

# NF- $\kappa$ B and AP-1 regulate activation-dependent CD137 (4-1BB) expression in T cells

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**Abstract** 4-1BB(CD137) is a member of the tumor necrosis factor receptor superfamily and provides a costimulatory signal by interaction with 4-1BB ligand expressed on antigen-presenting cells. The expression of 4-1BB is known to be activation-dependent. Here, we investigated the transcriptional machinery required for T cell receptor (TCR) activation-dependent induction of 4-1BB expression in CD3-CEM cells treated with phorbol myristate acetate and ionomycin. Using 5'-deletion constructs of 4-1BB promoter in luciferase reporter assays, we demonstrated that the transcriptional elements mediating 4-1BB upregulation were located in the region between ~0.9 and ~1.1 kb from the translational start site. Characterization of these sites by electrophoretic mobility shift assay and site-directed mutagenesis revealed that nuclear factor  $\kappa$ B (NF- $\kappa$ B) and activating protein-1 (AP-1) are involved. MEK and c-Jun N-terminal kinase-1 activity was required for activation-dependent 4-1BB upregulation. Thus, NF- $\kappa$ B and AP-1 are involved in the TCR stimulation-dependent transcriptional regulation of the 4-1BB promoter.

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**Key words:** 4-1BB; CD137; T cell activation; T cell receptor signal; Transcription factor

## 1. Introduction

Stimulation of T cells by T cell receptor (TCR) engagement activates a cascade of signaling events. Following the sequential activation of *lck*, *fyn*, and ZAP-70 protein tyrosine kinase [1,2], other biochemical signals are generated including those derived from the phosphatidylinositol pathway [3] and a Ras-activated cascade of serine/threonine kinases [4,5]. Stimulation of the Ras GTP exchange molecule initiates a cascade of kinase activity involving Raf, MEK and ERK kinases [6,7], which lead to the activation of several transcription factors, including activating protein-1 (AP-1) [8]. Phosphatidylinositol biphosphate is cleaved into diacylglycerol and inositol trisphosphate by activated phospholipase C $\gamma$ 1 [9]. Activation of

protein kinase C by diacylglycerol leads to activation of the transcription factor nuclear factor  $\kappa$ B (NF- $\kappa$ B) [10]. Inositol trisphosphate leads to intracellular Ca<sup>2+</sup> influx and subsequently the activation of the serine phosphatase, calcineurin [11], which, in turn, mediates the activation of nuclear factor of activated T cells (NFAT). These activated transcription factors regulate the transcription of a number of genes including cytokines like interleukin-2 (IL-2) [3], and many other genes that lead to immune response and clonal expansion or activation-induced cell death.

One such gene product is 4-1BB (CD137), a cell surface protein that is induced by various T cell activation signals and provides a costimulatory signal by interaction with its ligand (4-1BBL) expressed on antigen-presenting cells [12–18]. Signals delivered to the T cell by the interaction of 4-1BB with 4-1BBL can induce T cells to produce IL-2, proliferate, and differentiate [13,14,19,20], as well as protect T cells from activation-induced cell death [21,22].

4-1BB is inducible through the TCR in the presence of cytokines or in combination with CD28 ligation [23,24], or anti-TCR monoclonal antibody (mAb), or anti-CD3 mAb [25,26], or by stimulation with phorbol myristate acetate and ionomycin (P/I) [27]. This induction could be inhibited with cyclosporin A [25] or cycloheximide [26]. Actinomycin D reduced 4-1BB mRNA levels in activated lymphocytes by 50% within 30 min, demonstrating a relatively short half-life of this mRNA [16].

Despite these findings, the transcriptional machinery controlling 4-1BB induction has not been elucidated. Based on the analysis of genomic DNA, 4-1BB is localized on chromosome 1p36 which contains the genes for other members of the tumor necrosis factor (TNF) receptor superfamily such as TNF-RII, OX40, CD30, and TRAMP/Apo3 [27]. Genomic sequences proximal to the 4-1BB translational start site have an absence of conventional TATA boxes. These are characteristic of other members of the TNF-nerve growth factor receptor superfamily [28–31].

In this study, we have investigated the transcriptional machinery required for TCR activation-dependent induction of 4-1BB expression in CD3-CEM cells, a subclone of the CD4<sup>+</sup> T cell line, CCRF-CEM. 4-1BB was markedly upregulated in these cells by P/I stimulation. Using deletion constructs of the 4-1BB promoter in luciferase reporter assays, we demonstrate the presence of an activation-responsive sequence of ~200 bp containing putative binding sites for transcription factors NF- $\kappa$ B and AP-1. Characterization of these sites by electrophoretic mobility shift assay (EMSA) and site-directed mutagenesis revealed that transcriptional activation of the 4-1BB gene

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**Abbreviations:** TNF, tumor necrosis factor; TCR, T cell receptor; AP-1, activating protein-1; NFAT, nuclear factor of activated T cells; P/I, phorbol myristate acetate and ionomycin; mAb, monoclonal antibody;  $\beta$ -gal,  $\beta$ -galactosidase

upon stimulation with P/I requires NF- $\kappa$ B and AP-1 recruitment to the promoter site.

## 2. Materials and methods

### 2.1. Cell lines and reagents

The CD4<sup>+</sup> T cell line CCRF-CEM was obtained from ATCC and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100  $\mu$ g/ml penicillin, 100 U/ml streptomycin (Biowhittaker, Walkersville, MD, USA). A CD3-positive population of CCRF-CEM was isolated by MACS (data not shown) and was named CD3-CEM. For TCR stimulation, anti-human CD3 mAb (BD Pharmingen, San Diego, CA, USA) was immobilized on plastic culture dishes by incubating dishes at 4°C with 10  $\mu$ g/ml in phosphate-buffered saline overnight. Phorbol myristate acetate (20 ng/ml, Sigma, St. Louis, MO, USA) and ionomycin (0.5  $\mu$ M, Sigma) were also used to activate CD3-CEM. PD98059 was purchased from Sigma. A mouse mAb 4B4 against the human 4-1BB molecule [32] was used for flow cytometry, and MAG56 mAb [33] was used as an irrelevant, isotype-matched control antibody. Goat  $\gamma$ -globulin was purchased from Pierce (Rockford, IL, USA). pcDNA3-Flag-JNK1 (APF, T183A/Y185F) [34] was kindly provided by Dr. H.-R.C. Kim, Wayne State University (Detroit, MI, USA).

### 2.2. Flow cytometry

Cells were harvested at determined time points and analyzed as described in [35].

### 2.3. Reverse transcriptase polymerase chain reaction (RT-PCR)

CD3-CEM cells are cultured with P/I and harvested at 0, 12, 24, 36, and 48 h. Total RNA was isolated from the cells using the RNeasy mini kit (Qiagen, Valencia, CA, USA) as described in the user manual. Total RNA 2.5  $\mu$ g was reverse transcribed into cDNA and the enzymatic amplification by PCR was performed as described in [35]. Sense and antisense primers used in the PCR were as follows: for 4-1BB, 5'-GGATTGTAAACAAGGTCAAG-3' and 5'-CACATCCTCCTTCTTCTCT-3'. For PCR reaction, the sample was first denatured at 95°C for 3 min and amplified by 40 cycles of PCR (94°C, 30 s; 55°C, 30 s; 72°C, 1 min), followed by 7 min at 72°C for 4-1BB or by 30 cycles of PCR for GAPDH. Equal aliquots of RT-PCR product for 4-1BB (448 bp) and GAPDH (500 bp) were separated on 1.5% agarose gels and stained with ethidium bromide.

### 2.4. Construction of 4-1BB promoter-luciferase plasmids

Human peripheral blood mononuclear cells were obtained from peripheral blood of healthy donors by Ficoll density gradient centrifugation. Genomic DNA was isolated by the method of Gross-Bellard et al. [36] and ~1.7 kb of 4-1BB promoter was amplified by PCR using a *KpnI*-*HindIII*-flanked primer set derived from genomic sequences (GenBank accession number NT\_004454) upstream of the 4-1BB translational start site. The forward and reverse primers used for PCR amplification from human genomic DNA were as follows and underlines mean flanked *KpnI* or *HindIII* restriction enzyme sequences: forward, 5'-AGGTACCTGCCATGTTGGACGTCTAG-3'; and reverse, 5'-TAAGCTTGATGAAATCTGGCACAGGTA-3'. This ~1.7 kb PCR product was subcloned into luciferase-link reporter plasmid, pGL3-Enhancer (Promega, Madison, WI, USA). To generate smaller promoter fragments, plasmid pGL3E-1.7 kb was used as a template during PCR by various 5'-flanking sequences of the 4-1BB promoter gene as forward primers. For 1.1 kb, 5'-TGGTACCAATGCTTTGTCTCTGGAGAGC-3'; 1.0 kb, 5'-AGGTACCTGAACAGGAGGTTCTT-3'; 0.9 kb, 5'-TGGTACCGAGACCCCGCCCCTG-3'; 0.85 kb, 5'-AGGTACCTGAAGTCTCTCG-3'; 0.8 kb, 5'-AGGTACCAAGGTCTGTCCATCT-3'; 0.55 kb, 5'-AGGTACCTGAACAGGAGGTTCTT-3'; 0.5 kb, 5'-TGGTACCCAGCTAAAGGGGAAGATG-3'. The 3' end of each promoter fragment was the first nucleotide of the translational start site. All PCR-derived constructs were confirmed by fluorescent automated sequencing (Pangenomics, Seoul, Korea).

### 2.5. Generation of mutant 4-1BB promoter-reporter construct

Transcription factor binding mutants were created using overlap extension PCR with the above forward and reverse primers for 1.1 kb and the following primers to incorporate a transcription factor

binding site mutation: NF- $\kappa$ B forward, 5'-TGTGGTGC GAATT-TC CATGAG-3'; and reverse, 5'-CTCATGGGAAATTCGACCA-ACA-3'. Proximal AP-1 forward, 5'-GCCCTGGCTGAGTTGCC-GCACT-3'; and reverse, 5'-AGTGCGGCAACTCAGCCAGGGG-C-3'. Distal AP-1 forward, 5'-GTCATCTGTGACAGGTCCTG-3'; and reverse, 5'-CAGGACCTGTACAGATGAC-3'. All mutants are verified by fluorescent automated sequencing (Pangenomics).

### 2.6. Transient transfection and luciferase assay

Different 4-1BB promoter constructs were transiently transfected into CD3-CEM cells using DMRIE-C (Life Technologies) as described in the user manual. pCMV $\beta$  (BD Clontech, Palo Alto, CA, USA) was cotransfected to normalize transfection efficiencies. In all cases, the total amount of DNA in each transfection was kept equal by the addition of control DNA (pcDNA3) where appropriate. Cells ( $8 \times 10^5$  cells/well) were incubated with DNA (3  $\mu$ g) and DMRIE-C (3  $\mu$ l). For JNK1, pcDNA3-Flag-JNK1 (T183A/Y185F) was cotransfected with 1.1 kb promoter plasmid and pCMV $\beta$ . After 4–5 h, transfected cells were incubated with phorbol myristate acetate 20 ng/ml and ionomycin 0.5  $\mu$ M for 36–40 h. For anti-CD3 stimulation, transfected cells were transferred to the wells coated by 10  $\mu$ g/ml/well of anti-human CD3 mAb and cultured for 36–40 h. Cells were harvested and washed with phosphate-buffered saline, and lysed in 150  $\mu$ l of reporter lysis buffer (Promega). Cell debris was removed by centrifugation, and the supernatant was used in the luciferase assay and  $\beta$ -galactosidase ( $\beta$ -gal) assay. Luciferase activity was measured in triplicate using the Dual Luciferase Assay kit (Promega) following the manufacturer's recommendations using a luminometer, Luminoskan Ascent (Thermo Labsystems USA, Franklin, MA, USA). Luciferase activity was normalized by  $\beta$ -gal activity for each data point.

### 2.7. EMSA

CD3-CEM  $4 \times 10^7$  cells were stimulated with P/I and harvested at 0, 2, and 4 h. Nuclear extracts were prepared according to the method by Schreiber et al. [37]. Wild type and mutant oligonucleotides containing NF- $\kappa$ B, proximal AP-1 and distal AP-1 sequences of the 4-1BB promoter were synthesized (Cosmogentech, Seoul, Korea). The probe corresponding to the distal AP-1 site (−1067 to −1048) was 5'-GTATCTGTGACACATCCTG-3' (mutant: CA  $\rightarrow$  GG); NF- $\kappa$ B (−915 to −894), 5'-TGTGTGGGAATTTCCCATGAG-3' (mutant: G  $\rightarrow$  C); proximal AP-1 (−888 to −876), 5'-GCCCTGGCTGAGTCAACGCACT-3' (mutant: CA  $\rightarrow$  TG). The  $\kappa$ B site in the  $\kappa$  light chain enhancer region used as a control probe (5'-AGTTGAGGGG-GACTTTCCAGGC-3') and AP-1 control probe (5'-CGCTTGATGAGTCGCCGAA-3') were purchased from Promega. Oligonucleotides were annealed to make double-stranded target DNA and then end-labeled by [ $\gamma$ -<sup>32</sup>P]ATP. For EMSAs, 10  $\mu$ g of nuclear extracts was incubated with ~100 000 cpm (40 fmol) of the indicated  $\gamma$ -<sup>32</sup>P-end-labeled probes for 30 min at room temperature. For competition assays, excess unlabeled wild type, mutant, or control oligonucleotides for transcription factor binding sites were added. For antibody-mediated supershift assays, either anti-p50 or p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies were co-incubated. The reaction mixtures were loaded on a 4.5–5.5% polyacrylamide non-denaturing gels in 0.5 $\times$ TBE buffer and electrophoresed for 2 h at 150 V. The gels were dried and exposed to autoradiographic film.

## 3. Results

### 3.1. Induction of 4-1BB expression in CD3-CEM cells by T-cell activation

To study the transcription mechanisms controlling 4-1BB expression in response to signals through TCR, we first attempted to select a human T cell line, which highly expresses 4-1BB protein on the cell surface upon activation. Flow cytometry analyses have shown that 4-1BB induction was greater in CCRF-CEM than any other T cell lines, such as Jurkat, Molt-4, and SUPT-1 (data not shown). Therefore, we isolated CD3<sup>+</sup> population of CCRF-CEM (CD3-CEM) and used in this study. To assess the induction of 4-1BB following TCR stimulation, CD3-CEM cells were stimulated with anti-CD3 mAb or P/I that mimic TCR-mediated signaling events for

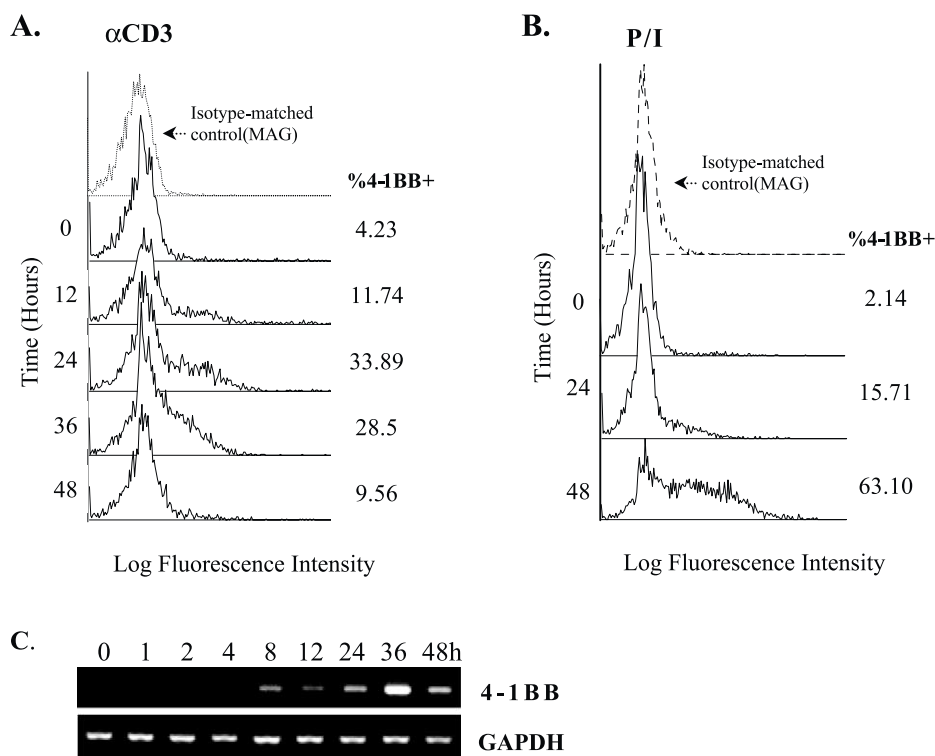


Fig. 1. Time course of induction of 4-1BB in CD3-CEM cells treated with anti-CD3 mAb or P/I. A: Cells were incubated in the tissue culture plates coated with anti-CD3 mAb and harvested at the indicated time, and 4-1BB expression was analyzed by flow cytometry. Isotype-matched control antibody, MAG56 staining is represented as a dashed line. B: Cells stimulated with P/I were harvested at the indicated time, and 4-1BB expression was analyzed as in A. C: Cells treated with P/I were harvested at the indicated time. The total RNA was extracted and analyzed for relative levels of 4-1BB mRNA by RT-PCR. GAPDH was used to normalize for template quantity. Data were representative of at least two independent experiments.

various times, and protein and mRNA expression was determined with flow cytometry and RT-PCR, respectively. Surface 4-1BB expression is upregulated after anti-CD3 mAb and P/I treatment. Increased 4-1BB was detectable at 12 h post stimulation, reached maximal levels at 24 h, and declined to baseline levels by 48 h in cells stimulated with anti-CD3 mAb (Fig. 1A). In cells treated with P/I, 4-1BB was induced after 24 h and the level reached 63% at 48 h (Fig. 1B). The amount of 4-1BB mRNA was also increased after P/I treatment. 4-1BB mRNA was hardly detected at 0 h, the level increased significantly at 8 h and the increased level was maintained up to 48 h (Fig. 1C). It was reported that 4-1BB mRNA has a relative short half-life, 30 min, and induction of 4-1BB mRNA was primarily due to increased transcription [16]. These results suggested that the induction of surface 4-1BB of CD3-CEM by TCR activation is due to the upregulation of transcription.

### 3.2. A 251 bp region in the 4-1BB promoter is required for transcriptional activation

Putative transcription factor binding sites, which include NFAT, NF- $\kappa$ B and AP-1 elements, were identified in the 5'-upstream region of the 4-1BB translational start site using MatInspector V2.2 software (Fig. 2). We cloned a 1.7 kb size fragment of the putative 4-1BB promoter by PCR from human genomic DNA and inserted it into the luciferase reporter plasmid, pGL3-enhancer. Within this 1.7 kb region, 5'-untranslated exon 1 and partial exon 2 are localized. To investigate the requirements for TCR-mediated expression of 4-1BB, the reporter construct was transiently transfected into

CD3-CEM cells and its expression was measured after cell stimulation. A 39 h stimulation with immobilized anti-CD3 mAb induced 4-1BB reporter activity six-fold over that in unstimulated controls (Fig. 3B). When the cells were stimulated with P/I, the response was comparable to that seen with anti-CD3 stimulation. To determine which segment(s) of the 5'-untranslated region of the 4-1BB gene is important for transcriptional activation, six deletion promoter constructs of various sizes (Fig. 3A) were transiently transfected and luciferase activity was measured after anti-CD3 mAb or P/I stimulation. The results shown in Fig. 3B indicate that stimulus-dependent transcriptional activity was detected in constructs containing promoter sequences from -866 bp to



Fig. 2. Nucleotide sequence of the 5'-upstream region of the human 4-1BB gene. The translational start site is designated +1 and numbers indicate nucleotide locations with respect to the translational start site. An arrow indicates the transcription start site and the exon is underlined. The boxes indicate the predicted transcription factor binding sites and gray shadowed regions represent the location of primers used in cloning of 4-1BB 5'-deletant promoters.

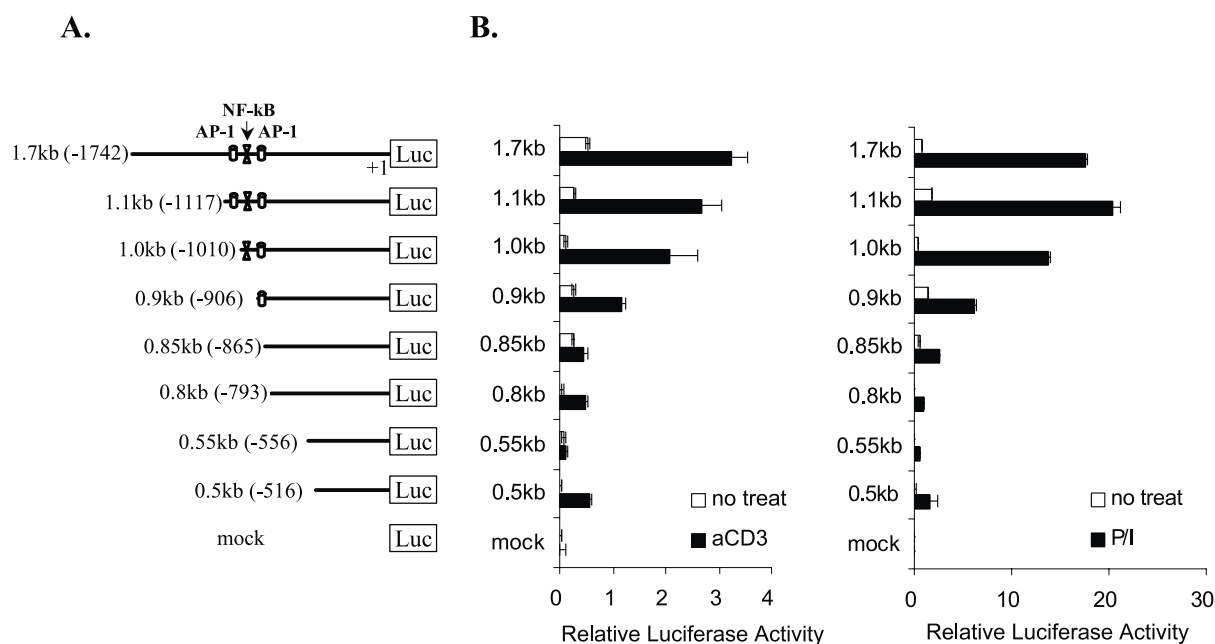


Fig. 3. Characterization of 4-1BB promoter by reporter analysis. A: 4-1BB promoter deletion constructs used in reporter analysis. 4-1BB promoter DNA sequences with various 5'-deletions were cloned upstream of the luciferase reporter gene in pGL3-enhancer. Numbers indicate nucleotide locations with respect to the translational start site of the 4-1BB gene. B: Analysis of the promoter region of the human 4-1BB gene. CD3-CEM cells were transiently transfected with various 5'-deletion promoter-reporter constructs and pCMVβ. Cells were treated with anti-CD3 mAb (left panel) or P/I (right panel) and reporter activity was determined after 39 h. Transfection efficiencies were normalized by β-gal activity and the results are represented as relative luciferase activity. Error bars represent the standard deviation of triplicate samples and data are representative of at least three independent experiments.

–1117 bp and a maximal induction of 23-fold was observed in the construct containing up to –1117 bp, after P/I stimulation. The transcription activity of –1741 bp is similar to that of –1117 bp. These results suggest that the region between –1117 and –866 relative to the translation initiation site is involved in signal-induced transcriptional activation and contains enhancer element(s) necessary for activation responsiveness. Sequence analysis within this region demonstrated the presence of consensus elements for several transcription factors including NF-κB, AP-1, GATA and SP1 (Fig. 2).

### 3.3. AP-1 and NF-κB bind to the putative 4-1BB promoter binding site

To analyze whether transcription factors are involved in the activation-dependent upregulation of the 4-1BB promoter within the –1117 and –866 region, EMSA analyses were carried out. Nuclear extracts prepared from unactivated and P/I-stimulated CD3-CEM cells were incubated with <sup>32</sup>P-labeled probe spanning the 4-1BB promoter regions of –1067 to –1048 (putative distal AP-1), –915 to –894 (putative NF-κB), and –888 to –876 (putative proximal AP-1). A time course analysis revealed the presence of complexes binding to these region and maximum binding was detected after 2 h stimulation with each probe (Figs. 4 and 5). The binding was competed out with 25- and 50-fold excess of unlabeled probes. Unlabeled probes containing mutations in the putative binding site (for distal AP-1, TGACACA → TGACAGG; for NF-κB, GGGAA → GCGAA; for proximal AP-1, TGAGTCA → TGAGTTG) did not interfere with DNA binding of the protein complexes (Figs. 4 and 5). To determine the nature of each complex binding, competition was also carried out with 25- or 50-fold excess of the oligonucleotides contain-

ing the NF-κB site in κ light chain enhancer region and AP-1 control elements. As shown in Fig. 4, putative distal and proximal AP-1 binding was inhibited by AP-1-specific oligonucleotides, but not NF-κB-specific oligonucleotides, indicating the specificity of AP-1 binding at the –1067 to –1048 and –888 to –876 sequences. As shown in Fig. 5A, putative NF-κB binding was inhibited by unlabeled probe (lanes 7 and 8), but not mutant oligonucleotide or AP-1 control DNA (lanes 9–12). To characterize the protein complex that binds to a putative NF-κB sequence, we repeated the EMSAs in the presence of antibodies against p65 and p50 of the NF-κB family. Antibodies specific for p65 and p50 caused the super-shifted, slower migrating DNA–protein complex (Fig. 5B). These results indicate that the nuclear protein complex, which binds to the putative NF-κB binding sequence, contains at least p50 and p65 of NF-κB and P/I stimulation increases p65 binding more than p50 binding. When we use the probes containing potential Sp1 and GATA-1 binding sites, DNA–protein complexes were not detected (data not shown).

### 3.4. AP-1 and NF-κB promoter elements are critical for regulation of 4-1BB expression in activated T lymphocytes

After showing that the AP-1 and NF-κB present in nuclear extracts are major components of the nuclear protein complex that binds to the 4-1BB promoter sequences, we next investigated whether these AP-1 and NF-κB binding sites are important for the regulation of 4-1BB expression. We mutated each distal and proximal AP-1 and NF-κB site in the context of the 1117 bp 4-1BB promoter-reporter. These mutant constructs were tested for their ability to induce reporter gene expression upon T cell activation with anti-CD3 or P/I treatment. As shown in Fig. 6, mutation of the NF-κB and AP-1



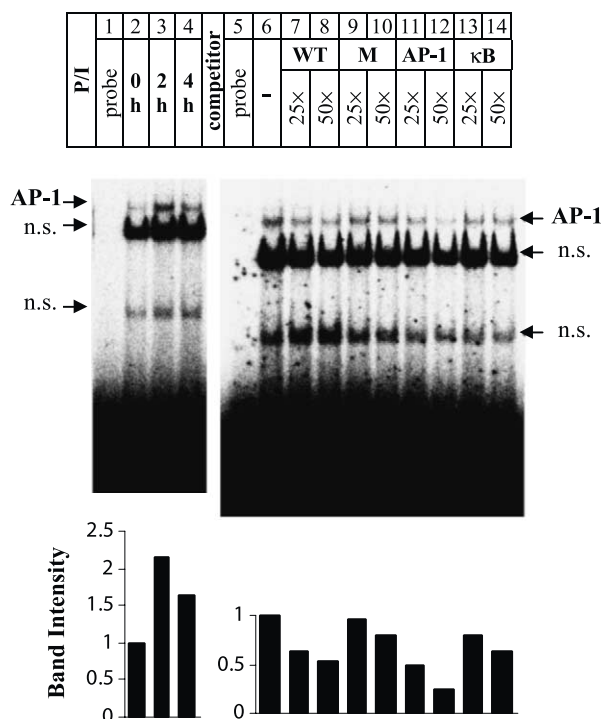
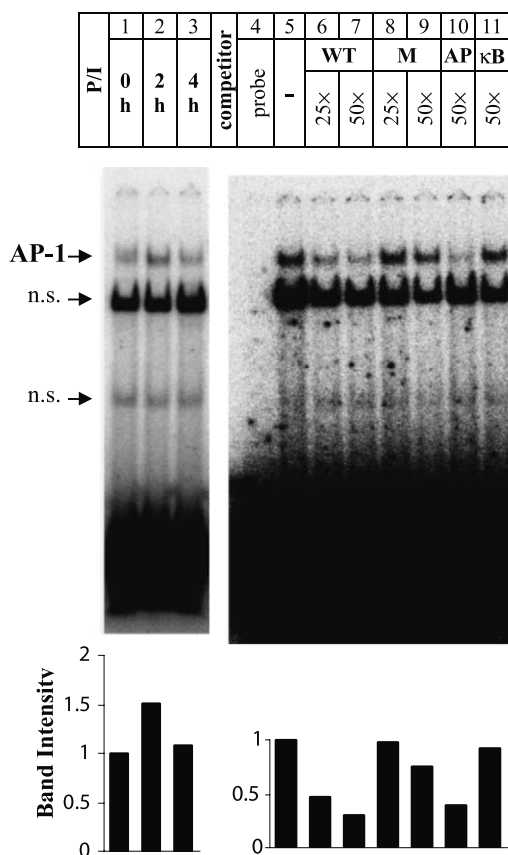
**A. Probe: putative distal AP-1 (–1067 to –1048)****B. Probe: putative proximal AP-1 (–888 to –876)**

Fig. 4. EMSA of putative AP-1 binding sites. CD3-CEM cells were activated with P/I, nuclear extracts were harvested, and EMSA analysis was performed with indicated  $^{32}$ P-labeled probe. A: Analysis of protein binding to a putative distal AP-1 site located at –1067 to –1048. Time course analysis (0, 2, 4 h) of DNA–protein complex binding is shown in the left panel, lanes 1–4. Cold competition was performed with 2 h activated CD3-CEM cell nuclear extracts with 25- and 50-fold excess of wild type probe (WT, lanes 7 and 8), mutant probe (M, lanes 9 and 10), consensus oligonucleotides for transcription factor AP-1 (lanes 11 and 12) or NF-κB (lanes 13 and 14). B: Analysis of protein binding to a putative proximal AP-1 site at –888 to –876. Time course analysis is shown in the left panel, lanes 1–3. Cold competition was performed with 2 h activated CD3-CEM cell nuclear extracts with 25- and 50-fold excess of wild type probe (WT, lanes 6 and 7), mutant probe (M, lanes 8 and 9), 50-fold of consensus oligonucleotides for transcription factor AP-1 (lane 10) or NF-κB (lane 11). n.s., non-specific binding.

sites had a profound effect on T cell activation-mediated 4-1BB expression ( $\leq 67.9\%$  reduction), further supporting the idea that AP-1 and NF-κB are required for optimal activation of the 4-1BB transcription induced by T cell activation signals.

### 3.5. MEK and JNK1 activities are required in activation-dependent 4-1BB upregulation

We explored the possible role of protein kinase MEK and JNK1 in the induction of 4-1BB. T cell receptor ligation activates the early genes *fos* and *jun* that heterodimerize and bind to the AP-1 site to regulate IL-2 and other cytokine gene transcription [38]. Since Erk contributes to *fos* activation, the potential role of the Ras/MEK/Erk pathway was defined by PD98059, a pharmacological inhibitor of MEK [39]. As shown in Fig. 7A, 1.1 kb of 4-1BB promoter activity was more reduced after P/I plus PD98059 than after P/I stimulation only. Phosphorylation of c-Jun by JNK/SAPK is required for AP-1-dependent promoter activity [38]. DN-JNK1 (T183A/Y185F) expression inhibited c-Jun phosphorylation effectively (data not shown). To study the effect of

JNK1 on 4-1BB promoter activity, we cotransfected the 1.1 kb of 4-1BB promoter with DN-JNK1 (T183A/Y185F) expression vector. P/I-activated CD3-CEM cells transiently transfected with DN-JNK1 showed a dose-dependent decrease in 4-1BB promoter activity with increasing DN-JNK1 concentration (Fig. 7B). As shown in Fig. 7C, the reporter activity was significantly reduced in anti-CD3 mAb stimulation as well as P/I activation. Taken together, these results strongly support that the MEK and JNK pathways are involved in TCR activation-dependent 4-1BB expression.

## 4. Discussion

In this study, we have shown that the transcription factors NF-κB, and AP-1 are involved in induction of 4-1BB expression through TCR signaling.

4-1BB is known to be expressed primarily on activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells and 4-1BB/4-1BBL binding delivers the costimulatory signals to T cells synergized with CD28 or in the absence of CD28 [17,40,41]. When signals through the TCR are weak, 4-1BB co-stimulation is not as effective as

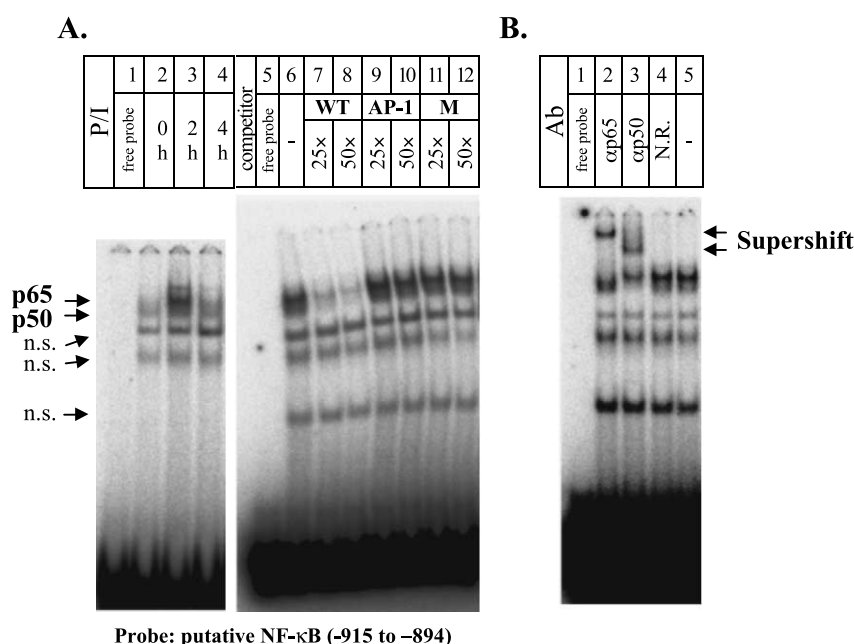


Fig. 5. EMSA of putative NF-κB binding site at -915 to -894. A: CD3-CEM cells were stimulated with P/I for the indicated time, nuclear extracts were harvested and EMSA was performed with  $^{32}$ P-labeled probe (left panel, lanes 2–4). Cold competition assay (lanes 5–12) was performed by incubating CD3-CEM nuclear extracts obtained from cells stimulated with P/I for 2 h with 25- and 50-fold excess of wild type probe (WT, lanes 7 and 8), AP-1 control oligonucleotide (lanes 9 and 10), or mutant probe (lanes 11 and 12). B: Antibody-mediated supershift analyses of NF-κB site binding proteins. CD3-CEM nuclear extracts prepared from cells of 2-h stimulation were subjected to EMSA analysis in the absence (lane 5) or presence of the indicated specific antibody of NF-κB subunit (lanes 2 and 3), or goat  $\gamma$ -globulin (N.R.). Arrows indicate the identity of the supershift complexes. n.s., non-specific binding.

CD28 costimulation in the induction of IL-2 [41]. This may be due to the fact that 4-1BB must be upregulated during the initial T cell activation event – in contrast to CD28, which is expressed constitutively. While much has been learned about the molecular mechanisms that transduce activation signals following 4-1BB/4-1BBL interactions [42–44], little is yet understood about how the expression of 4-1BB is regulated. Because of the important role played by 4-1BB in control of T lymphocyte activation, we became interested in how this molecule was regulated at the level of transcription in human T cells.

We therefore constructed 4-1BB promoter-reporter to address questions about the transcription factors that are involved in driving 4-1BB following TCR engagement. The 4-1BB surface expression level following P/I activation was 63% in the CD3-CEM cell line (Fig. 1), this was enough to elucidate the linkage between TCR activation and 4-1BB upregulation. Our first experiments were designed to show that the  $\sim 1.7$  kb reporter mirrored expression of the endogenous gene. We demonstrated this to be true in the CD3-CEM cell line whether cells are stimulated through their TCR or with pharmacological agents, P/I (Fig. 3). Although the transcrip-

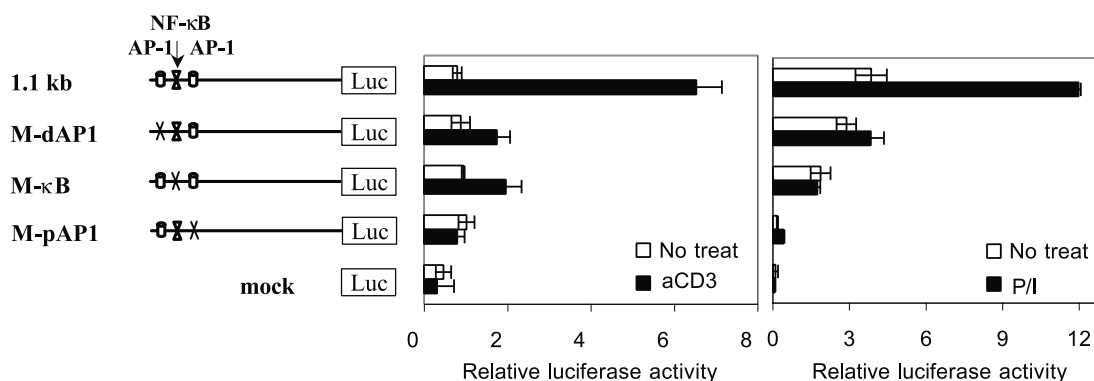


Fig. 6. The distal AP-1, NF-κB, and proximal AP-1 sites are required for optimal 4-1BB promoter activity in stimulated CD3-CEM cells. 1.1 kb of different mutant promoters were generated as described in Section 2. CD3-CEM cells were transiently transfected with the different 1.1 kb regions of promoter-reporter constructs which are the wild type 1.1 kb (1.1 kb), distal AP-1 mutant (M-dAP1), NF-κB mutant (M-κB), and proximal AP-1 mutant (M-pAP1), or with promoterless luciferase reporter (mock) and pCMV $\beta$ . The transfectants were left unstimulated (no treat), or were stimulated with P/I or plate-bound anti-CD3 mAb for 23 h and 45 h respectively. Cells were then lysed and assayed for luciferase activity. Data are represented as relative luciferase activity and transfection efficiencies were normalized by  $\beta$ -gal activity. One representative experiment of at least three performed is shown.

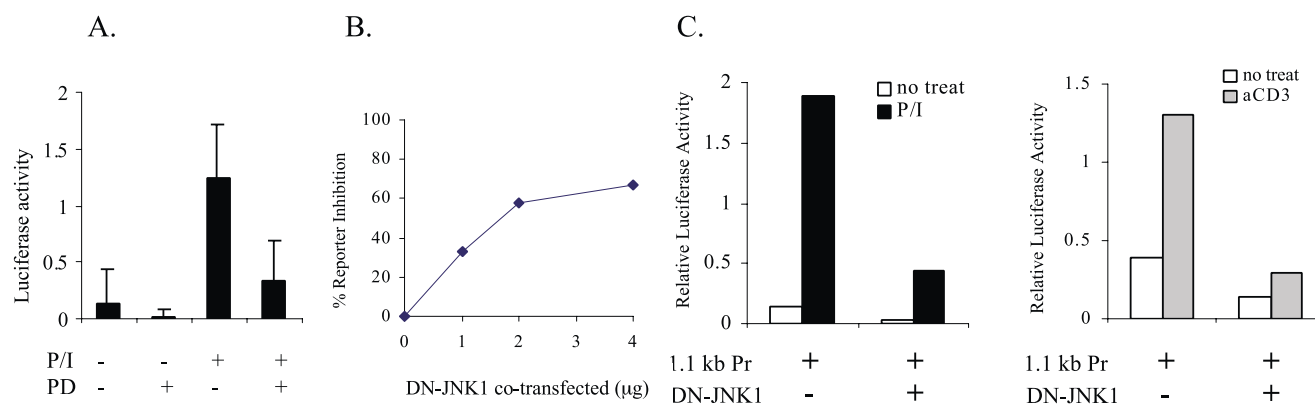


Fig. 7. MEK and JNK1 activity is required for activation-induced 4-1BB promoter activity. A: 4-1BB promoter 1.1 kb (1 µg) was transfected. The transfected CD3-CEM cells were stimulated with P/I or P/I plus PD98059 (50 µM). Results shown are the average of three independent experiments and the standard deviations are represented as error bars. B: Dose-dependent inhibition of P/I-inducible 4-1BB promoter activity by DN-JNK1. Increasing amounts of DN-JNK1 (T183A/Y185F) expression plasmid were cotransfected with 4-1BB promoter 1.1 kb (1 µg). The total amount of DNA in each transfection was kept equal by the addition of empty vector, pcDNA3. Transfected cells were activated with P/I and luciferase activity was measured after 22–24 h. C: 4-1BB promoter 1.1 kb (1 µg) and DN-JNK1 (4 µg) or control DNA (4 µg) were co-transfected. Cells were activated with P/I (left panel) or anti-CD3 mAb (right panel) and analyzed as in A. Transfection efficiencies were normalized by β-gal activity and the results are represented as relative luciferase activity. Results shown are representative of at least three independent experiments.

tion initiation site was not experimentally determined, the ability of this construct to drive luciferase expression suggests that a functional promoter is located within the 1.7 kb region. The comparison among the published 4-1BB mRNA sequences (GenBank accession numbers U03397, 1415 bp; L12964, 1419 bp; and NM\_001561, 1937 bp) supported that the transcription start site was at around 833 bp upstream from the translation start site. In experiments with 5'-deletion promoter constructs, the change in reporter activity in cells transfected with various constructs also paralleled in both stimuli as the promoter was shortened below 1.1 kb (Fig. 3). We revealed that the −866 bp to −1117 bp region of 4-1BB gene is important for signal-induced transcriptional activation and identified several potential regulatory regions including AP-1, NF-κB, GATA1 and SP1 in this region (Fig. 2). However, we could not detect P/I-inducible GATA1 or SP1 binding when these sites were used as probes in EMSA analyses (data not shown).

Our results demonstrated that the 4-1BB promoter contains a NF-κB binding site at position −911 to −898 relative to the 4-1BB translational start site. The prototypic form of NF-κB is a heterodimer complex containing NF-κB1-p50 or NF-κB2-p52 in combination with a transactivating subunit such as c-Rel or RelA(p65) [45]. Each NF-κB-Rel family member contains a conserved N-terminal region responsible for DNA binding, dimerization, and IκB interaction. NF-κB-responsive genes are transactivated by the C-terminal domain of dimeric NF-κB-Rel family members. Our EMSA analyses showed that nuclear proteins that bind to the 4-1BB NF-κB sequence are induced in CD3-CEM cells stimulated with P/I. Competition assay with excess unlabeled probe for NF-κB and control AP-1 or κB site in the κ light chain enhancer region identified the induced protein as NF-κB and indicated specificity of the binding.

An antibody-mediated supershift assay showed that the NF-κB probe (−915 to −894) was bound by p50 and p65 (RelA) (Fig. 5). Because the p50/p50 homodimer lacks the ability to activate genes, it is suggested that upon activation,

the p50/p65 heterodimer translocates into the nucleus and bind the 4-1BB site, and this, in turn, activates 4-1BB promoter activity. However, it remains possible that there is co-operation of other NF-κB family members such as p52, RelB, and c-Rel. The dramatic reduction in transcriptional 4-1BB promoter activity is observed with a 1.1 kb 4-1BB promoter-reporter containing site-directed mutagenesis in NF-κB binding site (85.4% reduction) (Fig. 6). Wang et al. reported that the level of 4-1BB expression and the percentage of 4-1BB-expressing T cells is higher in HIV-1<sup>+</sup> individuals after T-cell stimulation than in HIV-1<sup>−</sup> individuals [46]. This phenomenon might be due to the fact that both HIV-1 binding and gp120-mediated CD4 cross-linking can induce NF-κB [47]. This result further supports our suggestion that NF-κB plays a critical role in stimulus-induced 4-1BB gene expression in T cells.

Our result showed that two AP-1 binding sites located at the −866 and −1117 bp regions of the 4-1BB promoter are critically involved in the induction of 4-1BB in stimulated CD3-CEM cells. AP-1 complexes can consist of different dimeric Fos and Jun family members and exhibit variable DNA binding specificity. Although Jun/Jun homodimers and Fos/Jun heterodimers can transactivate AP-1 sites, Fos/Jun heterodimers are more potent transactivators [48]. In EMSA, the AP-1 probe-protein complex band intensity was not decreased by the competitor oligonucleotides containing the NF-κB binding site but AP-1 consensus elements (Fig. 4). This was confirmed by site-directed mutagenesis in distal and proximal AP-1 sites. Each AP-1 mutant promoter construct resulted in significant reduction of 4-1BB promoter activity (Fig. 6). The results show that the proximal AP-1 site is a more critical binding site than the distal site and suggest that the proximal AP-1 site could be involved in the constitutive activity of 4-1BB promoter as shown in EMSA, 0 time data (Fig. 4A). PD98059, a pharmacological inhibitor of MEK, or DN-JNK1 (T183A/Y185F) expression reduced 4-1BB promoter activity in activated CD3-CEM (Fig. 7), further supporting the idea that the transcriptional activation signal through

the TCR is mediated by the MEK/Erk and JNK/SAPK pathways, and the 4-1BB promoter is an AP-1-dependent promoter.

Collectively, our studies clearly demonstrate a critical role for NF- $\kappa$ B and AP-1 in 4-1BB upregulation through TCR activation, although other transcriptional or posttranscriptional processes may also be operative. The elucidation of the role of these transcription factors not only gives us insights into the relationships between TCR activation and signaling pathway into the costimulatory molecules, but also provides strategies aimed toward its manipulation in inappropriate 4-1BB and 4-1BBL interactions, including rheumatoid arthritis [49] and antigen-specific immune regulation [50].

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