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).

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\(^{2+}\) also has 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 NF\(\kappa\)B and AP-1: combined TCR/CD28 stimulation activates cJun kinase (JNK), p38 MAP kinase, and IkB kinase (IKK) pathways and increases nuclear levels of NF\(\kappa\)B/Rel and AP-1 proteins more strongly than TCR stimulation alone (Su et al., 1994; Harhaï 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 1A). 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-\(\gamma\), TNF-\(\alpha\), GM-CSF, and MIP-1\(\alpha\), 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-\(\gamma\) transcripts in lane 4 and of MIP-1\(\alpha\) 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) 3H-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. 3H-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.

regulated 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 Ca2+/calcineurin signaling directs a genetic program associated with anergy development in T cells, distinct
Figure 2. Stimulation with Ca\textsuperscript{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
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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.

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 OVA323–339 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 Ca2+/calcineurin-dependent pattern of gene expression. In vivo tolerized T cells also show basal elevation of intracellular Ca2+ 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 Ca2+/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-
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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. 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 [32P]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.

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$\alpha$ 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$\alpha$ genes, which require the cooperative interaction of NFAT and AP-1 (lane 4; Macian et al., 2000). PMA stimulation also further upregulated TNF$\alpha$, 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 NFAT$^{-/-}$ 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 6D).
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 (infected) and GFP (uninfected) 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.
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 Ca2+ or Ca2+/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 NF-κB; 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 Ca2+ 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 Ca2+ signaling, or participation of Ca2+–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 Ca2+/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., unpublished data).

Figure 7. A Model of Anergy Induction
For details, see text.
lished). These data suggest that NFAT2 and NFAT4 also participate in anergy induction, making increasing contributions as stronger energizing 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 I KK/NF-κB 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; Hoffmann 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; Lanou 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; Sabattini 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 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 non-composite 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 (Au-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 OVA323–339 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 kit and specific multiprobe probes (Pharmingen). Jurkat cells were selected after transfection with 10 μg/10⁸ 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 DNA 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, 3H-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

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 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 (Biotec). cDNA was synthesized using oligo-dT primers and Superscript polymerase (Invitrogen) following the manufacturer’s recommendations. Quantitative real-time PCR was performed in an iCycler (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 OVA323–339 was analyzed by measuring 3H-thymidine incorporation during a 16 hr pulse beginning 60 hr after stimulation with irradiated splenic APC pulsed with different concentrations of OVA323–339.

Acknowledgments
We thank members of the Rao and Byrne laboratories for valuable discussions, K. Murphy for the original version of the retroviral vector, and G. Nolan for the Phoenix Ecotropic packaging cell line. This

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work was supported by National Institutes of Health grants CA42471 and AI48213 (to A.R.)

Received: September 26, 2001
Revised: May 7, 2002

References


Supplementary Methods

RNA samples and DNA array procedures: T cells were stimulated for 2, 6 or 16 hours with 500 nM ionomycin, 20 nM PMA plus 500 nM ionomycin, or 1 mM CsA plus 500nM ionomycin. Total RNA was isolated with an RNeasy kit (QIAGEN). 10 mg of total RNA was quantitatively amplified and biotin-labeled as described (Byrne et al., 2000). Hybridization to Genechips (Affymetrix) displaying probes for 11,000 mouse genes/ESTs was performed at 40°C overnight in a mix that included 10 mg fragmented RNA, 6X SSPE, 0.005% Triton X-100 and 100 mg/ml herring sperm DNA in a total volume of 200 ml. Chips were washed, stained with SA-PE and read using an Affymetrix Genechip scanner and accompanying gene expression software. 11 labeled bacterial RNAs of known concentration were spiked into each chip hybridization mix to generate an internal standard curve, allowing normalization between chips and conversion of raw hybridization intensity values to absolute mRNA frequency (mRNA molecules per million) (see Figure 2B, 3C) (Lockhart et al., 1996; Hill et al., 2001). Two arrays were performed with RNA from D5 T cells, and one array each with wildtype and NFAT1-/- Th1 cells. The y-axis of the Self-Organizing Map (SOM) in Figure 2a is normalized mRNA Frequency. Genes are clustered on the basis of the kinetic pattern of expression over the 16 hour period of treatment, independent of expression magnitude, on the assumption that some of the genes displaying similar expression kinetics will be involved in the same biological process. The normalization was a “mean zero, variance one” normalization, and is accomplished as follows. (i) Compute the mean and standard deviation of the Absolute mRNA Frequency for a gene across all timepoints (0, 2, 6, 16 hours). (ii) Transform the data for that gene by subtracting the mean, then dividing by the standard deviation. To summarize in a single equation: Normalized Frequency = (Absolute Frequency – (Mean of Absolute Frequency)) / (Standard Deviation of Absolute Frequency). The method used for constructing the Self-Organizing Map has been described (Tamayo et al., 1999).

References for Supplementary Methods


**Supplementary Table 1:** Accession numbers and identities of the genes whose expression profiles are shown in Figure 2A.

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x53584   HSP60
X68193   Nucleoside diphosphate kinase B.
X70100   Ma1 mRNA for keratinocyte lipid-binding protein.
x97047   M2-type pyruvate kinase.
Y13071   26S proteasome non-ATPase subunit.
Z16078   CD53
Z31399   CCT eta subunit (chaperonin containing TCP-1).
Z31553   CCT (chaperonin containing TCP-1) beta subunit.
Z31555   CCT (chaperonin containing TCP-1) epsilon subunit.
Z31557   CCT (chaperonin containing TCP-1) zeta subunit.
z49085   Mouse developmental kinase 2 (MDK2).

Panel 3
af031486 Spermidine aminopropyltransferase (Mspmsy)
D42048 Squalene epoxidase
I34290 Guanine nucleotide regulatory protein beta 5
m13445 Alpha-tubulin isotype M-alpha-1
m60456 Cyclophilin
M96823 Nucleobindin
X04663 Tubulin, beta 5
X78683 BCR-associated protein 37
x06453 Protein disulfide isomerase
X70296 PN-1 (protease-nexin 1)
X90582 Signal sequence receptor delta

Panel 4
AB000733 AF1q
D50589 Prostaglandin E2 Receptor, EP2 Subtype
d85570 Proteasome subunit Z (PSMB7)
D87691 Eukaryotic translation termination factor 1 (eRF1)
d87911 Proteasome 28 subunit, 3
d90151 CArG-binding factor-A
j04633 Heat shock protein 86
K02236 Metallothionein II (MT-II)
L09754 Cd30 ligand
I32751 GTPase (Ran)
m17015 Lymphotoxin (LT)
U28404 Macrophage inflammatory protein-1 alpha receptor
X16857 HSP86 heat-shock protein
U16162 Prolyl 4-hydroxylase alpha(1)-subunit
U63648 Myb-binding protein (P160)
u69135 Uncoupling protein 2 (UCP2)
x06143 CD2
X54511 Myc basic motif homologue-1 (mbh1).
Z22593 Fibrillarin
Panel 5
AF030559  ATP synthase beta-subunit (beta-F1 ATPase)
d55720  Importin alpha2
d82019  Basigin
D85904  Apg-2
L15447  Small nuclear RNA (Rnu1a-1)
L19737  H+ ATP synthase subunit c
L25274  Transmembrane glycoprotein (DM-GRASP)
u13393  Delta proteasome subunit (Lmp19)
U27830  Stress induced phosphoprotein 1 (mSTI1)
U31758  Histone deacetylase 2
u43548  Transcription factor like protein 4 TCFL4
u70315  U1 snRNP-specific protein C
U96700  Proteinase inhibitor 6 (SPI6)
x04480  Preproinsulin-like growth factor IA.
x53526  OX45
X59047  CD81

Panel 6
aa161799  Phosphoglycerate mutase (Pgam1)
D10024  Calpactin 1 (annexin II)
D50031  TGN38A
I42115  Insulin-activated amino acid transporter mRNA
m14044  Calpactin I heavy chain (p36)
m18186  84 kD heat shock protein
X16151  Osteopontin
X53333  Triosephosphate isomerase
X01756  Cytochrome C gene
D10024  Protein-tyrosine kinase substrate p36 (calpactin I heavy chain)
M18186  84 kD heat shock protein
U77040  LIM protein 3 (mSLIM3)
X16151  Eta-1, osteopontin
x51834  Osteopontin.
x67914  PD-1

Panel 7
d78645  78 kDa glucose-regulated protein (grp78)
D87990  UDP-galactose transporter related isozyme 1
m94087  ATF4
U17343  Signal recognition particle receptor beta subunit
L15447  Small nuclear RNA (Rnu1a-1)
U11027  Sec61 protein complex gamma subunit
U12922  CD1 geranylgeranyl transferase beta subunit
u84211  Defender against death 1 protein (DAD1)
Y11666  Hexokinase II
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<td>Retinoblastoma-binding protein (mRbAp46)</td>
</tr>
<tr>
<td>X76850</td>
<td>MAP kinase-activated protein kinase 2</td>
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<tr>
<td>U39192</td>
<td>Heparin-binding epidermal growth factor-like growth factor</td>
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<tr>
<td>TNFa</td>
<td>TNFalpha</td>
</tr>
<tr>
<td>u10551</td>
<td>Gem GTPase (gem)</td>
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<tr>
<td>u11692</td>
<td>Lymphoid-specific interferon regulator factor (LSIRF)</td>
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<tr>
<td>u19118</td>
<td>ATF3</td>
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<tr>
<td>U44088</td>
<td>TDAG51</td>
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<tr>
<td>u59463</td>
<td>Caspase 11</td>
</tr>
<tr>
<td>U70139</td>
<td>Nocturnin</td>
</tr>
<tr>
<td>U88328</td>
<td>Suppressor of cytokine signalling-3 (SOCS-3)</td>
</tr>
<tr>
<td>x02611</td>
<td>Tumour necrosis factor (TNF).</td>
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<tr>
<td>X67644</td>
<td>gly96</td>
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<td>x85214</td>
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<td>z72000</td>
<td>BTG3</td>
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**Panel 12**

<table>
<thead>
<tr>
<th>Gene Accession</th>
<th>Description</th>
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<tr>
<td>ab000677</td>
<td>SOCS1</td>
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<tr>
<td>AF006492</td>
<td>Friend of GATA-1 (FOG)</td>
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<tr>
<td>d10061</td>
<td>DNA topoisomerase I</td>
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<tr>
<td>D13759</td>
<td>Mitogen activated protein kinase kinase kinase 8</td>
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<td>D87115</td>
<td>MAP kinase kinase 3b</td>
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<tr>
<td>L16956</td>
<td>Jak2</td>
</tr>
<tr>
<td>L16956</td>
<td>Jak2</td>
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<tr>
<td>l26489</td>
<td>Furin (FUR)</td>
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<tr>
<td>M32489</td>
<td>Interferon consensus sequence binding protein</td>
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<tr>
<td>m59821</td>
<td>Ier2, pip92</td>
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<td>M83380</td>
<td>Transcription factor relB</td>
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<td>M95200</td>
<td>Vascular endothelial growth factor</td>
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<td>M13945</td>
<td>Pim-1 protein kinase</td>
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<td>L42462</td>
<td>TGF-1</td>
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<td>U10102</td>
<td>Bcl-x long</td>
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<td>U29056</td>
<td>Src-like adapter protein SLAP</td>
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<tr>
<td>L48687</td>
<td>Voltage-dependent Na+ channel beta-1 subunit</td>
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<tr>
<td>L41352</td>
<td>Amphiregulin</td>
</tr>
<tr>
<td>u09507</td>
<td>p21 (Waf1)</td>
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<td>u20735</td>
<td>junB</td>
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</tbody>
</table>
Panel 13

D31967  Jumonji
ET62419  p56lck-associated adapter protein Lad
j02930  Laminin B2 chain
L12120  Interleukin-10 receptor (Il10r)
U09507  Cyclin dependent kinase inhibitor 1A (P21)
U19463  Zinc finger protein A20 (murine A20)
U04379  Protein tyrosine kinase ZAP-70
D90242  nPKC-eta
L15435  Mus musculus 4-1BB ligand
L15435  Mus musculus 4-1BB ligand
U88327  Suppressor of cytokine signalling-2 (SOCS-2)
X51829  MyD116

Panel 14

M64292  TIS21
X89749  TGIF
y07711  Zyxin.
z30940  Histone H2A

Panel 15

AA119194  Rab10
AA061283  DAGkinase alpha (Dagk1)
AA066032  DAGkinase alpha (Dagk1)
aa710204  Acetyl-CoA acetyl transferase
ET63241  Caspase-3
Ikaros  Ikaros
L03547  Ikaros
Y00309  LDH-A
u23921  Osmotic stress protein 94 (Osp94)
U44731  GBP-3
U67187  G protein signaling regulator RGS2 (rgs2)

Panel 16

af003695  Hypoxia-inducible factor 1 alpha (Hif1a)
d28530  Protein tyrosine phosphatase PTPT9 (rPTPs)
d38023   PIMT
D88315   Tetracycline transporter-like protein
L10106   R-PTP-kappa
I33768   JAK3
m21952   Macrophage colony-stimulating factor
M60474   Myristoylated alanine-rich C-kinase substrate (MARCKS)
X57024   Glutamate dehydrogenase
U05252   Nuclear matrix attachment DNA-binding protein SATB1
u10871   MAP kinase p38
u30464   Wnt-10B
u54803   Caspase 3
U61363   Groucho-related gene 4 protein (Grg4)
U83176   ROSA 26
X57024   GLUD (glutamate dehydrogenase)

Panel 17
AA089339   Leukocystatin
aa215251   GEG-154, RNF19 or Xybp
aa221219   Myoinositol 1-phosphate synthase A1 (IsynA1)
AF034610   Nuclear autoantigenic sperm protein
d78141    TRAF5
U25708    CD98 heavy chain mRNA, complete cds
X71642    GEG-154
u06948    Fas ligand
U83148    NFIL3/E4BP4
X14425    Profilin
x64068    Cation-dependent mannose-6-phosphate receptor.
x66081    Pgp-1 mRNA for CD44
X71642    GEG-154, RNF19 or Xybp
W90837    Chloride intracellular channel (Clic1)

Panel 18
ac002397   PTPN6
AF011644   Oral tumor suppressor homolog (Doc-1)
D10712     Nedd-1 protein
D11091     Protein kinase C theta
j02990     CD3-epsilon
L02526     MAPKK1
L10244     Spermidine/spermine N1-acetyltransferase (SSAT)
m64279     BMI-1)
m81477     Protein tyrosine phosphatase, non-receptor type 2
m97636     Helix-loop-helix transcription factor ME1
U19617     Ets-family transcription factor Elf-1
U41341     Calgizzarin
U72941     Annexin IV mRNA, complete cds.
Panel 19
AA125310    Elastase Inhibitor
AA145127    Serpinb1
aa242017    Enoyl coenzyme A hydratase (Auh)
AF013632    FAN protein
d14566       Lmp-2
ET62534      CD4
l25069       Catalase
m37761       Calcyclin
m55637       HAM1
m59470       Cystatin C
L25885       beta 1,4N-acetylgalactosaminyltransferase
L11613       Proteasome Lmp2
U07950       GDP-dissociation inhibitor (GDI-1)
U12283       USF2
X66449       Calcyclin
u04268       Sca-2
u06923       Signal transducer and activator of transcription (Stat4)
U07890       Flotillin 2
u12283       USF2
U19119       Interferon inducible protein 1
U19596       Cdk4 and Cdk6 inhibitor p18 protein
u24674       Max interacting protein 1 (Mxi1)
u57325       Presenilin 2
X66449       Calcyclin
x75129       Xanthine dehydrogenase
z14252       Acidic sphingomyelinase (ASM-1)

Panel 20
AF001863    FYN binding protein
AF001863    FYN binding protein
m89956      Calcium-binding protein (pp52)
J05261      Cathepsin A
M38381      Serine threonine tyrosine kinase (STY)
Z25524      Integrin associated protein CD47
u16985      Lymphotoxin-beta
U43673      IL-1Rtp
U49351      Lysosomal alpha-glucosidase
U69535      Semaphorin M-sema G
x03533      Tyrosine protein kinase p56-lck.
x73359      AES-1
Supplementary Figure 1: Expression profiles of the 37 genes activated by ionomycin in D5 and primary Th1 cells. The genes are grouped into eight categories based on function.