

# The MAP Kinases are Differently Utilized by CD28 and CD2 Adhesion Pathways in Superantigen-Activated Jurkat T cells

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## ABSTRACT

To mimic the two-signal requirements for T cell activation mediated by ligands, we exposed the superantigens SEA or SEE (signal 1) to T cells incubated with HLA-DR/LFA-3 or HLA-DR/B7-1-CHO transfected cells (signal 2). LFA-3 costimulation was able to induce T cell proliferation as well as IFN- $\gamma$  and IL-4 production at similar levels as in cells induced by B7-1. Analysis of the CD28RE of the IL-2 promoter showed specific transcription factor recruitment at the CD28RE element upon induction by B7-1/SEE. Further functional studies with an IL-2 enhancer-promoter carrying either wild type or mutated versions of the CD28RE site revealed that this element is necessary for full activation upon B7-1 costimulation. While both CD28/B7-1 and CD2/LFA-3 costimulation resulted in the up-regulation of IL-4 and IFN- $\gamma$  promoters, IL-2 promoter activity and production of IL-2 were only seen after B7-1 costimulation. However, contrary to what has been previously proposed, we show that costimulation with either B7-1 or LFA-3 further enhanced the ERK-2 activity and strongly activated the p38 MAPK pathway, but only B7-1 costimulation induced high levels of JNK-1 activity. These data suggest that the differential effect of CD28 vs. CD2 can be related to the difference in the ability of the two pathways to induce JNK-1 activity.

**Key terms:** CD28 response element; Staphylococcal Enterotoxin A-E; Extracellular signal regulated kinase; c-Jun N-terminal kinase; Interleukin-2.

## INTRODUCTION

In addition to T cell receptor (TCR) cross-linking (signal one), optimal T cell activation requires engagement of the costimulatory molecule CD28 by its ligand B7-1 (CD80) on Antigen presenting cells (APC) (signal two) (Parra et al., 1994; Schwartz 1992; Linsley et al., 1991; Faroudi et al., 2002; Parra, 2002). The ligand for CD28 is B7 (B7-1/CD80 and B7-2/CD86), which displays a restricted pattern of expression on APCs, including activated B cells, suggesting an important role for CD28 in the interaction between T and B cells (Freeman et al., 1989; Linsley et al.,

1990; Lenschow et al., 1996; Van Seventer et al., 1991). Furthermore, CD28 costimulation synergizes with TCR signals to increase IL-2 production and T-cell proliferation in a cyclosporine A-insensitive manner (Mattila et al., 1990; Williams et al., 1992).

Another receptor on T cells that can induce the secondary stimulus required for T cell activation is the CD2 receptor, which recognizes and binds its ligand on the surface of the antigen presenting cells (Bierer et al., 1988; 1989; Janeway and Golstein, 1993). Like CD28, CD2 has a relatively large and highly conserved cytoplasmic domain, which allows

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association with the Src-like kinases, Fyn and Lck (Rabb et al., 1995; Schneider et al., 1995). Despite that CD2 ligation may also transduce an independent signal, initiating T cell activation (Kabelit et al., 1990), it seems that a major role for CD2 molecules is to enhance the TCR/CD3- or CD28 mediated response, particularly in resting and naive T cells (Bierer et al., 1989; Kabelit et al., 1990). LFA-3, the natural human ligand for CD2, is a glycoprotein expressed on virtually all nucleated cells. It is also a member of the Ig super gene family. Several lines of evidence have demonstrated that CD2 binds to its ligand LFA-3 with high affinity and thus should play a significant role in stabilizing initial cell-cell interactions and adhesion prior to the TCR/MHC class II+complex interaction (Shaw et al., 1988; Springer et al., 1987; Springer, 1990).

The induction of signals one and two in T cells leads to the activation of several signal transduction pathways, including the MAP kinase family of enzymes (Nunes et al., 1996; Zhang et al., 1999). These enzymes play a critical role in a diverse number of signal transduction pathways in response to mitogenic stimuli, and they are directly implicated in the linking of cytoplasmic signaling cascades to the control of transcription in the nucleus (Robinson et al., 1996). The MAPK family is divided into two main groups. The Extracellularly Regulated Kinases (ERK group) (Boulton et al., 1991) and the Stress-Activated Protein kinase (SAPK group), which includes the c-Jun N-terminal Kinase (JNK) and p38 kinase (Hibi et al., 1993; Minden et al., 1994). Dual phosphorylation of these kinases on a TXY motif in their activation loop results in an open, catalytically active, conformation. The active kinases phosphorylate several transcription factors, including Elk-1, c-Jun, and ATF-2, which are involved in the regulation of c-fos and c-jun gene expression (Gille et al., 1992; Minden et al., 1995; Gupta et al., 1995; Karin, 1995). Several reports using other models, have shown the importance of the MAP kinases for cell proliferation (Leiva-Salcedo et al., 2002). The activation of c-Jun and c-Fos

proteins form a complex called "Activator protein-1" (AP-1) which was first identified as a transcriptional factor that binds to an essential cis-element of the human metallothioneine IIa (hMTIIa) promoter (Lee et al., 1987; Gille et al., 1992; Davis et al., 1994; Nebreda et al., 1994). Later the binding site for AP-1 was also recognized as the TPA response element (TRE) of several cellular and viral genes including human collagenase, SV40 and IL-2. Indeed, the AP-1 complex plays a critical role in controlling IL-2 gene transcription (Angel et al., 1987; 1991; Steal et al., 1991), by binding to three sites: the NF-AT, Oct-1, and AP-1 itself. The expression of the IL-2 gene is regulated by its 5'-flanking promoter sequence and its critical regulatory and inducible activity is contained within the 300-bp region immediately upstream of the start site (Parra et al., 1997; Rahmsdorf et al., 1990; Durand et al., 1988; Granelli-Piperno et al., 1991). The IL-2 promoter includes defined binding sites for the transcription factors NF-AT, NF- $\kappa$ B, AP-1, Oct-1, and CD28RC (Parra et al., 1997; Jain et al., 1992a, 1992b). Thus, activated T cells produce IL-2, express high affinity IL-2 receptors, and proliferate in an autocrine or paracrine fashion. However, the interaction of the CD28 and B7-1 molecules has been recognized as the major pathway in the regulation of IL-2 expression, and subsequently several reports have demonstrated its specificity to this respect (Parra et al., 1993; Fraser et al., 1991; Fraser and Weiss 1992). Early reports showed that CD3 cross-linking by antibodies could induce ERK-2 activity and that antibodies against CD28 failed to increase the activity of ERK-2 in Jurkat T cells (Minden et al., 1994; Steal et al., 1991). However, one major discrepancy of this study compared with others was that we could strongly increase the ERK-2 activity after TCR and LFA-3 or CD28 costimulation. This discrepancy may reflect differences in the use of CD3 and CD28 antibodies v/s a superantigen and B7-1 costimulatory molecule expressed in CHO-DR cells. A role for the JNK and ERK cascades in T cell activation has been described, but the links between receptor engagement and downstream events still need to be defined.

## MATERIALS AND METHODS

*Reagents*

Staphylococcal enterotoxins A and E (SEA, SEE) were purchased from Toxin Technology (Madsion, W I). Ficoll-isopaque, G418 and L-methionine sulfoximine (MSX) were purchased from Pharmacia Inc. (Uppsala, Sweden). The protease inhibitors phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin, aprotinin and bestatin were from Roche, USA. [ $\gamma$ - $^{32}$ P]ATP was from Amersham. T4 polynucleotide kinase and poly (dI-dC)<sub>2</sub>, were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Tris-Borate-EDTA buffer and acrylamide-bisacrylamide (29:1) were obtained from Bio-Rad (Richmond, CA). PHAS-1 was purchase from Strategene (La Jolla, CA). Luciferase assay reagent, lysis buffer and the pGL-2 luciferase vector were obtained from Promega (Madison, WI). Recombinants and mAbs to human IL-2 (5344.111 and B33-2), IL-4 (8D4-8, MP4-25D2) and IFN- $\gamma$  (NIB42, 4S.B3) were obtained from PharMingen (San Diego, CA). Phorbol-12-Myristate-13-Acetate (PMA) and Ionomycin were purchased from ICN Pharmaceuticals (Costa Mesa, CA). GST-cJun, GST-ATF-2 were a gift from Dr. Roger J. Davis (Howard Hughes Medical Institute, MA). Production and purification of GST-cJun and GST-ATF-2 proteins were performed as described (Hibi et al., 1993).

*Cell separation, culture and stimulation*

Peripheral blood lymphocyte (PBL) were isolated from buffy coats obtained from healthy blood donors by density centrifugation over Ficoll-Paque (Pharmacia Biotech, Uppsala Sweden) as previously described (Parra et al., 1995). T cells were enriched by separation over a gelatin column followed by positive selection of CD4<sup>+</sup> T cells by MACS (Miltenyi Biotech, Sunnyvale, CA) according to the manufacturer's description. The purity of the separated cells was

routinely checked by FACS analyses and was 99% CD4<sup>+</sup> cells.

The purified CD4<sup>+</sup> T cells were resuspended in RPMI 1640 supplemented with 2 mM glutamine and 10% FCS. The cells were stimulated with 10 ng/ml SEA presented by CHO transfectants at a T:CHO ratio of 20:1. After 72 h, the cultures were pulsed with 0.5  $\mu$ Ci [ $^3$ H]thymidine and harvested after an additional 4 h. The amount of incorporated [ $^3$ H]thymidine was determined by liquid scintillation counting. The human T leukemia cell line Jurkat was maintained at logarithmic growth in RPMI 1640 supplemented with 2 mM glutamine and 10% fetal calf serum. The transfected CHO cells were maintained in the same medium with G418 and/or MSX. Stimulation of the T cells with SEE was done at a concentration of  $1 \times 10^6$  cells/ml in the presence of  $0.1 \times 10^6$  cells/ml CHO cell transfectants at 37 °C as previously described (Parra et al., 1994; 1995).

*Transfected cell lines*

CHO cells stably transfected with the cDNAs encoding the human HLA-DR, B7-1 and LFA-3 cell surface molecules have been described in detail elsewhere (Parra et al. 1995). Single and double transfectants expressing similar levels of the transfected molecules were established by repeated cell sorting and they were periodically reanalyzed.

*Plasmids*

The human IL-2 promoter-enhancer fragment (Durand et al. 1988; Granelli-Piperno et al., 1991), nucleotides -500 to +60, was subcloned from pSV-IL-2-CAT into the luciferase reporter vector pGL2 (Promega, Madison, WI). The -500-to +60 fragment was prepared by PCR with appropriate primers creating a 5'-XhoI site and a 3'-HindIII restriction sites. The IL-2 promoter mutated in the CD28RE region was generated by PCR directed splicing overlap extension, to replace positions -159 to -164 with the sequence 5'-CCTCGA-3'. Constructs were confirmed by sequencing.

### *Preparation of nuclear extracts*

Human Jurkat leukemia CD4 T cells (3-5 x 10<sup>7</sup> cells) were stimulated with various irradiated transfected CHO cells (8000 rad) in the absence or presence of SEE (100 ng/ml) at 37°C (in a humidified atmosphere containing 5% CO<sub>2</sub>). The cells were harvested after 6 hr of culture and T cells were separated from CHO cells using a plastic adherence technique. The harvested T cells were re-suspended in 10 ml PBS and pelleted by centrifugation for 5 min at 1500 rpm. The pellet was resuspended in 1 ml PBS, transferred into an Eppendorf tube and repelleted by spinning for 15 sec in a microfuge. PBS was removed and the cell pellet was re-suspended in 500 µl cold buffer A (10 mM Hepes pH 7.8; 15 mM KCl; MgCl<sub>2</sub> 2mM; 0.1 mM EDTA; 1mM DTT; 1 mM PMSF). T cells were allowed to swell on ice for 15 min, 25 µl of a 10% solution of NP-40 was added, and the tube was vortexed vigorously for 10 sec. The homogenate was centrifuged and the nuclear pellet was re-suspended in 100 µl ice-cold buffer B (20 mM HEPES pH 7.9; 0.4 M NaCl; 1mM EDTA; 1mM EGTA; 1mM DTT; 1 mM PMSF) and the tube was rocked for 15 min at 4°C on a shaking platform. The nuclear extract was centrifuged for 5 min at 4°C and the supernatant was frozen in aliquots at -70°C. Before use of the buffers A and C, a mixture of the protease inhibitors was added: 0.5 µg Leupeptin/ml; 0.7 µg Pepstatin/ml; 1µg Aprotinin/ml and 40 µg Bestatin/ml. 1-2 µl of the nuclear extract (2-4 µg protein) was used for a gel shift assay in the presence of 3 µg poly dI-dC as previously described (Parra et al., 1997).

### *Electrophoretic mobility shift assay (EMSA).*

The double-stranded oligonucleotides corresponding to the wild type, mutated CD28RE of the IL-2 promoter were (coding strand): 5'-CTCAAGATCGAAATTCCA-AAGAGAC-3', and 5'-CTCAAGATCG-ACCTCGAAAAGAGAC-3', respectively (the mutation is underlined). One to 2 µl of nuclear extract corresponding to 5-10 µg of

protein were added to 4 µl binding buffer containing 2 to 3 µg poly (dI-dC)<sub>2</sub> as a non-specific competitor. The reaction mixtures were incubated at 37°C for 30 min with 15,000 cpm of double-stranded <sup>32</sup>P-labeled oligonucleotides in a final volume of 15 µl. The samples were electrophoresed on 5% polyacrylamide gels in 89 mM Tris, 89 mM boric acid, 2 mM EDTA. The gels were fixed in 40% methanol and 10% acetic acid for 15 min, dried, and visualized by autoradiography (Parra et al., 1997).

### *DNA transfection and Luciferase activity assay.*

Transfection of Jurkat cells was carried out by electroporation. Briefly, plasmid DNA was mixed with exponentially growing Jurkat cells (20 x 10<sup>6</sup> cells/ml) in complete medium and the cells were electroporated in an electro cell manipulator 600 (BTX, San Diego, CA.) using 130V/1700 µF capacitance. The transfected cells were cultured for 24 hrs before being stimulated with the different CHO-transfected cell lines with or without 100 ng SEE/ml. After various periods of time, cells from each independent well were harvested, washed twice in PBS and treated with lysis buffer (Luciferase Assay Promega, Madison, WI) for 5-10 min on ice. Lysates were spun down for 1 min and the total supernatants were analyzed using Luciferase Reagent (Promega) and measured as a duplicate in a luminometer (MicroLumat LB 96 P, Berthold) for 5 s. Background measurement was subtracted from each duplicate and experimental values are expressed either as recorded light units, Luciferase activity or as relative activity compared to extracts from unstimulated cells.

### *JNK/ERK/p38 Kinase Activity Assays*

Jurkat T cells were incubated with or without SEE and the different CHO cells (CHO-DR, CHO-DR/B7-1 and CHO-DR-LFA-3) for 30, 60, and 120 minutes (for ERK-2 and JNK-1) or 60, 120, and 180 min (for p38). After time periods the CHO cells were removed by plastic adherence and the Jurkat T cells were collected and lysed, and

the proteins of interest were immune precipitated using mAb to JNK-1 (Santa Cruz), ERK-2 and p38 and protein A-sepharose CL-4B beads for 3 to 4 h. Beads were washed 3 times with lysis buffer, twice with LiCl and twice with kinase buffer (25 mM HEPES, pH 7.4, 25 mM-glycerophosphate, 25 mM MgCl<sub>2</sub>, 25 mM DTT, and 0.1mM Na<sub>3</sub>VO<sub>4</sub>), and finally resuspended in 30 µl of kinase buffer. The beads were then incubated with human GST-c-Jun (1-79) for JNK-1, GST-ATF-2 (1-109) for p38, PHAS-I (Stratagene, La Jolla, CA) for ERK-2, and 1 µl of [ $\gamma$ -<sup>32</sup>P]ATP at 30°C for 30 min. One volume of 2x sample buffer (125 mM Tris, 6% SDS, 20% glycerol) was added, and the reaction mixtures were boiled at 100°C for 3 min. Phosphorylated proteins were analyzed by SDS-PAGE and autoradiography.

#### *Cytokine assays*

Cytokine levels in supernatants of unstimulated and stimulated T cells were determined by specific ELISAs using a sandwich technique as previously described (Sundstedt et al., 1997) and according to the manufacturer. Recombinant cytokines used for standard curves in ELISA and specific Ab pairs for each cytokine were obtained from PharMingen (San Diego, CA); IL-2 (5344.111 and B33-2), IL-4 (8D4-8, MP4-25D2) and IFN- $\gamma$  (NIB42, 4S.B3).

#### RESULTS

##### *B7-1 and LFA-3 costimulation in Jurkat cells induces distinct protein binding complexes that recognize the CD28RE sequence contained in the IL-2 promoter.*

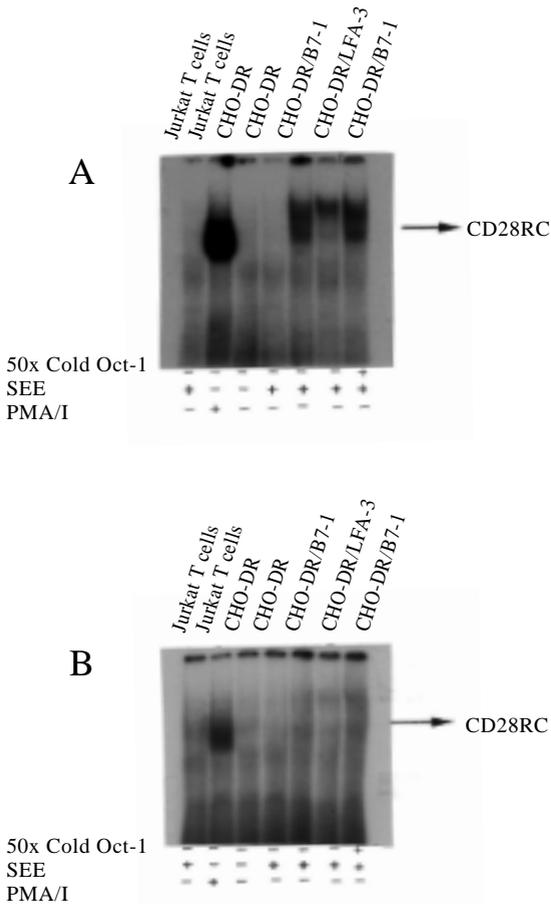
We first investigated the relative role of B7-1 and LFA-3 on the transcriptional regulation of the IL-2 promoter. The minimal inducible IL-2 enhancer region has been identified as a 300 bp region upstream of the transcription start site. A number of target sites recognized by various transcription factors have been identified within this region, including the CD28RE, AP-1, NF-AT and NF- $\kappa$ B (Jain et al., 1992a;

Parra et al., 1993). However, mutational analyses has demonstrated that the CD28RE plays a crucial role in the transcriptional activation of the IL-2 promoter.

Electrophoretic mobility shift assay (EMSA) was used with nuclear extracts prepared from costimulated Jurkat T cells and oligonucleotides encoding the wild type radiolabeled CD28RE of the IL-2 promoter to analyze the protein complexes that recognize this sequence. Figure 1 shows that costimulation with SEE and B7-1 or LFA-3 induces distinct nuclear factor or binding protein complexes which strongly bind to the CD28RE sequence (Fig.1A). Unstimulated and HLA-DR stimulated Jurkat cells did not contain detectable levels of nuclear factors (Fig. 1A). No binding was detected in Jurkat cells presented with HLA-DR either non-stimulated or treated with SEE. However, when cells were costimulated with B7-1 or LFA-3, strong protein binding complexes were detected, which were differently activated by both receptor pathways. These protein-DNA complexes were not competed with cold Oct-1 specific oligonucleotide, suggesting that the Oct-1 transcription factor is not present in these complexes. As a control, we stimulated Jurkat cells (not presented with CHO transfectants) with SEE or PMA/Ionomycin. As observed with the CHO transfectants, SEE alone did not induce any specific protein binding complex. However, PMA/I induced a strong complex with a higher electrophoretic mobility than that observed with B7-1/LFA-3 costimulation. When the same extracts were incubated with a mutated CD28RE sequence, the DNA-protein binding activity was completely abolished (Fig 1B). Only a residual binding activity was observed in Jurkat cells stimulated with PMA/Ionomycin.

##### *Strong costimulatory effect of B7-1 and LFA-3 on transcriptional induction of IL-2 promoter activity by B7-1 and LFA-3 required a functionally intact CD28RE.*

The functional importance of the CD28 response element was further dissected comparing the transcriptional response of transiently transfected Jurkat T cells with a



**Figure 1**

Jurkat T cells were incubated in RPMI supplemented with 10% FBS. The cells were stimulated with the various indicated CHO transfectants for 6 h, in the presence or absence of SEE 100 ng/ml, or with PMA/Ionomycin as a positive control. Nuclear cell extracts were prepared and analyzed by EMSA.

In A, protein binding to the CD28RE of the IL-2 promoter using a oligonucleotide corresponding to the 5'-CTCAAGATCGAAATTC AAGAGAC-3', of the IL-2 promoter. In B, the mutated version of the IL-2 promoter sequence showed above. 5'-CTCAAGATCGACCTCGA AAAGAGAC-3'. Sequence specificity of the CD28RE was assessed by incubating nuclear extract with radiolabeled CD28RE oligonucleotides in the presence of no competitor Oct-1 at 50-fold molar excess (5'-CGTCTCATGCGATGCAAATCACTTGAGATC 3'). The mutation is showed in bold face. The results from one of two similar experiments are shown.

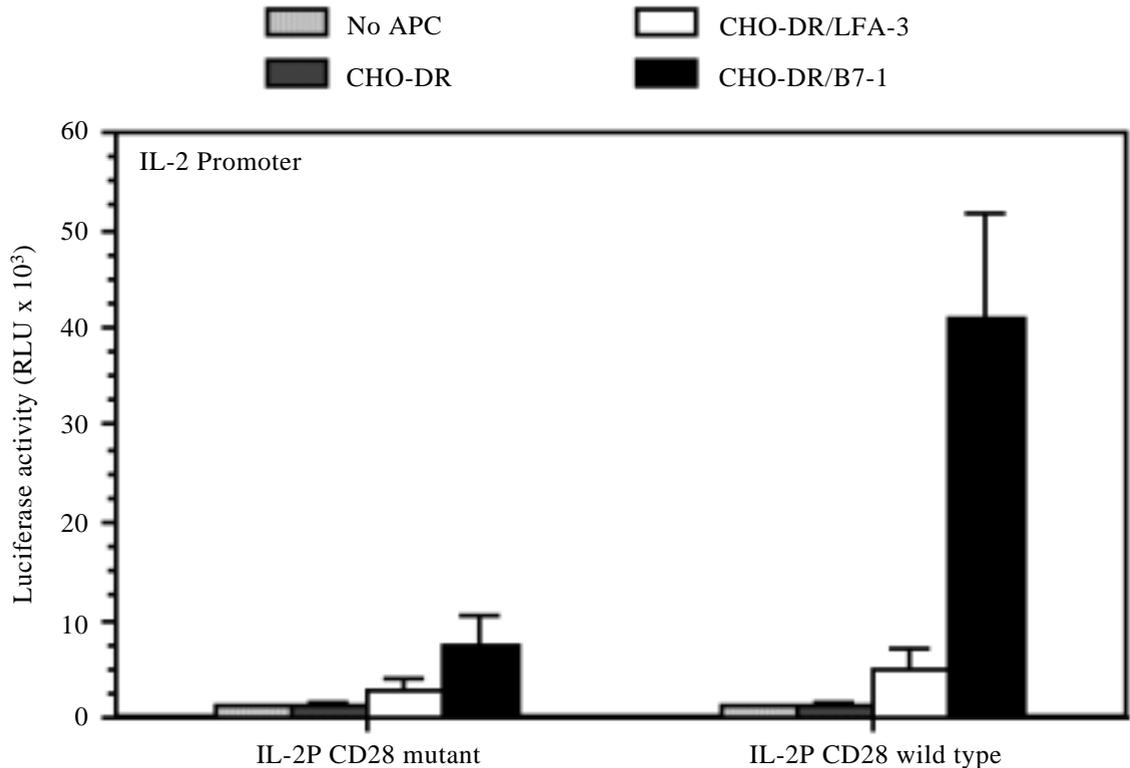
luciferase reporter-construct carrying the wild type IL-2 and the mutated version of this CD28RE. As we previously reported (Parra et al., 1997), a strong induction of the IL-2 promoter activity was observed by costimulation of Jurkat T cells with SEE and B7-1, but not with SEE and LFA-3 (Fig. 2). Importantly, this activity was severely reduced in the IL-2 promoter carrying a mutation in the CD28RE. We showed that the transcriptional activity of an IL-2 promoter reporter construct carrying a mutated version of the CD28RE was strongly reduced in B7-1 costimulated Jurkat T cells compared to the wild type (Fig. 2). These results further support a major role of the CD28/B7-1 pathway in the induction of IL-2 and T cell activation.

*Requirement of both signals for an optimal induction of JNK-1, ERK-2, and p38 kinase activities.*

To examine whether SEE (signal one) alone or together with LFA-3 or B7-1 (signals two) costimulation can activate the mammalian JNK-1, ERK-2 and p38 kinase cascades, we activated Jurkat T cells with and without SEE and costimulated with CHO-DR, CHO-DR/B7-1, or CHO-DR-LFA-3 for given periods of time.

JNK-1 was immunoprecipitated from the lysates (using a specific antibody, JNK1 (C-17): sc-474, Santa Cruz Biotechnology, Inc.) kinase assay with GST-c-Jun, (amino acids 1-79) as protein substrate (Fig. 3). We show that optimal induction of JNK-1 activity required the participation of both signals, since SEE alone did not affect basal levels of the kinase activity. Substantial activation of JNK-1 was already observed after 30 min of costimulation. This activity peaked around 60 min after B7-1 costimulation and decreased after 120 min (Fig. 3C).

To determine whether SEE (signal one) and LFA-3 (signal two) costimulation could also activate the mammalian ERK-2 and p38 pathways, similar assays were employed. ERK-2 and p38 were immunoprecipitated and their activities were determined by phosphorylation of either PHAS-1 protein or GST-ATF-2 respectively. As reported previously, ERK-



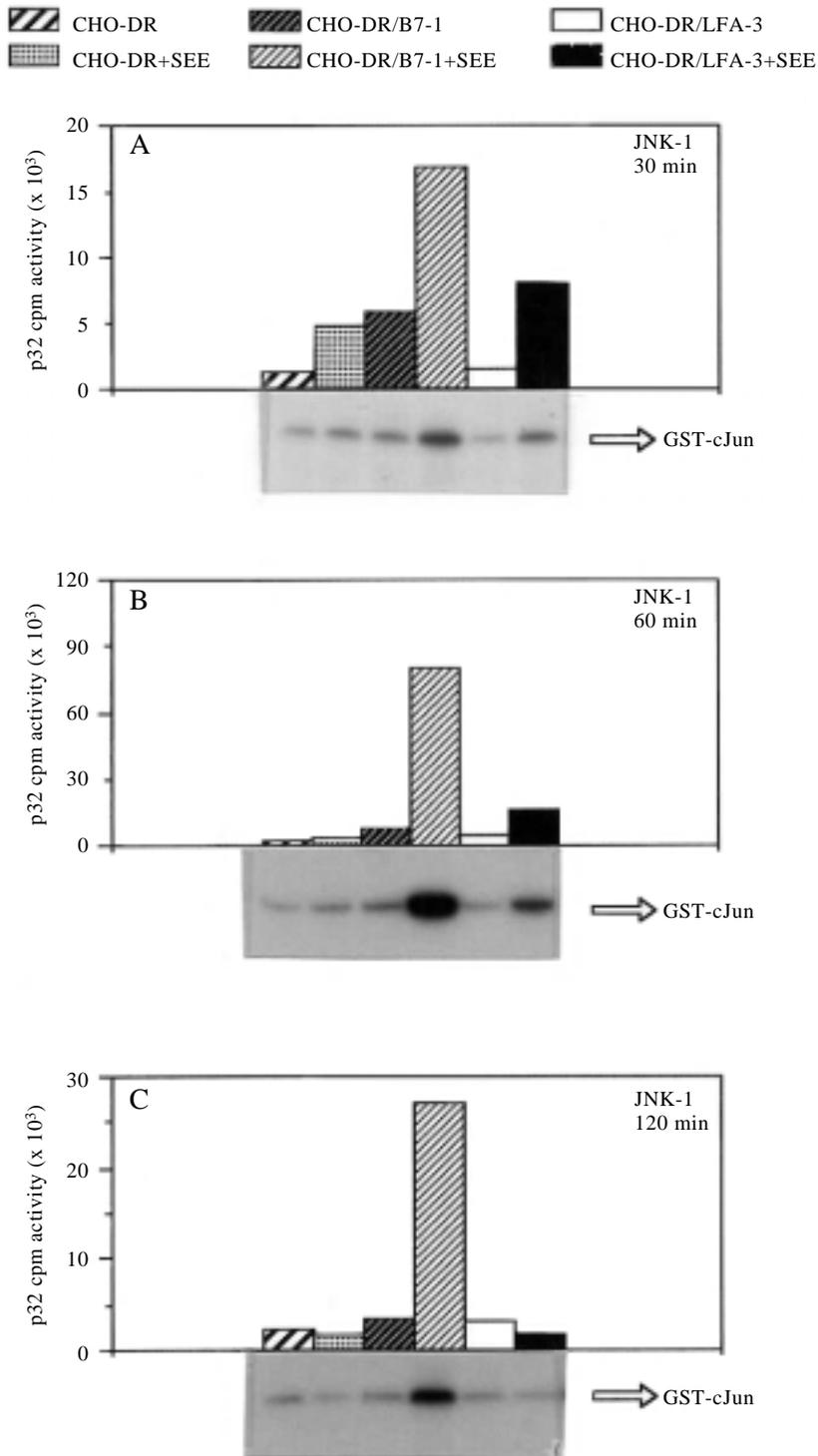
**Figure 2**

Functional demonstration of the specificity of the CD28RE in the transcriptional activity of a wild type and a mutated version of the IL-2 promoter. Jurkat T cells were transfected with the wild type IL-2 luciferase reporter gene or with the mutant version. After 24 h incubation the cells were stimulated with SEE and the CHO and CHO transfectants CHO-DR, CHO-DR/LFA-3 and CHO-DR/B7-1. Eight hours later, samples were harvested and analyzed for luciferase activity. The native CD28RE sequence of the IL-2 promoter 5'-AAATTC-3' was mutated to the 5'-CCTCGA-3' sequence (mutation shown in bold-face). Luciferase activity is expressed as arbitrary light units minus background units of buffer alone. Results from one of two similar experiments are shown.

activity was elevated 30 min of costimulation with signal one (CHO-DR/SEE) (Fig. 4A). However, in our system, LFA-3 and B7-1 costimulation (signal two) increased the activity of ERK-2 several fold, suggesting a principal role for signal two in the up-regulation of ERK-2 activity. The optimal activity peaked 60 min after DR, DR/B7-1 and DR-LFA-3 costimulation (Fig. 4B) and decreased after 120 min (Fig. 4C). Interestingly, LFA-3 costimulation proved to be more effective than B7-1 in increasing the levels of ERK-2 activity (Fig. 4B and C). These results suggest a synergistic role between TCR and CD2 to superinduce the levels of ERK-2 activity.

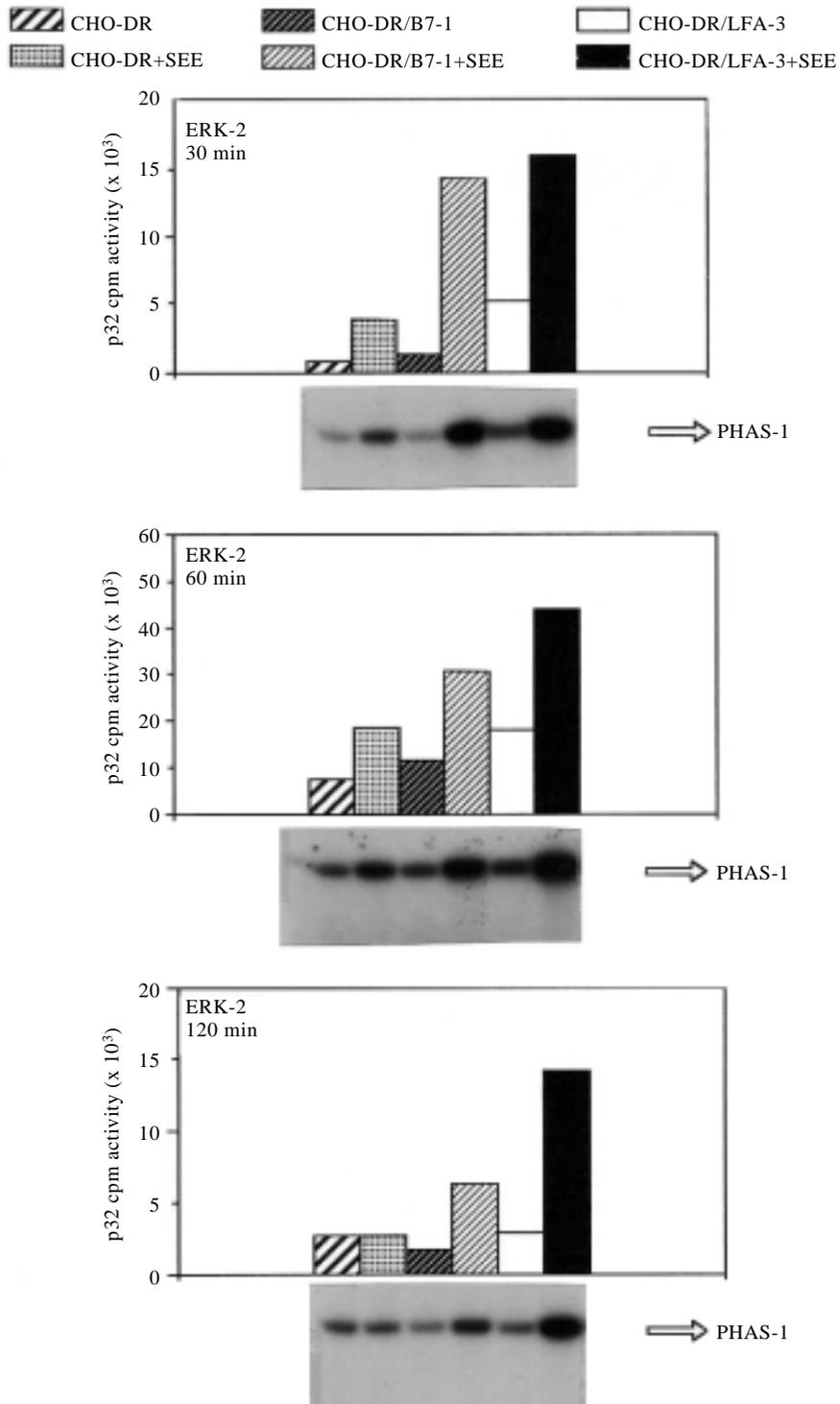
We finally investigated the activation of p38 kinase. Similarly to JNK-1, B7-1 costimulation strongly activated p38 kinase activity in Jurkat T cells (Fig. 5). No p38 kinase activity was detected in the absence of costimulation, suggesting the requirement of both signals to induce p38 activity. However, activation of p38 kinase activity was only observed after 120 min of costimulation with either B7-1 and or LFA-3 and decreased to basal levels after 180 min of costimulation (Fig. 5).

Western blot analysis confirmed that JNK-1, ERK-2 and p38 kinases were expressed at comparable levels (data not shown).

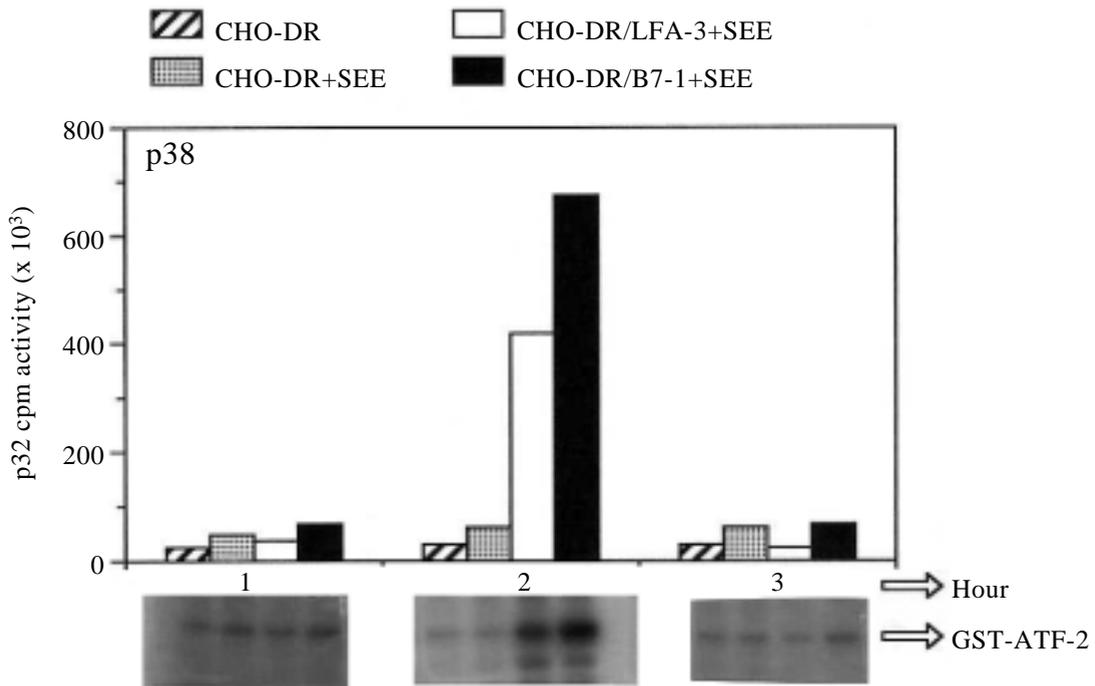


**Figure 3**

Activation of the JNK-1 MAP kinase member by SEE (100 ng/ml) and the CHO-DR, CHO-DR/B7-1 and CHO-DR/LFA-3. Jurkat cells were incubating in medium containing 10% FBS. The cells were stimulated with and without SEE and the various CHO transfectants for the indicated periods of time (in minutes). Whole cell extracts were prepared and assayed for JNK-1 using and immune complex kinase assay with GST-c-Jun as substrate. The cpm activity with respect to untreated cells is shown above the kinase assay panel.

**Figure 4**

Activation of the ERK-2 MAP kinase member by SEE (100 ng/ml) and the CHO-DR, CHO-DR/B7-1 and CHO-DR/LFA-3 in Jurkat T cells stimulated with and without SEE and the various CHO transfectants for the indicated periods of time (in minutes). Whole cell extracts were prepared and assayed for ERK-2 using and immune complex kinase assay with PHAS as substrate. The cpm activity with respect to untreated cells is shown above the kinase assay panel. These experiments were performed at least twice with identical results. A representative experiment is shown.



**Figure 5**

Activation of the p38 MAP kinase member by SEE (100 ng/ml) and the CHO-DR, CHO-DR/B7-1, CHO-DR/LFA-3 and Jurkat cells were incubating in medium containing 10% FBS. The cells were stimulated with and without SEE and the various CHO transfectants for the indicated periods of time (in minutes). Whole cell extracts were prepared and assayed for p38 using and immune complex kinase assay with GST-ATF-2 as substrate. The cpm activity with respect to untreated cells is shown above the kinase assay panel.

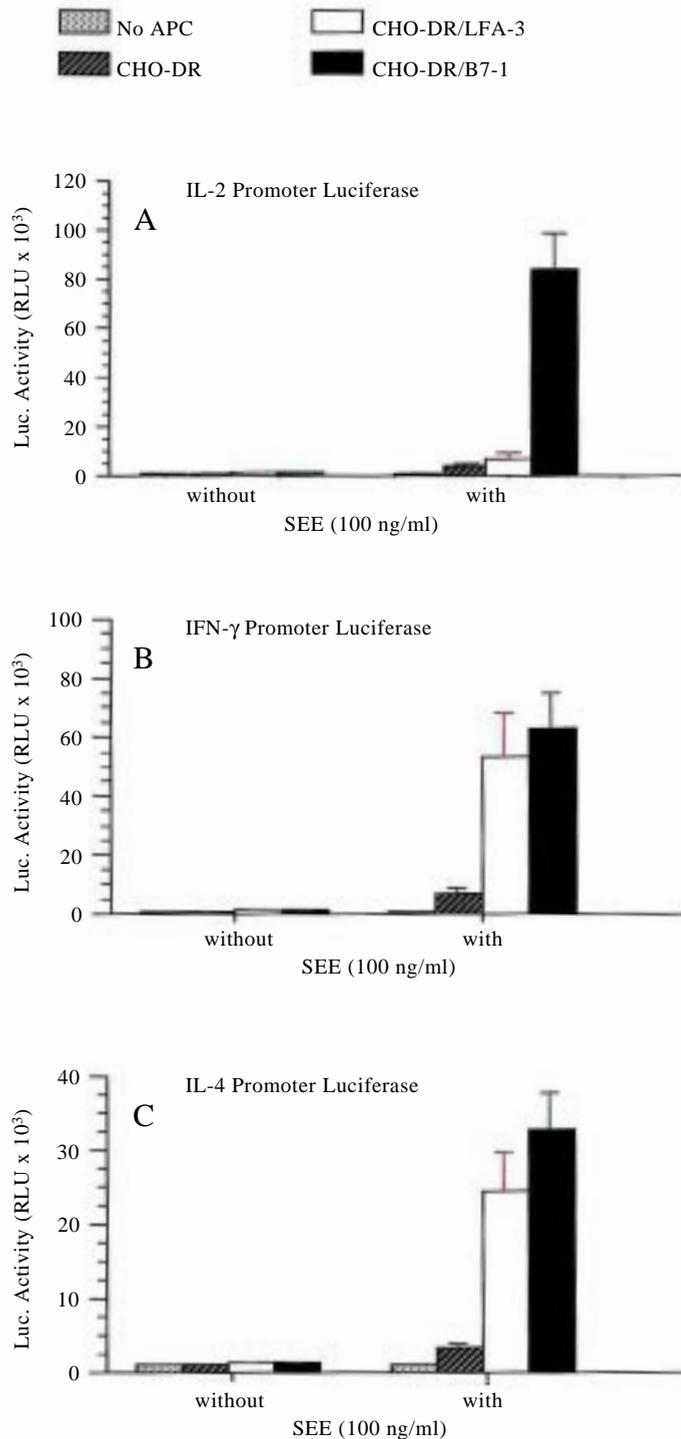
*Costimulation by B7-1 strongly induces the transcriptional activity of the IL-2, INF- $\gamma$  and IL-4 promoters-enhancer region in Jurkat T cells transiently transfected with luciferase reporter constructs.*

Based on the role of the CD28/B7-1 pathway in T cell activation, we wanted to analyze the effects of LFA-3 and B7-1 costimulation on the transcriptional activity of several promoters that play important roles in the immunological response, such as IL-2, INF- $\gamma$  and IL-4. Jurkat T cells were transiently transfected (electroporation) with the human IL-2 (Fig. 6A) INF- $\gamma$  (Fig. 6B), and IL-4 (Fig. 6C) promoter regions fused to the luciferase reporter gene. The transfected cells were then stimulated with SEE (100 ng/ml) and presented to the different CHO transfectants, after which the luciferase activity was measured. As shown in figure 6,

stimulation with SEE alone in the absence of signal two did not affect the transcriptional activity of the different promoters (Fig. 6 A, B, and C). Similarly, signal two (B7-1 or LFA-3) had no effect in the absence of SEE. However, costimulation with B7-1 resulted in a very strong transcriptional activation of all three promoters, IL-2, INF- $\gamma$ , and IL-4 (Fig. 6 A, B, and C). In contrast, costimulation with LFA-3 had no effect on IL-2 transcriptional activity, but it produced a strong activation of both INF- $\gamma$  (Fig. 6B) and IL-4 (Fig. 6C) promoter.

*Proliferative effect of costimulation on proliferation response and IL2 production of CD4<sup>+</sup> T cells to SEA and CHO-DR, CHO-DR/LFA-3 and CHO-DR/B7-1*

Finally, we investigated the effect of B7-1 or LFA-3 costimulation on the induction of

**Figure 6**

Comparison of the transcriptional regulation of the IL-2, IFN- $\gamma$  and IL-4 promoters in Jurkat T cells costimulated with CHO-DR, CHO-DR/LFA-3 and CHO-DR/B7-1 transfected cells. Jurkat T cells were transfected with the various luciferase promoter reporter genes and cultured in RPMI supplemented with 10% FCS. After 24 h the cells were treated with SEE and cultured for 8 h with the various CHO transfectants (1:10 ratio to T cells). Luciferase activity is expressed as RLU minus background units of buffer. The results shown are mean values from three similar experiments. Error bars indicate standard errors of the mean.

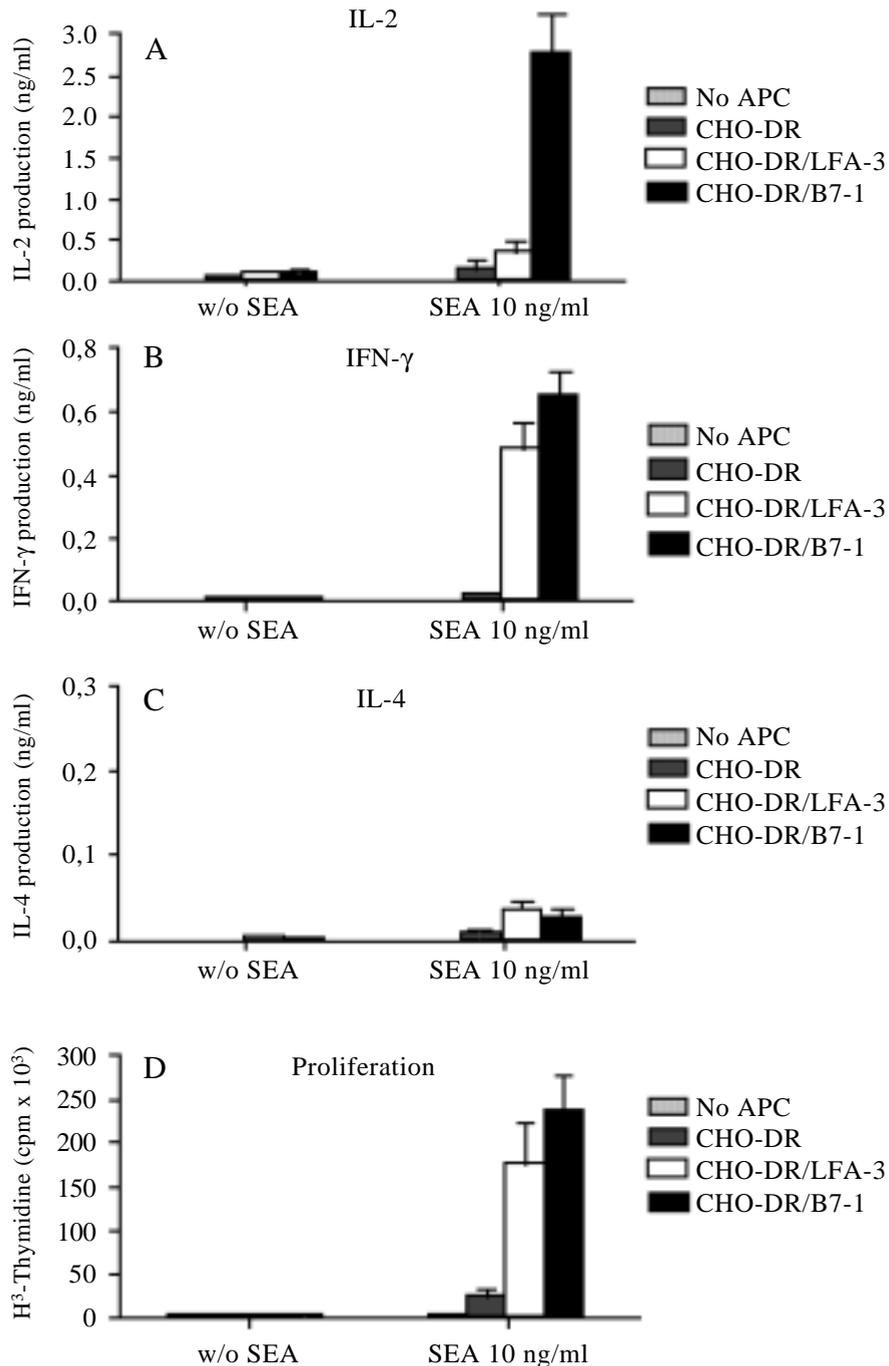
proliferation and the production of IL-2, IFN- $\gamma$  and IL-4 and correlated these results with the transcriptional activity of the MAP kinases ERK-2, JNK-1 and p38. T cell proliferation and IL-2, IFN- $\gamma$ , and IL-4 production were measured in freshly prepared CD4<sup>+</sup>T cells ( $1 \times 10^6$ /ml) co-cultured with 10 ng/ml SEA and with either CHO-DR, CHO-DR/LFA-3, or CHO-DR/B7-1 for 72 h at 37°C. (Fig. 7). B7-1 costimulation was required to induce large amounts of IL-2 (Fig. 7A) while both B7-1 and LFA-3 costimulation induced large amounts of IFN- $\gamma$  (Fig. 7B and C). However, only marginal levels of IL-4 were detected in supernatants of T cells costimulated with B7-1 and LFA-3. The proliferation activity that clearly correlated with the induction of IL-2 and IFN- $\gamma$ , also required B7-1 costimulation (Fig. 7D).

#### DISCUSSION

One of the most interesting signaling pathways involved in the activation of T cells is represented by the MAP kinase cascade. Signal one activates ERK-2 pathway and is involved in the survival of T cells (Minden et al., 1994; Nunes et al., 1996; Robinson et al., 1996). The second signal delivered via accessory molecules such as CD28 and CD2 led to the activation of the JNK and p38 pathways, responsible for induction of IL-2 receptor expression, IL-2 production and subsequent clonal expansion. Contrary to what has been previously proposed (Minden et al., 1994; Su et al., 1994; Robinson et al., 1996) we show that the requirement for at least two signals for the efficient activation of T cells is not unique to JNK, as the ERK is also fully activated by TCR engagement and B7-1 or LFA-3 costimulation. We showed by kinase assays that T cells activated with SEE (signal one) alone substantially activated ERK, but not JNK or p38 pathways. On the other hand, T cells activated with SEE and costimulated with CD28 or CD2 (signal two) showed a strong activation of all three pathways (ERK, JNK and p38). Our results also contrast with recent studies showing that the CD28 was

not required for JNK activation in normal murine T cells (Rivas et al., 2001). In this study high concentrations of anti CD3 mAb alone were sufficient for ERK and JNK activation, even in the absence of anti CD28. This discrepancy may reflect differences in the use of superantigens SEE (signal one) and CHO transfectants expressing the ligands for CD28 and CD2 (signal two) in our studies, as compared with the use of anti-CD3 and anti-CD28 antibodies to induce a similar response or our use of a more physiological system for activation of T cells. The MAP kinases regulate the activity of AP-1, a nuclear factor that plays a critical role in IL-2 gene transcription by binding to three different sites; NF-AT, Oct-1, and AP-1 itself (Durand et al., 1988; Granelli-Piperno et al., 1991). It has also been demonstrated that JNK activates the *c-fos* promoter through phosphorylation of TCF/Elk-1 (Gille et al., 1992) and that MEKK-1 kinase, an upstream kinase in the cascade of JNK, can also induce *c-fos* and *Elk-1* transcriptional activity in the presence of lower levels of ERK (Minden et al., 1994; 1995), suggesting the involvement of mechanisms other than the ERK signal in the activation of *c-fos*. In other systems, JNK, unlike ERK, is not activated by the phorbol ester alone, but rather requires the phorbol ester in combination with calcium ionophore (Nunes et al., 1996; Su et al., 1994; Robinson et al., 1996). Triggering of CD3 and CD28 also results in CsA-sensitive activation of JNK, whereas each stimulus alone results in little or no activation.

However, the dramatic reduction in transcriptional IL-2 promoter activity observed with a luciferase reporter driven by IL-2 promoter containing a mutated CD28RE further underscores the functional importance of the B7-1 costimulation for IL-2 transcriptional activity. These observations, along with the EMSA experiments, indicate the presence of a functional CD28RE in the IL-2 promoter. It is worth commenting that LFA-3, in contrast with B7-1, cannot costimulate IL-2 production, but can costimulate cell proliferation. The results suggest that the

**Figure 7**

Costimulatory effect of LFA-3 and B7-1 in induction of IL-2 (A), IFN- $\gamma$  (B) and IL-4 (C) production and proliferation (D) of SEA primed T cells. In A, CD4<sup>+</sup> T cells ( $1 \times 10^6$ /ml) were cultured for 3 days with RPMI supplemented with 10% FBS, CHO-DR, CHO-DR/LFA-3 and CHO-DR/B7-1 ( $0.1 \times 10^6$ /ml) with or without SEA (10 ng/ml). In A, B, and C, supernatants from the same culture were collected on day 2 and assayed by ELISA for the production of the above mentioned cytokines. In D, the [<sup>3</sup>H]TdR incorporation for proliferation was assessed the last 4 h of culture. Experiments were done at least three times and the most representative results were shown. Error bars indicate standard errors of the mean.

differential effect of CD2/LFA-3 vs. CD28/B7-1 on IL-2 regulation could be related to the difference of the ability of the two pathways to induce JNK activity. In this regard, other laboratories have reported that the activation of ERK, JNK and p38 is differentially sensitive to inhibitions of PI3K (Jascur et al., 1997). Both CD2 and CD28 are linked to the PI3K pathways, suggesting an alternative mechanism of activation of JNK and thus regulation of IL-2 gene expression. The adhesion of the complex induced by the CD2/LFA-3 pathway was also inhibited by the loss of the functional CD28RE. However, earlier reports indicated a central role of CD28RE in CD28-costimulated T cells, although several studies suggested that the CD28RC was not exclusively seen in CD28-stimulated T cells (Civil et al., 1992; Ghos et al, 1993). Using native B7-1 and LFA-3 ligands, we found that the CD28RC is detectable in EMSA by both B7-1 and LFA-3 costimulation. However, the B7-1 costimulation forms two distinct bands in the gel shift assay while the LFA-3 costimulation induces only the upper band. We speculate that the complex bound to the CD28RE, corresponding to the faster migrating band, may be involved in activation of IL2 promoter, as it is induced only by B7-1 and binds to CD28RE on its own as well as in combination with nuclear factors, such as AP-1. In our system, elevated ERK and JNK activities can be detected 1 h after SEE presentation to T cells, while p38 kinase induction was observed only after 2 h, particularly in the presence of B7-1 costimulation. Thus, early activation of these kinases may be required for the activation of *fos* and *jun* genes, while kinase activity at later time-points may be important for activation of the transactivating capacity of the Fos and Jun proteins. Consequently, the TCR signal alone or together with LFA-3 costimulation does not affect JNK-1 and does not cause a synergistic effect in the activation of JNK-1, although its synergy is observed for the activation of p38 and ERK-2.

However, despite the fact that LFA-3 costimulation could increase the activity of ERK-2 and p38, only B7-1 costimulation

correlates with high levels of IL-2 production, proliferation, transcriptional up-regulation of IL-2 and INF- $\gamma$  promoter activities, and the full induction of all three MAPKs. The high levels of proliferation observed in LFA-3 costimulated T cells imply that alternative pathways are involved in the activation of T cells acting as an optional signal two to activated T cells for clonal expansion and cytokine production. The signal delivered by CD28 pathway could account for a paracrine production of the IL-2 growth factor leading not only to clonal expansion of CD4<sup>+</sup> T cells, but also to the activation of other immunological cells such as B cells, CTL and NK cells. Our studies also clearly demonstrate a requirement for B7-1 costimulation for the full induction of transcriptional activity of the IL-2 and IFN- $\gamma$ . The results suggest specificity of the JNK-1 kinase when B7-1 is applied as the second signal. The strong correlation between JNK-1 activation and transcriptional activity induced via the CD28/B7-1 pathway clearly supports a major role for this pathway in T survival, IL-2 production, and proliferation, despite use of alternative pathways such as CD2/LFA-3 and LFA-1/ICAM-1 to deliver signal two. These observations suggest that the integration signals that lead to T cell activation and cytokine production may occur through the JNK/ERK pathway induced by B7-1 costimulation.

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