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LFA-1 binding to ICAM-I provides a costimulatory signal for CD8+ T cell activation that results in increased IL-2 mRNA levels and protein production to support proliferation. CD28 binding to its B7 ligands has the same effect, and the two costimulatory receptors activate some of the same intracellular signaling events, including up-regulation of phosphatidylinositol (PI) 3-kinase activity. However, costimulation by LFA-1 depends upon the activity of this enzyme, whereas costimulation by CD28 does not, as evidenced by differential effects of specific inhibitors of PI 3-kinase. When cells are costimulated with ICAM-1 in the presence of the inhibitors wortmannin or LY294002, proliferation is blocked, but increases in IL-2 mRNA levels and protein production are not. Costimulation also results in increased surface expression of CD25, which is essential for formation of an active IL-2R. This is blocked by the PI 3-kinase inhibitors when costimulation is via LFA-1 but not when it is via CD28. Finally, IL-2-driven proliferation is not blocked by the inhibitors once CD25 surface expression has increased. Thus, the PI 3-kinase-dependent step in CD8 T cell costimulation by LFA-1 is up-regulation of IL-2R expression. In contrast, CD28 engagement also increases IL-2R surface expression, but the up-regulation does not depend upon PI 3-kinase activity. The Journal of Immunology, 2001, 166: 6523–6529.

Activation of T cells is initiated by specific TCR recognition of Ag, but in most cases this is not sufficient to fully activate the cells to proliferate. Instead, additional interactions of receptors on T cells and ligands on APC are required to provide costimulatory signals for complete activation (1). The “second signal” provided by these costimulatory receptors stimulates production of IL-2, which is then used by the cell as an autocrine growth factor. A number of receptors on T cells can provide costimulatory signals, with the most thoroughly characterized being the CD28R that binds B7.1 and B7.2 ligands that are expressed on APC (2–7). LFA-1, a β2 integrin, can also provide costimulation for T cells upon binding its ICAM (8–16). This may be a particularly important costimulatory receptor for CD8+ T cells, because virtually all cells express class I MHC Ags, and ICAMs are widely expressed on both hematopoietic and nonhematopoietic cells rather than being limited to expression on professional APCs (17). In fact, ICAM-1 is much more effective in costimulating CD8+ T cells than in costimulating CD4+ T cells (13, 15).

The signaling pathways activated by costimulatory receptors and required for IL-2 production and proliferation are poorly understood. Again, CD28 has been most extensively studied, and numerous signaling events become activated when CD28 is either cross-linked with Ab or binds B7 ligand. One of the first to be demonstrated was recruitment and activation of phosphatidylinositol (PI) 3-kinase (18–20). When PI 3-kinase is activated, it phosphorylates PIs at the 3 position on the inositol ring, generating PI-3-monophosphate, PI-(3,4)-biphosphate, and PI-(3,4,5)-triphosphate, which have been suggested to act as physiological intracellular mediators (21). However, whether PI 3-kinase activation is important in signaling for costimulation of T cell proliferation has remained unclear, with conflicting results being obtained depending upon the experimental system being examined (reviewed in Ref. 22).

We examined the role of PI 3-kinase activation in B7-dependent stimulation of murine T cells using microspheres that had anti-TCR mAb and purified B7.1 ligand coimmobilized on the surface. This approach eliminates the potential for contributions from other receptor-ligand interactions, a potential that exists when cells are used as the stimulus. We found that specific inhibitors of PI 3-kinase completely inhibited the up-regulation of its enzymatic activity in response to B7.1-dependent costimulation but had no effect on IL-2 production or proliferation in response to this (23). Recently, we found that costimulation of CD8+ T cells with ICAM-1 also up-regulates PI 3-kinase activity (24). The results described here demonstrate that, in contrast to B7.1-dependent costimulation, the activity of PI 3-kinase is required for costimulation by LFA-1 binding to ICAM-1. This requirement appears to be at the level of IL-2R expression; blocking the enzymatic activity does not block production of IL-2 or proliferation in response to IL-2 after the IL-2R is expressed.

Materials and Methods

Mice and cell lines

Female C57BL/6 mice, 6–12 wk old, were purchased from Charles River Breeding Laboratories (Wilmington, MA). IL-2-deficient mice (C57BL/6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were maintained in a specific pathogen-free environment at the University of Minnesota (Minneapolis, MN). CTLL-2 cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 medium.

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3 Abbreviation used in this paper: PI, phosphatidylinositol.
**ROLE OF PI 3-KINASE IN LFA-1-DEPENDENT COSTIMULATION**

B7.1 (25) and ICAM-1 (26) ligands were purified by mAb affinity chromatography as previously described. Native B7.1 was purified from a Chinese hamster ovary-B7.1 transfectant (27), and a soluble form of ICAM-1 was purified from a transfected cell line (28). Microspheres for T cell stimulation were prepared by immobilizing B7.1, ICAM-1, F23.1 anti-TCR mAb (29), and BSA on the surfaces in various combinations. In all cases, the protein was covalently attached using glutaraldehyde (12). The amount of protein was determined spectrophotometrically and used to optimize the ratio of ligand to microsphere diameter. BSA was added to ensure that the size of the microsphere (12) was greater than the size of the T cells. The microspheres were then finished at 72°C for 10 min. followed by 28 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and final PCR products were analyzed by agarose gel to confirm the presence of a single band.

**CD8\(^+\) T cell purification and proliferation assay**

Lymph nodes were harvested from 6- to 12-wk-old female C57BL/6 mice, and CD8\(^+\) T cells were isolated by depletion followed by negative selection on Biotex columns (Edmonton, Alberta, Canada). Cells purified in this way are routinely >95% CD8\(^+\) T cells and <1% CD4\(^+\) T cells. The purified cells were resuspended in complete RPMI 1640 medium and plated in triplicate in 96-well flat-bottom culture plates at 5 \times 10^5 cells/well along with 10^5 microspheres in a final volume of 0.2 ml/well. Cultures were incubated at 37°C for 48 h and pulsed with 1 \mu Ci/well of [\(^{3}H\)]thymidine for the last 6–8 h. PI 3-kinase inhibition experiments were performed by pretreating cells with either 50 nM wortmannin (Sigma, St. Louis, MO) or 5 \mu M LY294002 (Lilly Research Laboratories, Indianapolis, IN) at 37°C for 10 min, followed by addition of microspheres.

**PI 3-kinase activity assay**

Cells (10^5/sample) were prepared as described above and incubated with 5 \times 10^5 microspheres at 37°C for 5 min and lysed in kinase lysis buffer (23). Cell lysates were then immunoprecipitated with anti-p85 antiseraum, and PI 3-kinase activity assay was performed as previously described (23).

**IL-2 protein and mRNA measurements**

Purified CD8\(^+\) T cells were stimulated as described above for cell proliferation assays. After 48 h, 0.5-ml aliquots of medium were collected from the cultures and analyzed by ELISA for murine IL-2. Reagents for the ELISA were obtained from BD PharMingen (San Diego, CA), and the assay was performed according to the protocol provided by the supplier. Recombinant murine IL-2 was used as the standard for quantitation.

For RT-PCR determination of IL-2 mRNA, total RNA was isolated from 2 \times 10^6 cells stimulated with 2 \times 10^6 microspheres for 48 h, using the TRizol system (Life Technologies, Grand Island, NY). Using total RNA as template and oligo(dT) as primer, the single-strand cDNA were synthesized using the Superscript premplification system (Life Technologies). The primers used included: for IL-2, sense, 5'-TCCACTTCACAGCTTACACCGGGAAAGCA-3', and antisense, 5'-TGGAGGCTTTGTGCTAAGATGATGCTGGT-3'; and for \(\beta\)-actin, sense, 5'-ATGGATGACGATATCGTCTGC-3', and antisense, 5'-GCTGGAAAGTGGACAGCTGAG-3'. PCR amplification was performed in pH 8.4 reaction buffer containing 10 pmol of each primer, 20 mM Tris-HCl, 50 mM KCl, 100 \mu M each dNTP, 6 mM MgCl\(_2\), and 1 U AmpliTag DNA polymerase (Perkin-Elmer, Norwalk, CT) in a volume of 0.05 ml. IL-2 DNA was denatured at 94°C for 10 min, followed by 28 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, then finished at 72°C for 10 min. \beta-actin RT-PCR was done in the same way, with the exception that 20 cycles were performed. Final PCR products were visualized using 1% agarose gels and staining with ethidium bromide.

**Immunofluorescence staining for CD25 expression**

CD8\(^+\) T cells were prepared and stimulated as described above. After stimulation for 48 h, cells were collected and stained for IL-2R \(\alpha\)-chain using FITC-labeled anti-CD25 mAb (BD PharMingen) or FITC-labeled rat IgG2a Ab as an isotype control Ab. Cells were also stained with 7-amino actinomycin D (Sigma) to allow gating for analysis of only viable cells (31). Analysis was done using a FACScan flow cytometer and CellQuest software (BD Biosciences, San Jose, CA).

**Results**

Costimulation of proliferation by LFA-1, but not by CD28, is blocked by PI 3-kinase inhibitors

Microspheres that have just F23.1 anti-TCR mAb on the surface stimulate minimal proliferation of CD8\(^+\) T cells, but coimmobilizing ICAM-1 on the same surface results in a strong proliferative response (13). ICAM-1 also has a significant effect on the up-regulation of total cellular PI 3-kinase activity, as determined by measuring the in vitro enzymatic activity of immunoprecipitates prepared from whole-cell lysates using an Ab specific for the p85 subunit of the enzyme. Some activity is present in cells stimulated with beads that have just F23.1 mAb on the surface (Fig. 1A, lane 2), but this is no greater than in cells stimulated with BSA-coated beads (Ref. 23 and Fig. 1A, lane 1). In contrast, a substantial increase in enzymatic activity is found when stimulation is with beads that have both F23.1 and ICAM-1 on the surface (Fig. 1A, lane 3). Wortmannin specifically inhibits PI 3-kinase when used at concentrations below 100 nM and has an IC\(_{50}\) for the enzyme in the range of 20 nM (32). Incubation of CD8\(^+\) T cells with 50 nM wortmannin for 10 min before stimulation with microspheres completely eliminated PI 3-kinase up-regulation in response to ICAM-1 and F23.1 (Fig. 1A, lane 4).

Up-regulation of PI 3-kinase activity by coimmobilized ICAM-1 and inhibition by wortmannin (Fig. 1A) is a result very similar to the that obtained when B7.1 was examined in the same way (23). Despite this, wortmannin had no effect on the B7.1-dependent proliferation of CD8\(^+\) T cells that occurred in response to beads bearing F23.1 and B7.1 (Fig. 1B). In contrast, wortmannin potently inhibited proliferation costimulated by ICAM-1 (Fig. 1B). F23.1 alone on beads stimulated only a minimal response, and ICAM-1 alone stimulated no detectable response. However, substantial proliferative response was obtained when both were present on the microspheres, and this response was almost completely eliminated by wortmannin. As expected, wortmannin had no effect when costimulation was with B7.1 (Fig. 1B). Thus, inhibition of the ICAM-1-dependent response is clearly not due to nonspecific toxicity of the drug.

Further evidence for a role for PI 3-kinase activity in ICAM-1-mediated costimulation was obtained using the inhibitor LY294002, which is structurally unrelated to wortmannin (33). At a concentration of 1 \mu M, LY294002 had little effect on proliferation but caused potent inhibition at 5 \mu M (Fig. 1C). Thus, costimulation with ICAM-1 up-regulates PI 3-kinase activity (Fig. 1A), and inhibiting the activity of this enzyme inhibits the proliferative response to anti-TCR mAb and ICAM-1 (Fig. 1, B and C).

Costimulation by LFA-1 depends upon IL-2 production and use

Costimulation of CD8\(^+\) T cells with ICAM-1 results in IL-2 production, and the cells can then presumably use this as an autocrine growth factor to support proliferation (13). That proliferation does in fact depend upon IL-2 is demonstrated by the finding that the addition of anti-IL-2 mAb PC61.5.3 reduces proliferation in response to beads that have F23.1 and ICAM-1 down to the minimal level seen when the beads have just F23.1 on the surface, whereas a control Ab has no effect (Fig. 2A). IL-2 dependence of the response was further confirmed by examining responses from CD8\(^+\) T cells from mice that were deficient in IL-2. ICAM-1 costimulated a strong proliferative response by cells from wild-type mice, but cells from IL-2--/- mice responded no better to beads with F23.1 and ICAM-1 than they did to beads having just F23.1 on the surface (Fig. 2B). As expected, the cells from the IL-2--/- mice were able to respond when exogenous IL-2 was added to the cultures.
Inhibition of PI 3-kinase activity does not block IL-2 production

The finding that ICAM-1-dependent costimulation of proliferation depends upon an autocrine IL-2 pathway suggested that the role of PI 3-kinase might be at one of three steps: production of IL-2, expression of IL-2R, or signaling through the IL-2R in response to IL-2. Therefore, we examined each of these possibilities. IL-2 mRNA levels were substantially increased in cells stimulated with F23.1 mAb and ICAM-1 on beads in comparison to levels in cells stimulated with beads that had just F23.1 on the surface (Fig. 3A).

Addition of 50 nM wortmannin, a concentration that effectively blocked proliferation (Fig. 1B), did not prevent this increase in response to ICAM-1 (Fig. 3A). Wortmannin also had no significant effect on production of IL-2 protein, as detected by ELISA of culture supernatants that occurred in response to costimulation with ICAM-1 (Fig. 3B). Thus, PI 3-kinase activity is not required for ICAM costimulation-dependent increases in IL-2 mRNA or protein levels.

PI 3-kinase inhibitors block LFA-1-dependent increase in CD25 (IL-2R) expression

Resting T cells express IL-2R β- and γ-chains, but not α-chain (CD25), on their surfaces. Because murine IL-2R requires all three chains to be functionally active, CD25 must be up-regulated on stimulated cells in order for IL-2-dependent proliferation to occur. Beads that had just F23.1 mAb on their surface stimulated some
increase in CD25 expression (Fig. 4A), and this was further increased severalfold when either B7.1 (Fig. 4B) or ICAM-1 (Fig. 4C) were coimmobilized with F23.1 mAb. Although the level of CD25 expression was comparable when either costimulatory ligand was used, the effects of wortmannin were very different; CD25 expression was affected marginally, if at all, when B7.1 was the costimulatory ligand (Fig. 4B), whereas expression was dramatically inhibited when ICAM-1 was the costimulatory ligand (Fig. 4C). Expression of β- and γ-chains did not increase significantly upon stimulation with F23.1 anti-TCR mAb alone or along with B7.1 or ICAM-1 costimulation, and expression levels were not affected by wortmannin (data not shown).

Inhibition of CD25 expression by wortmannin correlated well with its ability to inhibit proliferation in response to ICAM-1 (Fig. 1B) but not in response to B7.1 costimulation (23). Similarly, LY294002 inhibits proliferation (Fig. 1C) and blocks the increase in CD25 expression in response to ICAM-1 costimulation (Fig. 4D). Neither inhibitor completely blocked CD25 expression on the surface, but both consistently caused a large reduction in the level of expression (Table I) when this was assessed as either the number of cells expressing CD25 (Table I, % gated) or the expression level of the positive cells (Table I, Mean).

Because IL-2 at high concentrations can up-regulate IL-2R expression, it was important to determine whether the increase in CD25 upon costimulation with ICAM-1 was due to the IL-2 being produced, and whether the inhibition by wortmannin and LY294002 might therefore be at the level of IL-2 signaling for CD25 up-regulation. This was examined using CD8 T cells from IL-2-deficient mice. ICAM-1 costimulation resulted in an increase in CD25 expression by the IL-2−/− CD8 T cells and was inhibited by wortmannin (Fig. 5B). B7.1 costimulation also increased CD25 expression on these cells and was not affected by wortmannin (Fig. 5C). Thus, inhibition of ICAM-1-induced CD25 expression by PI

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**Table I. Effects of PI 3-kinase inhibitors on ICAM-1-dependent costimulation of CD25 expression**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Treatment</th>
<th>% Positive</th>
<th>Mean Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>25</td>
<td>320</td>
</tr>
<tr>
<td>2</td>
<td>50 nM wortmannin</td>
<td>15</td>
<td>153</td>
</tr>
<tr>
<td>3</td>
<td>50 nM wortmannin</td>
<td>13</td>
<td>169</td>
</tr>
<tr>
<td>4</td>
<td>5 μM LY294002</td>
<td>4</td>
<td>129</td>
</tr>
<tr>
<td>5</td>
<td>5 μM LY294002</td>
<td>31</td>
<td>461</td>
</tr>
<tr>
<td>5</td>
<td>None</td>
<td>31</td>
<td>210</td>
</tr>
<tr>
<td>5</td>
<td>5 μM LY294002</td>
<td>31</td>
<td>163</td>
</tr>
</tbody>
</table>

*CD8 T cells were stimulated for 40 h with beads having F23.1 mAb and ICAM-1 on the surfaces. The cells were then harvested, and immunofluorescence staining was done as described in Materials and Methods.

*Cells were examined by flow cytometry, and the percentages that stained positively for CD25 expression are given based on a gates set as shown in Fig. 4.

*The mean fluorescence of the CD25-stained cells is given.
3-kinase inhibitors is acting at the level of LFA-1-dependent signaling, not IL-2R signaling.

Inhibition of PI 3-kinase activity does not block IL-2R signaling for proliferation

It appeared that signaling for increased CD25 expression on the cell surface might account for the apparent role of PI 3-kinase in ICAM-1-dependent costimulation. However, it was also possible that PI 3-kinase might instead, or in addition, have a role in signaling by the IL-2R upon binding to IL-2. This was particularly important to examine because the inhibitors did not completely eliminate CD25 expression. In addition, a role for PI 3-kinase in signaling by the IL-2R was suggested by the finding that the enzyme is activated upon IL-2 binding in the CTLL-2 cell line (34). However, we found that 50 nM wortmannin did not inhibit the IL-2-dependent growth of the CTLL-2 line (Fig. 6A), nor did concentrations as high as 575 nM (data not shown).

To determine whether wortmannin could inhibit the proliferation of CD8+ T cells in response to IL-2 irrespective of effects on CD25 expression, we took advantage of the fact that CD25 is up-regulated within the first 24 h after stimulation. Wells were prepared that had either F23.1 anti-TCR mAb alone or F23.1 plus ICAM-1 immobilized on the surface. When CD8+ T cells were incubated in the wells for 48 h, a strong proliferative response occurred when ICAM-1 was present but did not occur in its absence (Fig. 6B, condition 1). If the cells were instead incubated in the ligand-bearing wells for 24 h and then transferred to wells coated with BSA for the second 24 h before pulsing with [3H]thymidine, very little proliferation occurred (Fig. 6B, condition 2), unless exogenous IL-2 was added at the time of transfer to the BSA-coated wells (Fig. 6B, condition 3). Thus, the 24-h incubation with F23.1 and ICAM-1 was sufficient to induce CD25 expression so that the cells could respond to IL-2, but continued stimulation during the second 24 h was necessary to allow sufficient IL-2 production for a response to occur. Wortmannin during the first 24 h blocked the ability of transferred cells to respond effectively to exogenous IL-2 during the second 24 h (Fig. 6B, condition 4). However, when cells were incubated for 24 h with ligands, transferred, and both wortmannin and IL-2 were added, the wortmannin did not significantly inhibit the response (Fig. 6B, condition 5). Thus, wortmannin inhibits CD25 expression that occurs during the first 24 h but does not inhibit the IL-2-dependent proliferation once CD25 has been expressed.
Discussion

Both CD28 and LFA-1 can provide “signal two” for CD8 T cell activation, with the costimulatory signal(s) acting along with TCR-dependent signals to increase IL-2 production, IL-2R expression, and IL-2-dependent proliferation. CD28 and its ligands, B7.1 and B7.2, have been most extensively studied, and there is considerable information regarding the signaling pathways activated upon CD28 engagement. Less is known about signaling via LFA-1, in part because its role as a costimulatory receptor has been more difficult to establish. Although it was shown some time ago that LFA-1 interaction with ICAM-1 could enhance TCR-dependent T cell responses (9, 26), the ability of LFA-1 to mediate strong adhesion made it difficult to distinguish enhancement due to increased adhesion and, thus, increased TCR engagement vs costimulatory signal generation. However, there is evidence that engaging LFA-1 can result in generation of transmembrane signals (24, 35–37), and a recent report has shown that it interacts with JAB1, a transcriptional coactivator, resulting in modulation of AP-1 activity (38). In fact, several signaling events that occur upon engaging both CD28 and the TCR also occur when LFA-1 and the TCR are engaged, including up-regulation of the activities of PI 3-kinase, sphingomyelinase, and c-Jun NH2-terminal kinase (24). These are likely candidates for being “costimulatory” signals because they are not up-regulated in response to engaging just the TCR.

Although CD28 and LFA-1 activate several of the same signaling pathways, they do not act identically in costimulation. This was suggested by the fact that engaging both receptors along with the TCR had a highly synergistic effect on T cell activation of IL-2 production (11, 13). Differences were confirmed at the signaling level with the observation that both CD28 and LFA-1 engagement caused an increase in total PI 3-kinase activity, but CD28 had no effect on the TCR-mediated increase in pS970-associated PI 3-kinase activity, whereas LFA-1 caused a decrease in the fyn-associated activity (24).

Furthermore, the importance of PI 3-kinase activity differed for the two receptors: costimulation of proliferation by LFA-1 was blocked by inhibitors of the enzyme, whereas costimulation by CD28 was not (Ref. 24 and Fig. 1).

The dependence of LFA-1-mediated costimulation on IL-2 production and use (Fig. 2) raised the possibility that a step in this pathway was dependent on PI 3-kinase activity (i.e., either IL-2 production, IL-2R up-regulation, or signaling through the IL-2R). IL-2 mRNA or protein levels resulting from costimulation through either CD28 or LFA-1 were unaffected when PI 3-kinase was inhibited using either of the specific inhibitors, wortmannin or LY294002 (Fig. 3). In contrast, the increase in IL-2R expression that occurred in response to costimulation with LFA-1 was strongly blocked by both inhibitors (Fig. 4). Consistent with the lack of effect on CD28-dependent proliferation, the inhibitors did not block the increase in IL-2R in response to costimulation by B7.1 (Fig. 4). That inhibition was at the level of LFA-1 signaling, and not IL-2-dependent signaling for CD25 up-regulation, was confirmed by the demonstration that ICAM-1 costimulation increased CD25 expression on IL-2−/−T cells, and this was inhibited by wortmannin (Fig. 5). This suggested that the role of PI 3-kinase activity in LFA-1-dependent costimulation was at the level of signaling for up-regulation of the IL-2R. However, it remained possible that inhibition of proliferation might also result from a role for PI 3-kinase in signaling through the IL-2R, because binding of IL-2 to its receptor can induce PI 3-kinase activity (34). However, activity of PI 3-kinase does not appear to be important for IL-2R signaling for proliferation in CD8+ T cells because inhibition of the enzyme did not block IL-2R-dependent signaling (Fig. 6).

The results reported here clearly demonstrate that the signaling events that mediate costimulation by CD28 and LFA-1 differ. Recently, Geginat et al. (16) also concluded that the two receptors have an impact on qualitatively different signaling pathways based on their finding that stabilization of IL-2 mRNA by LFA-1 signaling required cytoskeletal integrity, whereas stabilization by CD28 did not. Our results show that, while both CD28 and LFA-1 activate PI 3-kinase upon binding their ligands, activity of this enzyme is only needed for LFA-1-dependent costimulation of IL-2-driven proliferation, and its critical role appears to be in up-regulating expression of the IL-2R. Although CD28-dependent proliferation does not require PI 3-kinase activity, blocking of this activity does prevent CD28-mediated up-regulation of the Bcl-xL survival protein (39). Whether PI 3-kinase activation by LFA-1 might also have a role in up-regulating Bcl-xL, as it does in up-regulating IL-2R, has not yet been examined.

Acknowledgments

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