Murine CD4$^+$CD25$^+$ Regulatory T Cells Fail to Undergo Chromatin Remodeling Across the Proximal Promoter Region of the IL-2 Gene[1]

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CD4$^+$CD25$^+$ regulatory T cells (T_{reg}) acquire unique immunosuppressive properties while maintaining an anergy phenotype when activated in vitro under conditions that induce IL-2 production and proliferation in conventional CD4$^+$ T cells. We investigated the mechanism underlying one component of this naturally anergic phenotype, the inability of the T_{reg} cells to produce IL-2 following activation. Analysis of freshly isolated murine CD4$^+$CD25$^+$ T_{reg} and conventional CD4$^+$CD25$^-$ T cells following PMA/ionomycin stimulation demonstrated no differences in inducible AP-1 formation, an important transcriptional complex in regulating IL-2 gene expression. Although p38 MAPK and ERK1/2 protein kinases were phosphorylated with similar kinetics, we observed diminished activation of JNK in the CD4$^+$CD25$^+$ T_{reg} cells. However, lentiviral-mediated reconstitution of the JNK pathway using a constitutively active construct did not overcome the block in IL-2 synthesis. Using a PCR-based chromatin accessibility assay we found that the minimal IL-2 promoter region of CD4$^+$CD25$^+$ T_{reg} cells, unlike conventional CD4 T cells, did not undergo chromatin remodeling following stimulation, suggesting that the inability of CD4$^+$CD25$^+$ T_{reg} cells to secrete IL-2 following activation is controlled at the chromatin level. ***The Journal of Immunology*****, 2004, 173: 4994–5001.**

CD4$^+$CD25$^+$ regulatory T cells (T_{reg})[3] are important mediators of peripheral immune tolerance. In addition to their unique suppressive properties, CD4$^+$CD25$^+$ T_{reg} cells display key features of anergic T cells. When activated in vitro, CD4$^+$CD25$^+$ T_{reg} cells fail to produce IL-2 or undergo proliferation under conditions that normally induce robust IL-2 production and proliferation in conventional CD4$^+$ T cells.

IL-2 is a critical cytokine that is rapidly induced in Ag-stimulated naive T cells. The production of IL-2 in T cells is primarily regulated at the transcriptional level by a 300-bp region proximal to the transcriptional start site of the IL-2 gene. This promoter/enhancer region contains binding sites for several inducible (such as AP-1, NF-κB, and NF-AT) and constitutive transcription factors (Oct-1), integrating input from a variety of signaling pathways (1). In addition to the formation of appropriate transcription factor complexes, IL-2 transcription in primary T cells appears to be preceded by activation-dependent regulated changes in chromatin structure across the IL-2 locus. Studies have revealed the IL-2 promoter/enhancer region of T cells to become susceptible to cleavage by the endonuclease DNase I upon stimulation, implying a role of nucleosomal structure in regulating IL-2 expression (2, 3). Changes in histone acetyltransferase activity of nucleosomal DNA and demethylation of CpG dinucleotide sequences in the promoter of the IL-2 gene have also been shown to regulate IL-2 gene expression. The molecular processes of how TCR-mediated signaling pathways can modulate the chromosomal configuration of the IL-2 gene, possibly by recruitment of chromatin remodeling complexes or by posttranslational modification of DNA binding factors, is still unclear.

T cell anergy was initially described in T cell clones rendered unresponsive to subsequent restimulation by first activating them through the TCR (signal 1) without appropriate costimulation (signal 2) (4) and, more recently, using an altered peptide ligand for activation (5). Two characteristic features of this induced unresponsiveness were the inability of the anergic T cells to proliferate or produce IL-2 following subsequent restimulation. A number of molecular defects in TCR signaling pathways have been described in these "classical" anergy systems. An in vivo superantigen-mediated model of T cell anergy revealed decreased Fos protein levels, thereby reducing the formation of AP-1, a transcriptional complex critical in regulation of IL-2 expression (6, 7). Other studies have shown that T cells rendered anergic by TCR stimulation in the absence of costimulation through the coreceptor CD28 exhibit defects in the JNK as well as the ERK signaling pathways (8, 9). We asked whether the molecular mechanisms underlying the anergy phenotype of CD4$^+$CD25$^+$ T_{reg} cells were similar to those described in "classical" anergy models, or represent a novel mechanism of maintaining a naturally anergic state. Our results suggest that IL-2 transcriptional silencing in CD4$^+$CD25$^+$ T_{reg} cells involves the regulation of chromatin accessibility at the proximal promoter of the IL-2 gene locus.

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[3] Abbreviations used in this paper: T_{reg}, regulatory T; Ion, ionomycin; CHART-PCR, chromatin accessibility by real-time PCR; MNase, micrococcal nuclease; NGFR, nerve growth factor receptor.

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Materials and Methods

Mice

BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed under National Institutes of Health-approved guidelines in the Department of Comparative Medicine, Stanford University (Stanford, CA).

Cell preparations and flow cytometry

Isolation by MACS (Miltenyi, Auburn, CA) and FACS, and culture of primary $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were performed as previously described (10).

IL-2 ELISA and real-time PCR

Equal number of sorted cells was incubated either in the presence of APC, 0.5 μg/ml anti-CD3 (145-2C11) and 1.0 μg/ml anti-CD28 (37.51; BD Pharmingen, San Diego CA), or with 50 ng/ml PMA and 1 μM ionomycin (Ion; Sigma-Aldrich, St. Louis, MO) in 96-well plates. IL-2 level in cultured supernatant was determined by ELISA and IL-2 mRNA levels were determined on cells harvested after 4 h of stimulation using real-time quantitative PCR as previously described (11).

Immunoblot analysis

Following treatment, cells were lysed in buffer containing 20 mM HEPES, pH 7.4, 1% Triton X-100, 100 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM sodium-vanadate, and 1 mM PMSF. Lysates were centrifuged and protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA). Proteins were detected with c-Jun (Cell Signaling Technology, Beverly, MA), MKK7 (Zymed, South San Francisco, CA), JNK2 (Santa Cruz Biotechnology, Santa Cruz, CA), and c-Fos (Upstate Biotechnology, Lake Placid, NY). Phospho-specific p38, ERK1/2, and Jun Abs were purchased from Cell Signaling Technology. All blots were developed with HRP-conjugated secondary Abs and ECL.

Lentiviral transduction

MKK7-JNK was cloned into pHR′tripCMV-Ires-tNGFR (12). Lentivirus production in HEK 293T cells and transduction protocols were adapted from a previous report (12). For infection, purified T cells were resuspended at 10×10^6/ml in OptiMEM containing 10 μg/ml protamine sulfate and virus (multiplicity of infection range, 5–10). Cells were incubated at 37°C for 2 h and subsequently were resuspended in complete RPMI containing either IL-7 (20 ng/ml for $CD25^-$) or IL-2 (50 U/m) for $CD25^+$) to 1×10^6/ml. Forty-eight hours postinfection, nerve growth factor receptor (NGFR)-positive cells were stained with anti-human biotinylated NGFR and streptavidin-APC (BD Pharmingen), sorted and stimulated with PMA/Ion for 12 h.

EMSA

EMSAs was performed according to manufacturer protocol (Geneka, Montreal, Quebec, Canada). Nuclear extracts, prepared from a buffer containing 0.1% Nonidet P-40, 20 mM HEPES, pH 7.4, 10 mM KCl, 1 mM $MgCl_2$, 400 mM NaCl, and 10% glycerol, were incubated with [γ-^{32}P]ATP end-labeled consensus wild-type (5′-CGCTTGATGAGTCAGCCGGAA-3′) and mutant AP-1 probes. For supershift assays, indicated Abs were incubated with extracts before the addition of probes.

Chromatin accessibility assay and PCR amplification

Following 4 h of PMA/Ion stimulation, nuclei were isolated as previously described (3). Nuclei were subsequently digested with micrococcal nuclease (Mnase) and subjected to real-time PCR with the following conditions: 95°C for 10 min for one cycle; 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min for 40 cycles using a MX4000 thermocycler (Stratagene, La Jolla, CA). Primer sequences were as follow: SetB: forward, CACAGG TAGACTCTTTGAAAATATGTGTAA, reverse, CATGGGAGGCAATT TATACTGTTAATG; SetD: forward, CTTTTGTGTCTCCACCCCAAA, reverse, CACACTTAGGTGGCAGTTTTAATTCAT; SetF: forward, CA TGCAGAGTTTTTTGTTGTTTTCTAG, reverse, GCCTAAAGTCTCTC ACAAAGAACAGA; and β-actin: forward, GACGGCCAAGTCATCAC TATTG, reverse, AGGAAGGCTGGAAAAGAGCC. Standard curves were generated using genomic DNA, and data were normalized for genomic β-actin. Change in accessibility for individual primer sets was calculated as 1 − [(stimulated)/(unstimulated)].

Results

Highly purified $CD4^+CD25^+$ T_{reg} cells fail to produce IL-2 upon activation

We previously described a system using latex beads coated with anti-CD3 and anti-CD28 Ab as surrogate APC to study the interaction of $CD4^+CD25^+$ T_{reg} cells with conventional $CD4^+CD25^-$ T cells in vitro (11). While we could demonstrate loss of suppression in the coculture of $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells with increasing costimulation, the Ab-coated beads did not induce proliferation or IL-2 production in the $CD4^+CD25^+$ T_{reg} cells. Similarly, highly purified $CD4^+CD25^+$ T_{reg} cells (Fig. 1*A*) did not secrete IL-2 when stimulated in vitro with irradiated T cell-depleted splenocytes and soluble anti-CD3/anti-CD28 or with a combination of PMA and Ion (Fig. 1*B*). We hypothesized that the small amount of IL-2 produced was likely due to nonregulatory $CD25^+$ T cells isolated within the T_{reg} population. Currently, there are no surface markers available for reliable distinction between $CD4^+CD25^+$ T_{reg} and recently activated conventional $CD4^+CD25^+$ T cells. To estimate the amount of "contaminating" IL-2-producing nonregulatory $CD25^+$ T cells in our preparation, we stimulated admixtures of highly purified $CD4^+CD25^+$ T cells and increasing numbers of $CD4^+CD25^-$ T cells with PMA/Ion for 4 h and measured the induction of IL-2 mRNA with quantitative PCR. Data presented in Fig. 1*C* show a linear relationship between the percentage of admixed $CD4^+CD25^-$ T cells and IL-2 mRNA levels. Based on the intersection of the trend-line with the *x* coordinate, we estimate that ~5% of the $CD4^+CD25^+$ T cells are nonregulatory, recently activated T cells, responsible for the minimal IL-2 production seen in the purified T_{reg} population (Fig. 1*B*).

Activated $CD4^+CD25^+$ Treg cells exhibit normal p38 MAPK and ERK1/2 activation and inducible AP-1 binding activity

The MAPK family of proteins mediates a series of distinct signaling cascades that ultimately regulate a multitude of cellular functions including T lymphocyte proliferation and differentiation (13). The three major mammalian MAPK groups, the ERK, p38 MAPK, and JNK kinases, have all been implicated in mechanism(s) of T cell clonal anergy. T cell clones rendered anergic by TCR stimulation in the absence of costimulatory signals have been reported to display defects in ERK, p38 MAPK, and JNK activity (6–9). The MAPK family of kinases is activated by dual phosphorylation on the threonine (T) and tyrosine (Y) residues at the T-X-Y motif in the activation loop. A time-course treatment with PMA/Ion was performed on freshly isolated $CD4^+CD25^+$ T_{reg} and $CD4^+CD25^-$ T cells, and lysates were subjected to immunoblot analysis for MAPK activation using dual T/Y phosphorylation-specific MAPK Abs. In both populations of T cells, stimulation with PMA/Ion led to rapid and robust phosphorylation of p38 MAPK and ERK1/2 (Fig. 2*A*).

One downstream effect of ERK pathway activation includes formation and activation of AP-1, a transcriptional dimer complex comprised of Jun and Fos family members. In lymphocytes, AP-1 has been shown to be a key factor in regulating IL-2 transcription. Furthermore, this transcriptional complex has been suggested to be a unique target in T cell clonal anergy (7). In the in vivo staphylococcal enterotoxin A superantigen-induced T cell anergy model, the anergy phenotype has been attributed to defective AP-1 formation, due in part to impaired expression of the Fos and Jun family members (6). To investigate whether differences in Fos and Jun protein levels contributed to defective IL-2 expression in the $CD4^+CD25^+$ T_{reg} cells, whole cell lysates were prepared and subjected to Western blot analysis. Increased levels of c-Fos protein

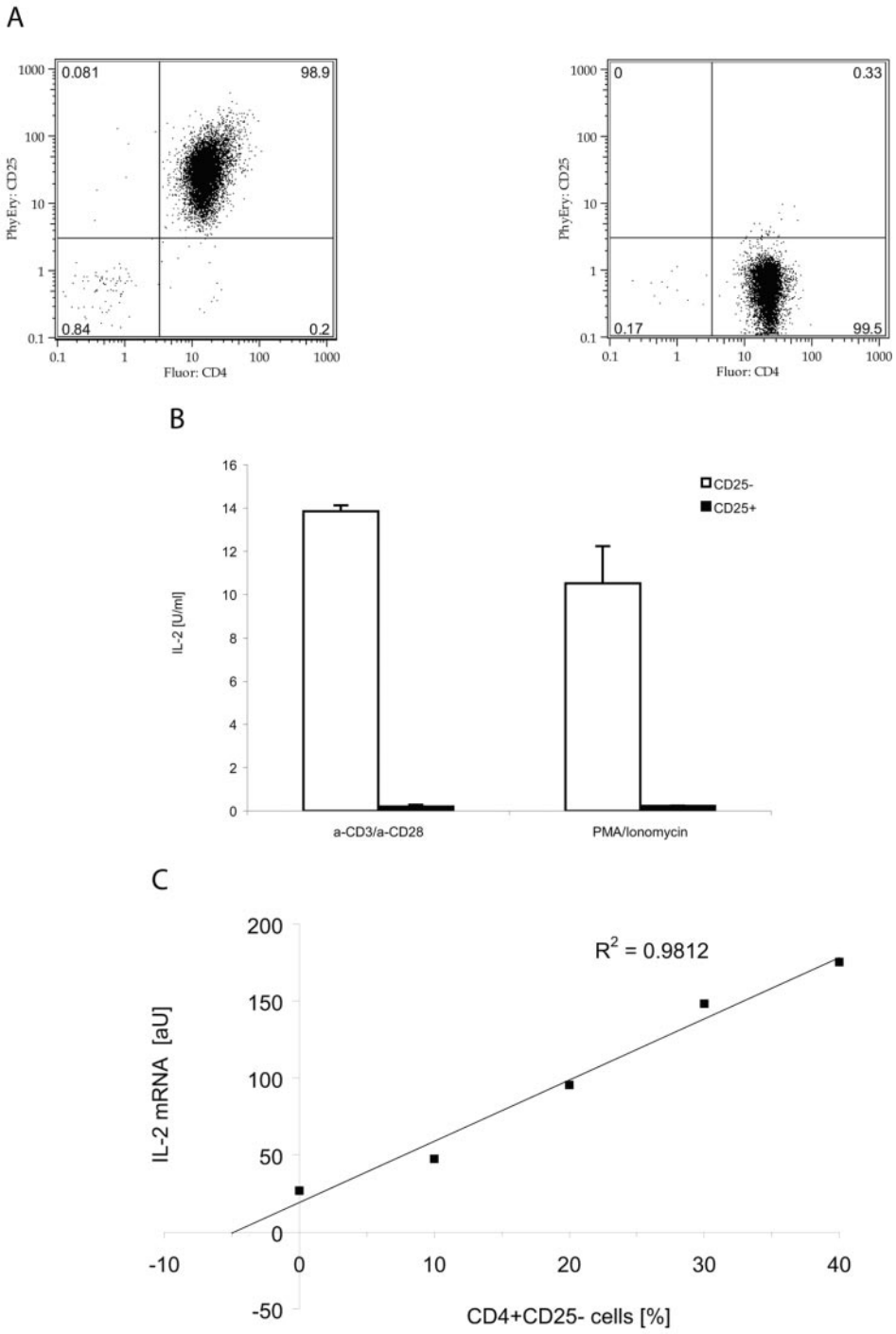

FIGURE 1. Lack of IL-2 production in activated CD4$^+$CD25$^+$ T_{reg} cells. *A*, Peripheral CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cells were sorted using a combination of MACS and FACS. The purity of the cell preparations was routinely >98% CD25$^+$ as indicated by postsort analysis. *B*, IL-2 production in activated CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cells. Cells were stimulated for 24 h with APC and soluble anti-CD3/CD28 (*left*), or with a combination of 50 ng/ml PMA plus 1 μM Ion (*right*). Levels of IL-2 in cultured supernatant were measured by ELISA. *C*, Direct correlation of IL-2 gene expression and percentage of input CD4$^+$CD25$^-$ T cells. Increasing numbers of CD4$^+$CD25$^-$ T cells were added to purified CD4$^+$CD25$^+$ T_{reg} cells. IL-2 mRNA levels were determined following 4 h PMA/Ion stimulation by real-time quantitative PCR. IL-2 levels of individual samples were normalized to β-actin.

were detected in both, CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cell populations within 30 min of PMA/Ion stimulation, increasing steadily for up to 2 h of treatment (Fig. 2*B*, *top*). Similarly, we noted comparable increases in c-Jun protein levels in both cell populations following 2 h of PMA/Ion stimulation (Fig. 2*B*, *bottom*). While the induced levels of Fos and Jun protein did not appear to differ between CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cells, defects in posttranslational modifications of these subunits could alter AP-1 complex formation. To examine possible perturbations in AP-1 formation, nuclear extracts were prepared from untreated and stimulated cells and subjected to EMSAs. Following 2 h of PMA/Ion treatment, there was no quantitative difference in the level of inducible AP-1 DNA binding activity between CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cells (Fig. 2*C*). Specificity of AP-1 binding activity in both cell populations was demonstrated by competition with an excess of unlabeled wild-type (WT) and mutant (MT) AP-1 probes. Defective formation of NF-AT and NF-κB complexes has also been reported in T cell anergy models (6, 14). However, gel shift assays did not reveal detectable differences in inducible formation of NF-AT and NF-κB complexes between CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cells (data not shown).

AP-1 complexes composed of different Fos and Jun family members can contribute to the regulation of cellular proliferation and differentiation. For example, the process of adipocyte differentiation has been shown to be regulated by AP-1 DNA binding activity mediated by c-Jun, JunB, and JunD, and the expression

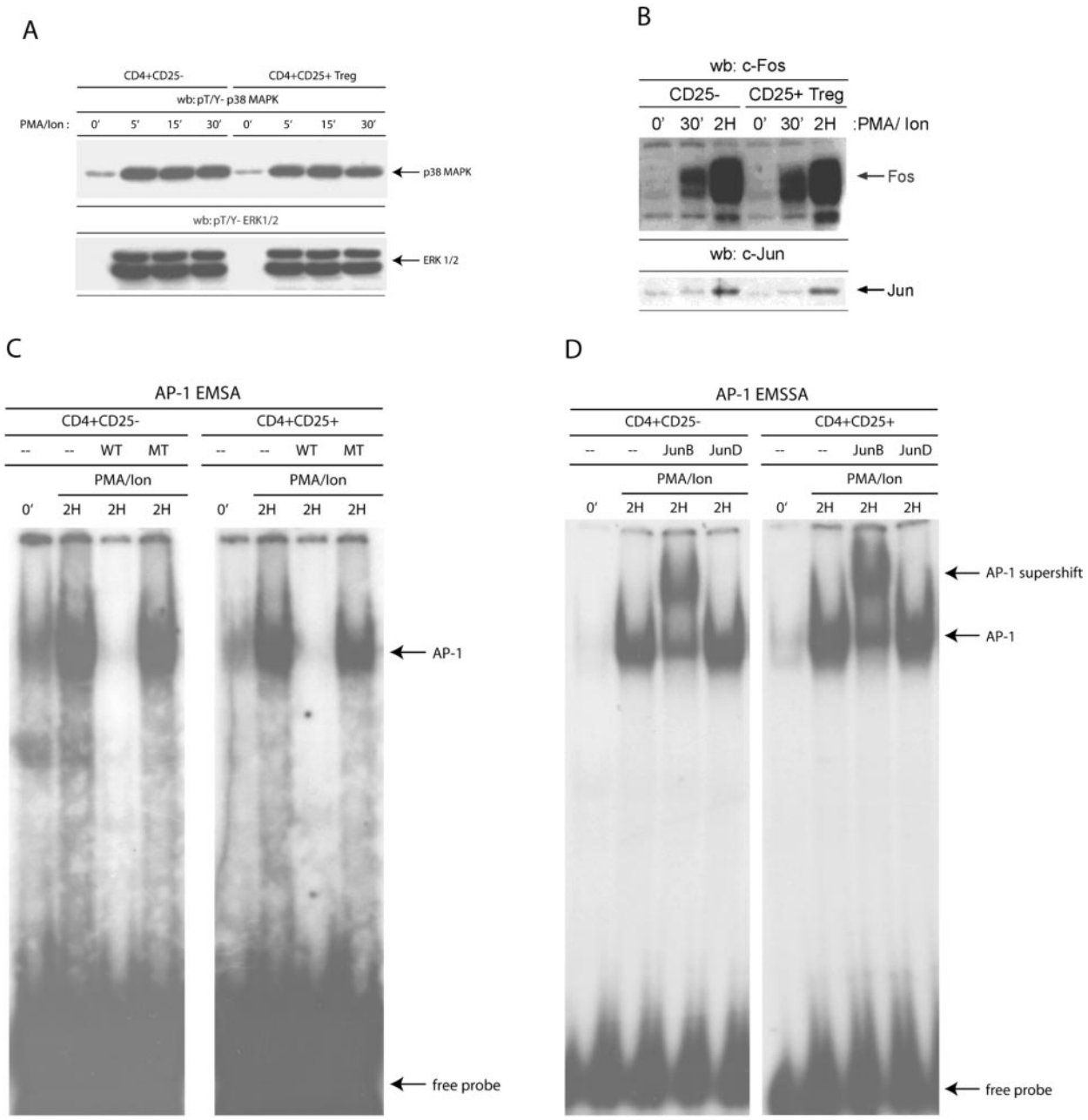

FIGURE 2. MAPK activation and inducible AP-1 formation in $CD4^+CD25^+$ T_{reg} cells. *A,* Similar levels of p38 MAPK and ERK1/2 kinase phosphorylation in activated $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells. Freshly isolated cells were treated with PMA/Ion for the times indicated. Whole cell extracts were subjected to immunoblot analysis with phosphorylation-specific p38 MAPK (*top*) and p44/42 ERK1/2 (*bottom*) Abs. *B,* Similar levels of Fos and Jun expression in activated $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells. Whole cell lysates of T cells were prepared after stimulation for the indicated times and subjected to immunoblot analysis with c-Fos (*top*) and c-Jun (*bottom*) Abs. *C,* Inducible AP-1 DNA binding activity in activated $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells. Freshly isolated cells were treated with PMA/Ion for 2 h. EMSA was performed with 2 μg of nuclear extract incubated with ^{32}P-labeled consensus AP-1 binding site oligo, in the absence or presence of 100× excess unlabeled wild-type (WT) or mutant (MT) AP-1 oligo. *D,* A supershift mobility assay reveals similar Jun subunit composition in inducible AP-1 DNA binding complexes. Cells were isolated and treated as in *C*. In indicated lanes, Abs to JunB and JunD were incubated with nuclear extracts before gel loading.

levels of these Jun proteins (15). To study the composition of the inducible AP-1 complex in the $CD4^+$ T cells, we conducted supershift EMSA with Abs to specific Jun family members. Incubation of $CD4^+CD25^-$ and $CD4^+CD25^+$ nuclear extracts with specific JunD Ab did not result in a shifted complex, while supershift assays in the presence of specific JunB Ab revealed that the AP-1 complex of both cell populations contained JunB (Fig. 2*D*). In summary, while perturbations in the formation of activation-dependent transcriptional complexes have been suggested as a potential mechanism for T cell anergy, we did not detect alterations in the formation of the AP-1 DNA binding complex in the activated $CD4^+CD25^+$ T_{reg} cells.

Activated $CD4^+CD25^+$ T_{reg} cells display defective JNK activation

While we observed no defects in p38 MAPK and ERK1/2 activation, PMA/Ion treatment resulted in dramatically reduced phosphorylation of JNK1/2 in the $CD4^+CD25^+$ T_{reg} cells compared

with conventional CD4$^+$ T cells, though both cell populations displayed comparable levels of JNK protein (Fig. 3*A*, *middle* and *bottom*). In addition, we observed a reduction in MKK4 phosphorylation, one of two upstream MAPK kinases known to activate JNK (16) (Fig. 3*A*, *top*).

The role of the JNK pathway in regulating IL-2 expression has been controversial. A study using a competitive inhibitor of JNK activity implicated the JNK pathway in regulating IL-2 gene transcription (17). Furthermore, anergic Th1 T cell clones have been demonstrated to lack JNK activity upon Ag receptor engagement or stimulation with PMA/Ion (8). However, investigations with JNK1- and JNK2-deficient T lymphocytes revealed a dispensable role for JNK protein kinases in IL-2 production (18). To assess the possible role of JNK in mediating IL-2 production in CD4$^+$CD25$^+$ T_{regs}, we chose to introduce a fusion construct of MKK7-JNK into the regulatory T cells. This MKK7-JNK fusion protein contains constitutive JNK activity as shown by phosphorylation of the endogenous substrate, c-Jun, in virally transduced HEK 293T cells (Fig. 3*D*) and as earlier reported (19). Due to the nonproliferative nature of freshly isolated CD4$^+$CD25$^+$ T_{reg} cells, we used a previously described bicistronic lentiviral system (12). Lentiviral transduction of freshly isolated naive CD4$^+$CD25- and CD4$^+$CD25$^+$ T_{reg} cells resulted in >50% transduction efficiency, based on bicistronic expression of NFGR (Fig. 3*B*). Forty-eight hours posttransduction, cells transduced with either empty (VEC) or MKK7-JNK (MKK/JNK) vector were sorted and exposed to PMA/Ion for 12 h. Reconstitution of CD4$^+$CD25$^+$ T_{reg} cells with MKK7-JNK resulted in increased endogenous c-Jun phosphorylation, as determined by protein microarray analysis (20), but did not result in IL-2 production following PMA/Ion treatment (Fig. 3*C*). Thus the defect in JNK activation does not provide an explanation for the inability of CD4$^+$CD25$^+$ T_{reg} cells to produce IL-2.

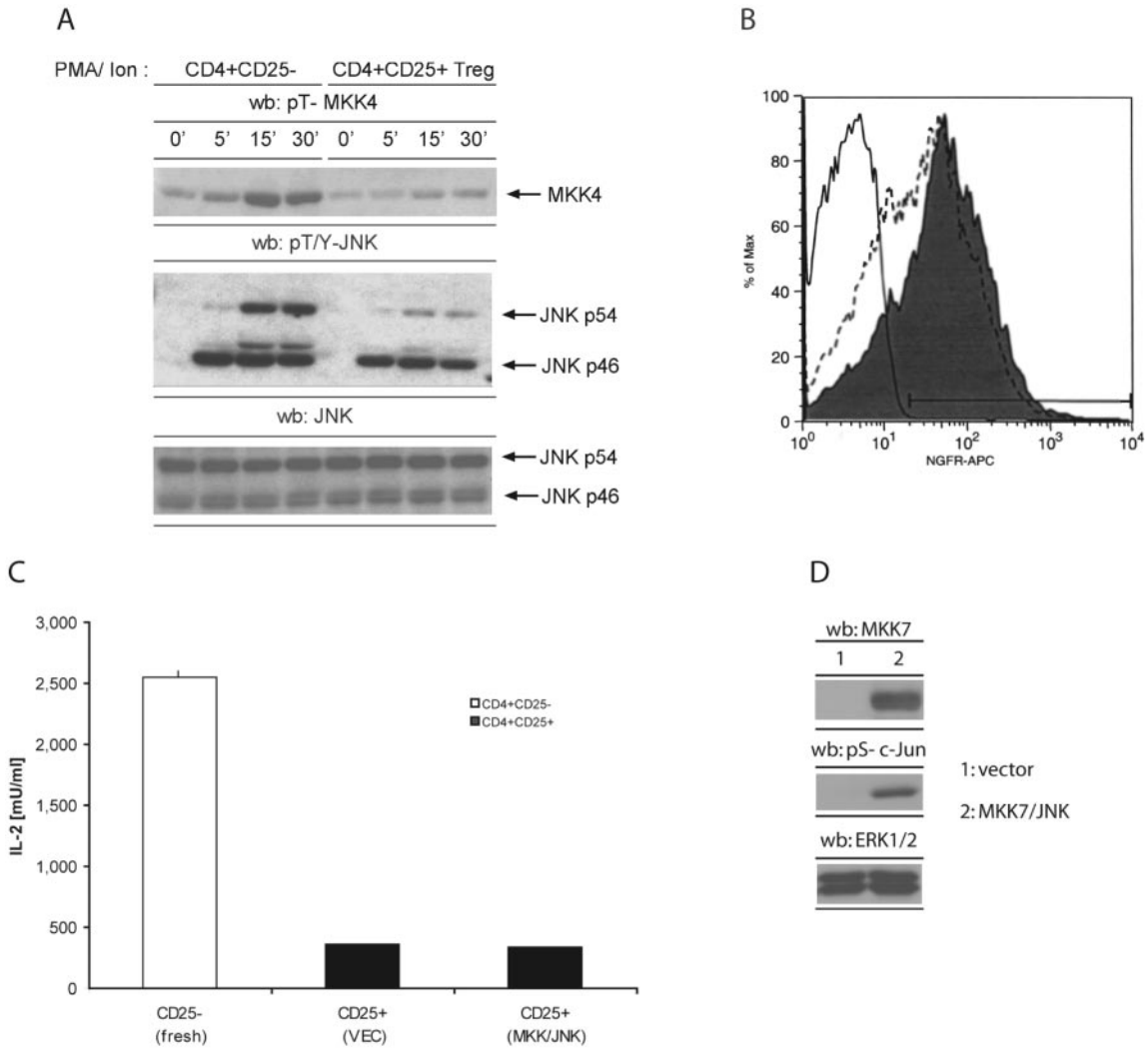

FIGURE 3. Impaired JNK signaling pathway does not account for the failure of IL-2 production. *A*, Impaired activation of JNK signaling pathway in CD4$^+$CD25$^+$ T_{reg} cells. Freshly isolated cells were treated with PMA/Ion for times indicated, and whole cell extracts were subjected to immunoblot analysis with phospho-specific MKK4 (*top*) and JNK1/2 (*middle*) Abs. Blot was reprobed for JNK2 (*bottom*). *B*, Lentiviral transduction of CD4$^+$ T cells with constitutively active JNK. Freshly isolated CD4$^+$ T cells were transduced with either empty lentiviral vector (dashed line) or with constitutively active MKK7-JNK construct (solid/shaded line). Forty-eight hours posttransduction, cells were analyzed and separated based on biscistronic NGFR surface expression. Transduction efficiency of >50% was observed for both CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cells. *C*, Reconstitution with constitutively active JNK does not abrogate block in IL-2 production in CD4$^+$CD25$^+$ T_{reg} cells. Freshly isolated CD4$^+$CD25$^-$ T cells, and CD4$^+$CD25$^+$ T cells transduced with either empty vector (VEC) or MKK7-JNK were stimulated for 12 h with PMA/Ion. Levels of IL-2 in cultured supernatant were measured by ELISA. *D*, MKK7-JNK possesses constitutive JNK activity. HEK 293T cells were transduced with either vector or MKK7-JNK construct. 48 h posttransduction, whole cell lysates were subjected to immunoblot analysis with MKK7 (*top*) or phospho-specific Jun (*middle*) Abs. Equal protein loading was verified by probing with ERK1/2 Ab.

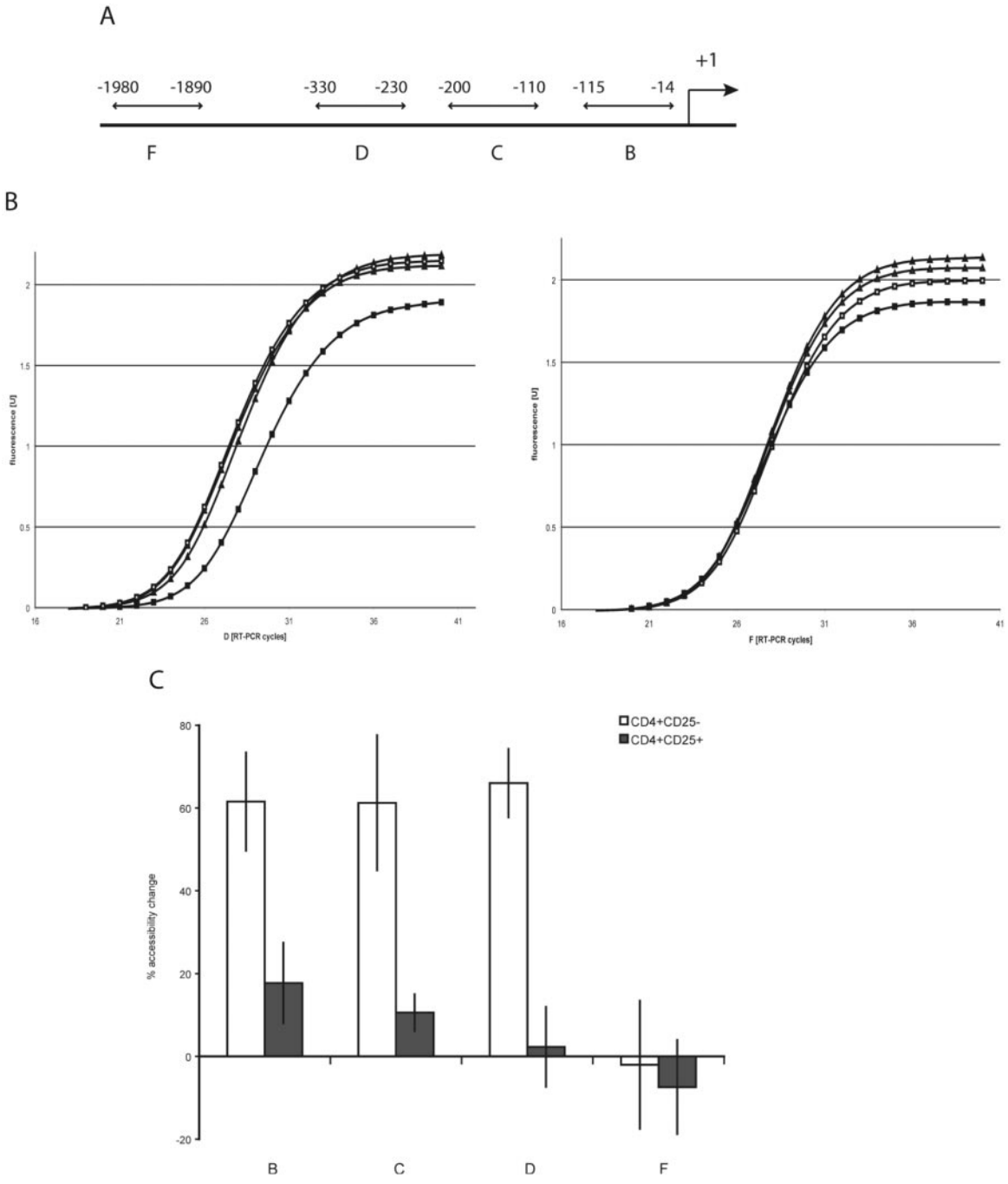

FIGURE 4. The IL-2 promoter region retains a closed chromatin configuration in $CD4^+CD25^+$ T_{reg} cells. *A*, Schematic diagram of murine IL-2 promoter/enhancer region. Primer sets used for accessibility assay are indicated, with transcriptional start site represented by (+1). *B*, Inaccessibility of selected IL-2 promoter regions in $CD4^+CD25^+$ T_{reg} cells. Nuclei of untreated $CD4^+CD25^-$ (□) and $CD4^+CD25^+$ (△), and PMA/Ion-stimulated $CD4^+CD25^-$ (■) and $CD4^+CD25^+$ (▲) T cells were treated with MNase for 5′ and genomic DNA was subjected to SYBR Green real-time PCR. $CD4^+CD25^-$ and $CD4^+CD25^+$ T cell samples were subjected to real-time quantitative PCR with primers spanning region D (*left*) and with primers spanning region F (*right*). *C*, Proximal IL-2 promoter of $CD4^+CD25^+$ T_{reg} cells does not undergo change in chromatin accessibility upon activation. Nuclei of stimulated $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were treated and subjected to real-time quantitative PCR as described in *B* using primers spanning indicated regions. Standard curves for individual primer sets were generated using serial dilutions of genomic DNA. Values were normalized for genomic β-actin. The data are plotted as the percentage of change in accessibility following 4 h of PMA/Ion stimulation. Mean data of four separate experiments are shown.

Minimal IL-2 promoter of $CD4^+CD25$ T_{reg} cells remains in a closed chromatin configuration upon activation

In addition to integration of signaling pathways and formation of transcriptional complex machinery, chromatin configuration of cell type-specific, stimulation-dependent genes forms an additional level of transcriptional control of cytokine gene expression in mammalian cells. The nucleosomal DNA structure of cytokine genes, not transcribed under basal conditions, is referred to as a "closed" chromatin configuration. Upon extracellular stimulation (i.e., Ag challenge or differentiation cues), areas of high transcriptional activity undergo reconfiguration of the nucleosomal DNA to achieve an "open" configuration, allowing chromatin accessibility

to transcriptional complexes. Previous studies have reported changes in chromatin accessibility of the IL-2 locus corresponding to increases in IL-2 gene expression. Nuclease digestion sensitivity assays have revealed increased accessibility within the proximal minimal IL-2 promoter upon activation in primary T cells and T cell clones, indicative of a change in chromatin configuration (2, 3, 21).

To investigate whether the proximal promoter region of the IL-2 gene in murine $CD4^+CD25^+$ T_{reg} cells becomes accessible to nuclease digestion upon activation, we used a recently described chromatin accessibility by real-time PCR (CHART-PCR) assay (3). In brief, nuclei isolated from unstimulated and stimulated cells are treated with MNase, a DNase that preferentially digests nucleosome-free regions of DNA. The extent of nucleosome remodeling as detected by MNase accessibility can be quantified by real-time quantitative PCR across the region of interest. Accessibility is inversely proportional to the amount of PCR product (threshold cycle value) obtained using primers encompassing the perturbed region of DNA. The CHART-PCR assay was particularly suited for our purposes as it can be performed with relatively few numbers of cells.

The positions of the PCR fragments relative to the IL-2 transcriptional start site we investigated are shown in Fig. 4*A*. The nonoverlapping primer sets B (−115 to −14 bp), C (−200 to −110 bp), and D (−330 to −230 bp) cover a region that was reported to show increased MNase accessibility upon PMA/Ion stimulation of conventional T cells, while no change was detected in the distal region, F (3). To validate the sensitivity of this in vivo chromatin remodeling assay, nuclei isolated from unstimulated and PMA/Ion treated $CD4^+CD25^-$ and $CD4^+CD25^+$ cells were subjected to MNase treatment, followed by real-time PCR. Using primers spanning region D, a clear shift is observed in PMA/Ion-stimulated conventional $CD4^+$ T cells, compared with untreated cells, indicative of an "open" configuration in this region. Interestingly, there is no observable difference in MNase accessibility between unstimulated and stimulated $CD4^+CD25^+$ T_{reg} cells (Fig. 4*B*, *left*). To demonstrate regional selectivity, no change is observed in the distal region F in either subpopulation of cells (Fig. 4*B*, *right*).

RT-PCR data of tested regions, normalized to an internal actin control, are shown in Fig. 4*C*. Conventional $CD4^+$ T cells treated with PMA/Ion display >60% increase in chromatin accessibility in the regions covered by primer sets B, C, and D. In contrast, no change in accessibility of these regions is observed in the $CD4^+CD25^+$ T_{regs} following PMA/Ion treatment. These data strongly suggest that the proximal IL-2 promoter in $CD4^+CD25^+$ T_{reg} cells remains in a closed nucleosomal structure upon activation and does not undergo the selective chromatin remodeling observed in conventional $CD4^+$ T cells following similar activation.

Discussion

$CD4^+CD25^+$ T_{reg} cells display two key properties of anergic T cells, namely the inability to proliferate or produce IL-2 upon activation. In this study, we investigated the mechanism of one component of this anergy phenotype in T_{regs}, the lack of IL-2 production following activation. Unlike previously described in vitro and in vivo models of T cell anergy, we did not observe defects in p38 or ERK1/2 signaling in the $CD4^+CD25^+$ T_{reg} cells. Analysis of activation-dependent transcriptional binding complexes reveal no differences in AP-1 DNA binding activity or subunit composition between $CD4^+CD25^+$ T_{reg} and conventional $CD4^+$ T cells. While diminished JNK signaling was noted, reconstitution of JNK activity was not sufficient to overcome the inability of these cells to produce IL-2. Although JNK phosphorylates and activates Jun family members, the reduction in JNK activity in the $CD4^+CD25^+T_{reg}$ cells does not necessarily preclude the formation of AP-1, as AP-1 DNA binding activity is intact in stimulated T cells of JNK1-deficient mice (22).

Our data from the CHART-PCR assay show that, in contrast to $CD4^+CD25^-$ T cells, the minimal 300-bp proximal promoter of the IL-2 gene in $CD4^+CD25^+$ T_{reg} cells does not undergo nucleosomal alterations following stimulation with PMA/Ion. While chromatin remodeling has been implicated as a mechanism for regulating cytokine expression during Th1 and Th2 cell differentiation, this is the first study to provide evidence that an anergic T_{reg} cell phenotype may involve regulation of chromatin structure. Current literature has suggested $CD4^+CD25^+$ T_{reg} cells are a separate thymus-derived T cell lineage; it is possible that the epigenetic modification of the IL-2 locus contributes to the developmental program of these cells.

The regulation of gene expression at the chromatin level requires a complex interplay of tightly coordinated events involving precise temporal and spatial recruitment of chromatin remodeling complexes. Many possibilities can provide plausible explanations of how the naturally anergic $CD4^+CD25^+$ T_{reg} cells maintain an inaccessible chromatin structure at the IL-2 promoter. These cells may lack functional (or translocation defective) promoter-specific remodeling complexes, such as mating type switching/sucrose nonfermenting-like remodeling complexes found associated with chromatin upon TCR engagement (23). Defective recruitment of remodeling complexes may be a consequence of an altered modification of DNA methylation status and/or posttranslational modification of histone proteins by acetylation or phosphorylation. For example, a role for dynamic DNA demethylation has been recently described for regulation of IL-2 expression in T lymphocytes following activation (24). Thus, the lack of chromatin remodeling due to defective DNA demethylation of CpG nucleotide sequences within the IL-2 promoter may contribute to the lack of IL-2 expression in $CD4^+CD25^+$ T_{reg} cells. The state of histone acetylation can also contribute to the observed anergy phenotype. IL-2 promoter binding elements, such as the cAMP response element modulator, have been reported to block histone acetyltransferase activity, which can result in chromatin condensation and decreased IL-2 production (25). Finally, presence of "anergy" factors that can regulate selective accessibility of the proximal IL-2 promoter may exist. For example, previous results have documented the presence of a yet-unidentified dominant-acting repressor molecule that abrogates signal transduction to the IL-2 gene in anergic T cells (26). While our results clearly suggest that regulation of chromatin structure plays a key role in inhibiting IL-2 gene expression in $CD4^+CD25^+$ T_{reg} cells, further studies will be necessary to provide further mechanistic insight into this phenomenon.

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