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*J Immunol* 1999; 162:5183-5189; ;
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Signaling Pathways Activated by Leukocyte Function-Associated Ag-1-Dependent Costimulation

Hsiao-Tzu Ni,* Matthew J. Deeths,* Wei Li,† Daniel L. Mueller, † and Matthew F. Mescher2*

LFA-1 binding to ICAM-1 can enhance TCR-dependent proliferation of T cells, but it has been difficult to distinguish contributions from increased adhesion, and thus TCR occupancy, versus costimulatory signaling. Whether LFA-1 ligation results in generation of a unique costimulatory signal(s) distinct from those activated by the TCR has been unclear. Using purified ligands, it is shown that ICAM-1 and B7.1 provide comparable costimulation for proliferation of CD8+ T cells, and that both ligands up-regulate the activities of phosphatidylinositol 3-kinase, sphingomyelinase, and c-Jun NH2-terminal kinase (JNK). These pathways are distinct from those activated by the TCR, and have previously been implicated in up-regulating IL-2 production in response to CD28-B7 interaction. Thus, under conditions in which ICAM-1 provides costimulation of proliferation, LFA-1 ligation activates some of the same signaling pathways as does CD28 ligation. LFA-1 and CD28 do not act identically, however, as indicated by differential sensitivity to inhibitors of phosphatidylinositol 3-kinase; LFA-1-dependent costimulation of proliferation is inhibited, while CD28-dependent costimulation is not. Given the broad distribution of class I and ICAMs on many cell types, the ability of LFA-1 to provide costimulatory signals has implications for where and how CD8+ CTL may become activated in response to an antigenic challenge. The Journal of Immunology, 1999, 162: 5183–5189.

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D28 is perhaps the best characterized costimulatory receptor on T cells. When CD28 binds B7.1 (CD80) or B7.2 (CD86), signals are generated that cooperate with TCR-dependent signals to up-regulate IL-2 production and proliferation (1–3) and activate transcription of the gene for the Bcl-xL survival protein (4). CD28 engagement has been shown to activate several signaling pathways that are distinct from those activated by the TCR, and are thus candidates for being specifically involved in costimulatory signaling. These include activation of phosphatidylinositol (PI)3-kinase (5–7), sphingomyelinase (8–10), and tyrosine kinases (11–14). Distal events stimulated by CD28 engagement include activation of c-Jun NH2-terminal kinase (JNK), a mitogen-activated protein kinase (MAPK) that has an important role in activating the AP-1 transcription factor that contributes to IL-2 gene transcription (15).

There is considerable evidence to suggest that the β2 integrin LFA-1 might also act as a costimulatory receptor for T cells upon binding to its ICAM ligands. Expression of ICAM on Ag-bearing cells enhances T cell responses (16–18), as does coimmobilization of ICAM or anti-LFA-1 Ab on a surface along with Ag or anti-TCR mAb (19–22). In many cases, however, interpretation of these effects is not straightforward since LFA-1 is also an adhesion molecule (23, 24). Thus, increased response might result simply from increased adhesion between the surfaces, leading to a higher TCR occupancy level, and thereby enhanced or prolonged TCR-dependent signals. However, there is evidence LFA-1 engagement can result in generation of costimulatory transmembrane signals that contribute to T cell activation (25–27).

When ICAM is coimmobilized with anti-TCR mAb and used to stimulate human CD4+ T cells, its effect is to prolong inositol phospholipid hydrolysis and sustain increased intracellular Ca2+ levels (25). Prolonged phosphorylation of phospholipase Cγ1 also occurs in response to coligation of TCR and LFA-1 (26). These pathways are activated via the TCR, and activation appears to be prolonged or enhanced when LFA-1 is also engaged. Tyrosine phosphorylation of cellular substrates has also been reported in T (27) and B (28) cells in response to LFA-1 engagement, and a recent report has shown that LFA-1 can induce tyrosine phosphorylation of p130Cas and subsequent association with crkll in a B cell line (29). The potential roles of these tyrosine phosphorylation events in lymphocyte costimulation have not been elucidated.

We have reported recently results of a study comparing the ability of purified B7.1 and ICAM-1 proteins to costimulate murine T cells when they are coimmobilized on cell-size latex microspheres along with anti-TCR mAb (30). Use of this well-defined system allows TCR, CD28, and LFA-1 contributions to be examined in the absence of additional receptor-ligand interactions that may occur when cells are used to present Ag. We found that ICAM-1 could provide costimulation to CD8+ T cells to support IL-2 secretion and IL-2-dependent proliferation of CD8+ T cells, and that these were comparable with the costimulation provided by B7.1. However, costimulation by ICAM-1 and B7.1 differed markedly in that only B7.1 supported substantial clonal expansion. Thus, costimulation of proliferation by ICAM-1 was effective, but the cells did not survive as well as when costimulation was provided by B7.1. We also found that B7.1 costimulated CD4+ T cells very effectively. In contrast, ICAM-1 provided little costimulation to these cells in comparison with that provided to CD8+ T cells. This is consistent with results recently reported by Zuckerman et al. (18), showing that ICAM-1 expressed on APC could costimulate
CD4+ T cells, but that the costimulation was incomplete and re-
sulted in tolerance rather than clonal expansion. ICAM costimu-
lation only a very transient increase in IL-2 mRNA expression in the CD4+ T cells, with message detectable at 2 h, but gone by 6 h.

Generation of signals that are unique from those activated via the TCR, and that can contribute to up-regulating IL-2 production and proliferation, is considered the hallmark of a true costimula-
tory receptor, as demonstrated by the CD28 receptor. Despite con-
siderable evidence that LFA-1 engagement can generate trans-
membrane signals (25–27), and that it can enhance T cell responses (16–22), it has remained unclear whether it activates such signaling pathways distinct from those activated by the TCR. In this study, we describe the results of experiments comparing the ability of ICAM-1 and B7.1 engagement by CD8+ T cells in ac-
tivating signaling pathways that have been implicated in CD28-
dependent costimulatory signaling.

Materials and Methods

**Mice and T cells**

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME), housed in the University of Minnesota animal facility according to National Institute of Health guidelines, and used at 6–12 wk of age. Lymph nodes were harvested into complete RPMI medium (RPMI 1640 supple-
mented with 10% Fetal bovine serum, 100 U/ml penicillin, 10 mg/ml streptomycin, 1 mM sodium pyruvate, 10 mM HEPES, 2 mM glutathione, and 100 mM 2-ME) with 10% FCS, pooled, and homogenized using a tissue homogenizer to yield a single cell suspension. Suspensions were treated with 11 mM KCNO3, 152 mM NH4Cl to remove RBC, centrifuged, and resuspended at 10^6/ml in RPMI medium, and adherent cells were depleted by adherence to plastic at 37°C for 1.5 h. T cell subsets were then purified by negative selection on Biotex (Edmonton, Canada) mouse cell enrichment columns according to the pro-
cedure provided by the manufacturer. Recovered CD8+ cells were greater than 95% CD8+ and less than 1% CD4+, and recovered CD4+ cells were greater than 95% CD4+ and less than 1% CD8+. For comparison of naive and memory cells, column-purified CD8+ T cells were sorted for CD44high and CD44low expression by flow cytometry following staining with FITC-conjugated anti-CD44 mAb clone IM7 (PharMingen, San Diego, CA), using 1 µg Ab per 2×10^6 cells.

**Ligand purification and microsphere preparation**

B7.1 was purified from Triton X-100 lysates of CHO-B7.1 transfectants by mAb affinity chromatography using the 16-10A1 hybridoma, as previously described (31). B7.1 was eluted from the affinity column using 15 mM carbonate buffer (pH 12) containing 0.5% deoxycholate and 250 mM NaCl, immediately adjusted to pH 8, and stored at −20°C. Protein was deter-
mined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rock-
ford, IL). A secreted form of murine ICAM-1 having the first four extra-
cellular domains and a portion of the fifth domain, but lacking the transmembrane and cytoplasmic domains, was purified by mAb affinity chromatography, as previously described (21), and stored at −20°C. Anti-
TCR mAb used for immobilization on microspheres included F23.1 anti-
Vβ8 mAb (32) and 2C11-145 anti-CD3ε mAb (33), and were used in purified form.

Abs and ligands were immobilized on 5-µm-diameter sulfate polysy-
trene latex microspheres (Interfacial Dynamics, Portland, OR) essentially as described previously (31). Briefly, mAbs in PBS were immobilized at the concentrations indicated in the figure legends by mixing with a suspension of 10^7 microspheres/ml for 1.5 h at 4°C, or at 37°C in the case of the 2C11 mAb. BSA was then added to a final concentration of 0.25%, and incor-
ubation was continued for an additional 30 min at 4°C to block any remaining sites on the beads. ICAM-1 and B7.1 were immobilized in the same way at 4°C. For coimmobilization of mAb and ligands, the mAb was first in-
cubated with the microspheres for 1.5 h. The beads were then pelleted by centrifugation for 10 min, and resuspended in PBS, and the B7.1 or ICAM-1 was added to the suspension. Incubation was then continued for 1.5 h, and the beads were then blocked with BSA, as described above. Immobilization of the proteins was done using sterile reagents and conditions. The final prepar-
ations were quantitated by counting using a hemacytometer.

Microspheres were characterized with respect to surface ligand density by flow cytometry using specific Abs. ICAM-1 was detected using FITC-
conjugated anti-ICAM-1 mAb (PharMingen) and B7.1 using FITC-conju-
gated anti-CD80 16-10A1 (PharMingen). All preparations of B7.1 and ICAM-1 were titrated in experiments to determine optimum levels for CD8 T cell costimulation, and all experiments reported in this study were done using optimum levels. In Fig. 1, F23.1 density is presented as mean equiva-
lent soluble fluorochrome (MESF), as determined relative to FITC-labeled standard microbeads (Flow Cytometry Standards, San Juan, PR). MESF is directly proportional to the amount of FITC-conjugated Ab binding to the latex-bearing microspheres, and nonspecific binding of isotype control mAb (31).

**Proliferation assay**

CD8+ or CD8+ T cells purified as described above were cultured in triplic-
icate in flat-bottom microtiter wells (Falcon, Franklin Lakes, NJ) at 5×10^6 cells/well with 1×10^6 microspheres in a final volume of 0.2 ml complete RPMI medium with 10% FCS. Cultures were incubated at 37°C for 48 h and pulsed with 1 µCi [3H]thymidine for the final 6 h. Blocking experiments were performed using M174 anti-LOA-1 mAb (anti-CD11a, PharMingen), BE29G1 anti-ICAM-1 mAb, and CTLA4-Ig (a gift from Robert W. Karr, Monsanto, St. Louis, MO). Rat IgG2a,k (PharMingen) was the isotype control used for M174 and BE29G1, and IgG from the UPC10 myeloma (Sigma, St. Louis, MO) for CTLA4-Ig. Wortmannin was purchased from Sigma.

**Assay of PI 3-kinase activity**

Cells were stimulated with microspheres for 5 min at 37°C, as described above. The cells were then lysed in 50 mM HEPES buffer, containing 1% Triton X-100, 0.15 M NaCl, 10% glycerol, 1.5 mM MgCl2, 1 mM EGTA, 100 mM NaF, 1 mM Na3VO4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM PMSF. Lysates were precleared by treatment with 0.05 ml of protein A-coupled Sepharose 4B (Sigma) at 4°C for 1 h. Immunoprecipitations were then done by adding 0.05 ml of either anti-p85 antisera (Upstate Biotechnology, Lake Placid, NY) or anti-fyn rabbit polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA) and 0.05 ml of protein A-
coupled Sepharose 4B, and incubating overnight at 4°C. Precipitates were then washed and assayed for PI 3-kinase activity exactly as described previously (34) using PI as the substrate. Reaction products were separated by TLC, identified using known standards, and visualized by autoradiography.

**Assay of acidic sphingomyelinase activity**

CD8+ or CD8+ T cells (2×10^6) were prepared as described above and incubated with 4×10^6 microspheres, pelleted by microfuge, and incubated at 37°C for the indicated times. Reaction was stopped by immersion in ethanol-dry ice bath, and the cells then pelleted in a microfuge. Pellets were lysed in 200 µl of 0.1% Triton X-100, and the lysate was then assayed for acidic sphin-
gomyelinase activity, as described by Boucher et al. (8), using [32P]sphin-
gomyelin (Amersham, Arlington Heights, IL) as the substrate. The reaction was allowed to proceed for 2 h, and the reaction mixture was then phase separated by the addition of 200 µl H2O and 800 µl of chloroform:metha-
nol (2:1) mixture. Radioactivity in the aqueous phase was measured by scintillation counting.

**Assay of JNK and ERK activity**

CD8+ or CD8+ T cells (1.5×10^5) were stimulated with 2C11 or F23.1 mAb immobilized in 24-well plates either alone or along with ligands. Cells were incubated in the wells for 25 min at 37°C, lysed by the addition of 150 µl HEPES buffer (pH 7.7) containing 0.1% Triton X-100, 0.3 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 20 mM β-glycerol phosphate, 0.1 mM NaVO3, 2 µg/ml leupeptin, and 100 µg/ml PMSF into each well, and incubated with rocking at 4°C for 30 min. Lysates were then clarified by centrifugation for 10 min. JNK and ERK were then precipi-
tated, and the precipitates were assayed for kinase activity, as previously described, with minor modification as described below (35). ERK was precipitated using a GST-Elk-1 fusion protein (kindly provided by Bob Hipskind, Institut de Genetique Moleculaire, Strasbourg, France), and JNK was precipitated using a GST-c-Jun (1–232) fusion protein bound to glutathione agarose beads. Enzymatic activity was determined by measuring 32P incorporation into the GST fusion proteins. Reaction was stopped by the addition of 0.02 ml of 2X Laemmli loading buffer and boiling for 3 min, and the phosphorylated substrates were separated by SDS-PAGE and vi-
ualized by autoradiography.

**Results**

**ICAM-1 costimulation of CD8+ T cell proliferation**

B7.1 and soluble ICAM-1 proteins both provide costimulation for purified CD8+ T cell populations (>95% CD8+, <1% CD4+) when coimmobilized on latex microspheres along with anti-TCR mAb at varying levels (Fig. 1). In all of the experiments reported
CD8<sup>+</sup> cell proliferation, as determined for each ligand preparation (data not shown). CD8<sup>+</sup> T cells were purified from the lymph nodes of mice genetically deficient in CD28 (Deeths and Mescher, unpublished results). Recent reports have demonstrated that CD28 ligation activates T cells from the lymph nodes of normal mice and placed in wells along with the microspheres, and proliferation was assessed by measuring [3 H]thymidine incorporation during the last 6 h of a 48-h culture period. The F23.1 anti-LFA-1 mAb was used (32); about 20% of the T cells bear this TCR b-chain. Both ligands stimulate responses that are substantially above the response stimulated by even very high levels of anti-TCR mAb alone (Fig. 1A), and B7.1 usually stimulates a somewhat greater response than ICAM-1 when the ligands are present at optimal surface densities on the microspheres. These results confirm that costimulation by ICAM-1 requires LFA-1, and suggest that recognition of B7.1 or B7.2 on the T cells does not contribute to the ICAM-1-dependent response. This conclusion has been confirmed by demonstrating that ICAM-1 can provide effective costimulation for CD8<sup>+</sup> T cells purified from mice genetically deficient in CD28 (Deeths and Mescher, unpublished results).

The CD8<sup>+</sup> T cells from the lymph nodes of C57BL/6 mice used in these experiments include both CD44<sub>low</sub> naive T cells and CD44<sub>high</sub> memory T cells. LFA-1 expression is increased on memory cells (38), raising the possibility that ICAM-1 might preferentially costimulate this subpopulation of CD8<sup>+</sup> T cells. This was examined by isolating naive and memory populations by staining with anti-CD44 mAb and sorting into CD44<sub>low</sub> (naive) and CD44<sub>high</sub> (memory) subsets. These results suggest that recognition of B7.1 or B7.2 on the T cells does not contribute to the ICAM-1-dependent response. This conclusion has been confirmed by demonstrating that ICAM-1 can provide effective costimulation for CD8<sup>+</sup> T cells purified from mice genetically deficient in CD28 (Deeths and Mescher, unpublished results).

Up-regulation of sphingomyelinase activity in response to ICAM-1 costimulation

Recent reports have demonstrated that CD28 ligation activates acid sphingomyelinase (A-SMase), and that this can lead to an increase in nuclear factor-κB (NF-κB), an enhancer-binding protein implicated in transcription of the IL-2 and the IL-2Rα genes (8–10). Cross-linking CD28 with Ab was shown to result in a rapid and transient increase in A-SMase activity, with maximal...
activity occurring at 2–3 min and being 1.5–3-fold above controls (8, 9). To determine whether the native CD28 ligand B7.1 could also stimulate this signaling pathway, and determine whether ICAM-1 would also activate it, we examined A-SMase activity in whole cell lysates of CD8⁺ T cells. Cells were stimulated for 0–4 min with microspheres having anti-TCR mAb alone, or along with either B7.1 or ICAM-1. Stimulation was then stopped by freezing the cells at −70°C, and sphingomyelinase activity was assayed, as described in Materials and Methods, using [14C]sphingomyelin as the substrate.

Stimulation of CD8⁺ cells with just anti-TCR mAb on the microspheres caused no detectable increase in A-SMase activity. However, costimulation with either B7.1 or ICAM-1 caused a significant increase in activity at 2–3 min, which then declined to basal levels (Fig. 3). In multiple experiments, B7.1 costimulation resulted in maximal responses of 130–350% of control, while ICAM-1 costimulation resulted in maximal responses of 125–240% of control. Thus, native B7.1 protein activates the A-SMase signaling pathway in a very similar manner to that reported for cross-linking with anti-CD28 Ab (8, 9), and ICAM-1-dependent costimulation results in a comparable activation of this pathway.

ICAM-1 costimulation increases JNK activity, but not ERK

Like NF-κB, the AP-1 transcription factor also regulates IL-2 gene transcription and increases upon CD28-dependent costimulatory signaling (15). The serine-threonine kinases ERK and JNK are MAPKs that are both thought to play an important role in activating AP-1. Anti-TCR mAb stimulation of CD4⁺ T cells results in increased ERK activity, and stimulation with both anti-TCR mAb and anti-CD28 mAb causes little or no additional increase in ERK activity, but stimulates a large increase in JNK activity (15, 35). Similar results were obtained when we examined responses of CD8⁺ T cells. ERK activity was up-regulated by TCR signaling alone and did not increase when B7.1 was also present, while JNK activity was strongly up-regulated in response to B7.1 (Fig. 4). ERK activity peaks at about 5 min and is falling by 25 min, the time examined in the experiment shown (Fig. 4A). ERK levels in response to anti-TCR mAb and anti-CD28 mAb were no greater than in response to anti-TCR mAb alone when examined at earlier time points (data not shown). The data shown (Fig. 4) are representative of three independent experiments using the anti-CD3ε mAb 145-2C11 as a source of TCR signaling.

The responses seen when ICAM-1 was coimmobilized with the anti-CD3ε mAb were the same as those seen in response to B7.1; ERK did not increase above the level reached in response to anti-TCR mAb alone, but ICAM-1 stimulated a large increase in JNK activity (Fig. 4). In addition, JNK activity was also strongly up-regulated when anti-CD28 mAb was coimmobilized along with anti-CD3ε mAb, and likewise, anti-LFA-1 mAb had the same effect (Fig. 4). 2C11 anti-CD3ε mAb was used in the experiment shown in Fig. 4. F23.1 mAb has given essentially the same results, although less consistently, probably because it binds TCR on only about 20% of the cells and thus results in a lower signal to noise ratio in the kinase assays. Consistent with the failure of ICAM-1 to provide strong costimulation for CD4⁺ T cells (30), no up-regulation of JNK could be detected in response to anti-TCR mAb and ICAM-1 for these cells, while coimmobilized B7.1 gave a strong response (data not shown). Thus, ICAM-1-dependent costimulatory signaling in CD8⁺ T cells can be seen at the level of JNK up-regulation. The failure of ICAM-1 to increase the TCR-dependent up-regulation of ERK strongly supports the conclusion that the LFA-1/ICAM-1 interaction is not simply increasing TCR occupancy by increasing adhesion.

Up-regulation of PI 3-kinase activity in response to ICAM-1-dependent costimulation and inhibition of ICAM-1-dependent costimulation by Wortmannin

One of the signaling events that occurs in response to CD28 ligation is activation of PI 3-kinase (5–7). Total PI 3-kinase activity in CD8⁺ cells costimulated with either B7.1 or ICAM-1 was examined by stimulating cells for 5 min with microspheres having anti-TCR mAb alone, or along with either B7.1 or ICAM-1. Cells were then lysed with detergent and PI 3-kinase was immunoprecipitated using an Ab specific for the p85 subunit of the enzyme. PI 3-kinase activity in the immunoprecipitates was then determined using PI as the substrate. Stimulation of CD8⁺ T cells with anti-TCR mAb alone does not result in a detectable increase in total activity (34). Costimulation with B7.1 resulted in a large increase in activity, and costimulation with ICAM-1 resulted in a smaller but still significant increase in activity (Fig. 5A). ICAM-1 consistently stimulated smaller increases in p85 activity than did B7.1 in these experiments.
Although stimulation with anti-TCR mAb alone does not result in a significant increase in total cellular PI 3-kinase activity measured in anti-phosphotyrosine precipitates (34), cross-linking just the TCR with Ab has been shown to cause an increase in PI 3-kinase associated with the fyn protein tyrosine kinase in T cells (39). To determine whether the costimulatory ligands affected this fyn-associated PI 3-kinase activity, cells were stimulated with microspheres for 5 min, detergent lysed, the fyn immunoprecipitated, and PI 3-kinase activity in the immunoprecipitates determined. As expected, fyn-associated PI 3-kinase activity increased in response to beads having just anti-TCR mAb on the surface (Fig. 5, B and C). No significant change in the level of activity occurred when B7.1 was also present on the beads, while the presence of ICAM-1 consistently resulted in a decrease in fyn-associated activity. These results demonstrate that costimulation by either B7.1 or ICAM-1 results in increased cellular PI 3-kinase activity, with a smaller increase occurring in response to ICAM-1. In addition, no increase in fyn-associated PI 3-kinase activity, above that which occurs in response to ligation of just the TCR, is seen when B7.1 or ICAM-1 is present; in fact, ICAM-1 results in a decrease in the fyn-associated activity. This result lends support to the conclusion that costimulation by ICAM-1 does not simply result from increased adhesion and consequently higher TCR engagement; were this the case, fyn-associated PI 3-kinase activity would be expected to increase when ICAM-1 is present.

The importance of CD28-mediated activation of PI 3-kinase in costimulating T cell proliferation has remained unclear (reviewed in Ref. 40). Studies of the effects of specific inhibitors of PI 3-kinase, wortmannin, and LY294002 have yielded conflicting results. In some experimental systems, blocking of CD28-dependent responses was found, while in others the inhibitors had no effect. In the system used in this study, purified B7.1 coimmobilized with anti-TCR mAb on microspheres, the inhibitors do not block B7.1-dependent up-regulation of IL-2 production or proliferation (Fig. 6) (34). In contrast, wortmannin (Fig. 6) and LY294002 (data not shown) completely block proliferation in response to ICAM-1-dependent costimulation. While results obtained using pharmacologic agents as inhibitors must be interpreted with caution, these results demonstrate that while B7.1 and ICAM-1 activate some of the same signaling pathways, they are clearly not identical in all respects.

Discussion

LFA-1 is an adhesion receptor that can mediate strong binding of T cells to ICAM-bearing cells, or to purified ICAM ligand on a surface (23, 24). This raises the possibility that LFA-1/ICAM interaction might enhance responses to Ag or immobilized anti-TCR Abs by increasing the adhesion between the surfaces and thus increasing TCR occupancy levels. There is also considerable evidence, however, that LFA-1 binding to ICAM can generate transmembrane signals. Signaling events previously shown to be affected by coimmobilized ICAM-1 or anti-LFA-1 Ab include prolonged inositol phospholipid hydrolysis, sustained increase in intracellular Ca\(^{2+}\) levels (25), and prolonged phosphorylation of phospholipase C\(_{\gamma}\) (26). The polyphosphoinositide hydrolysis pathway is activated by the TCR, and these studies thus show that LFA-1 engagement can modify TCR-dependent signaling, but do not provide evidence for a unique costimulatory signal being generated by LFA-1.

Some evidence for unique signaling via LFA-1 is provided by experiments demonstrating activation of tyrosine phosphorylation upon LFA-1 engagement (27, 28), but it has not been determined

FIGURE 5. Total PI 3-kinase activity, but not fyn-associated PI 3-kinase activity, is increased in response to costimulation with ICAM-1. A, CD8\(^{+}\) T cells were stimulated with microspheres coated with either F23.1 alone (lane 1), F23.1 plus B7.1 (lane 2), or F23.1 plus ICAM-1 (lane 3), and incubated at 37°C for 5 min. Following stimulation, cells were lysed, p85 immunoprecipitated using anti-p85 Ab, and activity determined as described in Materials and Methods. PI 3-P was quantitated by densitometry of autoradiographs, and the relative densities are shown. B, CD8\(^{+}\) T cells (10\(^{7}\)) were stimulated with microspheres coated with either BSA (lane 1), F23.1 alone (lane 2), F23.1 plus B7.1 (lane 3), or F23.1 plus ICAM-1 (lane 4), and incubated at 37°C for 5 min. Following stimulation, fyn was immunoprecipitated and PI 3-kinase activity in the precipitates was determined as described in Materials and Methods. C, Summary of results obtained in three independent experiments measuring fyn-associated PI 3-kinase as in B, with autoradiographs quantitated by densitometry. Open circles are the values obtained in the individual experiments, and the horizontal lines represent the averages.

FIGURE 6. Wortmannin inhibits proliferation in response to ICAM-1 costimulation, but not B7.1 costimulation. CD8\(^{+}\) T cells were placed in culture in the absence (shaded bars) or presence (filled bars) of 50 nM wortmannin and stimulated with microspheres having the indicated proteins immobilized on the surface. Proliferation was measured by pulsing with \(^{3}H\)thymidine for the last 8 h of a 48-h culture period. Bars represent the SDs of triplicate samples. The experiment shown is representative of four independent experiments.
whether this is related to increased IL-2 production or cell proliferation. Efforts to understand the basis for CD28-mediated costimulation have focused on signaling pathways that are not activated when TCR alone is engaged, but are activated when CD28 is also engaged, and several such pathways have been identified (1–3). It appeared likely that some of these same pathways might be activated by LFA-1 engagement on CD8+ T cells, since B7.1 and ICAM-1 costimulate these cells comparably with respect to proliferation (Fig. 1) and IL-2 production (30). The results described in this work demonstrate this to be the case.

Acidic sphingomyelinase (A-SMase) is a signaling enzyme up-regulated in response to CD28-dependent costimulation, but not TCR engagement alone; activity increases 1.5–3-fold within 2–3 min of stimulation and then returns to basal resting levels (8, 9). A-SMase cleaves sphingomyelin to yield phosphatidylcholine and ceramide, a lipid messenger intermediate, and a variety of evidence implicates the involvement of this pathway in costimulation, at least in part through activation of NF-kB. Addition of exogenous A-SMase (8, 9) or a cell-permeable analogue of ceramide (9) substitutes for CD28 ligation in stimulating T cell proliferative responses to anti-TCR Ab, overexpression of A-SMase in Jurkat T cells can substitute for CD28 in activating NF-kB (8), and inhibition of A-SMase activity by addition of chloroquine to cells inhibited NF-kB activation in response to B7.1 (10). Stimulation of CD8+ T cells with B7.1 and anti-TCR mAb on microspheres resulted in a rapid and transient activation of A-SMase activity (Fig. 3) comparable with that previously reported (8, 9). Costimulation with coimmobilized ICAM-1 increased A-SMase activity with a similar time course and to a similar extent as B7.1 (Fig. 3). This raises the possibility that LFA-1-dependent costimulation of CD8+ T cells may involve activation of this pathway to increase NF-kB levels, and thus increase transcription of the IL-2 gene.

The AP-1 transcription factor is also involved in regulating IL-2 gene transcription, and has been shown to increase in response to CD28-dependent costimulatory signaling (15). MAPKs are involved in formation of active AP-1 complexes, with ERK activity contributing to production of the functional fos subunit and JNK activity enhancing transactivation by the jun subunit. ERK activity is up-regulated in response to TCR ligation, and is not further up-regulated when CD28 is also engaged. In contrast, JNK activity is strongly up-regulated in response to CD28-dependent costimulation (15, 35). When B7.1 and ICAM-1 were compared for their effects on CD8+ T cells, neither ligand stimulated an increase in ERK activity above that seen in response to TCR ligation alone, while both stimulated comparable increases in JNK activity (Fig. 4). Thus, like the A-SMase lipid intermediate pathway, activation of this more distal component of the signaling pathways leading to up-regulation of IL-2 gene transcription appears to be a shared feature of costimulation by CD28 and LFA-1 in CD8+ T cells.

Activation of total cellular PI 3-kinase was one of the first signaling events shown to be up-regulated by CD28, but not by the TCR, and the cytoplasmic domain of CD28 includes a motif that mediates binding of this kinase (6–8). Activation of this enzyme is also observed in response to costimulation with ICAM-1 when total cellular PI 3-kinase is examined by immunoprecipitation of the p85 subunit from stimulated cells (Fig. 5A). A further effect of ICAM-1 costimulation on PI 3-kinase was observed when association of PI 3-kinase with fyn src tyrosine kinase was examined, an effect not shared with B7.1. TCR ligation results in an increase in PI 3-kinase activity associated with fyn (Fig. 5, B and C) (39). This increase was unaffected by coimmobilized B7.1, but was decreased substantially when ICAM-1 was coimmobilized with the anti-TCR mAb (Fig. 5C). The mechanism by which LFA-1 causes a decrease in fyn-associated activity is not known, but possibilities include conversion of the enzyme to a detergent-insoluble form or activation of a phosphatase. Thus, while both costimulatory ligands increase total p85 activity, they have differing effects on the TCR-dependent increase in fyn-associated PI 3-kinase activity. These results suggest that PI 3-kinase activity, if it functions in B7.1- or ICAM-1-mediated costimulation, is regulated by these costimulators in different ways.

The PI 3 phosphates produced by PI 3-kinase have been implicated as having several second messenger roles, but the importance of this pathway in costimulation by CD28 remains uncertain (reviewed in Ref. 40). Studies of the effects of specific inhibitors of PI 3-kinase, wortmannin, and LY294002 have yielded conflicting results, blocking in some cases and having no effect in others. When costimulation is provided by purified B7.1 coimmobilized with anti-TCR mAb on microspheres, the inhibitors do not block up-regulation of IL-2 production or proliferation (Fig. 6) (34). In contrast, they completely block proliferation when ICAM-1 is the costimulating ligand (Fig. 6), although IL-2 mRNA up-regulation and protein production still occur (Ni et al., manuscript in preparation). These results suggest, but do not prove, that PI 3-kinase activity may be important in LFA-1 signaling for costimulation. The inhibitory effects of pharmacologic agents must be interpreted with caution with respect to the specificity of the inhibitory mechanism. What these results do clearly demonstrate, however, is that B7.1 and ICAM-1 are not identical in their signaling for costimulation of proliferation.

Cloned effector CD8+ T cells undergo degranulation in response to TCR ligation with anti-TCR Ab or class I Ag, and interaction of LFA-1 with ICAM-1 can enhance this response. In this case, LFA-1 appears to contribute predominantly by increasing adhesion between the surfaces (41, 42). In contrast, the results described in this work argue strongly that LFA-1 delivers costimulatory signals to resting CD8+ T cells that are distinct from the signals generated by the TCR, and that act along with the TCR-dependent signals to activate IL-2 production and proliferation. If the interaction of LFA-1 with ICAM-1 were contributing simply by increasing adhesion and thus TCR occupancy, then the signals generated in response to TCR ligation alone would be expected to increase when ICAM-1 is coimmobilized on the surface, but this is not observed. Fyn-associated PI 3-kinase activity, and ERK activity are increased in response to just anti-TCR mAb stimulation, but neither is further increased in the presence of ICAM-1 (Figs. 4 and 5A).

Instead of increasing the levels of TCR-dependent signals, our results show that the LFA-1/ICAM-1 interaction activates distinct signaling pathways, including p85 PI 3-kinase, A-SMase, and JNK: pathways that are also up-regulated by CD28 and strongly implicated in costimulatory signaling in response to B7 ligands. Although LFA-1 and CD28 activate some signaling pathways in common, they do not appear to have identical effects. The response to costimulation by ICAM-1 is blocked by wortmannin, while the response to B7.1 is not (Fig. 6), and ICAM-1 decreases the TCR-dependent increase in fyn-associated PI 3-kinase activity, while B7.1 does not (Fig. 5). In addition, the extent of clonal expansion that occurs in response to ICAM-1 costimulation is not as great as that in response to B7.1; proliferation is comparable in response to both, but cells survive better when costimulation is with B7.1 (30). Finally, coimmobilization of both B7.1 and ICAM-1 along with anti-TCR mAb has a synergistic effect on IL-2 production, yielding levels of IL-2 that are not achieved with optimal densities of either ligand alone (30). Thus, while LFA-1 and CD28 activate in common several signaling pathways that may be involved in their costimulatory function, they clearly differ in significant respects. These differences probably provide the explanation for the synergy
observed by us and others (30, 43, 44) when B7.1 and ICAM-1 are present together, as they would be on fully activated professional APC.

Acknowledgment
We thank Paul Champoux for excellent technical assistance.

References