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Transcriptional Mechanisms Underlying Lymphocyte Tolerance

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Summary

In lymphocytes, integration of Ca^{2+} and other signaling pathways results in productive activation, while unopposed Ca^{2+} signaling leads to tolerance or anergy. We show that the Ca^{2+}-regulated transcription factor NFAT has an integral role in both aspects of lymphocyte function. Ca^{2+}/calcineurin signaling induces a limited set of anergy-associated genes, distinct from genes induced in the productive immune response; these genes are upregulated in vivo in tolerant T cells and are largely NFAT dependent. T cells lacking NFAT1 are resistant to anergy induction; conversely, NFAT1 induces T cell anergy if prevented from interacting with its transcriptional partner AP-1 (Fos/Jun). Thus, in the absence of AP-1, NFAT imposes a genetic program of lymphocyte anergy that counters the program of productive activation mediated by the cooperative NFAT:AP-1 complex.

Introduction

The antigen receptors of T and B cells recognize not only antigens derived from pathogenic cells and organisms, but also self-antigens expressed on the body's own tissues and nonpathogenic antigens responsible for allergic reactions. In healthy individuals, self-antigens do not elicit a significant immune response. Self-reactive lymphocytes are clonally eliminated during development; cells that survive this process are rendered tolerant to self-antigens in the periphery (Kamradt and Mitchison, 2001). There are at least two mechanisms for inducing peripheral lymphocyte tolerance. The first is anergy induction, an intracellular process in which antigen receptors become uncoupled from their downstream signaling pathways (Fields et al., 1996; Li et al., 1996; Boussiotis et al., 1997). The second involves regulatory T cells, which limit the responses of other lymphocytes to self- and environmental antigens, in part by producing immunosuppressive cytokines such as TGFβ and IL-10 (Maloy and Powrie, 2001).

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Activation of the cell-intrinsic mechanism of lymphocyte tolerance is closely linked to the cell-surface stimulus received. In both T and B cells, combined activation of antigen and costimulatory receptors leads to full activation of all TCR-coupled signaling pathways and culminates in a productive immune response. In contrast, tolerance is evoked, both ex vivo and in vivo, by unbalanced stimulation through antigen receptors without engagement of costimulatory receptors, or by stimulation with weak agonist antigens in the presence of full costimulation (Schwartz, 1996; Sloan-Lancaster and Allen, 1996; Goodnow, 2001). In each system, the process of tolerance induction may be conceptualized as occurring in two stages. The tolerizing stimulus first elicits partial or suboptimal activation; next, the partially activated lymphocytes enter a long-lasting unresponsive state, in which they paradoxically become refractory to subsequent full stimulation with antigen and costimulatory ligands (Schwartz, 1996; Sloan-Lancaster and Allen, 1996).

The most consistent feature of tolerizing stimuli is their ability to induce elevation of intracellular free Ca^{2+}. One of the simplest methods of inducing tolerance (anergy) in T cells is treatment with the Ca^{2+} ionophore ionomycin; conversely, anergy induction is blocked by the extracellular Ca^{2+} chelator EGTA and by the calcineurin inhibitor cyclosporin A (CsA) (Schwartz, 1996). Ca^{2+} has also been implicated in a well-established model of B cell tolerance in vivo: B cells bearing an anti-hen egg lysozyme (HEL) Ig transgene that have been tolerized to circulating antigen in vivo show a small but significant elevation in their basal levels of intracellular free Ca^{2+} and a concomitant increase in resting nuclear levels of the Ca^{2+}-regulated transcription factor NFAT (Healy et al., 1997). Ca^{2+} is also implicated in anergy imposed by altered peptide ligands, weakly agonistic peptide-MHC complexes that dissociate rapidly from the T cell receptor. Measurements of Ca^{2+} transients in single cells show that these weak agonist peptides elicit much lower levels of Ca^{2+} mobilization than strong agonist peptides, but increased Ca^{2+} levels are maintained for much longer times (Rabinowitz et al., 1996; Sloan-Lancaster et al., 1996).

A major consequence of Ca^{2+} mobilization is activation of the transcription factor NFAT (Rao et al., 1997; Crabtree, 1999). NFAT is a family of highly phosphorylated proteins residing in the cytoplasm of resting cells; when cells are activated, these proteins are dephosphorylated by the Ca^{2+}/calmodulin-dependent phosphatase calcineurin, translocate to the nucleus, and become transcriptionally active (Kiani et al., 2000; Okamura et al., 2000). In the nucleus, they cooperate with an unrelated transcription factor, AP-1 (Fos/Jun), to induce a large number of cytokine genes and other genes that are central to the productive immune response (Rao et al., 1997; Macian et al., 2001). Notably, NFAT activation does not require strong stimulation of antigen receptors on B and T cells: substantial nuclear localization of NFAT can be achieved with low, sustained levels of Ca^{2+} mobilization, such as those achieved by low concentrations

of Ca^{2+} ionophores, self-antigens, and low-affinity peptide-MHC complexes (Dolmetsch et al., 1997). Costimulatory receptors are not coupled to Ca^{2+} mobilization and contribute only weakly to activation of NFAT (Lyakh et al., 1997). Thus, NFAT activation occurs in response to Ca^{2+} signals or TCR stimulation alone, the precise conditions needed to evoke anergy. In contrast, costimulation is critical for optimal activation of NFκB and AP-1: combined TCR/CD28 stimulation activates cJun kinase (JNK), p38 MAP kinase, and IκB kinase (IKK) pathways and increases nuclear levels of NFκB/Rel and AP-1 proteins more strongly than TCR stimulation alone (Su et al., 1994; Harhaj and Sun, 1998).

Here we demonstrate that NFAT plays a central role in tolerance induction in T cells. Using a simple pharmacological method of inducing T cell anergy, we show that anergized T cells express a novel set of anergy-associated genes, distinct from those characteristic of the productive immune response. These genes are also upregulated in vivo in T cells from orally tolerized mice. T cells lacking a major NFAT protein, NFAT1 (NFATp, NFATc2), are resistant to anergy induction and show significantly lower expression of many anergy-associated genes. Conversely, T cells harboring a constitutively active NFAT1, under conditions where AP-1 is not activated or NFAT: AP-1 cooperation does not occur, show increased expression of anergy-associated genes and display an anergic phenotype of lowered TCR responsiveness. Thus, a single transcription factor, NFAT, regulates two contrasting aspects of T cell function, mediating nonoverlapping genetic programs of productive activation or anergy depending on the presence or absence of its transcriptional partner AP-1.

Results

Sustained Ca^{2+}/Calcineurin Signaling Attenuates Transcription of Effector Cytokine Genes

We used the murine antigen-specific Th1 clone D5 to set up a model of clonal anergy ex vivo (Figure 1). As previously reported for other T cell clones (Schwartz, 1996), pretreatment of D5 T cells with ionomycin greatly diminished their subsequent proliferative response to antigen or anti-CD3 (Figures 1A and 1C and data not shown) without inducing detectable levels of apoptosis (Figure 1B). As expected (Schwartz, 1996), anergy was overcome by exposure to IL-2 (Figure 1C). Anergy development was slow: decreased antigen responses were apparent after 4–6 hr of ionomycin pretreatment, but complete unresponsiveness was only achieved after 16 hr (data not shown). Ionomycin-treated D5 cells showed markedly decreased transcription of several inducible genes, including *IL-2*, *IFN-γ*, *TNF-α*, *GM-CSF*, and *MIP-1α*, in response to a second stimulation with anti-CD3/anti-CD28 or antigen/antigen presenting cells (APC) (Figure 1D; the apparent lack of downregulation of *IFN-γ* transcripts in lane 4 and of *MIP-1α* transcripts in lane 12 is due to probe saturation).

Ionomycin pretreatment also reduced cytokine gene transcription by primary T cells in response to TCR stimulation (Figure 1E), essentially abrogating mRNA induction by Th1 cells (lanes 2, 4, 10, and 12) and decreasing by ~70% the induction of *IL-4*, *IL-5*, and *IL-13* mRNAs by Th2 cells (lanes 6 and 8). Notably, Th1 and Th2 cells differed significantly with respect to *IL-10* gene expression: Th1 cells showed a striking decrease in *IL-10* transcript levels following ionomycin pretreatment (lanes 2 and 4), whereas Th2 cells were unaffected (lanes 6 and 8). Ionomycin pretreatment also did not affect *IL-10* mRNA induction by regulatory Tr1 cells, generated by culturing stimulated CD4 T cells in media containing IL-10 (Groux et al., 1997; S.-H.I., unpublished results). Thus, the net effect of a tolerizing stimulus on T cells is to skew the cytokine response toward production of the immunosuppressive cytokine IL-10 while downregulating production of multiple effector cytokines associated with a productive immune response.

As previously noted (Schwartz, 1996), anergy induction required calcineurin activity (Figure 1F). DO11.10 Th1 cells were stimulated overnight with immobilized anti-CD3 in the presence or absence of the calcineurin inhibitor CsA, then detached, washed thoroughly, cultured for 2–3 days to remove both drug and stimulus, and restimulated with antigen/APC (Figure 1F). The CsA washout was successful since it barely affected IL-2 production at later times (clusters 1 and 3); however, CsA strongly impaired anergy induction by immobilized anti-CD3 (clusters 2 and 4).

A Gene Expression Program Activated by Ca^{2+} and Calcineurin

Based on these data, we hypothesized that sustained Ca^{2+}/calcineurin signaling induced a distinct genetic program that correlated with the development of a long-lasting anergic state. To test the hypothesis, we evaluated the gene expression profile of D5 T cells stimulated for 2, 6, or 16 hr with ionomycin alone, which results in anergy induction; with ionomycin plus CsA, which blocks anergy induction; and with ionomycin plus PMA, which pharmacologically mimic complete stimulation through the TCR and CD28. Using Affymetrix oligonucleotide arrays, we identified 1358 genes and ESTs whose expression was altered at least 3-fold by any of the treatments at one or more time points. Genes with similar expression patterns were clustered into 36 panels, 20 of which (736 genes and ESTs) are shown in Figure 2A. Known genes in the 20 panels are listed in Supplementary Table S1 at http://www.cell.com/cgi/content/full/109/6/719/DC1.

This analysis established that the effect of ionomycin stimulation on gene expression was distinct from that of combined PMA/ionomycin stimulation, and moreover involved a much smaller number of genes (Figure 2A). Only 205 genes/ESTs could be considered to be ionomycin induced: ~165 were induced more strongly by ionomycin than by PMA/ionomycin (panels 15–19), while ~40 were equivalently induced by ionomycin and by PMA/ionomycin (panels 13 and 14 plus a few in panel 6; these are considered to be ionomycin-induced genes on which PMA had no additional effect). In contrast, 585 genes/ESTs were upregulated (panels 1–12 and three panels not shown) and 568 were downregulated (panel 20 and 13 panels not shown) in response to PMA/ionomycin stimulation, with little or no change in response to ionomycin alone. As expected (Teague et al., 1999; Glynne et al., 2000; Feske et al., 2001), genes upregu-

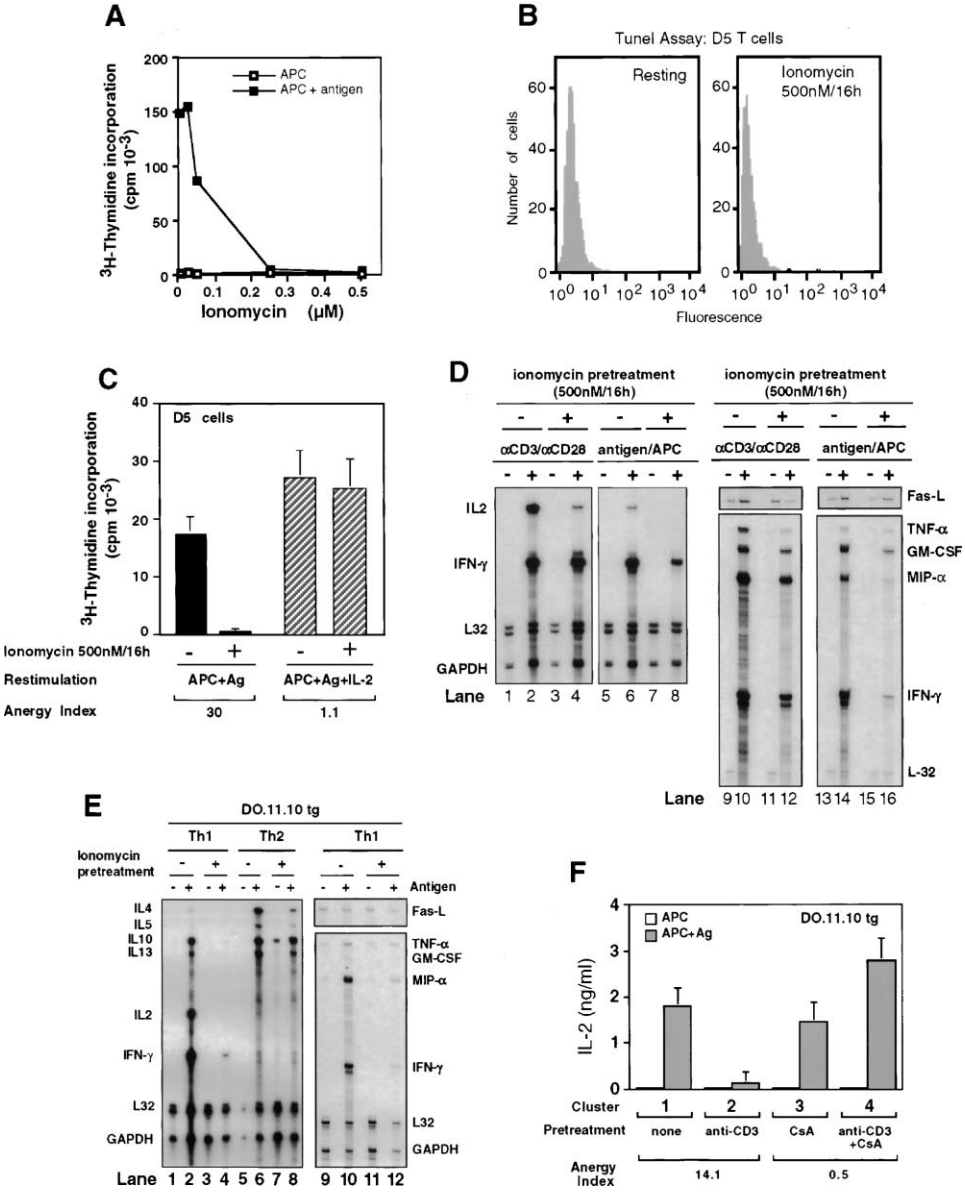

Figure 1. Ionomycin Pretreatment Attenuates T Cell Responses to Subsequent TCR/CD28 Stimulation

(A) ^{3}H-thymidine incorporation was measured in D5 T cells cultured with or without ionomycin for 16 hr, then washed and restimulated with antigen/APC. Unless otherwise indicated, all subsequent experiments were performed using 16 hr pretreatment with 500 nM ionomycin.

(B) TUNEL assay showing that ionomycin treatment does not result in cell death.

(C) Ionomycin-pretreated T cells remain responsive to IL-2. D5 cells were incubated with or without ionomycin, then washed and stimulated with antigen/APC with or without exogenous IL-2. ^{3}H-thymidine incorporation was measured. Results are mean and range of two experiments.

(D) Ionomycin pretreatment attenuates cytokine expression by D5 T cells. Cells were pretreated with ionomycin, then washed and stimulated for 4 hr. Cytokine mRNA levels were determined by RNase protection assay.

(E) Ionomycin pretreatment attenuates most cytokine expression by primary Th1 and Th2 cells. Cells were pretreated with ionomycin, then washed and stimulated with antigen/APC. Cytokine expression was determined by RNase protection assay.

(F) Anergy induction is inhibited by CsA. Th1 cells were incubated with or without 1 μg/ml plate bound anti-CD3 in the presence or absence of 1 μM CsA for 16 hr. After washing and resting for 48–72 hr, cells were restimulated with antigen/APC for 24 hr. IL-2 levels were determined by ELISA. Values are average ± S.E.M. of three independent experiments.

lated by PMA/ionomycin included cytokine, chemokine, and other inducible genes characteristic of the productive immune response (*IL-2*, *IFN-γ*, *GM-CSF*, etc.; see Supplementary Table S1 at http://www.cell.com/cgi/content/full/109/6/719/DC1). For almost all genes, alterations in expression were abolished by CsA, consistent with our previous findings using human T cells (Feske et al., 2001). The results support the hypothesis that Ca^{2+}/calcineurin signaling directs a genetic program associated with anergy development in T cells, distinct

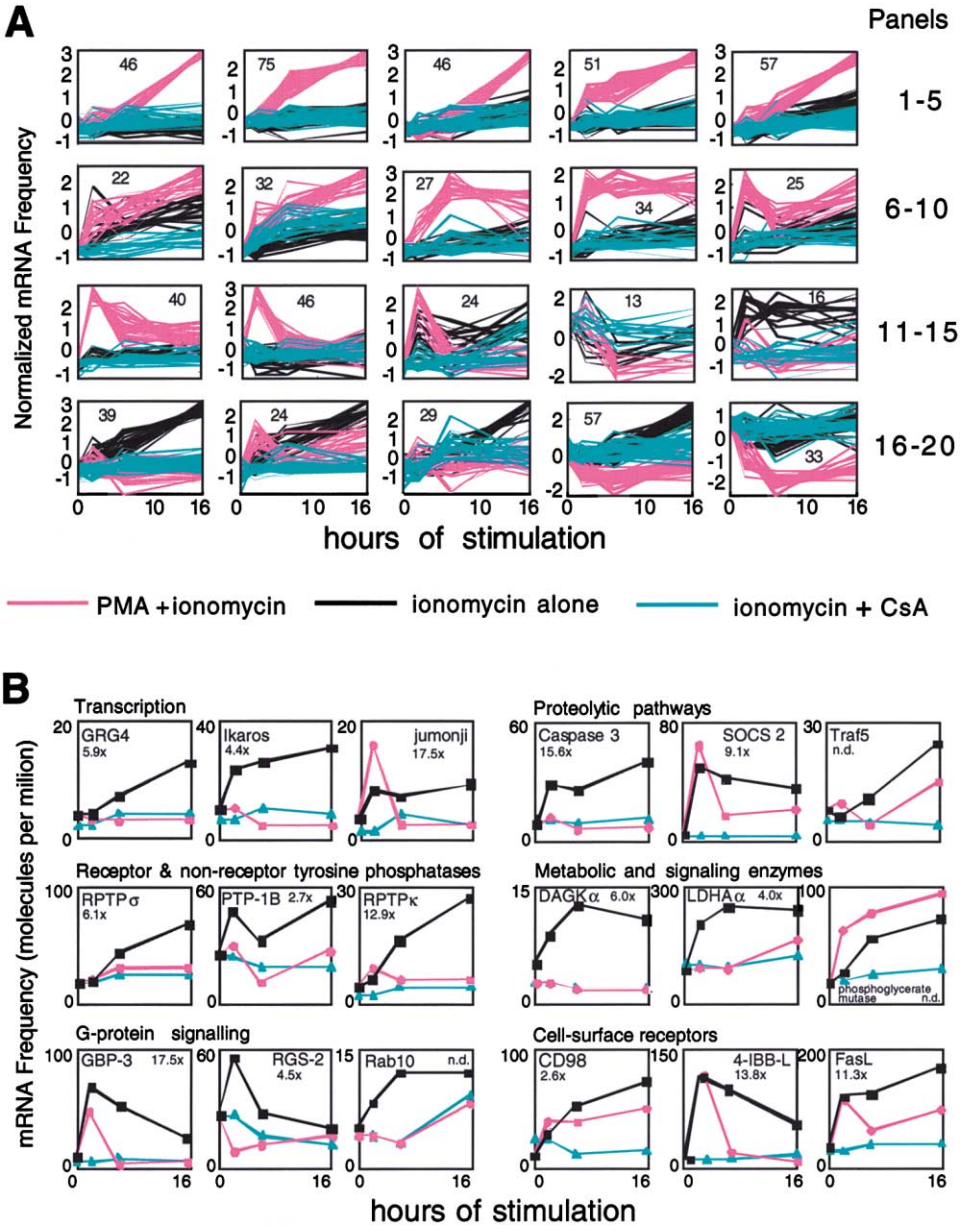

Figure 2. Stimulation with Ca^{2+} Ionophore Activates a Calcineurin-Dependent Program of Gene Expression Distinct from that Induced by PMA plus Ionomycin

(A) RNA was prepared from resting D5 T cells or cells stimulated for 2, 6, or 16 hr as indicated. Gene transcription profiles were evaluated using Affymetrix oligonucleotide arrays. Genes whose expression levels were altered at least 3-fold in response to any of the treatments were selected for clustering analysis using the self-organizing map (SOM) algorithm on the basis of kinetic expression pattern. The number of clustered genes and ESTs is indicated inside each panel.

(B) Expression profiles of 18 specific genes chosen on the basis of their strong activation by ionomycin. The genes are grouped into six categories based on function. Numbers within the panels indicate the fold induction of each transcript after stimulation with ionomycin for 2 hr, as confirmed by real time quantitative PCR. n.d. indicates not determined.

from that associated with the productive immune response (Glynne et al., 2000; Lechner et al., 2001). For the remainder of this study, the ionomycin-induced genes will be referred to interchangeably as "anergy-associated" genes.

By repeating the DNA arrays with RNA prepared from primary Th1 cells, we confirmed ionomycin inducibility of ~70 of the ~205 anergy-associated genes/ESTs (e.g., see Figure 3C). Of the 37 known genes in this category (see Supplementary Table S2 at http://www.cell.com/cgi/content/full/109/6/719/DC1), we selected 18 genes because of their robust and reproducible induction and because their encoded gene products fell into potentially interesting functional classes (Figure 2B). Like the

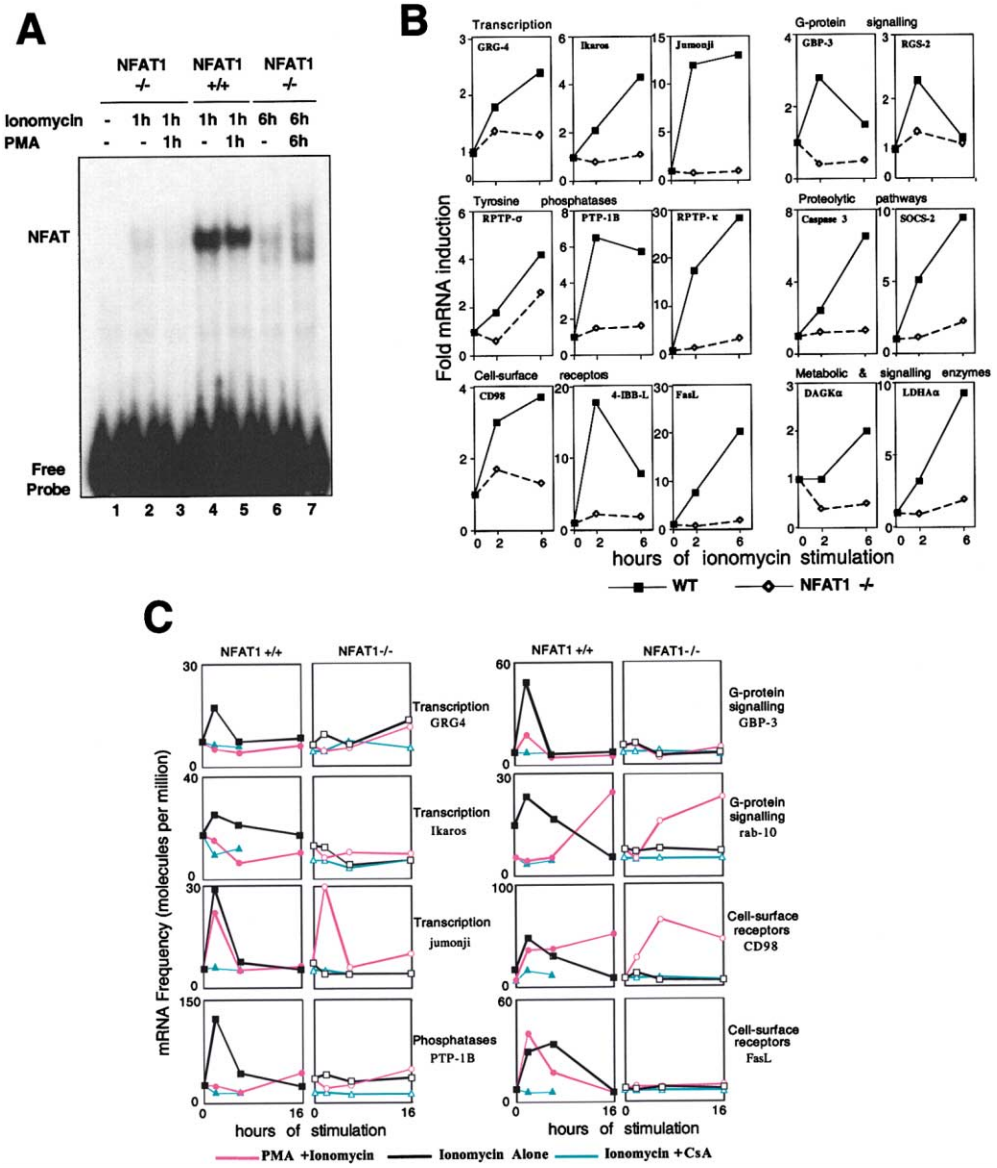

Figure 3. NFAT1$^{-/-}$ Th1 Cells Show Reduced Expression of Anergy-Associated Genes

(A) NFAT1 is the predominant NFAT protein in resting T cells. Nuclear extracts from wild-type and NFAT1$^{-/-}$ Th1 cells were tested in EMSAs using an NFAT probe. Comparison of lanes 2 and 3 with lanes 4 and 5 shows that NFAT1 accounts for ~90% of total NFAT DNA binding activity in wild-type T cells. A control EMSA with an octamer probe showed equivalent binding activity in all lanes (not shown).

(B) NFAT1 regulates expression of many anergy-associated genes. Expression of 15 of the ionomycin-induced genes shown in Figure 2B was examined by real-time quantitative PCR in ionomycin-stimulated wild-type and NFAT1$^{-/-}$ Th1 cells. Results are represented as fold increase over the levels of mRNA present in resting cells (set to 1). The average of two independent experiments is plotted.

(C) Gene transcription profiles of eight selected genes in wild-type and NFAT1$^{-/-}$ Th1 cells obtained using Affymetrix oligonucleotide arrays. All genes showed NFAT1-dependent induction in response to ionomycin. For jumonji, Rab10, and CD98, PMA/ionomycin-mediated induction was not NFAT1 dependent and may be mediated by inducible isoforms of NFAT2 (Lyakh et al., 1997).

Ca^{2+}-dependent genes described in a separate study (Feske et al., 2001), the ionomycin-induced genes displayed diverse expression patterns consistent with differential regulation by PMA- and ionomycin-induced signaling pathways (Figure 2B). To validate the array data, we evaluated expression of 15 of the 18 genes in D5 T cells by quantitative real-time PCR, and in every case we were able to confirm ionomycin-mediated induction (Figure 2B).

The Transcription Factor NFAT1 Participates in Anergy Induction

Since calcineurin activity is required for anergy induction, we tested the role of the calcineurin-regulated transcription factor NFAT. We first asked whether induction of the anergy-associated genes was regulated, directly or indirectly, by NFAT. To do this, we took advantage of the fact that the NFAT family member NFAT1 is the predominant NFAT protein in resting T cells (Figure 3A).

NFAT1$^{-/-}$ T cells do not show compensatory increases in other NFATs (F.M., unpublished); thus, these cells not only lack all NFAT1, but also contain only about 10%–15% of the normal levels of total NFAT. About 35 of the ~70 ionomycin-induced genes/ESTs, and 15 of the 18 selected anergy-associated genes (Figures 3B and 3C), showed significantly lower expression in NFAT1$^{-/-}$ relative to wild-type Th1 cells following ionomycin stimulation, consistent with participation in an NFAT1-dependent anergy program. Twenty to twenty-five genes were equivalently induced in wild-type and NFAT1$^{-/-}$ T cells, implying participation of transcription factors other than NFAT1 (see Discussion). The remaining genes were upregulated in NFAT1$^{-/-}$ relative to wild-type T cells, suggesting loss of an inhibitory signal mediated by NFAT1. The results implicate NFAT proteins, directly or indirectly, in a substantial proportion of ionomycin-induced gene transcription in T cells.

We asked whether NFAT1$^{-/-}$ T cells were also defective for anergy induction ex vivo (Figure 4). As expected, ionomycin pretreatment of DO11.10 TcR transgenic Th1 cells resulted in markedly decreased induction of *IL-2* and *IFN-γ* mRNAs (Figure 4A, lanes 1 and 2). Because of their lower levels of total NFAT, Th1 cells from NFAT1$^{-/-}$ DO11.10 mice showed somewhat lower induction of cytokine mRNAs compared to wild-type Th1 cells (1.5-fold and 2-fold decrease for *IL-2* and for *IFN-γ*, respectively, lanes 1 and 3), but they were much less susceptible to anergy induction, showing perceptible induction of *IL-2* and *IFN-γ* mRNA even after ionomycin pretreatment (Figure 4A, lane 4). Ionomycin-treated wild-type Th1 cells showed 18-fold and 12-fold decreases in *IL-2* and *IFN-γ* transcripts following stimulation, but NFAT1$^{-/-}$ T cells showed only 2.5-fold decrease in either case (Figure 4A, right). The anergized cells were fully responsive to PMA/ionomycin stimulation, which bypasses the membrane-proximal steps of TCR signal transduction (Figure 4A, lanes 5–8; note that the relative defect of the NFAT1$^{-/-}$ T cells is overcome under these "strong" stimulation conditions). NFAT1$^{-/-}$ Th1 cells did not become anergic in response to anti-CD3 pretreatment, compared to wild-type T cells, which were effectively anergized under these conditions (Figure 4B), again supporting a role for NFAT1 and possibly other NFAT proteins in anergy.

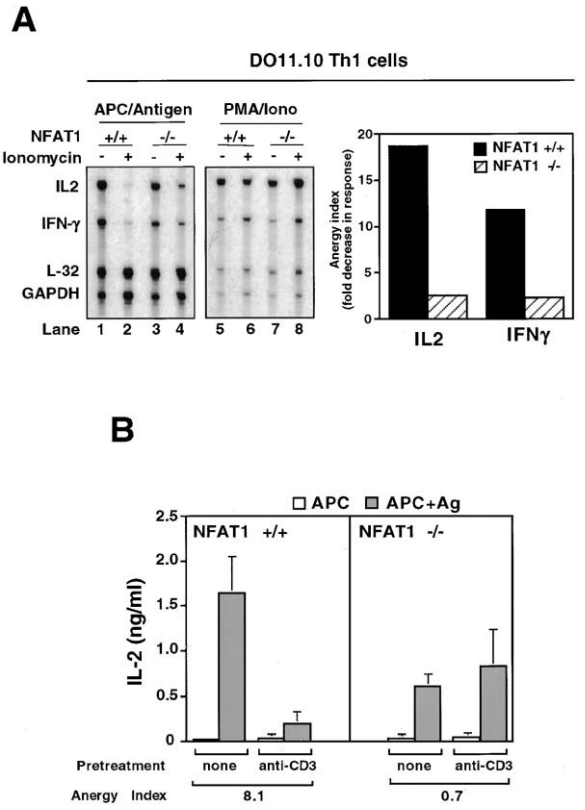

Figure 4. NFAT1$^{-/-}$ T Cells Are More Resistant to Anergy Induction than Wild-Type T Cells

(A) Wild-type or NFAT1$^{-/-}$ Th1 cells were incubated with or without ionomycin, then stimulated with antigen/APC or PMA/ionomycin for 4 hr. *IL-2* and *IFN-γ* transcript levels were determined by RNase protection assay (left). Unstimulated cells did not express detectable cytokine transcripts. The graph on the right shows the anergy index (fold reduction of cytokine transcript levels following ionomycin pretreatment) for *IL-2* and *IFN-γ* in wild-type and NFAT1$^{-/-}$ Th1 cells. The results are representative of two independent experiments.

(B) Th1 cells from wild-type or NFAT1$^{-/-}$ DO11.10 transgenic mice were incubated with or without 1 μg/ml plate bound anti-CD3 for 16 hr. After resting for 48–72 hr, cells were restimulated with antigen/APC, and IL-2 production was determined by ELISA. Values are average ± S.E.M. of three independent experiments.

Orally-Tolerized T Cells Upregulate the Expression of Anergy-Associated Genes

To ask whether anergy-associated genes were upregulated in tolerant T cells in vivo, we set up a model of oral tolerance in which administration of high doses of protein antigens induces systemic, antigen-specific T cell tolerance in mice (Garside and Mowat, 2001). Ovalbumin (OVA) was administered to DO11.10 TCR transgenic mice in their drinking water for 5 days, after which CD4 T cells were isolated from spleen and lymph nodes and their response to $OVA_{323–339}$ peptide was measured. As expected, T cells from OVA-fed mice showed profoundly diminished proliferation and IL-2 production compared to T cells from control transgenic mice that had not been given OVA (Figures 5A and 5B). In parallel we assessed expression of 14 selected ionomycin-induced genes by quantitative real-time PCR: the in vivo tolerized T cells showed upregulation of 13 of these 14 genes (Figures 5C and 5D). Thus, in vivo tolerized T cells exhibit a gene expression profile very similar to that observed in ex vivo anergized T cells, downregulating production of IL-2 and other effector cytokines while establishing a distinct Ca^{2+}/calcineurin-dependent pattern of gene expression. In vivo tolerized T cells also show basal elevation of intracellular Ca^{2+} levels (V. Heissmeyer, S. Feske, S.-H.I., and A.R., unpublished), suggesting strongly that T cell tolerance, like B cell tolerance (Healy et al., 1997; Goodnow, 2001), depends on sustained low-level signaling through the Ca^{2+}/calcineurin/NFAT pathway.

Anergy Is Induced by NFAT in the Absence of AP-1 (Fos-Jun)

NFAT:AP-1 cooperation is critical for transcription of most genes induced during the productive immune re-

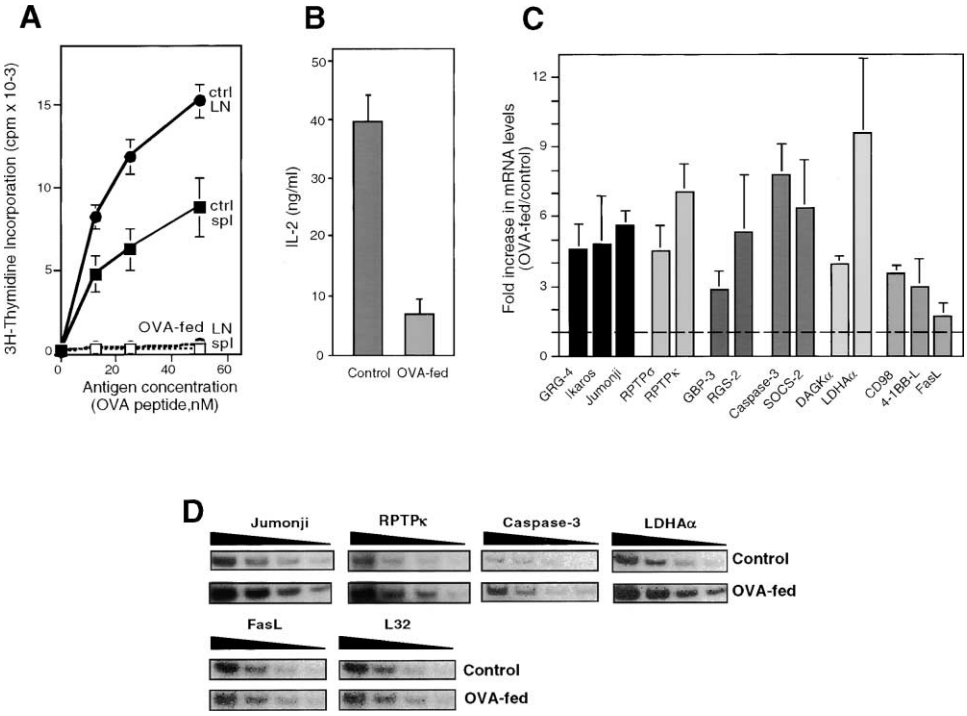

Figure 5. Orally-Tolerized T Cells Upregulate Most Anergy-Associated Genes

(A and B) CD4 cells, isolated from spleen (spl) and lymph nodes (LN) of unfed or OVA-fed DO11.10 mice, were stimulated with different concentrations of $OVA_{323-339}$. Tolerized T cells show greatly diminished ^{3}H-thymidine incorporation (A) and IL-2 production (B) of antigen responses compared to control (ctrl) T cells. One of four representative experiments is shown.

(C) Relative expression of 14 anergy-associated genes in tolerized versus non-tolerized T cells. Transcript levels were analyzed by real-time quantitative PCR in control and OVA-tolerized CD4 T cells. Bars are grouped according to the functional categories of Figure 2B. Results are average ± S.D. of four independent experiments.

(D) PCR analysis using [^{32}P]dCTP and 2-fold serial dilutions of cDNA. For the four genes displayed at top, the signal from tolerized T cells is higher than the signal from control T cells. There was no change in levels of FasL mRNA. L32 levels serve as an internal control.

sponse (Rao et al., 1997; Kiani et al., 2000; Macian et al., 2001). We asked whether NFAT:AP-1 cooperation was required for anergy induction as well. Electrophoretic mobility shift assays showed that ionomycin-induced anergy correlated with NFAT but not AP-1 or NFκB activation (Figure 6A, lanes 2, 5, and 8), while combined stimulation with PMA and ionomycin induced the cooperative NFAT:AP-1 complex, the AP-1 complex, and the p50/p65 NFκB complex as expected (closed symbols, lanes 3, 6, and 9).

To explore the role of NFAT:AP-1 cooperation in anergy induction, we utilized a constitutively active (CA) version of NFAT1 (Okamura et al., 2000). This protein bears alanine substitutions in 12 phosphorylated serines whose dephosphorylation is required for nuclear localization and is constitutively nuclear under conditions where endogenous NFAT proteins are fully localized to the cytoplasm (Okamura et al., 2000). We also generated CA-RIT-NFAT1, a CA-NFAT1 derivative engineered to be incapable of cooperation with AP-1. In addition to the 12 Ser to Ala substitutions present in CA-NFAT1, CA-RIT-NFAT1 contains three point mutations in its DNA binding domain that abrogate Fos-Jun interaction (R468A/I469A/T535G; Macian et al., 2000).

We first showed that CA-NFAT1 could activate the transcription of endogenous inducible genes (Figure 6B). Untransfected Jurkat cells showed no cytokine expression in response to PMA stimulation, as expected from the lack of activation of endogenous NFAT (lanes 1 and 2). Cells transfected with the CA-NFAT1 plasmid showed perceptible basal induction of the *TNF*α gene (lane 3), an NFAT-dependent gene that can be transcribed in the absence of NFAT-AP-1 cooperation (Goldfeld et al., 1993; Macian et al., 2000), as well as strong PMA-stimulated induction of the *IL-3*, *GM-CSF*, and *MIP-1*α genes, which require the cooperative interaction of NFAT and AP-1 (lane 4; Macian et al., 2000). PMA stimulation also further upregulated *TNF*α, a gene that normally is maximally activated under conditions of combined PMA/ionomycin stimulation (Macian et al., 2000).

Despite the ability of CA-NFAT1 to activate gene transcription, both CA-NFAT1 and CA-RIT-NFAT1 paradoxically reduced TCR responsiveness when retrovirally expressed in unstimulated *NFAT1*$^{-/-}$ Th1 cells (Figures 6C and 6D). Five to seven days after infection with IRES-GFP retroviruses, the ability of GFP^{+} (infected) and GFP^{-} (uninfected) cells to produce IL-2 in response to anti-CD3/anti-CD28 stimulation was assessed by intracellular cytokine staining. T cells expressing CA-NFAT1 or CA-RIT-NFAT1 showed markedly decreased IL-2 production, compared to control T cells expressing GFP alone (Figure 6C, compare top and bottom; the results of four independent experiments are presented in Figure

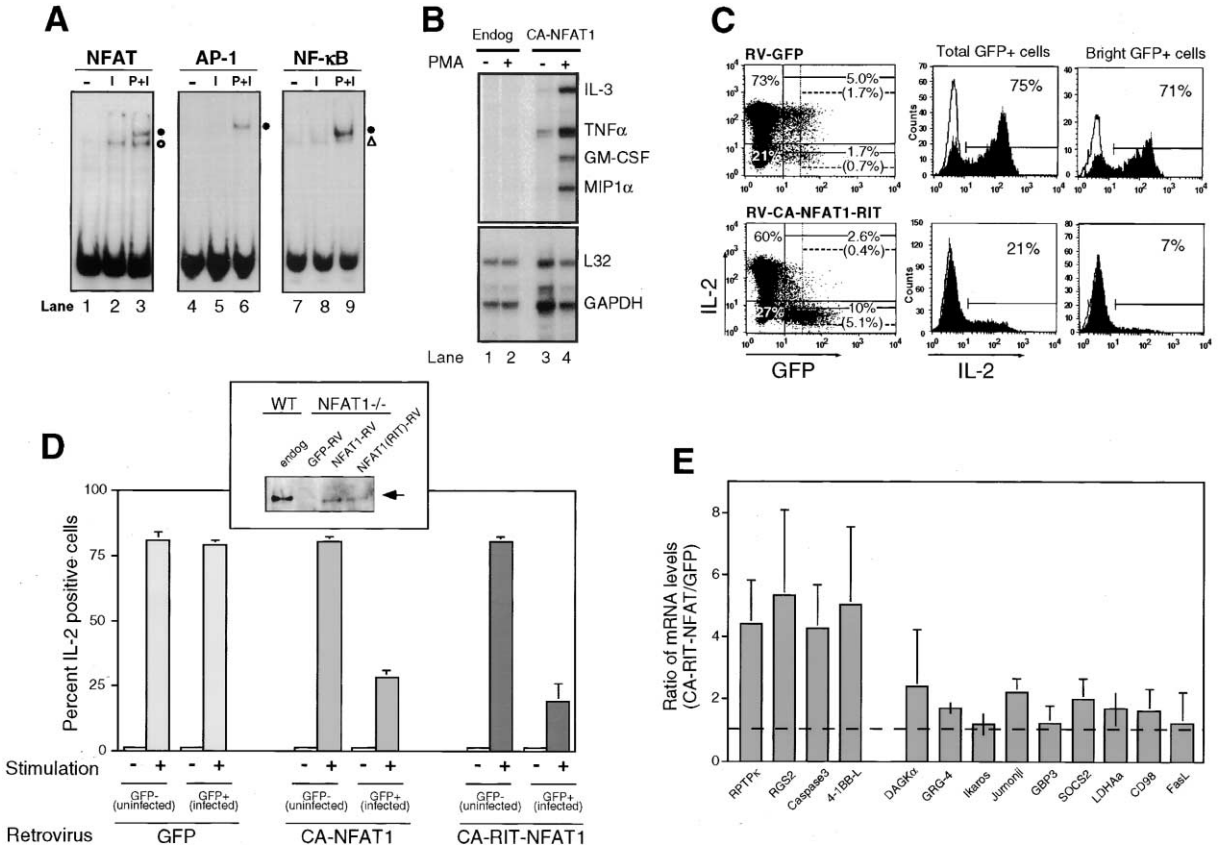

Figure 6. NFAT Induces T Cell Anergy in the Absence of AP-1 (Fos-Jun)

(A) Ionomycin treatment activates NFAT but not AP-1 or NFκB. EMSAs were performed with nuclear extracts of unstimulated or stimulated Th1 cells and labeled NFAT, AP-1, or NFκB probes. DNA-protein complexes are indicated as follows. Left: open circle, NFAT; closed circle, cooperative NFAT:AP-1 complex. Middle: closed circle, AP-1 complex. Right: open triangle, NFκB1(p50) homodimer; closed circle, NFκB1(p50)/RelA(p65) heterodimer.

(B) Constitutively active (CA) NFAT1 induces expression of endogenous cytokine genes. Jurkat T cells were cotransfected with expression plasmids encoding mouse CD4 and either GFP or CA-NFAT1. Productively transfected cells expressing mouse CD4 were isolated and stimulated with PMA as indicated, and cytokine transcript levels were analyzed by RNase protection assay. Endogenous (Endog) NFAT is unable to induce cytokine expression under these conditions (lanes 1 and 2).

(C) Constitutive expression of CA-RIT-NFAT1 renders T cells unresponsive to TCR/CD28 stimulation. NFAT1$^{-/-}$ Th1 cells were infected with GFP or CA-RIT-NFAT1/GFP retroviruses (RV), then cultured for 3 days with and 24 hr without IL-2. The cells were then stimulated for 4 hr with anti-CD3/anti-CD28, and the percentage of IL-2-producing cells in GFP$^+$ (infected) and GFP$^-$ (uninfected) cells was determined by intracellular cytokine staining. Left: dot plot showing GFP and IL-2 expression by individual T cells. Two different gates were used: total GFP$^+$ cells (continuous vertical line) and cells expressing high levels of GFP (dotted vertical line). Middle and right: open and closed histograms show IL-2 staining in unstimulated and stimulated cells gated for total or high GFP expression.

(D) Average ± S.D. of four independent experiments similar to that shown in part (C). Cells were infected with GFP, CA-NFAT1, or CA-RIT-NFAT1 retroviruses. Inset, CA-NFAT1 and CA-RIT-NFAT1 expression in infected populations of NFAT1$^{-/-}$ cells was estimated by immunoblotting with an anti-NFAT1 antibody. Expression levels are lower than those of endogenous NFAT1 in wild-type T cells.

(E) Constitutive expression of CA-RIT-NFAT1 induces expression of some but not all anergy-associated genes. NFAT1$^{-/-}$ Th1 cells were infected with GFP and CA-RIT-NFAT1 retroviruses and sorted for GFP expression. RNA was isolated from sorted cells, and levels of anergy-associated genes were determined by quantitative real-time PCR. mRNA expression in CA-RIT-NFAT1-expressing cells is plotted relative to that in control GFP-expressing cells. Results are average ± S.D. of three independent experiments except for GRG-4, Ikaros, and LDHA, for which the mean and range of two independent experiments is shown.

6D). When total and bright GFP$^+$ cells were compared, there was a clear correlation between the levels of GFP/CA-RIT-NFAT1 expression and the extent to which IL-2 production was reduced (Figure 6C). Note that the constitutively active proteins are not overexpressed (see inset in Figure 6D), ruling out a dominant-negative mechanism in which CA-RIT-NFAT1 competes with endogenous NFAT for binding to the *IL-2* promoter. This mechanism is unlikely in any case, since without AP-1, the affinity of NFAT for composite NFAT:AP-1 sites is at least 20-fold lower than its affinity when it forms a cooperative NFAT:AP-1 complexes on the same site (G. Powers and P.G. Hogan, personal communication; Jain et al., 1993).

Together, these results establish that continuous, low-level activation of NFAT1 induces a state similar to classical anergy, in which T cells are significantly less capable of producing IL-2 in response to TCR stimulation. Notably, NFAT:AP-1 cooperation is not required for this process of anergy induction. Stimulation of CA-RIT-NFAT1-expressing T cells with PMA and ionomycin re-

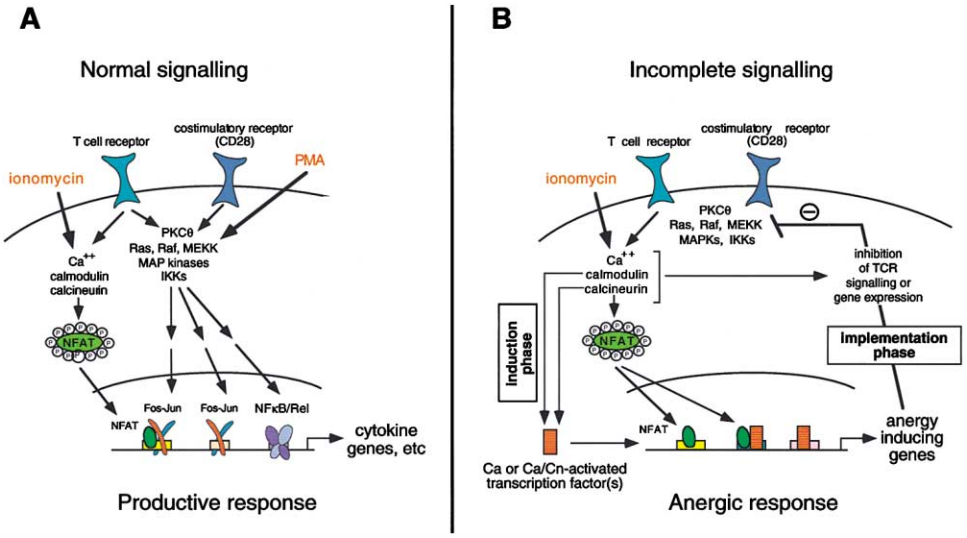

Figure 7. A Model of Anergy Induction
For details, see text.

stored IL-2 expression substantially (data not shown), indicating that the anergy involved a TCR-proximal block in signaling which could be overcome with pharmacological agents that bypassed the TCR.

Constitutive expression of CA-RIT-NFAT1 induced the transcription of specific anergy-associated genes (Figure 6E). CA-RIT-NFAT1 was retrovirally expressed in *NFAT1*$^{-/-}$ Th1 cells, GFP^{+} cells were isolated by cell sorting, RNA was prepared from the unstimulated cells, and expression of 13 anergy-associated genes was assessed by real-time PCR. At least 4 of the 13 genes showed increased expression in CA-RIT-NFAT1-expressing cells relative to cells expressing GFP alone (Figure 6E), indicating that NFAT1 is sufficient as well as necessary (Figure 3B) to induce expression of these genes. In contrast, NFAT1 did not induce (or only moderately induced) expression of 8 of the 13 genes (Figure 6E), although it clearly participated in their induction (Figures 3B and 3C). Thus, the transcriptional arm of the anergy program requires not only NFAT1, but also additional signaling pathways and transcription factors induced by Ca^{2+} or Ca^{2+}/calcineurin signaling.

Discussion

A Model of Anergy Induction

Based on our data, we propose that NFAT plays a central role, not only in productive activation of lymphocytes but also in lymphocyte tolerance. Our model of tolerance induction is depicted in Figure 7. Combined stimulation of TCR and costimulatory receptors results in balanced activation of NFAT, AP-1, and NFκB; the cooperative NFAT:AP-1 complexes formed under these conditions are necessary for transcription of cytokine genes and other genes critical for the productive immune response (Figure 7A). In contrast, TCR stimulation without costimulation results in higher activation of the Ca^{2+} arm of the TCR signal transduction pathway relative to the PKC/IKK/Ras/MAP kinase arm; these conditions lead to unbalanced activation of NFAT relative to its cooperating transcription factor AP-1 (Fos/Jun), thus diverting NFAT toward transcription of an alternate set of anergy-associated genes whose products together impose the tolerant state (Figure 7B). This model does not exclude the participation of nontranscriptional mechanisms dependent on Ca^{2+} signaling, or participation of Ca^{2+}-regulated transcriptional modulators other than NFAT.

The model is consistent with essentially all previous data on tolerance induction, both in vivo and ex vivo (see Introduction). Our supporting experimental data are as follows. First, T cells lacking NFAT1, the major NFAT protein expressed in resting cells, are more resistant than wild-type T cells to anergy induction ex vivo, consistent with previous findings of T and B cell hyperproliferation in mice lacking NFAT1 (Hodge et al., 1996; Xanthoudakis et al., 1996) or both NFAT1 and NFAT2 (Peng et al., 2001). Second, T cells anergized with ionomycin show selective NFAT activation as well as induction of a novel set of anergy-associated genes; these genes are distinct from those activated during the productive immune response and encode diverse categories of proteins that could plausibly impose an anergic state (discussed below). Third, the anergy-associated genes are also upregulated in T cells rendered tolerant to high dose oral antigen in vivo. Fourth, a substantial number of anergy-associated genes are direct or indirect targets of NFAT, since they are expressed at significantly lower levels in *NFAT1*$^{-/-}$ T cells following ionomycin stimulation. Fifth, constitutively active versions of NFAT1, which cannot cooperate productively with AP-1, are capable of downregulating IL-2 production when retrovirally introduced into *NFAT1*-deficient Th1 cells.

Anergy induction is likely to require not only NFAT1, but also other NFAT proteins and other nuclear factors induced by Ca^{2+}/calcineurin signaling. Although NFAT1$^{-/-}$ T cells are resistant to anergy induction (Figure 4), they can be rendered anergic by high concentrations of ionomycin or immobilized anti-CD3 (F.G.-C., F.M., unpub-

lished). These data suggest that NFAT2 and NFAT4 also participate in anergy induction, making increasing contributions as stronger anergizing stimuli are used. Moreover, half of the anergy-associated genes are not affected by NFAT1 deficiency, suggesting redundant effects of NFAT2 and NFAT4. It is likely that other Ca^{2+}/calcineurin-regulated nuclear factors also have a role: ionomycin pretreatment induces T cell anergy more effectively than CA-NFAT1 (Figures 1, 6C, and 6D), and CA-NFAT1 does not upregulate the entire panel of ionomycin-inducible genes (Figures 2 and 6E). Plausible candidates include Elk-1 and MEF2 family members, which are known to be regulated by calcineurin; NFAT-MEF2 cooperation has already been documented for other cell types and genes (Aramburu et al., 2000; Olson and Williams, 2000).

Relation between Anergy Induction and Cell Death

Our model also explains the fact that anergy is frequently associated with activation-induced cell death (AICD) (Li et al., 2000; Kamradt and Mitchison, 2001). Mice injected with high doses of soluble antigen or with superantigens (proteins that interact simultaneously with MHC Class II and the Vβ region of the TCR) delete large numbers of reactive cells, but the surviving cells are tolerant to subsequent stimulation (Garside and Mowat, 2001). Since all the signaling pathways shown in Figure 7A can be activated by TCR stimulation alone, we postulate that both NFAT and AP-1 are induced early in response to high circulating concentrations of antigen or superantigen, but while NFAT activation is relatively uniform, the extent of AP-1 activation depends on the strength of the stimulus encountered by the individual T cell, yielding a population of reactive cells that express a wide range of relative NFAT:AP-1 ratios. NFAT:AP-1 cooperation is required for AICD (Macian et al., 2000), and thus cells with the highest AP-1 levels would succumb to AICD while those with the lowest levels of AP-1 would become anergic. Costimulation would affect both aspects of this process: it provides a strong survival stimulus by activating PI-3 kinase and Akt and Bcl family members (Boise et al., 1995; Kane et al., 2001), thus promoting proliferation rather than cell death, and at the same time it potentiates MAP kinase/AP-1 and IKK/NFκB pathways (Su et al., 1994; Harhaj and Sun, 1998), thus diminishing the probability that a cell will become anergic. However, we emphasize that T cells anergized with ionomycin ex vivo showed no evidence of cell death in our experiments (Figure 1B), despite increased expression of FasL mRNA (Figures 2 and 3) and active caspase 3 protein (F.M., unpublished). This is likely to reflect their lack of AP-1 activation (Figure 6A) as well as the ability of Ca^{2+} signaling to downregulate Fas mRNA (Feske et al., 2001).

Mechanisms of Anergy Induction

Our data support the existence of distinct mechanisms of tolerance induction in lymphocytes. The first is simple interference with signaling pathways coupled to antigen receptors (Fields et al., 1996; Li et al., 1996; Boussiotis et al., 1997; Healy et al., 1997). This process could be mediated by the protein products of several of the anergy-associated genes we have identified, including soluble and receptor tyrosine phosphatases (Li and Dixon, 2000); diacylglycerol kinase-α, which metabolizes the diacylglycerol required to activate protein kinases C (Sanjuan et al., 2001); and the cell-surface receptor CD98, which is coupled to increased GTP loading of the small G protein Rap1 (Suga et al., 2001). Rap1 activation has been linked to impaired activation of the ERK MAP kinase pathway in anergic T cells (Boussiotis et al., 1997; Bos, 1998).

Our data also suggest that proteolytic mechanisms contribute to anergy induction. The procaspase 3 gene is robustly induced under conditions of anergy induction (Figures 2 and 3), and caspase 3 has been implicated in modulating lymphocyte responses under conditions where its activation does not appear to be associated with cell death (Alam et al., 1999). Thus, caspase 3 might implement T cell anergy in a manner unrelated to apoptosis by cleaving specific signaling proteins downstream of the TCR; its reported targets in the T cell activation pathway include Vav1, PKC-theta, the adaptor protein Gads, and the zeta chain of the TCR/CD3 complex (Datta et al., 1997; Gastman et al., 1999; Hofmann et al., 2000; Yankee et al., 2001). SOCS-2 and Traf5 are also products of anergy-associated genes; like the related proteins SOCS-1 and Traf6 (Kamizono et al., 2001; Wang et al., 2001), these proteins may be E3 ligases involved in ubiquitin transfer. Indeed, mice lacking the E3 ligases Itch and Cbl-b show a striking autoimmune phenotype (Perry et al., 1998; Bachmaier et al., 2000; Chiang et al., 2000), suggesting that protein degradation has a role in lymphocyte tolerance. Directed proteolysis of specific signaling components in anergic T cells could explain the long-lasting nature of anergy in vivo and ex vivo (Schwartz, 1996; Lanoue et al., 1997), as well as the finding that anergy is dominant in somatic cell fusion experiments (Telander et al., 1999).

Ex vivo, activation of NFAT without AP-1 blocks all Th1 cytokine production and skews the cytokine profile of Th2 cells toward IL-10 production (Figure 1E). A similar skewing toward IL-10 expression has been reported in tolerant T cells in vivo (Buer et al., 1998). Preferential IL-10 production by anergic T cells provides a link between the two current models of how peripheral tolerance is maintained: the cell-intrinsic mechanism of anergy induction would attenuate the antigen responsiveness of differentiated effector T cells, while the bias toward IL-10 production by Th2 and Tr1 cells would lead to some immunosuppression by itself but would also result, over the longer term, in generation of IL-10-producing regulatory T cells capable of suppressing any remaining productive response (Buer et al., 1998; Maloy and Powrie, 2001). The cell type- and gene-specific inhibition of cytokine gene transcription observed in anergic T cells is likely to be imposed in the nucleus by transcriptional modulators that act on specific genes, rather than in the cytoplasm by global interference with the TCR signaling complex. Candidate transcriptional modulators emerging from our screens include Ikaros, a family of proteins implicated in gene silencing (Brown et al., 1997; Sabbattini et al., 2001); the Groucho-related protein Grg4 (Eberhard et al., 2000); and the DNA binding protein jumonji that negatively regulates cell proliferation (Toyoda et al., 2000).

Therapeutic Implications

In transplant patients, interference with costimulatory pathways leads to tolerance induction; paradoxically, this process is blocked by CsA (Li et al., 1998). Our model explains both findings and offers an alternate strategy that might synergistically induce tolerance in combination with costimulatory blockade. The prediction is that even in the presence of ongoing immune stimulation, disrupting the interaction of NFAT with Fos and Jun would induce a long-lasting tolerant state: it would eliminate or severely disrupt transcription of genes involved in the productive immune response for which NFAT-AP-1 cooperation is essential, while at the same time switching the cell's genetic program toward transcription of the distinct set of anergy-inducing genes that are activated by NFAT in the absence of AP-1. A detailed molecular structure of the NFAT-Fos-Jun-DNA complex is available (Chen et al., 1998) and should facilitate identification of peptide and small molecule inhibitors that selectively disrupt cooperative NFAT:Fos:Jun complexes on composite NFAT-AP-1 sites, without affecting independent binding of NFAT or Fos:Jun to noncomposite sites.

Experimental Procedures

Mice

Mice were maintained in pathogen-free conditions in a barrier facility. BALB/cJ DO11.10 TCR transgenic mice were bred with NFAT1$^{-/-}$ mice (Xanthoudakis et al., 1996) or their isogenic wild-type controls to obtain NFAT1$^{-/-}$ or wild-type DO11.10 TCR transgenic mice.

Cell Culture

The murine Th1 cell clone D5 (Ar-5) was cultured as previously described (Agarwal and Rao, 1998). Primary CD4^{+} T cells were isolated from lymph nodes and spleen of NFAT1$^{-/-}$ or wild-type DO11.10 transgenic mice using magnetic beads (Dynal) and differentiated in vitro by stimulating for 1 week with irradiated APC and 1 μg/ml $OVA_{323-339}$ as previously described (Agarwal and Rao, 1998). Jurkat and Phoenix Ecotropic cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 10 mM HEPES, and 2 mM glutamine.

RNase Protection Assay

Total cellular RNA was analyzed using the RiboQuant multiprobe RNase protection kit and specific multiset probes (Pharmingen). Jurkat cells were selected after transfection with 10 μg/10^6 cells of a murine CD4 plasmid and pEGFPN1 (Clontech) or pNFAT1-NLS-(ST2+5+8) (Okamura et al., 2000) as previously described (Macian et al., 2000).

Electrophoretic Mobility Shift Assays (EMSAs)

Nuclear extracts were prepared from Th1 cells, unstimulated or stimulated for 1 or 6 hr with 500 nM ionomycin or 20 nM PMA plus 500 nM ionomycin. Binding reactions were performed as previously described (Macian et al., 2000) using probes for NFAT (distal murine IL-2 promoter), AP-1, and NF-κB (Goldfeld et al., 1993).

Retroviral Infections

Three different retroviral vectors were used: MSCV-containing GFP-KV-DV (Wyeth Research) that expresses GFP from an IRES sequence, GFP-KV-DV-CA-NFAT1, and GFP-KV-DV-CA-RIT-NFAT1. The latter two vectors were constructed by subcloning DNAs encoding murine CA-NFAT1 [(ST2+5+8) NFAT1; Okamura et al., 2000] with or without a R468A/I469A/T535G mutation (Macian et al., 2000) into GFP-KV-DV. The Phoenix ecotropic packaging cell line (kindly provided by G.P. Nolan) was transfected with the retroviral vectors and supernatants were collected 24 and 48 hr later, supplemented with polybrene (8 μg/ml), and used to spin-infect (1000 × g/90 min) NFAT1$^{-/-}$ Th1 cells 24 and 48 hr after stimulation with 1 μg/ml plate bound anti-CD3ε and 5 μg/ml anti-CD28 (Pharmingen) in media supplemented with 20 U/ml of IL-2. Cells were analyzed 72 hr postinfection and if necessary sorted for GFP expression. Infection efficiencies were similar for all three retroviruses, ranging between 10% and 40% in different experiments. Protein expression was confirmed by Western analysis.

ELISA

Supernatants were collected 24 hr after T cell activation, and IL-2 levels were measured in a sandwich ELISA (Pharmingen).

Immunoblotting

Whole cell extracts were prepared by boiling cell pellets directly in SDS to prevent proteolysis during cell lysis. Anti-NFAT1 antibodies have been described (Okamura et al., 2000).

Proliferation Assay

D5 or primary T cells were stimulated with APC and antigen, ^{3}H-thymidine (10 μCi/ml) was added, and incorporation was measured during a 16 hr pulse beginning at 24 hr following stimulation. DNA was collected using a cell harvester, and the amount of radioactivity incorporated was measured in a β counter.

Tunel Assay

Apoptosis was detected by the Tunel method using the In situ Cell Death Detection kit (Boehringer). Stained cells were analyzed on a FACSCAN (Beckton-Dickinson).

RNA Samples and DNA Array Procedures

See Supplementary Methods at http://www.cell.com/cgi/content/full/109/6/719/DC1.

Intracellular Cytokine Staining

T cells were stimulated for 4 hr with 1 μg/ml plate bound anti-CD3ε and 5 μg/ml anti-CD28. For the last 2 hours, Brefeldin A was added at 10 μg/ml to promote intracellular accumulation of IL-2. After stimulation, cells were fixed in 4% paraformaldehyde and permeabilized in PBS/1% BSA/0.5% saponin. Cells were then washed and incubated for 10 min with Fc-block (Pharmingen) and then for 30 more minutes with 10 μg/ml phycoerythrin (PE)-conjugated anti-mouse IL-2 antibody (Pharmingen) to detect intracellular IL-2. Stained cells were analyzed on a FACSCAN (Beckton-Dickinson).

Quantitative RT-PCR

Total RNA was prepared from resting or stimulated T cells using Ultraspec reagent (Biotecx). cDNA was synthesized using oligo-dT primers and Superscript polymerase (Invitrogen) following the manufacturer's recommendations. Quantitative real-time PCR was performed in an I-Cycler (BioRad) using a SYBR Green PCR kit from Applied Biosystems and specific primers to amplify 100–200 bp fragments from the different genes analyzed. A threshold was set in the linear part of the amplification curve (fluorescence = f [cycle number]), and the number of cycles needed to reach it was calculated for every gene. Melting curves and agarose gel electrophoresis established the purity of the amplified band. Normalization was achieved by including a sample with primers for L32.

Induction of Oral Tolerance

OVA (20 mg/ml) was administered to DO11.10 TCR transgenic mice in their drinking water for 5 days, after which CD4 T cells were isolated from spleen and lymph nodes of these mice and age- and sex-matched controls. The ability of the control and tolerized T cells to initiate a productive immune response to $OVA_{323-339}$ was analyzed by measuring ^{3}H-thymidine incorporation during a 16 hr pulse beginning 60 hr after stimulation with irradiated splenic APC pulsed with different concentrations of $OVA_{323-339}$.

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