IκB Kinase β Is Required for Activation of NF-κB and AP-1 in CD3/CD28-Stimulated Primary CD4+ T Cells

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Engagement of the TCR and CD28 coreceptor by their respective ligands activates signal transduction cascades that ultimately lead to the activation of the transcription factors NFAT, AP-1, and NF-κB, which are required for the expression of cytokines and T cell clonal expansion. Previous studies have demonstrated that in mature T cells, activation of AP-1 and NF-κB is dependent on protein kinase C θ, suggesting the existence of a common signaling pathway. In this study, we show that in human primary CD4+ T cells, exposure to the cell-permeable IKKβ inhibitor PS-1145 or genetic ablation of IKKβ abrogates cell proliferation and impairs the activation of NF-κB and AP-1 transcription factors in response to engagement of CD3 and CD28 coreceptor. In addition, we show that stimulation of T cells in the absence of IKKβ activity promotes the time-dependent and cyclosporine-sensitive expression of negative regulators of T cell signaling leading to a hyporesponsive state of T cells. *The Journal of Immunology*, 2012, 188: 2545–2555.**

Stimulation of T cells by engagement of the TCR and CD28 coreceptor activates a series of signal transduction cascades that ultimately lead to the activation of the transcription factors NFAT, AP-1, and NF-κB, which are necessary for T cell clonal expansion and differentiation (1, 2). TCR engagement in the absence of CD28 costimulation results in the induction of a state of hyporesponsiveness or T cell anergy, characterized by the sustained activation of NFAT in the presence of reduced activation of AP-1 and NF-κB (3, 4). Thus, the full activation of AP-1 and NF-κB appears to be a major mechanism of the anergy avoidance program induced by the CD28 pathway of costimulation.

NF-κB comprises a family of dimeric transcription factors involved in the regulation of T cells in response to foreign Ag. In the absence of an activating signal, NF-κB dimers are retained in the cytoplasm by tight binding to inhibitory IκB proteins. Upon engagement of the TCR and CD28 coreceptor, activation of the canonical NF-κB signaling pathway promotes serine phosphorylation and proteasomal degradation of IκBα resulting in the translocation of NF-κB dimers to the nucleus (5). The canonical NF-κB signaling pathway is centered on the trimeric serine kinase, IκB kinase (IKK), a 700-kDa complex composed of the catalytic IKKα and IKKβ subunits and the regulatory IKKγ subunit. All current evidence supports the view that the IKKβ subunit is the one necessary for activation of the canonical NF-κB signaling pathway (5). However, the identification of NF-κB and IκBα independent targets of IKKβ indicates that its function is not limited to the activation of the canonical NF-κB signaling pathway (6).

AP-1 transcription factors are dimers of Jun and Fos family proteins (7). cFOS and cJUN, the main AP-1 proteins induced in activated T cells, are low or undetectable in unstimulated cells but are rapidly induced upon T cell stimulation by the RAS-ERK1/2 signaling pathway (8).

Even though NF-κB and AP-1 transcription factors are regulated by different mechanisms, their activation in mature T cells is strictly dependent on protein kinase C θ (PKCθ), as evidenced by the fact that T cells from PKCθ$^{-/-}$ mice fail to activate both NF-κB and AP-1 in response to TCR/CD28 costimulation (2, 9–11).

The signaling pathway leading to NF-κB activation downstream of PKCθ has been studied in detail and has been shown to impinge on the PKCθ-regulated assembly of the lymphocyte-specific complex CARMA1–BCL10–MALT1 (CBM) essential for IKK activation (5, 12).

Relatively little, however, is known about the PKCθ signaling pathway that leads to AP-1 activation. Based on the findings from PKCθ$^{-/-}$ mice, it has been proposed that PKCθ regulates AP-1 activity at the level of cFOS and cJUN transcription (10).

Three NFAT proteins are expressed in T cells: NFAT1, which is the predominant NFAT family member in naive T cells; NFAT2, which is induced by a positive autoregulatory loop in activated T cells; and NFAT4, which is weakly expressed (13). Activation of NFAT transcription factors relies on the Ca^{2+}-calcineurin signaling pathway. Upon T cell activation, increased intracellular Ca^{2+} levels activate the calmodulin-dependent phosphatase calcineurin, which, by dephosphorylating phosphoserine residues within the regulatory region of NFAT, promotes NFAT nuclear translocation and subsequent DNA binding at the promoter regions of target genes. NFAT is capable of regulating two contrasting aspects of T cell function. In the presence of AP-1 proteins, NFAT–AP-1 cooperative complexes are formed at NFAT–AP-1 composite sites on DNA, thus driving the expression of a large number of genes essential to the productive immune response (14). In the absence of AP-1 proteins, NFAT directs the expression of a set of genes encoding negative regulators of TCR signaling, which are distinct from those characteristic of the productive immune response (3).

Because of the critical role played by NF-κB in inflammatory and autoimmune diseases and in cancer, selective IKK inhibitors have been developed (15). The cell-permeable IKKβ inhibitor PS-1145, a β-carboline derivative (16), has been shown to prevent T cell proliferation in vitro and to promote T cell tolerance in vivo (17–19). In this study, the function of IKKβ in T cell activation was investigated using a combination of pharmacological inhibition, RNA interference, and expression of a dominant-negative form of IκBα. We demonstrate that in human primary CD4⁺ T cells, IKKβ is required for the activation of NF-κB and AP-1 transcription factors in response to engagement of CD3 and CD28 coreceptor. Moreover, we show that sustained stimulation of CD4⁺ T cells in the presence of impaired IKKβ activity promotes the expression of negative regulators of T cell signaling and induces a state of T cell hyporesponsiveness, leading to the conclusion that IKKβ plays a critical role in the transduction of costimulatory signals necessary for anergy avoidance.

Materials and Methods

Reagents

PS-1145 [*N*-(6-chloro-9*H*-pyrido[3,4-b]indol-8-yl)-3-pyridinecarboxamide] (P6624), cyclosporin A (CsA) (C1832), protease inhibitor mixture (P8340), antibiotic–antimycotic solution (A5955), Laemmli 2× sample buffer (S3401), PBS (P5493), and proteinase K (P2308) were from Sigma-Aldrich. Recombinant human (rh)IL-2 was from Roche (11011456001). Abs to IKKα (sc-7218), p-AKT (sc-7985), AKT (sc-8312), ERK1/2 (sc-94), JNK (sc-571), cFOS (sc-52), cJUN (sc-45), casitas B-lineage lymphoma proto-oncogene b (CBLB) (sc-8006), p-ERK1/2 (sc-7383), and secondary Abs conjugated to HRP were from Santa Cruz. Abs to cABL (2862) and p-JNK (9251) were from Cell Signaling. Ab to diacylglycerol kinase-α (DGKα) (ab-88672) was from Abcam. Ab to IκBα (ALX-804-209) was from Alexis. Abs to FYN (AB-1378) and to IKKβ (05535), ECL reagent (WBKL-S0500), and polyvinylidene fluoride (Immobilon-P; IPVH00010) were from Millipore. BioWhittaker X-VIVO15 medium (BE04-418F) was from Lonza. Protein molecular markers (SM0671) were from Fermentas. All other chemicals were high grade from Sigma-Aldrich.

T cell isolation

Human primary CD4⁺ T cells depleted of CD25⁺ T cells (CD4⁺ T cells) were isolated by negative selection from PBMCs obtained by density gradient centrifugation of buffy coat leukapheresis residues of healthy donors as previously described (19).

T cell stimulation

Purified circulating CD4⁺CD25⁻ T cells (3 × 10⁶ cells) were maintained at 37°C in 5% CO₂ humidified atmosphere in 24-well plates in X-VIVO15 medium supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (culture medium). Cells were subjected to short-term (15 min to 8 h) or prolonged (24–96 h) stimulations with 1.5 × 10⁶ MACSiBeads loaded with anti-CD3 plus anti-CD28 mAbs (CD3/CD28 stimulation) as previously described (19). In restimulation experiments, immobilized anti-CD3/CD28 Abs were removed using MacSiMag Separator (130-092-168; Miltenyi). T cells were then washed with fresh culture medium, cultured for an additional 16 h, and restimulated for 24 h with immobilized anti-CD3/CD28 Abs in the presence or absence of 20 U/ml rhIL-2.

T cell proliferation assay

Cell proliferation was evaluated using the BrdU Cell Proliferation Assay Kit (HTS01; Calbiochem) as previously described (19). Where indicated, IL-2 (20 U/ml) was added to the cell cultures. Data are expressed as the ratio of the signals obtained from labeled versus unlabeled sample after subtraction of endogenous fluorescence.

Flow cytometry

Cells were washed in PBS and fixed on ice with cold ethanol to a final concentration of 75% v/v. Fixed cells were washed, resuspended in propidium iodide solution, and analyzed as previously described (19).

Inhibition experiments

CD4⁺CD25⁻ T cells (3 × 10⁶) were preincubated for 60 min with PS-1145 (3 μM) or vehicle (DMSO) and activated as described above. Where indicated, 1 μM CsA was added to the preincubation mixture.

Immunoblotting

Whole-cell extracts, prepared as previously described (19), were mixed 1:1 with Laemmli sample buffer and heated at 95°C for 5 min. Equal amounts of proteins (10 μg/lane) were resolved by SDS-PAGE using 12 or 10% polyacrylamide minigels and Tris/glycine buffer system as previously described (19). Separated proteins were electro-transferred (16 V, 12 h) at 4°C to polyvinylidene fluoride equilibrated in Towbin buffer (20). Membranes were blocked in 5% (w/v) BSA (sc-2323; Santa Cruz) in 20 mM Tris pH 7.6, 140 mM NaCl, 0.02% v/v Tween 20 (blocking buffer) and probed with primary Abs diluted to 0.4 μg/ml in blocking buffer. After washing, membranes were incubated with HRP-conjugated secondary Abs diluted in blocking buffer. Immunoreactive bands were detected by ECL using the G:Box Chemi-XT CCD gel-imaging system and GeneSnap image acquisition software (SynGene, Cambridge, U.K.). Relative band intensities were quantified using GeneTools image analysis software (Syngene).

Real-time PCR

Total RNA was extracted, reverse transcribed, and the indicated cDNAs quantified by real-time PCR as previously described (19). Primers (Supplemental Table I) were designed using PRIMER3 (http://frodo.wi.mit.edu/primer3/) and synthesized by MWG (Ebersberg, Germany).

RNA interference

Where indicated, CD4⁺ T cells were transfected with a pool of small interfering RNA (siRNA; 30 pmol/each) to IKKα (SI00605115; SI02654659), IKKβ (SI00300545; SI02777376), or nontargeting control siRNA (AllStar negative control) (scramble) from Qiagen using the Human T-cell Nucleofector Kit (VPA-1002) and Amaxa nucleofector II program U-014. After transfection, cells were cultured for 24 h in culture medium prior to stimulation with anti-CD3/CD28 Abs for 24–48 h.

EMSA

EMSAs were carried out using the LightShift Chemiluminescent EMSA kit according to the manufacturer's protocol (89880; Pierce). Nuclear extracts, 3 μl (2.5 μg protein), prepared as previously described (19), were added with 2 μl binding buffer (10×), 1 μl poly dI-dC (1 μg/μl), and biotinylated target DNA (1.25 pmol) in a final volume of 20 μl. To verify the specificity or the subunit composition of the DNA–protein complexes, unlabeled oligonucleotide competition reactions or supershift analyses were carried out. For unlabeled oligonucleotide competition reactions, nuclear extracts were preincubated for 20 min with excess non-biotinylated target DNA (250 pmol) prior to incubation with labeled DNA. For supershift analysis, nuclear extracts were preincubated for 20 min with specific Ab (1 μg) prior to incubation with labeled DNA. Reaction mixtures were incubated for 20 min at 23°C, combined with 5 μl 5× loading buffer, resolved by nondenaturing PAGE (100 V, 1.5 h) at 4°C on a 5% polyacrylamide minigel equilibrated in TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA), and electrotransferred (200 mA, 25 min) at 4°C to positively charged nylon membranes (15356; Fluka) equilibrated in TBE. Oligonucleotides were fixed to the membranes by UV cross-linking (312 nm, 10 min). Membranes were saturated in blocking solution and then incubated with streptavidin-conjugated HRP (1:5000 in blocking solution). Bands were detected by ECL as detailed earlier. Labeled and unlabeled oligonucleotides specific for NF-κB (5′-AGTTGAGGGGACTTTCC-CAGGC-3′), NFAT (5′-CGCCCAAAGAGGAAAATTTGTTTCATA-3′), or AP-1 (5′-CGCTTGATGACTCAGCCGGAA-3′) consensus sequences (one strand is indicated) were synthesized by MWG. Double-stranded DNA probes were generated by annealing complementary oligonucleotides in equimolar ratios by heating at 95°C for 5 min in a thermal cycler followed by cooling to 23°C at a rate of 0.5°C/min.

Expression of a dominant-negative form of IκBα

A clone encoding the human full-length IκBα (IMAGE ID:2957970) was obtained from Source Bioscience (Nottingham, U.K.), and the recombinant plasmid was purified from bacterial cultures using a Plasmid Midi Kit (12143; Qiagen). The sequence coding for an N-terminally truncated form of human IκBα comprising aa 37–317 (21) was amplified using the purified plasmid as template and PFU thermostable DNA polymerase (600670-51; Stratagene) in the presence of the forward primer 5′-CACCATGAAA-GACGAGGAGTACGA-3′, in which the 5′-CACC-3′ sequence comprising the topoisomerase I recognition site was fused to the sequence corresponding to positions 219–238 of human IκBα mRNA (NM_020529.2), and the reverse primer 5′-TCATAACGTCAGACGCTGGCCT-3′ corresponding to positions 1043–1064. The amplification product (846 bp) was

gel-purified using the Qiaquick Gel Extraction Kit (Qiagen), inserted into pcDNA3.1 directional TOPO expression vector (K4900-01; Invitrogen), and transformed into Top 10 chemically competent *Escherichia coli* cells. After isolation of positive transformants, the recombinant plasmid (IκBαΔN) was purified using EndoFree Plasmid Kit (12362; Qiagen) and sequenced at MWG. The purified plasmid was resuspended in sterile TE (10 mM Tris pH 7.6, 1 mM EDTA). Transfection of CD4$^+$ T cells (8×10^6) was carried out with 3 μg IκBαΔN or empty plasmid using Human T-cell Nucleofector Kit (VPA-1002) and Amaxa nucleofector II program V-024. After transfection, cells were cultured for 24 h in culture medium prior to stimulation with immobilized anti-CD3/CD28 Abs for 24–48 h.

Chromatin accessibility by real-time PCR

Chromatin accessibility by real-time PCR was carried out according to Rao et al. (22). Briefly, nuclei purified from 4×10^6 cells were suspended in 50 μl DNase I buffer (RDD buffer; Qiagen), DNase I added (0, 2.5, or 5 U) (79254; Qiagen), and incubated for 3 min at 37°C. Reactions were stopped by the addition of 50 μl stop solution (50 mM Tris pH 8, 100 mM NaCl, 0.1% SDS, and 100 mM EDTA pH 8) and digested overnight at 50°C with proteinase K (0.5 μg/ml). DNA was extracted, precipitated at −20°C, air dried, and resuspended in TE according to Moore (23). Purified DNA was quantified fluorometrically (365-nm excitation, 460-nm emission) according to Gallagher (24) using Hoechst 33258 (B2883; Sigma) and calf thymus DNA (D4764; Sigma) as standard and a Fluoroskan Ascent-Thermo microplate fluorometer. Relative levels of nuclease protection were measured by real-time PCR carried out as described earlier in the presence of 40 ng purified DNA. The region of genomic DNA upstream of cFOS 5′UTR (NM_005252.3) was amplified from −295 to −4 using the forward primer 5′-CCCCTTACACAGGATGTCCA-3′ and reverse primer 5′-GTAAACGTCACGGGCTCAAC-3′. The region of genomic DNA upstream of cJUN 5′UTR (NM_002228.3) was amplified from −288 to −102 using the forward primer 5′-CAGCCACCGTCACTAGACAG-3′ and reverse primer 5′-GCTCACGGGATGAGGTAATG-3′. All samples were assayed in triplicate and normalized using as a control the region of genomic DNA of the myelin associated glycoprotein (AC002132.1) amplified from −260 to +114 with respect to the 5′UTR using the forward primer 5′-CCAAGATTCCCTGAGGACAA-3′ and reverse primer 5′-TG-CCACCATACTCACTTGGA-3′.

Statistical analysis

Statistical significance was determined using Student *t* test, and *p* values <0.05 were considered statistically significant.

Results

In human primary CD4$^+$ T cells, IKKβ is required for the activation of NF-κB and AP-1 transcription factors

We have previously shown that signals from IKKβ, by promoting the expression of IL-2, IL-2 receptor α (IL-2RA), and a number of cell-cycle regulatory proteins, are required for the clonal expansion of human primary CD4$^+$ T cells (19). To extend these observations, we investigated the effect of the cell-permeable IKKβ-specific inhibitor PS-1145 on human primary CD4$^+$ T cells subjected to prolonged stimulation with anti-CD3 plus anti-CD28 Abs. PS-1145 blocked cell proliferation before DNA synthesis as evidenced by the negligible incorporation of BrdU into newly synthesized DNA (Fig. 1A) and by the absence of cell-cycle progression beyond the G0/G1 phase (Fig. 1B). Consistent with the mechanism of action of IKKβ inhibitors (15), PS-1145 impaired stimulation-induced IκBα degradation (Fig. 1C). Addition of exogenous rhIL-2 did not rescue T cell proliferation. Next, we explored the effects of PS-1145 on the activation of NFAT, AP-1, and NF-κB transcription factors. To study this, nuclear extracts of cells stimulated in the presence or in the absence of PS-1145 were prepared and subjected to EMSAs. Stimulation with PS-1145 did not affect the activation of NFAT; on the contrary, PS-1145 impaired the activation of NF-κB and, surprisingly, abolished AP-1 activation (Fig. 1D). Therefore, we evaluated the effects of PS-1145 on the expression of cFOS and cJUN, the main AP-1 proteins induced in activated T cells (8). In resting T cells, the expression of cFOS and cJUN proteins was at the detection

limit but was upregulated in response to CD3/CD28 stimulation (Fig. 1E). Exposure to PS-1145 prevented the induction of cFOS and cJUN both at the protein and mRNA levels (Fig. 1E, 1F).

These data suggest that IKKβ is a critical signaling protein controlling the induction of cFOS and cJUN. To support this conclusion further, human primary CD4$^+$ T cells were transfected with siRNAs specific for distinct regions of IKKβ or IKKα or with nontargeting control siRNA. As shown by real-time PCR and Western immunoblot analysis, by 24 h posttransfection, the expression of IKKβ and IKKα was diminished by ∼94 and 85%, respectively, and remained unvaried during CD3/CD28 stimulation (Supplemental Fig. 1A and Fig. 2A). Transfection with scrambled siRNAs had no effect. siRNA silencing of IKKβ recapitulated the effects of PS-1145 on T cell proliferation (Supplemental Fig. 1B, 1C), on IκBα degradation (Fig. 2A), and on the activation of NF-κB and AP-1 (Fig. 2B). In addition, silencing of IKKβ prevented the upregulation of cFOS and cJUN at both the mRNA and protein levels (Supplemental Fig. 1D and Fig. 2C). Consistent with previous studies (25), silencing of IKKα was ineffective on T cell proliferation and activation of the canonical NF-κB signaling pathway. In addition, silencing of IKKα did not affect the upregulation of cFOS and cJUN and AP-1 activation. Of note, silencing of both IKKα and IKKβ completely blocked NF-κB activation (data not shown), indicating that residual IKK activity provided by IKKα (5, 26) is responsible for the limited NF-κB activation detected in PS-1145–treated cells or in cells transfected with siRNA to IKKβ (Fig. 1D, *lanes 5, 9, 13,* and *17,* and Fig. 2B, *lanes 5* and *11*). These data collectively suggest that signals from IKKβ, in addition to controlling the activation of NF-κB, are required for the upregulation of cFOS and cJUN expression during T cell activation.

Activation of AP-1 transcription factor is independent of NF-κB nuclear translocation

To determine whether the effects of IKKβ blockade were dependent on NF-κB transcriptional activity, human primary CD4$^+$ T cells were transfected with an expression plasmid coding for a dominant-negative form of IκBα lacking aa 1–36 (IκBαΔN) and were then stimulated with anti-CD3 plus anti-CD28 Abs. Transfection with IκBαΔN, but not with the empty vector, induced the expression of a low molecular mass IκBα (Fig. 3A) that suppressed the expression of the physiological IκBα, which is an NF-κB–dependent gene (27). In cells transfected with IκBαΔN, but not in those transfected with the empty vector, T cell proliferation was prevented (Supplemental Fig. 2A, 2B). EMSA analysis showed that expression of IκBαΔN prevented the stimulation-induced nuclear translocation of NF-κB but preserved the activation of NFAT and AP-1 (Fig. 3B). Consistent with the latter result, in T cells transfected with IκBαΔN or with the empty vector, the CD3/CD28-induced upregulation of cFOS and cJUN was comparable (Fig. 3C and Supplemental Fig. 2C). The activation of NFAT and AP-1 in cells expressing IκBαΔN was verified by supershift analysis in the presence of anti-NFAT1, anti-cFOS, and anti-cJUN Abs (Supplemental Fig. 2D, 2E). Together, these data suggest that signals from IKKβ promote cFOS and cJUN upregulation independently of NF-κB transcriptional activity.

IKKβ is required for chromatin remodeling across the cFOS and cJUN promoters

Chromatin remodeling across the cFOS and cJUN promoter regions is a major regulatory mechanism of AP-1 expression in mouse naive CD4$^+$ T cells (28). Therefore, to investigate further the mechanisms of AP-1 inhibition caused by the blockade of IKKβ, DNase I sensitivity of the genomic DNA regions upstream
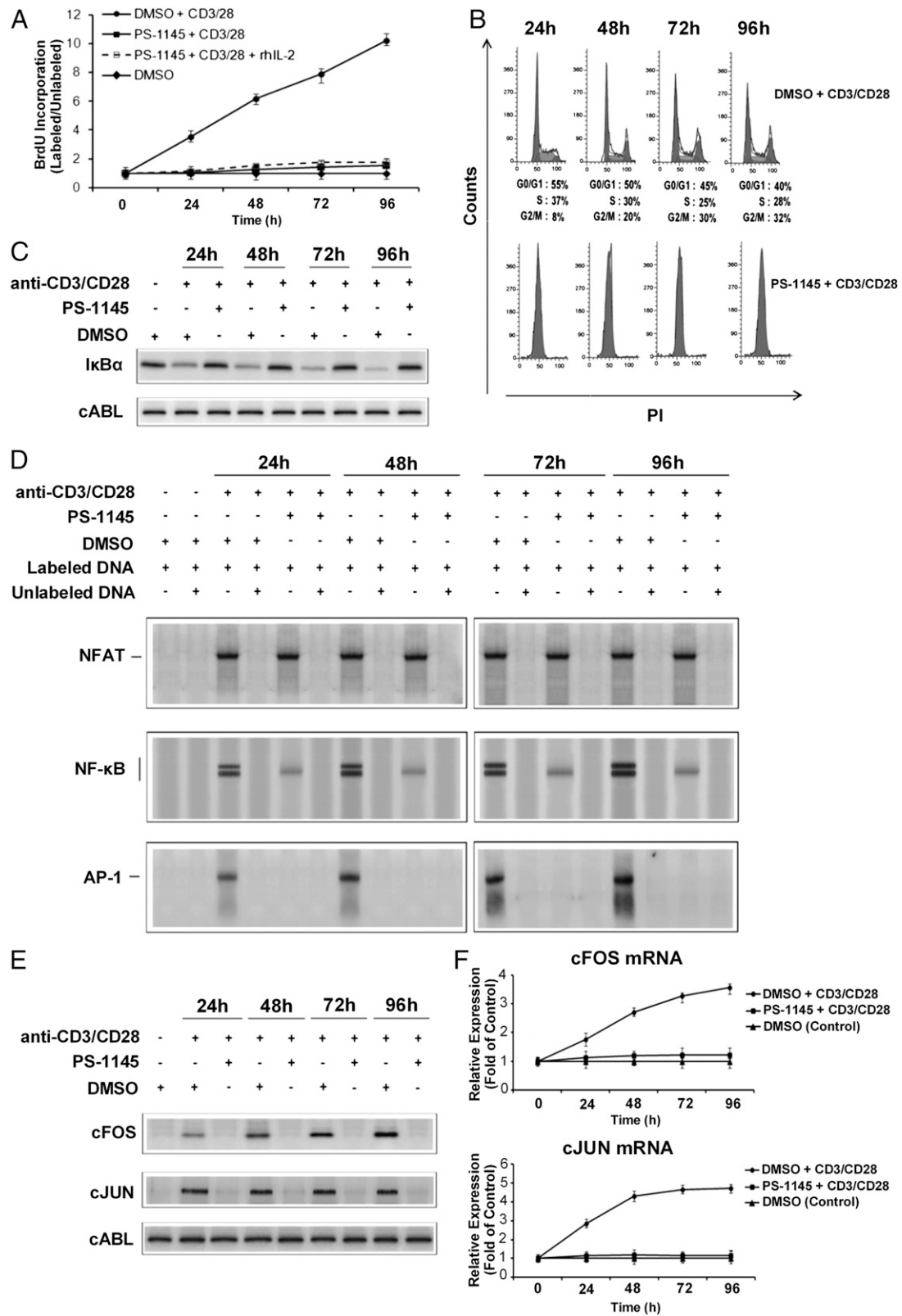
FIGURE 1. PS-1145 prevents the activation of NF-κB and AP-1 transcription factors. CD4^{+} T cells (3×10^6) were stimulated with immobilized anti-CD3/CD28 Abs in the presence or in the absence of PS-1145 for the indicated times. (**A**) Evaluation of cell proliferation by BrdU incorporation. Where indicated, rhIL-2 was added to the cell cultures. Values are means ± SD of three independent experiments each carried out in duplicate. (**B**) Flow cytometric analysis of DNA content. Distribution of cells in the different phases of the cell cycle is indicated as percent of the total cell population. A representative analysis from three independent experiments is shown. (**C**) Immunoblot analysis of IκBα protein levels. Proteins were resolved on a 12% polyacrylamide gel. Equal protein loading was verified by stripping and reprobing with an Ab to cABL. A representative Western blot from three independent experiments is shown. (**D**) Evaluation of the activation of NFAT, NF-κB, and AP-1 transcription factors by EMSA. A representative assay from three independent experiments is shown. (**E**) Immunoblot analysis of cFOS and cJUN protein levels. Proteins were resolved on a 12% polyacrylamide gel. Equal protein loading was verified by stripping and reprobing with an Ab to cABL. A representative Western blot from three (*Figure legend continues*)
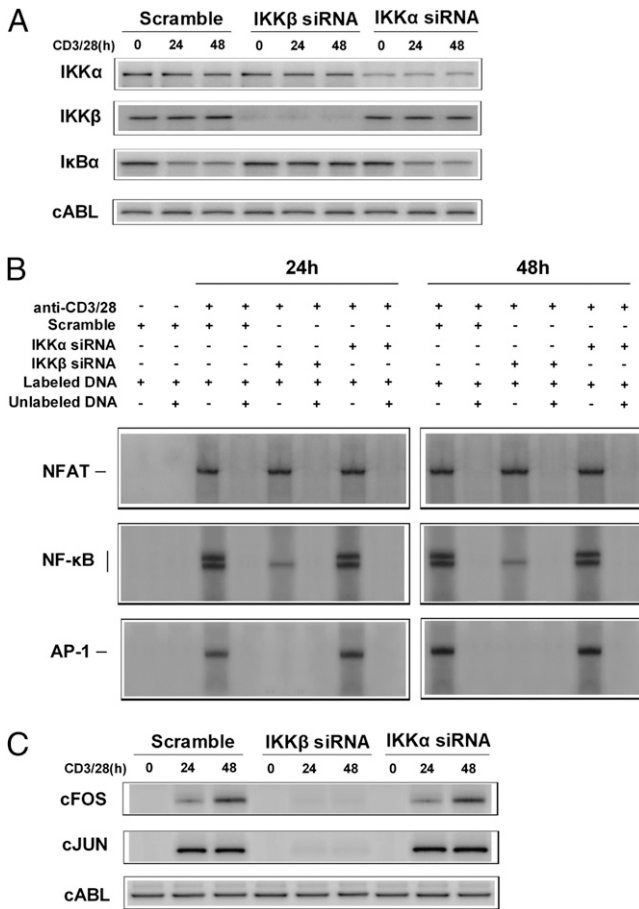
FIGURE 2. siRNA-mediated silencing of IKKβ recapitulates the effects of PS-1145 on the activation of NF-κB and AP-1 transcription factors. CD4^{+} T cells (3 × 10^{6}) were transfected with 60 pmol of a pool of siRNA against IKKα or IKKβ or with nontargeting control siRNA (scramble) and then stimulated for the indicated times with immobilized anti-CD3 plus anti-CD28 Abs. (**A**) Evaluation of the efficiency of IKKα and IKKβ silencing and IκBα degradation by immunoblot analysis. Proteins were resolved on a 10% polyacrylamide gel. Equal protein loading was verified by stripping and reprobing with an Ab to cABL. A representative Western blot from three independent experiments is shown. (**B**) Evaluation of NFAT, NF-κB, and AP-1 activation by EMSA. A representative assay from three independent experiments is shown. (**C**) Immunoblot analysis of cFOS and cJUN protein levels. Proteins were resolved on a 12% polyacrylamide gel. Equal protein loading was verified by stripping and reprobing with an Ab to cABL. A representative Western blot from three independent experiments is shown.

of the transcription start site (TSS) across the cFOS and cJUN promoters (Fig. 4A, 4B) were probed using chromatin accessibility by real-time PCR (22). Digestion of nuclei isolated from stimulated T cells resulted in reduced amplification of the cFOS and cJUN promoter regions compared with resting T cells, indicative of enhanced DNase I accessibility. In nuclei isolated from T cells exposed to PS-1145, the amplification of the cFOS and cJUN promoter regions was comparable with that of resting T cells (Fig. 4C, 4D). Silencing of IKKβ recapitulated the effects of PS-1145, whereas silencing of IKKα was ineffective (Fig. 4E, 4F). These data suggest that in CD3/CD28-stimulated T cells, signals from IKKβ are required for the modification of chromatin
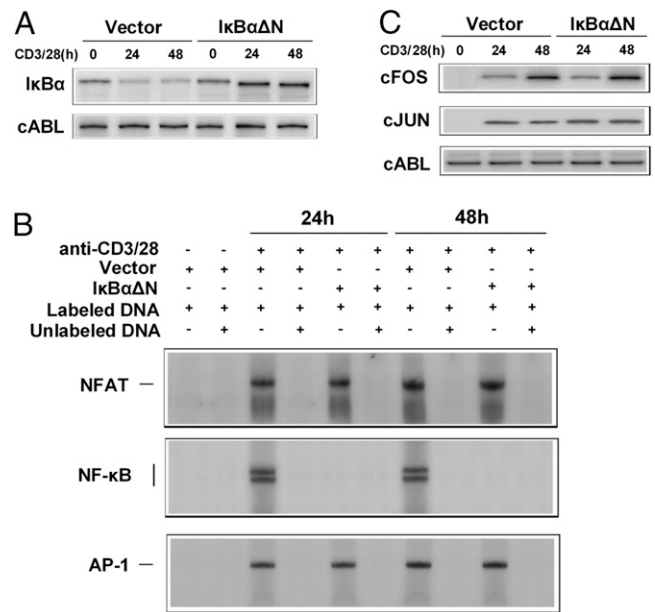

FIGURE 3. AP-1 activation is independent of NF-κB nuclear translocation. CD4^{+} T cells (3 × 10^{6}) were transfected with 3 μg IκBαΔN or empty plasmid (vector) and then stimulated for the indicated times with immobilized anti-CD3 plus anti-CD28 Abs. (**A**) Immunoblot analysis of IκBαΔN expression. Proteins were resolved on a 12% polyacrylamide gel. Equal protein loading was verified by stripping and reprobing with an Ab to cABL. A representative Western blot from three independent experiments is shown. (**B**) Evaluation of NFAT, NF-κB, and AP-1 nuclear translocation by EMSA. A representative assay from three independent experiments is shown. (**C**) Immunoblot analysis of cFOS and cJUN protein levels. Proteins were resolved on a 12% polyacrylamide gel. Equal protein loading was verified by stripping and reprobing with an Ab to cABL. A representative Western blot from three independent experiments is shown.

structure across the cFOS and cJUN promoters, which is essential for gene expression.

Blockade of IKKβ signaling promotes the expression of anergy-associated genes

In T cells, activation of the transcription factor NFAT in the absence of its transcriptional partner AP-1 promotes the expression of anergy-associated genes such as the lipid kinase DGKα and the E3 ubiquitin ligase CBLB, which act as negative regulators of T cell signaling (4, 29, 30). Moreover, increased expression of the Src-family tyrosine kinase FYN has been reported in anergic and tolerant T cells (31, 32), and the involvement of FYN in anergy maintenance has been proposed (33, 34). Prolonged stimulation of CD4^{+} T cells in the presence of PS-1145 resulted in the time-dependent upregulation of DGKα, CBLB, and FYN at the mRNA and protein levels, which was prevented in T cells exposed to PS-1145 and CsA in combination (Fig. 5A, 5B). Silencing of IKKβ recapitulated the effects of PS-1145, whereas silencing of IKKα or expression of IκBαΔN had no effect (Fig. 6A–C). Collectively, these results indicate that signals from IKKβ not mediated by NF-κB transcriptional activity are required for the repression of anergy-associated genes. Notably, DGKα protein was expressed at a detectable level in resting T cells, but its expression decreased upon engagement of CD3 and CD28 coreceptor (Fig. 5B), consistent with previous studies (35). In cells exposed to PS-1145 and CsA in combination, DGKα protein level

independent experiments is shown. (**F**) Real-time PCR evaluation of cFOS and cJUN mRNA levels. Expression is shown as fold difference compared with the same gene in unstimulated DMSO-treated cells (control). Values are means ± SD of three independent experiments.
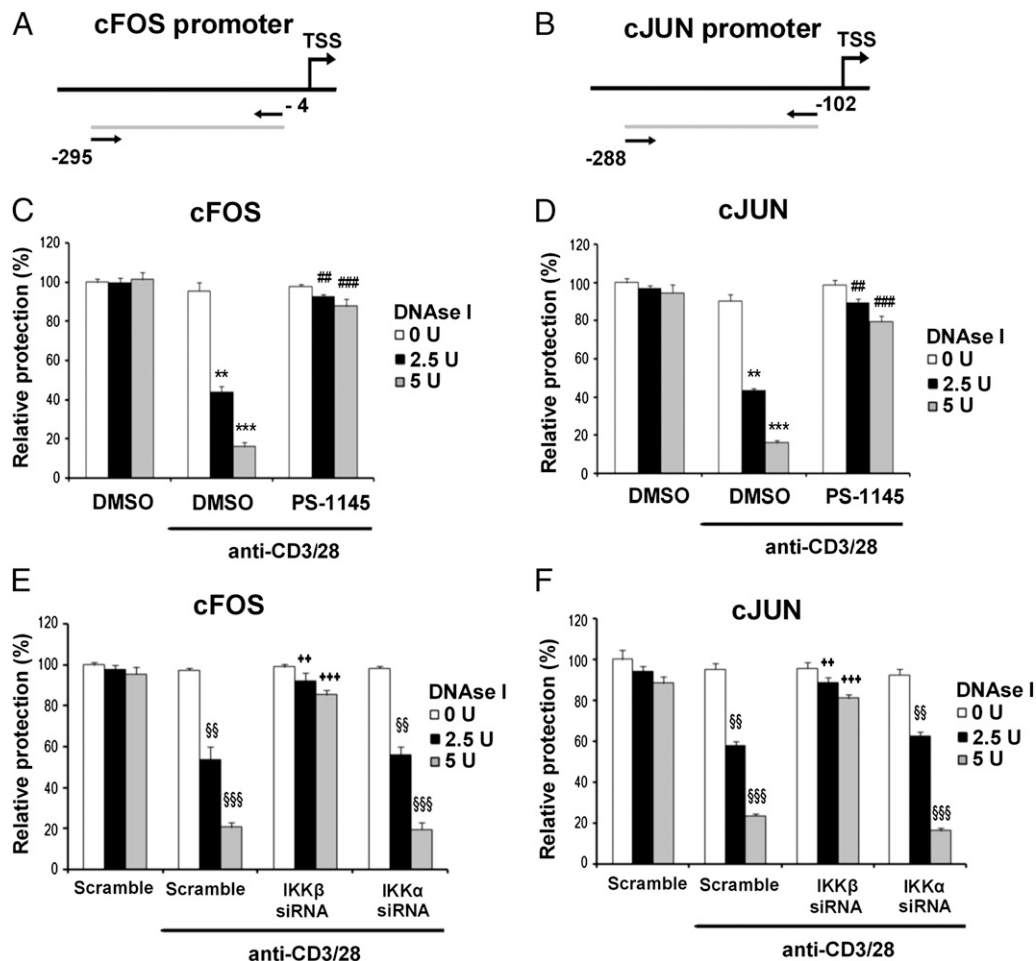
FIGURE 4. Effect of IKKβ blockade on chromatin accessibility across cFOS and cJUN promoters. (**A** and **B**) Schematic representations of human cFOS (A) and cJUN (B) promoters and location of the primer sets used in the chromatin accessibility by real-time PCR assay. Arrows at the bottom depict the position of the primer sets with respect to TSS. (**C** and **D**) Effect of PS-1145 on chromatin accessibility. CD4$^+$ T cells (3×10^6) were stimulated in the presence or in the absence of PS-1145 as in Fig. 1; nuclei were isolated, treated with increasing concentrations of DNase I, and subjected to real-time PCR analysis using the cFOS (C) and cJUN (D) primer sets. (**E** and **F**) Effect of IKKβ silencing on chromatin accessibility. CD4$^+$ T cells (3×10^6) were transfected and stimulated as in Fig. 2; nuclei were isolated and treated with increasing concentrations of DNase I and subjected to real-time PCR analysis using the cFOS (E) and cJUN (F) primer sets. Relative levels of nuclease protection are expressed as percent of control. Control: unstimulated DMSO-treated cells (C, D) or scrambled siRNA transfected, unstimulated cells (E, F). Values are means ± SD of three independent experiments. Statistical significance: **$p < 0.01$, ***$p < 0.001$ (versus DMSO-treated, unstimulated T cells); ##$p < 0.01$, ###$p < 0.001$ (versus DMSO-treated, stimulated T cells); §§$p < 0.01$, §§§$p < 0.001$ (versus scrambled siRNA-transfected, unstimulated cells); ++$p < 0.01$, +++$p < 0.001$ (versus scrambled siRNA-transfected, stimulated cells).

remained stable after stimulation, suggesting that signals from IKKβ are required for the downregulation of DGKα in response to CD3/CD28 stimulation (Fig. 5B, 5C).

Blockade of IKKβ signaling results in deficient ERK1/2 phosphorylation

The defective activation of the RAS-ERK1/2 signaling pathway is a major outcome of the expression of negative regulators of T cell signaling (36). In PS-1145–treated cells, the extent of ERK1/2 phosphorylation was substantially lower at each time point in comparison with that of untreated cells, whereas the phosphorylation of JNK and AKT was not significantly affected (Fig. 5D, 5E). Silencing of IKKβ recapitulated the effects of PS-1145 on ERK1/2 phosphorylation, whereas silencing of IKKα or expression of IκBαΔN were ineffective (Fig. 6D, 6E). JNK or AKT phosphorylation was not affected either by silencing of IKKβ or IKKα or by expression of IκBαΔN (Fig. 6D, 6E), thus suggesting that signals from IKKβ are required for sustained ERK1/2 activation.

In cells subjected to short-term stimulations (8 h or less), ERK1/2 phosphorylation was not affected by exposure to PS-1145

(Fig. 5F). cFOS and cJUN upregulation was instead prevented (Fig. 5G). These data, in agreement with the results reported by Bain et al. (37), exclude a direct inhibitory effect of PS-1145 on ERK1/2 activation. In addition, they provide evidence that in CD4$^+$ T cells, signals from IKKβ are required for rapid upregulation of cFOS and cJUN upon CD3/CD28 stimulation.

PS-1145–treated cells show reduced responsiveness to CD3/CD28 restimulation

Reduced cellular proliferation and reduced IL-2 expression on restimulation are characteristic of cells expressing negative regulators of T cell signaling (29). To study this, CD4$^+$ T cells were stimulated with immobilized anti-CD3 plus anti-CD28 Abs for 96 h in the presence or in the absence of PS-1145, rested for 16 h in fresh culture medium, and then restimulated in the presence or absence of rhIL-2. Cell proliferation and induction of IL-2 gene expression were significantly impaired in cells pretreated with PS-1145 (Fig. 7A, 7B). Moreover, in PS-1145–pretreated cells, ERK1/2 phosphorylation induced by restimulation was significantly lower with respect to control (Fig. 7C).
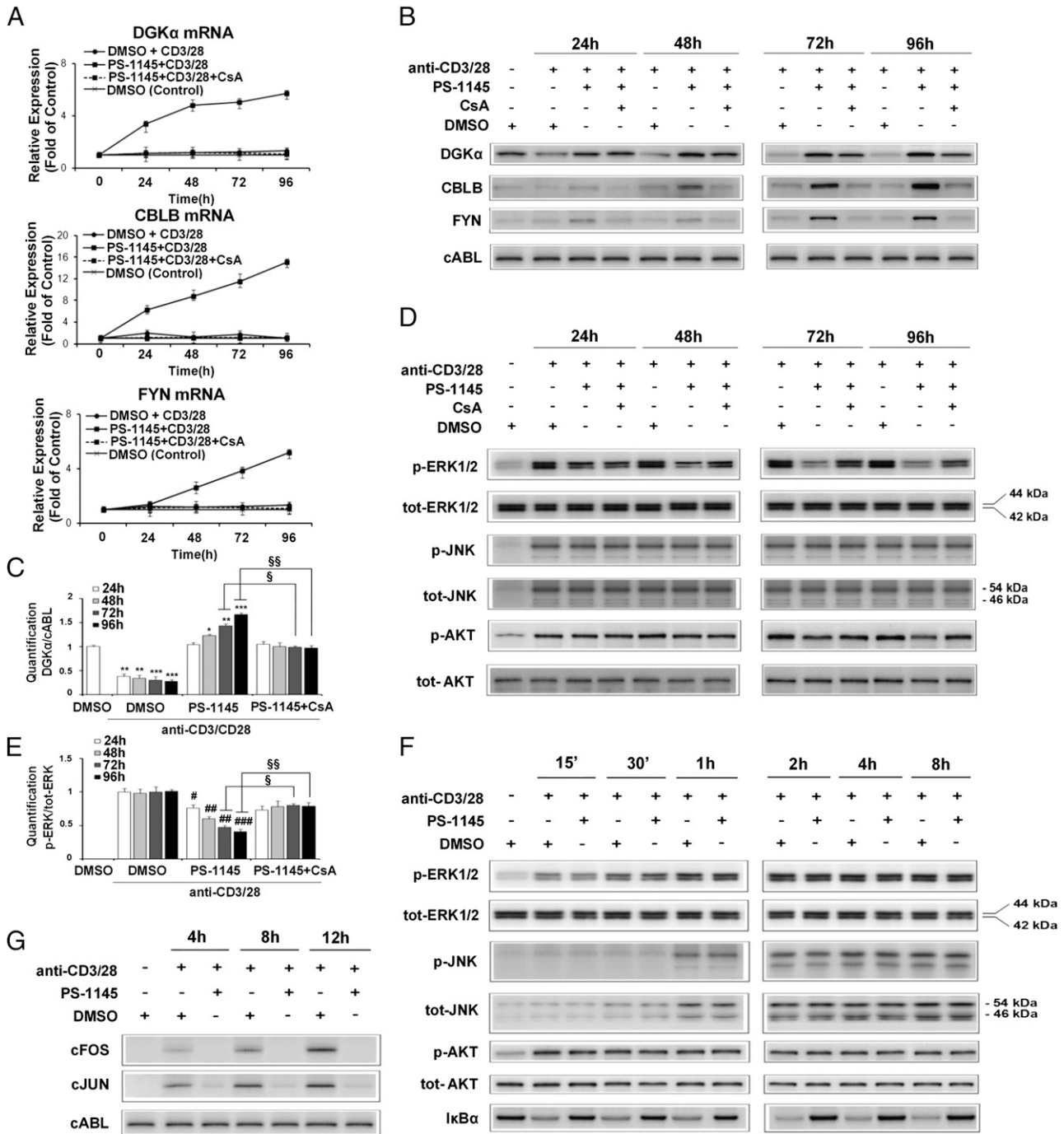
FIGURE 5. Effect of PS-1145 on the expression of anergy-associated genes and activation of signaling kinases. CD4^{+} T cells (3×10^6) were stimulated with immobilized anti-CD3/CD28 Abs in the presence or in the absence of PS-1145 for the indicated times. (**A** and **B**) Evaluation of the expression of DGKα, CBLB, and FYN by real-time PCR (A) and immunoblot analysis (B). (A) mRNA expression is shown as fold difference compared with the same gene in unstimulated DMSO-treated cells (control). Values are means ± SD of three independent experiments. (B) Proteins were resolved on a 10% polyacrylamide gel and immunoblotted with the indicated Abs. Equal protein loading was verified by stripping and reprobing with an Ab to cABL. A representative Western blot from three independent experiments is shown. (**C**) Blots of DGKα from three independent experiments were quantified, and the mean normalized values ± SD are shown. (**D** and **F**) Effect of prolonged (D) and short-term (F) cell stimulation on the phosphorylation of the signaling kinases ERK1/2, JNK, and AKT. Proteins were resolved on a 12% polyacrylamide gel and immunoblotted with the indicated Abs. Blots were stripped and reprobed with Abs to the nonphosphorylated forms (tot) to verify equal protein loading. A representative Western blot from three independent experiments is shown. To verify the effectiveness of the drug treatment during short-term stimulations, IκBα levels were evaluated. (**E**) Blots of p-ERK1/2 from three independent experiments were quantified, and the mean normalized values ± SD are shown. (**G**) Effect of short-term (4–12 h) stimulation on the expression of cFOS and cJUN proteins. Proteins were resolved on a 12% polyacrylamide gel. Blots were stripped and reprobed with an Ab to cABL to verify equal protein loading. A representative Western blot from three independent experiments is shown. Statistical significance: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (versus DMSO-treated, unstimulated T cells); $^\#p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ (versus DMSO-treated, stimulated T cells); $^\S p < 0.05$, $^{\S\S}p < 0.01$ (versus PS-1145–treated cells).

Stimulation of anergic T cell clones in the presence of exogenous IL-2 is reported to revert the anergic state (29, 38). T cell restimulation in the presence of exogenous IL-2 caused a significant increase (3.8-fold) in BrdU incorporation (Fig. 7A, compare *bars 2* and *4*) and upregulated the expression of mRNAs coding for G1/S regulatory proteins such as cyclin D2, cyclin E, and
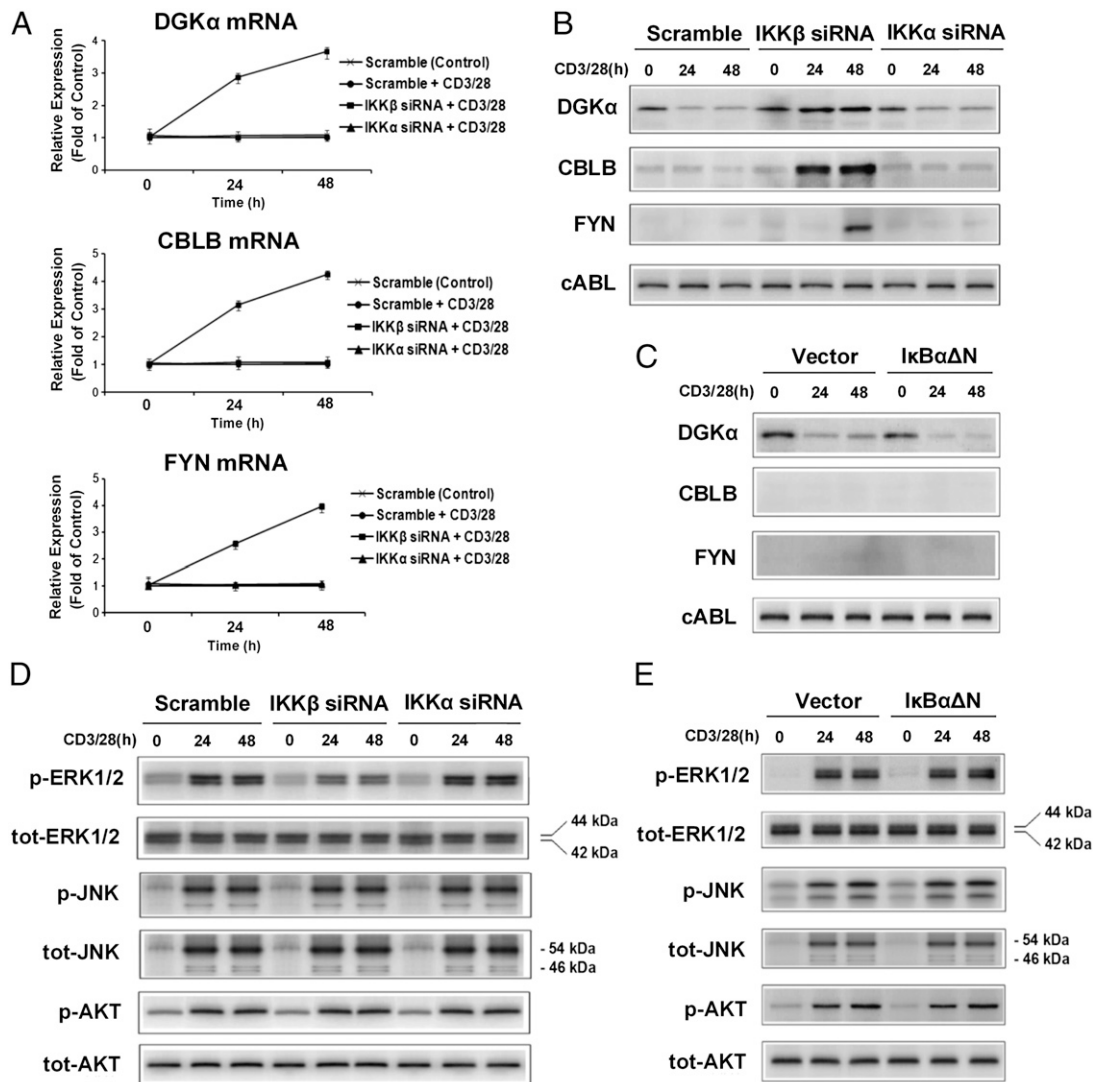
FIGURE 6. Transfection with siRNA to IKKβ but not with siRNA to IKKα or IκBαΔN promotes the expression of anergy-associated genes and impairs the activation of ERK1/2 signaling kinases. CD4^{+} T cells (3×10^6) were transfected with siRNA as in Fig. 2 and stimulated for the indicated time. (**A** and **B**) Evaluation of the expression of DGKα, CBLB, and FYN by real-time PCR (A) and immunoblot analysis (B). (A) mRNA expression is shown as fold difference compared with the same gene in scrambled siRNA-transfected, unstimulated cells (control). Values are means ± SD of three independent experiments. (B) Proteins were resolved on a 10% polyacrylamide gel and immunoblotted with the indicated Abs. Equal protein loading was verified by stripping and reprobing with an Ab to cABL. A representative Western blot from three independent experiments is shown. (**C**) Immunoblot analysis of DGKα, CBLB, and FYN protein levels in CD4^{+} T cells (3×10^6) transfected with IκBαΔN as in Fig. 3. Proteins were resolved on a 10% polyacrylamide gel and immunoblotted with the indicated Abs. Equal protein loading was verified by stripping and reprobing with an Ab to cABL. A representative Western blot from three independent experiments is shown. (**D** and **E**) Effect of transfection with siRNAs (D) or IκBαΔN (E) on the phosphorylation of ERK1/2, JNK, and AKT signaling kinases. Proteins were resolved on a 12% polyacrylamide gel and immunoblotted with the indicated Abs. Blots were stripped and reprobed with Abs to the nonphosphorylated forms (tot) to verify equal protein loading. A representative Western blot from three independent experiments is shown.

cyclin A (Fig. 7D) but did not completely revert the hyporesponsive state. To exclude that in our experimental settings T cell hyporesponsiveness was due to incomplete removal of PS-1145 after 16-h rest, freshly isolated CD4^{+} T cells were exposed for 96 h to PS-1145 or vehicle (DMSO), washed with fresh culture medium, and then cultured for an additional 16 h before CD3/CD28 stimulation for 24 h. As shown in Fig. 7E and 7F, the difference in BrdU incorporation and IL-2 gene expression between PS-1145–treated and DMSO-treated stimulated cells was not statistically significant, thus demonstrating the effective removal of the inhibitor.

In CD4^{+} T cells, PS-1145 is reported to inhibit the CD3/CD28-induced upregulation of IL-2 receptor α (IL-2RA/CD25), a RelA-p50-cRel–responsive gene (19, 39). Therefore, IL-2RA mRNA

levels were evaluated in hyporesponsive T cells. As shown in Fig. 7G, in cells subjected to 96-h stimulation in the presence of PS-1145 followed by 16 h rest, CD25/IL-2RA mRNA levels were ~6-fold lower with respect to cells stimulated in the absence of PS-1145, thus providing evidence for downregulated IL-2R signaling in T cells subjected to prolonged IKKβ blockade.

Discussion

Stimulation through CD28 coreceptor together with the TCR is required for full T cell activation. A major effect of CD28 costimulation is the activation of AP-1 and NF-κB transcription factors, a response that critically relies on PKCθ (10). The signaling pathway downstream of PKCθ that leads to the activation of NF-κB has been shown to impinge on the CBM complex-
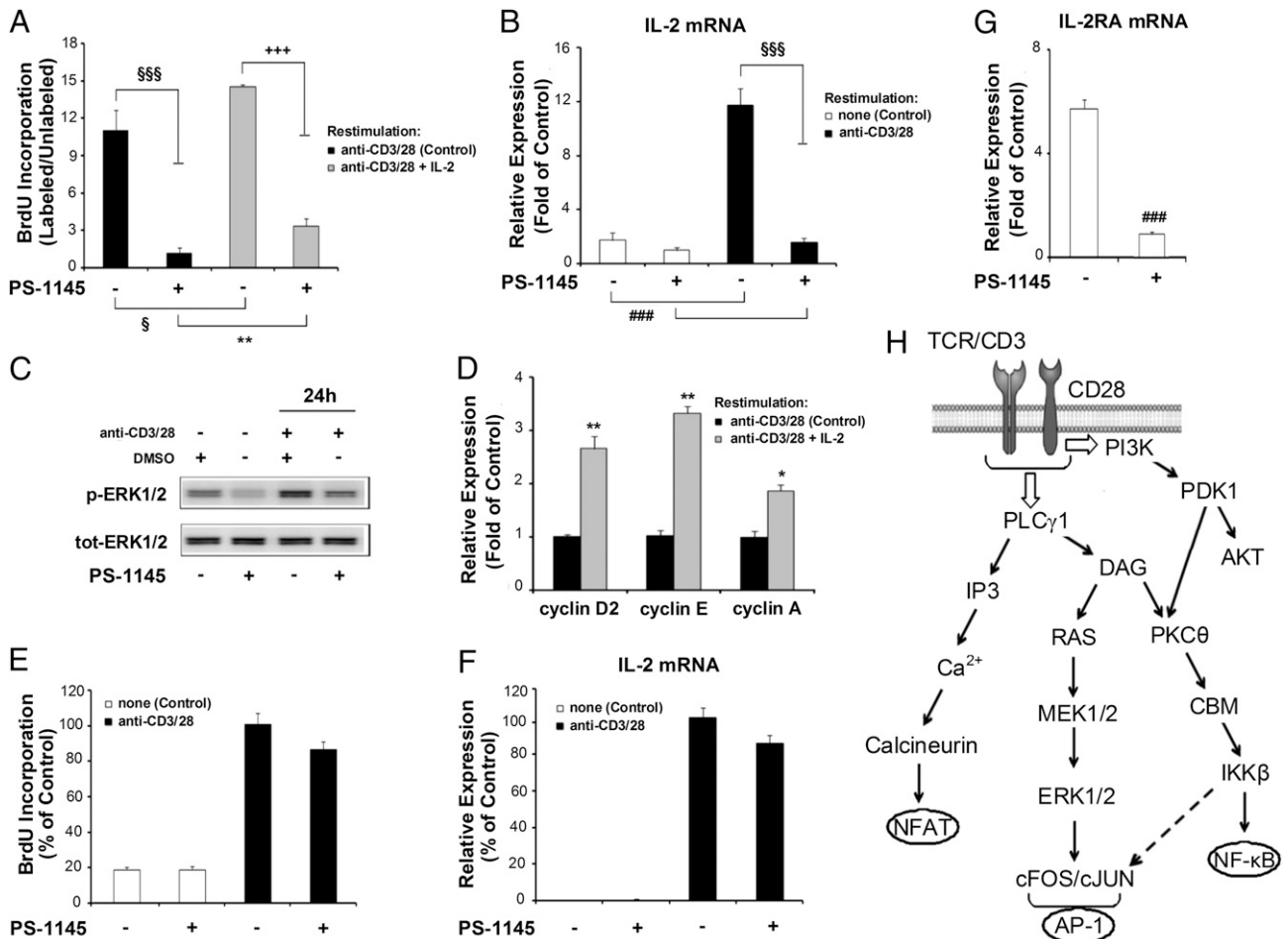
FIGURE 7. PS-1145 pretreatment attenuates T cell responses to subsequent CD28 costimulation. (**A–D**) CD4^{+} T cells (3×10^6) were stimulated in the presence or in the absence of PS-1145 as in Fig. 1. After beads removal, cells were washed and rested for 16 h in culture medium before CD3/CD28 restimulation in the presence or in the absence of exogenous rhIL-2. (A) Evaluation of cell proliferation by BrdU incorporation. Values are means ± SD of three independent experiments, each carried out in duplicate. (B) Real-time PCR evaluation of IL-2 mRNA levels. Expression is shown as fold difference compared with rested cells that were not restimulated (control). Values are means ± SD of three independent experiments. (C) Evaluation of the phosphorylation of ERK1/2 by immunoblot analysis. Proteins were resolved on a 12% polyacrylamide gel and immunoblotted with an Ab to p-ERK1/2. Blots were stripped and reprobed with Abs to the nonphosphorylated form (tot-ERK1/2) to verify equal protein loading. A representative Western blot from three independent experiments is shown. (D) Real-time PCR evaluation of cyclin D2, cyclin E, and cyclin A mRNA levels. Expression is shown as fold difference compared with pretreated cells restimulated in the absence of exogenous IL-2 (control). Values are means ± SD of three independent experiments. (**E** and **F**) Resting CD4^{+} T cells (3×10^6) were treated with PS-1145 or DMSO for 96 h, washed in fresh culture medium, and then cultured for an additional 16 h before CD3/CD28 stimulation for 24 h. (E) Evaluation of cell proliferation by BrdU incorporation. BrdU incorporation is shown as percent of control. Control: DMSO-pretreated stimulated cells. Values are means ± SD of three independent experiments, each carried out in duplicate. (F) Real-time PCR evaluation of IL-2 mRNA levels. Expression is shown as percent of control. Control: DMSO-pretreated unstimulated cells. Values are means ± SD of three independent experiments. Because of the large y-axis scale, control values (set to 1) are not appreciable. (**G**) Evaluation of IL-2RA mRNA level in hyporesponsive CD4^{+} T cells. Cells were stimulated in the presence or in the absence of PS-1145 as in Fig. 1. After beads removal, cells were rested for 16 h in culture medium, and IL-2RA mRNA levels were evaluated by real-time PCR. Expression is shown as fold difference. Values are means ± SD of three independent experiments. (**H**) Model of the proposed IKKβ signaling pathway in primary CD4^{+} T cells. Stimulation through TCR/CD3 and CD28 activates signaling cascades resulting in the full activation of NFAT, AP-1, and NF-κB transcription factors (53). Simultaneous signals from activated ERK1/2 and IKKβ are required for the upregulation of cFOS and cJUN expression and induction of AP-1 transcriptional activity. Statistical significance: *$p < 0.05$, **$p < 0.01$ (versus PS-1145–pretreated cells restimulated in the absence of exogenous IL-2); §$p < 0.05$, §§§$p < 0.001$ (versus DMSO-pretreated cells restimulated in the absence of exogenous IL-2); +++$p < 0.001$ (versus DMSO-pretreated cells restimulated in the presence of exogenous IL-2); ###$p < 0.001$ (versus DMSO-pretreated cells that were not restimulated).

dependent activation of IKKβ (12, 40). Less clearly understood is the mechanism by which PKCθ promotes AP-1 induction. Since the original observation of the importance of PKCθ in AP-1 activation (41), SPAK kinase is the only downstream effector of PKCθ described to date in this pathway (42).

In this study, we demonstrate that during the activation of primary CD4^{+} T cells stimulated by engagement of CD3 and CD28 coreceptor, signals from IKKβ are required for the activation of NF-κB and AP-1 transcription factors. Whereas the involvement

of IKKβ in the activation of NF-κB observed in this study is consistent with its role in the canonical NF-κB signaling pathway (5), the implication that IKKβ is involved in the induction of AP-1 constitutes a novelty of this study. In particular, we show that IKKβ is required for cFOS and cJUN upregulation. This conclusion is supported by experiments carried out on human primary CD4^{+} T cells exposed either to the IKKβ-specific inhibitor PS-1145 or transfected with siRNAs to IKKβ. In both experimental conditions, stimulation-induced cell-cycle entry was blocked, the

activation of NF-κB impaired, and the induction of cFOS and cJUN prevented. Induction of cFOS and cJUN was independent of NF-κB nuclear translocation, as the expression of a dominant-negative form of IκBα, which abrogated cell-cycle entry and NF-κB activation, had no effect on cFOS and cJUN expression or on AP-1 activation. On the basis of previous results showing that ERK1/2 activation is critical for the upregulation of cFOS and cJUN after mitogenic stimulation (43, 44), we conclude that signals from both ERK1/2 and IKKβ are required for cFOS and cJUN induction during primary T cell activation (Fig. 7H).

In the attempt to clarify the mechanisms by which IKKβ regulates cFOS and cJUN expression, we found that in CD28-costimulated primary T cells, signals from IKKβ are required for chromatin decondensation across the cFOS and cJUN promoters. As the SWI/SNF chromatin remodeling complex has been implicated in induction of AP-1 in naive mouse T cells (28), we hypothesize that signals from IKKβ are required for SWI/SNF chromatin remodeling activity in response to CD28 costimulation. Alternatively, simultaneous signals from IKKβ and ERK1/2 may be required for enhancement of histone acetylase activities associated with cFOS (45) and possibly cJUN promoters. Although further work is required to explore in greater detail the molecular mechanisms through which IKKβ promotes AP-1 activation, the results presented in this study establish a direct, previously unknown link between IKKβ and the induction of cFOS and cJUN and add new details with respect to the signaling pathway downstream of PKCθ that leads to the simultaneous activation of AP-1 and NF-κB (2, 9–11).

Activation of NFAT in the absence of AP-1 induces the expression of a set of anergy-associated genes that encode a number of negative regulators of TCR/CD28 signaling (29, 30). In agreement with this premise, we show that in human primary CD4+ T cells stimulated by engagement of CD3 and CD28 coreceptor for 24 h or longer, blockade of IKKβ signaling, but not inhibition of NF-κB activation alone, promotes the time-dependent expression of the E3 ubiquitin ligase CBLB, the lipid kinase DGKα, and the Src-family tyrosine kinase FYN. Taken together, these results provide evidence of the role played by IKKβ in the transduction of costimulatory signals required for the repression of anergy-associated genes. Even though in some T cell anergy models increased FYN activity has been linked to increased levels of activation (34), the increased FYN expression reported in this study is in agreement with the increased amount of enzyme detected in anergic (31) and tolerant T cells (32). Finally, it is of note that impaired degradation of IκBα has been reported in clonally anergic and adaptively tolerant T cells (32).

Deficient ERK1/2 phosphorylation is a relevant biochemical hallmark characterizing cells expressing negative regulators of T cell signaling (36, 46). In cells exposed to PS-1145 and subjected to prolonged stimulation, ERK1/2 phosphorylation decreased in a time-dependent manner beginning 24 h poststimulation. After 96-h stimulation, the ERK1/2 phosphorylation level in PS-1145–treated cells was comparable with that of resting T cells (Fig. 5D, compare *lanes 1* and *12*). ERK1/2 phosphorylation was, however, unaffected by PS-1145 in cells subjected to short (15 min to 8 h) stimulations (Fig. 5F). This result rules out a direct inhibitory effect of PS-1145 on ERK1/2 activation. In addition, as neither DGKα, CBLB, nor FYN was induced during short-term stimulations (data not shown), the data are consistent with an inverse relationship between expression of anergy-associated genes and ERK1/2 activation. In cells exposed to PS-1145 in combination with CsA, ERK1/2 phosphorylation was significantly, albeit not completely, preserved [Fig. 5D (*lanes 4*, *7*, *10*, *13*), 5E] even though CsA prevented DGKα upregulation. We speculate that

the intermediate level of ERK1/2 phosphorylation observed is a consequence of the Ca^{2+}-dependent translocation to the plasma membrane and activation of preexisting DGKα in the absence of increased gene expression (47).

The pharmacologic inhibition of ERK1/2 activation has been shown to block CD4+ T cell proliferation by suppressing the induction of AP-1 but is ineffective in inducing T cell anergy (48). In contrast, the block of T cell proliferation caused by inhibition of IKKβ signaling resulted in impaired cell proliferation and IL-2 expression after CD3/CD28 restimulation, thus demonstrating that the deficient ERK1/2 activation caused by IKKβ blockade is part of a hyporesponsive state of T cells, the induction of which requires inhibition of AP-1 induction and inhibition of the canonical NF-κB signaling pathway. Because this study used CD4+ T cells depleted of CD25+ cells, the observed T cell hyporesponsive state induced by IKKβ blockade should not be a consequence of immunomodulatory effects mediated by regulatory T cells (38). Further work will be required to evaluate the effect of the blockade of IKKβ signaling on the induction of anergy-associated genes and cell hyporesponsiveness in different subsets constituting human circulating CD4+ T cells (49).

Neither the induction nor the phosphorylation of JNK was impaired by blockade of IKKβ, thus excluding a role for JNK in AP-1 induction during the activation of primary T cells. This result is consistent with previous reports demonstrating that JNK activation is unaffected by the deletion of PKCθ (10) and with the notion that JNK is required for Th differentiation but not for T cell activation (46, 50). No detectable level of apoptosis was induced by the blockade of IKKβ or by expression of IκBαΔN, as shown by the absence of sub-G1 peaks in cell cycle analyses (Fig. 1B and Supplemental Figs. 1C, 2B). Because CD28 costimulation, through the activation of the PI3K/AKT signaling pathway, is reported to play a critical role in T cell survival (51, 52), the largely conserved AKT activation observed after blockade of IKKβ (Figs. 5D, 5F, 6D) may constitute a major mechanism driving the negligible levels of apoptosis observed.

In conclusion, by demonstrating that IKKβ is required for NF-κB and AP-1 activation in CD28-costimulated primary T cells, this study provides new data on the molecular mechanisms of T cell activation. In addition, by demonstrating that IKKβ is involved in the repression of anergy-associated genes, this study sheds light on the mechanism of the anergy avoidance program activated by the CD28/B7 pathway of costimulation. Finally, by showing the ability of the IKKβ-specific inhibitor PS-1145 to promote the expression of anergy-associated genes and to decrease T cell proliferation upon restimulation, this study sheds light on the molecular mechanisms of the immunosuppressive and tolerogenic effects promoted in vivo by inhibition of IKKβ signaling (17, 18).

Disclosures

The authors have no financial conflicts of interest.

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