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Functional Consequences of Costimulation by ICAM-1 on IL-2 Gene Expression and T Cell Activation

Linda A. Zuckerman,† Lara Pullen,† and Jim Miller‡

LFA-1 is a well-recognized adhesion molecule, but its role in providing costimulatory signals to T cells has remained controversial. We have compared the ability of class II-positive transfectants that do and do not coexpress ICAM-1 (ProAd and ProAd-ICAM) to activate Ag-specific Th1 clones and naive CD4-positive T cells isolated from TCR transgenic mice. Ag presentation by ProAd to Th1 clones can induce calcium-dependent signaling events after engagement of the TCR, as evidenced by the nuclear localization of the transcription factors NF-AT and NF-κB. Nevertheless, coexpression of ICAM-1 or B7-1 on ProAd is required to induce detectable levels of IL-2 gene expression in either Th1 clones or naive T cells. In Th1 clones, activation by ProAd-ICAM induces very transient IL-2 mRNA expression that does not result in detectable IL-2 secretion or T cell proliferation. In naive T cells, the duration of IL-2 mRNA expression is longer, allowing for a transient burst of IL-2 protein that is sufficient to drive the cells into the cell cycle. In spite of this initial response, Ag presentation by ProAd-ICAM is a tolerogenic signal to naive T cells, and responding T cells undergo apoptosis 4 to 5 days poststimulation. These data suggest that engagement of LFA-1 can provide sufficient costimulatory signals to induce T cell activation and IL-2 gene expression, but cannot protect against anergy induction or provide for T cell survival.


The surface of an APC contains accessory molecules that seem to be critical for the initiation of a T cell response (for review, see Ref. 1). These accessory molecules serve as ligands for receptors on the surface of T cells and provide two important functions: they provide adhesion to allow for the formation of stable T cell:APC conjugates, and they can provide discrete signals that work in concert with the TCR-signaling apparatus to promote T cell activation and differentiation (costimulation). All proteins that interact between the two cells will provide some contribution to intracellular adhesion. In contrast, only a subset of the accessory molecules has been clearly documented to provide effective costimulatory functions. Dissecting the specific contribution of each of these accessory molecules has been compounded by a number of issues, including the plethora of candidate molecules, the apparent functional redundancy of some of these molecules, the ability of some accessory molecules to impart both positive and negative effects under different conditions, the differences in specific requirements of accessory molecules for different subpopulations of T cells, and the relative import of accessory molecules on different readouts of T cell activation.

The best-defined costimulatory molecules are B7-1 and B7-2, which are ligands for CD28 (for review, see Ref. 2). CD28 costimulation has been shown to play an important role in activation of many different T cell populations, to up-regulate expression of IL-2 and other effector molecules, to protect against the induction of T cell anergy, and to provide important survival signals to newly activated T cells. Many other receptor-ligand pairs have also been implicated in provided costimulatory signals to T cells. These include (receptor/ligand): LFA-1/ICAM-1 (3–7), CD2/CD48 (CD58) (8–11), CD44/invariant chain-chondroitin sulfate (12), unknown/VAM-1 (13), unknown/heat-stable Ag (14), unknown/CD43 (15, 16), and TNFR/TNF family members such as CD70/CD27 (17, 18), CD40L/CD40 (19), 4-1BB/4-1BBL (20, 21), OX-40/OX-40L (22), and CD30/CD30L (23). However, none of these molecules induces all of the functions associated with CD28 signaling; each has some restriction either on the population of T cells that respond or on the specific activation events that can be induced. Nevertheless, the emerging view is that T cells encounter many different accessory molecules during interactions with different APC at different stages of T cell development and differentiation.

One of the important accessory molecules that mediates adhesion between T cells and APC is LFA-1. Adhesion mediated through LFA-1 is highly regulated during T cell activation through two distinct mechanisms (24, 25). First, LFA-1 undergoes a conformational change that has a higher affinity for ICAM-1 (26). Second, LFA-1 undergoes a signal-dependent release and reassociation with the cytoskeleton that results in LFA-1 clustering and a corresponding increase in avidity for ICAM-1 binding (27, 28). In addition to its role in adhesion, LFA-1 has been implicated in providing costimulation (3, 4) and in signal transduction (29–31). However, the relative importance of LFA-1 as a signaling molecule has been controversial, in part because in some studies it has been difficult to distinguish between direct effects on T cell signaling and indirect effects mediated through intercellular adhesion (32–34). For example, costimulation through CD28 can be provided on a surface separate from the TCR stimulus, an observation that has been used to argue that CD28 can transduce an independent signal to T cells. In contrast, although costimulation through

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3 Abbreviations used in this paper: ICAM, intercellular adhesion molecule; DAPI, 4,6-diamidino-2-phenylindole; ETBr, ethidium bromide; FAK, focal adhesion kinase.

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ICAM-1-LFA-1 interactions has been shown to function in a similar fashion in some studies (5, 7), this has not generally been the case (32, 34) (L. P. and J. M., unpublished observation). Thus, it remains unclear whether ICAM-1-LFA-1 interactions are mediating their effect on T cell activation entirely through adhesion or through a combination of adhesion and costimulation. In addition, unlike costimulation by CD28, the ability of LFA-1 to costimulate T cells is restricted to a subset of T cells. For example, LFA-1 engagement appears to have a selective, functional effect on naive T cells and a limited, if any, effect on activated T cells (5–7, 13, 35).

In this study, we have compared the ability of ICAM-1-LFA-1 interactions to provide costimulation to naïve T cells and to Th1 clones. In previous studies, we had found that Ag presentation by class II-positive, ICAM-1-positive transfectants does not induce Th1 T cell proliferation and rather induces T cell anergy (13). In this study, we have found that the same transfectants do induce proliferation from naive CD4-positive T cells, but, as in the Th1 clones, this is a tolerogenic signal, because these naïve T cells ultimately die of apoptosis. Interestingly, the ability of ICAM-1 transfectants to selectively activate naïve T cells does not appear to be a difference in the ability of naïve T cells and Th1 clones to respond to LFA-1-mediated costimulation, but rather a difference in the kinetics of IL-2 gene expression.

**Materials and Methods**

**Cells**

A panel of transfectants in the fibrosarcoma, 6132A-PRO (Pro), expressing I-A/Ad alone or in combination with B7-1 or ICAM-1 (13) and the murine CD4-negative Th1 T cell clone, pGL2 (36), has been described. All cell lines were maintained in DMEM supplemented with 10% FCS, 2 mM glutamine, 0.1 mM nonessential amino acids, 40 μg/ml gentamicin, and 50 μg/ml G418 (200 μg/ml) and/or MXH (250 μg/ml xanthine, 15 μg/ml hypoxanthine, and 6 μg/ml mycophenolic acid) were added to the culture media for maintenance of the transfectants. The pGL2 cells were maintained by weekly passage with irradiated BALB/c spleenocytes, 400 μg/ml OVA, 10 to 20 U/ml human rIL-2, and 200 U/ml rIFN-γ. CD4-positive T cells were purified from lymph nodes of DO11.10 TCR transgenic mice (37, 38) by negative selection. Class II-positive cells (using a mixture of mAbs M5/114, 25-9-17, and 3JP) and CD8-positive T cells (using mAb 53-6.7) were depleted by complement lysis (1/10 dilution of guinea pig complement from Accurate Chemical, Westbury, NY), followed by an equal number of sheep anti-mouse- and sheep anti-rat-coated M450 Dynal magnetic beads (Dynal, Oslo, Norway). Purity of CD4-positive T cells was determined by flow cytometry and/or by the lack of proliferation to 10 μg/ml Con A (Sigma Chemical, St. Louis, MO).

Flow cytometry

The transfectants were monitored for class II expression by binding to the Ab MKD6 (American Type Culture Collection (ATCC), Rockville, MD), ICAM-1 expression by binding to the Ab YN-1.7.1 (Abcam, and B7-1 by binding to CTLA4 Ab (Repligen, Cambridge, MA). Indirect immunofluorescence was performed with the appropriate FITC-coupled secondary Ab, FITC-coupled goat anti-mouse, and FITC-coupled goat anti-rat Ab. Nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma Chemical) at 1 μg/ml in PBT (PBS containing 1% Triton X-100). The cells were viewed by fluorescence microscopy using an Olympus BX-40 microscope with a 100-W mercury lamp, a x40 Fluorite objective, N.A. 0.75 (Leco #1-UBx), and a UV filter excitation (ex 330–385 nm at 5-nm wide band pass, Leco #U-M536). Images were photographed using an Oceanoptics low-light video camera (Leco #DEI-470TB) with a x2 coupler (Leco #HR200-CMT) and printed on a Sony UP 1800 MD printer.

**Western blot analysis**

To assay for the presence of Bcl-xL, 2.5 to 10×10^6 purified CD4-positive lymph node T cells were incubated with 10×10^5 transfectants overnight in the presence and absence of Ag. Cells were harvested and lysates were prepared, as previously described (40). Briefly, cells were lysed with NET-N (100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl (pH 8), and 0.2% Nonidet P-40), nuclei were pelleted, and the supernatants were adjusted to 2% SDS, boiled, and loaded onto 12.5 polyacrylamide gels for electrophoresis. Proteins were transferred for at least 5 h at 150 mA to nitrocellulose for blotting. Blots were blocked for at least 3 h in 5% nonfat milk with 1× PBS. Twine and developed with rabbit antiserum (1:5,000) or mAb against Bcl-xL (kindly provided by Dr. Craig Thompson, University of Chicago, Chicago, IL). Western blots were developed using enhanced chemiluminescence (Amersham, Arlington, Heights, IL).

**cDNA and RT-PCR assays**

Th1 clones or naïve CD4-positive lymph node T cells (2.5×10^6) were stimulated with an equal number of transfectants in the presence or absence of peptide Ag. RNA was isolated using 1 ml of TriZol reagent (Life Technologies, Gaithersburg, MD), and an aliquot of the RNA was electrophoresed on a 1% agarose/formaldehyde gel to determine quality and estimate quantity, as determined by EtBr staining of the 28S and 18S ribosomal RNA bands. Three to five micrograms of the extracted RNA were utilized to make cDNA using random hexamer primers and Superscript II reverse transcriptase (Life Technologies). The resulting cDNA was diluted 1/5 before use in PCR reactions. PCR was conducted essentially as described (41). Briefly, 1 to 5 μl of diluted cDNA was added to a 100 μl reaction mixture containing 10 mM Tris-Cl, 1.5 mM MgCl_2, 50 mM KCl, pH 8.3, 0.4 μM sense and antisense primers, 0.1 mM dNTPs, and 2.5 U Taq polymerase (Boehringer Mannheim, Indianapolis, IN). In most experiments, a control cDNA was added to the reaction as a positive control for the PCR reaction. Taq polymerase is inhibitory to the 32P-labeled 32 mer antisense primer at these conditions (34°C for 45 s, 60°C for 15 s, and 72°C for 45 s). The total number of cycles was 41. The resulting product was electrophoresed on a 2.5% agarose gel and stained with ethidium bromide. The quantity of hypoxanthine phosphoribosyltransferase (HPRT) PCR product in different samples was used to normalize the samples for equivalent amounts of cDNA. The HPRT reactions were done in the presence of a competitor cDNA to ensure linear amplification of PCR products (41). In some experiments, samples were also normalized to the amount of CD36 mRNA to ensure that equivalent amounts of T cell cDNA were assayed.

**Nuclear extracts and gel shifts**

Th1 clones were stimulated with Ag presented by Pro transfectants for 6 h. During this time, the majority of the transfection cells are lysed by the cytolytic Th1 cells (42), and the activated T cells have adhered to the plastic dish. Remaining transfectants were washed away and the T cells were briefly trypsinized before harvesting for assays. Only experiments that had greater than 75% recovery of total input T cells were considered for further analysis. The extracts themselves were made according to McCormick et al. (43) with few differences. Upon harvesting, the cells were resuspended at 2.5×10^7/ml in 10 mM HEPES, 1.5 mM MgCl_2, 10 mM KCl, 0.5 mM DTT, and 0.5 mM PMSF, and the cells were lysed by the addition of 0.1% Nonidet P-40 for 15 min at 4°C. The nuclei were collected by centrifugation and extracted with a mixture of 10 mM HEPES, 1.5 mM MgCl_2, 50 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 0.1% Nonidet P-40, and 25% glycerol for 30 min at 4°C. Cellular debris was pelleted, and supernatants were frozen and stored at −80°C. Protein was quantitated in nuclear extracts using the Bio-Rad Protein Assay (Richmond, CA) protein assay, and equivalent amounts of protein (5–10 μg) were used for each assay condition. The binding reactions were performed as previously described (44, 45). Between 5 and 10 μg of protein was used per reaction in a buffer containing 10 mM Tris (pH 7.5), 50 mM

**DAPI staining**

CD4-positive lymph node T cells (0.3–0.5×10^6) were incubated in 24-well plates with an equal number of transfectants for 3, 4, or 5 days. After the incubation, cells were harvested and spun down and resuspended in 100 μl of PBS or complete media. Aliquots of cells (50 μl) were mixed with 10 μl of DAPI (4′′,6-diamidino-2-phenylindole; Sigma Chemical) at 1 μg/ml in PBT (PBS containing 1% Triton X-100). The cells were viewed by fluorescence microscopy using an Olympus BX-40 microscope with a 100-W mercury lamp, a x40 Fluorite objective, N.A. 0.75 (Leco #1-UBx), and a UV filter excitation (ex 330–385 nm at 5-nm wide band pass, Leco #U-M536). Images were photographed using an Oceanoptics low-light video camera (Leco #DEI-470TB) with a x2 coupler (Leco #HR200-CMT) and printed on a Sony UP 1800 MD printer.
Costimulation through either B7-1 or ICAM-1 can induce proliferation of naive CD4-positive T cells. ProAd (triangles), ProAd-ICAM (squares), or ProAd-B7 (circles) were cocultured with the Th1 T cell clone, pGL2 (A), or with CD4-positive lymph node T cells purified from DO11.10 TCR transgenic mice (B) in the presence of varying concentrations of OVA peptide for 48 h. Thymidine incorporation was measured during the last 18 h of the assay. Data are represented as cpm × 10⁻³. Similar results on the ability of the Pro transfectants to activate Th1 clones have been published (13) and are shown here for comparison.

NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, and 4 μg polyd[dIdC] (Pharmacia, Piscataway, NJ). The reactions were incubated at room temperature for approximately 15 min with 10,000 cpm double-stranded [3H]-labeled oligonucleotides. After the binding reactions, the samples were subsequently electrophoresed on 4% polyacrylamide gels in the following buffer: 25 mM Tris, 190 mM glycine, and 1 mM EDTA. The oligonucleotides used in these studies were taken from the murine IL-2 gene (the 5'TT was added to the OCT site to increase radiolabeling) and were monomers of the binding sites: NF-AT (GCCCAAAGGGAAATTGTTCATACAG), OCT-1/2 (TTTATGTAAAACATCGTG), and NF-κB (AGAGGGATTTCACCTAAATCC).

Results

Costimulation through either B7-1 or ICAM-1 results in Ag-specific thymidine incorporation by naive CD4-positive T cells, but not Th1 clones

To determine whether costimulation through interaction of ICAM-1 and LFA-1 mediated distinct effects in naive T cells and Th1 clones, we assayed the ability of class II-positive transfectants in the costimulation-negative tumor cell line (6132A-PRO) that do and do not coexpress ICAM-1 or B7-1 to activate T cells (13). As predicted from their lack of detectable expression of costimulatory molecules, class II-positive transfectants (ProAd) do not stimulate Th1 T cell clones (Fig. 1A) or naive CD4-positive T cells, purified from the lymph nodes of DO11.10 TCR transgenic mice (Fig. 1B). As a positive control, Ag presentation by ProAd supertransfected with B7-1 (ProAd-B7) results in efficient activation of both naive T cells and Th1 clones (Fig. 1). In contrast, Ag presentation by ProAd cells supertransfected with ICAM-1 (ProAd-ICAM) does not induce proliferation of Th1 clones, but does stimulate proliferation of naive T cells (proliferation in this case is measured by [1H]thymidine incorporation, but see below). Ab blocking confirmed that ICAM-1 was mediating this effect through interaction with LFA-1 (data not shown). Thus, consistent with previous results (6, 7, 13), B7-1, but not ICAM-1, can function as a costimulatory molecule for previously activated T cells and for Th1 T cell clones. In contrast, Ag presentation in the context of either B7-1 or ICAM-1 is capable of stimulating entry of naive T cells into the cell cycle.

Activation of naive CD4-positive T cells following Ag presentation by ProAd-ICAM leads to cell death, rather than clonal expansion

Although in some experiments Ag presentation by ProAd-ICAM and ProAd-B7 induced similar levels of thymidine incorporation in naive T cells, in most cases Ag presentation by ProAd-ICAM was less efficient than ProAd-B7 (See Fig. 1B). The reduced responses are not due to reduced numbers of responding T cells, because both ProAd-B7 and ProAd-ICAM activated the majority of the cells in the culture and induced similar levels of the T cell activation markers, CD44 and CD69 (Table I). Likewise, propidium iodide staining indicated that similar numbers of T cells were driven into cell cycle in cultures stimulated with ProAd-B7-1 and ProAd-ICAM (data not shown). Reduced proliferation following Ag presentation by ProAd-ICAM did correlate with a 10-fold decrease in IL-2 production compared with ProAd-B7 (Fig. 2). This low level of IL-2 probably accounts for the reduced expression of CD25 (Table I) in the cultures stimulated by ProAd-ICAM, as addition of 50 to 100 U/ml of exogenous IL-2 up-regulated the level of CD25 expression to that seen in cultures stimulated with ProAdB7-1. In contrast, addition of exogenous IL-2–to cultures stimulated with ProAd-ICAM did not compensate for the difference in proliferation compared with T cells stimulated with ProAd-B7 (data not shown). Taken together, these data indicate that costimulation through ICAM-1 can activate naive T cells, but this activation is suboptimal, as evidenced both by reduced cytokine production and reduced proliferation in the presence of excess IL-2.

The inability of ProAd-ICAM to fully activate naive T cells is most dramatically seen when cells are recovered from culture. T cells stimulated with Ag presented by ProAd rapidly die within 1 to 2 days. In contrast, T cells stimulated with ProAd-ICAM and ProAd-B7 equivalently undergo blastogenesis and entry into the cell cycle. However, 3 to 5 days following stimulation, cultures stimulated with ProAd-B7 begin to expand, while T cells stimulated with ProAd-ICAM undergo a rapid demise, and by day 7, no viable cells are recoverable (Fig. 3). This is illustrated by staining T cells with the nuclear stain, DAPI. After 3 days, T cells stimulated with ProAd-B7 and ProAd-ICAM are indistinguishable; both

| Table I. Costimulation with either B7-1 or ICAM-1 up-regulates T cell activation markers |
|-----------------------------------------|----------------|----------------|----------------|
| Mean Fluorescence Intensity of CD4-positive T cells^a |
| Neg^b | CD44 | CD69 | CD25 |
| Unstimulated^c | 5 | 149 | 6^d | 6^d |
| ProAd-B7^c | 9 | 1132 | 213 | 2899 |
| ProAd-ICAM^c | 10 | 1100 | 291 | 846 |

^a Cells were gated for staining with CD4 and the mean fluorescence intensity for the individual markers in the CD4-positive subpopulation is shown.

^b Isotype-matched negative control antibody.

^c Unstimulated cells were freshly isolated lymph node cells from DO11.10 TCR transgenic mice. About 50% of the freshly isolated CD4-positive cells expressed the TCR transgene as indicated by staining with the clonotype specific mAb, KJ1.26.

^d In the unstimulated cells there was a small subpopulation (≤10%) that expressed high levels of these markers. The data shown are the mean fluorescence intensity of the major population.

^e Naive T cells were stimulated for 4 days with 20 μg/ml peptide presented by ProAd-B7 or ProAd-ICAM. At this time, >95% of the T cell express the TCR transgene as indicated by staining with the clonotype specific mAb, KJ1.26. Likewise, >95% of the activated cells express the markers shown.
populations have similar cell number, and the cells appear to be healthy (Fig. 4). In contrast, after 5 days, T cells stimulated with ProAd-B7 remain intact, while T cells stimulated with ProAd-ICAM have condensed and fragmented nuclei, characteristic of cells undergoing apoptosis (Fig. 4).

A number of factors have been implicated in promoting T cell survival, including IL-2 (46–48) and other cytokines and members of the Bcl-2 family, especially Bcl-XL (40). Addition of 50 to 100 U/ml of exogenous IL-2 to naive T cells stimulated with ProAd-ICAM can provide some T cell survival, but does not provide for clonal expansion (Fig. 3). Addition of excess IL-2 to cultures stimulated with ProAd-B7 does not enhance clonal expansion, indicating that IL-2 is not limiting in these cultures. We do not know whether the increased number of T cells in the cultures stimulated with ProAd-ICAM with exogenous IL-2 results from increased survival of individual cells or a shift in the balance of cell division and cell death. Nevertheless, this finding suggests that other factors independent of reduced IL-2 production are required to protect activated naive T cells from cell death. Consistent with this idea, we found that while ProAd-B7-stimulated T cells induced increased levels of Bcl-XL, ProAd-ICAM-stimulated T cells did not (Fig. 5). Together these data suggest that Ag presentation in the presence of ICAM-1 and the absence of B7 can induce T cell activation and entry into the cell cycle, but this stimulation leads to T cell death rather than clonal expansion.

Costimulation through ICAM-1 induces IL-2 transcription, but not accumulation of IL-2 mRNA

The ability of ProAd-ICAM transfectants to induce IL-2 production and proliferation in naive T cells, but not Th1 clones, raised the possibility that costimulation by ICAM-1 had a different effect on IL-2 gene regulation in these T cell populations. To test this possibility, we assayed for IL-2 gene expression by RT-PCR. Naive T cells stimulated with either ProAd-ICAM and ProAd-B7, but not ProAd, induced readily detectable levels of IL-2 mRNA by 2 to 3 h after stimulation (Fig. 6A–C). IL-2 mRNA continued to accumulate in the naive T cells stimulated by ProAd-B7 and was still present at maximum levels at 24 h (data not shown). However, after 3 h, IL-2 mRNA decreased in the ProAd-ICAM-stimulated T cells and was undetectable by 6 h. This pattern of expression is consistent with the level of functional IL-2 protein secreted in these cultures (see above).

Interestingly, a small, but detectable, burst of IL-2 mRNA is also seen in Th1 clones stimulated with ProAd-ICAM at 2 h (Fig. 7A). In comparison, ProAd-B7-stimulated T cell clones induced much higher levels of IL-2 mRNA at 2 h, and the message was detectable for up to 7 h poststimulation (Fig. 7B). To enhance the low level of IL-2 mRNA detected, we added the protein synthesis inhibitor, cycloheximide, to the T cell activation cultures. Cycloheximide-mediated superinduction of gene expression has been
attributed both to transcriptional and posttranscriptional events and can dramatically increase the level of cytokine mRNA (49-54). The low level of IL-2 mRNA expressed in Th1 clones (Fig. 7C) and in naive T cells (data not shown) following stimulation by ProAd-ICAM was increased significantly by superinduction with cycloheximide. In contrast, addition of cycloheximide had no effect on IL-2 mRNA levels following presentation by ProAd-B7 (Fig. 7D), and, notably, did not reveal IL-2 mRNA expression in ProAd-stimulated Th1 clones (Fig. 7C) or naive T cells (data not shown). Thus, it appears that costimulation through ICAM-1/LFA-1 interactions can initiate IL-2 transcription in both naive T cells and Th1 clones; however, only the naive T cells accumulate enough mRNA to produce functional levels of IL-2 protein that are sufficient to induce T cell proliferation.

Ag presentation by ProAd does induce TCR-mediated signal transduction in Th1 clones

Two possible explanations can account for the ability of ProAd-ICAM, but not ProAd, to induce T cells to produce IL-2 mRNA: 1) ICAM-1/LFA-1 interactions function through intracellular adhesion and stabilization of T cell:APC conjugates, allowing more efficient TCR signaling; 2) ICAM-1/LFA-1 interactions function through costimulation, modulating the nature of T cell signaling. The failure to detect IL-2 mRNA following Ag presentation by ProAd even in the presence of cycloheximide suggests that TCR ligation alone is not sufficient to induce IL-2 gene expression (see Fig. 7). However, these data do not indicate whether the TCR was engaged following Ag presentation by ProAd. Previous studies had shown that Ag presentation by ProAd to Th1 clones results in the induction of T cell anergy, an event that requires TCR occupancy (13). One caveat with the anergy induction studies is that it is possible that the level of TCR signaling that is necessary to induce anergy may be significantly less than that required to induce IL-2 transcription or T cell proliferation (55). To address this possible concern, we used gel-shift analysis to assay the expression of two transcription factors, NF-AT and NF-κB, which participate in the up-regulation of IL-2 gene expression. NF-AT is composed of a
constitutively expressed cytosolic factor that, upon a sustained calcium signal, is translocated to the nucleus (56, 57), where it assembles with a newly synthesized AP-1 family dimer to generate a complete complex (58, 59). In contrast, activation of NF-κB by displacement of the cytosolic inhibitor IκB requires a large transient rise in intracellular calcium (57). Thus, expression of these two factors is a stringent assay for effective TCR signaling and the induction of both phases of a calcium response. Resting T cells do not express any NF-κAT, and expression of the intact NF-κAT complex is equivalently induced following activation by ProAd or ProAd-B7 (Fig. 8). Resting T cells express a fast migrating complex is product derived from the control plasmid (C), and the bottom band is the wild-type IL-2 gene product (IL-2).

Discussion

In this work, we have shown that Ag presentation in the context of ICAM-1 and the absence of B7 can induce naive T cell activation, as evidenced by up-regulation of activation markers, induction of IL-2 transcription, and entry into the cell cycle. However, this activation does not appear to be complete; IL-2 mRNA does not accumulate, and [3H]thymidine incorporation in the face of activation of the entire T cell population is reduced. Furthermore, naive T cells stimulated by ProAd-ICAM cells ultimately die of apoptosis. Interestingly, Ag presentation in the context of ICAM-1 can result in the induction of IL-2 gene transcription from both naive CD4-positive T cells and Th1 clones. In the Th1 clones, IL-2 mRNA does not accumulate to sufficient quantities to induce detectable levels of IL-2 protein. The subsequent failure to secrete IL-2 and to drive proliferation may account for the induction of anergy in Th1 clones following Ag presentation by ProAd-ICAM cells (13, 62). In contrast, naive T cells stimulated with ProAd-ICAM induce IL-2 mRNA, which is present for at least 5 h. This short burst of IL-2 mRNA allows for the secretion of functional levels of IL-2 protein that are sufficient to induce naive T cells to enter the cell cycle. These differences in the temporal regulation of the IL-2 message expression may account for the different biologic responses observed between naive T cells and Th1 clones following costimulation through LFA-1/ICAM-1 interactions.

One issue that has been difficult to resolve is whether the ability of ICAM-1/LFA-1 interactions to enhance T cell activation is mediated through intracellular adhesion, allowing for more efficient

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FIGURE 6. Kinetics of IL-2 mRNA induction in naive CD4-positive T cells. ProAd (A), ProAd-ICAM (B), or ProAd-B7 (C) were cocultured with CD4-positive T cells from the DO11.10 TCR Tg in the presence of 20 μg/ml OVA peptide for the indicated times. Cultures were harvested, and total mRNA was extracted and assayed for IL-2 gene expression by RT-PCR analysis. The top band in the ETBr-stained gel is the product derived from the control plasmid (C), and the bottom band is the wild-type IL-2 gene product (IL-2).

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FIGURE 7. Kinetics of IL-2 mRNA induction in the Th1 clone, pGL2. A and B, ProAd-ICAM (A) or ProAd-B7 (B) transfectants were cocultured with an equal number of pGL2 in the presence of 20 μg/ml OVA peptide for the indicated times. C, ProAd, ProAd-ICAM, and ProAd-B7 were cocultured with pGL2 in the presence or absence of 20 μg/ml OVA peptide. As indicated, CHX at 100 μg/ml (+) or at 500 μg/ml (++) was added at 2 h and the cultures were harvested at 4 h. D, ProAd-B7 was cocultured with pGL2 with 20 μg/ml of OVA peptide, and CHX was or was not added at 2 h and the cultures were harvested at 4 h. In all cases, total mRNA was extracted at the end of the culture and assayed for IL-2 gene expression by RT-PCR analysis. The top band in the ETBr-stained gel product is from the control plasmid (C); the bottom band is from the wild-type IL-2 product (IL-2). Note that there was no control plasmid in the reactions shown in A and B.
TCR signaling, or through LFA-1-linked signaling events that work in concert with TCR signals. Studies that have attempted to measure the adhesive contributions of ICAM-1/LFA-1 interactions to T cell stimulation have suggested that this enhanced intracellular adhesion can contribute to a one to two log shift in the Ag dose response (32). In our experiments, this approaches the limit of detection between Ag presentation to naive T cells by ProAd and ProAd-ICAM: typically a minimum of 100-fold shift in the dose response (see Fig. 1). One concern with the naive T cells is that we have no indication that ProAd engages the T cells; the cells do not transcribe IL-2 mRNA, proliferate, nor induce cell surface activation markers. However, there is clear data that ProAd can form functional conjugates with Th1 clones. Stimulation of pGL2 cells with ProAd induces T cell anergy (13) and normal levels of NF-AT and NF-κB, two transcription factors that regulate IL-2 gene expression (see Fig. 8). Nevertheless, we cannot detect any IL-2 mRNA produced in pGL2 cells stimulated with ProAd cells, even following superinduction with cycloheximide. Thus, although ProAd cells can form functional conjugates with Th1 cells and effectively trigger the TCR, they do not stimulate IL-2 transcription. The ability of ProAd-ICAM to induce IL-2 transcription suggests that engagement of LFA-1 may provide additional signals that are not mediated through TCR engagement alone.

IL-2 gene expression is regulated in many ways, including sequestration of tissue-specific transcription factors in the cytoplasm (63), up-regulation of transcription factor expression (64–67), cooperative binding of transcription factors to the IL-2 promoter/enhancer region (68), transcriptional elongation (69), mRNA stabilization (70), and translational control (53). In this study, we have shown that TCR signaling following Ag presentation by ProAd in the absence of costimulation cells results in the translocation and assembly of NF-AT and NF-κB transcription factors. However, these events are not sufficient to induce detectable levels of IL-2 mRNA expression. The failure to detect IL-2 gene expression following TCR engagement differs from the prevailing view that TCR signaling in the absence of costimulation is sufficient to induce IL-2 transcription (71). For example, anti-CD3 has been shown to fully induce IL-2 transcription in T cell clones, as assayed through the use of reporter constructs and transcription factor induction (69, 72), and this level of transcription was not further increased when the Th1 cells were stimulated in the presence of anti-CD28 mAbs (69). However, most of the published experiments have utilized T cell tumors, which can differ from normal cells in how they regulate IL-2 gene expression (73), or have stimulated T cells with pharmacologic agents or with Abs as ligands for CD3 or CD28, which can recruit effector molecules that are not induced with natural ligands (74). In contrast, in our studies, we

![Figure 8](http://www.jimmunol.org/)

**Figure 8.** Ag presentation by ProAd induces NF-AT and NF-κB DNA-binding activity. Th1 clones were cocultured with an equal number of ProAd or ProAd-B7 cells in the presence of 100 μg/ml tryptic digest of OVA (equivalent to 4 μg/ml OVA peptide) for 6 h. Gel mobility shift assays were performed on nuclear extracts with the following probes, NF-AT, NF-κB, and OCT-1. Free probe indicates the mobility of the probe in the absence of any protein. Resting pGL2 cells were not stimulated with Ag and APC. Similar results were obtained using ProAd-ICAM cells as APC. Interestingly, we did not observe OCT-2 induction even with ProAd-B7 stimulation, although pGL2 cells do induce OCT-2 following activation by conventional APC (61). Although OCT-2 is not necessary for IL-2 gene transcription (104), these data suggest that some other signaling event, not provided by Pro cell transfectants, is necessary for induction of this transcription factor in T cells. Note that in this experiment fivefold less Ag was used than in the IL-2 mRNA analyses shown in Figures 6 and 7.

![Figure 9](http://www.jimmunol.org/)

**Figure 9.** Kinetics of NF-AT induction. The presence of NF-AT in nuclear extracts was detected at various times following Ag presentation by ProAd and ProAd-B7 with 50 μg/ml tryptic digest of OVA (equivalent to 2 μg/ml OVA peptide). In this experiment, T cells and APC were not separated before cell lysis, and nuclear extracts from ProAd in the absence of T cells (APC alone) were analyzed in parallel. The position of the NF-AT/DNA complex and the free probe is indicated on the right.

<table>
<thead>
<tr>
<th>Oligo:</th>
<th>NF-AT</th>
<th>NF-κB</th>
<th>OCT 1/2</th>
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<tr>
<td>Stimulation Conditions:</td>
<td>Free Probe</td>
<td>Resting ProAd</td>
<td>ProAd-B7</td>
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have assayed normal T cells stimulated with natural ligands expressed on the surface of live APC.

Coexpression of either ICAM-1 or B7-1 in the ProAd cells confers the ability to induce IL-2 gene expression, indicating that engagement of LFA-1 or CD28 can provide unique signals of their own that work in concert with TCR signals to up-regulate IL-2 gene transcription. The kinetics of IL-2 mRNA expression, the known ability of CD28 signaling to induce IL-2 mRNA stability (70), the selective superinduction of cycloheximide on ProAd-ICAM-stimulated T cells, and the demonstration that cycloheximide treatment can result in enhanced stabilization of IL-2 mRNA (49) raise the possibility that costimulation through either ICAM-1 or B7-1 induces IL-2 gene transcription, but only B7-1/CD28 interactions induce IL-2 mRNA stability. However, it is also possible that the rapid down-regulation of IL-2 gene expression following Ag presentation by ProAd-ICAM corresponds to the transient induction of a transcription factor, whose continued expression is necessary for sustained IL-2 transcription. Clearly, additional studies are necessary to determine the exact signals relayed through either the TCR or accessory/costimulatory molecules that regulate IL-2 gene expression.

Over the past decade, it has become clear that integrins play an important role in the regulation of cell growth and differentiation. Integrins were defined originally as adhesion molecules that mediate contact between cells and between cells and the extracellular matrix, but it is now clear that these proteins can function as bone fide cell surface signaling molecules. The importance of integrins as signaling molecules is understood better in nonlymphoid cells, where they play an essential role in interactions with the extracellular matrix through the formation of focal adhesions (for reviews, see Refs. 75–79). This focal adhesion signal is initiated by ligand binding of the integrin receptor, which induces a conformational change releasing the cytosolic domain of the β-chain from the regulatory sequence of the α-chain. The activated β-chain now is free to recruit the cytoskeletal components, α-actinin, talin, and vinculin and the tyrosine kinase, FAK, to the plasma membrane. This leads to phosphorylation of paxillin and actin polymerization, and through SH2/SH3 domain interactions, FAK can recruit src family kinases, grb2, PI-3-kinase, and other signaling components to the focal contact (80–82). Both the MAP-kinase and ras downstream signaling pathways can be induced following integrin-mediated cell signaling. In addition, small GTP-binding proteins such as Rho, Rac, and CDC42 have been shown to control the formation of focal adhesion complexes (83), and other integrin-binding proteins, such as cytohesin (84) and integrin-associated kinase (85), can regulate the affinity and/or avidity of integrin/ligand interaction. The focal adhesion therefore represents a large aggregate of receptors, signal-transduction molecules, and structural proteins that can facilitate the activation of signaling pathways and changes in cell-cell or cell-substrate contacts.

The integrin β-chain is well conserved among most family members, including the LFA-1 β-chain (82). Thus, signaling through LFA-1 could be mediated by a similar complex to that seen in focal contacts. Consistent with this possibility, many of the cytoskeletal proteins that are associated with focal adhesions have been shown to bind to the cytosolic tail of the LFA-1 β-chain, including talin, α-actinin, vinculin, and FAK (86–88). In addition, it has been shown recently that activation of FAK following engagement of β1 integrins can costimulate IL-2 production in T cells (89). However, to date, only talin and actin have been shown to be recruited to the T cell:APC adhesion complex (90). One difference between signaling through β1 or β2 integrins and signaling through LFA-1 is the fate of the activated cells. Integrin signaling in endothelial cells and fibroblasts is associated with an antiapoptotic signal (91), whereas we have shown that Ag presentation by ProAd-ICAM to naive T cells results in T cell death.

It is not clear whether the T cell death we have observed is actively induced by interaction between ICAM-1 and LFA-1 or is the result of the failure to induce essential survival factors. Activation-induced cell death is a well-recognized phenomenon in T cells (92) and is mediated primarily by fas following restimulation of previously activated T cells (93, 94) or TNF (95, 96). Damle et al. (97) has shown that coinhibition of ICAM-1 with anti-TCR Abs can enhance activation-induced cell death, but it is not clear whether this is mediated by signaling through LFA-1 or by enhanced TCR signaling. In most cases, activation-induced cell death is restricted to previously stimulated T cells and occurs with 24 h of T cell stimulation. The cell death that we have observed differs in that it occurs in naive T cells and takes 4 to 5 days. This is more similar to superantigen-induced cell death in vivo, in which peripheral naive T cells initially proliferate and then die (98, 99). Mice lacking ICAM-1 lose the proliferative phase of this response, but not the cell death phase, suggesting that cell death is not mediated through engagement of LFA-1 (100). However, these studies do not exclude the possibility that LFA-1 interactions with the alternative ligand, ICAM-2, may be important for superantigen-induced cell death. Superantigen-induced cell death is also mediated by fas (93, 94) and/or TNF (95) and is likely to be dependent on persistence of Ag and repeated stimulation of T cells (101). In this regard, the ability to induce cell death in naive T cells is similar to other models of activation-induced cell death, in which the initial exposure to superantigen induces T cell activation and secondary exposures of the now activated T cells lead to cell death (99). This scenario probably does not take place when we stimulate the naive T cells with ProAd-ICAM, because the mitomycin C-treated Pro cell transfectants do not survive in cell culture for more than 1 to 2 days, well before the T cells begin to apoptose.

Alternatively, the ability of ProAd-ICAM to induce T cell death may result from the failure to induce essential survival factors. Our finding that ProAd-B7-stimulated T cells induced increased levels of Bcl-XL, whereas ProAd-ICAM-stimulated T cells did not (see Fig. 5), is consistent with this possibility. Bcl-XL is an important T cell survival factor, and its absence has been implicated in the eventual death of activated T cells isolated from CD28-deficient mice (102, 103). Thus, it may be more likely that T cell death following Ag presentation by ProAd-ICAM is by neglect rather than induction by LFA-1 signaling. At present, we cannot distinguish these possibilities on the naive T cells because T cells stimulated by ProAd die rapidly without apparent activation.

Overall, our results underscore the complex role that accessory molecules play in the activation, maintenance, and down-regulation of an immune response. Thus, costimulation may not be merely an on or off event, but rather there may be a spectrum of T cell responses, and each response may depend on constellation of the initial exposure to superantigen induces T cell activation and secondary exposures of the now activated T cells lead to cell death (99). This scenario probably does not take place when we stimulate the naive T cells with ProAd-ICAM, because the mitomycin C-treated Pro cell transfectants do not survive in cell culture for more than 1 to 2 days, well before the T cells begin to apoptose.

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Overall, our results underscore the complex role that accessory molecules play in the activation, maintenance, and down-regulation of an immune response. Thus, costimulation may not be merely an on or off event, but rather there may be a spectrum of T cell responses, and each response may depend on constellation of individual accessory molecules and cytokines present in the environment as well as the activation status of the T cell itself. While this spectrum may include easily documented on or off signals, we imagine that the gray area of T cell activation will be important for our understanding of T cell responses and the way in which costimulatory molecules can affect them.

Acknowledgments

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References


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