

## **CD28 and T Cell Antigen Receptor Signal Transduction Coordinately Regulate Interleukin 2 Gene Expression In Response to Superantigen Stimulation**

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### **Summary**

Activation of an immune response requires intercellular contact between T lymphocytes and antigen-presenting cells (APC). Interaction of the T cell antigen receptor (TCR) with antigen in the context of major histocompatibility molecules mediates signal transduction, but T cell activation appears to require the induction of a second costimulatory signal transduction pathway. Recent studies suggest that interaction of CD28 with B7 on APC might deliver such a costimulatory signal. To investigate the role of CD28 signal transduction during APC-dependent T cell activation, we have used Staphylococcal enterotoxins (SEs) presented by a B7-positive APC. We used anti-B7 monoclonal antibodies and a mutant interleukin 2 (IL-2) promoter construct, unresponsive to CD28-generated signals, in transient transfection assays to examine the contribution of the CD28-B7 interaction to IL-2 gene activation. These studies indicate that the CD28-regulated signal transduction pathway is activated during SE stimulation of T cells and plays an important role in SE induction of IL-2 gene expression through its influence upon the CD28-responsive element contained within the IL-2 gene promoter. This effect is particularly profound in the activation of the IL-2 gene in peripheral blood T cells.

T lymphocytes are activated by antigen to initiate immune responses during a complex interaction with APC. The specificity of the T cell response is determined by the TCR, but interactions with other T cell surface molecules are required for activation (1, 2). It is believed that the TCR delivers one set of signals, and another receptor delivers a second signal that is required for T cell activation. One candidate for the receptor that mediates this second signal is CD28. Treatment of T cells with mAb directed against CD28 induces an unidentified but distinct signal transduction pathway that synergizes with TCR-generated signals to increase mRNA level and secretion of several T cell lymphokines, including IL-2, GM-CSF, IFN- $\gamma$ , IL-3, TNF- $\alpha$ , and lymphotoxin (3). CD28 signal transduction increases both the rate of IL-2 transcription by activating a specific element of the IL-2 promoter, and the stability of the IL-2 mRNA (4, 5).

A ligand for CD28, B7, has recently been identified (6). B7 was first identified on activated B cells and, like CD28, is a member of the Ig superfamily (7). Recent studies indicate that either B7-Ig C $\gamma$ 1 fusion proteins, or B7-transfected Chinese hamster ovary cells, can increase proliferation and IL-2 expression from suboptimally activated CD28<sup>+</sup> T cells (8, 9). These studies suggest that the interaction of CD28

with B7 can generate a costimulatory signal capable of amplifying T cell lymphokine expression.

To investigate the role of CD28 signal transduction during APC-dependent activation of the IL-2 gene, we have utilized Staphylococcal enterotoxins (SEs) presented by a B7-positive APC as a model system. SEs activate T cells by interacting with specific variable (V) regions of the TCR  $\beta$  chains (10). The SEs bind to class II MHC molecules and require the presence of class II MHC for efficient activation of T cells (10). Using anti-B7 mAbs and a mutant IL-2 promoter that is largely unresponsive to CD28-generated signals, we have examined the contribution of CD28 signal transduction to IL-2 gene activation. Our findings indicate that the CD28-regulated signal transduction pathway is activated and plays an important role in APC-dependent SE induction of IL-2 gene expression.

### **Materials and Methods**

*Cells.* The human leukemic T cell line Jurkat was maintained as described (11). Nonadherent PBLs were isolated by density-gradient centrifugation through Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) followed by plastic absorption for 2 h

at 37°C. The PBLs were suspended in RPMI 1640 medium with 10% FCS, penicillin G, streptomycin (Irvine Scientific, Santa Ana, CA), and PHA (100 ng/ml, Burroughs Wellcome, Research Triangle Park, NC) for 48 h before transfection.

**Immunoprecipitations and Western Blots.** Immunoprecipitates of TCR  $\xi$  were isolated and resolved on 15% SDS-PAGE gels, transferred to nitrocellulose, and analyzed for the presence of phosphotyrosine or TCR  $\xi$  as described (11).

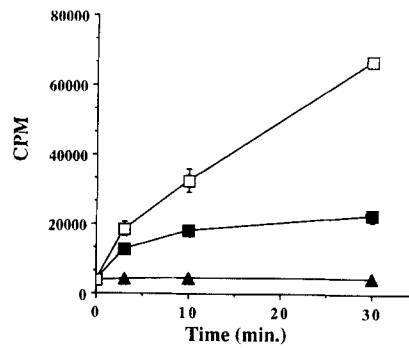
**Inositol Phosphate Assays.** Jurkat cells were loaded with [<sup>3</sup>H]myo-inositol as described (11). The Jurkat cells were mixed with either mAb anti-Leu4 (1/1,000 dilution of ascites), an equal number of Raji cells alone, or Raji cells and 100 ng/ml SED (Toxin Technology, Madison, WI), and the cells were pelleted to promote cell-cell contact. The cells were cultured for the indicated period of time at 37°C in the presence of 10 mM LiCl and then lysed. Soluble inositol phosphate generation was determined, as previously described (11).

**Transfections.** Jurkat cells were transiently transfected with either pIL-2-Luc or pM2-Luc using DEAE-Dextran, as previously described (12). PBMCs were transfected using electroporation as described (13). Transfection efficiency was equalized by cotransfecting with RSV-CAT.

**Luciferase and Chloramphenicol Acetyltransferase Assays.** The determination of luciferase and chloramphenicol acetyltransferase activity was performed as described (12).

## Results and Discussion

Since there have been conflicting reports regarding the signal transduction mechanisms by which "superantigens" such as the SEs stimulate T cell activation (14, 15), we attempted to characterize the TCR-generated signal transduction events after SE stimulation. The enterotoxin SED, when presented by the class II-positive B cell lymphoblastoid line Raji, acti-



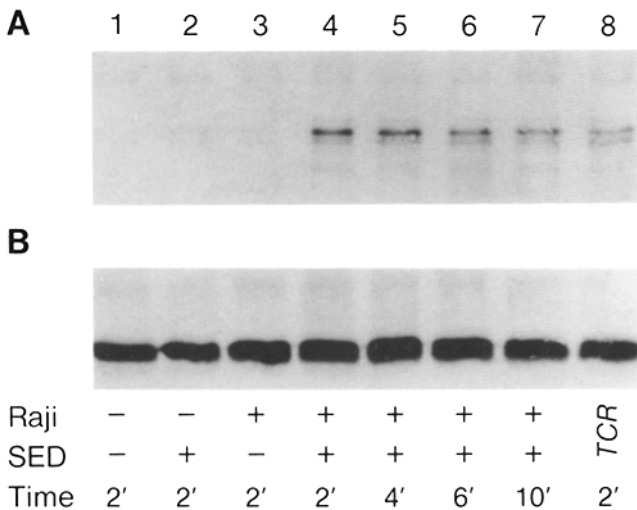
**Figure 2.** SE presentation induces inositol phosphate generation. Time course of total soluble inositol phosphates generation in response to incubation with Raji alone (▲), anti-TCR mAb C305 (■), or Raji and 100 ng/ml SED (□). Data points represent the mean  $\pm$  SD of triplicate samples.

vates the V $\beta$ 8-expressing T cell line Jurkat to secrete IL-2 (16). To determine whether SE-induced IL-2 expression involves a TCR-activated tyrosine kinase, we stimulated Jurkat cells with SED and immunoprecipitated the TCR  $\xi$  chain. Exposure of Jurkat cells to either Raji or SED cells alone did not significantly affect the phosphorylation state of the  $\xi$  chain, but stimulation with SED plus Raji cells induced rapid and prolonged phosphorylation of  $\xi$ , comparable to that induced by anti-TCR mAb (Fig. 1).

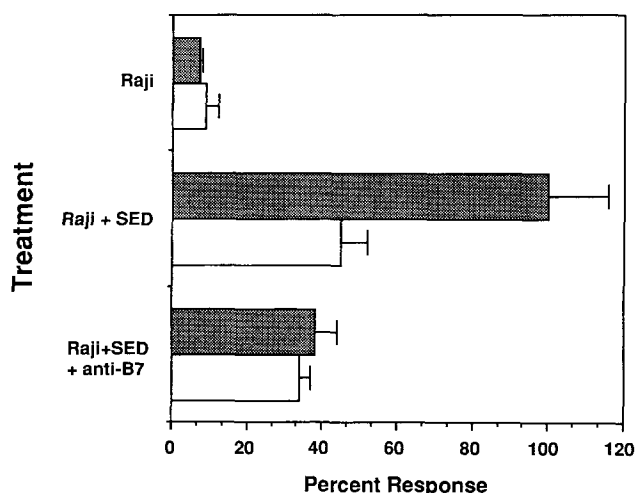
To investigate whether SED presented by Raji would also induce phospholipase C activity, we examined changes in inositol phosphate levels after incubation with either Raji cells alone, Raji cells coated with SED, or with anti-TCR mAbs (Fig. 2). Treatment of Jurkat cells with anti-TCR antibody induced a sixfold increase in the level of total inositol phosphates. Whereas incubation with Raji alone failed to induce a detectable increase in inositol phosphates, Raji plus SED induced a 17-fold increase over that seen in unstimulated Jurkat after 30 min. By these criteria at least, activation of T cells by SE on an APC induces TCR-generated signals qualitatively similar to that induced by nominal antigen or anti-TCR mAbs.

To determine if interaction of CD28 with B7 is involved in the induction of IL-2 secretion during SED presentation to Jurkat, we attempted to block binding of CD28 to B7 using mAbs directed to B7. Addition of either of two different mAbs to B7 decreased the SED-induced IL-2 secretion by 58–68% (data not shown).

CD28 stimulation induces the binding of a nuclear complex, CD28RC, to an element in the 5' flanking region of the IL-2 promoter that is responsible for most of the CD28-induced IL-2 promoter activity (4). To ascertain whether CD28RC activity was induced during SE stimulation, Jurkat cells were transfected with either the pIL-2-Luc construct, which contains sequences from -326 to +46 of the human IL-2 gene directing transcription of the firefly luciferase gene, or the pM2-Luc construct, which also includes the -326 to +46 region of the human IL-2 gene, but contains a 4-bp substitution in the CD28RE site at -159 to -156 (4). The pM2-Luc construct responds to either phorbol 12-myristate



**Figure 1.** The presentation of SE by an APC induces TCR-regulated tyrosine kinase activation. Immunoprecipitation and Western blotting of the TCR  $\xi$  chain. Jurkat cells were incubated with medium (lane 1), 100 ng/ml SED alone (lane 2), Raji cells alone (lane 3), Raji cells, and 100 ng/ml SED (lanes 4–7), or with the anti-TCR mAb C305 (lane 8) for the indicated times. Immunoprecipitates of the TCR  $\xi$  chain were isolated and analyzed by Western blot for phosphotyrosine (A) or TCR  $\xi$  (B).



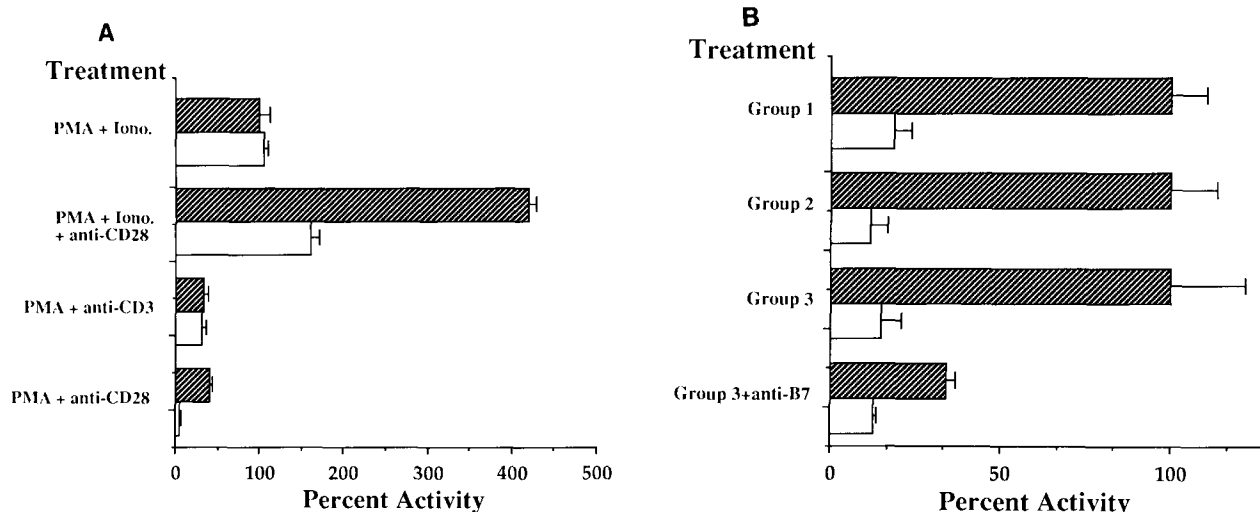
**Figure 3.** The CD28 response element is involved in SED-induced activation of the IL-2 gene promoter. Jurkat cells were transiently transfected with either the pIL-2-Luc (filled bars) or the pM2-Luc plasmids (open bars). After 40 h, the transfected Jurkat cells were incubated with Raji alone, Raji and 100 ng/ml SED, or Raji cells, 100 ng/ml SED, and mAb B7 (1:500 dilution of ascites). Data for the pM2-Luc-transfected cells are presented as the percent Raji plus SED-induced response of the wild-type pIL-2-Luc (mean of 1,118 light U/10  $\mu$ g). Results represent the mean  $\pm$  SD of triplicate samples from three independent experiments.

13-acetate (PMA) and ionomycin treatment, or anti-TCR-generated signals, as well as the wild-type pIL-2-Luc construct, however, CD28-induced activity is decreased by 80–90% (4). In Jurkat cells transfected with pIL-2-Luc, incubation with Raji cells and SED induced a 15–25-fold increase in luciferase activity compared with cells incubated with Raji alone (Fig.

3). In contrast, Jurkat transfected with the pM2-Luc construct responded only 41% as well. Addition of anti-B7 mAb caused a 62% decrease in the activity induced in the pIL-2-Luc transfected cells, but only minimally affected the SED-induced luciferase activity from the pM2-Luc-transfected cells, indicating that the B7 antibody treatment and the enhancer mutation were both influencing the same pathway.

To determine whether CD28 signal transduction also affected IL-2 transcription in peripheral blood T cells, PBLs were transfected with either the pIL-2-Luc or the pM2-Luc constructs, and stimulated with combinations of PMA, ionomycin, anti-TCR, and anti-CD28. As seen in the Jurkat T cell line, treatment with PMA and ionomycin, or with PMA and anti-TCR mAb induced similar levels of luciferase activity from either construct (Fig. 4 A). Addition of anti-CD28 mAb to PMA and ionomycin-treated cells increased the induced luciferase activity by 4.2-fold in the pIL-2-Luc-transfected cells. However, the anti-CD28 treatment only induced a 1.6-fold increase in the pM2-Luc transfected cells, 19% of the observed increase for the pIL-2-Luc-transfected cells. Moreover, treatment of pM2-Luc-transfected cells with PMA and anti-CD28 mAb induced only 14% of the luciferase activity elicited from lymphocytes transfected with the wild-type pIL-2-Luc plasmid. Thus, the comparison of the activity of the wild-type pIL-2-Luc to the activity of pM2-Luc containing the 4-bp mutation of the CD28 responsive element reveals a specific target of the CD28 signal transduction pathway in normal T cells, as was previously detected in Jurkat.

Activation of normal peripheral T lymphocytes is stringently regulated, perhaps more so than might be observed with the leukemic T cell line such as Jurkat. To assess the role of CD28 signal transduction in APC-dependent SE-



**Figure 4.** CD28 stimulation increases IL-2 promoter activity in peripheral blood T cells, and the CD28 signal transduction pathway is used during SE-induced IL-2 gene activation. Peripheral blood T cells were transfected with either the pIL-2-Luc (filled bars) or the pM2-Luc plasmids (open bars). (A) The transfected T cells were stimulated with the indicated combinations of PMA (5 ng/ml), ionomycin (1  $\mu$ M), anti-CD3 (anti-Leu 4, 100 ng/ml), and anti-CD28 (9.3, 100 ng/ml). The results are presented as the percent response of the PMA- and ionomycin-induced pIL-2-Luc-transfected cells (mean of 12,178 light U/10  $\mu$ g). (B) The transfected T cells were incubated with Raji cells and 100 ng/ml each SED, TSST, and SEC<sub>2</sub> (Group 1), Raji, and 100 ng/ml each of SEA, SEB, and SEC<sub>3</sub> (Group 2), or Raji and 100 ng/ml each of SEA, SEB, and SEE (Group 3) for 8 h. Anti-B7 antibody was used at a 1:500 dilution of ascites. Data for the pM2-Luc-transfected cells are presented as the percent response of the wild-type pIL-2-Luc (mean of 6,207 light U/10  $\mu$ g). Results represent the mean  $\pm$  SD of triplicate samples.

induced activation of peripheral T cells, mononuclear cells transfected with either the pIL-2-Luc or the pM2-Luc constructs were stimulated by SE presented on Raji cells. Combinations of SEs were used to stimulate large numbers of T cells contained within the polyclonal T cell population. The SE-induced luciferase activity from the pIL-2-Luc transfected lymphocytes ranged from 8 to 26-fold greater than the basal activity. In contrast, SE-induced activity in the pM2-Luc-transfected T cells was only 11–18% of that of the pIL-2-Luc-transfected cells (Fig. 4 B). Moreover, the addition of anti-B7 antibody caused a 66% decrease in the luciferase activity of the pIL-2-Luc-transfected cells, but without significantly affecting the activity of the pM2-Luc-transfected cells.

These studies indicate that B7 on the surface of an APC can activate CD28 signal transduction and that this pathway contributes substantially to the induction of IL-2 expression

in the context of TCR signal transduction. This effect was particularly pronounced in peripheral T cells induced by SE stimulation. This supports the notion that antigen-induced IL-2 gene expression is coordinately regulated by TCR- and CD28-generated signals that activate distinct transcription elements. Studies on murine T cell clones indicate that antigen-induced proliferation requires activation of a signal transduction pathway in addition to those activated via the TCR (1). In the absence of this second, or costimulatory signal, the T cells fail to secrete IL-2 and enter a prolonged nonresponsive or anergic state. Whereas the current studies do not directly address the role of the CD28 signal transduction in preventing induction of the anergic state, they indicate that CD28 signal transduction plays an important role in IL-2 gene activation and may act as this requisite costimulatory pathway.

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