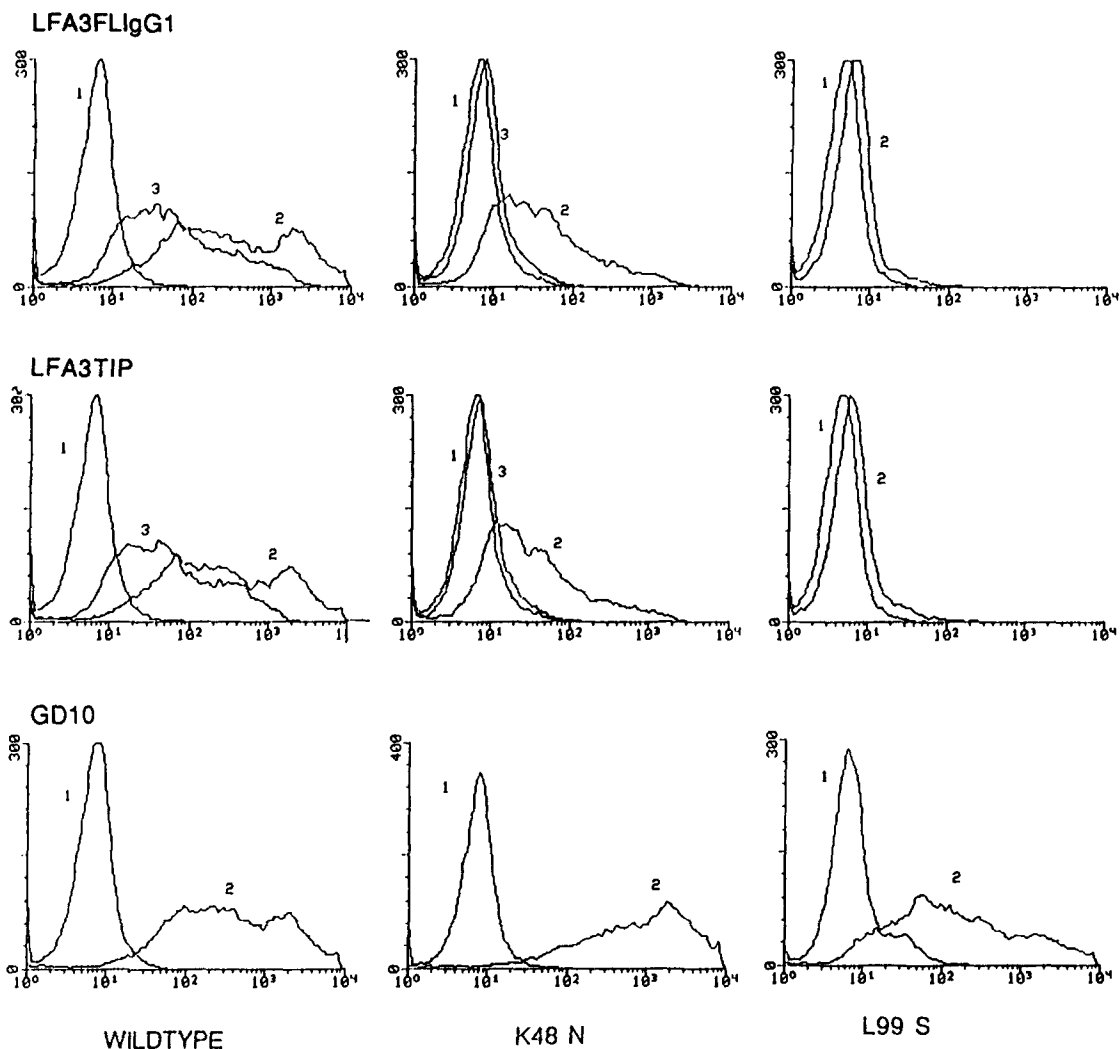


Figure 11. mAb and LFA3TIP binding analysis to mutant CD2 expressing COS cells. COS cells transfected with the wild-type or mutant CD2 cDNAs (K48N; L99S) were incubated with either mAb GD10, to demonstrate expression levels of CD2, or with LFA3TIP or LFA3FLIgG1. Binding of the IgG1 fusion proteins was detected by PE-conjugated goat anti-human IgG and binding of GD10 with FITC-conjugated goat anti-murine IgG. (Tracing 1) In all graphs represents background fluorescence. (Tracings 2) In each group, represent the fluorescence intensity obtained when cells were incubated with 10 $\mu\text{g}/\text{ml}$ LFA3TIP, LFA3FLIgG1, or mAb GD10. (Tracing 3) Binding of LFA3TIP or LFA3FLIgG1 at 1 $\mu\text{g}/\text{ml}$. (*y*-axes) Number of cells; (*x*-axes) arbitrary units of fluorescence.

respectively) did not compete with LFA3TIP binding (Fig. 10 *a*, tracings 3 and 4, respectively). To confirm that mAbs T11₂ and T11₃ bind to Jurkat cells in the presence of LFA3TIP, a two-color FACS[®] analysis was performed. Anti-CD2 mAbs were detected with FITC-conjugated anti-mouse IgG and LFA3TIP with PE-labeled anti-human IgG. Both mAbs and LFA3TIP are able to bind simultaneously to CD2 (data not shown), thus indicating distinct binding epitopes. The same binding properties were determined for LFA3-FLIgG1 (data not shown).

To fine-map the LFA3TIP binding epitope on human CD2, a series of CD2 variants with single amino acid substitutions either in epitope I, II, or III, as well as wild-type CD2, were expressed in COS-7 cells and used for binding analysis of LFA3TIP. Peterson and Seed (18) had developed these CD2 mutants to map the LFA-3 binding epitopes on CD2. We monitored expression levels of mutant or wild-type CD2 on the cell surface of CD2-transfected COS cells with anti-CD2 mAb GD10, which is specific to a newly defined CD2 epitope not involved in the LFA-3/CD2 interactions (Miller, G., and D. Hess, unpublished observations; binding data shown in Fig. 11, bottom row). Individual substitutions of CD2 residues in the T11₁ epitope (region II, domain 1) (L99 to S, K87 to Y, Y91 to Q, and Q95 to E), abolished LFA3TIP or LFA3FLIgG1 binding to these mutants even when added at high concentrations (10 $\mu\text{g/ml}$). Binding data for one of these mutants (L99 to S) is shown in Fig. 11, right column. Binding of mAb T11₁ to all of these mutants and binding of TS2/18 to all but substitution Q95E was also eliminated (data not shown). Binding of mAb T11₂ (specific for region I, domain 1) and mAb T11₃ (specific for region III in domain 2) to these T11₁ epitope mutants was not affected (data not shown). CD2 mutants in region I (T11₂ epitope) bound LFA3TIP and LFA3FLIgG1 with much lower affinity than wild-type CD2. These mutants had the following single amino acid substitutions: K48 to N, K48 to M, K46 to N, A50 to E, and Q51 to L. Only at high concentrations (10 $\mu\text{g/ml}$) and not at 1 $\mu\text{g/ml}$ was binding of LFA3TIP detectable (data for substitution K48N shown in Fig. 11, middle column, tracing 2 for 10 $\mu\text{g/ml}$ and tracing 3 for 1 $\mu\text{g/ml}$). Binding patterns for purified FITC-labeled PI-LFA-3 to all mutants tested were identical to that for LFA3TIP (data not shown).

Discussion

By deletion analyses of the extracellular region of LFA-3, we localized the CD2 binding site to the first domain of LFA-3. The extracellular regions of both CD2 and LFA-3 consist of two Ig-like domains and their primary and secondary structures show a high degree of homology. The structural similarities between this ligand/receptor pair are emphasized by our finding that the mutual interactions occur through their respective first domains. Using this binding information, we constructed an LFA-3 domain 1/IgG1 fusion protein and examined its effect on T cell responses.

Results described here suggest that under specific conditions, interactions between CD2 and LFA-3 can represent a potential negative regulatory mechanism for T cell responses.

The LFA-3/IgG1 fusion protein, LFA3TIP, is capable of inhibiting T cell responses in a series of *in vitro* T cell activation assays. LFA3TIP inhibited allogeneic and xenogeneic MLR, TT, HBsAg, and mAb OKT3-induced T cell responses, as well as T cell activation by PHA-P and Con A. The mechanism by which LFA3TIP inhibits T cells is not clear, but it appears to be distinct from that of mAbs to CD2 or LFA-3, which block CD2/LFA-3 interactions and cellular adhesion. In contrast with these mAbs, LFA3TIP can interfere with T cell activation in situations where CD2/LFA-3 interactions are not required. This was best demonstrated in a xenogeneic MLR where human PBLs proliferate in response to murine A20 cells. Costimulatory signals in this MLR are supplied by interactions of accessory cell surface antigens other than human CD2 and the murine LFA-3 analogue. mAbs to human CD2 and LFA-3 which block human allogeneic MLR therefore have no effect on T cell proliferation in response to A20 cells in these xenogeneic MLR responses. LFA3TIP, however, interacts with human T cells in such a manner that subsequent T cell activation signals by A20 cells have no effect. This inhibition by LFA3TIP, but not by mAbs to human CD2 or LFA-3, was even observed when LFA3TIP was added up to 4 d after the initial T cell stimulus in allogeneic MLR, which indicates that LFA3TIP is capable of inhibiting proliferation of already partially or even fully activated T cells.

In all T cell activation assays described here, LFA3TIP exhibited its effect only when T cell activation was, at least in part, elicited through simultaneous TCR/CD3 complex activation. T cell activation through direct stimulation of the CD2 molecule by a combination of activating anti-CD2 mAbs was not affected by LFA3TIP. A potential mechanism by which LFA3TIP could inhibit T cell activation is through the inhibition of CD2 conformational changes. It has been suggested that binding of the anti-CD2 mAb T11₂ to CD2 induces a conformational change of CD2 so that the second activation epitope T11₃ is upregulated (5). Furthermore, an anti-CD3 mAb S34, upon binding to CD3, has been reported to induce a similar conformational change of CD2, transforming it to its activated state (23). It can therefore be hypothesized that during the initial interactions between T cells and APCs, which are facilitated by the TCR-CD3 complex and the MHC-Ag complex, upregulation of the T11₃ epitope occurs. Subsequent interactions of CD2 with LFA-3 on the APC then transduces an activation signal to T cells. Binding of LFA3TIP to CD2 during these initial interactions potentially inhibits these conformational changes and thus prevents the second signal. In the *in vitro* assays described here, this inhibition is apparently overcome by the simultaneous presence of mAbs T11₂ and T11₃. However, several data described in this manuscript do not fully support this theory of interference by LFA3TIP with conformational changes. For example, the observation that LFA3TIP, but not mAbs to LFA-3 or CD2 can inhibit T cell proliferation even when added to cultures up to 4 d after the initial T cell stimulation has occurred, together with the observation that LFA3TIP can inhibit xenogeneic MLR responses, is an indication that LFA3TIP transduces a negative signal to T cells, independent of early LFA-3/CD2 interactions. Furthermore the

finding that LFA3TIP, but not blocking mAbs, can induce lasting T cell unresponsiveness to repeated stimuli, suggests a mechanism other than interference with the initial cellular interactions.

Clearly, the mechanism of inhibition by LFA3TIP is not merely a disruption of cellular adhesion, and thus is different from that of blocking mAbs to LFA-3 and CD2. This difference was demonstrated not only by the effect of LFA3TIP on xenogeneic MLR and by its induction of T cell anergy, but also by its inhibition of OKT3-induced T cell proliferation. Activation of T cell proliferation *in vitro* by mAbs specific for the CD3 complex are strongly dependent on the experimental design and on the anti-CD3 mAbs used. The effect of anti-CD2 mAbs on anti-CD3 mAb-induced T cell activation has been variable (23, 24). In our experimental setup stimulation of T cell proliferation by soluble anti-CD3 mAb, OKT3 was inhibited by LFA3TIP, but neither anti-CD2 nor anti-LFA-3 mAbs were able to inhibit this activation. It has been demonstrated by a number of different laboratories that the signal transduction events elicited through the TCR-CD3 complex and through CD2 are identical and functionally interact to regulate antigen-dependent T cell responses (9, 25, 26). CD2 is also functionally associated with other accessory cell-dependent T cell activation pathways. Increased pp56^{lck} tyrosine kinase activity has been reported in CD2-stimulated cells (27, 28) and the CD45-associated phosphatase activity has been suggested as a potential regulatory mechanism for CD2-induced phosphotyrosine kinase activity (29, 30). It is therefore likely that LFA3TIP through its binding to CD2, under conditions where TCR/CD3 occupation occurs, causes interference with the second T cell activation pathway required for optimal T cell responses. The inhibitory effect of LFA3TIP on PHA-P-induced T cell proliferation is supportive of this mechanism. Stimulation of PBLs by lectins such as PHA-P or Con A has been attributed to the involvement of the TCR/CD3 pathway and accessory cell-dependent pathways such as CD2 or CD45. It has been suggested that PHA-P directly binds to CD2 (31-33). In the presence of PHA-P, purified multimeric LFA-3 can replace accessory cell functions in the augmentation of T cell activation without a second ligand to CD2 (33, 34). LFA3TIP on the other hand inhibits, rather than costimulates PHA-P-induced T cell proliferation. Since LFA3TIP interacts with the same epitopes as PI-LFA-3, inhibition of PHA-P-induced T cell activation is most likely not due to competition between PHA-P and LFA3TIP for binding sites on CD2.

In all T cell activation assays described here, purified full-length PI-LFA-3 had a different effect on T cell responses than LFA3TIP. It had no effect on MLR or OKT3-induced T cell proliferation, and it augmented rather than inhibited PHA-P or mAb T11₂ stimulated T cell proliferation. Because of these dramatic differences between LFA3TIP and various other forms of LFA-3 proteins, it was of importance to determine whether LFA3TIP's inhibitory activity stemmed from the fact that it contained only one domain. In addition, Damle et al. (35) have recently reported that an immobilized two domain LFA-3/IgG1 fusion protein augmented anti-TCR-1 or OKT3-induced CD4⁺ T cell activation. We have

not examined the effect of LFA3TIP on the costimulation with these mAbs, but have found that LFA3TIP, when immobilized on plastic in combination with activating anti-CD2 mAbs, does not augment PBL proliferation (data not shown). Again, the difference in response obtained with these two LFA-3/IgG1 fusion proteins could be due to the fact that LFA3TIP contains only one domain of LFA-3, or it could be a function of the specific costimulatory mAbs used. We therefore constructed a two domain LFA-3/IgG1 fusion protein (LFA3FLIgG1) and found that this protein had identical MLR inhibitory properties and binding profile as LFA3TIP. The CD2 binding epitopes of all forms of purified LFA-3 (PI-LFA-3, LFA3FLIgG1, and LFA3TIP) were found to be identical in mAb competition assays, as well as in binding analysis to CD2 site-specific mutants. CD2 binding epitopes determined for LFA3TIP are the same as those published by Peterson and Seed (18) for LFA-3 expressed on the surface of human RBCs. We therefore cannot attribute the difference in activities of these proteins to different interactions with CD2.

So far, we cannot exclude the possibility that engagement of FcRs on accessory antigen-presenting or T cells with the IgG1 portion of LFA3TIP is a contributing factor to the activity of LFA3TIP. We have used control fusion proteins to show that engagement of FcR by itself is not responsible for T cell inhibition by LFA3TIP. Human IgG1 fusion proteins that do not bind to CD2 as well as purified human IgG have no effect on any T cell responses examined here and do not diminish LFA3TIP's inhibitory effect when added simultaneously to T cell activation assays. However, we cannot yet exclude the possibility that LFA3TIP, through its simultaneous interactions with CD2 on T cells and with FcRs on APCs (through its IgG1 portion), aligns both cells into a configuration that is either unfavorable for the transduction of the second positive T cell activation signal or is favorable for a negative signal either directly to T cells or through the induction of regulatory cytokines. The lack of inhibition by LFA3TIP of anti-CD2 antibody-induced T cell proliferation argues against a mechanism that relies solely on cross-bridging of T cells with their cognate partners or on an immobilization of CD2 polarization that occurs upon cellular interactions (7). Rather, our data described here suggest that LFA3TIP interferes with an event that functionally links the TCR/CD3 and the CD2 T cell activation pathways. Induction of a negative signal to T cells by anti-CD2 mAbs had been suggested before by Ohno et al. (11), as well as by Palacios and Martinez-Maza (10). However, experiments described by these authors do not unequivocally demonstrate that the inhibition of T cell responses is indeed independent of the interruption of CD2/LFA-3 binding.

The mechanism by which LFA3TIP inhibits T cell responses is under investigation. A LFA-3/IgG1 fusion protein, which lacks the functional FcR binding sequences has been produced and is currently being used to determine the contribution of the FcR engagement in the inhibition by LFA3TIP. The LFA3TIP activity is clearly different from that of purified LFA-3 and of mAbs to CD2 or LFA-3. Inhibition by LFA3TIP is not merely dependent on blocking cellular adhesion, but

occurs through a signal to T cells which renders them unresponsive to activation through the TCR-CD3 complex. The unique properties of LFA3TIP are being exploited to

induce antigen-specific anergy in organ transplants and autoimmune diseases.

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Address correspondence to Dr. Barbara P. Wallner, Immulogic Pharmaceutical Corp., 610 Lincoln Street, Waltham, MA 02154. Dr. Margaret D. Rosa is currently at BioTransplant, Inc., Bldg. 96, 13th Street, Charlestown Navy Yard, Charlestown, MA 02129. Glenn T. Miller is currently at Immulogic Pharmaceutical Corp.

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