

Contribution of Lymphocyte Function-associated-1/ Intercellular Adhesion Molecule-1 Binding to the Adhesion/Signaling Cascade of Cytotoxic T Lymphocyte Activation

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Summary

A rapid induction of adhesion to immobilized intercellular adhesion molecule (ICAM)-1 occurs when cytotoxic T lymphocytes (CTL) are stimulated with either soluble anti-T cell receptor (TCR) monoclonal antibodies (mAb) or with immobilized alloantigen, and this binding is blocked by the addition of anti-lymphocyte function-associated (LFA)-1 mAbs. Requirements for activating LFA-1 adhesion to ICAM-1 are similar to those found for induction of binding to immobilized fibronectin (FN), but distinct from those for activating CD8-mediated adhesion to class I major histocompatibility complex. A distinct role for LFA-1 in co-signaling for TCR-dependent degranulation could not be demonstrated. In contrast, both CD8 and the FN-binding integrin provide costimulatory signals for this response. Thus, if co-signaling via LFA-1 occurs, it clearly differs from that provided by CD8 or the FN-binding integrin. On the basis of antibody blocking effects, alloantigen-dependent activation of adhesion to ICAM-1 involves both the TCR and CD8. These results support a view of CTL activation as a cascade of adhesion and signaling events, with different coreceptors making distinct contributions.

Activation of CTLs involves not only the recognition of antigen via the TCR but also interaction of accessory receptors with their specific ligands. For several of these accessory receptors, engagement of the TCR results in increased binding to ligand. Thus, although the interaction of the receptor-ligand pairs is antigen-independent, the activation steps that lead to adhesion are dependent on occupancy of the TCR. The receptor-ligand systems shown to be activated via the TCR include CD8/class I MHC (1), LFA-1/intercellular adhesion molecule (ICAM-1) (2), very late antigen (VLA)-4/fibronectin (FN), VLA-5/FN, VLA-6/laminin (3, 4), and CD2/LFA-3 (5). Evidence is accumulating to indicate that, in addition to mediating adhesion, accessory receptors can generate costimulatory signals. LFA-1 and the VLA receptors have been shown to have costimulatory activity for antibody-activated proliferation of CD4⁺ T cells (6-8). Similarly, our previous studies of CD8/class I and VLA/FN interactions have demonstrated adhesion and costimulatory roles for these receptors in generation of CTL functional response (9).¹ Furthermore, these studies have suggested that

these two receptor ligand systems are involved in a cascade of signaling events, rather than sharing a common mechanism and redundant roles in the generation of CTL response.

Recent studies suggest that other T cell accessory receptors may also contribute costimulatory signals capable of influencing the CTL response. Studies of CD4⁺ T cells suggest that the high affinity adhesion of LFA-1 to ICAM-1 can deliver an activation signal to T cells in addition to mediating adhesion (6, 10). Here, we have examined the potential contributions of adhesion of LFA-1 to ICAM-1 to the functional response of CTL to soluble mAb and to purified antigen. The results described in the present report strongly suggest that the contribution of the LFA-1/ICAM-1 interaction to the adhesion/signaling cascade is distinct from that of either VLA/FN or CD8/class I.

Materials and Methods

Cloned CTL Lines. Clones 11 and 35, of (B10.BR × B10.D2)F1 origin, are specific for K^b alloantigen and have been described previously (11). Clone OE4 is of C57BL/6 origin and is specific for H-2^d alloantigen (12). Clones were maintained by weekly restimulation with irradiated (3,000 R) spleen cells (C57BL/6 or DBA/2)

¹ Ybarrodo, B., A. M. O'Rourke, J. B. McCarthy, and M. F. Mescher, manuscript submitted for publication.

and 10 U/ml rIL-2. Culture medium consisted of RPMI 1640 supplemented with 20 mM Hepes, 2 mM L-glutamine, 5×10^{-5} M 2-ME, penicillin-streptomycin (100 U/ml), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2.5% FCS, and 1.0% CMF-11 nutrient supplement (J. Brooks Laboratories, San Diego, CA).

Antibodies. mAb F23.1, a mouse Ab specific for V β 8 (13) was purified from ascites fluid by affinity chromatography on Protein A-Sepharose. Goat anti-mouse IgG (H and L chains) (Fisher Scientific, Pittsburgh, PA) was used in some experiments to further cross-link the F23.1 mAb. The anti-LFA-1 mAb FD441.8 was purified from hybridoma culture supernatant by ammonium sulfate precipitation. The anti-ICAM-1 mAb (10) was used as a hybridoma culture supernatant. The mAb anti-Lyt-2 53-6.7 (14) was purified by ammonium sulfate fractionation followed by DEAE chromatography. M1/42, a rat anti-mouse class I mAb (15), was purified from culture supernatant by ammonium sulfate precipitation followed by DEAE chromatography and used to detect immobilized K^b alloantigen by ELISA.

Ligands. A secreted form of the murine ICAM-1 protein (ICAM-1s) was generated as described previously from a truncated ICAM-1 cDNA coding for the first four extracellular domains and a portion of the fifth domain of the ICAM-1 protein, but lacking the putative transmembrane and cytoplasmic domains (10). Purified murine plasma FN was purchased from Telios Pharmaceuticals, Inc. (San Diego, CA). ICAM-1s and FN were diluted in PBS for use in functional assays. H-2 K^b was purified from TX-100 lysates of EL4 (K^bD^b) tumor cells by mAb affinity chromatography as previously described (15). H-2K^k was purified from TX-100 lysates of RDM4 (K^kD^k) cells (16). H-2 preparations were quantitated by protein determination and by ELISA relative to standard preparations. Purity of the Ag preparations was determined by SDS-PAGE.

Protein Immobilization. Purified H-2 antigens were stored at -20°C in 10 mM Tris buffer containing 0.5% deoxycholate and NaCl at concentrations of 20–160 mM. For use, class I protein was diluted directly into PBS (pH 7.4) to concentrations ranging from 0.1 to 2 $\mu\text{g}/\text{ml}$. 0.1 ml aliquot/well of diluted H-2 was then incubated for 1.5 h at 25°C in flat bottom wells of Microtiter III flexible assay plates (Falcon Labware, Oxnard, CA). Binding of H-2 increased linearly with input up to a level of 0.15–0.3 $\mu\text{g}/\text{ml}$, as determined by ELISA (17). After a 1.5-h incubation, the wells were washed with 0.1% BSA in PBS to remove unbound Ag and to block unoccupied sites on the plastic.

ICAM-1s and FN were diluted in PBS and immobilized by addition to microtiter plate wells and incubation at room temperature for 1.5 h. The wells were then washed and blocked with 0.1% BSA as described above. Co-immobilization of ICAM-1s and H-2 was accomplished by adding ICAM-1s to the wells first. After a 1.5-h incubation, the wells were washed with PBS to remove unbound ICAM-1s, and H-2 was immobilized as described above.

Assay of CTL Degranulation. The release of serine esterase activity in response to proteins immobilized in microtiter wells was determined by the addition of 1×10^5 CTL/well in 0.1 ml of RPMI supplemented with 2% FCS and 15 mM Hepes. Plates were centrifuged at 1,000 for 1 min and incubated at 37°C for times ranging from 30 min–5 h. After incubation, 0.02-ml aliquots of cell free supernatant were removed and serine esterase activity determined as previously described (11). All experiments included triplicate determinations and standard deviations are shown.

Assay of CTL Binding. CTLs were labeled by overnight incubation in complete medium at 37°C with 1 μCi [^3H]thymidine per $2\text{--}5 \times 10^5$ cells. Cells were washed and resuspended at $10^6/\text{ml}$ in RPMI with 2% FCS and 15 mM Hepes. Binding was initiated by adding 10^5 cells per well in 0.1 ml and centrifuging the mi-

croter plates at 1,000 rpm for 1 min. After a 2-h incubation at 37°C , binding was determined as described previously (9). In some experiments, SE release and binding were determined for the same cells. To accomplish this, 0.02-ml aliquots of supernatant were removed from the wells for esterase activity determination before proceeding with the binding assay. In blocking studies, purified mAb were added to CTL before initiation of the binding assay.

Assay of Phosphoinositide Hydrolysis. For determination of [^3H]inositol phosphate release, CTL clones were labeled overnight with [^3H]myo-inositol in inositol-deficient Eagle's basal medium supplemented with 10% (vol/vol) dialyzed FCS, containing 10–15 $\mu\text{Ci}/\text{ml}$ [^3H]myo-inositol (S.A. 60–100 Ci/mmol, Amersham). Labeled CTL were incubated with stimuli in RPMI 1690 containing 10 mM Hepes, 10 mM LiCl, and 0.1% (wt/vol) BSA. [^3H]inositol phosphates were extracted after 120 min by addition of methanol/chloroform/HCl and quantitated by Dowex (Dow Chemical Co., Midland, MI) (HCOO^-) ion-exchange chromatography as previously described (9).

Results and Discussion

Fluid-Phase Anti-TCR mAb Triggers Adhesion of CTL to Immobilized ICAM-1s. Murine ICAM-1s can be directly immobilized on plastic by dilution in PBS and incubation in microtiter wells. Under the conditions used, the amount of ICAM-1s immobilized is maximal upon addition of 0.3–0.5 $\mu\text{g}/\text{well}$ (Fig. 1A). Cloned murine CTL bound to immobilized ICAM-1s upon stimulation with soluble anti-TCR mAbs, F23.1, as seen in Fig. 1B. Unstimulated CTL showed a low but significant basal level of binding to ICAM-1s, which was significantly increased upon the addition of F23.1 anti-TCR mAbs. The addition of a second cross-linking Ab further increased adhesion to levels more than double those reached with F23.1 alone. The addition of an anti-LFA-1 blocking mAb (FD441.8) inhibited binding of CTL to immobilized ICAM-1s, confirming that the activated adhesion

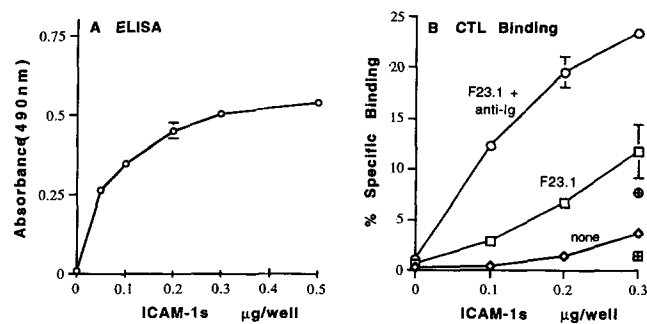


Figure 1. Soluble anti-TCR Ab triggers CTL binding to immobilized ICAM-1s. The indicated concentrations of ICAM-1s were immobilized in microtiter wells and the wells blocked with 0.1% BSA. (A) ELISA assay of immobilized ICAM-1s using BE29G1 anti-ICAM-1 mAb (1:13,000) dilution followed by HRP-linked goat anti-rat Ig as second Ab. (B) CTL clone 11 was either not stimulated ($-\diamond-$), stimulated with F23.1 mAb ($-\square-$), or with F23.1 and goat anti-mouse IgG (anti-IgG) ($-\circ-$). After a 2.5-h incubation at 37°C , percent specific binding was determined as described in Materials and Methods. For Ab blocking, anti-LFA-1 mAb FD441.8 was added to CTL at a concentration of 75 $\mu\text{g}/\text{ml}$ before addition of the cells to the ICAM-1 bearing wells. FD441.8 was added to CTL stimulated with F23.1 (\boxplus) or F23.1 and second Ab (\boxplus).

was mediated by LFA-1. Optimum binding was observed with a 2-h incubation and 0.2–0.3 mg/well immobilized ICAM-1s (data not shown). The Ab-triggered binding of CTL to immobilized ICAM-1s can be compared with previous studies with immobilized class I and FN. The augmentation of binding observed with addition of cross-linking Abs together with anti-TCR was also observed with immobilized FN¹. In contrast, activated adhesion to irrelevant class I MHC via CD8 was not augmented by the addition of a second cross-linking Ab, suggesting that the activation steps leading to triggered binding of CD8 to class I differ from those involved in triggered binding to ICAM-1s and FN.

Co-immobilized Suboptimal Levels of Alloantigen Trigger Adhesion to Immobilized ICAM-1s. To examine Ag-activated adhesion to ICAM-1s, a suboptimal level of H-2K^b alloantigen was immobilized together with a range of concentrations of ICAM-1s. The concentration of K^b used (0.02 μg/well) was insufficient to mediate CTL adhesion when immobilized alone. However, the presence of K^b resulted in induction of adhesion to co-immobilized ICAM-1s (Fig. 2), and the addition of blocking anti-LFA-1 mAb resulted in significant inhibition of adhesion. In addition to occupying the TCR, immobilized K^b is also a ligand for CD8. While the concentration of K^b immobilized is too low to support detectable CTL adhesion via CD8, it seemed possible that TCR-activated binding of CD8 to K^b could contribute to activation of binding to ICAM-1s. To assess this possibility, the ability of an anti-CD8 mAb to block binding was determined. The addition of anti-CD8 Abs substantially inhibited adhesion, suggesting that both TCR and CD8 interactions with class I MHC are involved in activating LFA-1 dependent adhesion to ICAM-1s.

Binding to Immobilized ICAM-1s, FN, and Nonantigenic Class I MHC Have Different Co-signaling Effects. Stimulation with soluble anti-TCR mAbs alone is sufficient to trigger adhe-

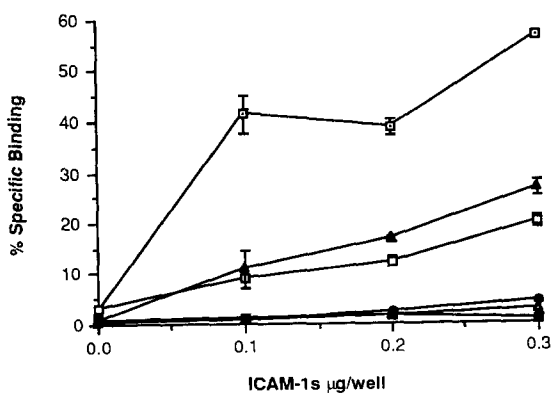


Figure 2. Suboptimal levels of alloantigen stimulate adhesion to immobilized ICAM-1s. CTL clone 11 was incubated in wells coated with the indicated concentrations of ICAM-1s alone or in wells coated with 0.02 μg/well of K^b together with ICAM-1s. Where indicated, anti-LFA-1 mAb at 75 μg/ml or anti-CD8 mAb at 10 μg/ml were present. Percent specific binding was determined as described. ● control; □ +K^b; ■ +anti-LFA-1; △ +K^b+anti-LFA-1; ▲ +anti-CD8; ▴ +K^b+anti-CD8.

sion of CTL to immobilized class I MHC (K^k), ICAM-1s, or FN (Fig. 3 A). The addition of a second cross-linking Ab resulted in increased adhesion to ICAM-1s and FN, but had no effect on the level of binding to K^k. Thus, as described above, activated adhesion to class I differed from adhesion to FN or to ICAM-1, which appear to have similar properties. In contrast, all three ligands were found to differ in

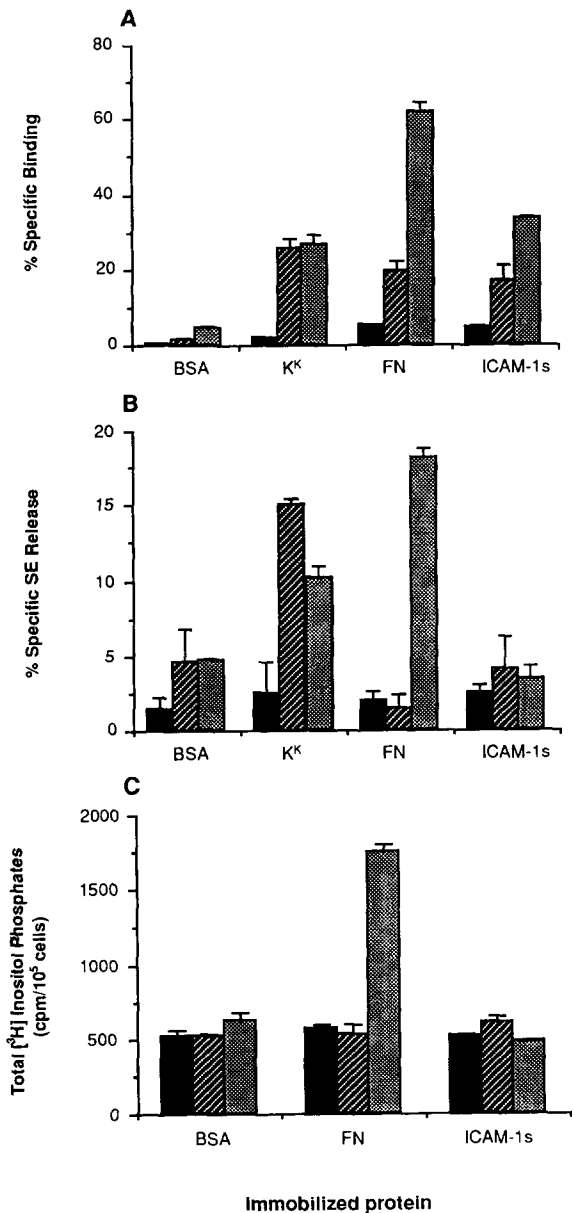


Figure 3. ICAM-1s, FN, and class I MHC have different co-signaling effects. CTL clone 11 was stimulated with the indicated Abs and added to wells coated with 0.5 μg/well of FN, 0.2 μg/well ICAM-1s, or 0.2 μg/well class I MHC (K^k). After a 5-h incubation, (A) CTL binding and (B) serine esterase release were determined as described. (C) Total [³H]inositol phosphates were determined in 10⁵/well [³H]inositol-labeled clone 11 after a 120-min incubation in the presence of 10 mM LiCl. Results are expressed as mean and range of duplicate samples. ■ No Ab; ▨ +F23.1; ▩ +F23.1+anti-Ig.

their ability to provide a costimulatory signal for degranulation (Fig. 3 B). When CTL were activated with anti-TCR alone, adhesion to class I MHC was sufficient to stimulate degranulation, and the level of serine esterase released was somewhat decreased upon the addition of a cross-linking Ab (Fig. 3 B). In contrast, anti-TCR activated CTL incubated with immobilized FN were unable to initiate a degranulatory response unless the second cross-linking Ab was also present (Fig. 3 B), as previously described. In further contrast, activated adhesion of CTL to ICAM-1s in the presence of anti-TCR alone or with the combination of anti-TCRs and cross-linking Abs did not result in the initiation of degranulation, despite the fact that adhesion was clearly occurring under these conditions. Additional experiments have demonstrated that co-signaling is readily detected using 0.1 $\mu\text{g}/\text{well}$ FN, while no co-signaling is found using up to 1 $\mu\text{g}/\text{well}$ ICAM-1s (i.e., well above saturating levels, see Fig. 1 A). Essentially the same results have been obtained in experiments using native ICAM-1 purified by affinity chromatography after detergent solubilization (data not shown). Similarly, a lack of costimulation with ICAM-1s was demonstrated with two other allospecific CTL clones, OE4 and 35, both of which undergo TCR-activated adhesion to ICAM-1s. Thus, although the requirement for activation of adhesion to FN and ICAM-1 appears to be the same, the requirements for costimulation as a result of binding clearly are not.

A recent study of activation of phosphoinositide (PI) hydrolysis in CTL under the conditions described above has suggested a possible explanation for the differential effects of class I and FN on costimulation. As reported, activation of murine CTL with fluid phase anti-TCR alone was unable to initiate detectable PI hydrolysis (9). Hydrolysis did occur if CTL subsequently underwent CD8-mediated adhesion to class I MHC, but not VLA-mediated adhesion to FN. Activating CTL with anti-TCR and cross-linking the TCR by adding a second Ab resulted in a low but detectable level of PI hydrolysis. If the CTL were subsequently allowed to adhere to FN, a dramatic increase in PI hydrolysis occurred, demonstrating that while FN was unable to initiate PI hydrolysis, it was capable of amplifying it once it had been initiated. Here, we have determined the effect of adhesion to immobilized ICAM-1s on PI hydrolysis in Ab-activated CTL. For comparison, CTL were also allowed to adhere to immobilized FN. CTL were stimulated with either F23.1 alone or with F23.1 together with cross-linking Ab and incubated in wells coated with BSA, 0.125 $\mu\text{g}/\text{well}$ ICAM-1s, or 0.25 $\mu\text{g}/\text{well}$ FN. After a 120-min incubation, PI hydrolysis was determined. As expected, binding to FN dramatically amplified PI hydrolysis in the presence of F23.1 and cross-linking antibody (Fig. 3 C). In contrast, binding to ICAM-1s had no effect on PI hydrolysis, consistent with the lack of degranulation under these conditions. While these results do not rule out a potential co-signaling role, they clearly demonstrate that LFA-1 does not make the same co-signaling contribution as either the FN-binding receptor or CD8.

ICAM-1s Enhances CTL Adhesion and Response to Alloantigen. To determine the effect of ICAM-1s on the functional response of CTL to antigen, ICAM-1s, and antigenic H-2K^b

were co-immobilized in microtiter wells. ICAM-1s was immobilized at 0.05 $\mu\text{g}/\text{well}$ or 0.1 $\mu\text{g}/\text{well}$ together with concentrations of K^b alloantigen ranging from 0.0125 to 0.2 $\mu\text{g}/\text{well}$. Adhesion and degranulation were determined in parallel. Immobilized ICAM-1s did not stimulate serine esterase release from CTL in the absence of alloantigen, but augmented alloantigen-induced serine esterase release from CTL when it was co-immobilized on the same surface at either 0.05 or 0.1 $\mu\text{g}/\text{well}$ (Fig. 4 A). The predominant effect of co-immobilized ICAM-1s was to decrease the surface density of K^b required to initiate a response, rather than to increase the maximal response level. The presence of ICAM-1s also increased the level of CTL binding to the ICAM-1s/alloantigen coated surface, as seen in Fig. 4 B. Increased binding was observed with 0.05 $\mu\text{g}/\text{well}$ ICAM-1s, with no further increase seen in the presence of 0.1 $\mu\text{g}/\text{ml}$. These results suggest that the LFA-1/ICAM-1 interaction may increase adhesion between the surfaces to promote higher TCR and CD8 occupancy at suboptimal class I density, but contribute no amplifying signal when TCR and CD8 occupancy is optimal.

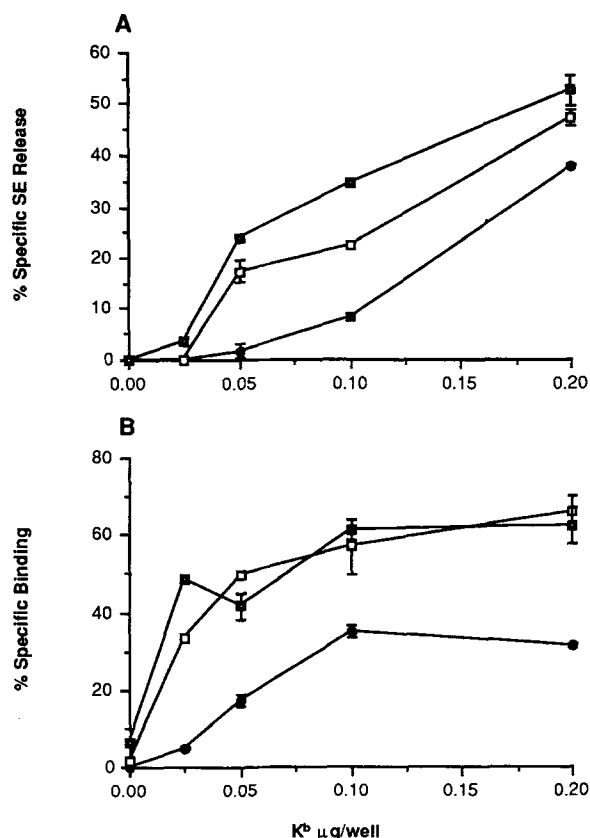


Figure 4. Co-immobilized ICAM-1s enhances CTL response to immobilized alloantigen. CTL clone 11 was cultured in wells coated with the indicated concentrations of K^b alone or in wells coated with K^b together with 0.05 $\mu\text{g}/\text{well}$ or 0.1 $\mu\text{g}/\text{well}$ ICAM-1s. After a 5-h incubation, percent specific serine esterase release (A) and percent specific binding (B) were determined as described in Materials and Methods. —●— K^b alone; —□— +0.05 μg ICAM-1s; —■— +0.1 μg ICAM-1s.

In contrast, co-immobilized FN substantially increases the response level even when class I density is optimal.¹

CTL activation appears to involve a cascade of adhesion and signaling events with different accessory receptors making distinct contributions. The findings reported here indicate that while CTL binding to ICAM-1, class I MHC, and FN are all activated through the TCR, the relative contributions of these interactions to functional response are different. CD8/class I and VLA/FN interactions clearly provide costimulatory signals leading respectively to initiation and amplification of PI hydrolysis (9). LFA-1-dependent adhesion can be acti-

vated by antigen, but this appears to depend on both TCR and CD8 (Fig. 2), suggesting a position downstream from these receptors in the activation cascade. Furthermore, if LFA-1 provides a costimulatory signal, as it can in some T cells (6, 10), then the signal clearly differs from those provided by binding to class I or FN (Fig. 3). At least in the case of CTL degranulation, one of the mechanisms for target lysis, the predominant contributions of LFA-1/ICAM-1 binding appears to be to increase adhesion with the antigen bearing surface, thus promoting increased occupancy of the other receptors.

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